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Optimization of cultural and nutritional conditions for selection of the most promising microalgae intended for wastewater treatment and biodiesel production by direct transesterification method

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Abstract

Recently, algae have become the latest feasible source being targeted for biofuel production since they exhibit several attractive features. An investigation concerning the treatment of wastewater and the production of biodiesel by different cyanobacteria and micro-algae has been undertaken. Twelve soil and water samples were collected from different localities in Egypt. Eleven microalgae were isolated from collected soil and water samples and subjected to different purification methods. The isolated microalgae were used in the treatment of wastewater samples, and physical and chemical properties of wastewater samples were evaluated before and after treatment. The results evoked the superior activity of the isolated microalgal strains in the treatment of wastewater. Determination of lipid content of all isolated microalgae was carried out and showed 2.10 up to 5.13% of lipid. Factors affecting the lipid content productivities by selected promising microalgae were investigated viz. pH, incubation periods, and nitrogen sources. Three isolates were selected for cultivation on large scale, *Oscillatoria chalybea* (2) with dry weight 1028.4 mg and lipid yield 0.72%, *O. chalybea* (3) with dry weight 480.4 mg and lipid yield 0.9%, and *O. chalybea* (5) with dry weight 524.4 mg and lipid yield 1.01%. Extraction of oil from microbial biomass was done by Bligh and Dyer method. The lipid composition was determined as fatty acid methyl esters (FAMEs) through the direct transesterification method. The conversion of the extracted oil into biodiesel was analyzed by standard gas chromatographic technique.

Keywords: Cyanobacteria, Oscillatoria, Wastewater treatment, Lipid content, Transesterification, Biodiesel.

1. Introduction

The amount of consumption in municipal and industrial wastewater in Egypt was calculated and forecasted based on the growth rate, and it is predicted to reach 7.9 billion cubic meters by 2030, up from 3.5 billion cubic meters in 1995. So, especially in underdeveloped nations, wastewater treatment is the only option for conserving water and reducing consumption. Nowadays, several atypical wastewater treatment procedures are used with the goal of lowering operating costs and generating high-quality treated water [1-4]. Now the world has been challenged by the global warming problem. The release of Carbon dioxide from the combustion of fossil fuels, the key contributor to the process has generated interest in promoting biofuel as one of the leading renewable energy sources **[5]**. The sustainable production of biofuel is a valuable tool in stemming climate change **[6]**, boosting local economies, particularly in lesser-developed parts of the world **[7,8]**, and enhancing energy security for all **[9,10].** Advancement in renewable biofuel sources; cling to solution key of the dual difficulties, running down the fossil fuel reservoirs and environmental pollution **[11].** Therefore, exploration of novel, renewable, environmentally friendly, clean, reliable, and economically feasible energy resources is a serious requirement of the day **[12]**.

The discharge of greenhouse gases through the burning of fossil fuels in the transport sector alters the natural equilibrium of the environment. The world has now started to realize the problem and syndromes created by conventional fuels **[13]**. Microalgae culture offers an interesting step for wastewater treatments because they provide a tertiary biotreatment coupled with the production of potentially valuable biomass,

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which can be used for several purposes. Microalgae cultures offer an elegant solution to tertiary and quandary treatments due to the ability of microalgae to use inorganic nitrogen and phosphorus for their growth. And also, for their capacity to remove heavy metals, as well as some toxic organic compounds, therefore, it does not lead to secondary pollution. In the current review, we will highlight the role of micro-algae in the treatment of wastewater **[14].** Microalgae are the main microorganisms used in the treatment of domestic wastewater in units such as oxidation ponds or oxidation ditches. Algae have also been deployed for low-cost and environmentally friendly wastewater treatment. The idea of coupling wastewater as a medium for biofuel production from algae is not innovative **[15].** Microalgae create/make different kinds of inexhaustible biofuels, for example, biogas shaped by anaerobic absorption of the algal biomass; biodiesel from microalgae oil and photograph natural creation of hydrogen. Green growth is more controlled and stable for the creation of energy contrasted with land-based biomass, algal culture can deliver bigger measures of biofuel with no utilization of good water or prolific land. **[16].**

Microalgae can be a rich source of carbon compounds, which can be utilized in biofuels, health supplements, pharmaceuticals, and cosmetics **[17].** They also have applications in wastewater treatment and atmospheric CO2 mitigation. Microalgae produce a wide range of bioproducts, including polysaccharides, lipids, pigments, proteins, vitamins, bioactive compounds, and antioxidants **[18].** Third-generation biodiesel from microalgae is a potential possibility with its ability to quickly build up lipid and chances for year-round gathering cycles, the talent of microalgae to fold up their biomass weight quickly, and utilizing sunlight, water, and CO2 [**19].** At present, an international algal biomass production stands up at 38 million litres **[20].** The interest in microalgae as a renewable and sustainable feedstock for biofuels production has inspired a new focus in biorefinery. The industrial cultivation of microalgae to produce biofuels and bioproducts has increased dramatically over the last few decades **[21].** Most of the microalgae species are favorable for biodiesel production due to high lipids contents 50–70% and may reach 80% such as in the case of the microalga *B. braunii* which accumulate up to 80% of oil in its biomass **[22].** Microalgae can produce algal oil 58,700 L/hac which can produce 121,104 L/hac biodiesels **[23].** The infeasibility of algal biodiesel is due to the associated high operational, maintenance, harvesting, and conversion cost **[24].** Microalgae are rapidly growing photosynthetic organisms having the potential of transforming 9–10% of solar energy (average sunlight irradiance) into biomass with a theoretical yield of about 77 g/biomass/m2 /day which is about 280 ton/ha/year **[25].** In the current study we will highlight on the role of micro-algae in the treatment of wastewater. Therefore, this study was conducted to evaluate the efficacy of different cyanobacteria and micro-algae strains for the treatment of wastewater and the Production of biodiesel.

2-Materials and Methods

1-Sample Collection:

Ten wastewater samples and two cultivated soils were collected from different localities in Egypt table (1)

2.1.1-Estimation of physicochemical properties of the collected samples:

Physical and chemical properties of the collected soils and water samples were conducted at Egyptian Petroleum Research Institute (EPRI) and National Research Centre (NRC). Anions and cations were determined according to the American Society For Testing and Materials **[26,27]**, respectively using ion chromatography. The instrument used was the Dionex IC model ICS 1100 equipped with high capacity columns (AS9 and CS12) for anion and cations, respectively. The trace metal was estimated very preciously according to **[28]** using Atomic absorption spectrophotometer Zeenit 700 p. Total Dissolved Solids and total suspended solids were determined according to **[29].** Standard Test Methods for Filterable Matter in Water. In addition, conductivity and resistivity were determined on-site using digital conductivity meter WTW 330I **[30].** Density and specific gravity were determined according to **[31].** pH was determined on-site according to **[29]** using Metler Toledo pH meter with a combined glass electrode. However, Alkaline species (CO_3, OH, HCO_3) were measured according to **[32].**

2.1.2-Media:

Blue - Green Medium BG11 **[33]** and Modified Watanabe medium **[34]** were used for the isolation of microalgae.

2.2-Isolation of microalgae from collected samples:

Ten ml of water sample was transferred to a 250 ml conical flask containing 100 ml of sterilized BG 11 and Watanabe media under aseptic conditions then incubated on a static incubator at 30°C with continuous illumination using white fluorescent light at intensities of 3000 lux for three weeks. Every two days, the flasks were examined for algal growth with the aid of the naked eye. For soil samples, about a loop of soil sample was placed in 250 ml Erlenmeyer flasks containing 100 ml of sterile broth media, and then incubated under the previously mentioned conditions of light and temperature.

Table (1): Description of samples

Number of Samples	Location of Sample	Sample symbol	Type of sample	Sample status
1	Al-Sharkia governorate	(SH 1)	Water Wells	Wastewater
2	Al- Behera Governorate, Itai El-Baroud city,	(AB 1)	Municipal	Wastewater
3	Kafr Al-sheikh Governorate, De Souq city	(KA)	Healthy Drainage	Wastewater
4	Al- Behera Governorate, Itai El-Baroud city, Ezbet EL-Shekh Ahmed	(AB 2)	Agricultural drainage	Wastewater
5	Al-Sharkia governorate, Abou Kebir	(SH 2)	House Drainage	Wastewater
6	Nasr City	(NC 1)	Healthy Drainage	Wastewater
7	Al-Sharkia governorate, Abou Kebir	(SH 3)	Municipal	Wastewater
8	Al-Sharkia governorate, Abou Kebir	(SH 4)	Agricultural drainage	Wastewater
9	Nasr City1, Faculty of Science Garden	(NC 2)	Soil	Cultivated
10	Giza Governorate	(GC)	Healthy Drainage	Wastewater
11	Helwan City	(AH)	Healthy Drainage	Wastewater
12	Nasr City2, Faculty of Science Garden	(NC3)	Soil	Cultivated

2.3-Purification of microalgal isolates and determination of microalgae growth:

Purification of microalgal isolates was carried out by inoculating 1 ml of culture solution onto Petri plates containing the same isolation media solidified with 1.5 % (w/v) of bacteriological agar. Petri plates were incubated at 30°C under continuous illumination for two weeks. The purity of the culture was confirmed by repeated plating and by regular observation under a microscope. The microalgal growth was determined by transferring algal isolates to wastewater on a large scale then incubated for 14 days at 30°c to produce biomass. By ending the incubation period, the microalgae biomass was harvested and allowed to dry at room temperature to determine the dry weight (DW) of microalgae biomass.

2.4-Wastewater treatment by microalgal isolates:

The microalgal isolates were allowed to grow on wastewater for 14 days on a large scale [35].

