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Ethyl Methane Sulfonate effect on total lipids of *Chlorella vulgaris* isolated from Nile River Egypt

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Chlorella vulgaris species was isolated from Nile River in Qalubia Governorates, Egypt. The highest constant of growth (μ=1.5), and the lower time of reproducing a generation (G=0. 2).The green alga was identified using 18S rRNA gene amplification followed by sequencing the resultant sequence was compared with those available on the NCBI website database through the BLAST bioinformatics tool. The percentage of lipids synthesized was 20 %. In order to increase lipid contents in this green alga was made chemical mutation by EMS. Using three concentrations from EMS1/10, 1/100 and1/1000 for two hours. The resulted in the treatment of concentration 1/10 decrease total lipid to 16.2 % in by concentration 1/100 almost no change the total lipids was equal with wild strain. By the concentration 1/1000 a significant increase in total lipids content as it reached 25% compared with wild strain.

Key words: *Chlorella vulgaris*,18S rRNA gene, EMS, and Total lipid.

Introduction

Chlorella Beijerinck is mono-cell green algae. They are everywhere, from land to sea, from cold water to warm water, to rocks and sand. They have served as model organisms for the leading physiological and biochemical studies in the process of photosynthesis and reduction of nitrates. (Huss et al., 1999). Moreover, collective cultures of *Chlorella* in agriculture has been used can a single cell for both humans and animals, in the field of biotechnology for waste recovery agents Treatment, and biofuel technology as microbial energy producers. (Golueke and Oswald., 1964, Fogg., 1971, Abbott and Cheney., 1982).From the most famous microalgal species around the world, *Chlorella*. After describing *Chlorella vulgaris*Beijerinck about 120 years ago, more than 100 species have been identified morphologically since species description. (John et al., 2003). The microalgae simple identify extremely hard because of the size is small Molecular techniques can be allowing an evaluation of the legitimacy of the morphological species idea for general microalgae and, finally, these techniques permit for partitioning and distribution of the seorganisms. For a lot of types of microorganisms, the gene most generality appoint for studies of diversity is the small-subunit ribosomal RNA gene (16S rRNA in prokaryotes and 18S rRNA in eukaryotes). (Fawley et al., 2004). Furthermore, many Chlorophyceae groups find in the genus *Chlorella* was switched to *Scenedesmus* according to 18S rRNA sequence analysis. (Hanagata., 1998). Although, a lot of studies have observed that the 18S rRNA is in many cases also conserved to differentiate between closely linked genera and species. (Luo et al., 2010). Ethyl methanesulfonate (EMS) is a single functional absorption agent found to be transplanted into a wide range of genetic testing systems from viruses to mammals. It has also been shown to be carcinogenic in mammals. AlkylaNucleophilic Cellular Sites are Caused by EMS via a Mixed Interaction Mechanism SN ~ / SN: While methylation of DNA occurs mainly in nitrogen sites in bases, Due to partial SN 1 reaction character, EMS is also able to produce high levels of alkali in oxygen such as 6 0 of guanine and in phosphate DNA groups. Genetic data obtained using microorganisms indicate that EMS can produce both GC to AT and AT to GC transition mutations. There is also some evidence to suggest that EMS can cause introductions or deletions between rules as well as more extensive deletions within the group. In higher living organisms, there is clear evidence that EMS is able to break chromosomes, although the mechanisms involved are not well understood. A hypothesis often cited is that the DNA bases interpreted by the EMS system (often the N-7 site of the guanine) gradually degrade from deoxyribose on the RNA backbone, leaving behind an unstable (or possibly ephedermine) site that can cause to break a strand of DNA. There are also data indicating that some chromosome proteins in some sperm in mice may be an important factor in causing chromosomal fractures. (Gary., 1984). Ethylmethanesulfonate (EMS) was the first factor found unequivocally to increase the proportion of mutants. (Loveless., 1958).

