IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 8, Issue 12 Ver. III (Dec. 2014), PP 58-64
www.iosrjournals.org

Enzymatic Decolorization of Malachite Green Dye by a Newly Isolated Bacillus Cereus Strain wwcp1

Wycliffe Chisutia Wanyonyi[*1], John Mmari Onyari[1], Paul Mwanza Shiundu[2], Francis Jackim Mulaa[3]

[1]*Department of Chemistry, College of Biological and Physical Sciences,University of Nairobi, P.O. Box 30197 - 00100, Nairobi, Kenya.*
[2]*Department of Chemical Sciences and Technology, The Technical University of Kenya, Haile Selassie Avenue, P.O. Box 52428 - 00200, Nairobi, Kenya*
[3]*Department of Biochemistry, College of Health Sciences, University of Nairobi, P.O. Box 30197 - 00100, Nairobi, Kenya.*

Abstract: *Enzymatic decolorization of Malachite Green (MG) dye was studied using crude enzyme from a newly isolated Bacillus cereus strain wwcp1. 98% decolorization efficiency was achieved within 24 hours using an initial dye concentration of 1.0 x 10^{-5}M. Batch experimental results revealed that the decolorization process was highly dependent on contact time, initial MG concentration, aqueous solution temperature and pH. Biodegradation of MG dye was monitored spectrophotometrically and metabolites confirmed by thin layer chromatography (TLC). The comparison of TLC chromatograms before and after decolorization confirmed that crude protease enzyme had the ability to degrade MG dye. The results provide evidence that the crude enzyme from Bacillus cereus strain wwcp1 is an effective and potential candidate for industrial wastewater treatment.*
Keywords: *Decolorization, Malachite Green Dye, Bacillus cereus strain wwcp1, Biodegradation, Enzyme.*

I. Introduction

Water pollution due to release of industrial wastewater has become a serious environmental problem in almost every industry using dyes to color products. Wastewater from textile, paper, leather, cosmetic, food, and plastic industries often contains synthetic dyes that are toxic, mutagenic, and carcinogenic [1, 2]. The strong color of industrial wastewaters containing dyes even at a small concentration has a huge impact on the aquatic environment due to their turbidity, increased chemical oxygen demand (COD), and reducing light penetration, which has adverse effects on photosynthetic phenomena. Among many classes of synthetic dyes used in the industries, triphenyl methane group of dyes such as malachite green and crystal violet constitute a major and versatile group that play a predominant role in almost every type of application [3].

Malachite oxalate green dye (MG) $[(C_{23}H_{25}N_2)\cdot(C_2HO_4)]2\cdot C_2H_2O_4]$, (Bis[[4-[4-(dimethylamino) benzhydrylidene]cyclohexa-2,5-dien-1-ylidene]dimethylammonium] oxalate, dioxalate; N,N,N',N'-Tetramethyl-4,4'-diaminotriphenylcarbenium oxalate) MW 929.03g (Figure 1) has been found to be highly toxic to mammalian cells; promote hepatic tumor formation in rodents and cause reproductive abnormalities in rabbits and fish [4]. It was nominated by the Food and Drug Administration as a priority chemical for carcinogenicity testing by the National Toxicology Programme of the USA [5]. The need to maintain a cleaner environment for the survival of both aquatic and terrestrial lives has forced many governments to established environmental restrictions with regard to the quality of colored effluents forcing dye houses to decolorize their effluents before discharging. Treatment of industrial wastewater containing dyes is an essential, but difficult process because dyes usually have a complex aromatic structure which makes them resistant to biodegradation by conventional biological treatments.

Several methods have been developed to remove synthetic dyes from wastewaters in order to decrease their impact on the environment [6, 7]. Conventional physicochemical methods such as adsorption with activated carbon, coagulation, precipitation, solvent extraction, membrane filtration, chemical oxidation, ozonation, and flocculation have been used to treat dye containing effluents; however, most of these methods are expensive or require additional chemicals [8]. The decolorization of dye in wastewater using microbial enzymes has been a subject of many studies in recent years due to their low cost of production and efficient application. Compared to physicochemical treatment methods, the enzymatic treatment of dyes have low energy cost and are more ecofriendly process although not commonly used in the textile industries [9].

