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OPTIMIZATION OF GROWTH CONDITIONS OF DIFFERENT ALGAL STRAINS AND DETERMINATION OF THEIR LIPID CONTENTS

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ABSTRACT

Aim of the present study was collection and identification of native algal species, optimization of their growth conditions and comparison of their oil content. From the collected 20 samples 3 different algal species were identified. The effect of growth media, temperature, pH level, light intensity and aeration on growth of *Spirogyra sp.* and *Oedogonium* sp. and *Chlorella* sp. was recorded. It was observed that both *Oedogonium* sp. and *Chlorella* sp. grow well in Blue green medium while *Spirogyra* sp. showed better growth in terms of fresh weight in Bold Basal medium. Algal growth optimum temperature was between 24-28 ºC. It was also observed that neither very low nor very high pH is suitable for algal growth and at pH 7.5 algae were able to grow at maximum with fresh weights of 4.89±0.091 g and 4.79±0.021 g for *Spirogyra sp.* and *Oedogonium* sp. respectively. Artificial fluorescent light resulted in an increased growth of algae as compared to sunlight or when algae placed near window and exposed to indirect sunlight. The oil content of the three algal species is in order *Chlorella* sp > *Oedogonium* sp. *Spirogyra* sp. Hence, *Chlorella* sp. was found to have the highest lipid content with 15.46±0.240% yield for lipid content.

Key words: Algae; Biofuel; *Chlorella.; Oedogonium*.; Optimization; Spirogyra.

INTRODUCTION

Algae are a group of organisms that have been generally described as photoautotrophic, simple microscopic or macroscopic, unicellular to multicellular plants and are competent converters of sun energy to useful biochemical products like oil (Schenk *et al.,* 2008). Algae are mainly water dwelling organisms lacking complex morphological organization (Barsanti and Gualtieri, 2006). Algae either macro or micro, have great potential for biofuel production. Microalgae are a varied group of single-celled organisms; also they are capable to offer a range of solutions for our energy demands through a number of ways (Bala, 2006). Algae efficiently use CO_2 from atmosphere and they are responsible for more than 50% of the total global carbon fixation (Feng, 2011). Algae are considered as third generation biofuel. They can increase biomass very quickly and few of algal species can double their biomass in as few as 5 hours while several species are revealing two doublings each day (Huber, 2009). Altogether, algae possess the capacity to yield energy-rich oils and a number of microalgae were found to naturally gather high oil content in their total dry weight (Rodolfi *et al*., 2008).

Algae are easy to grow and cultivate anywhere with less energy requirements and using very few of the nutrients. The ideal growth conditions for microalgal cultures are strain specific and the biomass productivity depends upon many factors. These include abiotic factors like temperature, minerals, carbon dioxide, pH, water quality, light cycle and intensity. The biotic factors include cell fragility and cell density. Mechanical factors include continuous mixing, gas bubble size and distribution and mass transfer, all these are of particular concern in photo-bioreactors (Schenk *et al.,* 2008). Light and temperature are the two most important factors that affect algae biomass productivity. The energy for growing algae is provided by light via photosynthesis. Sufficient light energy must be effectively utilized to achieve higher biomass productivity. Temperature influences the rates of all chemical reactions related to algal growth and its metabolism (Sandnes *et al.,* 2005). Change in temperature affects the biochemical composition of the cells specifically lipids and proteins. Thus light and temperature have a significant effect in the metabolism, enzyme activities and cell composition of algae. Algae cultivation also depends on pH levels and optimum pH influences the carbon availability, metabolism and biochemical composition of cells (Richmond, 2000). For efficient use of algae as a source of biodiesel it is very important to focus on the native algal species and to select that algal species which not only has a high growth rate but has greater lipid content. Identification of local algal species, optimization of conditions for native algal species and comparison of their oil contents is the part of this study. Hence, present study is a significant step forward in utilization of algae as a source of renewable energy.

MATERIALS AND METHODS

Sampling: During the present study, total 30 samples were collected from 10 different locations of Lahore

including ponds and damp soil places. The sampling time was from the month of September, 2011 to March, 2012.

Samples were collected in glass jars, Plastic bags and bottles and then transferred to various nutrient media standardized during the present work using flasks, jars and plastic bottles.