The present works aimed to isolation and genetic identification of rich oil content fresh water algae and develop total lipids for microalga Chlorella vulgaris strain isolated from Nile River in Qalubia Governorates, Egypt, to achieve this goal induced chemical mutation In order to increase total lipids content.

Materials and Methods

Source of the isolation

Chlorella vulgaris strain was isolated from fresh water of Nile River in Qalubia Governorate, Egypt. Isolation and purification of the microalgae were done by sub culturing and grown in BG-11 medium. Samples were isolated as the cultures were maintained at 25°C under illumination (120µ.e) cycle 16 h light and 8 h dark in a shaking growth chamber at 90 rpm and pH range (7.1).

Growth studies

Growth was estimated according (Stein., 1973) by measuring the OD650nm for the culture by spectrophotometer

Specific growth rate (µ), based on the equation µ=ln(Ny/Nx)/(ty−tx),

WhereNy and Nx are the numbers of cells (N) at the start (tx) and the end (ty) of the logarithmic growth phase (Levasseur et al., 1993).

Generation time (G) was determined using the following equation:

G= 0.301/ µ (Stein., 1973).

Determination of total Lipids

Lipids were determined according to the standard method A.O.A.C. (1984).A known weight was extracted with Petroleum ether (60-80°C) for 24 hours in a Soxhlet apparatus, after which the solvent was evaporated, and the residue was dried to a constant weight at 95°C.

%Total lipid = Sample before determination-Sample after determination X 100

Sample before determination

Extraction and purification DNA:

Total DNA was extracted according to the method outlined by Doyle and Doyle (1987)Samples were suspended in the (CTAB) extraction buffer (3% CTAB, 0.1 molTris-HCl, 0.01 mol EDTA, 1.4 molNaCl, 0.5% β-mercaptoethanol, 1% PVP) at pH 8.0. The mixture was incubated at 60°C for one hour with shaking for every fifteen minutes, and was cooled down to room temperature. DNA was then extracted with an equal volume of chloroform: isoamyl alcohol (24:1) and precipitated from the supernatant by the addition of one volume isopropanol. DNA extract was re-suspended in TE buffer. DNA quality was controlled by agarose gel electrophoresis.

18S rDNA Sequence Analysis

The genomic 18S ribosomal DNA region of microalgae was amplified by colony PCR as described previously by (Uclés., 2008)using the C-2: 5'> ATTGGAGGGCAAGTCTGGT<3'forward and D-2:5'> ACTAAGAACGGCCATGCAC <3'reverse primers.

The PCR reaction were performed for the 18SrDNA gene in 25 µl volume by mixing 30 ng genomic DNA with 2µl of primer (10 p mole/µl) and master max (Takara, Japan) and PCR water. The PCR reaction continued with denaturation for 30sec. at 95 °C, followed by 30 cycles of 30 sec. denaturation at 95°C, 1 min. annealing temperatures at 60°C, and 1.0 min. extension at 72°C. The 30 thermal cycles were followed by a final extension of 5 min. at 72°C. Following amplification, PCR products were electrophoresed on a 0.9% agarose gel and purified using a Thermo Scientific Gene Jet PCR purification Kit.

Sequence analysis

The current rDNA gene sequences were registered at DNA database under accession number**.** Sequences were compared with those available in the Gen-Bank database using Blast. Phylogenetic tree was constructed through two Bioinformatics Processes. In the first process, the nucleotide sequences of the recovered rDNA gene phenotypes and their homologues sequences, from the DNA database, were aligned using the online program "Clustal Omega". In the second process, the aligned sequences were submitted to the MEGA 7 software (http://www.megasoftware.net/) for drawing the phylogenetic tree. Phylogenetic tree was constructed by applying the algorithms maximum likelihood in MEGA software and used BioEdit version 7.2.5 in bioinformatics tools.

