FEATURES OF SPIRULINA PLATENSIS CULTIVATED UNDER AUTOTROPHIC AND MIXOTROPHIC CONDITIONS

Zahra Rasouli[1], *E-mail*: zahrarasouli87@gmail.com
Mahdi Parsa[1], *Email*: parsa@um.ac.ir, corresponding Author
Hossein Ahmadzadeh[2], *E-mail* h. ahmadzadeh@um.ac.ir
[1]Department of Agriculture, Ferdowsi University, Mashhad, Iran
[2]Department of Chemistry, Ferdowsi University, Mashhad, Iran

Abstract. Cultivation of *Spirulina platensis* in Zarrouk media containing 0–20 g l^{-1} glucose was studied in a photobioreactor for 30 days using a light intensity of 3 klux. Various parameters were measured to evaluate the enhancement of cell performance with glucose such as cell number, osmolarity, membrane stability, biomass productivity, doubling time, stress intensity, stress tolerance, chlorophyll, protein, carbohydrates, and lipid contents. Based on the results, we concluded that *S. platensis* is able to grow and produce some ingredients in Zarrouk media containing up to 20 g l^{-1} of glucose which is the first to be reported. The cell concentration of the mixotrophic cultures (80 cells per mm^2) corresponded well to the sum of the autotrophic cell concentrations (50 cells per mm^2), showing that the addition of carbohydrate positively effects on the microalgae growth. The continuous operation supplemented with 0.5 g l^{-1} of glucose (G0.5) led to the maximum cell concentration about 9.06 g l^{-1} wet and 1.32 g l^{-1} dry weights. The highest tolerance index, specific growth rate, biomass productivity, cell division, osmolarity and membrane stability index were respectively 102.5%, 0.15 d^{-1}, 0.04 g $l^{-1}d^{-1}$, 0.26 div d^{-1}, 0.87 osmol kg^{-1} and 93.8%, obtained in the same treatment. Chlorophyll (6.7 % in G0; 0.046 g l^{-1} in G0.5), protein (79.9 % and 0.884 g l^{-1} in G0.5), carbohydrates (55.5% in G20; 0.492 g l^{-1} in G6) and lipid (53.3% in G10; 0.636 g l^{-1} in G0) percentages and yields were mostly enhanced in the mixotrophic condition. This study indicated that mixotrophic growth of *S. platensis* is useful for commercial biomass production.

Keywords: microalgae continuous cultivation, glucose, autotrophic and mixotrophic conditions, biochemical composition.

Introduction. Formulation of the problem

Large-scale microalgal production has been studied for decades [1,2], given the wide variety of practical and potential metabolic products under different cultivation conditions. However, in spite of the recent progresses in the design of high efficiency photobioreactors, cultivation and extraction techniques, there still exist a large number of unsolved problems since there are many gaps to be filled out. The low productivity and high production costs of the most common procedures, photoautotrophic culture of *S. platensis* in open ponds, are the major obstacles to the successful commercialization. From the very beginning researchers have reported that *S. platensis* is an obligate autotroph [3]. Under autotrophic cultivation (in the light), the cells harvest light energy and use CO_2 as a carbon source. This organism has been found to utilize organic carbon substrates (i.e. glucose) for heterotrophic growth [4]. However, this form of growth is not suitable for the production of valuable chemicals due to a low specific growth rate and an extremely long lag phase [5]. Next, a new method has been suggested: mixotrophic cultivation (simultaneously in the light and on glucose supplement) which is the aerobic oxidative phosphorylation of an organic carbon substrates in the presence of light. The accessible light and carbon source as a dual limiting process might upgrade the yield [6] up to a limitation of massive growth and dense population. This way the biofixation potentially combines the capture of carbon from CO_2 and carbohydrate molecule, building a promising road for mitigation of greenhouse gas emissions, environmental impacts and cultivation costs.

