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Isolation of lipid producing *Spirulina* strain WS-41 and effect of various cultural factors on their lipid accumulation

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ABSTRACT

Total 25 strains of *Spirulina* were isolated from various sites of Meerut, India in order to determine the biomass yield and lipid content. Out of which, only 8 strains, namely WS-02, WS-26, WS-30, WS-41, WS-49, SS-07, SS-13 and SS-24 were found lipid producing, while the strain WS-41 was the maximum lipid producer (26.36±1.04 % of dry wt.). This strain was further investigated for the effect of various environmental factors viz., nitrogen source, phosphorous source, carbon source, NaCl, pH, Tween-80 on lipid content and biomass yield. Lipid content was determined as percentage of dry weight of biomass. A maximum lipid percentage (31.09±0.58 % of dry wt.) of WS-41 was observed at 0.2M KNO_3, 0.6 mM KH_2PO_4, 0.5 M NaCl and pH 9. Maximum biomass (3.43±0.25 g/l) was observed at 3.2 M KNO_3, 0.6mM KH_2PO_4, 0.15M NaCl and pH 9. Thus, it can be concluded that *Spirulina* strain WS-41 is a high lipid producer which can be a good source for food supplement as well as biofuel production in future.

Key word: *Spirulina*, biofuel, lipid content, biomass, cyanobacteria, microalgae.

1. INTRODUCTION

The continued use of fossil fuels is not sustainable, as they are finite resources (Srivastava, 2000) and their combustion lead to increased energy-related emissions ofgreenhouse gases (GHG) viz., carbon dioxide (CO_2), sulfur dioxide (SO_2) and nitrogen oxides (NOx). Biofuels can be a good alternative to supplement the continuously increasing fuel demands. Biofuels can be produced from various plants and microbial sources such as jatropha, rapeseed/canola, oilpalm, sunflower, mustard, soybean, cotton, corn and algae etc (Barnwal & Sharma, 2005). Plants are not able to fulfill the dependence of world on these fuels due to low growth rate, consumption of more quantity of nutrients and difficulty in growth in adverse conditions. However, algae are low input; high yield feed stocks to produce biofuels (Chisti, 2007). Microalgae are microscopic structures, either prokaryotic or eukaryotic cell type. They are capable of synthesizing a huge amount of lipids in their microscopic cell factory without consuming land as well as big amount of nutrients. They need only sunlight, water and CO_2 for their survival, which are freely available (Chisti, 2007; Pirt, 1986; Brown & Zeiler, 1993; Banerjee *et al.* 2002).

Cyanobacteria (blue green algae) contain significant quantities of lipids with a composition similar to vegetable oil. Many of these cyanobacterial fatty acids are essential component of human and animal diet and are important feed additives in aquaculture (Borowitzka, 1988). The lipids of some cyanobacteria are rich in fatty acids (Singh *et al*. 2002). They are photosynthetic and therefore autotrophic and reproduce by binary fission. The helical shape of the filaments (or trichomes) is characteristic of the genus and is maintained only in a liquid environment or culture medium. *Spirulina* is a member of the blue–green algae class (*Cyanophyceae*), a spiral filamentous mesophilic organism made up of microscopic prokaryotic cells. *Spirulina* contains high levels of gamma-linoleic acid. It is produced commercially as a food source in health foods and pharmaceutical industry. Microalgae are principal organisms which produce a wide variety of biological compounds, principally vitamins, pigments, proteins, minerals, lipids and polysaccharides. As compared to other living organisms, algae are very rich in some kinds of fatty acids such as polyunsaturated fatty acids (PUFA), ð-linoleic acid (GLA) (Borowitzka 1992; Cohen 1997). To find out the changes in fat and fatty acid composition of *Spirulina* stemming from various stress sources (Salinity, nitrogen, phosphorous, pH, temperature, etc.), a lot of studies have been carried out by various workers (Vonshak *et al.* 1996; Rafigul *et al*. 2003; Koru and Cirik, 2003; Işık *et al*. 2006; Ayachi *et al.* 2007; Vonshak *et al*. 1996; Rafiqul *et al*. 2003; Koru and Cirik, 2003, Isik *et al*. 2006; Ayachi *et al*. 2007).Temperature can also affect the biochemical composition of *Spirulina*. Growth and lipids content of *S. platensis* was affected in temperature ranges from 25 to 38^{0}C (Tedesco & Duerr, 1989).

In the present communication, we have isolated various *Spirulina* strains from various places of Meerut, India for investigating their lipids and biomass yield. Further, the effects of various factors viz., nitrogen source, phosphorous source, salinity, pH, carbon source, and Tween-80 on the degree of lipid content and biomass yield *Spirulina* strain have been investigated.

