Lipid production and molecular studies of *Anabaena torulosa* treated with different types of stress

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Abstract:

Lipid production in terms of oil percentage and fatty acid composition of *Anabaena torulosa* which was identified morphologically and genetically was studied to determine its ability for biodiesel production, in response to salinity, nitrogen and phosphorus starvation stress; salinity ranging from 50 to 250mM NaCl, nitrogen and phosphorus regime (50%, NO_3-N , 50% PO_4-P of BG11 media). The results indicated that there was change in physiological behavior under these different stresses and illustration of these differences genetically. The results of oil analysis revealed that the lipid production increased when nitrogen and phosphorus decrease (68.83% and 160.97% respectively) and 200mM of NaCl increase oil content by 120%. GC/MS analysis of fatty acid composition revealed that methyl linoleate is the main constituent of fatty acids ester 57.9% which classified as a biodiesel due to its long methyl ester chain and is also used as a fuel in standard diesel engines. All these results explained genetically by using RAPD technique which revealed new bands appeared and other bands disappeared. DNA sequence was changed leading to DNA polymorphism 41.25%. Also, the DNA pattern indicated that stress of nitrogen and phosphorus were more related to control, while the other stresses (salinity and combined stresses) caused more changes comparing to control. So that stress changes in physiology was confirmed genetically.

Keywords: *Anabaena torulosa*, lipid, fatty acids, salinity, RAPD.

Introduction

Many scientists in recent years turn their attention to the use of cyanobacteria as excellent organisms for renewable biodiesel production, it has high oil yield, and easily grown in non-arable land. Because the production of biodiesel depends primarily on the amount of oils found in these microorganisms, so that; the scientists went to study the factors that affect the production of oils.

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There are a lot of stress conditions that affect the oil production efficiency of the algal cells by increasing their oil content which includes; nitrogen and phosphorus starvation, high salinity stress and light wave lengths stress. A lot of studies carried out with such techniques in microalgae (**Hu *et al*., 2008; Salem *et al*., 2013;Elsayed and Salem, 2016**). **Nirupam *et al*.** (**2012**) showed that cyanobacteria; such as *Lyngbya majuscula*, *Phormidium valderianum*, *Synechocystis pevalekii* and chlorophytes such as *Chlorella vulgaris*, *Rhizoclonium riparium*, *Rhizoclonium africanum*, *Spirogyra orientalis* and *Cladophora crystallina*, having suitable fatty acid composition were identified for biodiesel production.

Salehzadeh and Naeemi (2017) proved that *Anabaena* is one of many promising species of Cyanobacteria which produced a mixture of saturated and unsaturated fatty acids that exceeded 40% of the weight of dry biomass, where Palmitic acid was defined after GC-Mass analysis of oil extracted from the *A. variabilis*, as a fatty acid that can be used as biodiesel. Nevertheless, nitrogen stress might not always promote lipid induction but can result in change in lipid composition as well with accumulation of fatty acids (**Rodolfi *et al*., 2009; *Tang et al*., 2011**).

Most species belonging to Cyanobacteria have long been known for their taxonomic complexity that basically depends on the morphological data. Hence, several generic lineages needed to be re-circumscribed to promote the enhancement of poor informative morphological data with molecular based data. Use of molecular markers for identification of cyanobacteria in diverse has gained considerable significance. Molecular datasets can effectively complement morphological characterization. However, **Wacklin *et al.* (2009)** and **Chio*et al.* (2018)** suggested classification of *Dolichospermum hangangense* using 16SrRNA phylogenetic analysis which confirmed morphology-based classification.

Other molecular markers such as restriction fragment length polymorphism (RFLP) and 16SrRNA to characterize *Anabaena* strains (**Prasanna*et al*., 2006**). Random amplified polymorphic DNA (RAPD) has also been used to differentiate among different Cyanobacteria species including *Anabaena* and *Nostoc* (**Perumal*et al*., 2009; Chakdar and Pabbi, 2017**). These molecular techniques have been employed in phylogenetic analysis and characterization of cyanobacterial diversity. The presence of highly conserved repetitive sequences in

the genomes of microorganisms makes them highly useful for strain differentiation and diversity analysis.

RAPD-PCR is a PCR based technique for identifying genetic variation by using single arbitrary primer in a PCR reaction resulting in the amplification of many discrete DNA products. This procedure detects nucleotide sequence polymorphism in a DNA amplification based assay using a single primer of arbitrary nucleotide sequence (**Okore*et al*., 2017**).

