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Bioremediation of Crystal Violet and Malachite Green Dyes by Some Algal Species

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BIOLOGICAL treatment of crystal violet and malachite green using blue-green algae (*Nostoc* sp., *Microcystis aeruginosa* and *Oscillatoria geminata*) and green algae (*Chlorella vulgaris* and *Scenedesmus* sp.) in order to assess the decolorization ability of these algae after incubation for 2 and 24 hr in three dyes concentrations at 10, 50 and 100 ppm. *Chlorella* has achieved the highest percentage of crystal violet decolorization after 2 hr at 10 ppm, but after 24 hr *Scenedesmus* gave the highest percentage of decolorization at 10 ppm. The highest proportion of malachite green decolorization after two hours using *Chlorella* at 50 ppm, but after 24 hr the higher decolorization percentage of malachite green obtained by *M. aeruginosa* at 100 ppm followed by *Scenedesmus* sp. at the same concentration. The green algae showed a high capacity for crystal violet decolorization than blue-green algae. Measuring the activity of laccase, manganese peroxidase and tyrosinase enzymes indicated that *Nostoc* sp. gave the highest laccase activity in all dyes concentrations, while the other two enzymes haven't any activity. Chlorophyll-a and phaeophytine-a values obtained showed significant differences between most treatments; the most negatively affected species was *O. geminata* which showed decreasing in chl-a content in the two dyes concentrations. In conclusion; the decolorization process of dyes by algae had been done by different mechanisms; one of them was enzymatic degradation as *Nostoc* sp. *C. vulgaris* and *Scenedesmus* sp. have a high ability to decolorize the two dyes so they might be used in wastewater treatment of fish farms contains these carcinogenic dyes as antifungal agents.

Keywords: Bioremediation, Triphenylmethane dyes, Malachite green, Crystal violet, Cyanophyta, Chlorophyta.

Monoazo triphenylmethane dyes (malachite green and crystal violet) are often used in fish farms to control some bacterial and fungal diseases. US Food and Drug Administration in 1991 prohibited malachite green (MG) exploitation in fish farming owing to its alleged carcinogenic possessions (Sudova *et al*., 2007). Malachite green is an N-methylated diaminotriphenylmethane dye had been extensively used as the most worthwhile antifungal agent in fish farming industry. Although the use of MG and crystal violet (CV) are not officially

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permitted, its worldwide application in aquaculture everywhere would undoubtedly be continued due to its relatively low cost, ready availability, and efficacy; and hence potential human exposure to both dyes is expected from either consuming fish grown in fish farms treated with MG and/or CV or being in touch with their ecosystems (Cha *et al*., 2001).

Omar (2008) illustrated how the variation of molecular structures and functional groups present in monoazo dye (Tartrazine) and diazo dye (Ponceau), affects decolorization capabilities of green algae, cyanobacteria and diatoms. Certain algae can degrade a number of azo dyes to some extent; the reduction rate appears to be related to the molecular structure of the dyes and the species of algae used. The azo reductase of algae is responsible for degrading azo dyes into aromatic amine by breaking the azo linkage and use of azo dyes as a sole source of carbon and nitrogen by the algae (Jinqi and Houtian, 1992). The mechanisms of algae in discoloration might be engaged to enzymatic degradation and/ or adsorption. Oxidative enzymes are also involved in the discoloration process. The competence of adsorption is highly influenced by the structure of the dye and the species of algae used (Rana *et al.* 2013).

Manel *et al*. (2009) and Murugesan *et al*. (2009) stated that laccase, Manganese peroxidase and Phenol oxidase were able to decolorize triphenylmethane dyes. The activities of these enzymes involved in the degradation of azo dyes were illustrated by Joshni and Kalidass (2011). Most of the azo dyes have sulphonate substituent groups and high molecular weight and they are unlikely to pass through cell membranes. Therefore, the reducing activity referred to the dye is not dependant on the intracellular uptake of the dye (Robinson *et al*., 2001). Andrea *et al*. (2005) showed that laccase modifies azo dye structures by destroying their chromophoric structure. This is observed visually in azo dye solutions as a discoloration.

