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Osmotic stress induced by salinity for lipid overproduction in batch culture of *Chlorella pyrenoidosa* and effect on others physiological as well as physicochemical attributes

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Keywords: NaCl-induced osmotic stress, heterotrophic cultivation, lipid,biomass, *Chlorella pyrenoidosa*.

Abstract

Effect of NaCl-induced osmotic stress on lipid production was investigated in batch culture of *Chlorellapyrenoidosa*. Based on the facts that NaCl stress improved lipid production but inhibited cells growth at thesame times, the novel strategies of multiple osmotic stresses with different NaCl Concentration wereadopted varying from 5 mM to 25mM for lipid overproduction. Results showed that after 15 days cultivation, lipid yield reached3.16±0.008 g/L and an intracellular lipid content was 43.84% with corresponding increase in biomass(0.19±0.016 to 1.53±0.012)at 25mM respectively, compared to the control. While, total chlorophyll and carbohydrate content increased in all the concentrations of NaCl as compared to control for the culture studied.

Introduction

Nowadays, the global energy system is predominantly based on utilization of fossil fuels, coal oil and naturalgas. This system has several problems, such as: 1) it creates pollution on local., regional and global scales,2) the reserves of fossil fuel are limited while on the other hand the demand for fossil fuel increases dramatically with the increasing population as a consequence creating a global energy crisis and 3)fossil fuel produces greenhouse gas emissions (NO_x,CO2 and So_x) that cause global warming and climatechange problems (Barbir, 2009). For the past ten years, fuel production from biomass (biofuel) has received considerable attention from researchers and scientists as it is a biodegradable, renewable and non-toxic fuel (Mutanda et al., 2010).Biofuel based on vegetable oil, bioethanol and biodiesel represent promising energy sources to displace fossil fuel (Lardon et al.,2009). Biodiesel, as a biodegradable and renewable fuel source, is considered as an ideal substitute for energy crisis (Lang et al., 2001; Antolin et al., 2002).

Biodiesel are monoalkylesters of long chain fatty acids which are trans esterified from vegetable oil or animal fat.Biodiesel from microalgae seems to be a promising renewable biofuel that has the potential to completely displace petroleum-derived transport fuel without adversely affecting the supply of food and other crop products (Christi, 2008; Xu et al, 2006; Bastianoni et al., 2008;Ma and Hanna, 1999)

FTIR Spectroscopy has been widely used to provide the information on range of vibrationally active functional groups (including O-H, N-H, C=O, =C-H,-CH_2,-CH_3, C-O-C,and >P=O)in biological specimens.(Stuart,1997). Although the technique has been largely used with isolated macromolecules and molecular complexes such as nucleic acid (Liquier, Taillandier,1996), Proteins (Stuart, 1997), Lipids (Lewis, 1996), Polysaccharides (Brandenburg and Seydel,1996), studies carried out on whole organism. The FTIR spectroscopy has successfully been established as a tool reailably, quickly and easily identifying microalgae (Bastert, 1999).

The most significant ecological factor is salinity that affecting the growth as well metabolic activities of plants and microorganisms. Several environmental factors such as pH, light, temperature and salinity are significantly affect the phytoplankton growth and cellular composition(Alam et al. 2001).The most important effect of salinity and pH on algal growth were the osmotic consequences of movement of water molecules along electrochemical gradient, and the

flow of ions along electrochemical gradients.(Lobban and Harrison,1994).Variation in the salinity of water distress the growth as well as metabolism and photosynthetic activity of phytoplankton organisms. (Moisander et al.,2002; Lartigue et al.,2003)