Photosynthetic bacteria, cyanobacteria and blue-green microalga, *Spirulina platensis*, have long been grown photoautotrophically for the production of algal health food because of their high cell growth rate, incapability of affecting the food chain, ease of harvesting and potential marketability. They have the potential of producing a diverse range and large quantities of valuable chemicals and biologically active compounds, such as 74% dry weight of proteins (essential amino acids in the proportions recommended by the WHO/FAO, except for methionine) [7], polyunsaturated fatty acids, polysaccharides, vitamins, carotenoid pigments [8], because of which it is used as a dietary supplement for both animals and humans, phycoremediation and green energy products [1,9]. *S. platensis* contains 13.6–50% of carbohydrates (from the photosynthetic fixation of CO_2), which comprise principally glucose along with rhamnose, mannose, xylosc, galactose and two unusual sugars (2-0-methyl-L-rhamnose and 3-0-methyl-L-rhamnose) [10]. Although it is generally agreed that glucose (produces 2.8 kJ mol^{-1} energy) [11] is the end product of photosynthesis and can be easily assimilated to produce energy-rich compounds, published works on the effect of glucose on value-added products are few, since it has been confirmed that microalgae cannot use external carbon sources efficiently and *S. platensis* has been considered to be an obligate autotroph [3,6]. Besides, it has been proved that heterotrophic growth is very problematic and cost intensive [6]. Although *Spirulina* sp. can ferment glucose only for the maintenance of metabolism during heterotrophic growth [5], its mixotrophic cultivation can change the common consensus among researchers. Moreover, 50% (w/v) of 14C-glucose in the medium is

converted into cell carbon [12] and produces 2.8 kJ mol^{-1} of energy (compared to 0.8 kJ mol^{-1} for acetate) [11]. On the other hand, this non-recalcitrant carbohydrate can be easily utilized by hydrogenogens for hydrogen production [13] and scarcely acts as a downstream overproduction. So, not surprisingly, far higher growth and respiration rates are obtained with glucose than with any other substrate, including sugars, sugar phosphates, organic acids, sugar alcohols and monohydric alcohols [14].

In this context, the **objective of this work** was to investigate the influence of glucose concentration on the mixotrophic growth and comprehensive biochemical analysis of *S. platensis*, focusing on the best biochemical composition of the biomass obtained. Several reports related to the growth of *Spirulina* in synthetic media with glucose have been published [6,15]. However, cultivation in modified media using precise amounts of glucose (in such high concentrations) and its influence on different metabolites is scanty. Furthermore, most of the previous works on the culture of *S. platensis* dealt with batch-wise operation. One of the first tasks, therefore, on the way to solve the mentioned problems is to find the best glucose concentrations through measuring numerous different features and ingredients. In addition, the task includes estimating the moderating influence of the treatments on the main and integrated effects of these features and considering the best effective treatment.

Research materials and methods

Microorganism and culture medium. The microalga *Spirulina platensis* was kindly provided by the Research Center of Biotechnology of Mashhad University, Iran. The microalga was grown in the culture medium of Zarrouk [16] having the following composition (per l): 16.8 mg NaHCO$_3$, 0.5 mg K$_2$HPO$_4$, 2.5 mg NaNO$_3$, 1 mg K$_2$SO$_4$, 1 mg NaCl, 0.04 mg CaCl$_2$.2H$_2$O, 0.2 mg MgSO$_4$.7H$_2$O, 0.01 mg FeSO$_4$.7H$_2$O, 0.08 mg EDTA, 1 ml of trace element solutions A5 (0.222 mg l^{-1} ZnSO$_4$.4H$_2$O, 0.079 mg l^{-1} CuSO$_4$.5H$_2$O, 0.018 mg l^{-1} Na$_2$MoO$_4$, 2.86 mg l^{-1} H$_3$BO$_3$, 1.81 mg l^{-1} MnCl$_2$.4H$_2$O) and B6 (0.023 mg l^{-1} NH$_4$VO$_3$, 0.096 mg l^{-1} K$_2$Cr$_2$(S0$_4$)$_4$.24H$_2$O, 0.048 mg l^{-1} NiSO$_4$.7H$_2$O, 0.018 mg l^{-1} Na$_2$WO$_4$.2H$_2$O, 0.044 mg l^{-1} Co(NO$_3$)$_2$.6H$_2$O, 0.04 mg l^{-1} Ti(SO$_4$)$_3$), supplemented with (0.5, 1, 1.5, 2, 3, 4, 6, 10, 20 g l^{-1}) or without glucose (Merk, Germany). All media were sterilized at 121 °C for 15 min before use, and the initial pH was adjusted to 9.5 with 1M NaOH. After cooling the media to room temperature, the photobioreactors were operated in continuous mode by adding 30 µg l^{-1} Streptomycin (Sigma-Aldrich) and 1 mg l^{-1} Benomyl (Melli Agrochemical Company, Iran).