Identification: Species were identified based on the morphological characteristics. These were studied by preparing slides and observing under by 10X and 40 X powers of Microscope Irrmeco GmbH Gemany model IM - 800.

Cultivation: Blue-green (BG) medium and Bold basal (BB) medium (Bold 1949) were tested for the algal growth and support. Two growth medium are mostly adapted to freshwater algae. Growth experiments were performed in conical flasks of 250 ml. Each flask was filled with 100 ml of media and initially 2 g of algal sample was added to each flask. Every experiment was performed in replicates and both the media were compared for each culture conditions.

Optimization of different culture conditions: The effect of different parameters on the growth rate of algae was assessed by following completely randomized experimental design. In these experiments only one factor was variable while all other conditions were kept constant. The culture conditions which were controlled for the algal growth were nutrients, light, temperature, pH and constant mixing or aeration.

Culture medium/Nutrients: For monitoring the best growth of algae both the media (BB and BG) were used for culturing algal cells and the best growth rate was estimated after a required period of time has been passed. The first calculation was made on 7^{th} day and the rate was estimated on the basis of the rate of increase in fresh weight of biomass. The cell mass was separated through filtration and then weighted after blotting the excess water.

Temperature: The temperatures were maintained at 16, 20, 24, 28 and 32^{o}C. Temperature variations were monitored regularly using thermometer. Changes were recorded and the algal growth rates were found by measuring their fresh weights.

pH: Both the culture media were adjusted with various pH ranges i.e., 6.5, 7, 7.5, 8, 8.5, and 9. Algae samples were grown within these pH ranges and the effect was observed and measured by calculating the fresh weight of samples after the specified time period has been elapsed.

Light: Algal cultures were placed out door in open sunlight, inside the lab near window and under artificial light provided by fluorescent tubes. The effect of light on the growth of algae was calculated by measuring the algal biomass.

Aeration/mixing: Different means of mixing were used for this purpose. For mixing orbital shaker OS 5 basic yellow line was used and the flasks were kept over the shaker at 300 rpm. Aeration was supplied through aerating pumps. These cultures were placed indoor where constant sunlight was given. The growth results were then compared and cultures with maximum growth were continued

Oil Extraction: The algal cultures grown on optimized conditions were further tested for their oil contents. Filamentous algae were dried by spreading over blotting paper and placing the material under running fan until all of the water content was removed. In case of *Chlorella* sp. unicellular algae the cell biomass was collected together and dried in oven at 65 ^{o}C for 3 hours. All types of algae were ground to fine powder with motor and pestle. To extract oil from the algal samples Bligh and Dyer method (1959) was used with slight modifications. Five grams of dried sample was homogenized in 15 ml of chloroform-methanol (1:2, v/v). After this the mixture was centrifuged for 10 min at 3000 rpm and then filtered. The residual biomass was re homogenized with 5 ml of chloroform and then centrifuged again (10 min, 3000 rpm). This mixture was filtered and collected together with the previous filtrate and centrifuged. The lower phase was collected and kept for drying to evaporate all the chloroform and the remaining content was measured by using the following formula

Lipid content (%) = lipid extracted (g)/original sample (g) × 100

RESULTS AND DISCUSSION

Increased oil prices, depletion of fossil fuels and high energy demand have made the researchers to look for an alternative source. Algal fuel might be an alternative to fossil fuel because these feedstocks are much more reliable and using them as biofuel production can decrease the dependence on fossil fuels (Bansal and Sharma, 2005; Kulkarni and Dalai, 2006). Many algae biofuel studies have shown that the quantity and quality of lipids within the algal cell biomass can vary as a result of changes in growth conditions (temperature, pH and light intensity) or nutrient media characteristics (concentration of nitrogen, iron and phosphates) (Illman *et al.,* 2000; Liu *et al.,* 2008).

Objective of this study was to collect and optimize the growth conditions and determine the oil contents of different algal strains. During the present work 30 samples were collected from 10 different locations of Lahore. These samples were identified based on morphological characters as given by (Sharma, 1986) and a voucher specimen *Spirogyra* sp., *Oedogonium.* sp. and *Chlorella* sp. was preserved as BT-Od-1, BT-Sp-1 and BT-Ch-1 in Algal Biotechnology Lab, Department of

Biotechnology, Lahore college for women University. Lahore.