It was reported that high concentration of salinity can stimulate accumulation of intracellular lipid in microalgae (Rao et al., 2007).Although salt-induced osmotic stress can stimulate lipid accumulation,its effects on cell growth is scarcely known. On the other hand, high salt conditions have been found to significantly enhance lipid formation. Upon changing the sodium chloride concentration from 10 to 20 gl^{-1} in a culture of N. laevis, the synthesis of total lipids, the production of eicosapentaenoic acid (EPA) and the accumulation of polar lipids increased while the synthesis of neutral lipids decreased.(Chen et al. 2008). Plant cells are generally able to live within a certain range of enhanced salt concentrations or changing salinities, since most probably all life originated in the oceans, i. e. a highly saline environment. However, during evolution, the degree ofsalt resistance and salt tolerance became very divergent among the present-dayaquatic organisms. Algae (and cyanobacteria) have attracted considerable attentionin this respect, since they are inhabitants of biotopes characterized by changingsalinities and can serve as model organisms or a better understanding of salt acclimationin the more complex physiological processes of higher plants. (Bohert and Jensen,1996,Fogg 2001, Bohnert, H.J., Sheveleva, E.1998).The aims of the present study was to know the effect of NaCl concentration on the lipid and chlorophyll, biomass and other cellular components of Chlorella sps.in the laboratory conditions. All experiments were conducted in triplicate, and results were expressed as means of the replicates along with standard deviation (± SD).

Material and methods

Isolation of algal species

The experimental organism green microalga*Chlorellasp*. was isolated from water sample collected from a freshwater pond from village Shahidaawaali, Sirsa (Haryana).Purified culture of *Chlorella*spp. was obtained by repeated streaking and plating at pH 7.0±1 using standard isolation and culturing techniques in BG-11 medium. The purified algal sample was cultivated on BG-11 medium and maintained by regular sub culturing. To study the impact of NaCl, the algal species was cultured in BG-11 medium modified with varying 5 level salt concentrations (5 mM to 25 mM). To investigate the effect of salinity on *Chlorella*spp. the experiments were carried out in 250 ml Erlenmeyer flasks each containing 100 ml of BG-11 medium incubated at 25ºC in an orbital shaker set to120 rpm in BOD incubator cum shaker for 15 days and control culture in BG-11 media was also run parallel. The samples were drawn on 15th day and were subjected to analysis for various physiological and biochemical parameters. All the experiments were carried out in triplicates.

Identification of algal isolate

The algal cells were observed under light microscope for their morphological features and other cellular details**.**Purified algal species was identified with the help of algal identification guide on the basis of morphological features under the light microscope.

Nile Red fluorescence microscopy:

Thirty two algal samples that had high lipid content and biomass were selected for further study.

Based on preliminary procedure for improved Nile red staining, Microalgal cells (0.5 ml) were collected by centrifugation at 5000 rpm (Rotation per minute) for 10 min and washed with distilled water after that washed with physiological saline solution (0.5 ml) several times. Further algal samples immersed in Nile red solution (0.5 mg/ml^{-1} in acetone), mixed with 50 ml glycerol: water mixture (75:25),gently vortex for 1min.After 15 minutes of incubation in darkness, the fluorescence of algal samples was measured with fluorescence Olympus Magnus microscope having 420 nm to 580 nm absorption and emission wavelength respectively.

Fourior transform infrared analysis (FTIR)

A known quantityof lyophilized dried biomass was taken, mixed with KBR powder and ground well to fine mixture. The mixture was pressed to a disc using aHydraulic press in to tablets. The disc was subjected to FTIR spectral measurement in the frequency range of 4000−400 cm^{-1}.The algal powder was characterized using Fourier Transfer Infrared Spectrophotometer.

Estimation of cellular components

Lipid extraction and estimation

Total lipids were extracted by mixing methanol-chloroform (2:1.5 v/v) with the algal samples using slightly modified version of Bligh and Dyer's method (Bligh and Dyer 1959). Algal biomass pellet was collected by centrifuging 50 mL of the algal culture at 5,000 rpm for 10 min. The supernatant was discarded, and the algal biomass was incubated for 24 h at 25 °C in a mixture of 2 mLmethanol and 1.5 mL chloroform. The mixture was then vortexed for 2 min, followed by the addition of 1.5 mL of chloroform and agitation again for 1 min. The mixture was amended with 1.8 mL distilled water followed by 2 min of vigorous agitation. It was then centrifuged for 10 min at 2,000 rpm, and a lower lipid layer was separated carefully using Eppendroff micropipettes in a clean previously dried (104 °C) and preweighed 15-mL glass centrifuge tube. The chloroform phase was evaporated near to dryness in a water bath at 70 °C, and the residue was dried further at 104 °C for 30 min. Lipid content was described as percentage dcw.