In the present study, molecular analyses were performed with 16SrRNA and 16S-23S ITS sequences to conform the morphological identification of *A. torulosa* obtained from Dr. Olfat M.A Salem. (Phycology lab - Helwan University) and also the present study aimed to assess the effects of nitrogen was and phosphorus deficiency and salinity stress on lipid production and their corresponding fatty acid profiling of *Anabaena*, finally we assess the effect of these types of stresses on genetic markers of the species using RAPD.

Materials and Methods

1. Organism and growth conditions

Anabaena torulosa isolated from Wadi El-Natron (longitudes 30° 18' 50" E and latitudes 30° 24' 10" N) was purified by algae lab, Faculty of Science, Helwan University. It was identified morphologically by light microscopy (Olympus IX, Tokyo, Japan), and the optimization growth conditioned were examined; It was cultivated in BG11 medium (**Allen, 1968**). For the production of biomass, exponentially growing algae culture was transferred into fresh sterile medium [10% (v/v) of inoculums]. Cultures were illuminated by tubular fluorescent lamps with light intensity of 25 µmol photons m^{-2} s^{-1}. Examination of the species growth optimization takes place as follow; pH intervals from 4,5,6,7,8,9 to identify the optimum initial pH, photoperiod (light/ dark) intervals (continuous light, 12h:12h, 16:8), the different growth temperatures (15, 20, 25, 30, 35◦C). The optimum incubation period was examined by measuring the organism growth every 3 days for 30 days. All experiments mentioned above were performed in triplicates using 1 L Erlenmeyer flasks. The growth of the organism was determined by dry weight estimation.

2. Stress experiments

The experiments were designed by inoculation of *A. torulosa* in BG11medium with optimized growth condition (as mentioned above) in Three sterile Erlenmeyer flasks (500 ml) each containing 200 ml medium treated as follow: strain cultured in BG11 medium (control), three types of stress were selected; BG11 media with 50%$NaNO_3$, media with 50%$K_2HPO_4.3H_2O$, Salinity stress with different concentrations of NaCl (50, 100, 150, 200 and 250mM) and finally the combined stress(media with 50% N, 50% P and 200 mM NaCl).

3. Oil extraction and analysis

The oil extraction takes place according to **Halim *et al*. (2011)**. A mixture of n-hexane and isopropanol (3:2) was added to 4 g of dried *A. torulosa* powder. The extraction mixtures were agitated at 800 rpm in shaking incubator (LSI-30 16/LSI-30 16R) for 7.5 hs. Cell residue was removed by filtering through Whatman GF/C paper. The filtrate was transferred into a separating funnel and sufficient hexane and water (approximately 40 ml each) were added to induce biphasic layering. After settling, the solvent mixture was partitioned into two distinct phases: a top hexane layer containing most of the extracted lipids and a bottom isopropanol layer containing most of the co-extracted non-lipid contaminants. The hexane phase was collected to enable gravimetric quantification of the lipid extract. The most effective stress treatment and the control were analyzed by GC/MS.

4. GC/MS fatty acid composition analysis:

Preparation of fatty acids methyl esters takes place according to **Rozes *et al*. (1993)**; 100 µl of oil sample were added in the test tube and dissolved in 0.5 ml hexane, and then sodium methoxide (0.5 ml) was added and mixed for 1 min. The upper phase (hexane layer) was removed and injected into the chromatography. The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m × 0.25 mm internal diameter and 0.25 µm film thickness). Analyses were carried out using helium as the carrier gas at a

flow rate of 1.0 ml/min at a split ratio of 1:10, injection volume of 1 µl and the following temperature program: 50 °C for 1 min; rising at 20 °C /min to 200 °C and held for 5 min; rising at 3 °C/min to 230 °C and held for 23 min. The injector and detector were held at 250°C. Mass spectra were obtained by electron ionization (EI) at 70 eV and using a spectral range of m/z 20-550 and solvent delay 1.8 min. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

5. DNA extraction and sequencing.

1. **Genomic DNA extraction:-**

Genomic DNA was extracted according to **Rogers and Bendish, 1998** from 0.5 g dried algal biomass by using CTAB method after minor modifications (incubation 40 min instead of one hour). The quantity of DNA was estimated by comparison with known standards in ethidium bromide stained 0.8% agarose gels.

