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Determination of *Anabaena oryzae* Survival by Chlorophyll-a and Carotenoid under Using Treated Sewage Water

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ABSTRACT

In this study, the impact of treated sewage water was tested using 6 isolates of *Anabaena oryzae* by determination of Chlorophyll-a, Carotenoids and dry weight. In this study, treated sewage water was utilized to grow *Anabaena oryzae* for 6 weeks compared with *Anabaena oryzae* grow in BG-11 Medium as a control. The results showed the sewage water effects and correlation on Chlorophyll-a, Carotenoids contents and dry weight, As a result adaptations, resistance to organic pollutants and nutrients showed that *Anabaena oryzae* able to survive because these isolates modification with growth condition. The results by Gel electrophoresis showed genetic variations among *Anabaena oryzae* isolates DNA by using OPC-01 random primer.

INTRODUCTION

Cyanolichens set cyanobacteria and their targets for gene expression and metabolic adaptation changing light, environmental factors, and circadian status were studied. (Schroeter, 1994).

The cyanobacteria, photosynthetic system is strongly linked to other main metabolic pathways and itself is a main metabolic sink for carbon skeletons iron and nitrogen. Therefore chlorophyll fluorescence pointer can enhance, continuous data information on photosynthesis and all adaptation states of cyanobacteria. (Leisner *et. al*., 1997)

Prokaryotic organism cyanobacteria are metabolize, with several key structural and metabolic singularity whom highly affect the nature and performance of their fluorescence signals distinguishing features of fluorescence signals of cyanobacterial resulted from these photosynthesis/ respiration interactions, including their special method of nonphotochemical and photochemical of cyanobacterial predominate. Regulation and role, photosynthesis lead to possibility and determination for chlorophyll fluorescence analysis of cyanobacteria. (Douglas *et al.,* 1998).

Cyanobacterial determination with microscopic examination of a sample is very useful even when exact counting was not being done. The obtained data regarding to the cyanobacteria determination can observation that hurtful cyanotoxins existing. This facts can determine the elect of analytical or bioassay technique suitable for define toxin levels (Chapter 13 WHO 1999).

Blue-green algae, cyanobacteria are photosynthetic bacteria present in most marine systems and. fresh water. Chlorophyll-a concentration is one of the most used variants in Limnology to define the phytoplanktonic collection biomass, to describe environments, in experiental works and even in observation scheme for the study of aqueous ecosystem regulation (DOS *et. al.,* 2003).

Kaushik and Murti (1981) Found that the reduction in alkaline pH with inoculation by Cyanobacteria from 9.5 to 7.6 is very important in the bioremediation process of alkaline soil.

The physiological, characteristics of algae, such as active photosynthesis, the ability to directly utilize nitrogen and phosphorus ions and other biogenic elements, and to withstand the high concentrations of toxic substances and metabolites, permits using recirculating aquaculture systems (RAS) waste water of various origin as culture media (Makarova *et al*. 2009, Woertz *et al*. 2009)

The increase of *Cyanosarcina fontana* has confirming the use of wastewater as a development medium than *Anabaena oryzae* compared to standard medium (Fawz and Issa 2016).

Chlorophyll content differs depending on the cell physiological state and the species, possible damage to the photosystems a change in the pigment pattern. The switch of Chl-a to possible damage to the photosystems (Sakshaug, 1981).

Chlorophyll extraction with acetone is the better method if there is no information on the structure of the sample, in addition to protect the chlorophyll products from increase degradation (Prezz and Bate, 1991).For the limitation of chlorophyll and pheopigments the concentration was limitation by the traumatic process in comparison with the ones by fluorimetry and after chromatography. The variation between the concentrations calculated with Lorenzen and tricromatic equations are correlated positively with the pheophytin concentration (Arar 1997a).

Liquid chromatography is wide recent developed method for other vegetal pigments and chlorophyll-a determination, while the first examine is nearly twenty years old. However the cost of the apparatus and time are disadvantages in the use of this method. (Arar, 1997b).