2.5-Selection of the high lipid content microalgae isolates:

For this purpose, all microalgal isolates were cultivated as usual under optimal conditions for 14 days, to determine algal lipid content as follows:

The lipid of segregated microalgae was extricated by a change of dry extraction strategy for Bligh and Dyer **[36]**. Dry biomass was blended in with chloroform: Methanol: H2O (1:1:1 v/v). The combination was sonicated for 10 min. Then, at that point, the combination was centrifuged for 5 min at 4°C and 4000 rpm. The lower layer of 5 ml chloroform + separated lipids was pipetted out for lipid examination. The excess cell pellets were re-extricated rehashing the strategy. Likewise, the lipid of dry biomass was extricated with Hexane: Isopropanol (3:2 v/v). The combination was sonicated for 10 min. then, at that point centrifuged for 5 min at 4°C and 4000 rpm. The top layer of hexane + lipids was pipetted out for lipid

investigation. The excess cell pellets were re-removed repeating the scheme **[37].**

2.6-Optimization of cultural and nutritional conditions for selection of the most promising microorganisms with the highest biomass and lipid content:
Different factors were considered to optimize biomass and lipid production for the selection of the most promising algal isolate(s), *viz*. different incubation periods, different pH values, different $NaNO_3$ concentrations, different K_2HPO_4 concentrations, different $CaCl_2.2H_2O$ concentrations, and different $MgSO_4$ concentrations [35].

2.7-Cultivation of selected microorganisms on a large scale:
To obtain large scale production of biomass and lipid content, the selected most promising isolates which exhibited the highest lipid content were cultivated in wastewater medium with large quantities (10 liters) and incubated on a static incubator at 30°C under continuous illumination using white fluorescent light at intensities of 3000 lux and for 10 days. At the end of the incubation period, the cells were harvested and dewatered for the determination of microalgal cells biomass as well as lipid content **[35].**

2.8-Biodiesel Production from most promising microalgal isolates:
The fatty acid composition was determined by the conversion of oil to fatty acid methyl esters prepared by adding 1.0 mL of n-hexane to 15 mg of oil followed by 1.0 mL of sodium methoxide (0.4 mol), according to the modified method of **[35,37]**. The mixtures were vortexed for 30 seconds and were allowed to settle for 15 minutes. The upper phase containing the FAMEs was recovered and analyzed by gas chromatography (GC-FID).

2.9-Fatty acid profile analysis:
Fatty Acid Analysis was determined by GC Perkin Elmer Auto System XL Equipped with flame ionization detector (FID), fused silica capillary column ZB-Wax (60 ml x 0.32 mm i.d). The oven temperature was maintained initially at 50°C to 220 °C at rate 4°C / min., injector and detector temperature was 250 °C. This step was carried out at National Research Center (NRC) Cairo, Egypt **[37].**

3-Results and discussion
The results indicated that soil sample NC2 had the highest values of salinity, total dissolved solids, total hardness, and conductivity. However, soil sample NC3 had the highest values of resistivity. Hydrogen ion concentration (pH) values of both soil samples were slightly alkaline as shown in **table (2)**. Soil sample NC2 contains the highest values of Ca^{++}, Br^-, Cl^- and SO_4^{--} (324.85, 52.30, 141.42, and 817, respectively) in comparison to soil sample NC3. Moreover, soil NC3 showed the highest values of Li^-, NH_3^+, Sr^+, Ba^-, F^- and NO_3^+ (0.42, 6.77, 7.12, 15.72, 15.96, and 44.75, respectively) in comparison to soil sample NC2 as shown in **Table (3)**.
Physical and chemical analysis of the collected water samples were illustrated in **table (4).** The results indicated that, KA followed by AH water samples had the highest values of both Ca^{++} and Mg^{++} cations, while SH1 had the highest value of Na^+. While SH1 followed by GC and AH exhibited the highest K^+ cations among all water samples; KA followed by SH1 had the highest values of SO_4^{--} anions, whereas SH1 had the highest values of Cl^- and HCO_3^- anions. The rest of the water samples showed a moderate value of cations and anions as represented in **Table (5).**

Table 2: Physical properties of collected soil samples.

Physical Properties	NC2 sample	NC3 sample
Total Dissolved Solids (mg/l)	1540.6	799.0
Salinity (as NaCl), (mg/l)	233.3	190.7
Alkalinity (as $CaCO_3$), mg/l	50.0	50.0
Total Hardness (as $CaCO_3$), (mg/l)	945.2	436.2
pH	7.53	7.54
Conductivity (mmohs/cm)	0.16880×10^{-2}	0.04630×10^{-2}
Resistivity (Ohm-m)	5.92	21.59

Table 3: Inorganic chemical properties of collected soil samples.

Chemical ions	NC2 soil sample		NC3 soil sample	
	(mg/L)	(meq/L)	(mg/L)	(meq/L)
Li^-	0.005	0.001	0.420	0.060
Na^+	64.760	2.816	62.650	2.724
NH_3^+	3.085	0.171	6.770	0.375
K^+	28.110	0.719	29.990	0.767
Mg^{++}	32.560	2.679	26.290	2.163

Ca^{++}	324.850	16.210	131.350	6.554
Sr^{+}	0.245	0.006	7.120	0.163
Ba^{-}	3.850	0.056	15.720	0.229
F^{-}	11.440	0.602	15.960	0.840
Cl^{-}	141.420	3.984	115.590	3.256
Br^{-}	52.300	0.655	6.200	0.078
NO_3^{-}	Nil	Nil	44.750	0.722
SO_4^{--}	817.000	17.018	275.210	5.733
OH^{-}	Nil	Nil	Nil	Nil
CO_3^{--}	Nil	Nil	Nil	Nil
HCO_3^{-}	61.000	1.000	61.000	1.000

Where meq means milliequivalent

Table 4: Physical properties of collected water samples.

Water sample	**Physical Properties**	
	pH	**EC (ds/m)**
AB1	9.8	0.49
AB2	9.5	0.57
KA	8.1	1.95
SH 1	8.1	2.83
SH2	8.9	0.44
SH3	9.3	0.47
GC	7	1.74
AH	6.9	1.34
NC1	8.7	0.48

Table 5: Inorganic chemical properties of the collected water sample.

Water sample	**Cations and Anions of Water Samples**						
	Ca^{++}	**Mg^{++}**	**Na^{+}**	**K^{+}**	**SO_4^{--}**	**Cl^{-}**	**HCO_3^{-}**
AB1	1.25	0.75	2.2	0.3	0.9	2.5	1.1
AB2	1.5	1	3	0.3	2.3	2.5	1
KA	3.75	9	6	0.5	12.65	5.5	1.1
SH1	2.5	8.5	16.5	0.6	10.3	12.2	5.6
SH2	2	1.2	1.5	0.2	2	2	0.9
SH3	2.1	1	1.7	0.14	1.74	2	1.2
GC	3	4.5	8.5	0.48	7.68	7.5	1.3
AH	3.5	4.5	5.2	0.43	5.63	5.5	2.5
NC1	2.5	1	1.4	0.15	2.1	1.75	1.2

3.1-Isolation and purification of microalgae isolates

Ten axenic microalgae isolates were obtained from collected soil and water samples and sub-cultured on Petri-dishes of its specific isolation media. Photos (1 and 2).

3.2-Determination of microalgae growth:

Determination of dry weight of isolated algal species in different times showed that KA isolates had the highest dry weight in a sample (2), AB2 had the highest dry weight in a sample (2), NC1 had the highest dry weight in a sample (1), SH3 had the highest dry weight in a sample (3), NC2 had the highest dry weight in a sample (2), GC(1) had the highest dry weight in a sample (4), GC(2) had the

highest dry weight in a sample(3), AH had the highest dry weight in a sample (2), NC3(1) had the highest dry weight in a sample (2) and NC3(2) had the highest dry weight in a sample(3) as summarized in **table (6)**. Water sample no (4) was selected to complete the present investigation. Additionally, this study was carried out to evaluate the capability of developing green growth utilizing wastewater as a supplemented medium. There was generally high supplement take-up for phosphorous and alkali. Complete nitrogen take-up was a lot lower since natural nitrogen was undoubtedly not absorbed by the way of life. The mean usefulness got for the whole development time frame was 3.3 ±1.5 g dry wt. $m^{-2}d^{-1}$. These outcomes were in harmony with a study by **Woertz et al [38],** who reported an algal production rate of 3 g dry wt. m^{-2} d^{-1} for *Chlorella* sp. grown on wastewater.

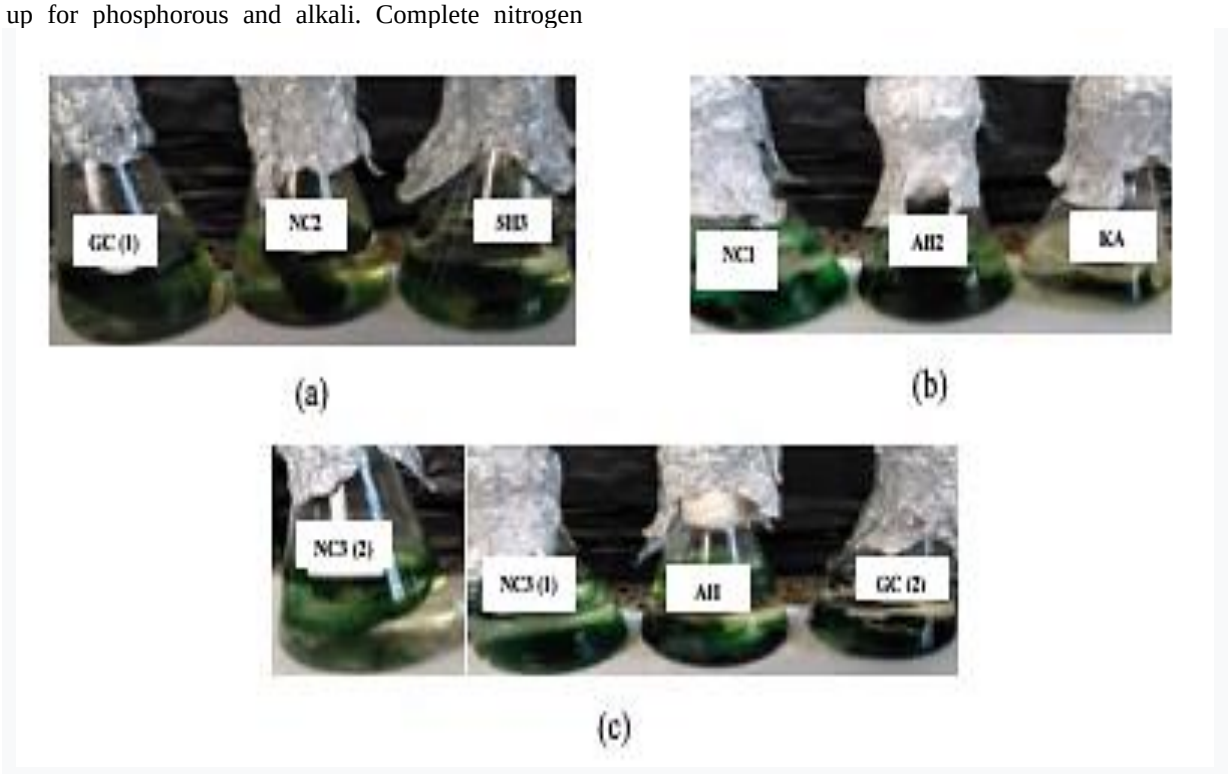


Photo (1): Axenic broth cultures of micro-algal isolates.

