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Effect of glucose supplementation and mixotrophic effects of glycerol and glucose on the production of biomass, lipid yield and different physiological, biochemical attributes of *Chlorella pyrenoidosa*

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Abstract

The objective of the current study was to investigate the effect of using glucose as a sole carbon source as well as in combination with glycerol as a complex carbon substrate in BG-11 media, to produce microalgal biomass (gL^{-1}), lipid (dcw%) and biochemical components, such as total soluble carbohydrates ($mgmL^{-1}$) and proteins ($mgmL^{-1}$) by *Chlorella pyrenoidosa*, over a cultivation period of 12 days. The present study revealed that using glucose as sole carbon source at various concentrations ranging from 1 to 20 (gL^{-1}), total lipid, total biomass, total protein and total carbohydrates increased. In comparison to control showed increased biomass gL^{-1} (0.29±0.021 to 0.53±0.012), while Lipid content (DCW %) enhanced from (4.87± 0.021 to 14.09±0.016). But it has no stimulatory effects found on photosynthetic pigment i.e. total chlorophyll (μgmL^{-1})

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In another batch experiment, results showed that *Chlorella* species can utilize glycerol as a source of sole carbon source, showed less biomass lipid and carbohydrates but its effect are more promising when cultured in mixture of glucose and glycerol over a cultivation period of 15 days. It was found that biomass and total lipid content enhanced with mixed concentration of glycerol and glucose whereas decreased when chlorella used glycerol as sole carbon source. But it has also stimulatory effects on total carbohydrates.

Keywords: *Chlorella pyrenoidosa*, Glucose, mixotrophic cultivation, biomass, lipid, protein, carbohydrates

Introduction

Microalgae have drawn more attention of researchers as they show rapid growth rate and provide enormous amount of lipid fraction, high biomass and other cellular composition (Song *et al*., 2013, Chisti, 2008). The photoautotrophic mechanism in microalgae cells can convert atmospheric CO_2 into biomass, protein and lipid, as well as other biologically active substances; one of them is chlorophyll (Chisti, 2007; Spolaore *et al*., 2006). It is well accepted that certain species of microalgae, particularly *Chlorella* (Lv *et al*. 2010), *Scenedesmus* (Li *et al*. 2011) and *Nannochloropsis* (Rodolfi *et al*. 2008), have relatively faster growth rates, easier cultivation characteristics and great oil producing capabilities (Song *et al*. 2013). Glucose stimulates rapid growth of the algae because it is simple sugar and can be easily assimilated to produce acetyl-CoA, which can be then utilized in multiple pathways including the synthesis of fatty acids (Stewart, 1974).

Lee (1997) found that compared with other heterotrophic cultures, commercial heterotrophic cultivation of *Chlorella spp*. in conventional stirred tank fermenters is common and that glucose and acetate are the most utilized carbons in fermentation process. According to Salim (2012), heterotrophic *Spirogyra sp.* can be cultivated by providing cassava starch hydrolysate (CSH) as a carbon source under dark condition.

Yeh *et al*. (2010) reported that the biomass production by *Chlorella vulgaris* ESP-31 increased with increasing sodium bicarbonate concentration in the culture medium. There are some reports suggesting that *Chlorella minutissima* can use organic carbon, such as glucose, acetate and methanol as a heterotroph (Vazhappilly and

Chen, 1998; Kotzabasis *et al*. 1999). In addition, Liu *et al*. (2009) concluded that organic carbon sources such as glycerol, acetate and glucose considerably increase specific rate of growth of a marine phytoplankton *Phaeodactylum tricornutum.* Recently, a process using crude glycerol as a substrate for the fermentation of the microalga *Schizochytrium limacinum* has been developed (Chi *et al*. 2007). The oleaginous *S. limacinum* is capable of producing significant amounts of total lipids and docosahexaenoic acid (DHA, C 22:6 n-3), especially when grown on a variety of carbon sources such as glucose, glycerol or fructose (Yokochi, 1997). As reported by Morales-Sánchez *et al* 2013, *N. oleoabundans* was able to grow under strict heterotrophic conditions, using glucose and cellobiose as sole carbon source.