Dry Biomass estimation

Dry cell biomass was measured as the cell density (dcw, g/l) at OD625 of an 11-day-old culture at dilutions ranging from 0.2to 1.0. The dry biomass was calculated using the regression
equation as the relationship given by Yount (2006): $y = 0.1015x + 0.2071$, $R^2 = 0.9456$

Total chlorophyll estimation

Chlorophyll content of the algae was estimated spectrophotometrically at 650 and 665nm Chlorophyll (MacKinney, 1941).The concentration of chlorophyll was calculated using the formula:Total chlorophyll (µg/mL) = 2.55×10^{-2} E650 + 0.4 1×0^{-2} E665$\times 10^{3}$

Carbohydrate Estimation

Carbohydrate was determined at 625 nm by Anthrone reagent method (Dubois et al. 1956).
Standard curve prepared by using graded conc. of glucose dilution ranging from 0.2 to 1
$y = 0.636x + 0.0592$, $R^2 = 0.9595$

Total Protein estimation

Protein content was estimated at 660 nm by the method of Lowry and coworkers (Lowry et. al.1951). Protein concentration was calculatedfrom the standard curve prepared with bovine serum albumin (BSA).
$y = 0.1097x - 0.0005$, $R^2 = 0.9989$

Result and discussion

In the present investigation, fresh water green microalga has been isolated from enriched mixed culture by standard isolation technique. Further characteristics and morphological features of the isolate have demonstrated its close similarity with genus *chlorellapyrenoidosa*.Its cells characteristicsare emerald-green coloured spherical, unicellular in shape. Nile red staining showed bright yellow to yellow-gold fluorescent are round bodies signifies that *Chlorella* species have substantial amount of lipid content.

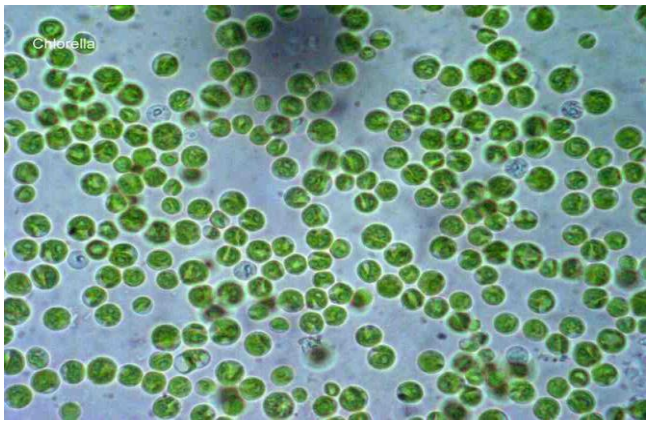

Figure1.Light microscope image of *Chlorella pyrenoidosa*.(100 $_x$)with immersion oil

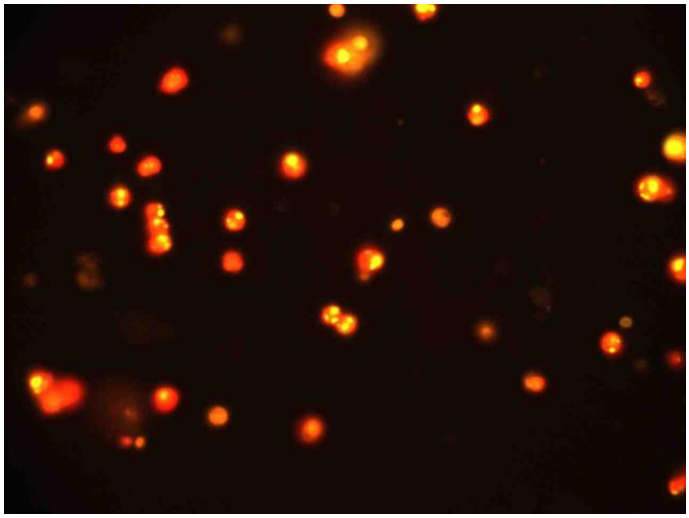

Figure 2. Nile red fluorescence of representative microalgal cells. All cells were observed for yellow-gold fluorescence with nile red stain using excitation band pass filter of 420 nm and emission band pass filter of 585 nm. The bright yellow to yellow-gold fluorescent are round bodies.