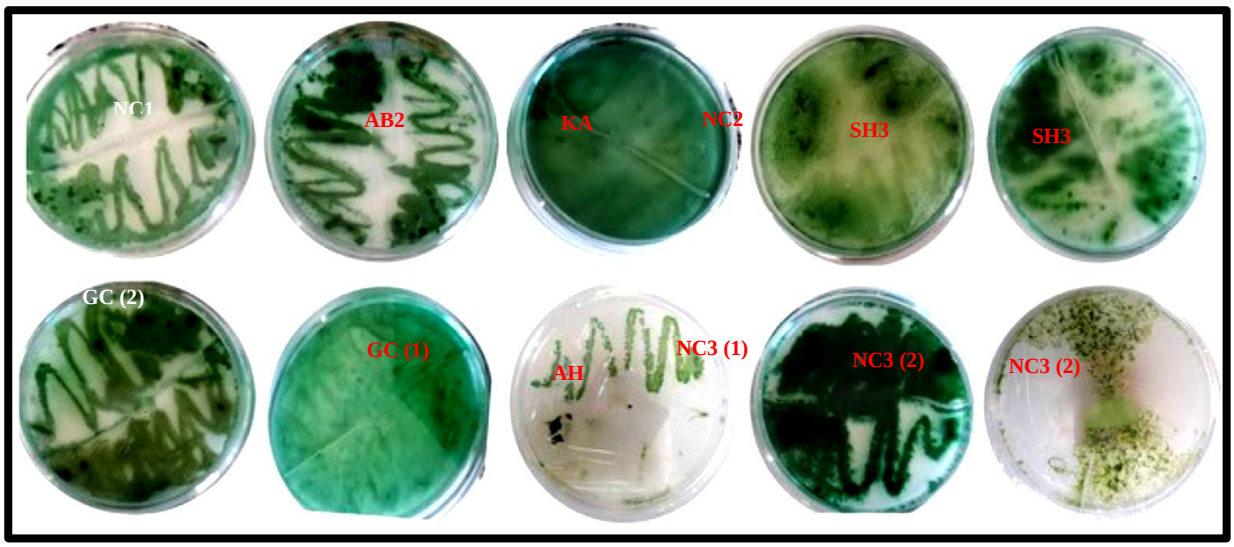


Photo (2): Axenic solid cultures of micro-algal isolate.

Table 6: Determination of Dry Weight for all Algal & Cyanobacteria Isolates grown in Wastewater samples for ten days.

Isolates	Wastewater Samples			
	Sample (1)	**sample (2)**	**Sample (3)**	**sample (4)**
AB 2	380±0.30	986.6±0.76	540±0.35	490.6±0.44
KA	280.6±0.28	1060±0.93	653.3±0.83	512.6±0.37
SH 3	326±0.09	400±0.20	960±0.10	506.6±0.18
GC (1)	--	273.3±0.17	420±0.60	950.6±0.74
GC (2)	--	320±0.33	853.3±0.45	688.6±0.88
AH	--	986.6±0.41	720±0.60	656±0.43
NC 1	503.3±0.3	320±0.38	306.6±0.11	296±0.95
NC 2	400±0.17	706.6±0.64	600±0.44	600±0.50
NC3(1)	--	673.3±0.77	440±0.35	511.3±0.15
NC3(2)	--	320±0.59	326.6±0.62	200±0.39

By Comparing the physical properties of wastewater before and after treatment by micro-algal biomass, data recorded in the **table (7)** showed that pH values were ranged from neutral to slightly alkaline with all used isolates. Electrical conductivity had the highest values with GC (2), the electrical conductivity of GC was lowered from 1.74 to 1.62 &1.3 dsm $^{-1}$ and electrical conductivity of SH1 was lowered from 2.83 to 1.38 dsm $^{-1}$. Recently, algae have become significant organisms for biological purification of wastewater since they are able to accumulate plant nutrients, heavy metals, pesticides, organic and inorganic toxic substances, and radioactive matters in their cells/bodies **[15].** The removal could be caused by the precipitation of phosphorus through the formation of hydroxyapatite due to elevated pH levels occurred as a result of microalgae activity **[39].** Various studies have shown the possibility for oleaginous microalgal culture in wastewater for the production of biodiesel, and microalgae showing significant effectiveness in eliminating pollutants from wastewater as well as the ability to accumulate lipid in the cell body **[40,41].** Microalgal species can be cultured in both municipal wastewater and industrial effluents for example, in starch wastewater and wastewater encompassing heavy metallic element **[42,43,44,45].** *Chlorella* sp., for example, was reported to remove 75% of the chemical oxygen demand (COD) from textile effluent while storing 20% lipid in dry mass **[46].** Moreover, *Botryococcus braunii* get rid of 100 and 65% of total phosphorous and nitrogen, correspondingly in wastewater **[47].**

Moreover, data presented in **Table (8)** indicated that the analysis of cations led to the fact that Na^{+} was lowered from 16.5 to 7.2 meq/l, by using SH1 isolate. Ca^{++} & Mg^{++} was lowered from 3.75 and 9.0 to 2.0 and 2.8 meq/l, respectively by KA isolate. NC2 lowered Ca^{++} from 16.210 to 3 meq/l, On the other hand, the highest values of Mg^{++} and K^{+} were attained by GC (2) and SH3 respectively. Also, analysis of anions showed that Cl^{-} & HCO_3^{-} were lowered from 12.2 &5.6 to 8.8 & 3.3meq/l in the presence of SH1 isolate, SO_4^{--} was lowered from 12.65 to 1.93meq/l by KA isolate and from 17.018 to 1.01meq/l by NC2.

Table 7: Comparison of physical properties of wastewater before and after treatment by micro-algal biomass.

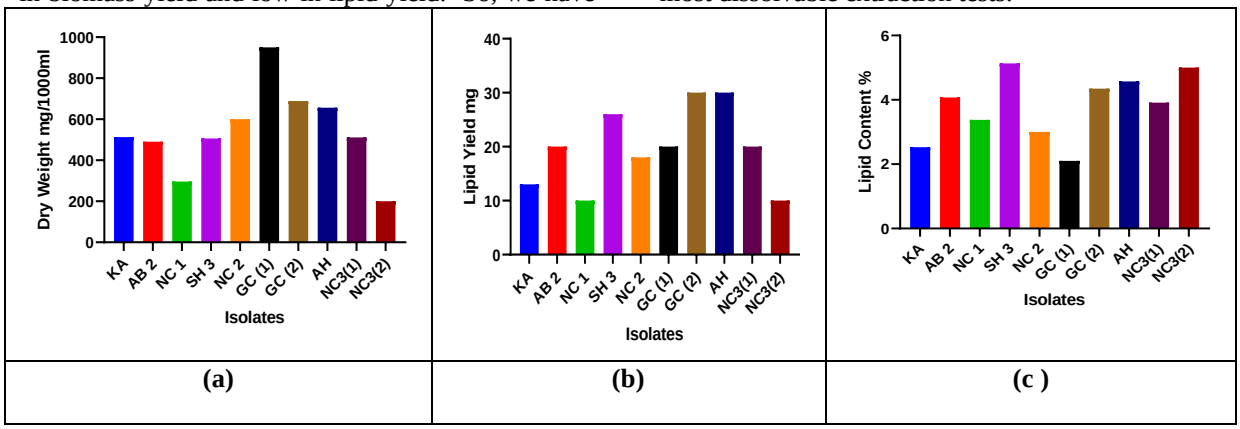
Isolates	After Treatment		Before Treatment	
	pH	**Electrical Conductivity (dsm^{-1})**	**pH**	**Electrical Conductivity (dsm^{-1})**
AB 2	7.7	0.77	9.5	0.57
KA	7.7	1.31	8.1	1.95
SH 1	7.9	1.38	8.1	2.83
SH 3	7	1.25	9.3	0.47
GC (1)	7.6	1.3	7	1.74
GC (2)	7.7	1.62	7	1.74
AH	7.9	1.52	6.9	1.34
NC 1	7.6	1.01	8.7	0.48
NC 2	7.6	1.16	7.53	0.16880 x 10^{-2}
NC3(1)	7.2	1.5	7.54	0.04630 x 10^{-2}
NC3(2)	7	1.22	7.54	0.04630 x 10^{-2}

Table 8: Comparison of cations and anions of wastewater before and after treatment by micro-algal biomass.