The damage in the phoytosystems causes a change in the pattern of pigment. Switch from Chl a causes uses more Chl b. This is depended on indication of stress and associated to Microcystin-LR (MC-LR) exposure (Stephan 2002). *Anabaena oryzae* tolerate Cu at 1 ppm and on the eightth day gave most carotenoid concentration (6.652 µg mL-1) in *A. oryzae*. The Hg treated cells showed lethality at (1, 10 and 100 ppm). There was a gradual rise of carotenoid after twelve days, essentially at (1 and 10 ppm) of Co^{2+} and Zn^{2+} in *Anabaena oryzae*. This indicated the chance of application of this species for removing toxic of effluents (Chakilam 2012).The content of carotenoids is regularly relevant to the cell stress level (Tomáš *et. al.*, 2015).

The present work aims to determine and compare the survival of *Anabaena oryzae* that can fix nitrogen and grow in waste water by chlorophyll, carotenoids determined by the spectrophotometer, and dry weight.Different isolates of *Anabaena oryzae* isolated from different freshwater samples from Alex and Sadat city under BG-11 medium and Sewage water. Also,this work intends to compare the genetic variation of *Anabaena oryzae* isolates used in this study.

MATERIALS AND METHODS

Sample of sewage water

Treated sewage water sample was collected from Sadat City. The collected sample was transferred into a sterile glass container.

BG-11 Medium

(Robert 2005) the medium contains/litre: $K_2HPO_4$0.04 g, $NaNO_3$1.5 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, Citric acid 0.006 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, Ferric ammonium citrate 0.006 g, $NaCO_3$0.001 g and 0.02 g, EDTA (disodium salt) Trace metal mix A5 1.0 ml, pH 7.1. Distilled water up to1.0 L

Trace Metal Mix A5/L:

The mix of trace metal A5 contains/litre H_3BO_3 2.86 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $NaMoO_4 \cdot 2H_2O$0.39 g, $Co(NO_3)_2 \cdot 6H_2O$49.4 mg, $CuSO_4 \cdot 5H_2O$ 0.079 g, Distilled water used up to 1.0 L

Sterilization

Anabaena oryzae isolated from pure Freshwater stored in light from Alex isolates (1, 2. 3) and Sadat city isolates (4, 5, 6).

***Anabaena oryzae*:**

Cultures were cultivated in 1000-mL flasks that were provided with 500 ml of suspension (BG-11 Medium or sewage water) and closed with a cotton plug and sub cultured at regular 6 weeks the cell sterilized by hypochlorite to geta pure culture. Erlenmeyer flasks were incubated at chamber temperature (25 ± 1 °C) on regulor shaking at 50 rpm, under normal day and night light.

Cyanobacteria Detection in Water

Cyanobacteria detected in water using the method of algae plate growth is to take samples for microscopic identification test and morphology examination (www.turnerdesigns.com).

***Anabaena oryzae* dry weightlimitation**

A definite volume (1/2 L) of each *Anabaena oryzae* suspension centrifuged to obtain there maining pellet, washed with distilled water and dried at 60 °C till constant weight. The data were given as (dry weight) g/L (suspension).

Chlorophyll and Carotenoids extraction of *Anabaena oryzae*:

The procedures for extracting chlorophyll from *Anabaena oryzae* are as follows. Add A definite weight of *Anabaena oryzae* fresh pellet o.1 g closed sample containing 10 ml of solvent (80% cold acetone. The extraction carried out by using ultrasonic for 3 min,centrifugation at 5000 rpm for 15 min, To clear the chlorophyll solution, the supernatant poured into a new glass tube The final volume of each supernatant was then adjusted to 6 ml by adding the 80% cold acetone before detection by spectrophotometer in glass cuvette.1 ml for the chlorophyll determination sample absorbance were measured at wavelengths (450A, 645A and 663A), chlo A= 12.7 (A663) - 2.69(A645). Carotenoids=A450 (Fd)/135310 , [(d = 1cm the width of the cuvette (usually 1 cm). and F a dilution factor].By spectrophotometer Ultraviolet (visable UV 200-R). JYH et. al.,(2002) with modification.

Genetic variation among *Anabaena oryzae* isolates

Genomic DNA extraction

Procedures of G^- bacteria DNA extraction from *Anabaena oryzae* isolates were, according to DNA extraction mini kit Intron Biotechnology Cat. No (17361).