Experimental Procedure. Preliminary batch tests were performed to select the operating conditions of our photobioreactor. Then 30-day experiments were performed continuously in sterilized 1 liter photobioreactors varying the composition of glucose to determine the optimal growth conditions. Cultures were continuously aerated (10%, v/v) to mix and exchange gases appropriately. Photobioreactors were illuminated by 3 fluorescent lamps that provided a light intensity of 3 klux for 16 h d^{-1} (8 h of dark period), which corresponded to 760 and 600 kJ m^{-2}d[-1] of PAR and IPAR, respectively. The photon flux density measurement was carried out using an integrated quantum meter LI-250A (Li-Cor Inc., USA) with its sensor located inside the photobioreactors. The photobioreactors were initially inoculated with a biomass concentration of 0.7 g l^{-1} active cells corresponding to 0.2 optical density (OD).

Analytical Procedures. The cell concentration in the culture fluids was daily determined turbidimetrically at 750 nm in a spectrophotometer (Photonix Ar 2015, Iran). To avoid interferences due to the different growth media employed, the optical density of the blank was zeroed for different glucose concentrations. To obtain an accurate estimate of the cell density, the cells were evenly suspended in the culture medium and then quickly the aliquot of cells were removed for further analysis. They were loaded on the hemocytometer using a micropipette. The total number and length of cells, average number, maximum number and length of helices were measured using a Neubauer (Marienfeld, Germany) and a microscope (Olympus, Japan) with 100x magnification. Dry weights were measured in each phase by taking samples every two days and drying at 80 °C in a vacuum oven until constant weight was obtained. The values were also compared with the previously prepared standard calibration curve of optical density versus *S. platensis* biomass dry weight.

The measurement of parameters such as pH (Milwaukee 151, Martini, USA), electrical conductivity (EC, mS cm^{-1}; 712 Conductometer, Metrohm, Switzerland) and osmolarity (osmol kg^{-1}; Osmometer 802, Vogel, Germany) was performed on previously filtered growth media. The membrane stability index (MSI) was calculated using the following equation [17]:

$$MSI = 1 - \frac{EC_1}{EC_2} \times 100, \% \qquad (1)$$

where EC_1 and EC_2 refer to *EC*s of 10 ml filtered sample in 10 ml of distilled water a day before and after autoclaving (1 bar, 120 °C, 15 min), respectively.

Growth kinetics and stress parameters of *S. Platensis* were calculated using the following equations [18-21]:

Biomass productivity $P_x = \frac{W_2 - W_1}{T_2 - T_1}$, g·l^{-1}d[-1] (2)

Specific growth rate $\mu = \frac{\ln W_2 - \ln W_1}{T_2 - T_1}$, d[-1] (3)

Doubling time $DT = \ln 2 \div \frac{\log(\frac{W_2}{W_1})}{T_2 - T_1}$, d[-1] (4)

Cell division $K_2 = \frac{\ln N_2 - \ln N_1}{T_2 - T_1} \div \ln 2$, div d[-1] (5)

Stress tolerance $TOL = TDW_{stress} - TDW_{control}$, g (6)

Yield sustainability index $YSI = \frac{TDW_{stress}}{TDW_{control}}$ (7)

Tolerance index $TI = \dfrac{TDW_{stress}}{TDW_{control}} \times 100$ (8)

Stress intensity $SI = 1 - \dfrac{TDW_{stress}}{TDW_{control}}$ (9)

Where T_1 and T_2 (d) are the initial and final time of growth, W_1 and W_2 (g) are the initial and final dry weights, N_1 and N_2 are the initial and final cell numbers, and TDW refers to total dry weight (g l^{-1}).

