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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2021 18(4): 2851-2862. **OPEN ACCESS**

Spirulina platensis cultivation under mixotrophic condition by using different concentrations and types of sugar

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Spirulina platensis is a biomass related to cyanobacteria, cultivated in worldwide due to high major advantage to human, such as dietary supplement or whole food, feed additive in the aquaculture, aquarium, and poultry, and it has immunomodulation, antioxidant, anti-microbial properties. So the world searches to enhancement the S*. platensis* biomass. Mixotrophic cultivation of *S. platensis* gives a very promising alternative for producing a high yield. In this work determined the effects of different types of sugars (glucose, sucrose, lactose, fructose, mannose and starch) with different concentrations 1,2 and 3 gm / liter media under continuous illuminations on the S*. platensis* growth and different type of pigments concentration. Results revealed that after 21 days of cultivations 1 gm sucrose 2, 3gm glucose and 1 gm fructose induced high yields of biomass in comparison to other types and concentrations of sugars. No significant increase in chlorophyll and carotenoids contents with addition of different types and concentrations of sugars, meanwhile different types and concentrations of sugars have different effects phycobilins concentrations. Biomass of S*. platensis* yield was enhanced by different types of sugar with possible used low cost sugar supplement.

Keywords: S*. platensis*, growth, biomass, pigments, phycobilins

INTRODUCTION

Spirulina platensis is a blue green alga that contains biochemical compounds of exceptional values which exceed those of all plant species studied (Hendrickson, 1989). It is rich food nutrients for human and animals referring to its contents of high quality protein (50-70%), vitamins, minerals, carbohydrates, carotenoids, phenolic acids and fatty acids (Anupama, 2000 and Chu, et al. 2010). The constituents of *S. platensis*is acquire it great properties for treatment and prevention of many diseases and disorders as in anemia referring to its high content of iron element and vitamins as vitamin B complex especially B-12 (cobalamin) and vitamin B-9 (folic acid) (Kapoor and Mehta, 1998). *S. platensis*is species also contains bioactive compounds such as polysulfated polysaccharides that have antiviral activities (Ghosh et al. 2004), phycobilliproteins and carotenoids considered as natural antioxidants (Piñero Estrada et al. 2001), mycosporine and scytonemin used as

photoprotectants (Rastogi and Sinha, 2009), polyunsaturated fatty acid (PUFA) improve serum lipid profile by reduction of serum lipids levels and increasing HDL-cholesterol level (Colla et al. 2008) and Gamma-linolenic acid (GLA) used to treat rheumatoid arthritis (Zurieret al. 1996). Also it contains sterols that act as anti-microbial agents (Kumar et al. 2013). *Spirulina*-based products are utilized by athletes as anti-fatigue and amino acid supplier, and for their anti-aging detoxifying and antioxidant properties in cosmetic products referring to its high content of natural pigments, especially carotenoids (Rao and Rao, 2007).

It acts as immunostimulant by enhancing antibodies production (Premkuram et al. 2004) and it has hypoglycemic (Parikh et al. 2001), anti-cancer (Eriksen, 2008), antifungal (Duda-Chodak, 2013) effects, *S. platensis*is used in agriculture as biofertilizers (Vaishampayan et al. 2001) and applications in biotechnology (Eriksen, 2008). *S. platensis* grow under phototrophic and mixotrophic conditions but, the growth was promoted when culture with light and glucose (mixotrophic) (Ogawa and Terui, 1970; Marquez et al. 1993). *S. platensis* are able to cultivate under heterotrophic condition by consuming crude oil as sole carbon sources (El-Sheekh and Hamouda, 2013). Thirty-five axenic *Arthrospira* strains were assayed to grow under heterotrophic conditions on six carbon sources; some strains were lost when cultivated with fructose, and others are well grew; some of strains grew with glucose and maltose, but none with fructose or sucrose under photoheterotrophically (Mühling et al. 2005).Biomass yielding of *S. platensis* was encouraged by molasses; this led to industrial by-product could be economic supplement for the growth of *S. platensis* (Andrade and Costa, 2007). *S. platensis* can be cultivated using whey under mixtrophic conditions without addition any expensive nutrients (Pereira et al. 2019). The aim of this work is to study the influence of different types and concentrations of carbon sources (glucose, sucrose, lactose, fructose, mannose and starch) on the *S. platensis* growth, chlorophyll, carotenoids and phycobilins (phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE) and Phycobiliprotein).

MATERIALS AND METHODS

2.1. Protective alga: Spirulina platensis (SP)

Spirulina platensis (SP) was collected from Al-Natron valley, Egypt. SP was isolated after repeated light migrations on Zarrouk's medium (Zarrouk, 1966) and purified according to the method described by Rippka (1988) and identified according to Vonshak (1997). It was grown in Erlenmeyer flasks containing 200 ml standard Zarrouk's medium at 25±2°C, pH 9.5 with continuous illumination using cool white fluorescent tubes (2500 Lux) and twice daily shaking for 15 days. This step was repeated several times to obtain pure stock used in the experiment

2.2. Cultivation of *S. platensis on* Zarrouk's medium with addition of different sources of carbohydrates

S. platensis, was cultivated on standard Zarrouk's medium at 25±2°C, pH 9.5 with continuous illumination using cool white fluorescent tubes (2500 Lux) and twice daily shaking by hand for 21 days (Zarrouk, 1966) and kept as control medium. In the same time this step was repeated with addition of different concentrations of carbohydrates 1, 2, 3 gm/L (glucose, sucrose, lactose, fructose, mannose and starch)