However, there are few reports on the effects of carbon sources, especially on the biomass production and algal cell constituents under mixotrophic cultivation (Andrade and Costa, 2007, Hayward 1968). In present study, the algal culture were supplemented with a predefined glucose concentration. Initially effect of glucose was investigated as a sole carbon source and heterotrophic mix effects of glycerol and glucose on the enhancement of biomass, lipid and total soluble carbohydrate production by *C. pyrenoidosa* were investigated.

Materials and Methods

Isolation purification and maintenance of algal species

The experimental organism green microalga *Chlorella sp*. was isolated from water samples 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 composition of BG-11 media are: (gL^{-1}): $NaNO_3$ 1.5; K_2HPO_4 0.04; $MgSO_4 \cdot 7H_2O$ 0.075; $CaCl_2 \cdot 2H_2O$ 0.036; Citric acid 0.006; Ferric ammonium citrate 0.006; EDTA (disodium salt) 0.001; Na_2CO_3 0.02; and 1ml of trace elements solution having composition (gL^{-1}): H_3BO_3 2.86; $MnCl_2 \cdot 4H_2O$ 1.81; $ZnSO_4 \cdot 7H2O$ 0.222; $NaMoO4 \cdot 2H_2O$ 0.39; $CuSO_4 \cdot 5H_2O$ 0.079; Co $(NO_3)_2 \cdot 6H_2O$ 0.0494.

The microalgal cells were ascertained underneath microscope for its morphological characteristics and alternative cellular details with the help of algal identification guide and finally purified species confirmed by Dr. R. Dhandapani, Department of Microbiology, Periyar University, Salem (Tamil Nadu). The axenic algal culture was maintained in culture lab at 25±1^{o}C. To study the impact of carbon source, approximately 100 ml of BG11 media prepared in 250 ml conical flasks. Appropriate dose of glucose ranging (0.1, 0.5, 1.0, 1.5, 2.0 %) were amended in to media as an additional sole carbon source and control culture in BG-11 media without glucose run parallel in BOD cum shaker.

To investigate the effect of mixotrophic cultivation of glucose and glycerol, the algal species was cultured in BG-11 medium modified with varying 5 level concentrations of glucose and glycerol. To investigate the effect of mix tropic effect of glucose and glycerol as a sole energy source on *Chlorella spp*. the experiments were carried out in 250 ml Erlenmeyer flasks each containing 100 ml of BG-11 medium incubated at 25^{o}C in an orbital shaker set to120 rpm in BOD incubator cum shaker for 15 days and control culture in BG-11 media without glucose and glycerol was conjointly run parallel. The experimental culture were drawn on 15th day and were subjected to analysis for various physiological and biochemical parameters. All the experiments were carried out in triplicates.

Nile Red staining

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 $mgmL^{-1}$ in acetone), mixed with 50 ml glycerol: water mixture (75:25), gently vortex for 1 min. 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)

Lyophilized dried biomass was mixed with KBR powder and ground well to fine mixture. The mixture was pressed to a disc using a Hydraulic press in to tablets. The disc was subjected to FTIR spectral measuring within the

frequency varies of 4000−400 cm−1.The algal powder was characterized victimization Fourier rework Infrared spectrophotometer.

Estimation of cellular components

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). Dry cell biomass was measured as the cell density (dcw, g/l) at OD625 of an 11-day-old culture at dilutions ranging from 0.2 to 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. 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 (μgmL^{-1}) = 2.55×10^{-2} E650 + 0.4×10^{-2} E665 × 10^3.

Total Carbohydrate was determined at 625 nm by Anthrone reagent method (Dubois et al. 1956).Protein content was estimated at 660 nm by the method of Lowry and coworkers (Lowry et. al.1951). y = 0.1097x - 0.0005.,R^2 = 0.9989.

Results and discussion

In the present investigation, fresh water green microalga were isolated from enriched mixed culture by standard isolation technique. Further characteristics and morphological features of the isolate have demonstrated its close similarity with genus *chlorella*. Its cells characteristics are emerald- green coloured spherical, unicellular in shape.