2. **Identification using amplification of 16S rRNA gene of *Anabaena***

16S rRNA gene fragment was amplified with universal primers E528F (5′ -CGGTAATTCCAGCTCC-3′) and Univ1517 (5′ -ACGGCTACCTTGTTACGACTT-3′) (**Lyra*et al*., 1997; Chakdar and Pabbi, 2017**). The polymerase chain reaction was carried out in a final volume of 25 µl containing 2X RED master mix, 16S primers (FD1 and RP2) 2.5 pmol each and 50 ng of genomic DNA. Amplification was carried out in Biometrathermocycler programmed for initial denaturation (94^{o} C for5 min) followed by 35 cycles, composed of denaturation (94^{o} C for 30 s), primer annealing (64^{o} C for 45 s), extension (72^{o} C for 2 min) followed by a final extension of 7 min at 72^{o} C and subsequent cooling at 4 ^{o}C temperature.

Amplified PCR product was separated along with a molecular weight marker (GeneRuler, 1 kb, Fermentas, USA) by electrophoresis on 1.2% agarose (Vivantis, USA) gel run in 1X TBE (Tris–Borate–EDTA) buffer, stained with ethidium bromide for a period of 1 h at 100 V. These were visualized under UV light and gel photographs using VilberLourmat Gel

Documentation System. The amplification product sizes were estimated using BIO-RAD Quantitiy One software.

The DNA band was extracted using a QIA quick Gel Extraction Kit following the manufacture instructions. The product was sequenced in GATC Company for sequencing which uses the ABI 3730xl DNA sequencer by using forward and reverse sequences, by combining the traditional Sanger with the new 454 technology. A nucleotide blast search was performed with the sequences using sequence alignment by NCBI blast and Geneious. Pro.v4.8.4 software.

3. RAPD-PCR and electrophoretic analysis

This molecular technique was performed for *Anabaena* treated with different treatments comparing with control. The RAPD-PCR assay was carried out at Genetic laboratory, Botany and Microbiology Department, Faculty of Science, Helwan University. The assay was carried out using 4 RAPD oligo nucleotide primers (OPA-11, OPA-13, CRA-22 and Hip-TG). The standard, optimized PCR was performed in a total volume of 25 µl containing 1 µl of each primer, 50 µg/ml of DNA and 12.5 µl of 2X RED Taq PCR master mix. This bioassay was performed using Biometrathermocycler. PCR programing was as follow: initial denaturation (94^{o} C for 5 min) followed by 40 cycles, of denaturation (94^{o} C for 30 s), primer annealing (32-34^{o} C for 45 s according to the primer), extension (72^{o} C for 1 min) followed by a final extension of 10 min at 72^{o} C and cooling at 4^{o} C temperature. The product was examined on 1.2% Agarose gel electrophoresis with ethidium bromide. After running, the gels were photographed under UV radiation in gel documentation system.

For amplification of the 16 min. whole genomic DNA was extracted using the MoBio Ultra Clean Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA bands were examined on a 1% agarose gel run at 100 volts for 40-23S rRNA gene product, previously published primer pairs were E528F (5′-CGGTAATTCCAGCTCC-3) Univ1517 (5′ - GCTACCTTGTTACGACTT-3′) (**Lyra *et al.*, 1997** and **Chakdar and**

Pabbi, 2017). Genomic DNA was extracted according to **Rogers and Bendish, 1998** from 0.5 g dried algal biomass by using CTAB method after minor modifications (incubation 40 min instead of one hour). The quantity of DNA was estimated by comparison with known standards in ethidium bromide stained 0.8% agarose gels.

6. Statistical analysis

Hierarchical cluster analysis was made by the complete linkage method with genetic distance measure for evaluating the relatives among the different studied treated *Anabeana*. The data was analyzed using Bio Rad Quantity One (**Shuaib *et al*., 2007**).