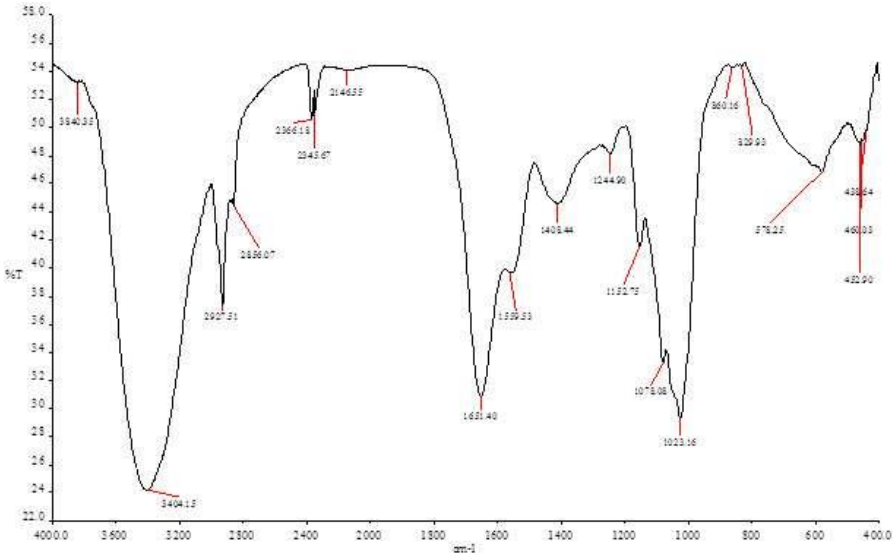

Figure3. FTIR spectra for *Chlorellapyrenoidosa*

FTIR spectra (Figure 2 in relation to specific functional groups (Table1).Each peak consigned a functional group. The molecular assignments of FTIR bands are based on published data phytoplankton, bacteria and other biological materials. In this study chlorella vulgaris protein spectra characterized by strong peaks 1651 cm^{-1} (amide I) and 1559cm^{-1} (amide II). These bands were due primarily to C=O stretching vibration and a combination of N-H and C-H Stretching vibrations in amide complexes. Lipid and carbohydrates were characterized by strong vibrations the C-H 2927cm^{-1},due to –CH2 symmetric as well as asymmetric stretching.C-O-C of polysaccharides at 1078 cm^{-1},1023 cm^{-1}respectively(Brandenburg, Seydel, 1996) while carbohydrates are the strongest absorbers between 1244 and 1023 cm^{-1}. Several other classes of compounds, such as nucleic acids have functional groups with absorption bands in the same region of the spectrum. The strongest peaks 1559 and 1408 shows that bending modes of methyl groups of protein. The peak 1244 shows carboxylic acid present in chlorella spp.(Benning,et al.,2004). In this study ,the close correlation between the peaks and the existence of with band 2 (29.27) suggested that lipid content very high and also carbohydrate, nucleic acid also present in *chlorellapyrenoidosa.*

Table.1Tentative assignment of bands found in FTIR spectra of *Chlorella vulgaris*

Band	Main peaks in cm^{-1}	Typical band vibration	Wave number range cm^{-1}
1	Water V(O-H) stretching Protein V(N-H) stretching	3404	3029-3639
2	Lipid –carbohydrate mainly Vas (CH2) and Vs (CH2) stretching	2927	2809-3012
3	Protein amide I band mainly V(C=O) stretching	1651	1583-1709
4	Protein amide II band mainly σ(N-H)bending V(C-N) stretching	1559	1481-1585
5	Protein σas (CH2) and σ s(CH3) bending of methyl lipid as (CH2) bending of methyl	1407	1400-1477
6	Nucleic acid (other phosphate containing compounds) Vas> P=0 stretching of phosphodi-esters	1244	1191-1356
7	Carbohydrate V (-O-C) of polysaccharides. Nucleic acid (other phosphate containing compounds) Vas> P=0 stretching of phosphor - diesters	1078	1072-1099
8	Carbohydrate V(C-O-C) of polysaccharides	1023	980-1072

Table.2 Effect of salinity (mM) on physiochemical components of *Chlorella* sp.