The present work, aims to find out the ability of algae under study on the decolorization of CV and MG and the most effective biosorbent of these algae that can be used in the biological treatment process for water fish farms containing these dyes.

Material and Methods

Algae and culture conditions

The green algae species; *C. vulgaris* and *Scenedesmus* sp. were obtained from the algae lab, Botany and Microbiology Department Faculty of Science, Helwan University, while Cyanobacteria strains *M. aeruginosa*, *Nostoc* sp. and *O.geminata* from Agricultural Microbiology Department, National Research Centre, Egypt. All algal species were grown in 250 ml Erlenmeyer flasks, each containing 100 ml of modified BG11 medium (Hoballah *et al.*, 2012). The cultures were incubated under shaking condition (150 rpm) and light regime at 16hr. light and 8 hr. dark for 2 weeks, in photo chamber at light intensity 950 lux at 28°C (Hoballah *et al.*, 2012).

Dyes and chemicals

Malachite Green, Crystal violet and ABTS [2, 2'–Azino-bis (3-ethyl benzothiazoline-6-sulfonic acid)] diammonium salt were obtained from the Sigma – Aldrich Chemical Company, (USA). Catechol was obtained from BDH Chemicals (Mumbai, India) and Pyrogallol was obtained from Oxford Lab. (Mumbai, India). All chemicals used were available in its highest purity and analytical grade.

Decolorization experiments

Stock solutions of 1g/100 ml distilled water (10000 ppm) of the tested dyes were prepared. The respective dyes stock solutions were added separately to 250 ml Erlenmeyer flasks each one containing 100 ml modified BG11 medium at concentrations of 10, 50 and 100 ppm. Dye removal was monitored by means of color at two time intervals, *i.e.*, 2 and 24 hr as mentioned by Sudova (2007) for short bath malachite green treatment. The aqueous media containing the tested dye were separated from the biomass using Whatman No. 1 filter paper and the residual dye concentration in the filtrates was filtered by millipore filter then determined colorimetrically, using a calibration curve prepared at the corresponding optimum wavelengths (588 nm for CV and 619 nm for MG) using spectrophotometer (Shimadzu UV-2401 PC, Japan) according to the procedures outlined in the standard methods (APHA, 1999). Control experiments were also carried out without algae. Thereafter decolorization was calculated as given by Kalyani *et al*. (2008) as follows:

% Decolorization= (InitialOD - Final OD)/ Initial OD × 100, where OD is the optical denisity

Chlorophyll a and phaeophytin a estimation: Chlorophyll a was estimated in the presence of phaeophytin-a by acetone extraction spectrophotometrically by reading optical density (OD) at 750 and 664 nm and then acidify extract with 0.1N HCl. Gently agitate the acidified extract and read OD at 750 and at 665 nm, 90 s after acidification according to APHA (1999).

Enzymes assay

The activities of laccase, manganese peroxidase and tyrosinase enzymes were determined, laccase activity was estimated by ABTS as a substrate in the presence of sodium acetate buffer (pH 5), the OD was measured at 420 nm, while manganese peroxidase activity estimated at 238nm by using $MnSO_4$ as a substrate and sodium tartarate buffer (pH 4.5) the former two enzymes determined as mentioned by Hatvani and Mecs (2001), while tyrosinase was determined by using pyrogallol as a substrate in the presence of phosphate buffer (pH 7) and OD measured at 450nm according to Zhang and Flurkey (1998). Enzyme activity was measured at 28 ºC in the filtrates of algae aqueous media after cells or trichomes removal, after 15-30 min. of incubation, it was expressed in International Units (IU) defined as the amount of enzyme required to oxidize 1µmol substrate in 1 min.

Statistical analysis

Results of chlorophyll-a and phaeophytin-a were statistically analyzed by SPSS (2006).