Results

Anabaena torulosa was cultured in BG 11 media. The optimizing condition experiments indicated that optimum pH was 7.0 and the optimum incubation period was 27 days at 25 ±1°C, 18/6 light/dark cycle with light intensity of 25 µmol photons m^{-2} s^{-1}. The oil content of cells grow in the control and treated cultures was measured, and the oil percentage gave some changes from control as shown in Table (1) phosphorus stress (T2) showed the highest percentage of increase in oil percentage 160.97%, followed by salinity stress with 200mM NaCl (120.86%), followed by 250mM NaCl (83.58%), and finally nitrogen stress giving 68.83% increase in oil, The combined stress (T8) had 44.1%

The composition and distribution of fatty acidsanalyzed by GC-MS in *A. torulosa* grown in BG11 medium (control) and treated with 50% K_2HPO_4 (The most effective stress treatment)are represented in Table (2); most fatty acids were unsaturated, although saturated fatty acids were detected as well. As can be seen, linoleic acid was the major fatty acid in the lipids extracted; the methyl linoleate represent 57.09 % in *A. torulosa* cultured in control media and 56.73% in stress medium. Elaidic acid methyl ester (isomer of oleic acid) followed linoleate and record 23.91% in control and 23.53% in treated organism.

Table (1): The oil percentage of *A. torulosa* cultured in BG11 medium under different stress conditions

	Treatments	Oil Percentage (mean± SD)	Percentage of increase
BG11 media	Control	15.53±0.11	-
Nitrogen stress	T1 (50%$NaNO_3$)	26.22±0.24	68.83
Phosphorus stress	T2 (50%$K_2HPO_4.3H_2O$)	40.53±0.25	160.97
Salinity stress mM NaCl	T3 (50)	17.19±0.41	10.68
	T4 (100)	19.62±0.25	26.33
	T5 (150)	22.93±0.14	47.64
	T6 (200)	34.3±0.01	120.86
	T7 (250)	28.51±0.26	83.58
Combined stress	T8 (50% N and 50%P and 200mM NaCl)	22.38±0.25	44.1

Table (2): Fatty acid profile of *A. torulosa* cultured in BG11 medium (control) and treated with 50% K_2HPO_4

Type of fatty acid	Fatty Acid Name	Lipid number	Common name	Formula	Area percentage	
					control	50% K_2HPO_4
Saturated	Hexadecanoic acid, methyl ester	C16:0	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	14.31	15
	Octadecanoic acid, methyl ester	C18:0	Stearic acid methyl ester	$C_{19}H_{38}O_2$	3.79	3.89
Poly Unsaturated	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	C18:2	Linoleic acid methyl ester	$C_{19}H_{34}O_2$	57.09	56.73
Mono Unsaturated	9-Octadecenoic acid, methyl ester, (E)-	C18:1 (n-9)	Elaidic acid methyl ester	$C_{19}H_{36}O_2$	23.91	23.53
Mono Unsaturated	9-Octadecenoic acid (Z)-, methyl ester	C18:1(n-9)	Oleic acid methyl ester	$C_{19}H_{36}O_2$	0.91	0.85

1. Identification using amplification of 16SrRNA gene of *Anabaena*

The results of prokaryotic identification using 16SrRNA showed that there is a single band at 515 bp. This band was sequenced and indicated that the sample was *A. torulosa*. The percent of similarity was 98% comparing to the data on NCBI.

2. RAPD-PCR and electrophoretic analysis

A total of 19 bands were amplified among four different treated *A .torulosa* compared to control by using four RAPD primers (OPA-11, OPA-13, CRA-22 and Hip-TG). Table (3) indicated the sequence, melting temperature (Tm) and GC% which characterized the used four primers.

Table (3) Codes, sequences, Ta and GC% for the four random primers used in RAPD- PCR analysis

No.	Primer name	Primer sequence	Ta	GC%
1	OPA-11	CAATCGCCGT	32	60
2	OPA-13	CAGCACCCAC	34	70
3	CRA-22	CCGCAGCCAA	34	70
4	Hip-TG	GCGATCGCTG	34	70

These amplifications were showed in Plate (1) and Table (4) which illustrated the bands from the different treatments through the different four primers comparing with control. The total bands are 19, 8 of them are polymorphic with polymorphism percentage of 43.75% and 11are monomorphic bands. So, the later bands could be specific bands of genus of Cyanophyta.

There were unique bands appeared only in control *Anabaena* comparing with all treatments such as band at 60 bp with Hip-TG primer and band at 230 bp with CRA-22 primer, these explain that these bands quite sensitive to any change

in media. Also, there is a new band appeared only with the different treatments and not exists at control like the one at 150 bp with primer Opa-13, these indicate there were some change happened with DNA. The most affected treatments were different sodium chloride concentration (T3-T7) and combined stress (T8) by appearing and disappearing some bands, these mean that salinity can affect directly on the DNA and proteins which can affect directly on physiological reaction like oil percentage and fatty acid ratio.