NaCl, Conc. (Mm)	Biomass g/l	Total lipid g/l	Lipid (DCW%)	Total chlorophyll mg/ml	Total Carbohydrates mg/ml	Total Protein mg/ml
Control	0.19±0.016	1.92±0.012	10.19±0.85	1.64±0.065	0.35±0.024	0.14±0.0057
5mM	0.32±0.016	2.59±0.016	12.57±0.56	3.61±0.41	0.41±0.016	0.13±0.0053
10 mM	0.37±0.008	2.70±0.026	13.59±0.18	3.50±0.36	0.47±0.036	0.11±0.024
15 mM	0.43±0.008	2.78±0.008	15.46±0.24	3.21±0.45	0.51±0.024	0.098±0.0028
20 mM	1.23±0.008	3.16±0.008	40.66±2.1	2.72±0.19	0.54±0.012	0.084±0.0021
25 mM	1.53±0.012	3.49±0.016	43.84±0.40	3.72±0.20	0.52±0.037	0.064±0.0028

Each value is the mean of three replicates ± standard deviation. Significant difference with respect to the corresponding control. p≤ 0.05)

In the present study effect of salinity on *Chlorella sp*. have been investigated for various physicochemical components of chlorella spp. It have been found that with increasing level of salinity, biomass (g/l)and total lipid(dcw%) contents also increased at various level of salinity ranging from 5 mM to 25 mM .The result indicated that highest algal biomass concentration was found to be 1.53±0.012 at 25 mM as compared to control, it subsequently

increased with increasing concentration of salinity as shown in Table 2.,Fig.4.Lipid content(Dcw%) also enhanced from(12.57±0.56 to 43.84±0.40)with increasing the salinity.(Table2.Fig.5). The increase in lipid content at higher NaCl concentration may be due to adaptation under stress conditions which help in accumulation of lipid content and these results are in accordance with the finding of Takagi and his coworkers (Takagi *et al.,*2006) in *Dunaliella* cells. Xu et al 2012,investigated Effect of NaCl-induced osmotic stress on lipid over production in *Chlorella vulgaris*.

Initially total chlorophyll content declined up to 20 mM level of salt concentrations ,further again increased at 25mM.(Table.2,Fig.6).According to Moradi and Ismail (2007), reduced chlorophyll contents at higher salinities are due to decrease in photosynthetic rate because of salt osmotic and toxic ionic stress. Many previous studies reported that the cultivation with higher saline concentrations had lower chlorophyll and protein contents (Vonshak et al. 1996). It has also been reported that chlorophyll is the primary target to salt toxicity limiting net assimilation rate, resulting reduced photosynthesis and reduced growth (Rai 1990; Rai and Abraham 1993).
Carbohydrate contents increased in all the concentrations of NaCl except 25mM for all the cultures studied (Table 2,Fig 7). Many previous studies reported that carbohydrates synthesis was stimulated by stress conditions (Warr et al. 1985; Tomaselli et al., 1987). Gill et al. (2002) made an observation that soluble sugars play an important role in the osmotic regulation of cells during reproduction and stress conditions.

Chlorella sp. exhibited total protein concentration also declined at various level of salinity from 0.14±0.0057 to 0.064±0.0028) in comparison to control.(Table 2,Fig.8).According to Hiremath and Mathad,2010,total protein concentration decreased at various concentration of salinity(0.1 to 2) in *Chlorella* Beijerinck. The present results are in agreement with the results of sheik et al. (2006). Hageman et al. (1990) found complete blockage of protein synthesis in cyanobacteria. Many previous studies reported that stress cells have lower protein synthesis capacity increasing lipid and carbohydrate metabolism (Warr et al. 1985; Tomaselli et al. 1987).