Control and supplemented media were sterilized in the autoclave under moist temperature at 121ºC for 20 minutes after wrapping with aluminum foil before SP cultivation. In case of glucose, one liter of standard Zarrouk's medium was prepared without addition of $NaCO_3$ and then divided into two equal parts in a glass flask. Glucose 1gm was added to a part of the medium and the other part sodium carbonate was added to avoid burning of glucose in alkaline medium of $NaCO_3$ during sterilization. Then sterilization of the two parts in the autoclave under moist temperature at 121ºC for 20 minutes after wrapping with aluminum foil was done. After sterilization, mixing two parts of media under a laminar flow and divide this liter in flasks each contain 200 ml of the medium with repeating of this step with other concentrations of glucose. After vigorous shaking of *S. platensis* stock solution, 50 ml was added to each flask under a laminar flow and incubated at 25±2°C, with continuous illumination using cool white fluorescent tubes (2500 Lux) and twice daily shaking for 21 days.

2.3. Estimation of SP growth rate and pigments content:

After cultivation, samples were collected periodically for analysis of growth and content of chlorophyll, carotenoids and phycobilins by readings the absorbance at different wave lengths using spectrophotometer. Samples of SP

suspension from each flask were homogenized separately by sonication at room temperature, 40 kHz for 1 hour and used in estimation of growth and pigment contents.

2.4. Growth estimation

Growth was estimated in each sample by measuring the biomass turbidity of SP homogenized suspension at wave length 660 nm using spectrophotometer (LW UV-200-RS), it was expressed in dry mass per liter of suspension (Seely et al. 1972).

2.4.1. Chlorophyll (Chl-*a*) and Carotenoids estimation

Ten ml homogenized suspension of each sample (volume extract) for each replicate (volume culture) was taken and centrifuged for 10 minutes at 4000 rpm**.** After throwing the supernatant and taking the precipitate, we added 10 ml 90% acetone on precipitate and placed the covered tube in the dark for 24 hours. Then the tube was centrifuged for 15 minutes at 5000 rpm with collection of the supernatant for measuring of Chl-*a* content by recording absorbance using spectrophotometer. The reading of absorbance of supernatant was taken at 630 nm, 645 nm and 665 nm against 90% acetone as blank. Calculating the concentration of Chl-*a* by using the following equation (Parson and Strickland, 1965).

2.4.2. Chl-a concentration in sample C = 11.6 A665–1.31A645–0.14 A630

The concentration of Chl-*a* in a given volume of culture can be determined by equation:

$$\textbf{Chl-}a\textbf{ (mg/ml)} = \frac{C \times Ve}{Vc}$$

C=Value obtained from above equation
Ve=Volume of extract (10 ml)
Vc=Volume of culture (1000 ml)
Carotenoids (Cart) concentration calculated by using this equation **(**Jensen, 1978)

$$\textbf{Cart concentration (mg/ml)} = \frac{A450 \times Ve \times f \times 10}{2500}$$

Ve=Volume of extract (10 ml)
f=Dilution factor

Phycobilins estimation

Ten ml from homogenized suspension of each sample was taken and centrifuged for 10 minutes at 4000 rpm. After aspiration of the supernatant the precipitate was washed three times by distilled water then adding 10 ml from PBS (0.05 M, pH 6.7) and exposed to three times repeated freezing and thawing. The tube was centrifuged for 15 minutes at 10,000 rpm. The collected supernatant is used for measuring phycobilins at 562 nm, 615 nm, and 652 nm against PBS as blank by using spectrophotometer. Then the concentrations of phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE) and Phycobiliprotein in the sample were calculated by using the following equations (Bennett and Bogorad,1971)

$$\textbf{PC (mg/ml)} = \frac{A615 - 0.474(A652)}{5.34}$$

$$\textbf{APC (mg/ml)} = \frac{A652 - 0.208(A615)}{5.09}$$

$$\textbf{PE (mg/ml)} = \frac{A562 - 2.41(PC) - 0.849\,(APC)}{9.62}$$

The concentration of phycobiliproteins in a total volume of culture can be determined as follows:

$$\textbf{Phycobiliprotein (mg/ml)} = \frac{C \times Ve}{Vc}$$

C=Values of PC, APC and PE together obtained from above equations
Ve=Volume of extract (10 ml)
Vc=Volume of culture (1000 ml)

2.5. Statistical analysis:

Values are presented as mean **±** standard error of mean (SEM). Statistical analyses were performed by one way ANOVA (Analysis of Variance) followed by Duncan's multiple range test using SPSS (Statistical Package for Social Sciences) Version 16 released on 2007. Statistical significances between different means were considered at P<0.05.

RESULTS

3.1. Effect of different concentrations of sugars on growth of *Spirulina platensis* at 7th, 14th, and 21th days of cultivation

The effects of different concentrations (1, 2 and 3gm/L media) of sugars such as glucose, fructose, sucrose, lactose, mannose and starch on *S. platensis* growth measured by optical density O.D (nm) at 7th, 14th and 21th days of culture are presented in table (1) and graphically illustrated in figures (1a,b and c).
At 7th day, the results indicated that 3gm, mannose and 1gm sucrose, followed by glucose 2g caused significant increase in the growth of SP in comparing to the control and the other sugar types and concentrations at P<0.05.