Chlorophyll a [22], protein (y=0.856x+0.5686) [23], carbohydrate (y=4.8494x+0.1421) [24] and lipid [25] contents were measured using a centrifuge (Routine 16, Behdad, Iran), a vortex (V1$^+$, Boeco, Germany), ultrasonic (SB-103D Ultra, Korea), a weighing scale (Gemini GR, A&D, USA) and a spectrophotometer (Photonix Ar 2015, Iran). Harvest indices of all qualitative measurements were calculated according to the equation [2-10]. All measurements were carried out seven times during 30 days of the experiment to obtain each phase effect on different characteristics.

Harvest index $HI = \dfrac{Metabolite\_weight}{TDW} \times 100$, % (10)

Statistical Analysis. Statistics software MSTATC was used to determine the mean value, standard deviation and significant differences amongst the treatments at P < 0.05.

Results of the research and their discussion

Photosynthetic metabolism and aerobic respiration have been reported to occur both independently and simultaneously within the cells in mixotrophic *Spirulina* cultures [26,27], but the presence of organic carbon can alter both conditions. Besides, cultivation conditions such as lower light and new ingredients composition can favor autotrophy or heterotrophy pathways regarding the presence of light or organic substrate [6]. Fig. 1 represents the effects of initial glucose concentration on auto- and heterotrophic growth of *S. platensis* and indicates that cultivation period is a highly important parameter that has been mostly ignored in many researches. The growth curves started to look significantly different at the very early stages, as cultures containing 0.5, 1 and 1.5 g l^{-1} of glucose (G0.5, G1 and G1.5) did not show any obvious lag phase and continued on to an exponential phase, these cultures showed more divisions than the control (0 g l^{-1} or G0) and other treatments. This lack of lag-phase phenomenon was previously reported [28] and seemed to be relevant to environmental conditions. Thus, these three treatments proved to have better environmental conditions for the production of different ingredients and growth. Linear phases of G10 and G20 took higher places than cultures containing 2–6 g l^{-1} of glucose. This phase was followed by a subsequent rise to the final stationary state, and it was observed in all curves. The stationary phase began with well-defined trends, followed by the best ones for 1, 0.5, 1.5 and 0 g l^{-1} glucose, respectively. However, the trends were changed in favor of 0.5 and 0 g l^{-1} of glucose after about the 10th day of the second growth cycle. The cultivations remained in the first and second stationary phase for about eight days of growth, whereas the whole cycle lasted for about 14 days. The highest optical densities were observed on days 16 and 28 when two growth cycles were completed, except for treatments of G10 and G20. This observation agrees with higher growth under mixotrophic conditions in comparison with control experiments [29]. In these cultures, glucose is used for up-regulation of growth genes called *bkt*, *chy-b*, and *pds* [30].
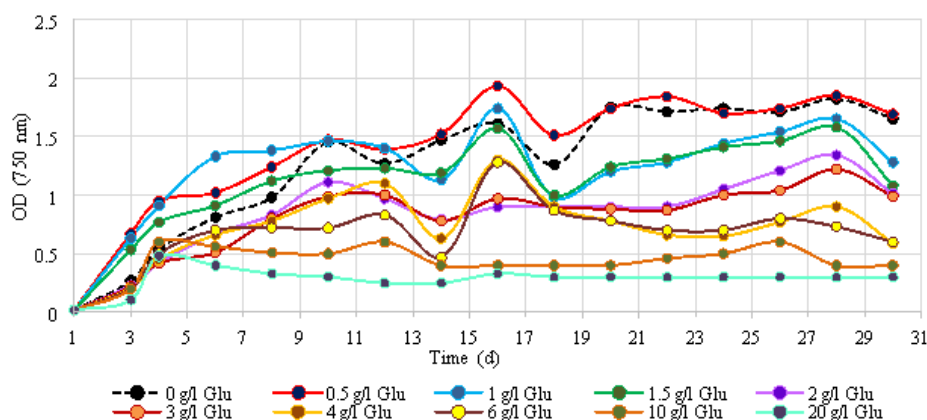

Fig. 1. Growth curves of the *S. platensis* in photoautotrophic and mixotrophic cultures with different glucose concentrations in aerated photobioreactors

