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Qualitative and quantitative determination of lipid content in microalgae for biofuel production

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Abstract
Isolation of high neutral lipid containing microalgae is a key for biofuel production. The florescent method has been successfully applied to the determination of lipids in certain microalgae, but has been un successful in many others, particularly those with thick, rigid cell walls that prevent the penetration of the dye. The cellular neutral lipids were determined and quantified using florescent Olympus microscope CKX41. Conventional method for quantitative lipid determination was done to verify the results of Nile red. Among the investigated species which gave the highest lipid percentage were *Microcystis aeruginosa* and *Chlamydomonas variabills* gave 30%±0.3 and 21%±0.5 respectively.

Introduction

Algal lipids have been suggested as a potential fuel substitute (**Abdeshahian *et al*., 2010; Jegannathan *et al*., 2011**) due to their accumulation inside the cells at the end of the growth stage (**Mohammady *et al*., 2012; Mohammady and fathy, 2007**). Biodiesel and different biohydrocarbons are most constituent fuels extracted from algal lipid (**Chisti, 2006**). Therefor, microalgal strains with high lipid content are of great interest in the search for a suitable feedstock for the production of biofuels (**Sharma *et al*., 2012).**

The most common lipids are composed of a glycerol molecule bound to three fatty acids, known as triacylglycerol (TAG), or to two fatty acids with the third position taken up by a phosphate (phospholipids) or carbohydrate (glycolipids) group. Fatty acids consist of a long unbranched carbon chain. They are classified according to the number of carbon atoms in the chain and the number of double bonds, for example saturated (no double bonds), monounsaturated (one double bond) or polyunsaturated (more than one double bond). Microalgae commonly contain fatty acids ranging from C12 to C24, often with C16 and C18 unsaturates. Certain species contain significant amounts of polyunsaturated fatty acids. Storage lipids, generally in the form of TAG, accumulate in lipid vesicles called oil bodies in the cytoplasm **(De la Pena, 2007)**

Most reports regarding lipid quantization in algae are based on indirect methods such as gravimetric measurement of crude lipid extracts, Nile Red fluorescence or related dye-partition assays, or gas chromatography (GC) analysis of lipid-derived fatty acid methyl esters (FAME).

Nile red can be applied to cells in an aqueous medium, and it does not dissolve the lipids it is supposed to reveal the formation of cytoplasmic lipid droplets is a normal cellular process. The droplets are neutral lipids, usually triacylglycerols or cholesteryl esters. The former serve as fatty acid energy reserves and the latter function as storage depots for excess cellular cholesterol **.** Nile red is intensely fluorescent and, if proper spectral conditions are chosen, it can serve as a sensitive vital stain for the detection of cytoplasmic lipid droplets **(Greenspan *et al*., 1985).**

Nile Red fluorescence has become one of the most popular methods for determining neutral lipids in algae **(Cooksey *et al*., 1987)**. At a minimum, Nile Red data must be confirmed by other more definitive techniques.

Numerous methods for extraction of lipids from microalgae have been applied; but most common methods are expeller/oil press, liquid–liquid extraction (solvent extraction), supercritical fluid extraction (SFE) and ultrasound techniques **(Harun *et al*., 2010)**.

Materials and Methods

Culture Condition

Algal species were isolated from River Nile water which concentrated via phytoplankton net (80μm mesh size) and using BG11 media and Diatom Medium, Modified for algal isolation **(Carmichael, 1986) (Cohn *et al*., 2003)** as mentioned in **Abdo *et al*., 2013.**

Cultivation of the Isolated Strains

Cultivation was carried out in sterilized 1 liter conical Shoulder flasks containing 600ml of the corresponding culture medium under continuous illumination. The cultivation time differed from one strain to another depending on the optimum growth rate till reaching stationary phase which always ranged between (15-20) days.

Harvesting of Cells

When the culture reached stationary phase, the biomass was harvested dried in hot air oven at 60∘C.

