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Biodiesel Production and Biotechnological Applications from Microalgae Isolated from Water System of Riyadh, Saudi Arabia

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Microalgae are a potential source of biodiesel. Isolates of the present study were isolated from Wadi-henifa, Rhiyad, Saudi Arabia. The urgent need for an alternative and sustainable energy has created renewed interest to analyse the microalgae for biodiesel production. The greatest lipid content reached 20.2, 16.4, 9.7 and 12.3 % under the optimal conditions of nitrate concentration (0.75 g/l), temperature (24 and 30 °C), salinity (0.05 and 0.001 mole/l) and pH (8 with *Chlorellavulgarus* and 9 with other strains), *Chlorella vulgarusArthrospiraplatensisGomont and Spirulina major*, respectively. It was demonstrated that the obtained model was effective for predicting lipid productivity of the isolated microalgae. The maximum protein content was at 24°C for *Chlorella vulgarus,ArthrospiraplatensisGomont and Spirulinamajor*kütz, (53, 56.8 and 54 % respectively), while the maximum protein content of*Arthrospira maxima* was at 30°C (56.2 %).The optimum protein content was found at pH9for*ArthrospiraplatensisGomont , Spirulina major* kützand*Arthrospira maxima*(48.47, 55.47 and 63.25 % respectively) while in case of *Chlorella vulgarus*was at pH 8 (51 %).The maximum protein content was 76.96 % at 0.001 moleNacl/L, 54, 75.38 and 75.09 % at 0.05 mole/L for *Chlorella vulgarus,Spirulina major* kütz, *Arthrospira maxima* and*ArthrospiraplatensisGomont* respectively. Results of this study revealed thatthe mention optimization conditions enhanced protein content of the tested isolates. *Arthrospiraplatensis*Gomont, *Spirulinamajor*kützand*Arthrospira maxima* are promising organisms with high nutritional value for animal and human beings.

Key words: *Arthrospira*, Biomass, Salinity

Microalgae are used for different applications, such as biofuel production (Scott *et al.,* 2010), extraction of high value food additives and pharmaceutical products oras food for aquaculture (Spolaore *et al.,* 2006). The continued use of petroleum sourced fuels is now widely recognized as unsustainable because of the depletion supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment leading to increase of global warming. In the last ten years, many studies have been conducted on biofuels for substituting fossil fuels and reduce the greenhouse gas emission (Bastianoni *et al.,* 2008). Biodiesel from oil crops, waste cooking oil and animal fat cannot realistically satisfy even a small fraction of the existing demand for transport fuels. Recent researches involved not only the existing renewable sources available from land plants, but also those coming from aquatic

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systems. Algae (macro and micro) have been taken in consideration as a residual biomass ready to be used for energy purposes. Algae, especially microalgae, were found to be the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels (Chisti, 2007 and 2008). The idea of using algae as a source of fuel is not new (Sawayama *et al.,* 1995), but it is now being taken seriously because of the increasing price of petroleum and more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Gavrilescu and Chisti, 2005). Microalgae can provide several different types ofrenewable biofuels which include, methane, biodiesel (methyl esters) and biohydrogen (Spolaore*et al.,* 2006). Oil productivity of many microalgae greatly exceeds the oil productivity of the best producing oil crops (Shay, 1993).

Arthrospira (*Spirulina*) is an economically important filamentous cyanobacterium. The high content of proteins, essential amino acids, vitamins, minerals and fatty acids makes it an ideal healthy (Ciferri O, 1983). It has been used as a food (Dillon *et al.,* 1995) because of its quantity of proteins, vitamins, essential amino acids, minerals and essential fatty acids (Campanella*et al.,* 1999; Mendes *et al.,* 2003).