The growth of algal samples was observed by calculating the increase in fresh weight of algae. *Spirogyra* sp. grown in both medium has shown varied results. It is evident from table 1 that medium has a significant effect on fresh weights of algal biomass. Initial weight of algae taken for each experiment was 0.5 g which almost became doubled (1.02±0.029 g) after one week when algae were grown in BB medium. It was also observed that after 1 week fresh weight of algal biomass in BG medium was greater (1.54±0.042 g) as compared to BB medium (Table 1). Generally the same trend was observed with the increase in time interval at week 2, 3 and week 4. Similarly *Oedogonium* sp. was cultured in BB and BG medium. The results have depicted that both the medium have significant effect on algal growth and increased their cell biomass by 1.28±0.022 g after 1 week. *Oedogonium* sp. grown in BB medium was having more increased growth rate as compared to BG medium i.e. 1.28±0.022 g and 1.03±0.032 g respectively. At week 4 the biomass became much denser with 4.75±0.020 g for BB medium while for BG medium 3.03±0.020 g (Table 1).

In case of unicellular algae i.e. *Chlorella.* sp. growth was measured in terms of absorbance at 600 nm using spectrophotometer *Chlorella* sp. cells were suspended in BB and BG medium and their absorbance was measured daily in order to find the better results for both medium. Figure 4 shows the values for absorbance at 600 nm and it is evident from the results that *Chlorella sp.* cells grow better in BG medium as compared to BB medium. These results show similarity with those revealed by Held (2011). Cultures of *Chlorella* sp. and *Spirogyra* sp. were grown in BG medium and light scattering and fluorescence calibration curves were plotted for growth rate. Results indicated that *Chlorella sp.* cells did not show increase in growth curve while *Spirogyra sp.* were able to grow and increase in biomass (Grobbelaar, 2000; Henderson *et al.,* 2008. Farooq *et al* (2013) also reported that synthetic medium gives good results for algal growth.

Effect of temperature on algal growth: Algae were grown in variable temperature ranges. These were 16, 20, 24, 28 and 32ºC. All other factors were made constant and the cultures were observed with varied temperature effect. The effect of temperature was found to be significant on algal growth and both the filamentous forms of algae have shown obvious effect of temperature on their growth rate in terms of increase in fresh weight. Minimum temperature was set at 16ºC and the growth rate was observed to be not very high i.e. 0.96±0.012 g for *Spirogyra* sp. and 0.99±0.040 g for *Oedogonium* sp. at week 1. The growth continued and after three weeks it was measured 2.89±0.36 g and 3.05±0.133 g for *Spirogyra sp.* and *Oedogonium* sp. respectively. Although growth was observed to increase by increasing the temperature from 16 ºC to 20 ºC thus the fresh weight of *Spirogyra sp.* at week 4 became 3.93±0.240 g at 20 ºC while at 24ºC it was calculated as 4.79±0.097 g (Table 2).

Similarly *Oedogonium* sp. was found with increased growth at 20 ºC (4.59±0.021 g). *Oedogonium sp.* was observed to best grow at 24 ºC and the fresh weight was measured as 4.96±0.089 g at week 4. At 28 ºC the effect was also significant and both the algae were grown with increased growth 4.66±0.226 g and 4.91±0.052 g for *Spirogyra* sp. and *Oedogonium* sp. respectively. Above this range i.e. at 32 ºC the algae again showed decrease in growth and it was found to be 2.93±0.087 g for *Spirogyra sp.* and 3.59±0.069 g for *Oedogonium sp.* (Table 2). Similar results were revealed for effect temperature changes on algal cells in a work on *N. oculata* and *Chlorella sp.* cells. The growth appeared to be affected at temperature above 30 ºC. At temperature of 35 ºC the microalgae did in fact exhibit a 17% decline in its growth and further increase in temperature (38 ºC) led to death of algal cells (Coverti *et al.,* 2009). In another study scientists have found that the optimal temperature for growing for most species of algae is between 20 ºC to 30 ºC (Konopka and Brock, 1978)