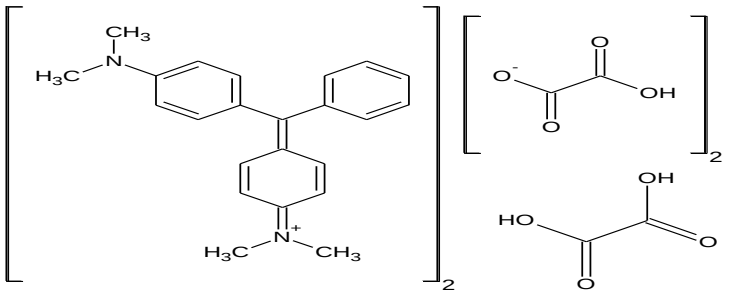

Figure 1: Structure of Malachite Green Oxalate (MG) dye

Extremophile like alkaliphiles bacteria exhibit the ability to grow at the extremely harsh environmental conditions such as high pH and temperature, high levels of salinity or salt, and pressure which critically influence their growth. Products of industrial importance from alkaliphiles have been commercialized, the most successful of which have been in the detergent and food industries [10]. However, relatively few studies have been reported on the application of alkaline enzyme in decolorization of organic dyes. The aim of present study is to investigate the ability of crude protease enzyme from newly isolated Bacillus cereus strain wwcp1 in decolorizing MG dye. Batch experiment were contacted to investigate and optimize the effects of contact time, pH, initial MG concentration, aqueous solution temperature on the decolorization of MG dye by crude protease enzyme. The resultant metabolites were analyzed by thin layer chromatography.

II. Materials And Methods

A cationic basic dye, malachite green oxalate, was obtained from Loba-Austria and used without further purification. MG solution was prepared from stock solution of 1.0×10^{-4}M (92.903 mg/L) by dilution. All solutions were prepared in double distilled water and pH adjusted by adding either 0.1 M HCl or NaOH.

1.1 Microorganism Isolation and enzyme production

Dye-decolorizing bacteria were isolated from mud water samples obtained from Lake Bogoria. One gram of the contaminated soil sample was weighed aseptically into 100 ml of sterile distilled water, agitated for 45 minutes on a shaker and 0.2 ml spread on casein agar plates before incubating at 37°C for 2 days. Protease producing strains were selected by spotting the bacterial cultures on culturing medium containing Reese Agar medium [11]. Four positive colonies were identified by the formation of zone of clearance around the colony. The zone was made clearer by flooding the plates with a solution of 5% Trichloroacetic acid (TCA). The diameter of the bacterial colony and the total zone of enzyme activity including the growth diameter were measured in each case. Bacillus cereus strain wwcp (GenBank accession No. KM201428) exhibited maximum clear zone around the colony indicating high protease activity and thus selected for enzyme production. The medium for crude enzyme production contained 0.5 % casein and 0.25% glucose. 500mL Erlenmeyer flasks containing 100mL of media plugged in cotton and aluminum foil was sterilized in autoclave at 121ºC (15 lb) for 15 min and after cooling the flask was inoculated with 5% over night grown seed bacterial culture. The inoculated medium was incubated at 45°C on a rotary shaker (140 rpm) incubator. After 72 hours, the culture medium was centrifuged at 5000 rpm and 4°C for 15 min to obtain the crude extract which served as crude enzyme source.

1.2 Assay of Enzyme Activity

Protease activity in the crude enzyme was assayed by the modified Tsuchida procedure [12] using 1% casein as substrate. Casein solution (1.0 ml) was mixed with an equal volume of crude protease enzyme solution and incubated at 45˚C for 10 min. The reaction was terminated by the addition of 4 ml of 10% (w/v) chilled trichloroacetic acid and reaction mixture allowed to stand in ice for 20 min to precipitate the insoluble proteins. The mixture was centrifuged to obtain the supernatant. 5ml of 0.4M Na_2CO_3 and 1 ml of one fold diluted Folin ciocalteau reagent was added to the supernatant which was further incubated for 30 minutes to develop the color. The absorbance was measured against an appropriate blank at 660 nm using a UV-VIS spectrophotometer.