RAPD analysis

RAPD PCR was Utilizing OPC-01 random primer (TTCGAGCCAG) the polymerase chainreaction (PCR) with a mixture total volume of 25 µl (1 µl of primer (10uM), 0.5µl of Taq DNA Polymerase, MgCl2 1µl, 50ng of genomic DNA1µl, PCR buffer 10X 2.5 µl , dNTPs 2µl (2.5 mM) and ddH_2O up to 25µl. PCR condition was settled for 35 cycles after initial denaturation for 3 min at 94°C. Each cycle of PCR consisted of denaturation at the 94 °C for 1 min.; annealing at 36 °C for 1 min., extension at 72 °C for 2 min and a final extension step at 72 °C for 10 min. PCR products were separated on 1% agarose gel and photographed.

Statistical analyses.

The results variance were analysis. Means of three replicates among treatments were determined using LSD test at 5%by ANOVA Completely Randomized, Duncan's Multiple rangeTest.

RESULTS AND DISCUSSION

Anabaena oryzae microscope identification test as a final examination

The microscope identification test showed the morphology of *Anabaena oryzae* of 6 isolates used in this study as shown in figure(1).Chlorophyll and Carotenoids extraction of *Anabaena oryzae*. The extraction chlorophyll and Carotenoids from *Anabaena oryzae* isolates are a clear supernatant (Figure 2).

Effect of sewage water on chlorophyll-a

The results presented in (Table 1) and (Figure3) showed chlorophyll-a content *in Anabaena oryzae* after 6 weeks from the growth. The maximum chlorophyll-a content *Anabaena oryzae* were 184.4 and 129.8 mg /g in treated isolate 2 and normal isolate 1 respectively. This effect might be attributed to adjustments of the bacteria under the growth condition.

The results according to the table (1) showed that the difference between the treated isolates is significant, the difference between the normal isolates is more significant, the differences between the normal isolates and treated isolates were highly significant.

The chlorophyll a amount is connected with the cell's physiological case (Sigleo *et al*., 2000).

Chlorophyll a only is present in cyanobacteria.Chlorophyll a predominante in the higher Cyanobacteria, maximum of the existing pigments are in the form of chlorophyll a (DOS *et al.,* 2003).The waste water from recirculating aquaculture systems (RAS) is rich in nutrients, particularly in various forms of nitrogen, and can be considered as a culture medium. The possibility of cultivating the blue-green algae *Anabaena hassalii* in RAS waste water has been demonstrated (Cheban *et. al*., 2014).

Table1. Effect of sewage water on Chlorophyll-a (mg/g) of *Anabaena oryzae*

No. of samples	1	2	3	4	5	6	Mean
Normal	129.8	58.9	57.4	79.4	57.1	39.6	70.37 X
Treated	42.3	184.4	60.4	58.1	32.6	29.5	67.88 Y
Mean	86.07 B	121.65 A	58.58 D	68.78 C	44, 86 E	34.53 F	69.13

The values are the mean of 3 replicates
Values connected with the same letter are not significantly different
The LSD (0.5) = 2.07 between the isolates . The LSD (0.5) = 3.59 between the normal isolates and treated isolates

Effect of sewage water on carotenoids content in *Anabaena oryzae*:

The results in (Table 2) and (Figure 4) showed that the carotenoids content of *Anabaena oryzae* after 6 weeks growth. The highest content of carotenoids were 21.1 and 20.2 in isolate2 treated and normal isolate 1 respectively. This effect may refer to the resistance and adaptations to organic pollutants.

Table 2. Effect of sewage water on Carotenoids (mg/g) of *Anabaena oryzae*

No. of samples	1	2	3	4	5	6	Mean
Normal	20.2	14.3	13.3	19.3	13.1	12.4	15.43 X
Treated	13.1	21.1	14. 8	14. 1	11. 6	11. 2	14.32 Y
Mean	16.62 B	17.90 A	13.96 C	16.7 B	12.75 D	11.8 E	14.95

The values are the mean of 3 replicates
Values connected with the same letter are not significantly different
The LSD (0.5) = 0.039 between the isolates . The LSD (0.5) = 0.67 between the normal isolates and treated isolates

According to the results obtained from table (2) the differences between the treated isolates were highly significant, the differences between the normal isolates were highly significant, the differences between the normal isolates and treated isolates were highly significant.