Isolates	Cations and anions of wastewater (meq/l)													
	After Treatment							Before Treatment						
	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	SO_4^{-}	Cl^{-}	HCO_3^{-}	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	SO_4^{--}	Cl^{-}	HCO_3^{-}
AB 2	1.5	0.5	5.3	0.40	0.7	5	2	1.5	1	3	0.3	2.3	2.5	1
KA	2	2.8	7.5	0.53	1.93	7	3.9	3.75	9	6	0.5	12.65	5.5	1.1
SH 1	2.5	3.3	7.2	0.48	1.38	8.8	3.3	2.5	8.5	16.5	0.6	10.3	12.2	5.6
SH 3	2.5	1	9	10.3	0.9	10	1.9	2.1	1	1.7	0.14	1.74	2	1.2
GC (1)	2	2.3	7.6	0.45	1.35	9	2	3	4.5	8.5	0.48	7.68	7.5	1.3
GC (2)	2.5	3.5	9.8	0.48	4.78	10	1.5	3	4.5	8.5	0.48	7.68	7.5	1.3
AH	2.8	3.3	9.3	0.48	2.08	11	2.8	3.5	4.5	5.2	0.43	5.63	5.5	2.5
NC 1	2.3	1	6.2	0.41	0.81	6.5	2.6	2.5	1	1.4	0.15	2.1	1.75	1.2
NC 2	3	3	5.2	0.41	1.01	7	3.6	16.210	2.679	2.816	0.719	17.018	3.984	1.0
NC3(1)	3.5	1	10.5	0.2	1.9	10	3.3	6.554	2.163	2.724	0.767	5.733	3.256	1.0
NC3(2)	2.5	2.2	7.4	0.3	0.8	8	3.6	6.554	2.163	2.724	0.767	5.733	3.256	1.0

3.3-Selection of the most potent micro-algal strains

Determination of the most intense microorganisms was done by lipid extraction from algal strains. Dissolvable extraction has generally been utilized to remove lipids for investigation in microbial science methodology. Dissolvable extraction includes the utilization of a dissolvable that coordinates with the extremity of the objective compound **[48]**, in this case, non-polar lipids. The results recorded in fig.1 indicated that GC (1) has the highest biomass yield of 950.6 mg/1000 ml followed by GC (2) with biomass yield of 688.6 mg/1000 ml, AH with biomass yield of 656 mg/1000 ml and NC2 with biomass yield of 600 mg/1000 ml. At the same time, there are strains high in biomass yield and low in lipid yield. So, we have chosen AB2 with a lipid yield of 20 mg and lipid content 4.07%, NC1 with a lipid yield of 10 mg and 3.378%, SH3 with lipid yield of 26 mg and 5.13%, GC (2) with a lipid yield of 30 mg and 4.35%, AH with lipid yield of 30 mg and 4.57%, NC3 (1) with lipid yield of 20 mg and 3.91% and NC3 (2) with lipid yield of 10 mg and 5%. The solvent should likewise connect with the lipids within the cell **[49]**, which for the most part requires a second, polar, dissolvable that can break the cell divider and layer. Bligh and Dyer **[35]** proposed a system utilizing chloroform, methanol, and water as cosolvents for separating and filtering lipids. Normally alluded to as the Bligh and Dyer technique, this strategy is utilized as a gauge for examination in most dissolvable extraction tests.



(a) (b) (c)

Figure 1: Selection of the most potent micro-algal strains according to dry weight and lipid content. Where (a) dry weight content, (b) lipid yield, and (c) lipid content.

3.4-Identification of the most potent micro-algal Strains

Identification of algal isolates was carried out in the microbiology and Botany Department, Faculty of Agriculture, Al-Azhar University, and as reported by **[50,51,52].** The four algal isolates were identified as *Oscillatoria chalybea, Oscillatoria brevis, Oscillatoria jenesis, and Phormidium fovealarum* (photo 3.) The selection of the most potent isolates was carried out according to lipid extraction from algal strains. Concerning the morphological characteristics of *Oscillatoria brevis,* the trichome of *Oscillatoria brevis* are blue green, straight, not choked at the cross dividers, closes momentarily lessened, all the less twisted, not capitates, 6.1µ expansive, 3.8µ long not granulated at the septa; end-cell adjusted, calyptra missing **[53].** On the other hand**,** the thallus of *Phormidsium foveolarum* is flimsy, dull green; trichome flexuous, contracted at the cross-dividers, at the closures not weakened, about 1.5 µ wide, light blue green; sheath boring, diffluent in a formless thick adhesive, not shaded violet by chlor-zinc-iodide; cells almost quadrate or to some degree more limited than wide, 0.8-1.8 µ long, septa not granulated; end cell adjusted, calyptra missing**.** Moreover, *Oscillatoria chalybea thallus* (Mertens) Gomont **is** dark blue green, trichome almost straight, tightened at cross dividers, weakened at the peak-end twisted, living trichomes show forward and rotatory development, 3-12 mm expansive cells, 1/2-1/3 times as long as wide or almost quadrate (4.5-5.5 mm wide), 2-8 mm long, septa not granulated, end cells harsh not capitates, without calyptra, gas vacuoles present **[54].** *Oscillatoria Jenesis* appeared as a total mass of dark brown trichomes 19.8-24.9 µm wide not contracted at the cross dividers, dim brown to some degree spiraled at the finishes, cells short end, dividers awry raised, not knobbed, or thickened, no granulation at the cross dividers.

3.5. Optimization of cultural and nutritional conditions for selection of the most promising microorganisms with the highest biomass and lipid content

In the present investigation, studying the environmental and nutritional factors which affect the vegetative growth of microalgae is carried out, then selecting the most potent microalgae isolates which have the highest biomass and lipid content. At the same time, studying wastewater treatment by axenic species of microalgae.

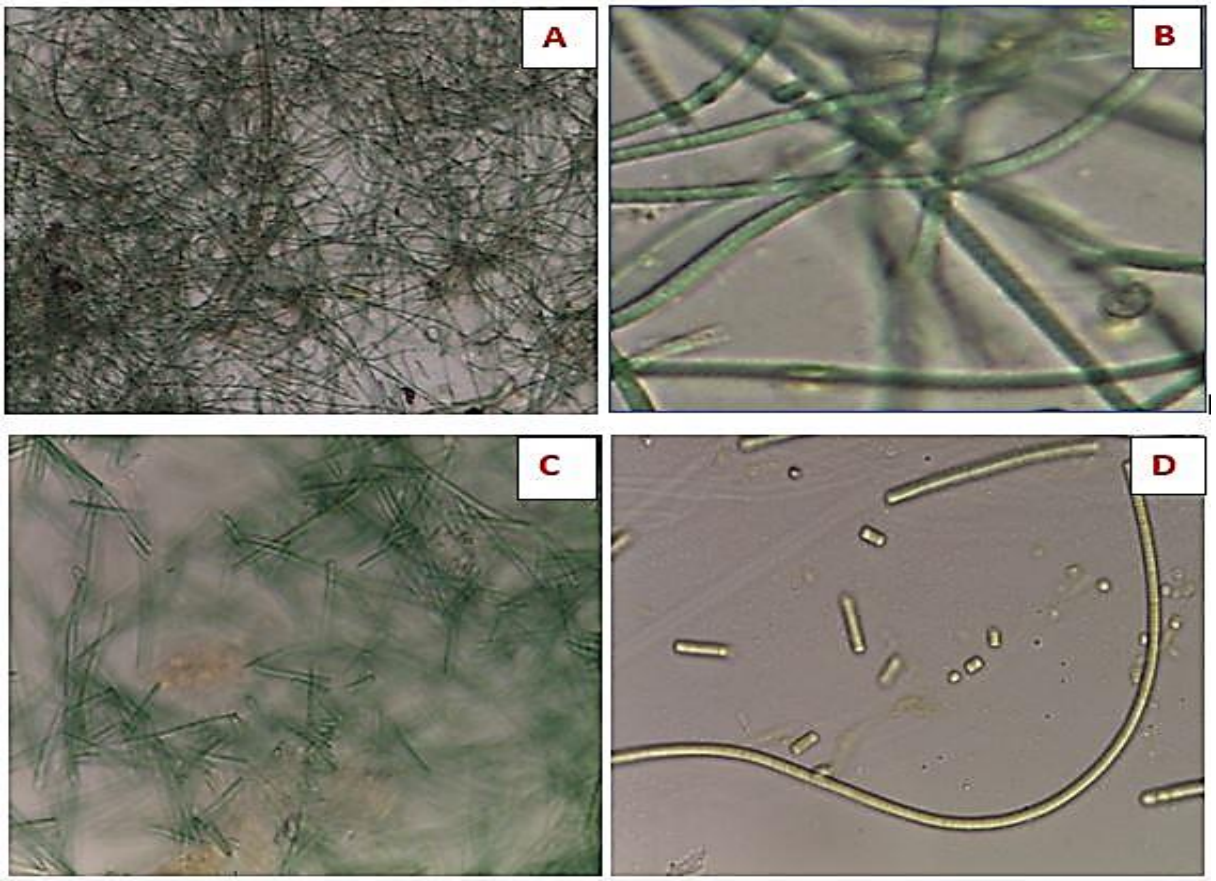


Photo (3): Purified microalgal isolates under the microscope (A): *Phormidium Foveolarum,* (B): *Oscillatoria Chalybea,* (C): *Oscillatoria Brevis, (*D) *Oscillatoria Jenesis*

The effect of different incubation periods on micro-algal isolates growth was studied. Different incubation periods were (7, 14, and 21 days). Results illustrated in **Fig. (2)** showed that microalgal isolates emphasize gradual growth from 7 days incubation to 21 days incubation.

In the present investigation, the effect of different concentrations of sodium nitrate on the growth of micro-algal isolates was tested. Different sodium nitrate concentrations (0.5, 1, 1.5, 2, and 2.5 g/l) were introduced in the BG-11 medium separately to study their effect on micro-algal isolates. Results recorded **Fig. (3)** showed that micro-algal isolates emphasize gradual growth from 0.5g to 2g $NaNO_3$ concentration then decline happened except for *Oscillatoria Chalybea (1)* has high dry weight (270.9 mg/200 ml) and high lipid content 2.69%. *Oscillatoria Chalybea (5)* has the highest dry weight (557.1 mg/200 ml) but is low in lipid content (0.59%) followed by *Oscillatoria Chalybea (3)* with a dry weight of (303.3 mg/200 ml) and high in lipid content (2.11%) and *Oscillatoria Chalybea (2)* with a dry weight of (299.5 mg /200 ml). Whereas, *Oscillatoria Jenesis* has high lipid content (2.16%) but low in dry weight (235.9 mg/200 ml). Currently, algal cultivation predominantly uses nitrogen fertilizer produced from the Haber–Bosch process **[55].** Enhanced biomass output from Nostoc cultures at low nitrogen levels appears to be linked not to enhanced cellular production or intracellular metabolite accumulation, but to an increase in exopolysaccharide formation, which is characteristic in cyanobacteria strains facing nutritional stress **[56].** Since there is no easy way to calculate dry weight from free cells that lack capsular polysaccharide, it was included in the dry weight value **[57].**