2. MATERIAL AND METHODS

Collection of samples

For isolation of *Spirulina* strains, total 20 samples (10 soil and 10 water samples) were collected in December- 2009, from lakes (2 soil + 2 water), brackish (2 soil + 2 water) and freshwater ponds (2 soil + 2 water), paddy fields (2soil + 2 water) and wetlands soils (2 soil + 2 water) of different places of Meerut, India. Isolation medium (K_2HPO_4 0.039, $MgSO_4.7H_2O$ 0.075, Na_2CO_3 0.020, $CaCl_2.2H_2O$ 0.027, $Na_2SiO_3.9H_2O$ 0.058, EDTA 0.001, Citric acid 0.006, Fe Citrate 0.006 g/1000 ml distilled water and 1 ml ofA_5- micronutrient solution) H_3BO_3 2.86, $MnCl_2$. $4H_2O$ 1.81, $ZnSO_4.7$ H_2O 0.222, $Na_2MoO_4.2H_2O$ 0.391, $CuSO_4$. $5H_2O$ 0.079gm/1000 ml distilled water) was used for the isolation of *Spirulina* from various water and soil samples (Hughes *et al*. 1958).

Isolation of *Spirulina*

Soil specimen of 10 g was mixed with the medium to give a total volume of 50 ml and agitated for 30 minutes on a rotator shaker. Ten fold serial dilutions were prepared from each of the 10 water samples and 10 soil samples separately and used to inoculate the two sets of isolation media (Hughes *et al*. 1958). All inoculated sets were incubated at 28°C ±2 under 2500-3000 lux light intensity with 18:06 hr photoperiods and bubbled with air for maximum period of 15 days. Observations for algal growth were examined after incubation of 10-15 days.

Microscopic examination of isolates

All isolates were examined under microscope such as (45X, 100X) for their morphological characteristics and cultural behavior in liquid medium. The colour of thallus, benthic nature and type of growth. The morphological and cultural characteristics were compared with the standard keys described by Desikachory (1959). All isolated strains were grown and maintained on Zarrouk medium ($NaHCO_3$ 18.0, $NaNO_3$ 2.5, K_2HPO_4 0.5, K_2SO_4 1.0, NaCl 1.0, $CaCl_2$ 0.04, Na_2EDTA 0.08, $MgSO_4.7H_2O$ 0.20, $FeSO_4.7H_2O$ 0.01 gm/1000 ml distilled water and pH 8.2, 1 ml solution of trace elements (H_3BO_3 2.86, $(NH_4)_6$ Mo_7O_4 0.02, $MnCl_2.4H_2O$ 1.80, $CuSO_4.5H_2O$ 0.08, $ZnSO_4.7H_2O$ 0.22 g/1000 ml distilled water) under discontinuous illumination of 16hrs: 8 hrs light/dark cycles at 2500-3000 lux light intensity with cool white fluorescent tubes at 28 ± 2 ^{0}C (Zarrouk, 1966).

Screening of isolated strain for lipid production

For selection of most perfect lipid producing isolated *Spirulina* strains, screening was carried out by following method. 1 ml of 14 days old cultures of all isolated *Spirulina* strains with O.D. of 0.9-1.0 at 560 nm were grown in 500 ml of Zarrouk medium separately for lipid production. All flasks were incubated under discontinuous illumination of 16hrs: 8hrs light/dark cycles at 2500-3000 lux light intensity with cool white fluorescent tubes at 28 ±2 ^{0}C in a culture room. Observations were recorded after 20 days and percentage of lipid content and biomass yield estimation was carried out according to Bligh and Dyer method (Bligh & Dyer, 1959).

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Effect of various factors on lipid accumulation and biomass yield of selected strain (*Spirulina*WS-41)

After screening, selected potent isolate (*Spirulina*WS-41) was cultured in various cultural conditions in order to obtain the optimal parameters for high lipid production.

Effect of nitrogen

To see the effect of nitrogen, four concentrations of KNO_3 viz., 0.2, 0.8, 3.2, and 12.8 mM were maintained in 500 ml Zarrouk medium contained in 1000 ml flask separately.1 ml of 14 days old culture with 0.9-1.0 O.D. at 560 nm was used as inoculum. All flasks were incubated at same conditions as described previously in section 2.3 above. Observations were recorded after 20 days and percentage of lipid content and biomass yield estimation was carried out according to Bligh and Dyer method.