Effect of various pH levels on algal growth: Algae when grown in different pH ranges show significant effect of it. Table 4 gives the results for effect of pH on algal growth and it is evident that growth was influenced by adjusting the pH at different ranges. The experiments were conducted with different pH ranges including 6.5, 7.0, 7.5, 8.0, 8.5, 9.0. It was observed that at minimum pH level tested during the present work (6.5), *Spirogyra sp.* and *Oedogonium* sp. were observed with low growth in terms of fresh weight with 3.23±0.022 g and 3.16±0.021 g fresh weight respectively after week 4. They were grown best and to the maximum at pH 7.5 and 8.0. *Spirogyra sp.* was having almost the same values for fresh weight i.e. 4.89±0.091 g and 4.81±0.052 g at pH 7.5 and 8.0 respectively (Table 3). The same effect was observed for *Oedogonium* sp. when their fresh weights were measured and they were found to be 4.79±0.021 g and 4.70±0.021 g at pH 7.5 and 8.0 respectively. At pH 9 growth was decreased and it was measured as 2.88±0.065 g for *Spirogyra sp.* and 3.52±0.015 g for *Oedogonium* sp. (Table 3). The findings of our work are in agreement with those publicized by Zhu (2010) whose results depicted that algae grew best in media adjusted with an initial pH of 7.10 and thus algae have shown fastest growth when the pH was near 8. He also concluded that in growth medium with a pH of 5.0 and 9.0 algae did not show apparent growth, thus the desired pH for growing algae is 7-8. Similar results were found in another study where microalgae was able to grow at pH 7.5 and above 8 the cells showed decline in growth rate (Garcia *et al.,* 2000).

It is also reported that pH is the third most important factor after temperature and light, for growing algae (Bajhaiya *et al.,* 2010).

Effect of light on algal growth: Algal cultures were placed under different light conditions (direct sunlight, indirect sunlight light, fluorescent light and under dark). No growth was recorded for any algal species under complete dark conditions so it was concluded that light is an essential factor for algal growth. Algal cultures kept in the room near window showed growth to some extent with mean value of 3.13±0.017 g for *Spirogyra* sp. as well as *Oedogonium sp*. But this was less as compared to fresh weights of algae when placed under direct sunlight. When algal cultures were exposed to direct sunlight 4.30±0.044 fresh weight was recorded both for *Spirogyra sp.* and 4.32±0.040 g for *Oedogonium* sp. Most suitable light condition for fresh and healthy growth of algal biomass found during the present study was florescent light (Table 4). At all the time intervals cultures placed under florescent light had higher fresh weights. Hence highest biomass recorded during present work for *Oedogonium* sp. was 3.98±0.020 g at week 4. And for *Spirogyra sp.* it was 4.52±0.018 g. Thus it is evident from results that light has significant effect on growth of algae (Table 4).

The growth of algae declines due to damage in light pigments at high light intensity (Janssen *et al.,* 1999). Other important findings have graphed out the importance of light intensity and its effect on algal growth. Their results have concluded that very high and intense light can inhibit the growth of algae, also the sun light sometimes can harm the algal cells (Converti *et al*., 2009. Thus it is depicted that light exposure, intensity and penetration are important factor for algal cultivation (Sharma *et al.,* 2008). Another work done by Hu *et al.,* (2008) have concluded that algae when grown at various light intensities have shown remarkable changes in their growth. They placed algal cultures in direct sunlight and under fluorescent (artificial) light. They have observed that although algae were able to grow and abundance in their biomass under sunlight but after few days algae started to deteriorate because too much of sunlight was not favorable for long survival of algae. Although when observed under artificial light, it was found that algal biomass exhibited better growth. Thus these results are in accordance with our proposed effect of light (Newsted, 2004).

Effect of aeration on algal growth: Unicellular form of algae was provided with constant aeration through pumps. This is an important factor because in the absence of any aeration or mixing means, the cells became dead and no more growth can be recorded. A comparison was made to see the effect of aeration by supplying on set of *Chlorella sp.* cultures with aeration pumps and the other set was left static (Figure 5). Absorbance was measured at 600 nm and the increase in cell number was observed in terms of increased absorbance rate. At day 1 the absorbance was measured 0.31±0.003 with aerating pump while 0.27±0.145 was found when *Chlorella sp.* were grown without aeration. At day 5 the growth increased and cell number were found to be more by calculating the absorbance as 0.70±0.009. Results were found to be significant and it was observed that *Chlorella sp.* cells can only grow when they are provided with constant aeration. Several researchers have reported the same effect of aeration and they said that it is highly recommended to provide algal culture with some mixing or aeration mechanism (Garcia *et al.,* 2005; Chen *et al.* 2006). Ip *et al.,* (2004) used several means of aeration in their experiments and paddle wheels were used in photobioreactors and thus algal culture were continuously mixed when wheel were rotated inside the cultures.