1.3 Effect of pH on crude proteases enzyme activity

In order to determine the optimum pH for protease activity, the crude enzyme was assayed in 1% Casein (w/v) as substrate dissolved in buffers of different pH ranging from 4 to 12. The pH was adjusted using the following buffer systems at 0.1M concentration: acetate (pH 4, 5), sodium phosphate buffer (pH 6, 7), Tris HC1 buffer (pH 8) and glycine-NaOH (pH 9 - 12). Reaction mixtures were incubated at 45°C for 30 min before measuring the enzyme activity.

1.4 Decolorization Assay

1.4.1 Determination of wavelength of maximum absorbance (λ_{max}) for Malachite green

A 1.0 x 10^{-5}M solution of MG dye was prepared from the stock solution of 1.0 x 10^{-4}M. A sample of dye solution in a cuvette was placed in the UV-VIS spectrophotometer that had been set to the absorbance mode. Starting from the lower wavelength (400 nm), the wavelength was adjusted and the wavelength together with the corresponding absorbance recorded over the entire wavelength range. The spectrophotometer was then set to the maximum wavelength value (617 nm) for all the absorbance measurements thereafter.

1.4.2 Determination of the effect of contact time on MG dye decolorization

10ml of crude protease enzyme was mixed with 40 ml of 1.0 x 10^{-5}M MG dye solution at room temperature in 100ml conical flask on a Thermolyne Orbital shaker at 150 rpm. The pH of the crude enzyme and dye solution was adjusted to pH 8.5. After a time interval of 10 minutes, aliquots from the reaction mixture were analyzed for residual MG concentrations using a UV–Vis spectrophotometer (UV-min 1240 SHIMADZU) monitoring changes in absorbance at 617 nm. Control tests were conducted where crude protease enzyme had been replaced by deionized water. All experiments were performed in triplicate and results expressed as the mean values.

1.4.3 Determination of the effect of initial MG dye concentration on decolorization

The effect of initial dye concentration was investigated at room temperature (25 °C) and pH 8.5. 40 ml of MG dye of concentrations at various increasing concentrations ranging from 1.0 x 10^{-6}M, to 1.0 x 10^{-5}M was placed in 100ml conical flask and 10 ml of crude protease enzyme added and placed on an orbital shaker. Samples were collected from the flask at various time intervals and analyzed for residual MG concentration. The rate of decolorization was expressed as the percentage decrease in absorbance at the peak wavelength.

1.4.4 Determination of the effect of temperature on MG dye decolorization

The effect of temperature on MG dye decolorization was investigated at temperature range of 25 - 70˚C and pH 8.5. 40 ml of MG dye of initial concentration of 1.0 x 10^{-5}M was mixed with 10 ml of crude protease enzyme both pre equilibrated at the study temperature for 30 minutes before mixing in 100ml conical flask. Absorbance reading was taken at an interval of 10 minutes till equilibrium was attained.

1.4.5 Determination of the effect of pH on MG dye decolorization

The effect of pH was investigated at room temperature (25˚C) and a pH range of 4-11 for 5 and 12 hours. 10 ml of crude enzyme at appropriate pH was mixed with 40 ml of 1.0 x 10^{-5}M MG dye solution in 100ml conical flask. The rate of decolorization was expressed as the percentage decrease in absorbance at the peak wavelength.

1.4.6 Assay of metabolites formed from the biodegradation

After complete decolorization, the metabolites from biodegraded products were extracted with equal volume of ethyl acetate (25: 25 ml). The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The dry crystals were placed in 25 ml conical flask and 5ml of methanol added to dissolve the metabolites for TLC analysis. Metabolite formation was examined by thin layer chromatography (TLC) using silica gel activated in chloroform. The solvent system used was n-propanol: ethyl acetate: acetic acid: distilled water (6:1:1:2 v/v). The separated products were visualized in iodine chamber.

II. Results And Discussion

2.1 Effect of pH on crude proteases enzyme activity

The crude protease enzyme from Bacillus Cereus Strain wwcp1 showed good protease activity over a broader pH range in alkaline media. The optimum pH was found to be 11 (Figure. 2), indicating that the enzyme is alkaline protease. Similar results were also observed in the protease produced by Aspergillus clavatus [13].