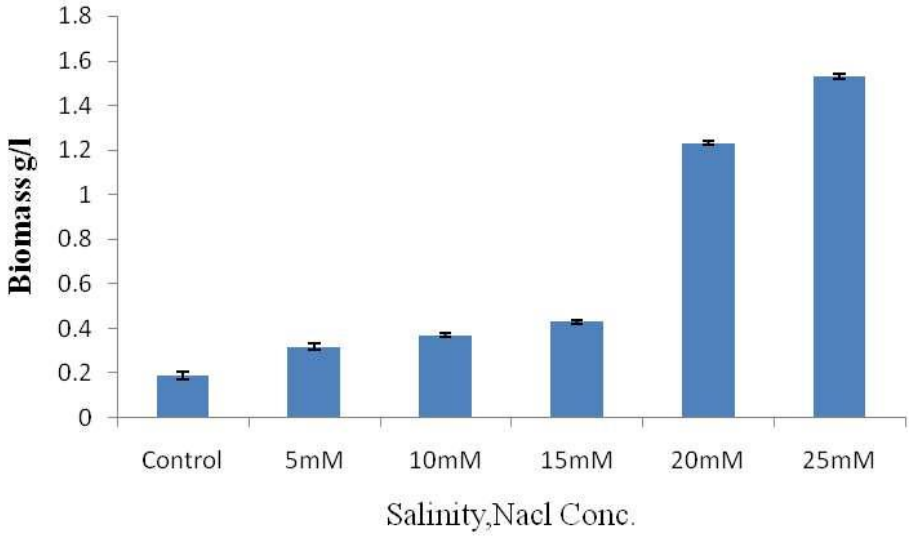

Figure 4.Effect of salinity on biomass of *Chlorella* sp. Error bars represent the SD from three replicates.

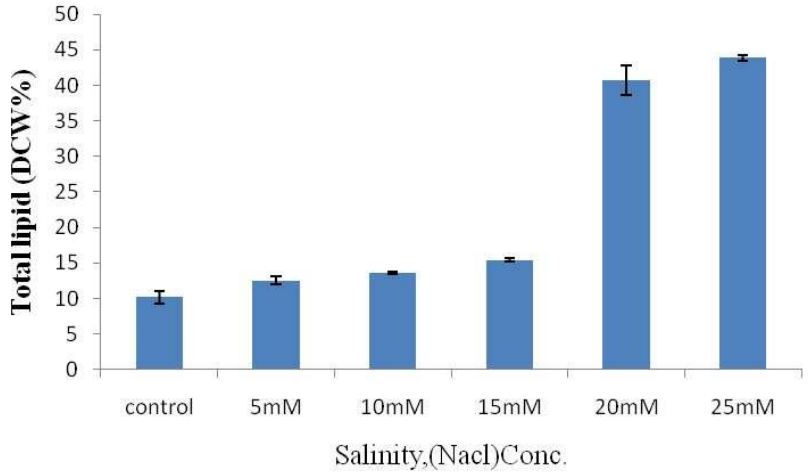

Figure 5.Effect of salinity on total lipid content(DCW%). Error bars represent the SD from three replicates.

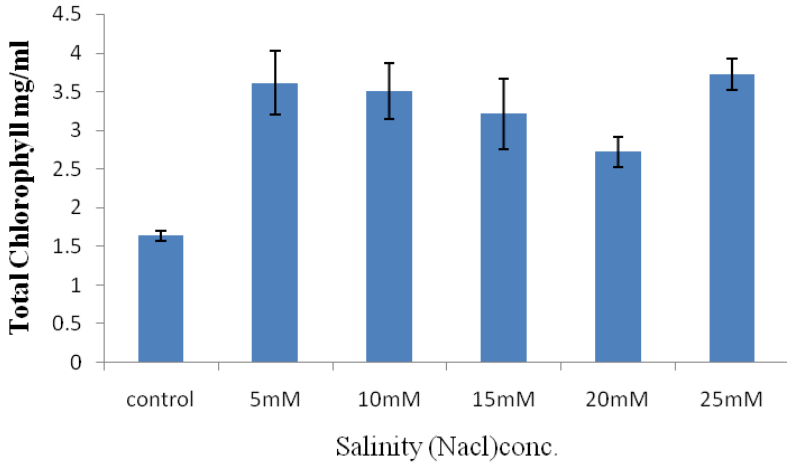

Figure 6.Effect of salinity on total chlorophyll (µg/ml). Error bars represent the SD from three replicates.