Proteins are the most abundant biological macromolecules occurring in all cells and parts of cells, exhibits enormous diversity of biological function inside an organism and represent great part of human and animal nutrition. Taking into consideration another important current issue the shortage of usable space, obtaining proteins through the cultivation of microorganisms can be more advantageous when compared to usual sources (animal and vegetable proteins). There are several studies about biomass composition or specific components of cyanobacteria and microalgae (Piorreck M. *et al.,* 1984 &Hongsthong A. *et al.,* 2007). There is previous study showed that *A. maxima*, and specifically its protein extract could protect against HU-induced teratogenicity in mouse embryos (Jorge Vázquez-Sánchez, *et al.,* 2009). Other important components of cyanobacteria and microalgae biomass are carbohydrates (De Philippis R and Vincenzini M, (1998) and lipids (Materassi*et al.,* 1980).

It has been reported in some reviews that *Arthrospira* have several pharmacological activities (Belay *et al.,* 2002; Khan *et al.,* 2005), of which antioxidant effect is one of the most important. Its antioxidant property is found in the protein extract, specifically some phycobiliproteins such as C-phycocyanin (CP) and allophycocyanin (Wu *et al.,* 2005; Lu *et al.,* 2006).

Arthrospira(*Spirulina*) *maxima* and *S.platensis* have a long history of use as food for human. Traditionally, they have been used for food during the Aztec civilization in mexico and more recently by natives in the lake chad area (Ciferri&Tiboni, 1985). Previously study evaluated the performance of continuous cultivations as well as to establish relationships between the rate of nitrogen source supply and protein and lipid contents in *A.platensis* (Sassano, C.E.N. *et al.,* 2010).The present study aims at evaluate the chemical composition of *A. maxima* such as proteins and carbohydratesat optimum conditions.

MATERIALAND METHODS

Isolation, Purification, Identification and growth conditions

Water samples were collected from Wadi-Hanifa, Addriyah, Rhiyad, Saudi Arabia.100ul of water sample was transferred to SP medium and BG11 containing plates (Schlosser, 1982), at light intensity 3000 lux. Three plates were prepared for each sample; the plate's cultures were incubated at 24°C ±2 for 15 days. Developing cultures were identified according to Desikachary (1959) and Bischoff and Bold, 1963.Pure isolates were maintained on SP medium for further studies. Thestrainswere also cultivated in SP medium, with photoperiod of 12 hours light/dark provided by fluorescent lamps at a light intensity of 3000 luxand temperature of 27 ± 2 ºC.

Determination of exponential phase for growth of target microbial strains

The strains were grown in suitable medium at 27 ± 2 ºC 8/12 hours light/dark provided by fluorescent lamps at a light intensity of 3000 lux. Fresh weight was determined by fresh weight of the strains at different times.

Nile red staining

Isolated pure cultures were further used for screening their lipid production using nile red staining method om which 200 µl of algal samples were added with50 µl of nile red dye (1 mg/ml DMSO stock) and incubated for 10 min. at room temperature followed by washing with double distilled water. Finally the slides with algal culture were prepared and observed under fluorescence microscope (Mahishi LH *et al.,* 2003) at 465 nm excitation.

Extraction of oil

A known weight of each ground dried algal species was mixed the extraction solvent mixture, hexane/ether (1:1 v/v), kept to settle for 24 hrs, followed by filtration according to Hossain and Salleh(2008).

Biomass preparation

The biomass was harvestedat exponential phase by centrifugation (2200rpm, 5 min), washed with a 1% aqueous NaCl solution, centrifuged again and freeze-dried. The dry biomass was analyzed immediately or stored at 6 20° C for up to 10-days prior to analysis (Cynthia, V. G. L.*et al.,* 2010).

Biomass pretreatment

The following pretreatment methods were tested: milling for 5-min with a pestle and mortar without grinding elements prior to suspension in buffer solution (Cynthia, V. G. L. *et al.,* 2010).

Determination of proteins

The protein content of culture filtrate was determined accordingto Lowery *et al.,* (1951) using bovine serum albumin as standard.