Chemical mutation by EMS (Ethyl Methane Sulfonate, 99% Target organ: Iungs, kidneys! Aldrich chemical company. Inc. USA

- Samples of each algal culture (20ml each)
- Three concentrations of EMS each concentration Its size final 1ml

a) 100µl EMS +900 µl dH_2o

b) 100µl (a) +900 µl dH_2o

c) 100µl (b) +900 µl dH_2o

-Each of concentration three Treatment on Liquid culture 20ml culture /100µl EMS.

-Put each Treatment in time 0.5hour, 1hour, 2hour

- Stop treatment: - Preparation of a sterile solution of 5% sodium Sayo sulfate

Put 1ml at the appropriate time to stop.

Results and Discussion

Algal Isolation for this study

Green alga species was isolated from Nile River in Qalubia Governorates, Egypt. That isolate was characterized by 20% of total lipids. Oil determination was performed at the stationary growth phase. This green alga was tentatively identified as *Chlorella vulgaris* belonging to *Chlorophyta*.**(Figure1).**

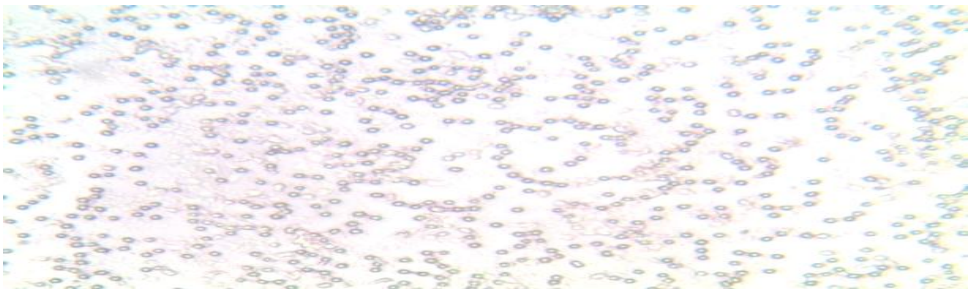

Figure1. Microscopic Image

The *Chlorella vulgaris* isolate is optioned for this study morphologically circular small cell and the color is green.

Growth study

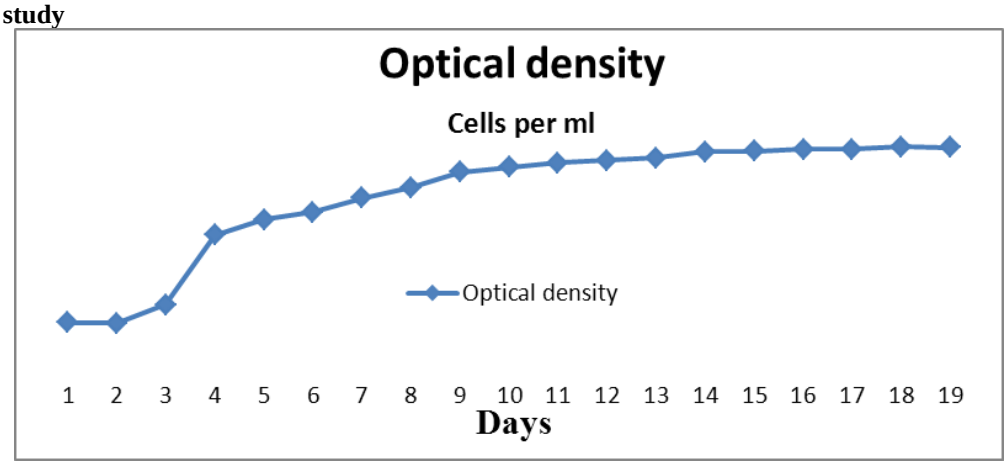

Figure2. Growth curve for the isolate optioned for this study

Beginning logarithmic growth phase after the third day to the eleventh day then outset stationary growth phase to the eighteenth day then in the nineteenth day start death growth phase. The highest constant of growth (μ =1.5), and the lower time of reproducing a generation (G=0.2). Growth phase is important for know the appropriate time for the experiment. Content of lipids and the composition of fatty acids are also susceptible to changes during the growth cycle. In many algae species examined, observed increase in TAGs during the stationary growth phase (Bigogno et al., 2002 and Mansour et al., 2003).