Table (4) Polymorphism and data analysis of the used primers with *A. torulosa* under different stress conditions

Treatments	1 Control	2 (50%N)	3 (50%P)	4 200 mMNaCl	5 combined stress 50% N+ (50%P + 200mM NaCl)	Total no. of bands for each primer	Total no. of polymorphic bands for each primer	Polymorphism percentage (%)
OPA-11	3	4	4	3	3	5	1	20
OPA-13	4	4	4	3	2	4	3	75
CRA-22	5	4	4	4	4	5	3	60
Hip-TG	5	2	2	4	4	5	1	20
Total no. of bands for all primers						19		
Total no. of polymorphic bands for all primers						8		
Total polymorphism percentage						43.75%		

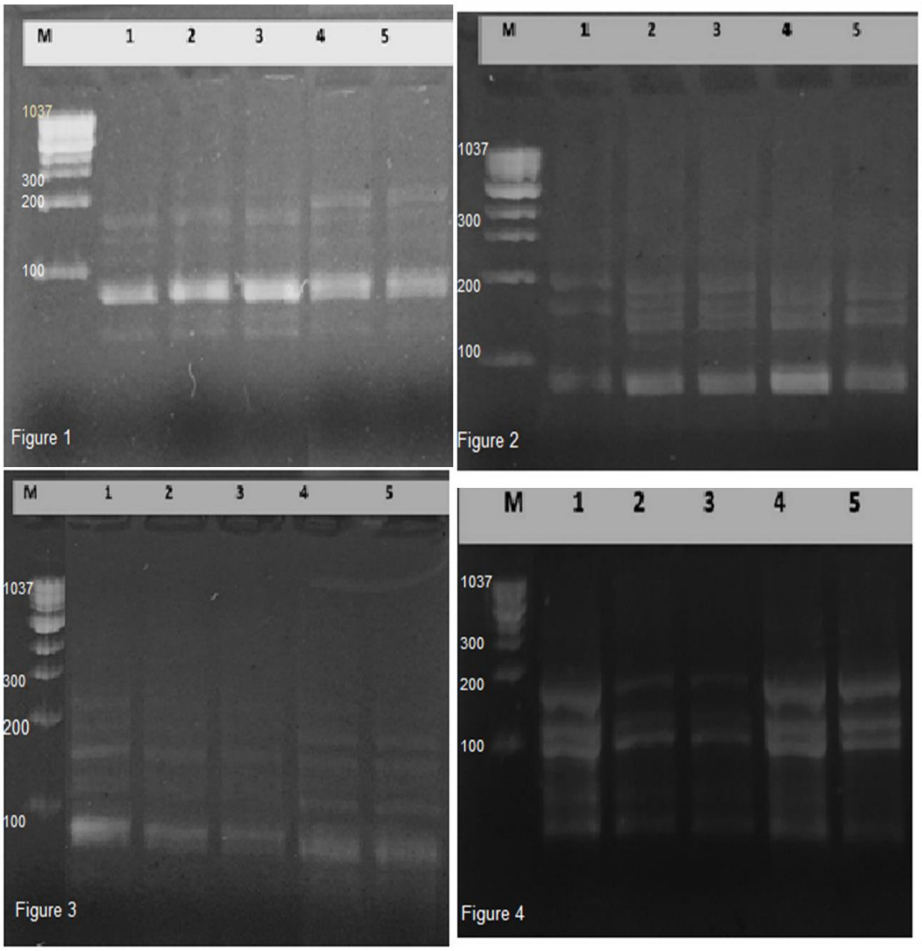

Plate (1): Agarose gel electrophoresis of four random primers under different stress conditions of *A. torulosa*. A: tested with primer OPA-11, B: tested with primer OPA-13, C: tested with primer CRA-22, D: tested with primer Hip-TG. Each figure contains 6 lanes; lane:M: Marker (in all figures), lane 1: control *A. torulosa*, Lane2: A. *torulosa* treated with 50% $NaNO_3$, lane 3: *A. torulosa* treated with50% K_2HPO_4, lane 4: *A. torulosa* treated with 200 mMNaCl, lane 5: *A. torulosa* treated with 50% $NaNO_3$ + 50% K_2HPO_4 + 200mM NaCl.