Determination of carbohydrates

Carbohydrates were determined following the phenolsulphuric acid method of Masuko*et al.,* (2005) using glucose as standard.

Effect of pH on lipid, protein and carbohydrate content

The effect of pH on lipid,protein and carbohydrate content was carried out using different pH like 8, 9, 10 and 11. The optimization media with the different pH (8, 9, 10, 11) were inoculated with the test samples at 24 °C (*C. vulgarus*, *A. platensis*and*S. major*) while *A. maxima* was incubated at 30 °C and the protein and carbohydrate assay was done for exponential phase. Using the assay method described in the earlier section.

Effect of temperature on lipid, protein and carbohydrate content

The test sampleswerecultivated at various temperatures like 22°C 24°C 27°C and 30°C for exponential phase. Lipid, protein and carbohydrate content wasestimated by using the assay methods described in the earlier section.

Effect of salinity on lipid, protein and carbohydrate content

The test samples were cultivated in SP or BG medium containing on different salinities at 0.0005, 0.001, 0.05 and 0.1moleNacl/L for exponential phase. Lipid, protein and carbohydrate content were estimated by using the assay methods described in the earlier section.

Effect of different concentration of $NaNO_3$ on lipidcontent

The test samples were cultivated in BG medium containing on different nitrogen concentrations at 1.5 g/L, 0.75 g/L and 0.0 g/L of $NaNO_3$ for exponential phase. Lipid, protein and carbohydrate content were estimated by using the assay methods described in the earlier section.

Statistical analysis

For statistical analysis, a standard deviation for each experimental result was calculated using the Excel Spreadsheets available in the Microsoft Excel.

RESULTS

C.vulgarus, *A. maxima*, *A.platensis*Gomont and *S.major*kütz were isolated from Wadi-Hanifa, Riyadh, Saudi Arabia. They were identified by its morphological characteristics. Our results in figure 1 showed the duration of the exponential phase for the strains, *A. maxima*, *A. platensis* and *S. major* that were cultivated in SP medium varied between 14 to 18 days depending on the species. *C. vulgarus* was cultivated in BG11 medium with the exponential phase was at 6 days. Biomass productivity at late exponential phase for the strains, *C. vulgarus*, *A. maxima*, *A. platensis* and *S. major* was 3.3, 11.2, 9.4 and14.8 mg/L, respectively.

In order to select a media that facilities high biomass productivity of microalgal strains under photoautitrophic condition, each strain was cultivated in three media, and the medium in which the highest biomass was selected for further studies. Our study showed that highest biomass for the strains, A. maxima, A. platensis and S. major was in the SP medium while the highest biomass of C. vulgarus was in BG11 medium (Table 1).

To select a strains which have ability to produce lipid, each stain was stained by Nile red stain, and the target strain was selected for

Table 1. Growth of *C. vulgarus*, *A. platensis*, *S. majr* and*A. maxima* which weregrowingat different media

Strainmedium	BG11	Cu10	Spirulina medium (SP medium)
C. vulgaris	+++	++	+
A. maxima	++	+	+++
A. platensis	++	+	++
S. major	++	+	+++

Table 2. Lipid content of *C. vulgarus*, *A. platensis*, *S. major*and *A. maxima* which were growing at different temperatures

StrainTm	20 °C	24 °C	27 °C	30 °C
C. vulgaris	5.5 ±0.9	11.5±0.4	8±1.2	4.2±0.3
A. maxima	4 ±1.1	5.8±0.7	7.5±0.5	10±0.9
A. platensis	3.5±1.0	5.75±0.2	5±0.4	2.5±0.5
S. major	5.2±0.6	6.8±0.1	3.3±0.3	3.6±0.2

Table 3. Lipid content of *C. vulgarus*, *A. platensis*, *S. major* and *A. maxima* which weregrowing at different salinity