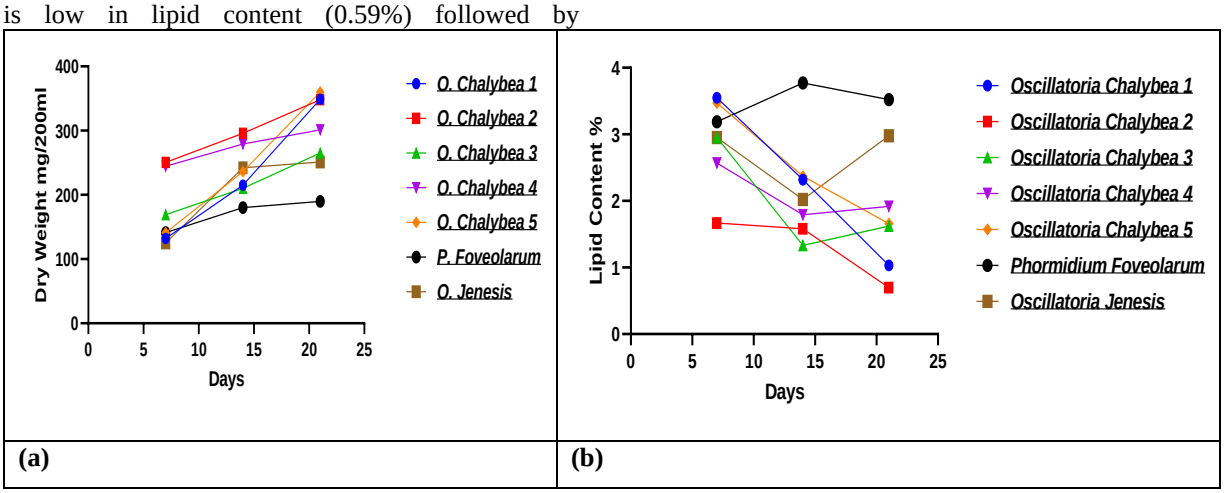


Figure 2: Effect of different incubation periods on micro-algal isolates. (a) On dry weight content (b) on lipid content

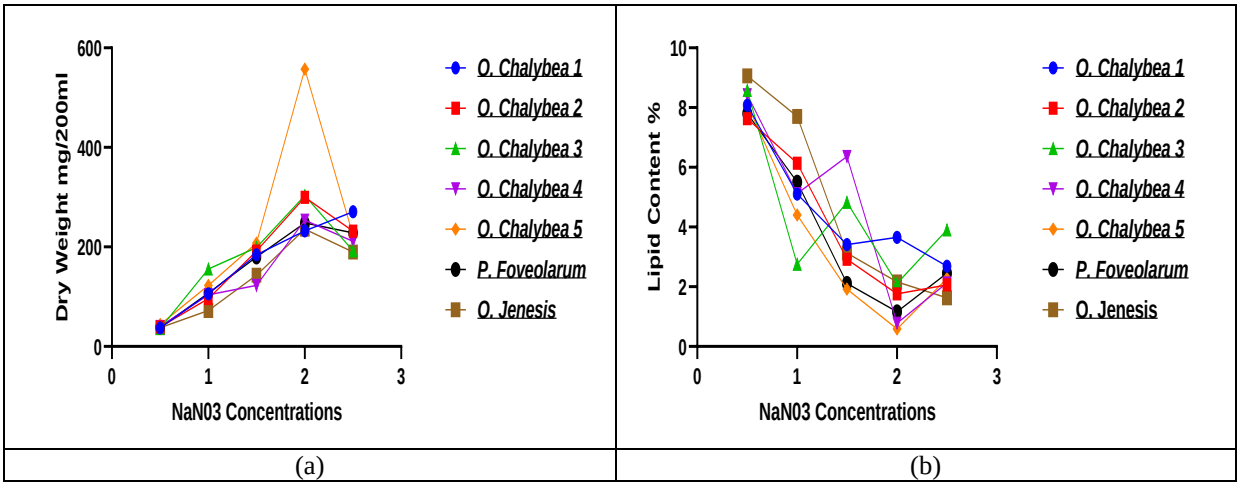


Figure 3: Effect of different $NaNO_3$ concentrations on most promising isolates to dry weight and lipid content.

Concerning the effect of different pH values on the growth of microalgal isolates, **Fig. (4)** showed that *Oscillatoria chalybea (1)* has the highest dry weight with pH 7.1, *Oscillatoria chalybea (2)* has the highest dry weight with pH 6.1, *Oscillatoria chalybea* (3) has the highest dry weight with pH 8.4, *Oscillatpria*

chalybea (4) has the highest dry weight with pH 7.1, *Oscillatoria chalybea (5)* has the highest dry weight with pH 7.1, *Phormidium fovealarum* has the highest dry weight with pH 8.4 *and Oscillatoria jenesis* has the highest dry weight with pH 7.1. *Oscillatoria chalybea (1)* has high lipid content (3.54%) but low in dry weight (132.4 mg/200 ml), *Oscillatoria jenesis* has high lipid content (3.23%) but low in dry weight (130 mg/200 ml), *Oscillatoria chalybea (3)* has high dry weight (214 mg/200 ml) but low in lipid content (0.84%) and Oscillatoria *chalybea (5)* has high dry weight (417 mg/200 ml) but low in lipid content (0.31%). Microalgae absorb nitrogen, which raises the pH of the medium since every nitrate ion converted to ammonia creates one OH- ion **[58].** Higher pH values may induce phosphate precipitation throughout the medium, but it can be stopped by lowering the pH value through respiration, which does not involve CO2 assimilation. The impact of pH on microalgae in wastewater report shows that the medium at a steady pH value of 7.0 has the fastest growth of microalgal species **[59].**

Different sets of K_2HPO_4 (10, 40, and 70 mg/l) were used to investigate the effect of K_2HPO_4 on the growth of microalgal isolates using BG-11 medium, the original K_2HPO_4 concentration in the medium was 40 mg/l. Results in **Fig. (5)** indicated that micro-algal isolates emphasize a gradual increase in growth from 0.01g to 0.07 g K_2HPO_4 concentrations except for *Oscillatoria chalybea* (4) which showed a decrease in growth from 0.01g to 0.04 g then an increase in growth from 0.04 g to 0.07 g. *Oscillatoria chalybea* (4) had the highest lipid content 6.84%. Phosphate is indeed a component of the DNA and RNA backbones, which are crucial macromolecules for any and all living cells. Phosphates, and nitrates, are important nutrients in our ecosystems on land and in water **[60,61].**

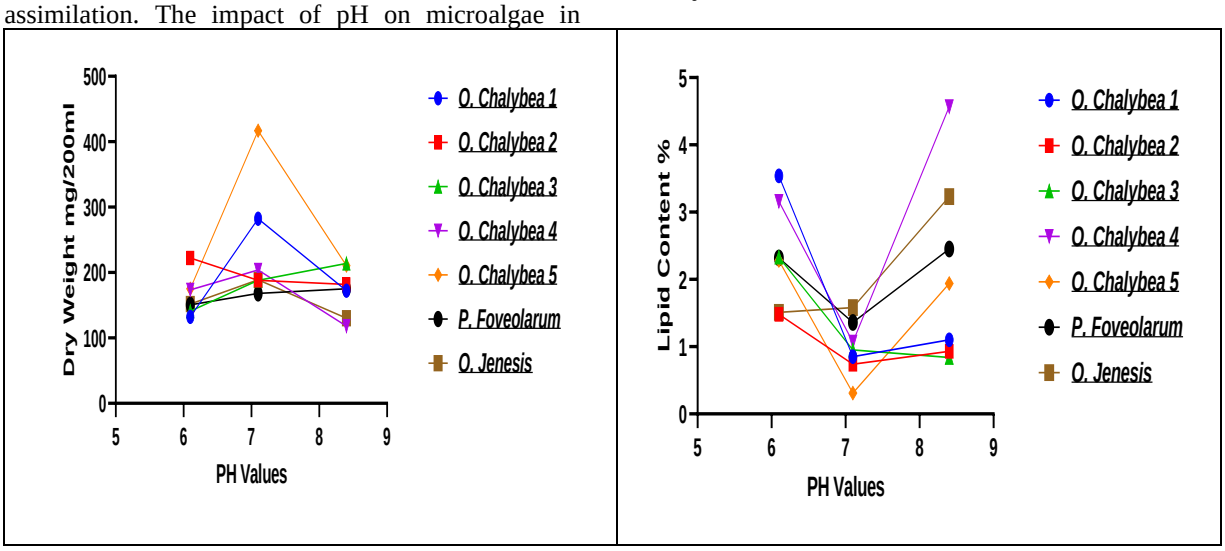


Figure 4: Effect of different pH values on dry weight and lipid content of microalgal isolates (a) On dry weight (b) on lipid content.

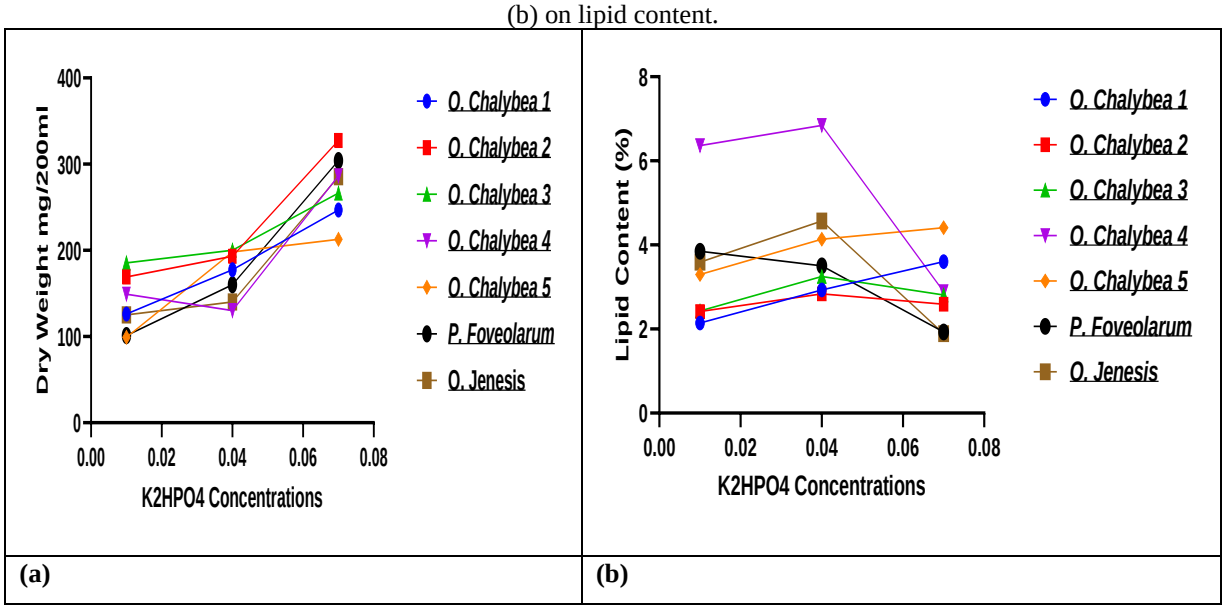


Figure 5: Effect of different K_2HPO_4 concentrations on dry weight and lipid content of microalgal isolates. (a) dry weight (b) lipid content.