Table 1: Mean values of optical density (nm) for *Spirulina platensis* growth with different concentrations of various sugars supplement at 7th, 14th and 21th days of culture

O.D (660nm)		7th day	14th day	21th day
Control		1.19 ± 0.06^{de}	1.32 ± 0.06^{de}	1.34 ± 0.03^{cde}
Glucose (gm)	**1**	0.95 ± 0.04^{hi}	1.01 ± 0.02^{h}	0.93 ± 0.03^{h}
	2	1.49 ± 0.07^{b}	1.62 ± 0.06^{ab}	1.61 ± 0.07^{ab}
	3	1.33 ± 0.07^{cd}	1.39 ± 0.04^{cd}	1.58 ± 0.04^{ab}
Fructose (gm)	**1**	1.33 ± 0.04^{cd}	1.53 ± 0.03^{bc}	1.56 ± 0.06^{b}
	2	1.03 ± 0.01^{fgh}	1.39 ± 0.04^{cd}	1.41 ± 0.01^{cd}
	3	1.23 ± 0.03^{cde}	1.24 ± 0.01^{ef}	1.11± 0.05^{fg}
Sucrose (gm)	**1**	1.7 ± 0.03^{a}	1.64± 0.04^{ab}	1.72± 0.01^{a}
	2	1.36 ± 0.01^{c}	1.74 ± 0.06^{a}	1.23 ± 0.07^{ef}
	3	1.13 ± 0.01^{ef}	1.39± 0.01^{cd}	1.37± 0.03^{cde}
Lactose (gm)	**1**	0.98 ± 0.003^{gh}	1.08 ± 0.04^{gh}	1.09 ± 0.05^{fg}
	2	0.93 ± 0.02^{hi}	1.37 ± 0.04^{de}	1.11 ± 0.01^{fg}
	3	0.78± 0.01^{i}	1.17± 0.01^{fg}	1.11± 0.01^{fg}
Mannose (gm)	**1**	1.11 ± 0.05^{efg}	1.39 ± 0.03^{cd}	1.49 ± 0.09^{bc}
	2	1.18 ± 0.04^{e}	1.42 ± 0.02^{cd}	1.34 ± 0.03^{cde}
	3	1.78 ± 0.05^{a}	1.45 ± 0.01^{cd}	1.35± 0.03^{cde}
Starch (gm)	**1**	0.82± 0.004^{ij}	1.13± 0.02^{fgh}	1.25± 0.06^{def}
	2	0.78 ± 0.04^{i}	1.47 ± 0.09^{cd}	1.04 ± 0.03^{gh}
	3	0.89 ± 0.08^{ij}	1.38 ± 0.06^{de}	1.11 ± 0.01^{efg}

Data are presented as means ± SEM (n=3). Values having different superscripts within the same columns are significantly different (P<0.05).

At P<0.05, the results after 14 days of culture showed that sucrose (2, 1gm) and glucose 2gm followed by1gm fructose induced significant elevation in growth of SP in comparison with other sugar types, concentrations and also control. After 21days**,** results recorded that sucrose 1gm and glucose (2, 3gm) followed by 1gm fructose induced significant increase in growth of SP compared to control and other types and concentrations of sugars at P<0.05.

3.2.Effect of different concentrations of sugars on pigment contents of *S.platensis* through 7, 14 and 21 days of cultivation

The effect of different concentrations (1, 2 and 3gm) of glucose, fructose, sucrose, lactose, mannose and starch each added to 1L media at the 7th day of culture on SP pigment contents represent by chlorophyll; carotenoids and phycobilins (phycocyanin, phycoerythrin, allophycocyanin, phycobiliprotein) are investigated in graphically in Figure (2). In the 7th day, the results revealed significant elevation of chlorophyll content in SP grown in control media, starch 3g caused significant increase in carotenoids. Starch 1g and 2g showed significant elevation in phycocyanin, mannose 2gm recorded significant increase in allophycocyanin, lactose 3g significantly increased phycoerythrin but starch 2gm and mannose 2gm increased phycobiliprotein at P<0.05.

3.3.Effect of different concentrations of sugars on pigment contents of *Spirulina platensis* at 14th day of growth

The effect of various concentration (1, 2 and 3gm/L media) of glucose, fructose, sucrose, lactose, mannose and starch each after 14th day on the chlorophyll, carotenoids and phycobilins as phycocyanin, phycoerythrin, allophycocyanin, phycobiliprotein concentrations (mg/ml) contents of SP are presented in figure (2).