Oil Extraction: Bligh and Dyer (1959) extraction method was performed to extract the lipids from algae. All the three types of algae (Spirogyra, Oedogonium and Chlorella.) were extracted by using this method. The results for oil extraction using Bligh and Dyer method are shown in Figure 5. It is evident from the results that *Chlorella sp.* sp. has the highest lipid content followed *Oedogonium* sp. and *Spirogyra* sp. Similar results were found by Hossain *et al.,* (2008) and showed that biodiesel production was found maximum in Oedogonium and minimum in Spirogyra Bligh and Dyer method was studied by Woertz (2007) and his results were in agreement with those proposed by our findings and the total lipid content was found to be 14% for Oedogonium while Chlorella was having higher lipid content and was given as 24%. In a recent study oil extraction has been made from Pakistani algal species and reported highest lipid production in oedogonium. (Khola and Ghazala, 2012).

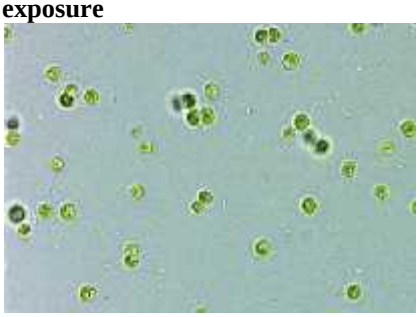
Fig. 1(a) Algae sample from Jillani Park

(b)Algae samples from Jillani Park

Fig 2. Algal cultures placed near window for light exposure

***a) Spirogyra* sp.**

b) *Oedogonium* sp.



c)*Chlorella sp.*

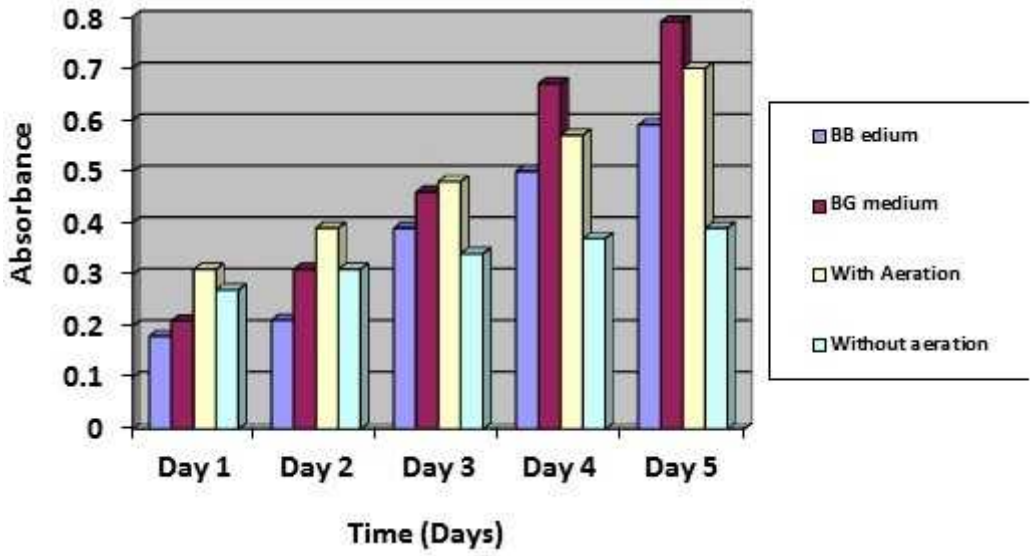


Fig.3 (a) *Spirogyra* sp., (b) *Oedogonium* sp., (c) *Chlorella* sp.