Several microalgae like *Chlorella vulgaris* and *Auxenochlorella protothecoides* were previously found to tolerate this amount of glucose [31,32], yet information was lacking on the *Spirulina* species. As shown in Table 1, *S. platensis* survived in all culture media containing glucose from 0 to 20 g l^{-1}, which is the first to be reported. However, its weight was greatly reduced by adding more than 4 g l^{-1} of glucose. Wet and dry weight followed the same trend in all cultures and reached the plateau in treatment G0.5 with 9.063 and 1.316 g l^{-1} (1.022 and 1.025 fold more than G0), respectively. G0, G1 and G1.5 were next in order after 30-day cultivation. These values were much higher than those of the others reported which were reduced significantly with increasing glucose concentration [7,9]. Despite this downturn, surviving of algae in 20 g l^{-1} of glucose is notable. This

stress results in a reduction of the cellular ionic concentration and water potential, but the ion ratios owing to selective uptake remain constant, and cell performance continues [33]. No significant differences were seen between G2 and G3 (1.67 times lower than G0) or G4 and G6 (2.85 times lower than G0).

The biomass accumulation of *S. platensis* is shown in Fig. 2a. The algae in all treatments steadily accumulated algal biomass during the first two weeks, except for G10 and G20 which showed reduced biomass after day 5. It was noticed that algae in treatment of G1 had the highest biomass in the first 10 days and stood higher than G0 along with treatments of G0.5 and G1.5. G0.5 and G1 reached the maximum on day 15 and reduced significantly afterwards. As G0.5 met G0 on day 20, G1 and G1.5 were significantly lower. The association between light and minor amounts of organic carbon could promote the algal growth in comparison with higher concentrations, whereas both autotrophic and mixotrophic cultivations promoted an increase in the stationary phase of growth.

In another study, carbon sources affected significantly the algal growth [34]. Although G4 and G6 showed higher peaks than G2 and G3, their biomass averages were lower and these later treatments reached the same significant level on day 30. It was reported that the biomass of *C. protothecoides* increased 14.6 g l^{-1} to 23.5 g l^{-1} in 6 days with 3 g l^{-1} of glucose [35].

Comparing the stress parameters demonstrates that a carbon source can act as a stress factor in cultures containing more than 0.5 g l^{-1} of glucose (Table 1). The stress tolerance and tolerance index of G 0.5 were 0.032 g and 2.47% higher than G0, and its stress intensity value did not denote any stress during 30-day cultivation. Values of other treatments ranged 0.293 g to 1.068 g stress tolerance, 0.228 to 0.831 stress intensity and 77.23% to 16.86% of tolerance index for treatments G1–G20, while there were statistically significant differences between G2 and G3 or G4 and G6. The tolerance index was reduced to less than 50 % of the control by the addition of 4 g l^{-1} of glucose.

Table 1 – Growth kinetics of *S. platensis* in Zarrouk modified media using different glucose concentrations;
WW – Final Wet Weight; DW – Final Dry Weight; TOL – Stress Tolerance; SI – Stress Intensity; TI – Tolerance Index; P_x – Biomass Productivity; μ_{max} – Maximum Specific Growth Rate; DT – Doubling Time; K_2 – Cell Division

Glucose (g l^{-1})	WW (g)	DW (g)	TOL (g)	SI	TI (%)	P_x (g l^{-1} d^{-1})	μ_{max} (d^{-1})	DT (d)	K_2 (div d^{-1})
0	8.864 B	1.284 B	0 B	0 G	100 B	0.042 A	0.148 A	4.673 I	0.232 C
0.5	9.063 A	1.316 A	0.032 A	-0.025 H	102.47A	0.043 A	0.149 A	4.648 J	0.255 A
1	7.023 C	0.992 C	-0.293 C	0.228 F	77.23 C	0.032 B	0.140 B	4.961 H	0.238 B
1.5	6.028 D	0.834 D	-0.451 D	0.351 E	64.91 D	0.027 C	0.134 C	5.176 G	0.226 D
2	5.680 E	0.778 E	-0.506 E	0.394 D	60.60 E	0.025 C	0.132 C	5.266 F	0.216 E
3	5.580 E	0.762 E	-0.522 F	0.406 D	59.37 E	0.025 C	0.131 C	5.293 E	0.215 E
4	3.590 F	0.446 F	-0.838 G	0.653 C	34.73 F	0.014 D	0.113 D	6.130 C	0.199 F
6	3.640 F	0.454 F	-0.830 G	0.646 C	35.34 F	0.015 D	0.114 D	6.099 D	0.193 G
10	2.645 G	0.296 G	-0.989 H	0.770 B	23.02 G	0.009 E	0.099 E	6.975 B	0.171 H
20	2.148 H	0.217 H	-1.068 I	0.831 A	16.86 H	0.007 E	0.089 F	7.788 A	0.159 I