Figure (5) illustrate the relationship between the control of *Anabaena* and the different stress treatments based on UPGAMA phylogenetic tree method. As the treatments 50%P and 200 mMNaCl are closer together on their effect so they were separated in the same clade. The treatments 50%N and combined stress are closely on their effect and nearly related to control *Anabaena*.

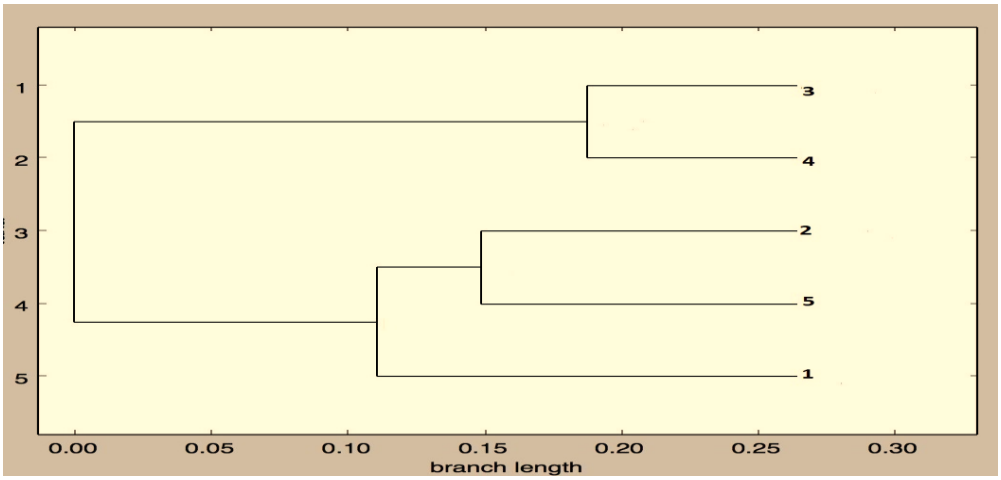

Figure (5) UPGAMA phylogenetic tree of *A.torulosa* under different stress conditions, 1:control, 2: A. *torulosa* treated with 50% $NaNO_3$, 3: *A. torulosa* treated with50% K_2HPO_4, 4: *A. torulosa* treated with 200 mMNaCl, 5: *A. torulosa* treated with 50% $NaNO_3$ + 50% K_2HPO_4 + 200mM NaCl.

The relation between results obtained from the result of oil content (biofuel production) and RAPD cluster analysis indicated that *A. torulosa* with 50%P and 200 mMNaCl stresses have significant effect. That significance in oil production may be related to appear of new band at 160bp with OPA-11 primer. Also, appear of two new bands at 130 and 190 bp with CRA-22 primer.

The low production of oil content from the treatments of 50%N and combined stresses was also indicated in the cluster analysis resulted from RAPD bioassay. The control is separated in a clade due to disappear of band at 150bp with OPA-13 which may explain the low oil content.

Discussion

The lipid production by *Anabaena* gave 15.53 % these rates are similar to or exceed the previously recorded rates for related species. **Cordiero *et al*. (2017)** reported that the lipid production by different *Microcystis* species ranged from 14.3 to 40.5 % so that cyanobacteria constitute a raw material for biofuel production. The changes in the nitrogen and phosphorus contents in BG11 medium affected the lipid yield. Results of this study are corroborated by those of **Salem *et al*.(2013)**, they found that the oil content of *Phormidium* increased 40% in media with 50% nitrogenand61.27% in mediawith50% phosphorus

The nitrogen stress on *A. torulosa* gave the lowest increase that may be due to its capability to diazotrophic growth, which means that these strains don't need a nitrogen source in the culture medium for growth (**Whitton and Potts, 2012**). The increase in lipid content at higher NaCl concentration may be due to adaptation under stress conditions which help in accumulation of lipid content and these results are in accordance with the finding of **Takagi *et al.* (2006)** in *Dunaliella* cells, **Salem *et al.* (2013)**, find 85.95% increase in oil content of *Phormidium* sp. treated with 100 mMNaCl. **Battah *et al*. (2014)** also reported that high salinity is directly related to minimum growth and over production of lipids.

Methyl linoleate (unsaturated fatty acids) in our results recorded 56-57 in control and stressed culture, linoleate is classified as a biodiesel due to its long methyl ester chain and is used as a fuel in standard diesel engines. This finding is in accordance with **Sallal *et al*. (1990)** who reported that, in lipids of *A. cylindrical*, palmitic, linoleic and linolenic acids were predominant. Also **Salem *et al.* (2013)** showed that palmetic, stearic, oleic, linolenic and linoleic acids were the most common fatty acids in *Phormidium* sp. on the other side **Gazal *et al* (2016)** found that in *Anabaena laxa, Anabaena fertilissima* linoleate not exceeds 1%.