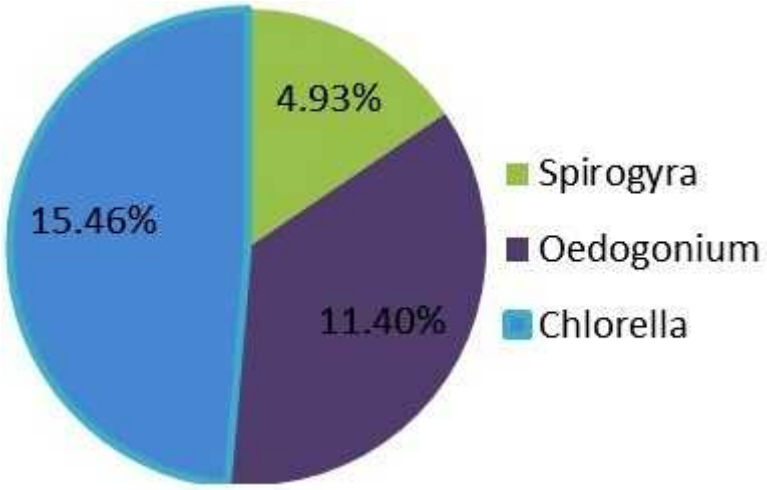


Figure 4. Effect of Medium and aeration on growth of *Chlorella* sp.

Figure 5: Percentage yield obtained from algal isolates by Bligh and Dyer method

Table 1. Effect of medium on growth of *Spirogyra* sp. and *Oedogonim* sp.

	Fresh weight (g)							
Growth medium	***Spirogyra* sp.**				***Oedogonim* sp.**			
	Week1	**Week2**	**Week3**	**Week4**	**Week1**	**Week2**	**Week3**	**Week4**
Distilled Water	0.5± 0.012	0.86± 0.101	1.01± 0.022	1.06± 0.015	0.65± 0.022	0.87± 0.121	1.05± 0.122	1.10± 0.015
BB medium	1.02± 0.029	1.42± 0.101	2.33± 0.061	3.02± 0.021	1.28± 0.022	2.41± 0.023	3.54± 0.011	4.75± 0.02
BG medium	1.54± 0.042	2.12± 0.056	3.54± 0.045	4.69± 0.071	1.03± 0.032	1.41± 0.101	2.33± 0.061	3.03± 0.02
Effect of medium (with 1 and 8 df)	*	*	*	*	*	*	*	*

Initial weight of each sample was 0.5 g
Data were recorded for 10 replicates for each experiment
*Significant at 5% probability level according to on-way ANOVA with df mentioned.

Table 2. Effect of temperature on growth of *Spirogyra* sp. and *Oedogonium* sp.

	Fresh weight (g)							
Temperature	***Spirogyra* sp.**				***Oedogonium* sp.**			
	Week1	**Week2**	**Week3**	**Week4**	**Week1**	**Week2**	**Week3**	**Week4**
16 °C	0.96±0.012 c	1.54±0.155 c	2.17±0.233 c	2.89±0.360[c]	0.99±0.040 c	1.66±0.112 c	2.40±0.179 c	3.05±0.133 c
20 °C	1.20±0.003 b	2.00±0.049 b	3.17±0.111 b	3.93±0.240 b	1.29±0.074 b	2.29±0.137 b	3.45±0.124 b	4.59±0.021 b
24 °C	1.73±0.142 a	2.80±0.074 a	3.82±0.033 a	4.79±0.097[a]	1.46±0.055 a	2.80±0.049 a	3.88±0.061 a	4.96±0.089 a
28 °C	1.51±0.061 a	2.62±0.137 a	3.52±0.109 a	4.66±0.226[a]	1.46±0.015 a	2.92±0.051 a	3.65±0.164 a	4.91±0.052 a
32 °C	0.92±0.062 c	1.65±0.006 c	2.13±0.089 c	2.93±0.087 c	1.06±0.032 c	1.80±0.061 c	2.39±0.168 c	3.59±0.069 c
Effect of temp (with 4 and 10 df)	*	*	*	*	*	*	*	*

Table 3: Effect of pH on growth of *Spirogyra* sp. and *Oedogonium* sp.

	Fresh weight (g)							
	***Spirogyra* sp.**				***Oedogonim* sp.**			
	Week1	**Week2**	**Week3**	**Week4**	**Week1**	**Week2**	**Week3**	**Week4**
6.5	0.94±0.020	1.72±0.022	2.40±0.006	3.23±0.022	1.03±0.017	1.77±0.023	2.58±0.021	3.16±0.021
7.0	1.22±0.015	2.01±0.049	3.29±0.013	4.16±0.026	1.19±0.029	2.12±0.030	3.25±0.079	4.57±0.035
7.5	1.88±0.015	2.88±0.022	3.76±0.026	4.89±0.091	1.73±0.035	2.70±0.026	3.70±0.015	4.79±0.021
8.0	1.57±0.032	2.76±0.066	3.63±0.012	4.81±0.052	1.37±0.012	2.24±0.020	3.34±0.038	4.70±0.021
8.5	1.13±0.015	1.48±0.018	2.95±0.023	3.50±0.044	1.44±0.029	2.82±0.038	3.78±0.045	4.65±0.043
9.0	0.088±0.023	1.66±0.015	2.04±0.026	2.88±0.065	1.00±0.032	1.72±0.045	2.21±0.015	3.52±0.015
Effect of pH (with 5 and 12 df)	*	*	*	*	*	*	*	*