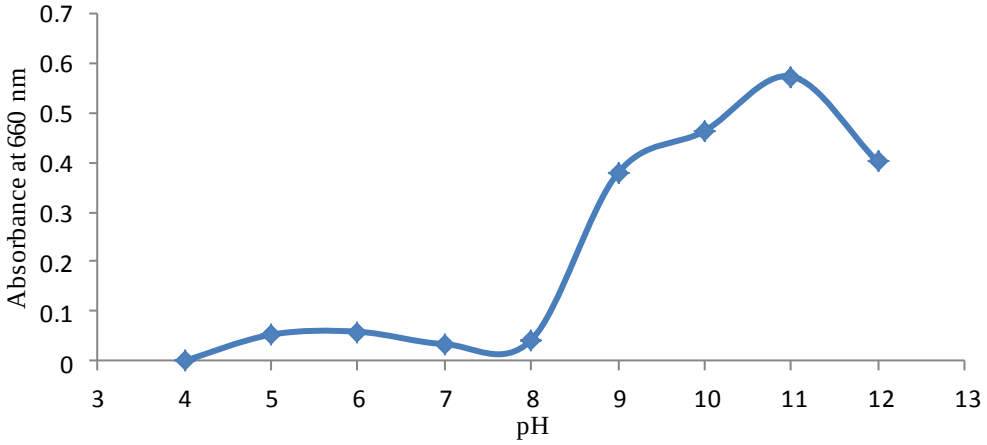

Figure 2. Effect of pH on Bacillus Cereus Strain wwcp1 protease activity

2.2 Determination of the effect of contact time on MG decolorization

The effect of contact time on MG dye decolorization by crude enzyme was examined by varying the time of incubation and the results presented in Figure 3. The results show that dye decolorization was rapid within the first 4 hours with approximately 82 % dye decolorization. The change in percentage decolorization after the 4th hour became relatively gradual attaining equilibrium at the 20th hour with 97.5 % dye decolorization. This observation suggested that the initial four hours was significant for dyes decolorization but quite slow after fourth hour which may be probably due to products inhibition. These results were in agreement with earlier published work of decolorization of textile dyes [14].

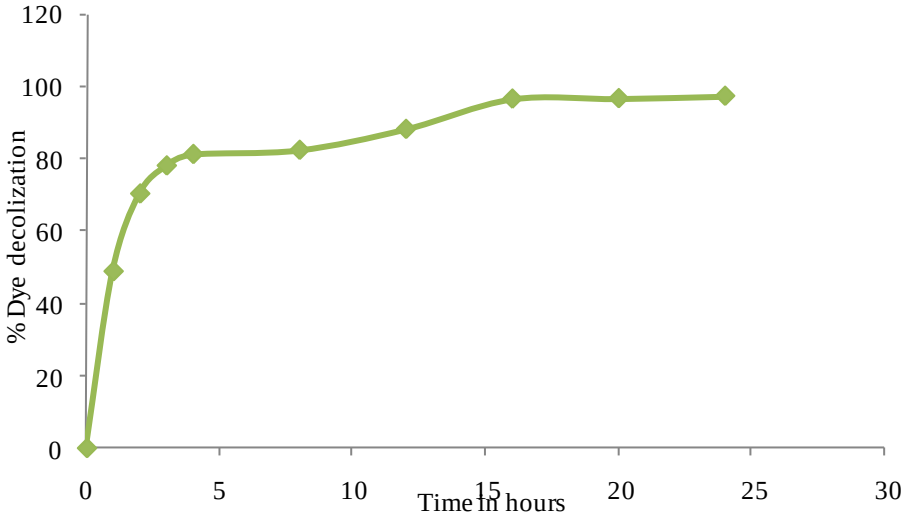

Figure 3: Effect of contact time on MG dye decolorization

2.3 Effect of pH on Malachite Green dye Decolorization

Enzymes are greatly affected by variation in pH. The effect of pH on decolorization of MG dye by crude protease enzyme was investigated at various pH values and results presented in Figure 4. It can be inferred that at a pH below 5, there was significantly very low decolorization rate but when the pH increased above 6.0, the rate of decolorization rapidly increased from 21% at pH 6 to 98% at pH 11. The result shows that the optimum pH for efficient MG dye decolorization was between pH 9 and 11. These results obviously present an advantage from industrial application point of view since most of dye effluents are characterized by alkaline pH under which crude protease enzyme works optimally. These findings are consistent with related studies done on decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria [15].