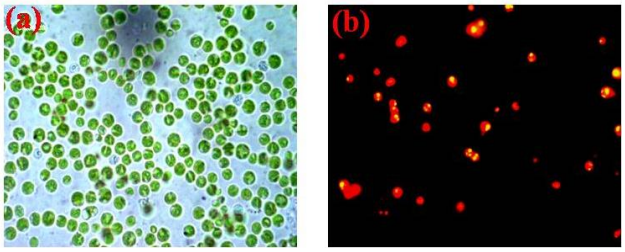


Figure1. (a). Light microscope image of *Chlorella pyrenoidosa* (100 x) with immersion oil, (b). Nile red fluorescence of representative microalgal cells.

All cells were discovered for yellow-gold visible fluorescence with Nile red stain pursuit excitation band pass filter of 420 nm and emission band pass filter of 580 nm. The brilliant yellows to yellow-gold fluorescent are spherical bodies.

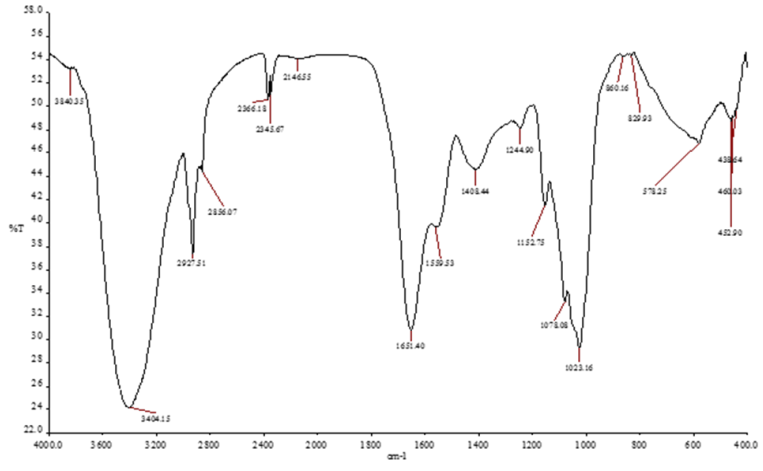


Figure 2. FTIR spectra for *Chlorella Pyrenoidosa*

In FTIR spectra (Figure 2) with regard to specific functional group, each peak consigned a functional cluster. The molecular assignments of FTIR bands square measure supported revealed information of plant life, microorganism and alternative biological materials. In this study, *Chlorella pyrenoidosa* protein spectra characterized by strong peaks 1651 cm^{-1} (amide I) and 1559cm^{-1} (amide II). These bands were primarily due to C=O stretching vibration and a combination of N-H and C-H Stretching vibrations in amide complexes. Lipids and carbohydrates were reported to be 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 cm^{-1} show the 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 is very high and carbohydrate, nucleic acid also present in *Chlorella pyrenoidosa.*

Table.1 Tentative assignment of bands found in FTIR spectra of *chlorella pyrenoidosa*

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 V as (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 σ as (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

Effects of glucose supplementation on *Chlorella pyrenoidosa*

Till date, dose effects from glucose on microalgae are intensively explored. In general, the mixotrophic conditions and supplementation of organic carbon supply influence the biomass, lipid content and reducing sugar

concentrations with coincidental reduction within the pigments and protein. In the present study, cultures supplemented with 0.1 to 2.0 % glucose concentration showed subsequently higher overall productivity than control treatments at any point during the experiment. Figure 3 (A) and Table 2 shows that glucose supplementation treatments had higher biomass and lipid content (Dcw %) as compared to control. Biomass concentration increased (from 0.29±0.021 to 0.53±0.012gL^{-1}), wheareas lipid content enhanced (from 4.87±0.021 to 14.09±0.016) with increasing the supplementation of glucose as shown in Figure 3 (A, B) Table 2.