Table 4: Effect of light on growth of *Spirogyra* sp. and *Oedogonium* sp

	Fresh weight (g)							
	***Spirogyra* sp.**				***Oedogonim* sp.**			
	Week1	**Week2**	**Week3**	**Week4**	**Week1**	**Week2**	**Week3**	**Week4**
Direct sunlight	1.14±0.025^{b}	2.08±0.013^{b}	3.16±0.032^{c}	4.30±0.044^{b}	1.11±0.006^{c}	2.11±0.041^{b}	3.14±0.035^{a}	4.32±0.040^{b}
Indirect sunlight	0.95±0.028^{c}	1.57±0.029^{a}	2.12±0.020^{b}	3.13±0.017^{c}	0.95±0.028^{b}	1.57±0.032^{c}	2.09±0.047^{c}	3.13±0.017^{c}
Fluorescent light	1.29±0.026^{a}	2.81±0.049^{c}	3.68±0.043^{a}	4.52±0.018^{a}	1.28±0.023^{a}	2.80±0.047^{a}	3.69±0.045^{a}	3.98±0.020^{a}
Effect of light (with 2 and 6 df)	*	*	*	*	*	*	*	*

Data were recorded for 10 replicates for each experiment

*Significant at 5% probability level according to on-way ANOVA with df mentioned.

Table 5. Effect of aeration on growth of *Chlorella* sp.

Aeration conditions	**Absorbance by *Chlorella sp.* sp. at 600 nm**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Aeration pump	0.31±0.003	0.39±0.006	0.48±0.006	0.57±0.186	0.70±0.009
Without pump	0.27±0.145	0.31±0.012	0.34±0.006	0.37±0.006	0.39±0.006
Effect of light (with 1 and 4df)	NS	*	*	*	*

Data were recorded for 10 replicates for each experiment

*Significant at 5% probability level according to on-way ANOVA with df mentioned.

Conclusion: From the present work it can be suggested that *Spirogyra sp.* and *Oedogonium* sp. and *Chlorella* sp. are common species of Lahore. Each of the algal species requires a different set of growth conditions if cultured in the laboratory. The comparison of oil contents indicate that *Chlorella* sp. has a higher oil content as compared to other algal species tested during the present work.

REFERENCES

Bajhaiya, A.K., S.K. Mandotra, M.R. Suseela, K. Toppo, and S. Ranade (2010). Algal Biodiesel: the next generation biofuel for India. Asian. J. Exp. Biol. Sci. 1(4): 728-739.

Bala, B.K. (2006). Studies on biodiesel from transformation of vegetable oils for diesel engines. Energy Educ Sci. Technol. 15: 1-45.

Bansal, B.K., and M.P. Sharma (2005). Prospects of biodiesel production from vegetable oils in India. Renew. Sustain. Energy. Rev. 9: 363-378.

Barsanti, L., and P. Gualtieri (2006). Algae biotechnology. In: *Algae: Anatomy, Biochemistry, and Biotechnology* CRC Press Taylor and Francis Group, Boca Raton. pp: 324-359.

Bligh, E and W. Dyer (1959). A rapid method for the total lipid extraction and purification. Can. J. Biochem. Physiol. 37 911-917.

Bold, H.C. (1949). The morphology of *Chlamydomonas chlamydogama* sp. Bull Torrey Bot Club 76:101-108.

Chen, F. (1996). High Cell Density Culture of Microalgae in Heterotrophic Growth. Trends in Biotechnology 14(11): 421-426.

Converti, A, A.A. Casazza Ortiz EY *et al*., (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process 48: 1146–1151.

Farooq, A., A. U. Khan and A. Yasir. (2013). Transesterification of oil extracted from different species of algae for biodiesel production. Afr. J. Environ. Sci. Tech. 7 (6) 358-364.