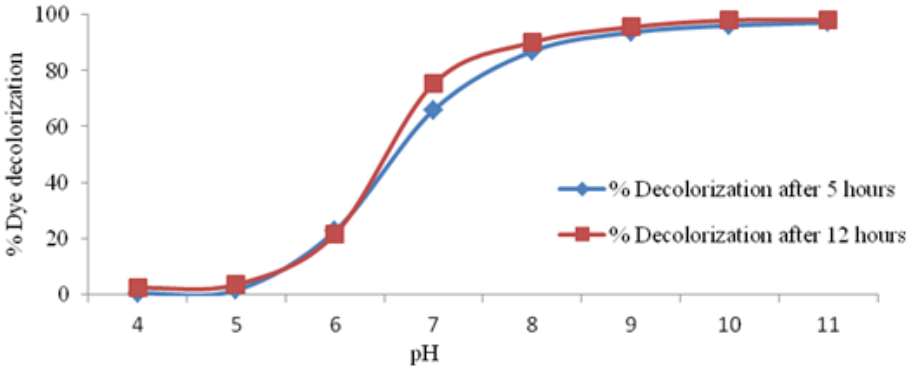

Figure 4: Effect of pH on the decolorization of MG dye

2.4 Effect of Temperature on Malachite Green dye Decolorization

Temperature plays an important role in enzyme activity. Effect of temperature on decolorization of MG dyes was investigated at temperature range of 25 - 70˚C and results presented in Figure 5. The results show that the rate of MG dye decolorization increased with increase in temperature between 25 ˚C, and 40˚C with decolorization efficiency increasing with increase of incubation time. The optimum temperature for crude protease enzymes was found to be 40˚C with 83% decolorization efficiency after 4 hours. At elevated temperature above 50°C, the rate of dye decolorization increased during the first 20 minutes but after one hour, percent dye decolorization remarkably decreased. This can be attributed to the denaturation of enzyme and thermal inactivation of enzyme under the operating temperature.

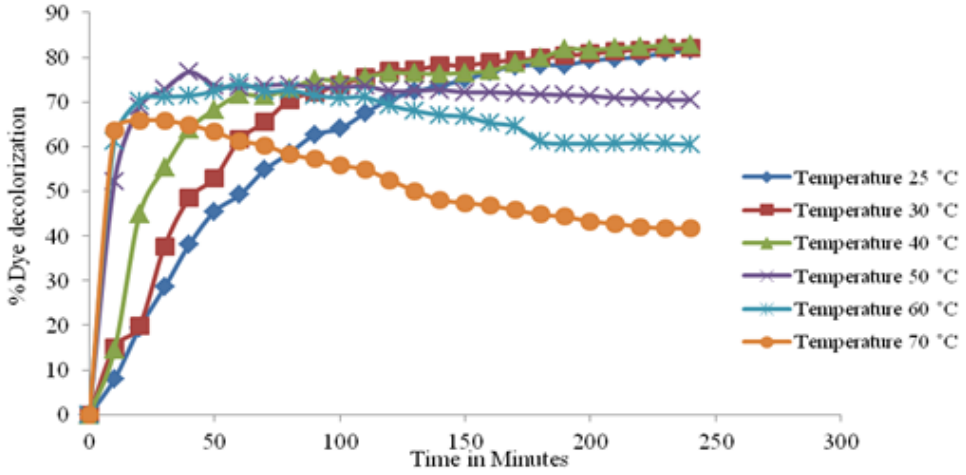

Figure 5: Effect of temperature on the decolorization of MG dye

2.5 Effect of initial Malachite Green dye concentration on decolorization

The decolorization of MG was studied at various increasing concentration of dye from 1.0 x 10^{-6}M, to 1.0 x 10^{-5}M and the results presented in Figure 6. It could be inferred from the results that the rate of decolorization decreased with the increase in initial MG dye concentration. However, the crude protease enzyme was able to decolorize higher concentration in the range of 83–100% during 24 h incubation period. The decrease in decolorization efficiency at high concentration might be due to the toxic effect of dye through the inhibition of metabolic activities. Similar results were observed by Murugesan et al [16] who investigated the decolorization of reactive dyes by a thermostable laccase produced by Ganoderma lucidum in solid state culture and indicated that decolorization of RB-5 and RBBR dyes decreased with increasing dye concentration.