Strainsalinity	0.0005 mole/L	0.001 mole/L	0.05 mole/L	0.1 mole/L
C. vulgaris	4.5±0.4	9.5±0.4	6±0.8	3±0.1
A. maxima	3±0.1	4.5±0.6	7.7±0.7	5±0.2
A. platensis	3.3±0.2	5.5±0.3	5.9±0.5	2.1±0.1
S. major	2.9±0.8	7.1±1.2	3.6±0.7	1.8±0.1

Table 4. Lipid content of *C. vulgarus*, *A. platensis*, *S. major* and *A. maxima* which weregrowingat different pH

StrainpH	8	9	10	11
c. vulgaris	10.5±0.4	5.5±0.7	4.2±0.3	No growth
A. maxima	7.8±0.6	8.7±2.2	3.5±0.6	1.2±0.2
A. platensis	3.1±1.1	6.1±1.1	4.3±0.9	1.6±0.4
S. major	3.7±0.9	6.9±0.8	5.9±0.5	No growth

Table 5. Lipid content of *C. vulgarus*, *A. platensis*, *S. major* and *A. maxima* which weregrowing at different concentration of $NaNO_3$

Strain $NaNo_3$	1.5 g/L (control)	0.75 g/L	0.0 g/L
c. vulgaris	13.75±0.8	20.2±4.5	15±1.7
A. maxima	9.4±0.3	16.4±2.9	10.7±0.7
A. platensis	5.78±0.9	9.7±3.7	7.6±1.3
S. major	7±1.1	12.3±2.7	8.5±0.5

production of biodiesel. Our study showed four strains gave positive result for lipid accumulation through Nile red staining method. Lipid inclusions were seen as bright orange intracellular granules shown in the figure 2.

Our results in Table 2 showed maximum lipid content was 11.5, 5.75 and 6.8 % in case of *C. vulgarus, A. platensis*and*S. major* at 24 °C while lipid content of *A. maxima* was 10 % at 30°C.

Effect of salinity on the lipid content of the strains was studied. We found that maximum lipid content of *C. vulgarus* and *S. major* was 9.5 and 7.1 % at 0.001 mole/L salinity while lipid content of *A. maxima* and *A. platensis* was 7.7 and 5.9 % at 0.05 mole/L salinity, respectively (Table 3).

In Table 4, results showed optimum pH for production of lipids from *C. vulgarus* was 8 at which lipid content was 10.5 %. In case of *A. maxima,A. platensis*and*S. major,* optimum pH was 9 and lipid content was 8.7, 6.1 and 6.9 %, respectively.

Results of lipid content at different concentration of $NaNO_3$revealed that deficiency of nitrogen led to increase its. Optimum concentration of nitrogen for lipid content of *C. vulgarus*, *A. maxima,A. platensis*and*S. major* was 0.75 g/L compared as the control 1.5 g/L. Lipid content of *C. vulgarus*, *A. maxima,A. platensis*and*S. major* was 20.2, 16.4, 9.7 and 12.3 % at 0.75 g/L of $NaNO_3$, while at control (1.5 g/L of

Table 6. Protein and carbohydrate content of *C. vulgarus*, *A. platensis*, *S. major* and *A. maxima* which were growing at different temperatures

StrainTm	20 °C		24 °C		27 °C		30 °C	
	C. content	p. content	C. content	P. content	C. content	P. content	C. content	P. content
C. vulgaris	5.5±1.0	40.5±3.7	6.5±0.9	53±0.9	5.4±0.8	51±2.1	4.2±0.9	35±3.6
A. maxima	0.25±0.02	39.3±2.8	0.39±0.1	43.6±1.7	0.27±0.03	46.8±1.8	0.43±0.1	56.2±1.2
A. platensis	0.19±0.01	41.6±1.9	0.21±0.04	56.8±2.4	0.22±0.01	55.1±2.0	0.49±0.1	41.7±2.7
S. major	0.53±0.1	45.2±2.1	0.11±0.01	54±1.9	0.54±0.1	40.4±0.9	0.54±0.2	35.3±1.5