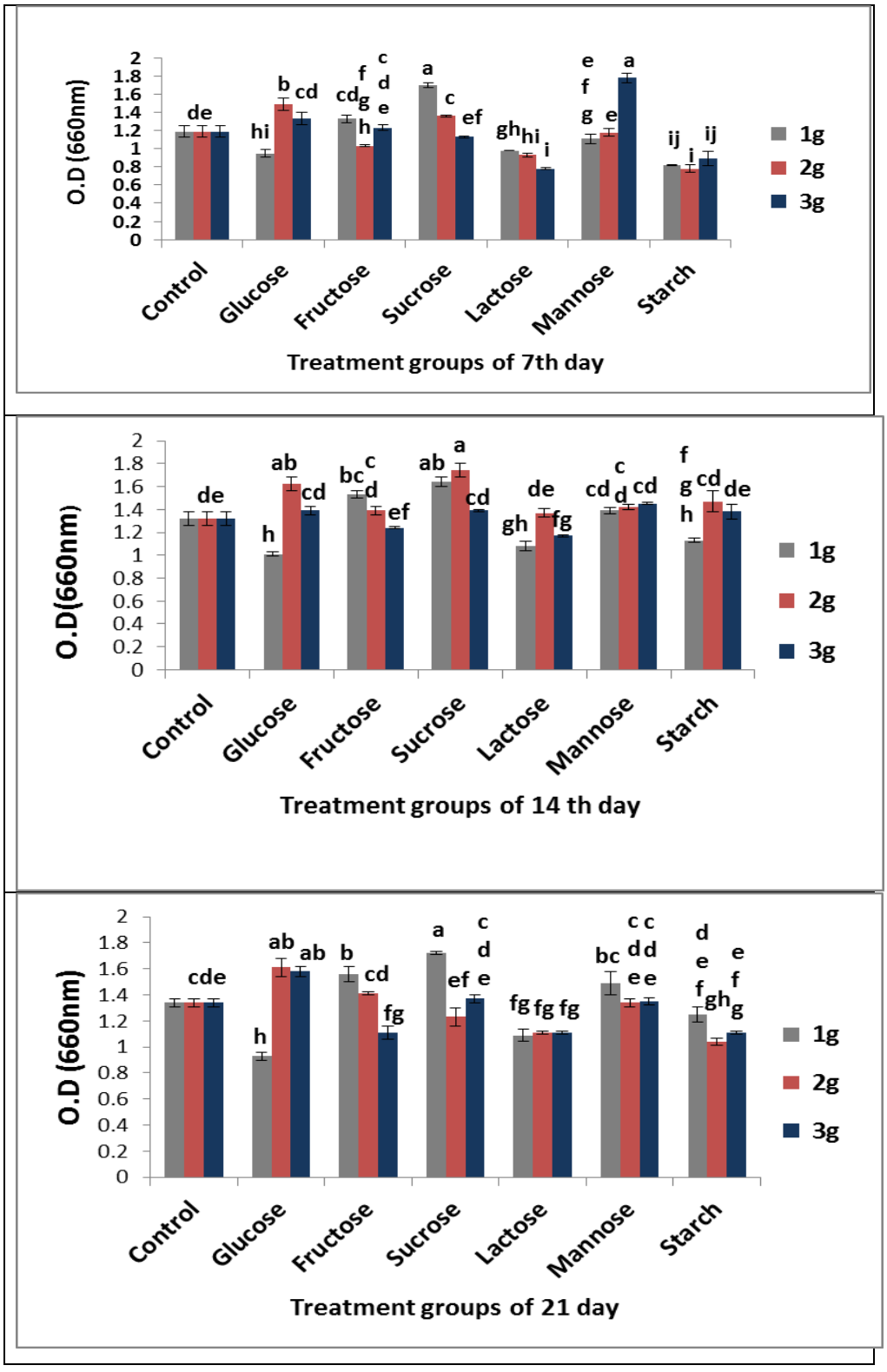


Figure 1: Effects of different concentrations (1, 2 and 3gm/L media) of various sugars on *S. platensis* growth measured by optical density (nm) at 7th day of culture (a) 14th (b) 21th (21). Different letters mean significant change between groups (P<0.05).