Effect of different $MgSO_4$ concentrations on isolated algae was shown in **Fig. (6)** and the results revealed that *Oscillatoria chalybea* (1), (2) and (3), *Phormidium foveolarum* and *Oscillatoria jenesis* showed a gradual increase in $MgSO_4$ concentrations from 0.045 g to 0.075 g then growth reduced from 0.075g to 0.105g. *Oscillatoria Chalybea* (4 and 5) showed a decline in growth from 0.045g to 0.075g then growth raised from 0.075g to 0.105g. *Oscillatoria Chalybea* (5) had the highest lipid content 5% and the highest dry weight 384.1mg. Magnesium is necessary for the proliferation of microalgal cells. Chlorophyll is made up of a hydrocarbon phytol chain and a tetrapyrrole ring structure that surrounds a chelated Mg ++ ion, which is essential for light intake in green plants. This indicates that one Mg ++ molecule is needed per each molecule of chlorophyll generated **[62].**

Data represented in **Fig. (7 a &b)** revealed that *O. chalybea (5)* showed the highest dry weight with $NaNO_3$ among all the tested nitrate sources tested in this study, whereas *O. chalybea (3)* showed both the highest dry weight and lipid content %. However, NH_4Cl exhibited a stimulatory effect on the dry weight of all the tested isolates *O.chalybea (2), O. chalybea (3), O. chalybea (4), O. chalybea (5), P. fovealarum*, and *O. jenesis* with 240.3, 260.3, 257.3, 287.1, 236.8, and 239.5 mg/200ml, respectively except isolate *O. chalybea (1)*. On the other hand, NH_4Cl has a repressive effect on lipid content production with all tested isolates. Moreover, NH_4VO_3 promote lipid production but inhibit dry weight formation with all the tested isolates. The fantastic result was obtained with $NaNO_3$ which stimulate both dry weight and lipid content *O. chalybea (3)* with 198.7 mg/200ml and 4.83% respectively. Nitrogen (N) exists in water in a variety of soluble forms. These N forms have a variety of effects on algae and cyanobacteria populations, owing to their ability to convert different N sources into biomass and competing with other species. Cyanobacteria can convert N to ammonium (NH4+) within the cell before they can use it for biomass or toxin production, regardless of the type of N. Ammonium also is the simplest form of nitrogen to get and move into the cell for photosynthetic organisms. Nitrate and nitrite (NO3/NO2) must be actively carried into in the cells and transformed to ammonium, which necessitates the use of energy and micronutrients like iron **[63].**

Accordingly, after all the previously optimized conditions a rescreening was carried out to select the most potent isolates of high dry weight, lipid content, and lipid yield %. *Oscillatoria chalybea* (2), *Oscillatoria chalybea* (3), and *Oscillatoria chalybea* (5) according to this system determination as shown in **Fig. (8) and photo 4.** From the illustrated results, *Oscillatoria chalybea* (2) showed the highest dry weight (1028.4 mg/2000ml) and also moderate lipid yield (0.72%), so it was selected for further investigations. Also, *Oscillatoria chalybea* (5) produced the highest lipid yield (1.01 %) and moderate dry weight (524.4 mg/2000 ml), while *Oscillatoria chalybea* (3) produced a remarkable lipid yield (0.9 %) with moderate dry weight content (480.4 mg/2000ml).

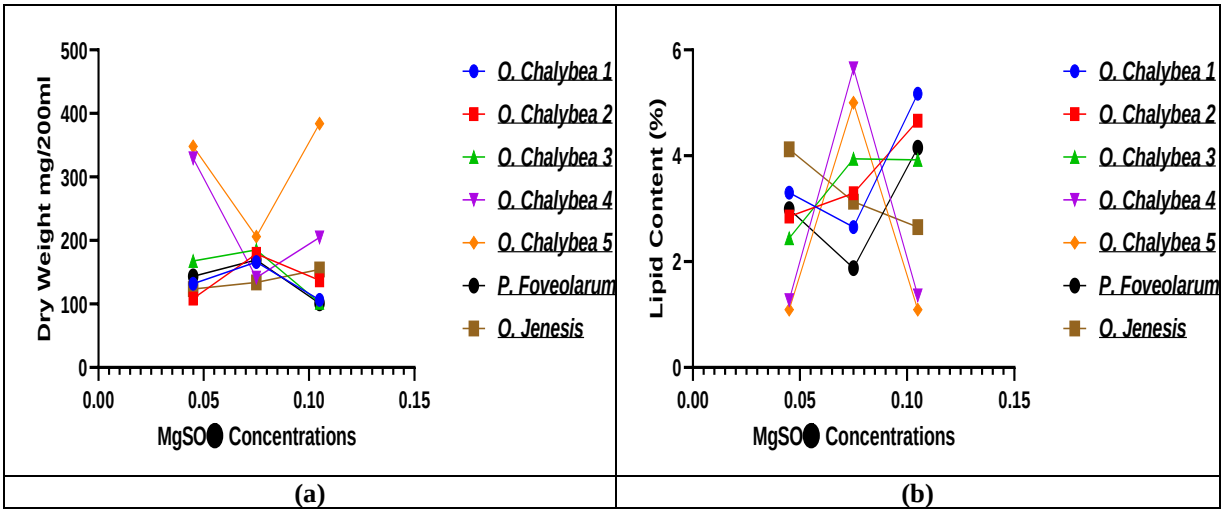


Figure 6: Effect of different $MgSO_4$ concentrations on dry weight and lipid content of microalgal isolates. (a) dry weight (b) lipid content.