Intracellular Lipid Identification by Nile Red Staining.

Nile Red Staining

To isolate the microalgal cells (0.5 ml) from the collected water samples were achieved through centrifuge at 1,500 rpm (Rev./min.) for 10 min and sediments were washed with physiological saline solution (0.5 ml) for several times. The collected microalgal cells were suspended in 0.5 ml of Nile red solution (0.1 mg of Nile red/ml of acetone) and incubate for 10 min at room temperature. After washing once with water, stained microalgal cells and the intracellular lipid contents observed by using fluorescent microscopy. **(Matsunaga *et al*., 2009; Elumalai *et al*., 2011).**

Lipid Extraction

All species after harvesting were subjected to two methods for oil extraction:

A- Modified method of Bligh and Dyer **(Bligh and Dyer, 1959).**

B- Hexane-Isopropanol Extraction method **(Halim *et al*., 2011).**

Results and Discussion

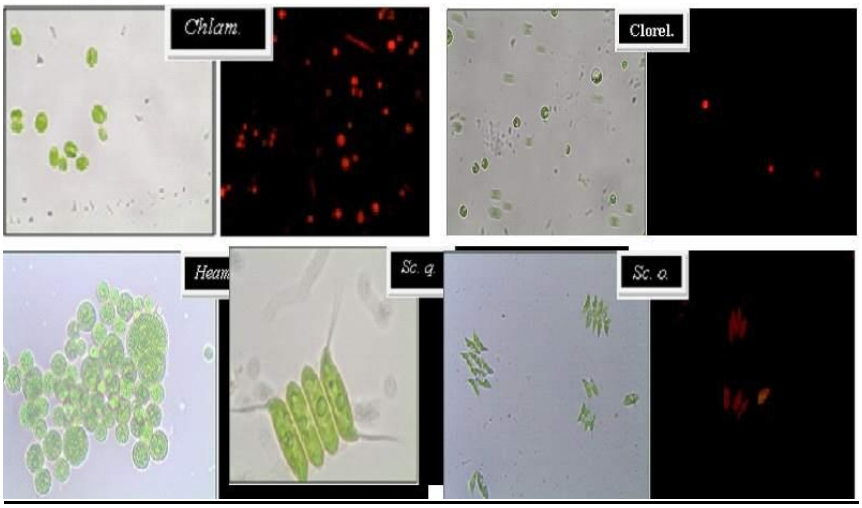
Four green microalgal isolates were used *Chlamydomonas variabills*, *Chlorella vulgaris Haematococcus pluvialis, Scenedesmus obliquus* and *Scenedesmus quadriquada*), six blue green isolates *Chroococcus turgidus*, *Merismopedia elegans* (isolated from wastewater samples) *Microcystis aeruginosa*, *Oscillatoria limnetica*, *Oscillatoria limosa, Phormidium rimosum* and two diatom isolates *Navicula cuspidata and Nitzschia linearis*. Intracellular lipid droplets of were observed by Nile Red staining under fluorescent microscope with excitation at 450–490-nm and emission at 515-nm. Neutral lipid or triglycerides appeared as yellow dots, whereas polar lipid and chlorophyll were stained in red colour cells were observed by Nile Red staining under fluorescent microscope with excitation at 450–490-nm and emission at 515-nm.

Nile red staining: Nile red (9-(Diethylamino) -5H benzo [α] phenoxazin- 5-one) staining is specifically used to identify and confirm the intracellular lipid droplets from the biological samples **(Greenspan *et al*., 1985)**.