Each type of algae contains a complex of pigments in differing ratios. Carotenoids (astaxanthin, keto-carotenoids canthaxanthin) (Pirastru *et al.*2012) and chlorophyll a and b (Sanchez *et al.* 2008).

Effect of sewage water on dry weight of *Anabaena oryzae*:

The results in (Table 3) and (Figure 5) showed that the effect of sewage (pH 7.8) on the dry weight of *Anabaena oryzae*. The results indicated that the constant dry weight of *Anabaena oryzae* after 6 weeks from the growth. The high constant dry weight were1.40, 1.28 and 20.2 in isolate2 treated and normal isolates 1 and 4 respectively. This effect may refer to modification, nutrients of *Anabaena oryzae* to able to survive in growth condition.

Table 3. Effect of sewage water on dry weight g/L of *Anabaena oryzae*

No. of samples	1	2	3	4	5	6
Normal	1.28	0.84	0.74	1.12	0.70	0.66
Treated	0.68	1.40	0.98	0.78	0.64	0.58
Mean	1.12 A	0.98 B	0.95 B	0.83 C	0.67 D	0.62 D

The values are the mean of 3 replicates Values connected with the same letter are not significantly different
The LSD (0.5) = 0.061 between the isolates . The LSD (0.5) = 0.105 between the normal isolates and treated isolates

The result in table (3) Showed that the differences between the treated isolates were non significant, the differences between the normal isolates were highly significant, the differences between the normal isolates and treated isolates were highly significant.

From the results obtained the correlation between dry weight and carotenoids were (0.94). The correlation between dry weight and Chlorophyll-a was (0.89). The correlation between carotenoids and Chlorophyll-a was (0.91).

Cyanobacteria capability of fixing N2 and their resistance to desiccation play a key role in nutrient cycle (Potts 1999).

The algae can increase in presence of organic pollutants, thus, they can be applied as a favorable microorganism able of biodegrading and biotransforming aromatic pollutants that are ordinarily found in the environment. Algae can improve the removal of these pollutants from the environment (Chaillan *et al*., 2004).

Samples of effluent were taken from oxidation ponds of Alexandria and Sadat City and estimate for different physicochemical parameters like Electrical conductivity (E.C) (2.74, 4.53), pH (7.58, 7.78) osmotic pressure (98.64 %, 163.08 %) concentrations of salts mg /L (1753.6, 2899.2), COD (94,. 65) and BOD (35, 65) The pH was 7.78 and 7.58, respectively compared with tap water (control) pH from Alexandria and Sadat City 7.2-7.4 , respectively.The concentrations of metals in the effluent samples were determined by Atomic Absorption Spectrophotometer (AAS). whereas the highest amount of Zn > Fe > Cu > Pb > Mn and Cd are represent in SadatCity Alexandria were Zn > Fe > Pb > Cu > Mn > Cd. The concentration of Pb is higher in Alexandria than Sadat City (Hanan and Ragaa 2014).

Cultivating *Desmodesmus armatus* in recirculating aquaculture systems (RAS) waste water permits obtaining acitive growing cultures characterized by constant biomass growth and high contents of total proteins, complex proteinogenic amino acids, and the main photosynthetic pigments. At the same time, the effectiveness of cultivation in RAS waste water in not significantly different from that in artificial Fitzgerald's medium. Using RAS waste water as a culture medium, helps to reduce significantly the costs of algal mass biotechnology and it cleans the water of nutrients (Larisa *et al*. 2015).Genetic variations among *Anabaena oryzae* using random primer.

Organisms differ from one to another, and therefore show different reactions to treatments. The

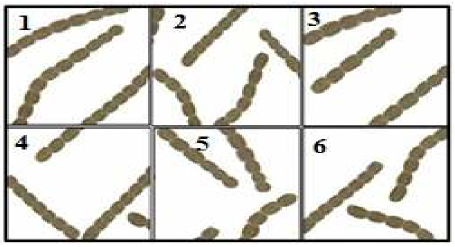

Fig. 1. photomicrographs of *Anabaena oryzae* isolates

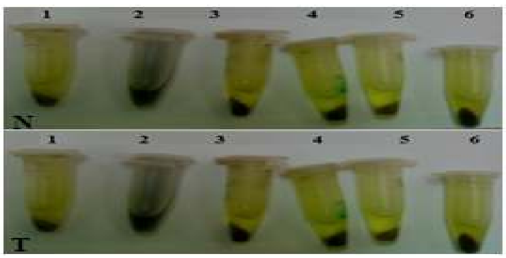

Fig. 2. *Anabaena oryzae* isolates contain a complex of pigments in differing ratios. Chlorophyll and Carotenoids. Normal (N) and Treated (T).