Effect of phosphorus

Phosphorus was supplied as KH_2PO_4 with the concentrations of viz., 0.3, 0.6, 1.2mM and 1.8 mM into different flask containing 500 ml Zarrouk medium in 1000 ml flask. 1 ml of 14 days old culture with 0.9-1.0 O.D. at 560 nm was used as inoculum. The flasks were incubated at the same conditions as described previously in section 2.3 above. Observations were recorded after 20 days and percentage of lipid content and biomass yield estimation was carried out according to Bligh and Dyer method.

Effect of salinity

To observe the effect of salinity on lipid content and biomass yield of selected potent isolate (*Spirulina*WS-41), four levels of salinity viz., 0.15, 0.4, 0.5, and 0.7 M of NaCl were maintained in different flasks containing 500 ml of Zarrouk medium in 1000 ml flask. 1 ml of 14 days old culture with 0.9-1.0 O.D. at 560 nm was used as inoculum. The flasks were incubated at the same conditions as described previously in section 2.3 above. Observations were recorded after 20 days and percentage of lipid content and biomass yield estimation was carried out according to Bligh and Dyer method.

Effect of pH

To observe the effect of pH on lipid content and biomass yield of selective potent isolate (*Spirulina*WS-41), 4 flasks containing500 ml of Zarrouk medium of 1000 ml were taken. The pH of each of the four flasks was maintained at 4.0, 7.0 using citrate phosphate buffer (Mellvaine, 1921) and pH 8.2 (control) & 9 using boric acid borax buffer (Holmes, 1943) 1 ml of 14 days old culture with 0.9-1.0 O.D. at 560 nm was used as inoculum. The flasks were incubated at the same conditions as described previously in section 2.3 above. Observations were recorded after 20 days and percentage of lipid content and biomass yield estimation was carried out according to Bligh and Dyer method.

Effect of carbon

To observe the effect of Carbon source (glycerol) on lipid production and biomass yield of selective potent isolate (*Spirulina*WS-41), glycerol was suspended with three different concentrations viz., 1.25 mM, 6.86 mM and 12.5 mM. Cultures were maintained in 500 ml Zarrouk medium contained in 1000 ml flask. 1 ml of 14 days old culture with 0.9-1.0 O.D. at 560 nm was used as inoculum. The flasks were incubated at the same conditions as described previously in section 2.3 above. Observations were recorded after 20 days and percentage of lipid content and biomass yield estimation was carried out according to Bligh and Dyer method.

Effect of Tween-80

To observe the effect of Tween-80 on lipid content and biomass yield of selective potent isolate (*Spirulina*WS-41), with one of the three different concentrations of Tween-80 viz., 0.1%, 1.0% and 5%.Cultures were maintained in 500 ml Zarrouk medium contained in 1000 ml flask. 1 ml of 14 days old culture with 0.9-1.0 O.D. at 560 nm was used as inoculum for mass production. The flasks were incubated at the same conditions as described previously in section 2.3 above. Observations were recorded after 20 days and biomass yield estimation was carried out according to Bligh and Dyer method.

3. RESULTS

Total 25 strains of *Spirulina* were isolated from various sites of Meerut, India in order to determine the biomass yield and lipid content. Out of which, only 8 strains, namely WS-02, WS-26, WS-30, WS-41, WS-49, SS-07, SS-13 and SS-24 were found lipid producing, while the strain WS-41 was the maximum lipid producer (26.36±0.58 % of dry wt.), (Figure 1). Although there is no

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linear relation of lipid content with biomass yield. Various strategies were taken into consideration for higher lipid production. Out of four different concentrations of KNO_3 as nitrogen source, a minimum concentration (0.2 M) of nitrogen source was observed statistically best for maximum lipid (31.09±1.04 % of dry wt.) accumulation and biomass 0.41±0.02 g/l, as in nitrogen starvation lipid accumulation increases in algal cells (Figure 2) (Feiyan *et al,* 2010).