Results and Discussion

The selected five algal species were used to decolorize different concentrations of malachite green and crystal violet. Results shown in Fig. 1 confirm that the highest incidences of CV decolorization after 2 hr was 51.6 % obtained by *C. vulgaris* treated with 10 ppm, after 24 hr *Scenedesmus* sp. gave 86.9% which represent the highest value at 10 ppm, followed by 80.6 obtained at100 ppm. It is also clear that the lowest incidence of CV decolorization reaching 4% was achieved after 2 hr of incubation with *M. aeruginosa* under treatment with 100 ppm and after 24 hr (25.8%) by *O. geminata* at 50 ppm.

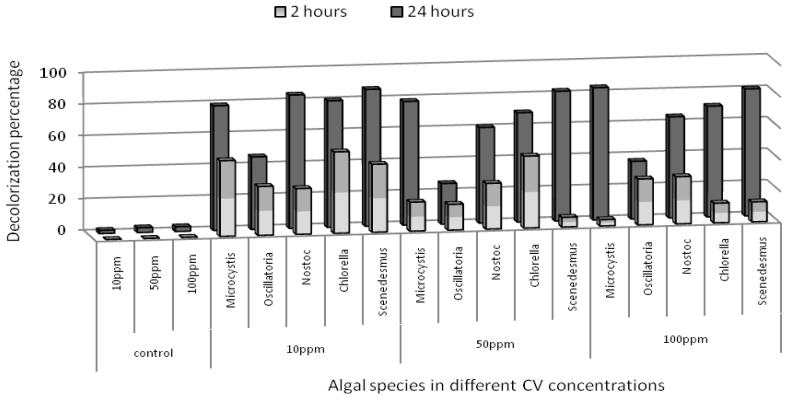

Fig. 1. Decolorization percentage of crystal violet after treating with algal species.

Results shown in Fig. 2. indicated that the highest incidence of MG decolorization after 2 hr reached 58.5 %obtained by *C. vulgaris* at 50 ppm followed by 57.3% obtained by the same species at 100 ppm .

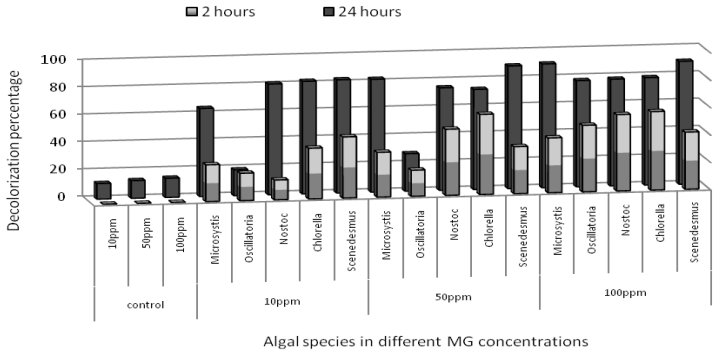

Fig. 2. Decolorization percentage of malachite green after treating with the tested algal strains.

After 24 hr the highest incidences of MG decolorization was 90.4% obtained by *M. aeruginosa* treated with 100 ppm followed by *Scenedesmus* sp. treated with the same concentration which gave 89.8%. The lowest incidence after 2 hr was 14.4% obtained by *Nostoc sp.* under a concentration of 10 ppm and 18.8 after 24 hr by *O. geminata* at 10 ppm. Results confirm that MG was decolorized by small proportions without treatment after 24 hr, while CV don't show any decolorization therefore, it is possible to say that the MG more sensitive to light than CV. These results revealed that the tested algal strains have the ability to decolorize CV and MG after 2 and 24 hr incubation at the three concentrations (10, 50 and 100 ppm) These findings are partially in parallel to those by Jinqi and Houtian (1992) confirming that *Chlorella vulgaris* has a high incidence to degrade some azo dyes. Also Dinesh Kumar *et al.* (2014) stated that *Chlorella* sp. removing 43% of dyes from textile waste water. Banat, *et al.* (1996); Forgacs *et al.* (2004) and Rana *et al.* (2013) that *Chlorella*, *Nostoc* and *Oscillatoria* species have the capacity to degrade azo dyes which are used as sole sources of carbon and nitrogen for these algae. All decolorization abilities, however, of the tested algal strains against CV and MG exhibited a noticeable increase after 24hr incubation compared to 2 hr, this suggest that there is different mechanisms in algae to bioremediate the two dyes. Green algae decolorize CV at a higher rate than blue green algae. These findings are in agreement with The decolorization was dependent on the types and the chemical structure of dyes; in our results *Chlorella* showed high activity to decolorize CV and MG, especially after 2 hr and *O. geminata* showed low activity especially with MG, while El-Sheekh *et al*. (2009) stated that *Oscillatoria* had the abilities to decolorize and remove azo dyes as methyl red, orange II and G-Red compared to *Nostoc* and *Chlorella* that did not had any ability to degrade those dyes in their culture media.