Table 2. Effect of Glucose on biochemical and physiological components of *Chlorella pyrenoidosa*

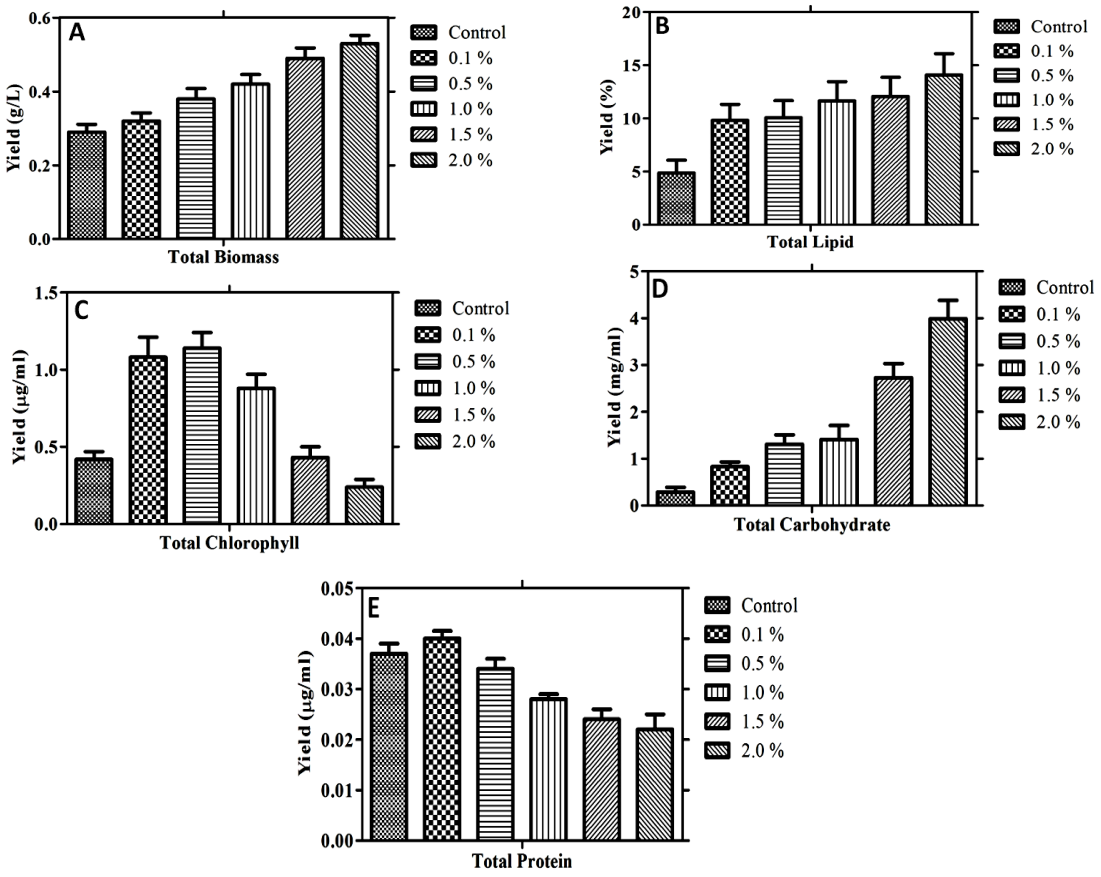
Glucose concentration	Biomass gL^{-1}	Lipid dcw%	Total chlorophyll µgmL^{-1}	Total carbohydrates mgmL^{-1}	Total protein mgmL^{-1}
Control	0.29±0.021	4.87±0.021	0.42 ±0.020	0.29±0.016	0.037±0.0012
0.1%	0.32±0.012	9.83±0.024	1.08±0.033	0.83±0.024	0.04±0.021
0.5%	0.38±0.008	10.08±0.02	1.14±0.020	1.31±0.030	0.034±0.0017
1.0%	0.42±0.016	11.66±0.08	0.88±0.024	1.41±0.008	0.028±0.002
1.5%	0.49±0.008	12.07±0.05	0.43±0.008	2.73±0.012	0.024±0.0012
2.0%	0.53±0.012	14.09±0.016	0.24±0.016	3.99±0.015	0.022±0.0008