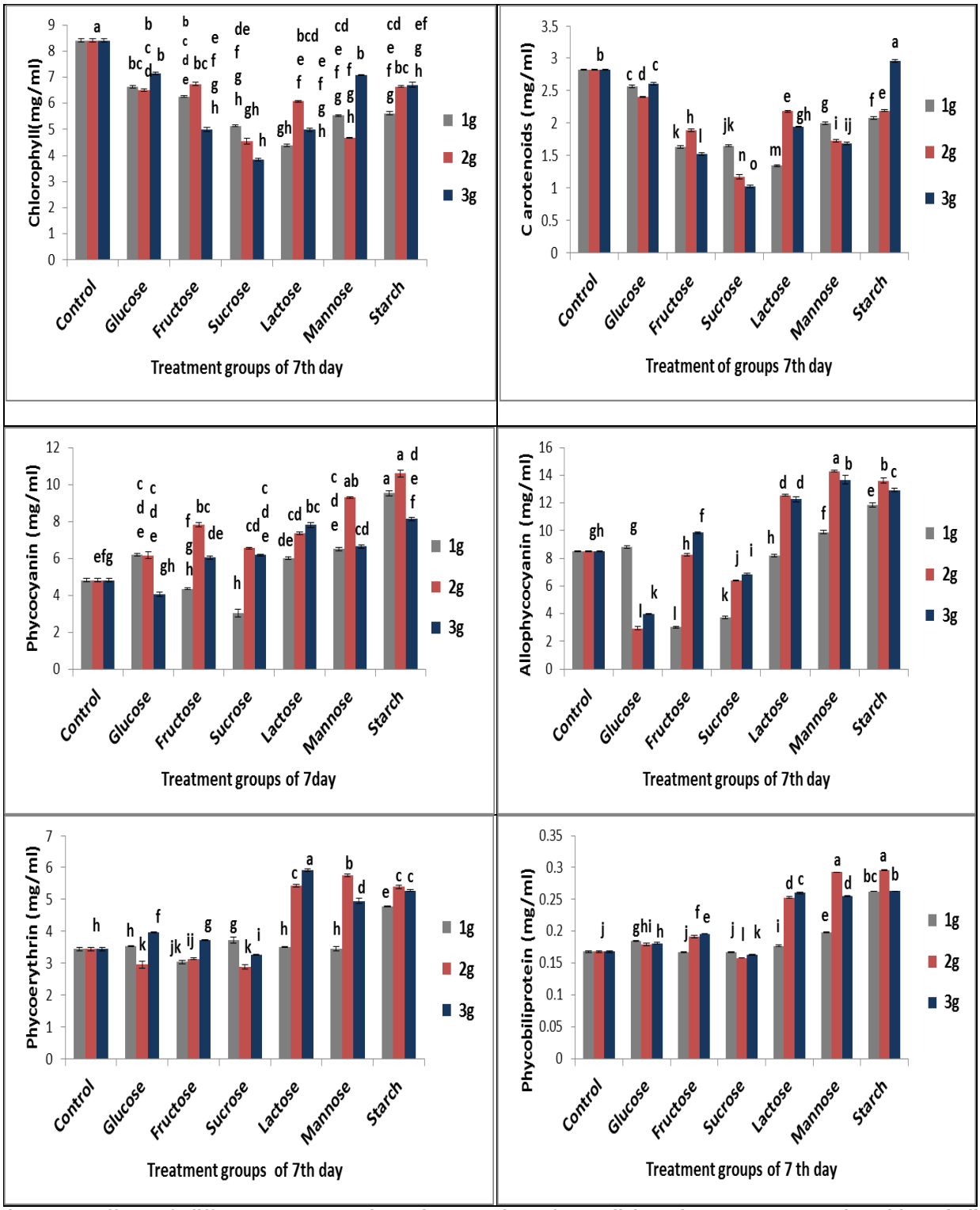
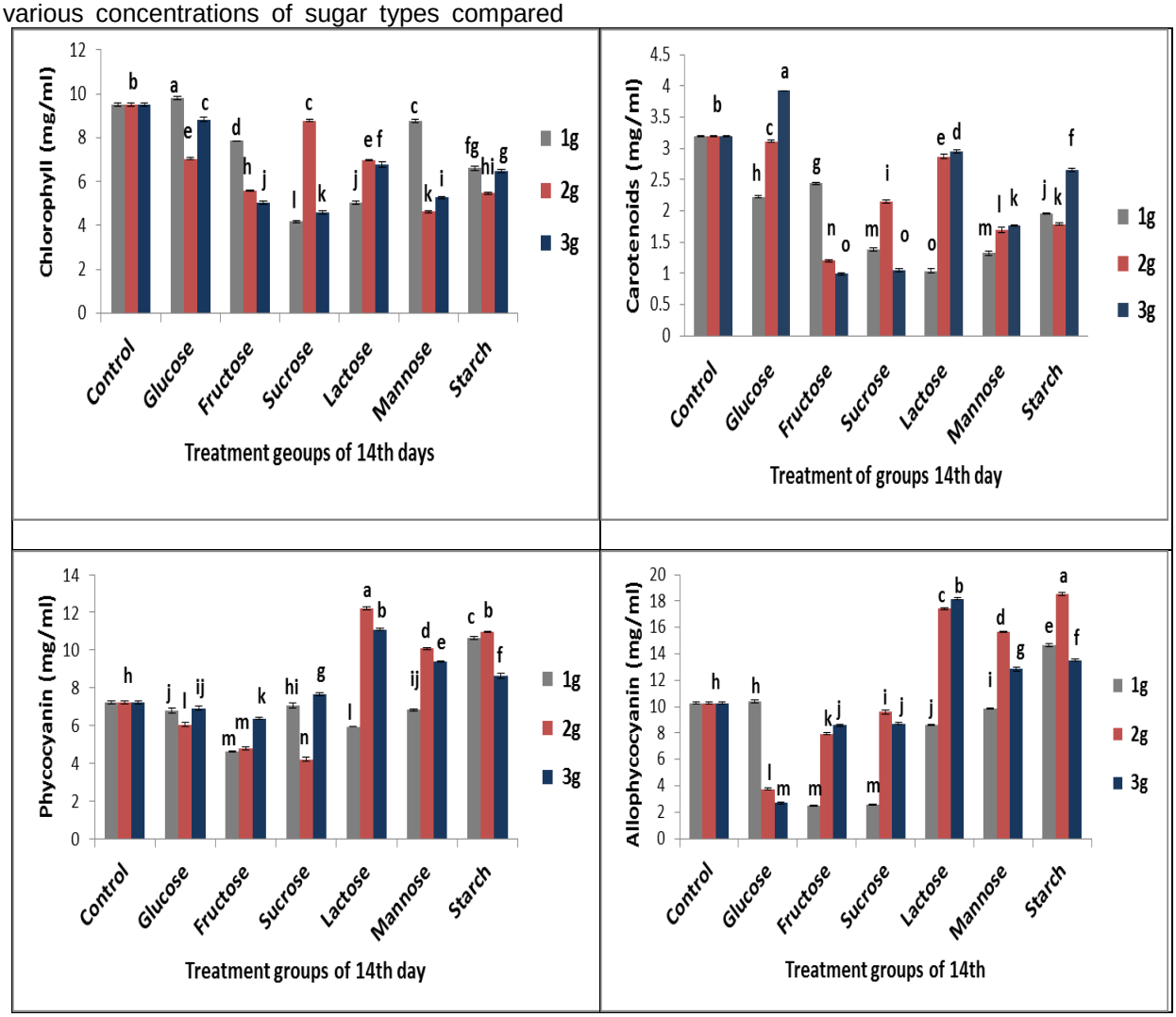


Figure 2: Effect of different concentrations (1, 2 and 3 g/L media) various sugars on the chlorophyll, carotenoids and phycobilins represent by phycocyanin, phycoerythrin, allophycocyanin, phycobiliprotein (mg/ml) contents of *Spirulina platensis* at 7th day of growth. Different letters mean significant change between groups (P<0.05).