The enzymes activity assay did not show any induction in manganese peroxidase or tyrosinase enzymes. Only laccase enzyme activities were observable in control and during decolorization process by all tested algae strains at different levels as shown in Fig. 3. *Nostoc* sp. was the only tested alga displayed high positive laccase activity, this activity on the tested dyes exhibited strong decolorization effects at all concentration of either CV or MG. our results were compatible with Telke *et al*. (2010), they demonstrated that laccase from Pseudomonas sp. was the key enzyme responsible for Congo Red docolorization. On the other hand, the other tested algal strains showed weak laccase activity after 2 hr despite they possessed high ability to decolorize the tested dyes indicating that the decolorization process had been done by different mechanisms. In most treatments, except *Nostoc* the removal of dyes exhibited (reversible) recurrent increase indicating that one of decolorization mechanisms was the adsorption of dyes on algal cell walls. Mona *et al.* (2011) and Gajare and Menghani (2012) (mentioned that *Nostoc linckia* waste biomass and its dried powdered biomass had discoloration ability towards crystal violet and malachite green as a biosorbent. Tsai and Chen), (2010) stated that *Chlorella*-based biomass from algae-manufacturing waste might be used as a low-cost biosorbent for the malachite green .

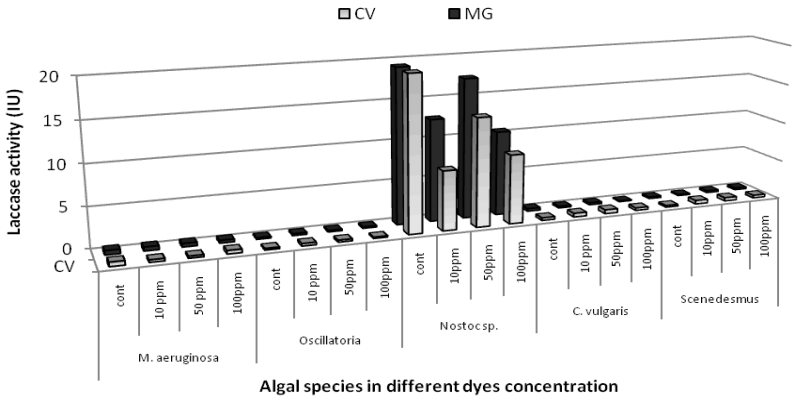

Fig. 3. Laccase enzyme activity in treated and untreated algal cultures with varied concentrations of crystal violet and malachite green.

The effect of CV and MG on the chlorophyll a and phaeophytin-a content in the tested algal species is given in Table 1, the data was expressed as mean of 3 replicates ±SD and the means having different letters are significantly different at $p<0.05$. pheophytin a is a common degradation products of chlorophyll a, can interfere with the determination of chlorophyll a because they absorb light in the same region of the spectrum as does chlorophyll a. Results in Table 1 illustrated that the chlorophyll a content showed significantly different values in the five algal strains treated with CV these differences occurred according to the concentration of dye and also the response of each species. *M. aeruginosa* chlorophyll a content showed significant increase at the two treatments of CV and MG at 10 ppm, giving 1.64± 0.09 and 0.82± 0.04 respectively, while control was 0.70±0.01 phaeophytin a also increased significantly in all conc. of CV and at conc.100ppm of MG (0.97±0.08, while treatment with10 ppm gave 0.67±0.08 which non significantly different from control (0.61±0.02) and 50 ppm decreased significantly to 0.11±0.01.