18S rRNA Sequences and phylogenetic tree

18S rDNA Sequences of the isolate the base size was 744 bp.

The DNA sequences of this isolate showed 100% identity to *Chlorella vulgaris.*

Although, morphological examination by light microscopy revealed that the strain hadconsistent morphology to *Chlorella vulgaris.*

The result obtained was compared in the gene bank.

The Sequences of accession number **LC333291** and10 accession numbers from genebank was aligned using the online program "Clustal Omega" and phylogeny tree was submitted to the MEGA 7 software Analyses of Phylogenetic for the 18S rRNA sequences observed the relationship between The Sequences of accession number**LC333291** and 10 accession numbers from gene bank.

The result showed that the present specimens were grouped in Phylum Chlorophyta**.** (Fig. **2**). The phylogenetic tree deducted from the sequence comparison of 18S rRNA region showed that the length of the branch that represents an amount genetic change of 0.0050, and The 18S rDNA-based phylogeny tree included five clades. The clades grouping had low support (bootstrap value between 7-87%).

and showed that the present specimen was placed in *Chlorella vulgaris* Sequence ID: LC333291 neighbor to *Chlorella vulgaris* Sequence ID: KX094755, *Chlorella sp* Sequence ID: - JX097060 and *Chlorella vulgaris* Sequence ID: KU720636.and *Chlorella vulgaris* Sequence ID: KX094753 and distant on *Chlorella vulgaris* Sequence ID: KT250599 , *Chlorella sp*Sequence ID: X73992,*Chlorella minutissima* Sequence ID: AB006046 ,*Chlorella saccharophila* Sequence ID: X63505 ,*Chlorella kessleri* Sequence ID: X56105 and *Chlorella sp*Sequence ID: LC037427.

That is mean that is sequences common in Phylum Chlorophyta in the world in the different environment and under different conditions.

Table1. Percentage divergence between wild simple and10accession number from the gene bank list

Accession number	Percentage divergence (/100)	Rate of transition	Rate of trans version
Wild simple	-	-	-
KX094755,	0.0013	0.0013	0.0000
JX097060	0.0013	0.0000	0.0013
KU720636	0.0027	0.0013	0.0013
KX094753	0.4355	0.0995	0.3360
KT250599	0.0699	0.0188	0.0511
X73992	0.0067	0.0027	0.0040
AB006046	0.0242	0.0040	0.0202
X63505	0.5992	0.1296	0.4696
X56105	0.0094	0.0040	0.0054
LC037427	0.0054	0.0027	0.0027