The results showed that glucose 1gm recorded significant increase in the chlorophyll but glucose 3gm showed significant elevation in carotenoid compared with control and other groups. Lactose 2gm showed elevation in phycocyanin but mannose 2gm followed by starch 2gm induced significant increase in phycoerythrin compared with control. Moreover starch 2gm lead to significant increase in allophycocyanin and phycobiliprotein at P<0.05.

3.4. Effect of different concentrations of sugars on pigment contents of *S. platensis* at 21th day of growth

Chlorophyll, carotenoids and phycobilins represent by phycocyanin, phycoerythrin, allophycocyanin, phycobiliprotein concentrations contents of SP were changed when grown with various concentrations of sugar types compared with control after 21 days of inoculation as represent graphically in **Figure (4).** The results showed that control SP contained the most significant levels of chlorophyll and carotenoids compared to other groups, lactose 3gm showed significant increase in the phycocyanin and phycobiliprotein, starch 3gm and mannose 2gm showed significant elevation of allophycocyanin but starch 2g showed significant increase in phycoerythrin content in comparison with other tested sugar and control groups at p<0.05. Although sucrose and glucose followed by fructose enhanced SP growth rates, SP grown on control medium induced significant elevation in the chlorophyll and carotenoids contents and supplemented media with lactose, mannose or starch induced significant elevation in phycobilins contents at the end of the experiment.



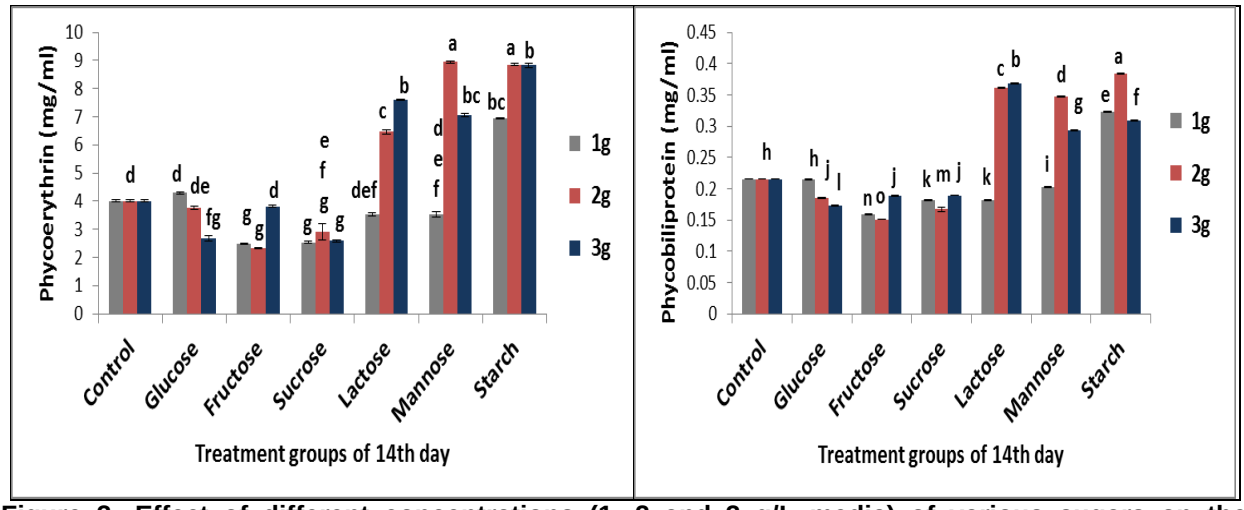
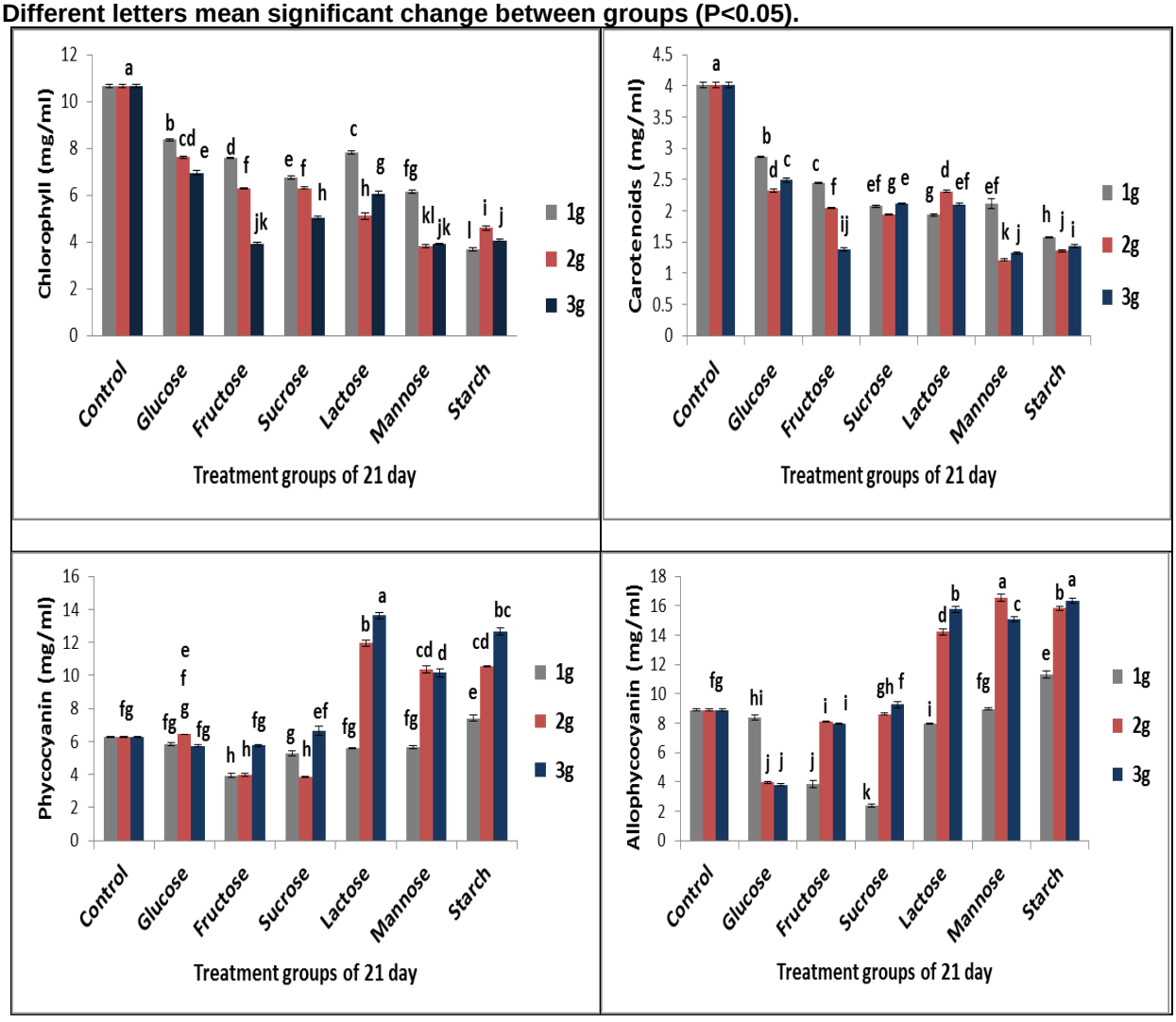