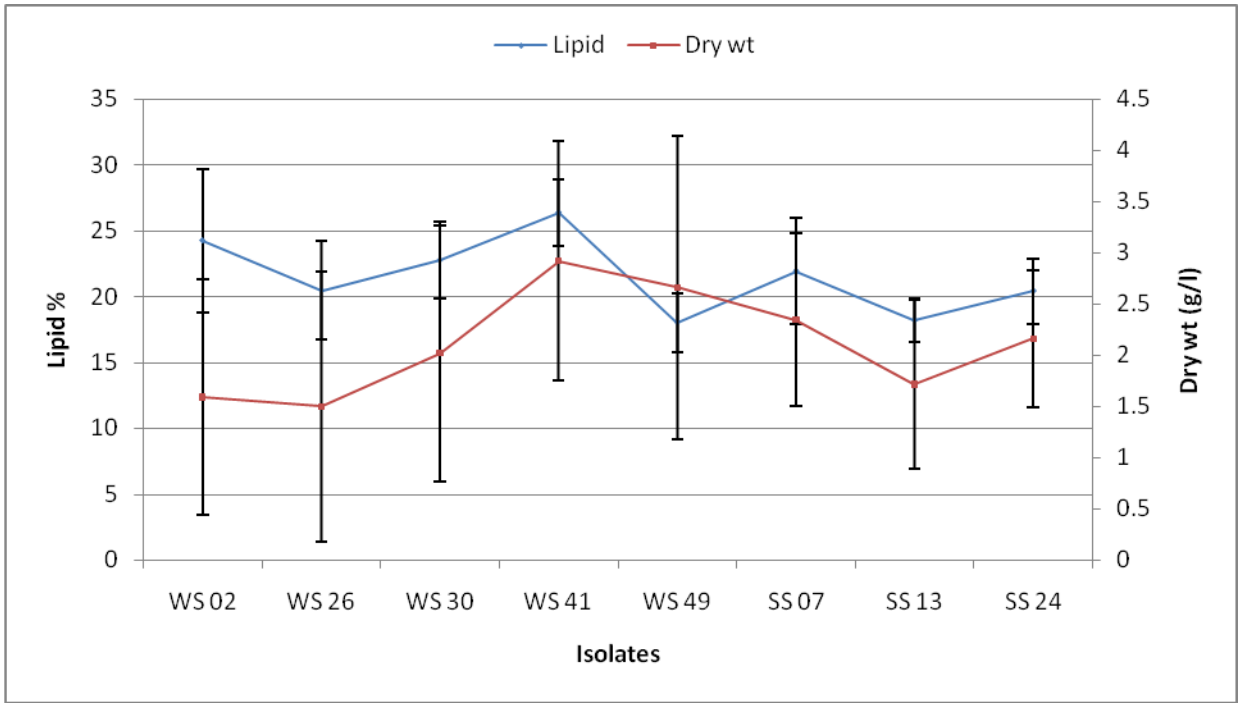

Figure 1

Screening of isolated *Spirulina* strain for high lipid production

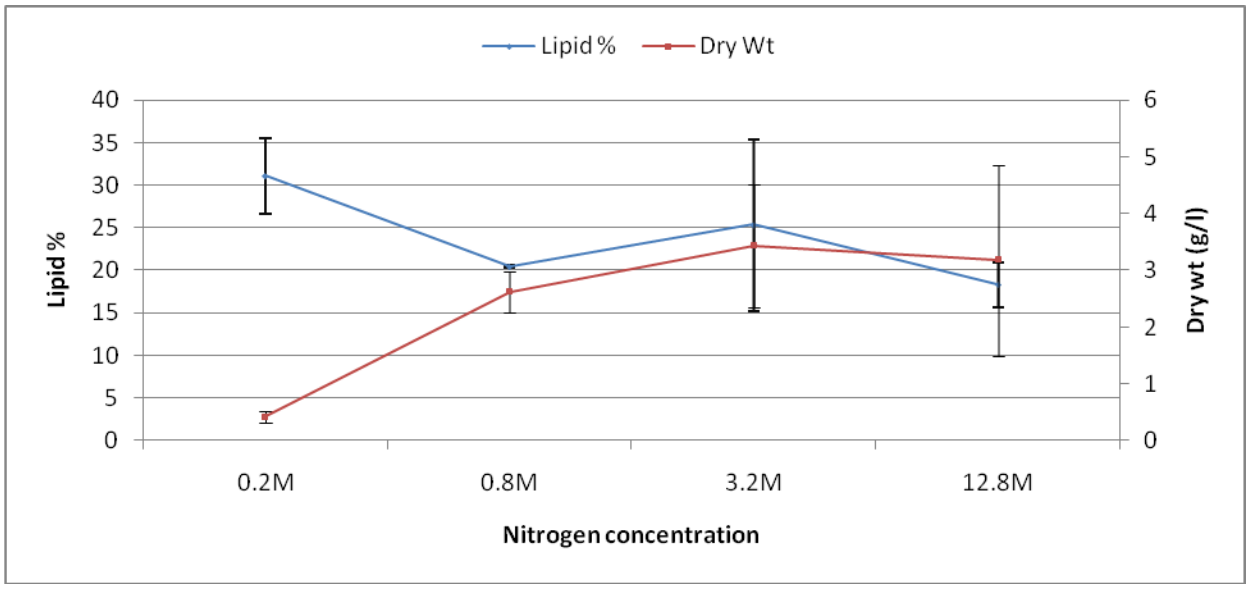

Figure 2

Effect of nitrogen on lipid content and biomass of WS-41 isolate

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The results of paired t- test showed the statistically significant variations between the means of control versus 0.2 molar KNO_3 conc. ($P< 0.05$), however the mean values of other KNO_3 conc. (0.8M, 3.2M & 12.8M) found not statistically significant with the mean value of control. The 0.8M and 12.8M KNO_3 conc. for lipid accumulation found statistically significant ($P<0.05$) except 0.02M KNO_3 conc. ($P<0.099$) and 3.2M KNO_3 conc. ($P<0.744$) versus control by paired t- test. The highest lipid (28.04±0.31%) accumulation was found at 0.6Mm conc. of K_2HPO_4 followed by 0.3Mm (26.77±0.80%), 1.2Mm (24.71±1.03%) & 1.8Mm (22.99±0.37%). The result of paired t- test showed the statistically significant variations between the means of control and 1.8 Mm K_2HPO_4 conc. ($P=0.005$) (Figure 3).