Neighbor-joining Method (Saitou and Nei., 1987)
Neighbor-joining Method

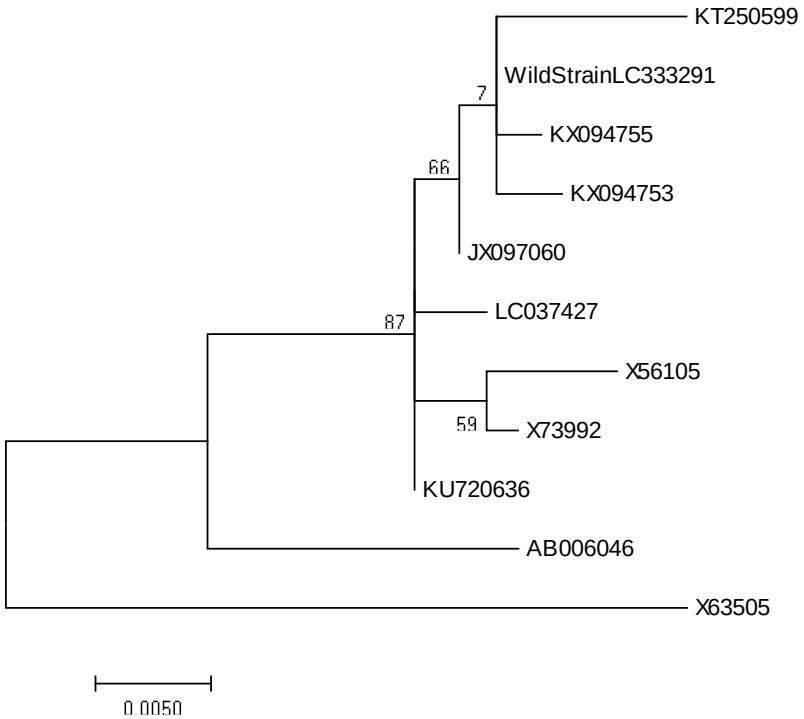

Figure 3. The Relationship between The Sequences of accession number**LC333291** and 10 Accession numbers from gene bank. The Phylogenetic tree was constructed by applying the algorithms Neighbor-joining Method

In this Phylogenetic found that:
Average percent standard deviation = 2.59436
Ln Likelihood = -3284.83696
Empirical Base Frequencies:
A 0.24065
C 0.20617
G 0.29601
T 0.25717
Conserved regions found
Region 1: Position 1 to 66
1ATTGGAGGGCAAGTCTGGTGCCAGCAGCCG CGGTAATTCCAGCTCCAATAGCGTATATTTA AGTTG 66
Region 2: Position 68 to 99
68
TGCAGTTAAAAAGCTCGTAGTTGGATTTCGG G 99
And other regions contain little difference in DNA sequences.

Chemical mutation by EMS Ethyl Methane Sulfonate

Genetic data obtained using microorganisms indicate that EMS can produce both GC to AT and AT to GC transition mutations (Gary., 1984).

In case of exposure to chemical mutation using EMS as an effort for total lipids improving.Using three concentrations from EMS1/10, 1/100 and1/1000 for two hours. The resulted in the treatment of concentration 1/10 decrease total lipid to

16.2 % in by concentration 1/100 almost no change in the total lipids was equal with wild strain. By the concentration 1/1000 a significant increase in total lipids content as it reached 25% compared with wild strain.

Table 2. The output of chemical mutation

Exposure %	Total Lipids %
wild strain(0.0)	20
EMS (1)	16.2
EMS (2)	20.3
EMS (3)	25

That is result disaccord with the result (Kavakli et al., 2017) the percentage of total lipids was increased in *Chlorella vulgaris* after EMS treatment with the ratio of 50%.

References

Abbott IA and Cheney DP. (1982) .Commercial uses of algal products: introduction and bibliography. In: Rosowski JR, Parker BC, editors. Selected Papers in Phycology II. Lawrence, KS, USA: Phycological Society of America, pp. 779–787.

A.O.A.C. (1984) official methods of analytical chemists.14th ed. Washington, DC.

Bigogno, C.**;** Khozin-Goldberg, I., Boussiba, S., Vonshak, A. and Cohen, Z. (2002) .Lipid and fatty acid composition of the green oleaginous alga Parietochlorisincisa, the richest plant source of arachidonic acid. Phytochemistry, 60, 497–503.

Doyle J. J. and Doyle, J. L. (1987): A rapid DNA isolation procedure for small

quantities of fresh leaf tissue. *Phytochem Bull* 1987; 19: 11-15.

5-Ehsan Sarayloo., Mehmet Tardu., YigitSabriUnlu., SalimSimsek.,GulCevahir.,Can Erkey. and Ibrahim HalilKavakli . (2017). Understanding lipid metabolism in high-lipid-producing Chlorella vulgaris mutants at the genome-wide levelAlgal Research 28 (2017) 244–252.

6-Fawley MW.,Fawley KP. andBuchheim MA. (2004). Molecular diversity among communities of freshwater microchlorophytes. Microbial Ecol 48: 489–499.

7-Fogg GE. (1971). Recycling through algae.P Roy SocLond B Bio 179: 201–207.