Figure 3: Effect of different concentrations (1, 2 and 3 g/L media) of various sugars on the chlorophyll, carotenoids and phycobilins represent by phycocyanin, phycoerythrin, allophycocyanin, phycobiliprotein (mg/ml) contents of *Spirulina platensis* at 14th day of growth. Different letters mean significant change between groups (P<0.05).



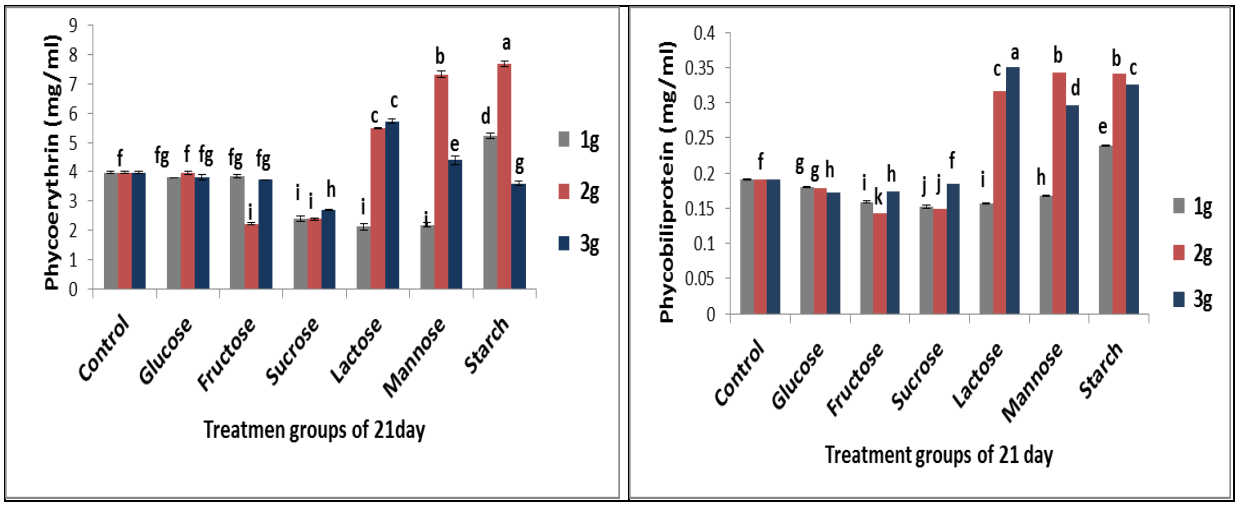


Figure 4: Effect of different concentrations (1, 2 and 3 g/L media) of various sugars on the chlorophyll, carotenoids and phycobilins represent by phycocyanin, phycoerythrin, allophycocyanin, phycobiliprotein (mg/ml) contents of *Spirulina platensis* at 21th day of growth. Different letters mean significant change between groups (P<0.05).