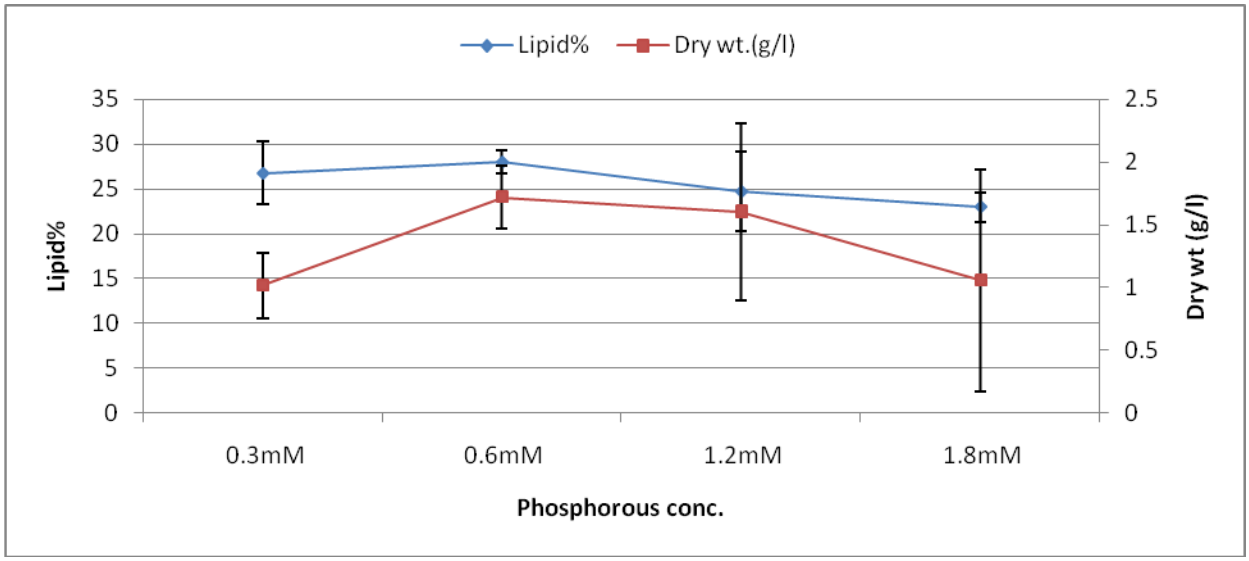

Figure 3

Effect of Phosphorous conc. on lipid content and biomass of WS-41 isolate

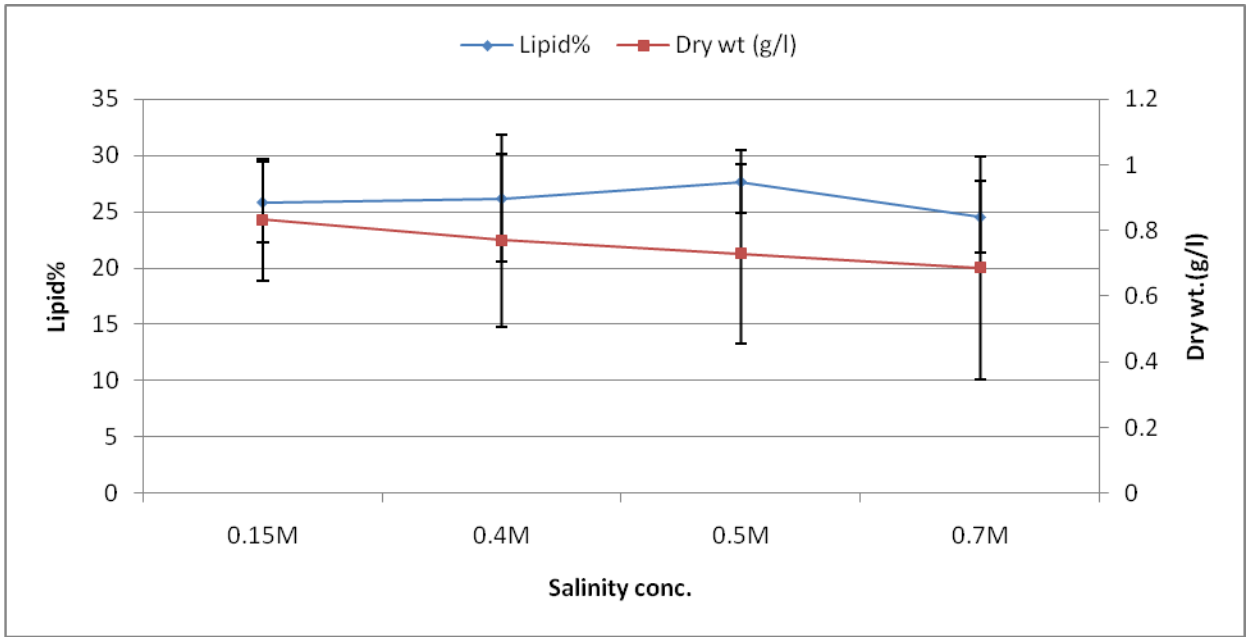

Figure 4

Effect of salinity conc. on lipid content and biomass of ws-41 isolate

The maximum lipid accumulation (27.66±0.64%) was found at 0.5 M NaCl conc. which is not statistically significant in comparison to control ($P> 0.05$). However the biomass yield was found statistically significant ($P<0.05$) at all tested conc. of NaCl

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with comparison to control (Figure 4). The lipid accumulation was highest at significantly highest (29.46±0.65%) at pH 9. The highest biomass was yielded at pH 8.2 which is not statistically significant (P>0.05) with comparison to control, however the biomass at pH 4, 7 and 9 were found statistically significant (P<0.05), (Figure 5). The lipid as well as biomass accumulation was significantly (P<0.05) highest at 1.25Mm conc. of carbon source (Glycerol). The biomass as well as lipid accumulation was significantly decreased (P<0.05) with increased carbon source conc. (Figure 6). The lipid accumulation and biomass was found significantly (P<0.05) highest at lowest conc. of Tween-80. However both the lipid accumulation and biomass production was significantly (P<0.05) decreased with all tested concentrations of Tween-80 (Figure 7).