The results indicated that not all algal species could be affected by Nile red staining since oil droplets were not clear and the whole cells were stained in red. This was the situation with green isolates, while certain blue green isolates were affected by the dye including *Microcystis aeruginosa* and *Phormidium rimosum* where the yellow stained parts were clear. Referring to *Chroococcus turgidus*, *Oscillatoria limnetica* and *Oscillatoria limosa* the cells showed yellow florescent color under florescent microscope even without adding the dye. So this gives false results when referring to lipid content. Diatoms were stained well with the dye and the oil drops were clear. Since the staining method may not be accurate

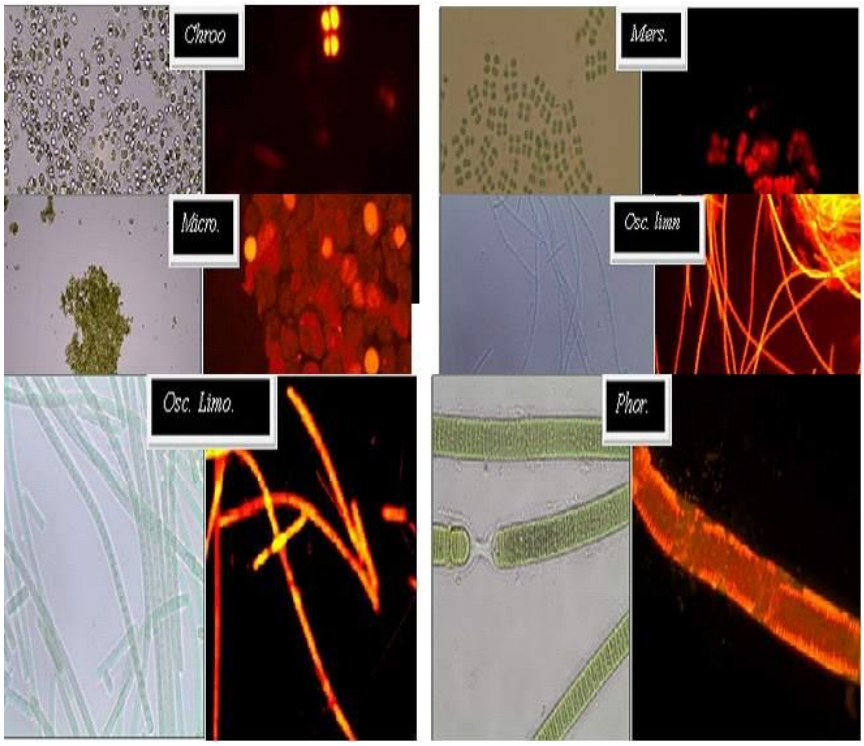
The florescent method has been successfully applied to the determination of lipids in certain microalgae, but has been unsuccessful in many others, particularly those with thick, rigid cell walls that prevent the penetration of the dye **(Held 2011).** Since Nile red method was not accurate in determining lipid content in microalgal cells so lipid content was determined using conventional extraction method using two organic solvents.

Green Isolates



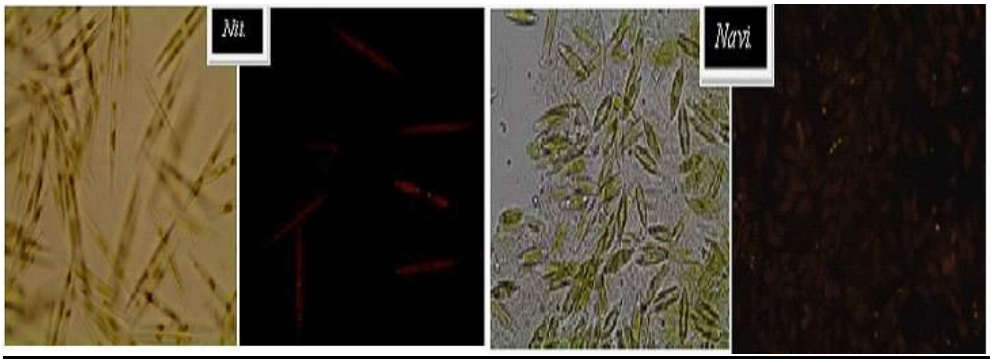
Chlam.; Chlamydomonas variabills, Chlor.; Chlorella vulgaris, Haem.; Haematococcus pluvialis, Sc.o.; Scenedesmus obliquus, Sc.q.; Scenedesmus quadriquada.