Table 7. Proteinandcarbohydrate content of *C. vulgarus*, *A. platensis*, *S. major* and *A. maxima*whichweregrowingat different pH

Strain	8		9		10		11	
pH	C. content	p. content	C. content	P. content	C. content	P. content	C. content	P. content
C. vulgaris	5.7±2.2	51±1.7	3.4±0.8	35±1.7	2.5±0.7	25±1.1	No	No
A. maxima	0.63±0.1	48.3±1.2	0.75±0.2	63.3±2.6	0.86±0.2	39±1.3	0.42±0.1	11.7±1.1
A. platensis	0.3±0.1	31.2±1.6	0.75±0.1	48.5±2.4	0.33±0.01	34.4±1.0	0.15±0.02	6.8±2.7
S. major	0.52±0.1	19.6±2.3	0.88±0.2	55.5±0.7	1.1±0.3	43.5±2.1	No	No

Table 8. Proteinandcarbohydratecontent of *C. vulgarus*, *A. platensis*, *S. major*and*A. maxima*whichweregrowingat different salinity

Strain	0.0005 mole/L		0.001 mole/L		0.05 mole/L		0.1 mole/L	
salinity	C. content	p. content	C. content	P. content	C. content	P. content	C. content	P. content
c. vulgaris	6.7±1.8	41±1.7	6.2±1.7	49±2.3	6.1±1.1	54±0.7	4.1±0.8	38±3.7
A. maxima	0.81±0.1	52.2±2.4	0.89±0.1	75.4±8.9	0.75±0.3	75.4±2.0	0.76±0.1	73.9±2.7
A. platensis	0.72±0.3	39.3±1.5	0.74±0.1	48±3.7	1.55±0.1	75.1±1.7	0.78±0.1	41.6±1.5
S. major	0.61±0.1	40.5±2.2	1.27±0.3	77±9.7	1.1±0.2	46.8±3.5	0.93±0.2	37.3±2.7

$NaNO_3$), lipid content was 13.75, 9.4, 5.78 and 7 %,respectively (Table 5).

In the study, different temperatures were tested for protein and carbohydrate content of *A. platensis*, *S. major* and *A. maxima*. The maximum protein content of*C.vulgarus*,*A. platensis*, *S. major* was53, 56.8 and 54 %, respectively at 24 °C wherever the optimum temperaturefor*A. maxima* was at 30 °C and protein content was 56.2 % (Table6).Table6also showed that the optimum temperaturefor*A. platensis*, *S. major* and *A. maxima* was at 30 °C, in case of *C. vulgarus* was at 24°C.

The maximum protein of *A. platensis*, *S. major* and *A. maxima* was 63.3, 48.6 and 55.5 % at pH 9 respectively, and in case of *C. vulgarus* was 51 % at pH 8 (Table 7). There was no growing of S. major and *C. vulgarus* at pH 11. The results in Table7 alsorevealed that the carbohydrate content of *A. platensis*, *S. major* and *A. maxima* at pH 9 was more than at pH 8, while carbohydrate contentof*C. vulgarus was* the highest at pH 8.

In this study we had tested 4 different concentrations of sodium chloride on the protein and carbohydrate content of *C. vulgarus, A. platensis*, *S. major* and *A. maxima*. In case of *S. major*, the maximum protein content was 77 % at 0.001 mole/L while in case of *C. vulgarus*,*A. platensis*and *A. maxima* was 54, 75.1 and 75.4 % at 0.05 mole/L, respectively. The results in Table 8 also showed that the optimum salinity for carbohydrate content of *A. maxima* and *S. major* was at 0.001 mole/L, while in case of *A. platensis*was at 0.05 mole/L.