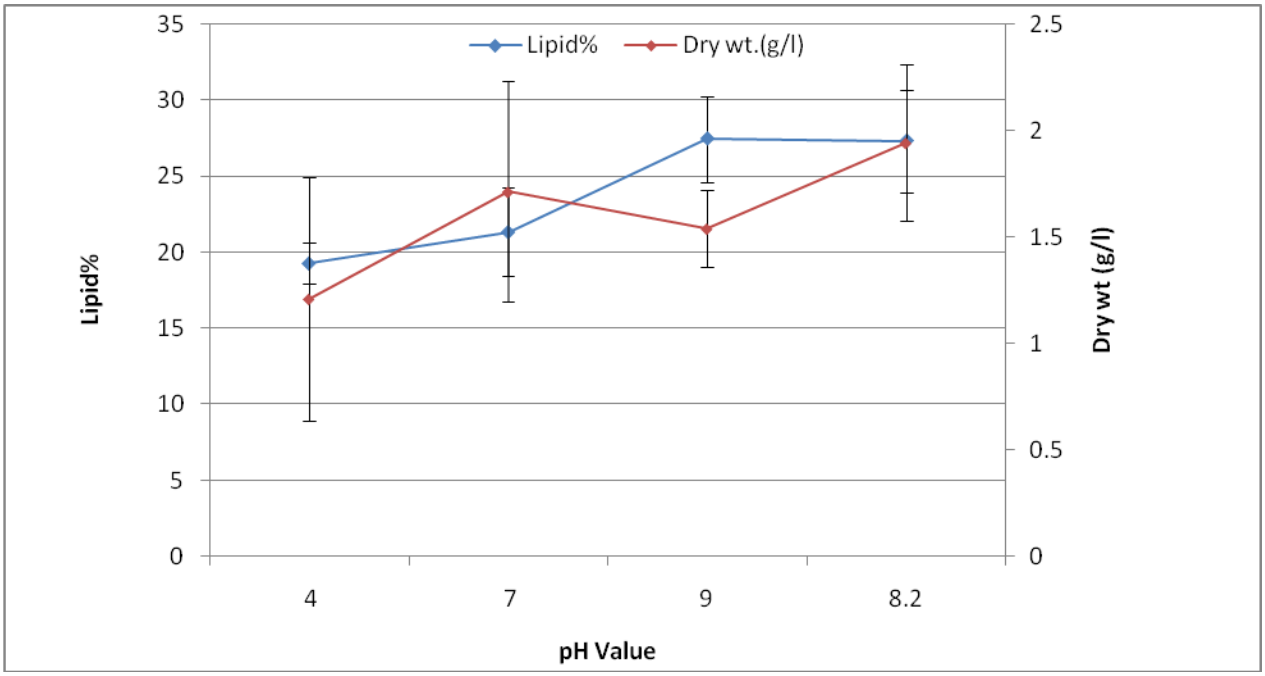

Figure 5

Effect of pH on lipid content and biomass of WS-41 isolate

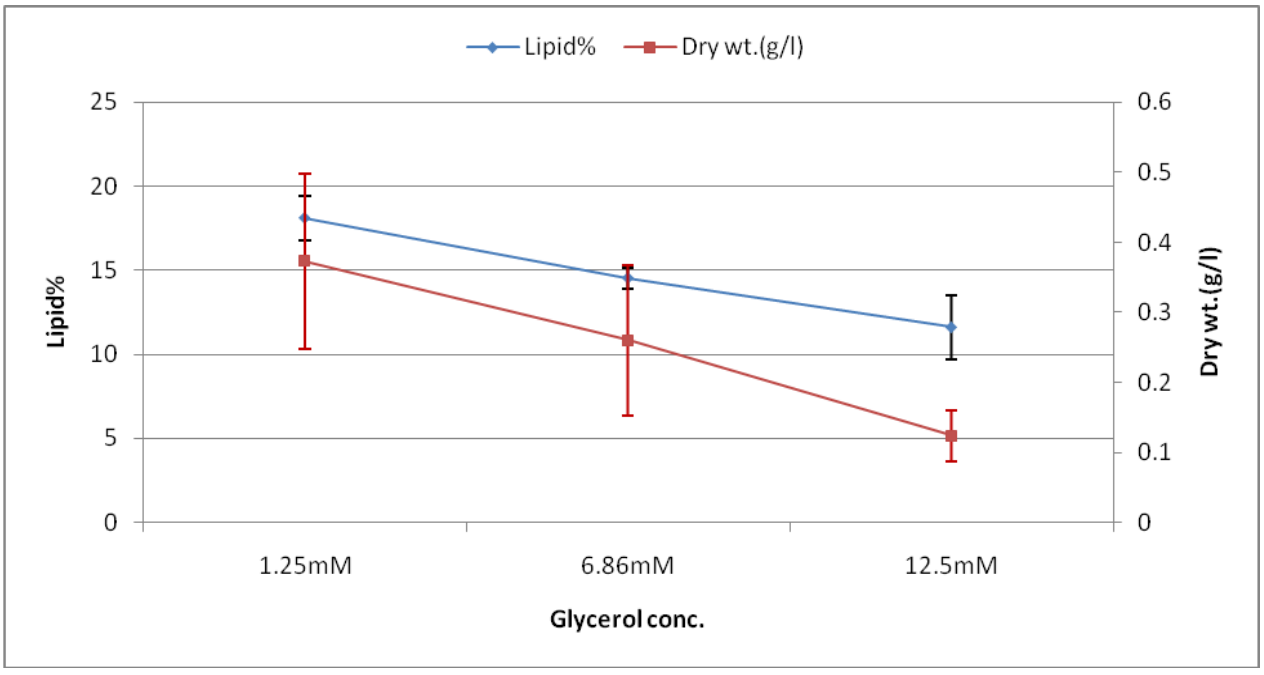

Figure 6

Effect of Carbon source (Glycerol) on lipid content and biomass of WS-41 isolate

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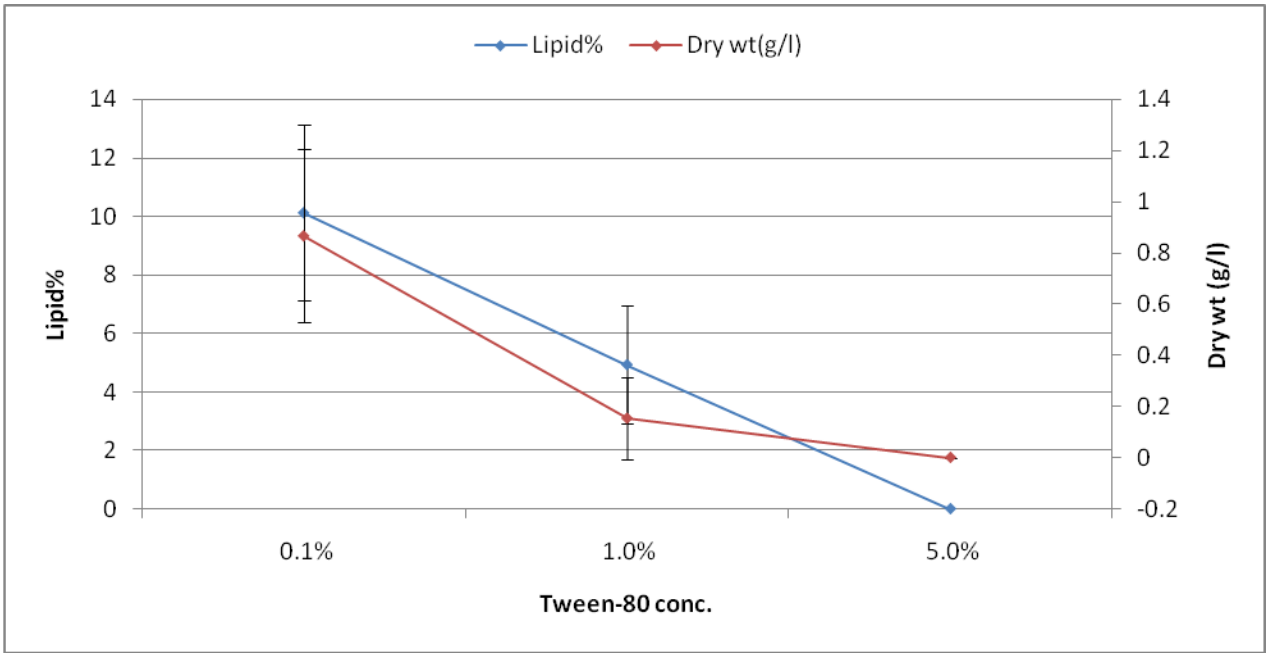

Figure 7

Effect of Tween-80 on lipid content and biomass of WS-41 isolate

4. DISCUSSION

The medium used for isolation of microalgae in this study was developed by Hughes *et al*. (1958). For most of the isolates of microalgae obtained from fresh water and lake water, Zarrouk medium was most suitable Zarrouk (1966). At the lowest concentration of N and P, the microalgal cell tended to produce high lipid contents. This result may be due to their influence on growth inhibition (Feiyan et al. 2010). Furthermore, Lombardi & Wangersky (1991) mentioned that in some species of microalgae, the growth rate is set by nitrogen or phosphorus availability. In the present investigation, at the initial KNO_3 concentration of 12.8 M, the lipid content for all tested isolates was decreased. This is because