Nostoc sp. chlorophyll a content showed significant increase at treatments with 10, 50 ppm, while significantly decreased from 0.24±0.01of control to 0.08±0.01 at 100ppm of CV, all MG concentrations treatment increased significantly chl.a. Phaeophytin a showed significantly increase at 50 and 100ppm of CV giving 2.67±0.08 and 1.72±0.04 and also at 50ppm of MG gave 0.88±0.09, while showed significant decrease at the other treatments. Chl- a of *O. geminata* showed significant decrease in all treatments of CV and 10, 50 ppm of MG, while treatment of 100 ppm MG gave significant increase. The phaeophytin content showed significant increase in all concentrations of the two dyes. From the previous presentstion of cyanophytes results it is clear that *O. geminate* was the most sensitive algal species to the two dyes, they cause inhibition in growth measured by decrease in chlorophyll a. Chlorophyll-a content in *C. vulgaris*,

showed significant increase at all treatment except the treatments with 10 ppm CV it gave 0.12±0.05 and at treatment with 50 ppm MG (0.12±0.01). the phaeophytin-a content showed increase in all treatment except 50 ppm CV which significantly decreased from 0.45±0.02 of control to 0.14±0.01. *Scenedesmus* sp. showed significant increase at all treatment except the treatments with 50 ppm MG it decreased significantly from 0.19±0.02 to 0.112±0.01. The phaeophytin-a content showed increase in all treatments. We can showed that *C. vulgaris* and *Scenedesmus* sp. have a high ability to resist the influence of the two dyes on their growth which evaluated by chlorophyll content, and they have a high bioremediation potential in all dyes concentrations. From the previous data we noticed that the ability of algae to decolorize the two dyes depends on the algal species and its ability to resist the impact of dyes on their growth, while the difference in the concentrations of the dye is not significantly affecting the results, these disagree with Acuner and Dilek (2004) stated that the algal growth showed a decreasing trend with increasing dye concentration. In some instances, the initial algal concentration declined to 50% of growth value within the first 20hr. As our results showed that *O. geminate* was the most sensitive algal species to the two dyes; they cause inhibition in growth measured by decrease in chlorophyll-a also Omar (2008) noticed that Tartrazine and diazo dye (Ponceau), affects decolorization capabilities of diatoms and the culture of the diatom *Nitzschia perminuta* was completely died after 2 days of incubation.

TABLE 1. Algal chlorophyll a and phaeophytin a estimations mg/m³ a with crystal violet (CV) and malachite green (MG) after 24 hr of treating.

	Algal strains	Control 0.0	CV conc. (ppm) 10	50	100	MG conc. (ppm) 10	50	100	LSD
Chl a mg/m³	*M.aeruginosa*	0.70± 0.01c	1.64± 0.09e	0.11± 0.01a	0.31± 0.01b	0.82± 0.04d	0.24± 0.12b	0.6± 0.03c	0.05
	Nostoc sp.	0.24± 0.01b	0.41± 0.01c	0.67± 0.08e	0.08± 0.01a	0.4± 0.04 c	0.55± 0.04d	0.42± 0.01c	0.009
	O.geminate	0.47± 0.01e	0.09± 0.02a	0.24± 0.02c	0.15± 0.01b	0.46± 0.01e	0.65± 0.03f	0.28± 0.03d	0.006
	C. vulgaris	0.326± 0.03b	0.12± 0.05a	0.87± 0.01e	0.51± 0.02d	0.5± 0.03 d	0.12± 0.01a	0.45± 0.02c	0.0026
	Scenedesmus sp.	0.19± 0.02 b	0.41± 0.02c	0.51± 0.01d	0.85± 0.05f	0.22± 0.03b	0.112± 0.01a	0.55± 0.03d	0.025
Phaephytin mg/m³	*M.aeruginosa*	0.61± 0.02 b	1.5± 0.03 e	1.49± 0.02e	1.09± 0.08d	0.67± 0.08b	0.11± 0.01 a	0.97± 0.08c	0.03
	Nostoc sp.	0.59± 0.02 c	0.22± 0.01a	2.67± 0.08f	1.72± 0.04e	0.42± 0.01b	0.88± 0.09 d	0.16± 0.01a	0.06
	O. geminate	0.56± 0.04 a	1.72± 0.04de	1.84± 0.05e	1.66± 0.07d	1.42± 0.09c	0.87± 0.09 b	1.99± 0.09f	0.06
	C. vulgaris	0.45± 0.02 b	1.08± 0.06 d	0.14± 0.01a	0.57± 0.01c	0.63± 0.04c	1.12± 0.02de	1.19± 0.1 e	0.04
	Scenedesmus sp.	0.14± 0.01 a	0.38±0 .01 d	0.21± 0.02b	0.37± 0.01d	0.26± 0.004c	0.47± 0.04 e	0.5± 0.003f	0.008