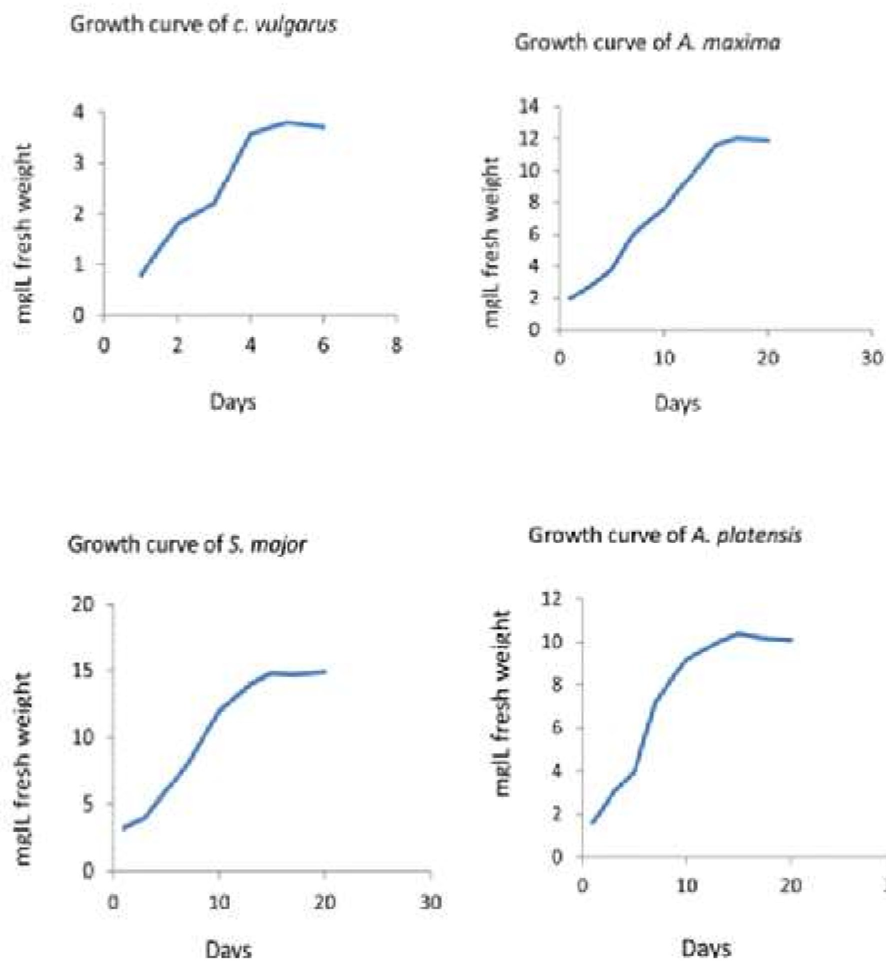

Fig. 1. Growth curve of lipid content of *C. vulgarus*, *A. platensis*, *S. major* and *A. maxima* which weregrowing at optimum conditions