Feng, P.Z. (2011). Lipid accumulation and growth of *Chlorella zofingiensis* in flat plate photobioreactors outdoors Biores Technol 102(22): 10577-10584.

Garcia, C.M.C., J.M.F. Sevilla, F.G.A. Fernandez, E. M. Grima, and F.G. Camacho (2000). Mixotrophic Growth of *Phaeodactylum tricornutum* on Glycerol: Growth Rate and Fatty Acid Profile. J Appl Phycol 12: 239-248.

Grobbelaar, J.U. (2000). Physiological and technological considerations for optimising mass algal cultures. J. Appl. Phycol. 12: 201-206.

Held, P. (2011). Monitoring Algal Cell Growth Using Absorbance and Fluorescence. BioTek Application Note.

Henderson, R, S.A. Parsons, and B. Jefferson (2008). The impact of algal properties and pre-oxidation on solid/liquid separation of algae. Water. Res. 42: 1827-1845.

Hossain, A.B.M.S., A. Salleh, A.N. Boyce, P. Chowdhury and M. Naqiuddin. 2008. Biodiesel fuel production from algae as renewable energy. Am. J. of Biochem. Biotech., 4(3): 250-254.

Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, and A. Darzins (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant. J. 54: 621–639.

Huber, G.W., and B.E. Dale (2009). Biofuels: Grassoline at the Pump. Sci American. 301(1): 52.-59.

Illman, A.M., A.H. Scragg and S.W. Shales (2000). Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol 27: 631–635.

Ip, P.F., K. H. Wong, and F. Chen (2004). Enhanced production of astaxanthin by the green microalga *Chlorella zofingiensi*s in mixotrophic culture. Proc. Biochem. 39(11): 1761-1766.

Janssen, M., T.C. B. Kuijpers, B. Veldhoen, M.B. Ternbach, J. Tramper, L.R. Mur and R.H. Wijffels (1999). Specific growth rate of *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* under medium duration light dark cycles: 13-87. J. Biotechnol. 70(1-3): 323-333.

Khola, G and B. Ghazala (2012). Biodiesel production from algae Pak. J Bot. 44 (1): 379-381

Konopka, A., and T.D. Brock (1978). Effect of temperature on blue-green algae (Cyanobacteria) in lake Mendota. Appl. and Environ. Microbiol 36(4): 572-576.

Kulkarni, M.G, and A.K. Dalai (2006). Waste cooking oil-an economical source for biodiesel: A review. Ind. Eng. Chem. Res 45: 2901-2913.

Liu, Z.Y., G.C. Wang, and B.C. Zhou, (2008). Effect of iron on growth and lipid accumulation in *Chlorella. vulgaris*. Bioresour. Technol. 99: 4717–4722.

Newsted, J.L. (2004). Effect of light, temperature, and pH on the accumulation of phenol by *Selenastrum capricornutum*, a green alga. Ecotoxicol. Environ. Safet. 59(2): 237-243.

Richmond, A. (2000). Microalgal biotechnology at the turn of the millenium: a personal view. J. Appl. Phycol. 12: 441–451.

Rodolfi, L., G. Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, and M. Tredici (2008). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102(1): 100-112.

Sandnes, J.M., T. Kallqvist, D. Wenner, and H.R. Gislerod (2005). Combined influence of light and temperature on growth rates of *Nannochloropsis oceanica*: linking cellular responses to large-scale biomass production. J. Appl. Phycol. 17: 515–525.

Schenk, P.M., S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, and B. Hankamer (2008). Second generation biofuels: High-Efficiency microalgae for biodiesel production. Bioenerg Res 1: 20-43.

Sharma, Y.C., B. Singh, and S.N. Upadhyay (2008). Advancement in development and characterization of biodiesel: A review. Fuel, 87: 2355-2373.

Stanier, R.Y., R. Kunisawa, M. Mandel, and G. Cohen- Bazire (1971) Purification andproperties of unicellular blue- green algae (Order Chroococcales). Bacteriol Rev 35: 171- 205.

Zhu, M. P.P., Zhou, and L.J. Yu (2002). Extraction of lipids from *Mortierella alpina* and enrichment of arachidonic acid from the fungal lipids. Bioresour. Technol. 84: 93–95.