at those levels of N, the optimal concentration of nitrogen dissolved in the medium was exceeded. Excess nitrogen nutrition in microalgae inhibits the protein production (Taguchi & Smith, 1993; Ben-Amotz *et al*. 1985). A recent study has shown that the lipid composition, especially the polar lipids, can be changed by salinity (Hilal *et al*. 2010). In our study, same results were observed, and found that salinity certainly affect the accumulation of lipid during cell growth. Therefore, the salinity manipulation may be used as a tool to yield algal biomass containing desired lipid composition. The high lipid production 29.0 % and 28.7 % was reported by Gouveia & Oliveira (2009) in *Neochloriso leoabundans and Nannochloropsis spp*, respectively. To our knowledge, the present study showed that such high lipid content (31.09 % of dry wt.) in a *Spirulina* isolate has been observed for the first time. In this study a maximum cell growth and lipid content was observed at alkaline range pH 9. High lipid and fatty acid accumulation in microalgae typically occurred during period of environmental stress, including growth under nutrient deficient conditions, but this is not always the case (Taguchi & Smith, 1993).

5. CONCLUSION

The current effort was focused on *Spirulina* strain (WS-41) for lipid accumulation and biomass yield. The optimal culture conditions for maximum lipid production were nutrients (nitrogen 0.2 M and phosphorous 0.6mM), salinity 0.5 M and pH 9. In this regard, the level of the nutrients, especially KNO_3 must be strictly controlled in highly productive system at full concentration. The results of nitrogen and phosphorous indicated that N-deficient cultures will develop higher amount of lipid content than N-sufficient culture conditions and all other factors should be kept in optimum range. Despite of all studied cultural factors, it is concluded that some specific environmental and cultural conditions can be fruitful for better biofuel production.

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Conflicting interests
The authors declare that there are no conflicts of interests.

Data and materials availability
All data associated with this study are present in the paper.

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