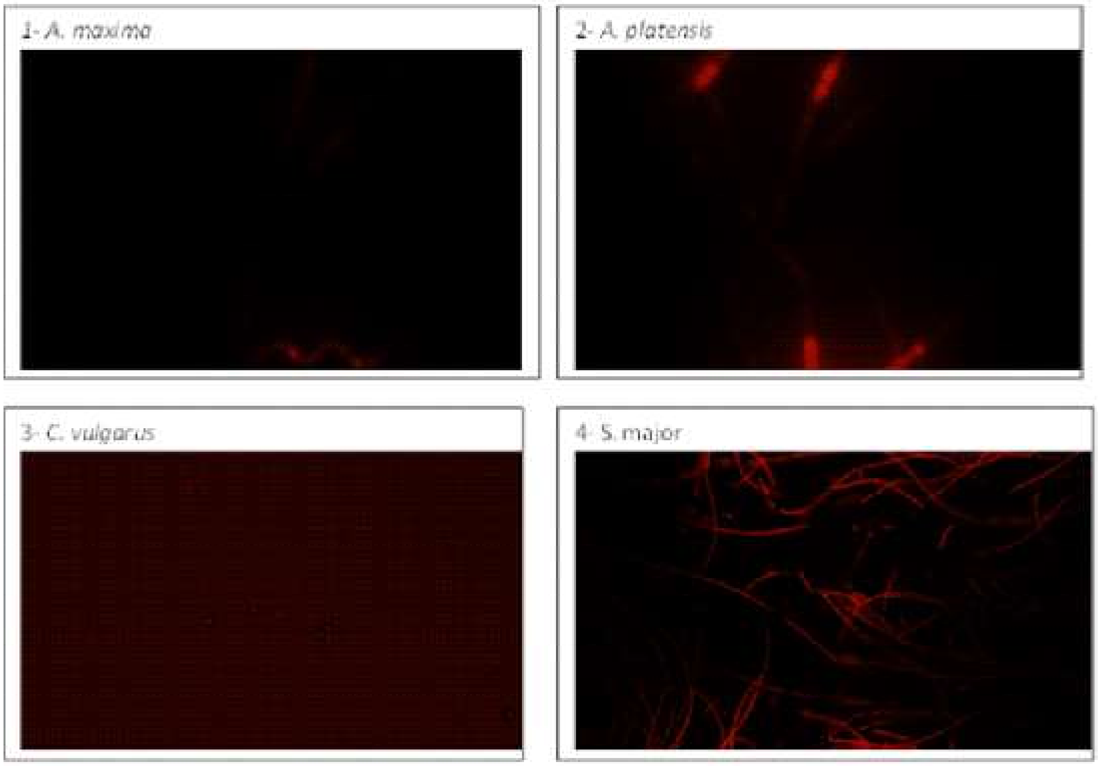

Fig. 2. Nile red stained *A. maxima*, *A. platensis, C. vulgarus* and *S. major*containing lipid inclusions

DISCUSSION

Nile red staining method was used for the detection of lipids in the isolated microalgae. There were reports supporting for this the method (Cooksey *et al.,* 1987; lee *et al.,* 1998 and Elsey*et al.,* 2007). High lipid content is one main criterion for the selection of microalgae strains as a renewable source for the production of biofuel. Our results in Table 2 showed maximum lipid content was 11.5, 5.75 and 6.8 % in case of *C. vulgarus, A. platensis*and*S. major* at 24 °C while lipid content of *A. maxima* was 10 % at 30°C In Table 4, results showed optimum pH for production of lipids from *C. vulgarus* was 8 at which lipid content was 10.5 %. In case of *A. maxima,A. platensis*and*S. major,* optimum pH was 9 and lipid content was 8.7, 6.1 and 6.9 %, respectively. We found that maximum lipid content of *C. vulgarus* and *S. major* was 9.5 and 7.1 % at 0.001 mole/L salinity while lipid content of *A. maxima* and *A. platensis* was 7.7 and 5.9 % at 0.05 mole/L salinity, respectively (Table 3). This was due to that temperature, salinity and pH could guarantee to enzyme activities, determination of microbial growth and production process, and under salt stress conditions the algal metabolism was altered with over production of carbon skeleton which were partly directed towards the production of substances with beneficial role in algal tolerance or defense mechanism as polyols, carbohydrate, methylated These results were similar to previous reports (Jiang and Chen, 2000 and Ruangsomboon, 2012). Maximum lipid contents were recorded in 50 % absence of nitrate (nitrate starvation) from the nutritive medium (20.2, 16.4, 9.7 and 12.3 % respectively) as illustrated in Table 5. These results may be explained by the fact that, under nitrate starvation, all the carbon structures produced during metabolic process might be directed towards lipid production which in turn converted to biodiesel by transesterification process. While in presence of nitrogen, most of the carbon structures were incorporated in nitrogenous compounds as amino acids, protein, nucleic acids or alkaloids. The data obtained in this investigation were in good agreement with results published by Widjaja (2009) and Afify et al (2010) who reported that the green microalga Chlorella vulgaris accumulated high lipid content when cultivated in nitrogen depletion condition (0.02 mg/l nitrate). Our results also went parallel with those obtained by Lardon*et al.,* (2009) who found that, the control of nitrogen stress during the culture and optimization of wet extraction led to maximum biodiesel production from the microalgal culture.