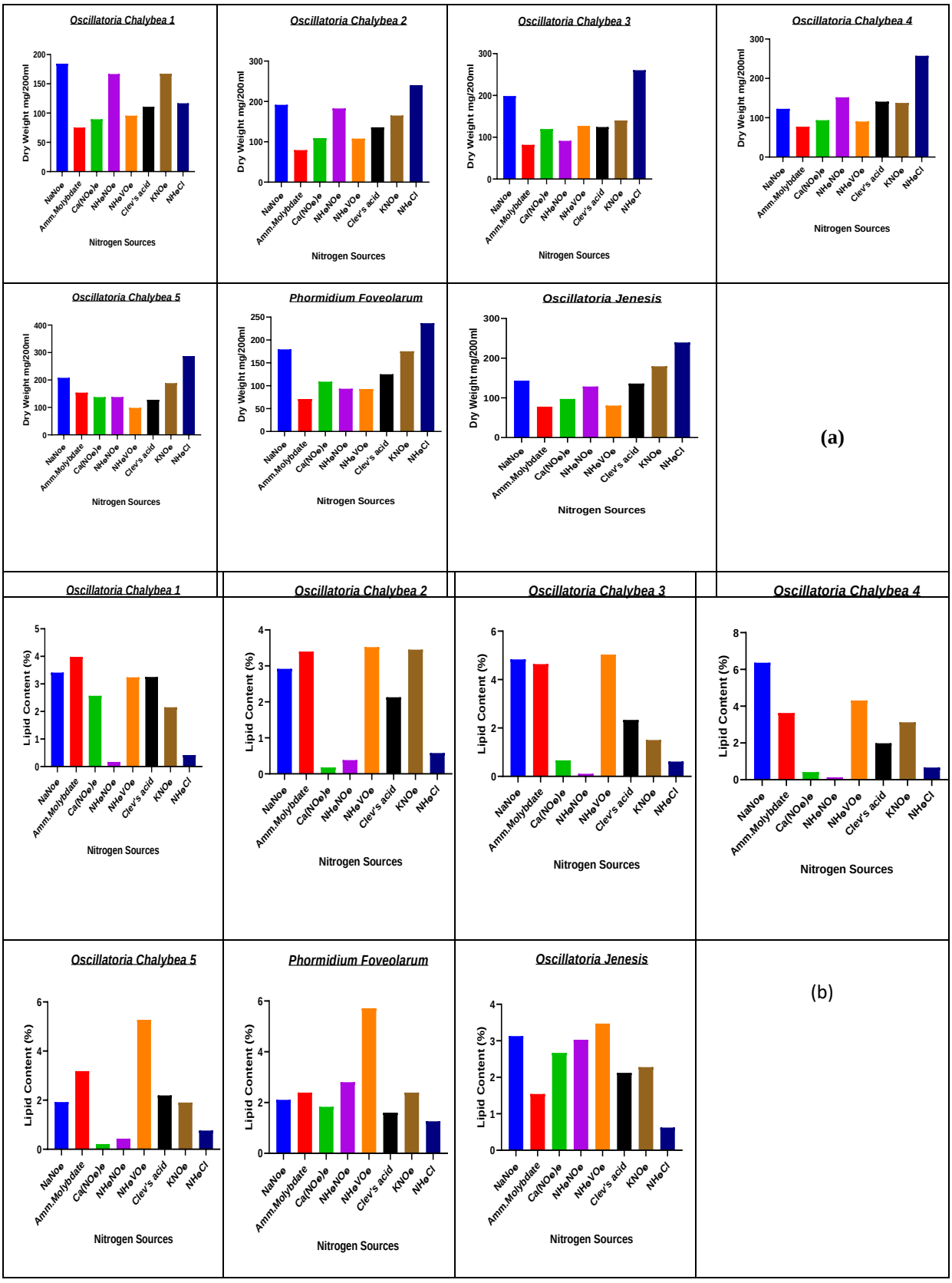
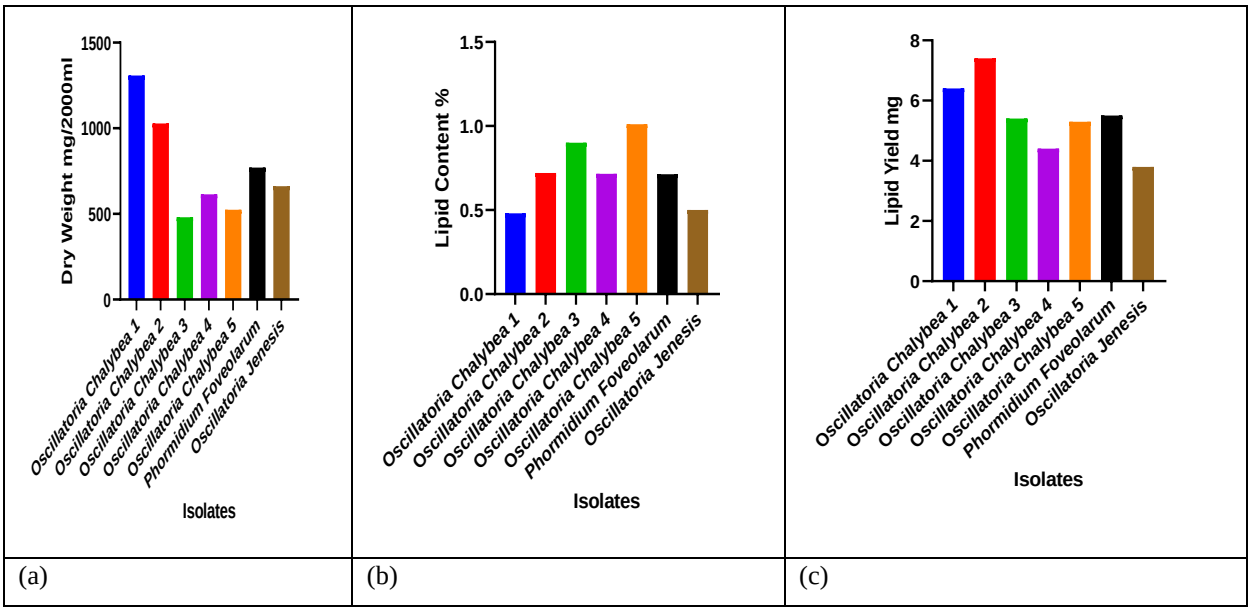
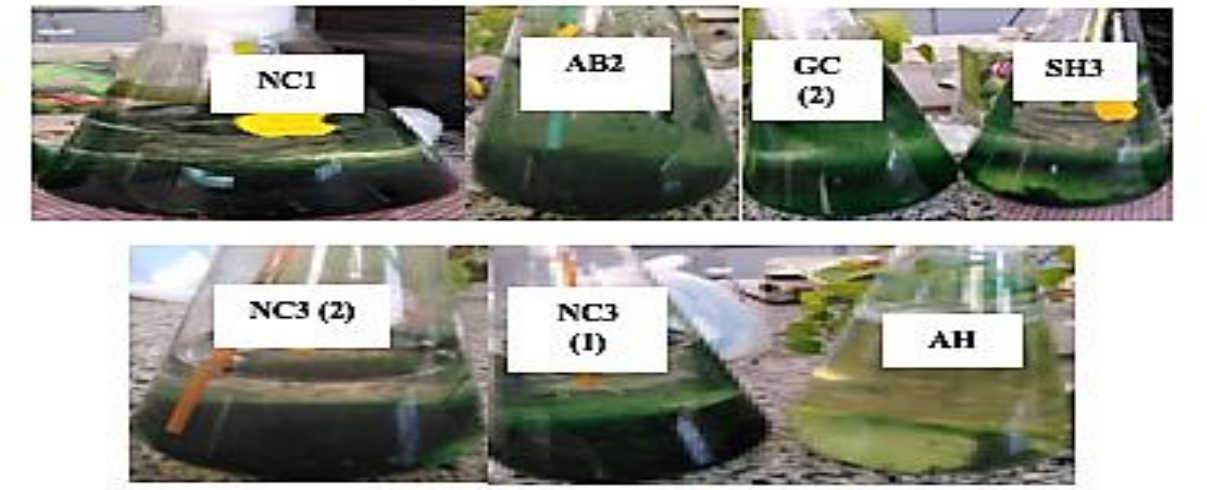


Figure 7 (a&b): Effect of different nitrogen sources on dry weight and lipid content of microalgal isolates.

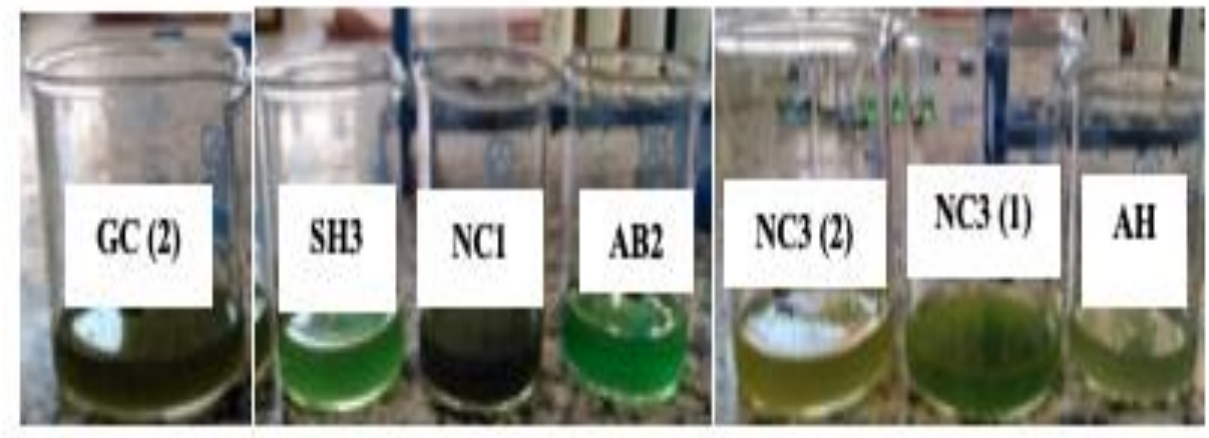


(a) (b) (c)

Figure 8: Rescreening for selection of the most potent isolate for further investigations. According to dry weight (a), lipid content (b), and lipid yield (c).



A



B

Photo 4: Biomass yield (a) and extracted lipid content from (b) of the most promising isolates.

3.6-Biodiesel Production

In the present study, local microalgae with a high growth rate and lipid content were selected and evaluated according to their dry weight, lipid content, and yield **(Table 9).** The lipid content of isolates *Oscillatoria chalybea (2), (3),* and *(5)* was extracted to determine fatty acid methyl ester as shown in **photo 5.** Because even though microalgae are not currently used during industrial oil production, experts think that advances in system biology, genetic manipulation, and modern technology will enable the advancement of a cost-effective industrial process for producing biodiesel from microalgal oils within next 10–15 years. The buildup of neutral lipids in Oleaginous microalgae ranges from 20% to 50% of the dry mass of the cell. Green algae Chlorophyta and diatomic algae Bacillariophyta have the ability to amass the greatest amount of lipids, are easy to culture, and show promise for industrial usage **[64].** Microalgae produce/generate a variety of renewable fuels, including biogas from anaerobic digestion of microalgae biomass, biofuel from microalgae oil, and photochemical hydrogen generation **[65].** As the fourth largest power source next to fossil-based sources, biomass and combustible material renewable sources are promising candidates with the potential to meet global energy **[66, 67, 68, 69].** Additionally**,** over than 80% of global biofuel is made up of biodiesels, which are mixes of fatty acid methyl esters generated by transesterification methods of biomasses **[70,71].**

Table 9: Total Dry Weight of *Oscillatoria chalybea* sp.

Isolates	Dry Weight (g/10L)
Oscillatoria chalybea (2)	**7.7421**
Oscillatoria chalybea (3)	**2.6115**
Oscillatoria chalybea (5)	**2.0814**

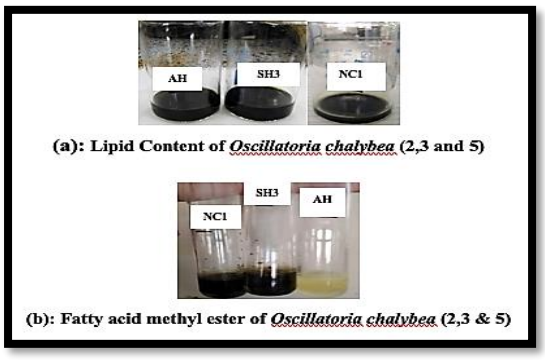


Photo 5: Lipid content and fatty acid methyl ester of *Oscillatoria chalybea* (2,3& 5).