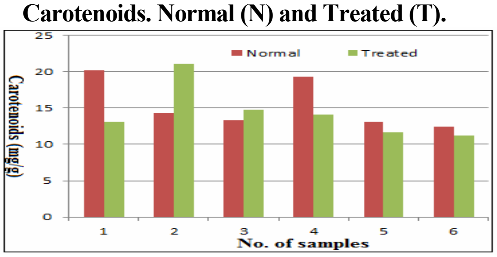

Fig. 3. Effect of sewage water on Chlorophyll-a of *Anabaena oryzae*

genetic variations among *Anabaena oryzae*. RAPD analysis was carried out using Opc-01 random primer. The number of producing bands per isolates varied between 4 and 8 bands. No genetic similarity found at the base of their bands in RAPD that demonstrated there is no genetic similarity between the isolates used in this study. (Figure 6).

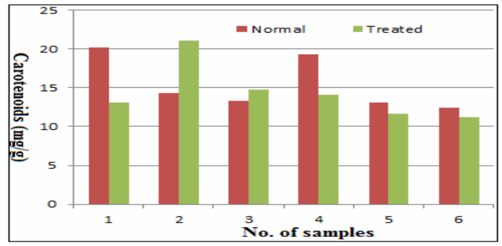

Fig. 4. Effect of sewage water on Carotenoids of *Anabaena oryzae*

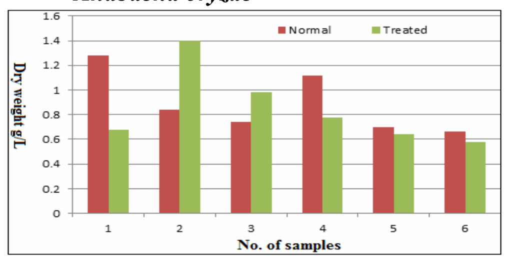

Fig. 5. Effect of sewage water on dry weight of *Anabaena oryzae*

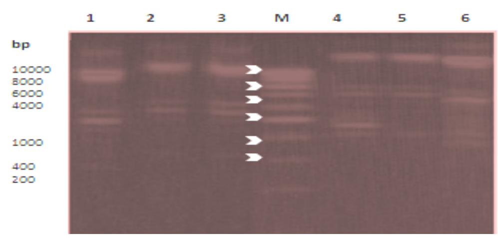

Fig. 6. genetic variations between *Anabaena oryzae* using OPC-01 random primer by gel electrophoresis.

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دراسة تحديد حيوية سيانو بكتريا أنابينا أوريزاى التى تنمو فى مياه الصرف الصحي المعالجة باستخدام دليل الكلوروفيل-أ و كاروتينات

حنان حسن احمد و عادل كمال عقبة
قسم البيوتكنولوجيا الميكروبية – معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية – جامعة مدينة السادات

تم دراسة تاثير مياة الصرف المعالجة على حيوية طحالب (سيانو بكتريا) انابينيا اوريزا باستخدام دليل الكلوروفيل (أ) ,الكاروتينات والوزن الجاف . وتم دراسة الاختلافات الوراثية بين العزلات.فى هذة الدراسة اختبر تاثير مياة الصرف المعالج على حيوية عزلات انابينيا اوريز بتقدير الكلوروفيل(أ) ,الكاروتينات والوزن الجاف وذلك بتنمية العزلات فى بيئة النمو مقارنة بنموها فى مياة الصرف المعالج لمدة ستة اسابيع. وقد اوضحت النتائج وجود علاقة ارتباط قوية بين تاثير مياة الصرف المعالج ومحتوى الكلوروفيل (أ) والكاروتينات بالاضافة الى الوزن الجاف. كما بينت دراسة التفريد الكهربى للحمض النووى لهذة العزلات وجود اختلافات بين العزلات المدروسة باستخدام البادئ العشوائى Opc-01.