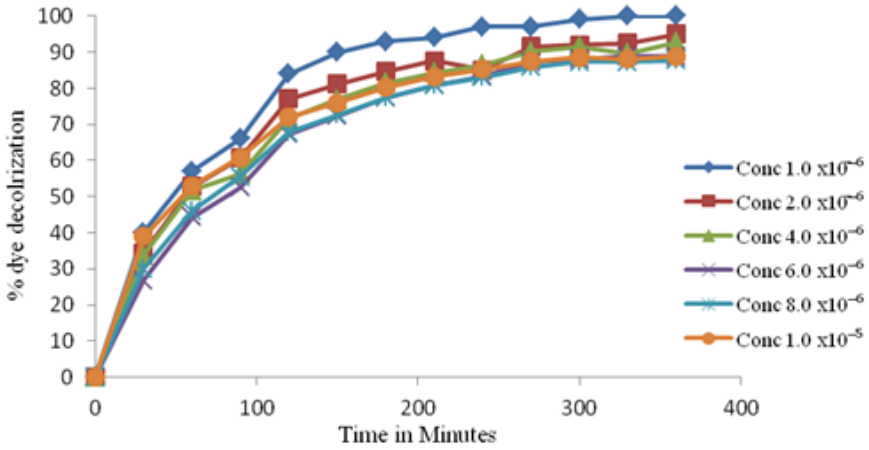

Figure 6: Effect of initial Malachite Green dye concentration on decolorization

2.6 Analysis of MG degraded metabolites

The metabolites produced during the biodegradation of malachite green dye were analyzed by thin layer chromatography and the results presented in Figure 7a and 7b. The comparison of TLC chromatograms before and after decolorization by crude protease enzyme in iodine chamber showed the appearance of three additional bands (M1, M2, and M3 of retardation factor (Rf) values of 0.96, 0.92 and 0.90 respectively (Figure 7b) as compared to control Rf value of 0.87 which might have been originated from the degraded dye metabolites. TLC results suggested that crude protease enzyme was able to degrade MG dye giving rise to three main metabolites which accounted for color disappearance.

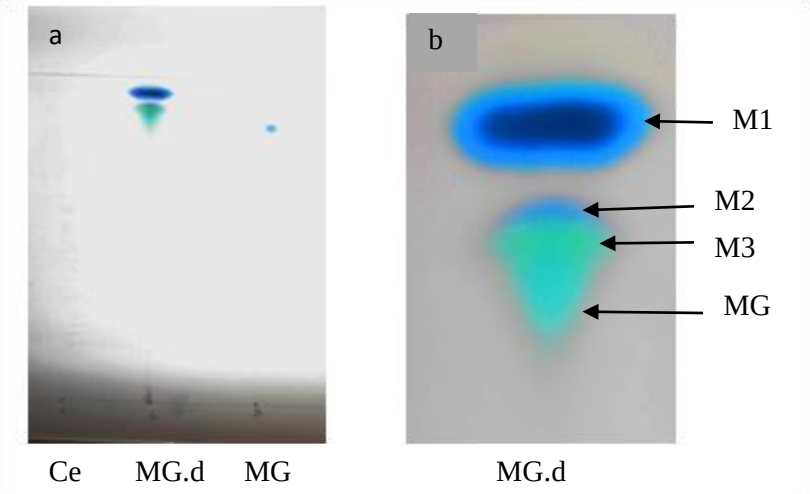

Figure 7. TLC Chromatograms of MG dye before and after degradation by crude protease enzyme **(a)** column 1,2 & 3 represent negative control with enzyme alone (Ce), degraded Malachite green dye (MG.d) and positive control with untreated Malachite green dye (MG)respectively; **(b)** Three metabolites (M1, M2, & M3) of degraded Malachite green dye.

III. Conclusion

Application of conventional physicochemical waste water treatment requires enormous cost and continuous input of chemicals which becomes uneconomical and causes further environmental damage. In the present study, our results clearly demonstrated that crude protease enzyme isolate from Bacillus Cereus Strain wwcp1 exhibit a novel alkaline protease properties with ability to decolorize and degrade MG dye. Taking these results into consideration, it can be concluded that newly isolated Bacillus Cereus Strain wwcp1 can be exploited for bioremediation of triphenyl methane group of dyes as a cost effective alternative technology for dye removal in wastewater treatment processes.