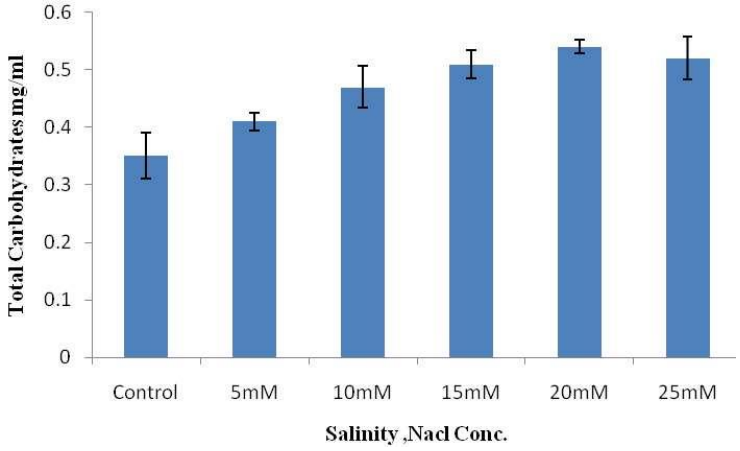

Figure 7.Effect of salinity on total carbohydrates (mg/ml). Error bars represent the SD from three replicates.

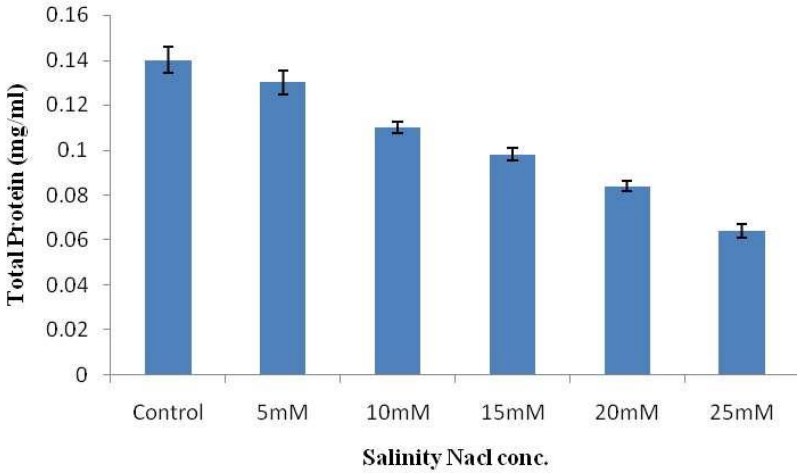

Figure 8.Effect of salinity on total protein (mg/ml). Error bars represent the SD from three replicates.

Conclusions

The effect of various concentrations of NaCl on the isolated algal species of *Chlorella pyrenoidosa.* showed, increased biomass yield at various level of NaCl concentration(5mM to 25Mm) as compared to control. Increase in concentration of salinity stimulated in lipid accumulation in chlorella sp. was ranging from lipid content 1.92±0.012 to 3.49±0.016 and lipid yield ranges 10.19% to 43.84% respectively. While, total chlorophyll and carbohydrates content raised all told the concentrations of NaCl as compared to control for the culture studied. Chlorella exhibited in turn decline within the total proteins content at the NaCl concentrations as compared to regulate. These helpful properties indicated that, adaptation of the algae to salinity was characterized by the buildup of chlorophyll, carbohydrates and protein. In this study ,FTIR spectra shows ,the close relationship between the peaks and the existence of with band 2 (29.27) suggested that lipid content very high and also carbohydrate, nucleic acid also present in *chlorellapyrenoidosa.*

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