As a result of this study it was suggest that, despite the inherent problems, taxonomic identification of cyanobacterial species could be improved through a combination of morphological and molecular analysis, taking into consideration both wild and cultured material. **Valerio *et al.* (2009)** and **Keshari *et al*. (2015)** support our results. Many molecular markers were used to differentiate between different strains of *Anabaena* or the effect of different treatments looks like our study. **Prasanna *et al.* (2006)** used molecular markers as LTRR and STRR for characterization of different 30 strains of *Anabaena*. Some people used RAPD analysis for differentiation among strains of cyanobacteria; **Shalini *et al*. (2007)**, **Perumal *et al.* (2009)** and **Chakdar and Pabbi, (2017)**.

The difference in bands resulted from using different primers explained as a follows: Generally; the different stress treatments resulted from deficiency of different elements so these treatments may cause some mutations which made change in the DNA sequence and follow change of protein. All these made change in algal physiological behavior. Some polymorphic and unique bands were appeared and disappeared, so it might be attributed to effect of different treatments of nitrogen, phosphorus content and salinity stress which affecting the vital physiological pathways.

The use of UPGAMA cluster analysis agreed with **Prasanna *et al.* (2006)** who used this relation analysis to differentiate a set of 30 *Anabaena* strains, isolated from diverse geographical regions of India.

The low production of oil content from the treatments of 50%N and combined stresses was also indicated in the cluster analysis resulted from RAPD bioassay. To sum up, the different stresses of the treated *A. torulosa* lead to variation in the oil content and so biofuel production. All that was explained genetically based on RAPD-PCR bioassay. This was obvious in the 50%P stress which gave the highest amount of oil content.

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إنتاج الدهون والدراسات الجزيئية لطحلب *Anabaena torulosa* معامل بأنواع مختلفة من الإجهاد

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تمت دراسة إنتاج الدهون من حيث نسبة الزيت وتكوين الأحماض الدهنية من طحلب *Anabaena torulosa* المعرف شكليا ووراثيا لتحديد مدى قدرته على إنتاج الديزل الحيوي وذلك باستجابته للإجهاد الناتج عن الملوحة ومجاعة النيتروجين والفوسفور. تتراوح الملوحة من 50 إلى 250 مللي مول كلوريد الصوديوم ، تركيز النيتروجين والفوسفور (50٪ نترات صوديوم ، 50٪ فوسفات البوتاسيوم فى مكونات الوسط الغذائىBG11) . وقد أشارت النتائج إلى وجود تغير في السلوك الفسيولوجي تحت هذه الضغوط المختلفة وتم توضيح هذه الاختلافات وراثيا. كشفت نتائج تحليل الزيت أن إنتاج الدهون زاد عندما انخفض النيتروجين والفوسفور (68.83 ٪ و 160.97 ٪ على التوالي) و تركيز 200مللى مول من كلوريد الصوديوم أدى الى زيادة محتوى الزيت بنسبة 120 ٪. كما كشف تحليل / GC MSلتكوين الأحماض الدهنية أن ميثيل لينيوليت هو المكون الرئيسي لأسترات الأحماض الدهنية حيث كانت نسبته 57.9 ٪ والتي تصنف على أنها وقود الديزل الحيوي بسبب سلسلة استرات الميثيل الطويلة وتستخدم أيضا كوقود في محركات الديزل القياسية. تم توضيح كل هذه النتائج وراثياً باستخدام تقنية RAPDالتي كشفت عن ظهور حزم جديدة واختفاء حزم أخرى. وقد تم تغيير تسلسل الحمض النووي مما أدى إلى تعدد أشكال الحمض النووي بنسبة 41.25 ٪. أيضا ، أشار نمط الحمض النووي إلى أن إجهاد النيتروجين والفوسفور كان أكثر ارتباطا بالعينة الحاكمة ، في حين أن الضغوط الأخرى (الملوحة والضغوط مجتمعة) تسببت في مزيد من التغييرات مقارنة بالعينة الحاكمة. بحيث تم تأكيد التغييرات الفسيولوجية الناتجة عن الإجهاد وراثيا.