Acknowledgements

We are thankful to National Commission for Science, Technology and Innovation (NACOSTI) research endowment fund (Project no. NCST/ST&I/RCD/4TH CALLPhD/140), World Federation of Scientists

(WFS) Scholarship through the International Centre for Insect Physiology and Ecology (ICIPE) and Department of Chemistry, University of Nairobi for supporting and providing the infrastructure facilities for this study.

References

[1]. P. Kaushik and A. Malik, Comparative performance evaluation of Aspergillus lentulus for dye removal through bioaccumulation and biosorption. Environmental Science and Pollution Research, 20(5), 2013, 2882–2892
[2]. P. M. K. Reddy, S. K Mahammadunnisa, B. Ramaraju, B. Sreedhar, and C.H. Subrahmanyam, Low-cost adsorbents from bio-waste for the removal of dyes from aqueous solution. Environmental Science and Pollution Research 20(6), 2013, 4111–4124
[3]. B Moturi and M. A Singara-Charya, Decolorization of crystal violet and malachite green by Fungi. Sci World J 4(4), 2009, 28 – 33
[4]. C. Cha, D.R. Doerge, and C. E. Cerniglia, Biotransformation of malachite green by the Fungus Cunninghamella elegans. Appl Environ Microbiol 67(9), 2001, 4358 – 4360
[5]. S. J. Culp, and F.A. Beland, Malachite green: A toxicological review. J Am Coll Toxicology 15, 1996, 219-238
[6]. W.C. Wanyonyi, J. M Onyari, and P.M. Shiundu, Adsorption of methylene blue dye from aqueous solutions using Eichhornia crassipes. Bull Environl Contam Toxicol 91(3), 2013, 362 – 366
[7]. W.C. Wanyonyi, J. M Onyari and P.M. Shiundu, Adsorption of Congo red dye from aqueous solutions using roots of Eichhornia Crassipes: Kinetic and equilibrium studies. Energy Procedia 50, 2014, 862 – 869
[8]. K. Deepa, P.Chandran, and S. K Sudheer Bioremoval of Direct Red from aqueous solution by Pseudomonas putida and its adsorption isotherms and kinetics. Ecological Engineering 58, 2013, 207–213
[9]. P. Pengthamkeerati, T. Satapanajaru, N .Chatsatapattayakul, P.Chairattanamanokorn, and N. Sananwai Alkaline treatment of biomass fly ash for reactive dye removal from aqueous solution. Desalination 261,2010, 34 –40
[10]. I. P Sarethy, Y. Saxena, A. Kapoor, M. Sharma, S.K. Sharma, V.Gupta, and S. Gupta, Alkaliphilic bacteria: applications in industrial biotechnology. Journal of industrial microbiology & biotechnology 38(7), 2011, 769 –790.
[11]. E.T Reese, R.G.H. Siu, and H. S Levinson, The biological degradation of cellulose derivatives and its regulation to the mechanism of cellular hydrolysis. J. Bacteriol 59, 1950, 485- 489
[12]. O. Tsuchida, Y. Yamagata, and J. Ishizuka, An Alkaline proteinase an alkalophilic Bacillus sp. Curr. Microbiol 14,1986, 7-12
[13]. R. Celia, Tremacoldi, and C.C. Eleonora Production of extracellular alkaline protease by Aspergillus clavatus, World J. of Microbiology and Biotechnology, 21,2007, 169-172
[14]. G. Arabaci and A. Usluoglu, The enzymatic decolorization of textile dyes by the immobilized polyphenol oxidase from quince leaves. The Scientific World Journal, 2014, 1-5
[15]. S. Asad, M.A. Amoozegar, A. Pourbabaee, M.N. Sarbolouki, and S.M.M. Dastgheib, Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. Bioresource technology 98(11), 2007, 2082-2088.
[16]. K. Murugesan, M. Arulmani. I.H. Nam, Y.M. Kim, Y.S. Chang, and P.T. Kalaichelvan, Purification and characterization of laccase produced by a white rot fungus Pleurotus sajor-caju under submerged culture condition and its potential in decolorization of azo dyes. Appl Microbiol Biotechnol 72(5), 2006, 939-946