8-Gary A. Sega.(1984). A review of the genetic effects of ethyl methanesulfonate*Mutation Research,* 134 (1984) 113-142 113 Elsevier.

9-Golueke CG.and Oswald WJ. (1964). Role of plants in closed systems.Annu Rev Plant Phys 15: 387–408.

10-Hanagata N. (1998). Phylogeny of the subfamily Scotiellocystoideae(Chlorophyceae, Chlorophyta) and related taxa inferred from18S ribosomal RNA gene sequence data. J Phycol 34: 1049-1054.

11-Huss, V. A. R., Frank, C., Hartmann, E. C., Hirmer, M., Kloboucek, A., Seidel, B. M., Wenzeler, P. and Kessler, E. (1999). Biochemical taxonomy and molecular phylogeny of the genus Chlorella sensulato(Chlorophyta). J Phycol 35, 587–598.

12-John DM.,Whitton BA. and Brook AJ. (2003). The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae. The Natural History Museum and the British Phycological Society. Cambridge, UK: Cambridge University Press.

13-Levasseur M., Thompson PA.and Harrison PJ. (1993) .Physiological acclimation of marine phytoplankton to different nitrogen sources. J Phycol 29:587–595.

14-Loveless, A. (1958) .Increased rate of plaque-type and host-range mutation following treatment of bacteriophage in vitro with ethyl methanesulphonate, Nature (London), 181, 1212-1213.

15-Luo, W., Pröschold, T., Bock, C. and Krienitz, L. (2010).Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). – Plant Biology 12: 545–553.

16-Mansour, M.P., Volkman, J.K. and Blackburn, S.I. (2003) .The effect of growth phase on the lipid class, fatty acid and sterol composition in the marine dinoflagellate, Gymnodinium sp. in batch culture.Phytochemistry, 63, 145–153.

17-Saitou, N. and Nei, M. (1987) The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. Mol. Biol. Evol., 4(4), 406-425.

18-Stein, J.R. (1973). Hand book of phycological methods. Cambridge Univ. Press. Cambridge., UK.

19-Uclés, R. M. (2008): Identification of algal strains by PCR amplification and

evaluation of their fatty acid profiles for biodiesel production, Master of Science,

Louisiana State University.

تأثير Ethyl Methane Sulfonate علي المحتوي الكليمن الدهون لطحلب *Chlorella vulgaris* المعزول من نهر النيل بمصر

عزل الطحلب الأخضر *Chlorella vulgaris* من نهر النيل ,محافظة القليوبية , مصر. تم قياس معدل النمو للطحلب إذ يبدأ بالطور الأسي بعد اليوم الثالث من زراعتة حيث تحصل زيادة مضطردة في عدد خلاياه, ويستمر تقريبا لما بعد اليوم الحادي عشر ومن ثم يبدأ طور الأستقرار بعد اليوم الثاني العاشر الذي أستمر الي اليوم الثامن عشر وبعدها بدأ طور الهبوط في اليوم التاسع عشر . ولقد أظهر الطحلب ثابتا للنمو مقداره (μ =1.5) بينما قيمة زمن تكاثر الجيل هو (G=0.2) . تم تعريف العزلة المختارة وراثيا بأستخدام بادئات 18S rRNA ثم تم عمل تحليل تتابعات للعزلة وقورنت تلك السلالة مع 10 سلالات متاحة من بنك الجينات بواسطة أدوات المعلوماتية الحيوية. وقدرت أنتاجية الطحلب من الدهون وكانت 20%.في محاولة لزيادة محتوي الطحلب من الدهون الكلية تم أستحداث طفور كيماوي بأستخدامEMSبعمل ثلاث تركيزات0.1,0.01,0.001 لمدة ساعتين . في حالة التركيز 0.1 أنخفضت نسبة الزيت الي 16.2% بينما التركيز 0.01 تقريبا لم يحدث تغير مقارنة بالسلالة البرية أما التركيز 0.001 زادت نسبة الزيت زيادة معنوية الي 25% مقارنة بالسلالة البرية.