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

These results are in consistent with findings of Salim 2012, the treatment of CSH (15 g/L) could produce the highest biomass concentration and lipid content of *Spirogyra* sp. Autotrophic growth of *Chlorella vulgaris* provides higher cellular lipid content that is 38%, but its lipid productivity was a lot of not up to those in heterotrophic growth with acetate, glucose, or glycerol (Liang *et al.*, 2009) .Glucose supplementation provide rapid growth of the algae because it is simple sugar and can be easily assimilated to produce acetyl-CoA, which was then utilized in multiple pathways including the synthesis of fatty acids (Stewart, 1974)

In present study, no stimulative effects have been found in case of total chlorophyll as total chlorophyll content decreased with increasing dose of glucose as shown in (Table 2 & Figure 3(C). According to Xiong *et al* (2010), heterotrophic growth of the culture results in degradation of chlorophyll in chloroplasts. Decrease in chlorophyll content may lead to higher light transmitting inside the algal culture, inflicting higher photosynthetic rates, providing there is still sufficient chlorophyll for photosynthesis. However with increasing biomass and lipid content in *chlorella sp*. An increase in carbohydrates content and reduction in protein biosynthesis with supplementation of glucose was also been observed (Table.2, Figure 3 (D,E). According to Kong *et. al* 2013, when protein content in *C. vulgaris* is decreased, both lipid and carbohydrate content increased.

Figure 3. Effect of Glucose on Biochemical and physiological components of *Chlorella pyrenoidosa* (A). Total Biomass, (B). Total Lipid, (C) Total Chlorophyll, (D) Total Carbohydrate, (E). Total Protein



Effect of mixotrophic cultivation on *Chlorella pyrenoidosa*

The mixotrophic conditions (Glucose +Glycerol) and supplementation of organic carbon sources promoted the biomass, lipid and carbohydrate production, while reduced the pigment and protein biosynthesis, which implicit that the mixotrophic conditions modified the metabolic pathways of nitrogen and carbon. In batch flask experiments, biomass gL^{-1} increased (0.32 ± 0.008 to 1.06±0.021) as compared to control in mixotrophic culture of glucose and glycerol instead of using glycerol as a single organic carbon source as shown in (Table 3 & Figure 4 A). An earlier report showed that mixotrophic growth offered a possibility to increase greatly the microalgal cell concentration and volumetric productivity in a batch system. (Yamane 2001). Similar observations were made by Kong *et al* 2012 that *C. vulgaris* can utilize glycerol as a sole carbon substrate, but its effect is not prominent as that of the mixture of glycerol and glucose.

Table 3. Mixotrophic effect of (Glucose and glycerol gL^{-1}) on biochemical and physiological attributes of *Chlorella pyrenoidosa*

Mixotrophic carbon sources	Biomass(gL^{-1})	Lipid (dcw%)	Total Chlorophyll (μgmL^{-1})	Total Carbohydrates ($mgmL^{-1}$)	Soluble Protein ($mgmL^{-1}$)
Control	0.32±0.008	11.74±0.24	4.33±0.012	0.12±0.012	0.032±0.0008
Glycerol 1g	0.38±0.012	12.75±0.35	4.51±0.028	0.17±0.016	0.035±0.0012
Gly1+Glu 2	0.45±0.022	13.41±0.47	5.46±0.029	0.22±0.008	0.030±0.0012
Glycerol5 g	0.48±0.012	15.79±0.36	4.28±0.024	0.2±0.021	0.086±0.0029
Gly 5+Glu 2	0.55±0.024	17.45±0.40	9.46±0.041	0.27±0.020	0.073±0.0028
Glycerol 10	0.96±0.012	19.46±0.20	10.39±0.35	0.17±0.012	0.085±0.0033
Gly10 +Glu 2	1.06±0.021	20.89±0.37	11.13±0.016	0.45±0.022	0.096±0.0020

Each value is the mean result of three replicates ± standard deviation. Significant difference with respect to the corresponding control. ($p \leq 0.05$)