*Spirulina*and*C.vulgarus*is the common name for human and animal food supplements produced primarily from two species of cyanobacteria: *A.platensis*, and *A.maxima*. These and other *Arthrospira* species were once classified in the genus *Spirulina*. There is now agreement that they are a distinct genus, and that the food species belong to *Arthrospira*; nonetheless, the older term *Spirulina* remains the popular name. *Arthrospira*and*C. vulgarus*is cultivated around the world, and is used as a human dietary supplement as well as a whole food and is available in tablet, flake, and powder form. It is also used as a feed supplement in the aquaculture, aquarium, and poultry industries (Vonshak, A. 1997).Proteins are the basis of many animal body structures (e.g. muscles, skin, and hair). They also form the enzymes that control chemical reactions throughout the body. Each molecule is composed of amino acids, which are characterized by inclusion of nitrogen and sometimes sulphur(these components are responsible for the distinctive smell of burning protein, such as the keratin in hair). Our study showed that the optimum protein content of *C. vulgarus,A. platensis*Gomont*,S.major*kütz was 53, 56.8 and 53.95 at 24 °C and *A. maxima* CCAP 1475/ 9 was 56.6% at 30 °C. Similar values for total protein, ranging from 46% to 50% in dry weight, were reported by Richmond (1990).There are several studies about biomass composition or specific components of cyanobacteria and microalgae (Piorreck M. *et al.,* 1984 &Hongsthong A. *et al.,* 2007). There is previous study showed that *A. maxima*, and specifically its protein extract could protect against HU-induced teratogenicity in mouse embryos (Jorge Vázquez-Sánchez, *et al.,* 2009). Other important components of cyanobacteria and microalgae biomass are carbohydrates (De Philippis R and Vincenzini M, (1998) and lipids (Materassi*et al.,* 1980). In this study, we have tested salinity on protein content of*A. platensis*. The *C. vulgarus* ,*A. platensis* and *A. maxima,* the optimum values were 0.05mole/L (54, 75.1 and 75.4) respectively, *S. major* at 0.001mole/L salinity but the values in desalinator wastewater were lower than that presented by Oliveira *et al.,* (1999). Pelizer*et al.,* (2003), studying different initial inocula, reported 55.0 - 61.0% protein content. Similar values were found by Rafiqul*et al.,* (2005), who found 58.6% of total protein content when using Zarroukmedium.Our study showed that the maximum protein content of *A. platensis, S. major* and *A. maxima* was at pH 9 but *C. vulgarus*at pH 8. This results were similar to previous study was to evaluate the different media for the growth of *S.* maxima and temperature on the protein and chlorophyll a, maximum specific growth rate and productivity of *S.* maxima at different media, The protein content of *S.* maxima was 62.0 % on Zarrouk medium, 55.2 %on Rao's medium, 61.0 % on CFTRI medium, 58.4 % on OFERR medium, 40.2 % on Bangladesh medium no. 3 and 60.4 % on Revised medium 6 (Pandeyi, *et al.,* 2010).Previous study showed that cultivation of *A. platensis* in wastewater medium (protein content = 48.59 %) and in salinated synthetic medium (protein content = 56.17 %), evaluating the amino acid profile and the protein content of the cells (Harriet, *et al.,* 2008). Previous study showed that protein content in *S. major* was 66.72% (Nagle, *et al.,* (2010); Ogbonda, *et al.,* 2007). Other studies had also been done by various workers reported that chlorophyll a content and protein content of cyanobacteria was also maximum in pH 9 (Carvallo, *et al.,* 2002& Kim, *et al.,* 2007).

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