Means with same letters denote statistically significant differences (P < 0.05)

As shown in Fig. 2b, the osmolarity of most treatments was significantly enhanced by the addition of glucose, with the maximum on days 5 and 15 (the beginning of the first and second stationary phase). The osmolarity of G0.5 was significantly higher than that of all other treatments (0.867 osmol kg^{-1}), followed by G1, G1.5 and G2. These values were much higher than that of the control experiment, explaining how nutrition in mass cultures of microalgae can change the growth conditions and be one of the key factors that controls the productivity. The membrane stability index (MSI) was influenced not only by glucose concentration but also by osmolarity (Fig. 2c). When increasing glucose concentration and reducing osmolarity reduce MSI, this would mean that a stronger and more efficient membrane would be required to achieve the high biomass concentration. This instability is a major cause of productivity loss in long-term cultivations, as the minimum MSI was observed in G20, G10 and G6 with the highest glucose concentration and the lowest yield sustainability index (YSI, Fig. 2d). YSI of G0.5 was higher than that of the control experiment during the whole cultivation period, prov-

ing that this amount of glucose is required for an algal medium. YSI of G1 and G1.5 were also more than G0 in the first cycle of growth, but they lost their positions in longer cultivation periods. Therefore, 0–1.5 g l^{-1} glucose increases algal growth and productivity in cultivations with short-term goals.

Biomass productivity (P_x), the maximum specific growth rate (μ_{max}), doubling time (DT) and cell division (K_2) are also presented in Table 1. P_x can be defined as cell concentration per unit of reactor volume and time, and μ_{max} was determined from the slope of the growth curve in its linearity region. Both parameters were significantly affected by the substrate concentration. P_x and μ_{max} of G0.5 were 0.043 g $l^{-1}d^{-1}$ and 0.149 d^{-1} with an insignificant difference to G0. G1 ranked the second place, then G1.5, G2 and G3 were in the third level. Values for other treatments were considerably lower, which proved that the risk of glucose concentrations over 4 g l^{-1} is very high and should be considered in laboratory works focused on the production of algal metabolites. Incubation in higher glucose concentrations affects the internal os-

motic potential, which is essential for maintaining turgor pressure as the driving force for growth [36], and leads to decreased P_x due to an almost complete loss of oxygen production and evolution [37]. DT of G0.5 was 4.648 divisions per day (slightly lower than G0) and its K_2 was 0.255 division per day (slightly higher than G1 and G0). Higher glucose concentrations have severed negative effects upon the life cycle of *Spirulina* cells.

Structural features described as the number and size of the cells and helices in photoautotrophic (G0) and different mixotrophic cultures, are compared in Table 2. Microscopic observations of these factors are also presented in Fig. 3. The highest cell number and length were 80 cell μl^{-1} and 0.45 mm, attained under G0.5. The maximum number, average number and average length of helices in this treatment were 12 helices $cell^{-1}$, 9 helices $cell^{-1}$ and 0.05 mm, respectively. While the presence of organic carbon in the mixotrophic treatments (except for G0.5 and G1) decreased the total cell number after 30-day cultivation, the association between autotrophy and heterotrophy supply in the mixotrophic cultivation enhanced the cell length (except for G6, G10 and G20). Although this enhancement was due to an increase in both the helices number and length, the effect of increasing the length of each helix compared to the total number