DISCUSSION

The Detection of natural antioxidant sources for nutraceutical and pharmaceutical effects has recently increased. Microalgae were proved to have endogenous antioxidant compounds as some vitamins, carotenoids and polyphenols that play an important role in prevention of oxidative damages caused by free radicals via their scavenging activities (Wang et al. 2017). Antioxidants help in improving all physiological functions of human, thus they can protect against diseases. Antioxidants from plants aid in prevention of degenerative neuropathies, diabetes, cardiovascular disease and cancers; also they provoke anti-inflammatory, anti-viral, and anti-ageing activities (Scalbert et al. 2005)

Microalgae carry out the photosynthesis process to increase their biomass and pigment contents (Olivieri et al. 2010). Carbon from carbon sources is utilized by organisms in metabolism and in biomass building (Reece et al. 2014 ; Tortora et al. 2018). When both inorganic carbon from carbon dioxide (CO_2) and organic carbon from carbohydrates are utilized by the organism in presence of light to carry out the photosynthesis process this is called mixotrophic cultivation (Chen and Zhang 1997 and Kang et al. 2004). In mixotrophic culture, photosynthesis and oxidative glucose metabolism worked simultaneously (Marquez et al. 1993). The light served as an energy source for synthesis of cell contents during mixotrophic growth and subsequent maintenance (Shi and Chen, 1999).

In this study *S. platensis* was grown on Zarrouk's medium contained various concentrations (1g, 2g and 3g/L) of sugars such as glucose, fructose, sucrose, lactose, mannose and starch. After 21 days of cultivation, among different concentrations of sugars the most significant O.D for growth was recorded in sucrose (1gm/L), glucose (2gm and 3gm/L) and fructose (1g/L) in order.

The findings of this study revealed that sucrose; glucose and fructose could increase growth rate or biomass of SP. Mishra et al. (1985) showed that the maximum growth of wild type *Nostoc linckia* was at glucose then fructose and sucrose. They referred the failure of growth at other carbon sources to absence of required enzymes needed for these compounds metabolism. Also Cerón García et al. (2006) demonstrated that the best biomass induction of *Phaeodactylum tricornutumon* was achieved when it grown under mixtrophic conditions with adding of glycerol then fructose and glucose. Borsari et al. (2007) showed that sugar cane molasse was the best substrate for cultivation of Nostoc species as it is rich in nutrients, vitamins and nitrogenous compounds. Our results are apparently constant with these results as sucrose is the main sugar in sugar cane molasse. It was proved that most of algae can utilize sucrose as a carbon source due to availability of sucrose transporter and ability to hydrolyze sucrose into monosaccharide (Wang et al. 2017). Moreover Perez-Garcia et al. (2011) showed that glucose was the best organic carbon source for growth of *Chlorella vulgaris* because

glucose has effect on metabolic carbon assimilation pathways, cell's size, vitamins and cellular contents of chlorophyll.

Velu et al. (2015) studied the effect of glucose, fructose, sucrose, lactose and galactose on *Nannochloropsis salina*, *Dunaliella tertiolecta* and *Tetraselmis suecica* (marine algae) and observed that the biomass of yield was high with sucrose and low with fructose in mixotrophic culture. In general Shyam and Sarema (2017) noted that stimulation of growth was dependent on concentration of sugar at studying the effect of organic carbon compounds on the growth of *Nannochloropsis salina.*

After 21 days of SP cultivation in the present study, control Zarrouk's medium without adding any sugars induced significant increase in chlorophyll and carotenoids contents.

Shyam and Saramma (2017) recorded decreasing chlorophyll and carotenoids content of *Nannochloropsis salina* at adding glucose and referring this to regulation of photosynthesis process as there is availability of glucose in the medium. Loss of chlorophyll content with fructose was referred to decrease in oxygen evolution that keeps microearobic condition needed for nitrogen fixation (Harry and Spiller, 1981).

Lactose 3gm/L showed significant elevation in the contents of both phycocyanin and phycobiliprotein. The content of allophycocyanin recorded significant increase at using mannose 2gm/L and starch 3gm/L as carbohydrates supplement meanwhile Phycoerythrin content elevated significantly at adding starch 2g/L in this study. Ogbonna and Tanaka, (1998) recorded decreasing in production of photosynthetic pigments as compared with the amounts present in the absence of organic carbon source due to alter both the photosynthetic and heterotrophic metabolism in mixotrophic cultivation.

CONCLUSION

The mixotrophic cultivation of *S. platensis* using different types and concentrations of sugars has been investigated. The results denoted that 3gm of mannose after 7 days of cultivations give the best growth in comparison with other sugar. Mixotrophic cultivations of *S. platensis* reduce the amount of chlorophyll and carotenoid but promoted phycobillins. Lactose, mannose and starch (3 and 3 gm) are the best sugar and concentrations for productions of phycocyanin, allophycocyanin, phycoerythrin and phycobiliproten. The results highlight for possible cultivations of *S. platensis* under mixtrophic conditions using different sugar sources.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

ND shared in planning the work and revised the manuscript. BElb (corresponding author) shared in writing the manuscript and data analysis. NG performed the experiments, analyzed data and wrote the manuscript. RH designed the experiment, reviewed the manuscript and applied the plagiarism.

All authors read and approved the final version.

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