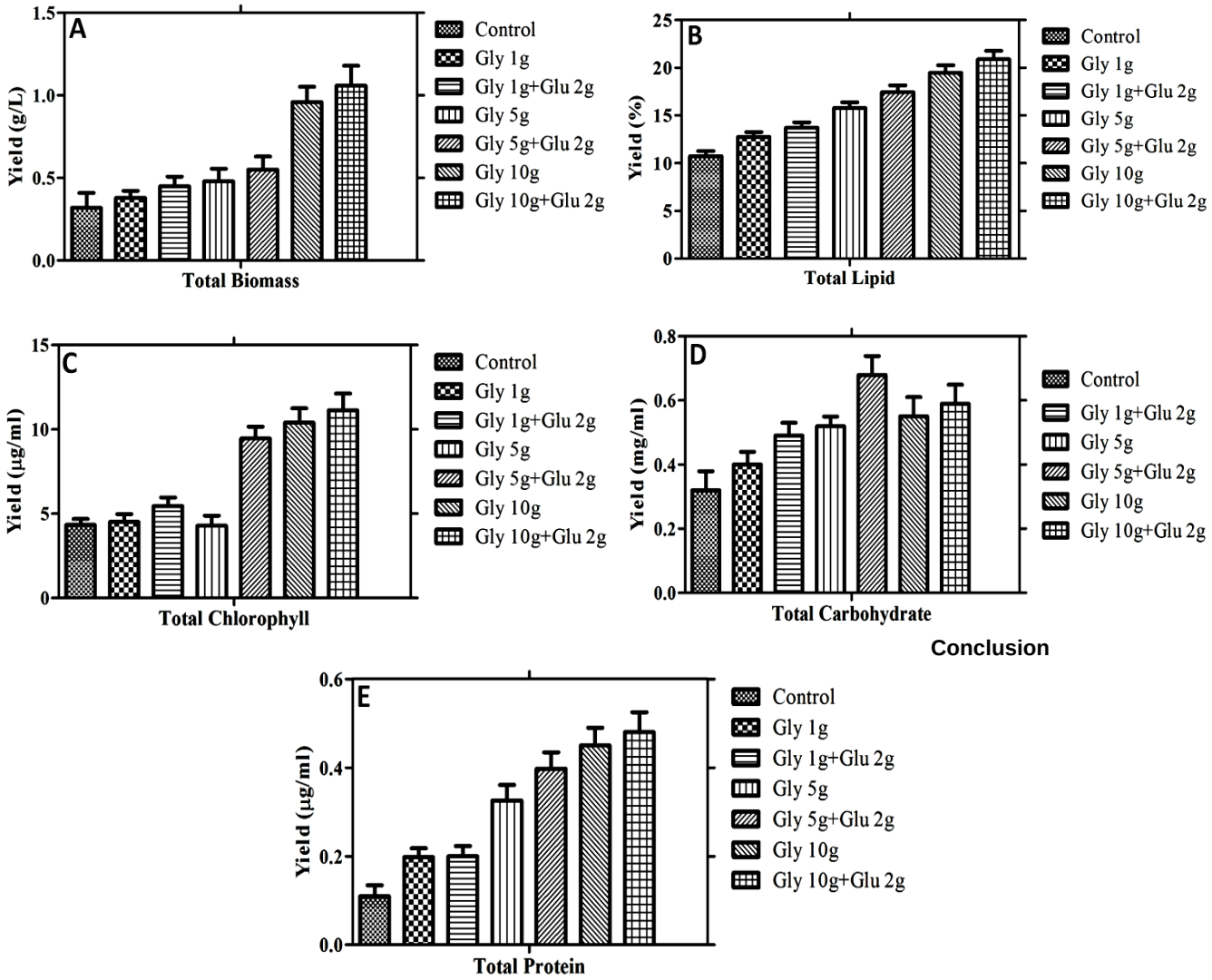
As summarized in Figure 4(B) & Table 3, mixotrophic effect of glycerol and glucose on lipid productivity are more promising as it has been enhanced from 11.74% to 20.89% as compared to control. With increase in concentration of glycerol 10 g/l and glucose 2g/l, a slight increase in lipid content has been investigated in culture with glycerol. In the mixotrophic samples, the lipid yield was higher when complex carbon sources were used rather than adding different concentrations of glycerol as the sole organic carbon source in BG-11 medium. These results are strongly supported by previous studies (Kong et al 2012, Andrade and Costa, 2007, Hayward 1968. The recent study by Grady and Morgan, 2011 has demonstrated simultaneous high growth rates and lipid yields by *Chlorella protothecoides* heterotrophically grown on mixtures of glycerol and glucose