Means with the same letters are not significant.

It seems reasonable to conclude that the decolorization process of dyes by algae had been done by different mechanisms; one of the algae mechanisms might be engaged to enzymatic degradation, such as *Nostoc* sp which showed high laccase activity. All decolorization abilities, however, of the tested algal strains against CV and MG exhibited a noticeable increase after 24 hr incubation compared to 2 hr. We can showed that *Chlorella* and *Scenedesmus* have a high ability to resist the influence of the two dyes and continue to increase growth and decolorize their colors, treatment of fish farms waste water with these species might be help in reusing of this water in agricultural irrigation.

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المعالجة البيولوجية لأصباغ الكريستال البنفسجى و الأخضر الملكيت باستخدام بعض أنواع الطحالب

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أجريت المعالجة البيولوجية لصبغتي الكريستال البنفسجي والأخضر الملكيت باستخدام الطحالب الخضراء المزرقة (النوستوك، الاوسيلاتوريا والميكروسيستس) والطحالب الخضراء (الكلوريلا و السيندسمس) من أجل تقييم قدرة هذه الطحالب على إزالة لون الصبغتين بعد فترة حضانة ساعتين و٢٤ ساعة في ثلاثة تركيزات لهذه الصبغات وهى ١٠ و ٥٠ و ١٠٠ جزء في المليون . وقد حقق طحلب الكلوريلا أعلى نسبة إزالة للون الكريستال البنفسجي بعد ساعتين عند ١٠ جزء في المليون ،أما بعد ٢٤ ساعة فقد أعطى السنديسمس أعلى نسبة إزالة عند ١٠ جزء في المليون . أما أعلى نسبة إزالة للون الأخضر الملكيت بعد ساعتين باستخدام الكلوريلا في ٥٠ جزء في المليون ،أما بعد ٢٤ ساعة كانت أعلى معدلات إزالة للون الأخضر الملكيت بواسطة الميكروسيتس عند تركيز ١٠٠ جزء في المليون، يلى ذلك السيندسمس المعامل بنفس التركيز. ولقد أظهرت الطحالب الخضراء قدرة عالية على إزالة الكريستال البنفسجى أكثر من الطحالب الخضراء المزرقة. وقد دل قياس نشاط الإنزيمات الحيوية ؛ البيروكسيديزوانزيم التيروزينيزوإنزيم اللا كيز على أن طحلب النوستوك له أعلى نشاط لانزيم اللا كيز في كل التركيزات للصبغتين فى حين أن الإنزيمين الآخرين لم يسجلا أى نشاط. وأظهرت قيم الكلوروفيل أ و الفيوفيتين االتي تم الحصول عليها فروقا معنوية بين معظم المعاملات و كانت الاوسيلاتوريا أكثرالأنواع تضرراً حيث أظهرت انخفاضاً في محتوى الكلوروفيل أ في تركيزات الصبغتين والخلاصة إن عملية إزالة لون الصبغتين يتم بواسطة آليات مختلفة فى الطحالب واحد من هذه الاليات هوالتكسير بواسطة انزيم اللاكيز مثل النوستوك وأن طحلب الكلوريلا والسيندسمس لديهم قدرة عالية على إزالة لون هاتين الصبغتين بحيث يمكن استخدامهما فى معالجة المياة المستعملة فى المزارع السمكية والتى تحتوى على هذه الصبغات المسرطنة كعوامل مضادة للفطريات.