3.7-Fatty acid profile analysis

The lipid composition was determined as fatty acid methyl esters (FAMEs) through the direct transesterification method proposed by **[72].** The major fatty acid composition of the tested ***Oscillatoria chalybea*** isolates was determined using a GC analysis. Results recorded in **Table (10)** and **Fig. (9)** provide detailed information about ***Oscillatoria chalybea*** isolates lipid profile during lipid accumulation. Caproic acid (C6:0), Caprylic acid (C8:0), Capric acid (C10:0), Undecanoic acid (C11:0), Laueic acid (C12:0), Tridecanoic acid (C13:0), Myristic acid (C14:0), Myristoleic acid (14:1), Pentadecanoic acid (C15:0), Cis-10-Pentadecenoic (C15:1), Palmitic acid (C16:0), Palmitoleic acid (C16:1n 7), Palmitoleic acid (C16:1n 9), Heptadecanoic acid (C17:0), Stearic acid (C18:0), Oleic acid (C18:1n9c), Elaidic acid (C18:1n9t), Linoleic acid (C18:2n6c), Arachidic acid (C20:0), γ- Linolenic acid (C18:3n6), Cis-11-Eicosenoic acid (C20:1), Linolenic acid (C18:3n3), Heneicosanoic acid (C21:0), Cis -11,14-Eicosadienoic acid (C 20:2), Cis-8,11,14-eicosetrienoic acid (C20:3n6) and Cis-11,14,17-Eicosetrienoic acid (C20:3n3) were recognized as the most common fatty acids contained in isolates of ***Oscillatoria chalybea*** biodiesel. Data also showed that, ***Oscillatoria Cha 9lybea*** **(2)** has the highest values of Umdecanoic acid (C11:0) 24.23%, Myristoleic acid (14:1)1.63%, Cis-11- Eicosenoic acid (C20:1)7.20% and Linolenic acid (C18:3n3) 7.05% , ***Oscillatoria Chalybea*** **(3)** has the highest values of Caprylic acid (C8:0) 2.21%, Laueic acid (C12:0) 1.68%, Tridecanoic acid (C13:0) 3.19%, Myristic acid (C14:0) 1.64%, Pentadecanoic acid (C15:0) 3.90%, Cis-10-Pentadecenoic (C15:1) 2.08%, Palmitoleic acid (C16:1n 9) 4.27%, Heptadecanoic acid (C17:0) 15.04%, Arachidic acid (C20:0) 1.85%, γ- Linolenic acid (C18:3n6) 3.29%, Heneicosanoic acid (C21:0) 6.92%, Cis -11,14-Eicosadienoic acid (C 20:2) 5.53% and Cis-8,11,14-eicosetrienoic acid (C20:3n6) 4.69%, while ***Oscillatoria Chalybea*** **(5)** has the highest values of Caproic acid (C6:0) 3.03%, Palmitic acid (C16:0) 15.60%, Palmitoleic acid (C16:1n 7) 7.69%, Stearic acid (C18:0) 3.47%, Oleic acid (C18:1n9c) 10.19%, Elaidic acid (C18:1n9t) 5.85%, Linoleic acid (C18:2n6c) 9.56% and Cis-11,14,17-Eicosetrienoic acid (C20:3n3) 3.40% .

Given the findings of other investigators, the detected fatty acids were recognized as the most common fatty acids contained in microalgal lipids as reported by **[73].** In the present study, Palmitoleic acid (C16:1n 9) and Cis-11,14,17-Eicosetrienoic acid (C20:3n3) don't Present in ***Oscillatoria Chalybea*** **(2)**, Capric acid (C10:0), Cis-11-Eicosenoic acid (C20:1) and Cis-11,14,17-Eicosetrienoic acid (C20:3n3) don't Present in ***Oscillatoria Chalybea*** **(3),** where Capric acid (C10:0), Myristoleic acid (14:1), Cis-10-

Pentadecenoic (C15:1), Arachidic acid (C20:0), γ-Linolenic acid (C18:3n6) and Linolenic acid (C18:3n3) don't Present in ***Oscillatoria Chalybea*** **(5).** Therefore, the highest content of Umdecanoic acid (C11:0) of tested ***Oscillatoria Chalybea*** **(2)** makes it most suitable for the production of good quality biodiesel. Laboratory investigations on lipid extraction with such a wet feedstock like algae revealed that hexane alone would be insufficient to fully recover lipids **[74, 75].**

Table 10: Fatty acid composition of *O. chalybea* isolates

No.	Type of fatty acid	Fatty acids	Relative concentration (%)		
	Saturated. and Unsaturated.		*O. chalybea* (2)	*O.chalybea*(3)	*O.chalybea*(5)
1.	Sat.	Caproic acid (C6:0)	1.72	2.39	3.03
2.	Sat.	Caprylic acid (C8:0)	1.48	2.21	1.88
3.	Sat.	Capric acid (C10:0)	0.62	---	---
4.	Sat.	Umdecanoic acid (C11:0)	24.23	18.55	10. 29
5.	Sat.	Laueic acid (C12:0)	0.62	1.68	---
6.	Sat.	Tridecanoic acid (C13:0)	1.36	3.19	2.20
7.	Sat.	Myristic acid (C14:0)	0.65	1.64	1.61
8.	Monounsat.	Myristoleic acid (14:1)	1.63	1.49	---
9.	Sat.	Pentadecanoic acid (C15:0)	2.42	3.90	1.65
10.	Monounsat.	Cis-10-Pentadecenoic (C15:1)	0.50	2.08	---
11.	Sat.	Palmitic acid (C16:0)	6.75	6.01	15.60
12.	Monounsat.	Palmitoleic acid (C16:1n 7)	5.24	1.27	7.69
13.	Monounsat.	Palmitoleic acid (C16:1n 9)	---	4.27	1.37
14.	Sat.	Heptadecanoic acid (C17:0)	11.65	15.04	9.12
15.	Sat.	Stearic acid (C18:0)	1.75	1.73	3.47
16.	Monounsat.	Oleic acid (C18:1n9c)	4.00	5.08	10.19
17.	Monounsat.	Elaidic acid (C18:1n9t)	1.40	2.10	5.85
18.	Polyunsat.	Linoleic acid (C18:2n6c)	2.65	3.97	9.56
19.	Sat.	Arachidic acid (C20:0)	1.46	1.85	---
20.	Polyunsat.	γ- Linolenic acid (C18:3n6)	2.50	3.29	---
21.	Monounsat.	Cis-11- Eicosenoic acid (C20:1)	7.20	---	2.85
22.	Polyunsat.	Linolenic acid (C18:3n3)	7.05	1.14	---
23.	Sat.	Heneicosanoic acid (C21:0)	6.72	6.92	1.88
24.	Polyunsat.	Cis -11,14- Eicosadienoic acid (C 20:2)	3.09	5.53	5.30
25.	Polyunsat.	Cis-8,11,14-eicosetrienoic acid (C20:3n6)	3.32	4.69	1.49
26.	Polyunsat.	Cis-11,14,17-Eicosetrienoic acid (C20:3n3)	---	---	3.40
Saturated.% Unsaturated %			61.43:38.58	65.11:34.91	50.73:47.7

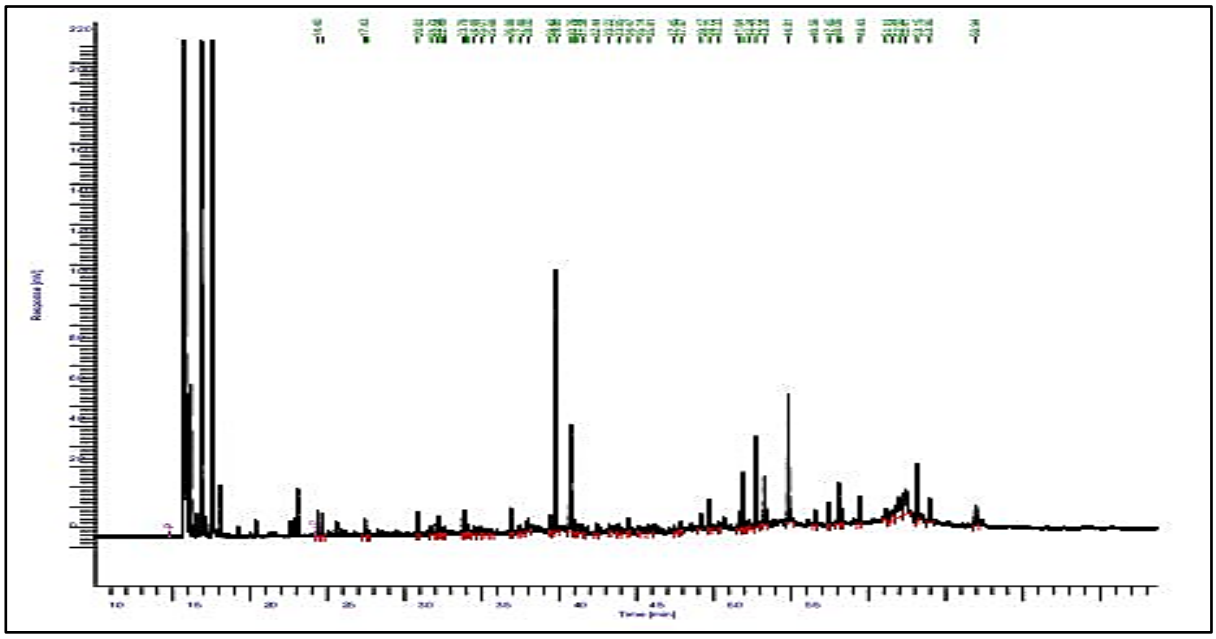


Figure. 9: Determination of fatty acid composition of the Oscillatoria chalybea isolates by GC.

The TFA relative composition did not show any significant difference ($p > 0.05$, ANOVA) in each culture conditions as it is summarized in **(Table 11).** The most abundant fatty acids were palmitic acid, oleic cis and oleic trans acids with a relative percentage of 24.5%, 22.4% and 20.4%, respectively. The C16/C18 fatty acid groups reached up the 89% of total fatty acid, according to what observed in *S. obliquus* CNW-N when cultured in nutrient deficient medium **[71].** moreover, in our results oleic acid is important indicator of biodiesel quality, occupied over 42% of total fatty acids, a better result compared with what achieved by **[72]** in a nutrient-deficient medium and 10% of CO2 feeding concentration.

Table 11: Summary of the total fatty acid composition (%) representative for each culture conditions.

Fatty acids	Relative composition
Hexadecadienoic	14.3%
Hexadecatrienoic	4.1%
Palmitic	24.5%
Linoleic	12.3%
Oleic Cis	22.4%
Oleic Trans	20.4%
Stearic	2.0%

Conclusion

Recently, researchers in the field of non-traditional sources of energy received a lot of world attention because of the large gap between the world's reserves of conventional energy sources and its needs. Algae have become the latest feasible source being targeted for biofuel production since they exhibit several attractive features. This study falls under the banner of one of these attempts, or what they call the third generation of biofuels. In the present work, the environmental and nutritional factors which affect the vegetative growth of microalgae are carried out, then selecting the most potent microalgae isolates which have the highest biomass and lipid content. At the same time, studying wastewater treatment by axenic species of microalgae. The results evoked the superior activity of the isolated microalgal strains in the treatment of wastewater Moreover, Oil is extracted from microbial biomass and converted into biodiesel by a trans-esterification method. Our results indicated high content of Umdecanoic acid (C11:0) of tested *Oscillatoria Chalybea* **(2)** which makes it most suitable for the production of good quality biodiesel.

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