Blue green Isolates



Chroo.; ***Chroococcus turgidus, Mer.; Merismopedia elegans, Mic.; Microcystis aeruginosa, Osc.limn.; Oscillatoria limnetica, Osc. Limo.; Oscillatoria limosa, Phor.; Phormidium rimosum.***

Diatoms group



Nit.; Nitzschia linearis, Nav.; Navicula cspidata

Total lipid content of selected isolates using conventional extraction methods.

Two solvent systems were used for extraction of lipids from microalgal isolates to evaluate their percentage of lipid content. The results in table (1) indicated that hexane- Isopropanol mixture is better for lipid extraction than methanol- chloroform mixture in most of the strains. Also the results revealed that *Microcystis aeruginosa* has the highest percentage of total lipid 30%±0.3 these results were in harmony with the results of **Da rós *et al*., 2012,** who stated that Microcystis *aeruginosa* has total lipid content 28.1%. *Chlamydomonas variabills* 21%±0.5. A result similar to those recorded by **Feinberg, 1984** who found that *Chlamydomonas* species has a total lipid percentage of 23%. *Chlorella vulgaris* showed a total lipid percentage 13.6% while **Gouveia and Oliveira, 2009** reported that *Chlorella vulgaris* has percentage of 14-56. **Becker, 1994** reported that *Scenedesmus obliquus* contained lipid percentage 12-14 while the result in this study showed that total lipid content of *Scenedesmus obliquus was* 10%. In addition *Scenedesmus quadricauda* reported to have a percentage of 19.9% as mentioned in **Mohapatra, 2006.** This percentage is higher than that found in this study where the percentage was 16%.

Two diatoms species were investigated in this study *Nitzschia linearis* and *Navicula cuspidata*. The two species showed relatively low percentage of oil 6% and 10% for *Nitzschia linearis* and *Navicula cuspidata* respectively. *Nitzschia sp*. was investigated by **Chisti 2007** and found to have lipid percentage of 45-47%. On the other hand **Renaud *et al*., 1999** found that *Nitzschia frustulum* has a total lipid percentage of 13.9%. This percentage near to that presented in this study.

Table (1) Total lipid content of selected isolates

Microalgal isolates	**Total lipid content %**	
	Hexane- Isopropanol	**Methnol- Chloriform**
Green isolates		
Chlamydomonas variabills	**21± 0.5**	**14±0.7**
Chlorella vulgaris	**12±0.2**	**13.6±0.6**
Haematococcus pluvialis	**10±0.3**	**8±0.0**
Scenedesmus obliquus	**7.9±0.3**	**10±0.1**
Scenedesmus quadriquada	**11±0.26**	**16±0.5**
Blue green isolates		
Merismopedia elegans	**10±0.7**	**2.3±0.0**
Microcystis aeruginosa	**30±0.3**	**28±0.6**
Oscillatoria limnetica	**11±0.5**	**5±0.3**
Oscillatoria limosa	**16±0.3**	**8±0.4**
Phormidium rimosum	**8±0.1**	**7±0.5**
Diatoms		**3.7±0.2**
Nitzschia linearis	**6±0.1**	
Navicula cuspidate	**10.5±0.4**	**10±0.1**

Coclusions:

1- Renewable biofuels are needed to displace petroleum derived transport fuels, which contribute to global warming and are of limited availability.
2- Nile red staining is not an effective method for oil detection in microalgal strains since it was successful with certain isolates while unsuccessful with others and the fluorescence intensity in all treatments was similar and no considerable lipid accumulation was observed.
3- Hexane reported to be more efficient than chloroform when extracting oil from microalgae especially when this oil was used for biodiesel production.
4- *Microcystis aeruginosa* was detected to have the highest total lipid percentage 30% and can be used as a source of biofuel.

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