Figure 4(C) & Table 3 shows that cultivation of *Chlorella sp*. with mixotrophic carbon sources have significant amount of chlorophyll content ($mgmL^{-1}$) in comparison with glycerol supplementation alone. Nieva and Valiente 1996, observed a decreased rate of CO_2 fixation under mixotrophic cultivation suggesting that some aspects of CO_2 metabolism may be modulated by organic compounds. This may be the reason for the inhibition of photosynthetic pigment synthesis under mixotrophic growth. These results are favored by many authors (Kong *et al* 2012., Xiong et al 2010)

Figure 4(D)& Table 3 shows the effect of mixotrophic supplementation of glycerol on soluble carbohydrates. In the present study, when protein content in *C. pyrenoidosa* decreased, both lipid and carbohydrate content

increased (Figs.4 A, D). These changes in the algal cell constituents are in agreement with reports of other authors who mentioned that there was a reduction in the protein content in mixotrophic *C. vulgaris* UAM 101 cells, which was compensated by an increase in lipid and carbohydrate content. Carbohydrates are found as the intermediary reserves in some algae, due to the fact that they are required when the nitrogen becomes limited in the lipid synthesis (Orus *et al* 2011). According to Kong *et al* 2013, when protein content in *C. vulgaris* decreased, both lipid and carbohydrate content increased. These changes in the constituents are in agreement with reports of other authors who mentioned that there is a reduction in the protein content in mixotrophic *C. vulgaris* UAM 101 cells, compensated by an increase in lipid and carbohydrate content (Orus et al 1991). Previous work reported that nitrogen (nitrate) was essential for astaxanthin accumulation in *Haematococcus pluvialis*. The authors suggested that nitrogen was required for continuous synthesis of the protein responsible for supporting the pigment formation (Boussiba and Vonshak 1991). A study by Tam and Wong, 1996 also reported that higher chlorophyll and protein content was found in *C. vulgaris* cultures with higher ammonium concentrations and the algal growth was accompanied by a decrease in nitrogen content in the medium, indicating that nitrogen removal was due to the algal uptake and assimilation.

Figure 4. Mixotrophic effect of (Glucose and glycerol g/l) on biochemical and physiological attributes of *Chlorella pyrenoidosa* (A). Total Biomass, (B). Total Lipid, (C) Total Chlorophyll, (D) Total Carbohydrate, (E). Total Protein



Conclusion

Chlorella pyrenoidosa was able to grow better under strict heterotrophic condition with glucose. Moreover, the current study has demonstrated the promising biomass concentration and lipid content yields of *Chlorella* sp. obtained heterotrophically grown on glucose. It also has significant effect on other physicochemical parameters such as protein and soluble carbohydrates. In comparison to control showed increased biomass (g/l (0.29±0.021 to 0.53±0.012) and lipid content (DCW % 4.87± 0.021 to 14.09±0.016). But it showed antagonistic effects on photosynthetic pigment, total chlorophyll and protein biosynthesis.

Mixotrophic cultivation results showed that *Chlorella* species can utilize glycerol as a source of sole carbon source, showed less biomass, lipid and carbohydrates but its effect are more promising when cultured in mixture of glucose and glycerol. It has been found that biomass and total lipid content enhanced with mixed concentration of glycerol and glucose whereas decreased when chlorella used glycerol as a sole carbon source. It has also stimulatory effects on total carbohydrates. FTIR results revealed high lipid content and also presence of carbohydrate and nucleic acid in *Chlorella pyrenoidosa.*

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