of helices per cell was greater and increased with increasing glucose concentration. This observation was consistent with decreasing the MSI and osmolarity in these treatments. In fact, more open helices (0.04–0.06 mm) can be seen in treatments containing up to 4 g l^{-1} of glucose, considering that higher osmotic values result in better water holding capacities. In contrast, cultures supplemented with higher amounts of organic carbon reacted differently and the helices were more contracted (0.025–0.03 mm), where helices number per cell decreased, too. So, the best response of the constant internal physical and chemical conditions achieved through responding to the culture condition, transforming algae energy is indeed to follow a threshold strategy due to more SI, in which helices structure comes to aid cell survival (Fig. 3). Plasmolysis effects have also occurred at 4 g l^{-1} of glucose (0.355 osmol kg^{-1}). As algae achieved homeostasis, relatively, and reproduction [38], this homeostasis could be helpful in reestablishing the cellular architecture upon replasmolysis. In cells undergoing plasmolysis, the periplasmatic space among the cell walls was filled with membrane enclosed structures, the protoplasts were retracted from the cell wall, the cytoplasm appeared dense, and vacuoles were fragmented [37].

Table 2 – The apparent structural features of *S. platensis* cell and helix for the cultivation in Zarrouk modified media using different glucose concentrations

Glucose (g l^{-1})	Cell		Helix		
	Number (cell μl^{-1})	Length (mm)	Maximum Number (helix $cell^{-1}$)	Average Number (helix $cell^{-1}$)	Average Length (mm)
0	50 C	0.18 E	8 C	6 C	0.03 D
0.5	80 A	0.45 A	12 A	9 A	0.05 B
1	57 B	0.35 C	10 B	7 B	0.05 B
1.5	44 D	0.35 C	10 B	7 B	0.05 B
2	36 E	0.42 B	10 B	7 B	0.06 A
3	35 F	0.36 C	10 B	6 C	0.06 A
4	25 G	0.24 D	8 C	6 C	0.04 C
6	22 H	0.125 F	7 D	5 D	0.025 E
10	14 I	0.125 F	6 E	5 D	0.025 E
20	11 J	0.12 F	6 E	4 E	0.03 D

Same letters denote statistically significant differences ($P < 0.05$)

Different nutrients such as carbon closely correlate to the growth rate and pigment contents, by affecting the biochemical composition of microalgae [39]. This fact by itself indicates a broader amplitude for photosynthesis in the examined strain. The changes in nutrients and harvest indices of 10 cultures during the 30-day continuous cultivation of *S. platensis* are shown in Fig. 4 and 5, respectively. Comparing the biochemical composition of the biomass under mixotrophic and autotrophic conditions, it can be seen in Fig. 4 that the values range 0.086 (G20) to 0.884 (G0.5) g l^{-1} of proteins, 0.115 (G20) to 0.492 (G6) g l^{-1} of carbohydrates, 0.121 (G20) to 0.636 (G0) g l^{-1} of lipids, and 0.008 (G20) to 0.046 (G0.5) g l^{-1} of chlorophyll. However, for the harvest index, a different result is observed. It is shown in Fig. 5 that values range 34.6 (G20) to 79.9 (G0.5) % w/w proteins, 30.1 (G0) to 55.5 (G20) % w/w carbohydrates,

25.1 (G1.5) to 53.3 (G10) % w/w lipids, and 4.5 (G20) to 6.7 (G0) % w/w chlorophyll. Based on the previous experimental results from the batch culture of *S. maxima*, 76.5% of proteins, 6.4% of carbohydrates, and 11.5% of lipids were obtained on 1% of vinasse [40].

The greater effects of G0, G0.5 and G1.5 on protein yield as compared to those in other cultures reflect that these treatments contain the most suitable concentration of glucose for producing proteins (Fig. 4a). Another point to consider is the fact that the production of protein was accelerated in G0.5 and G1.5 (days 5 and 15) compared with the control (day 20). These results indicated that protein production and growth rate are related and can stimulate each other, as higher protein concentrations caused more raw materials and faster growths. Experimental results are in agreement with the recent reports,

which studied the growth of *S. platensis* cultured with 1–2 g l^{-1} of beet vinasse (up to 77% of protein content) [40-42]. However, in other studies, the protein expression patterns under the autotrophic and mixotrophic conditions were very similar with no significant difference [43] and could even inhibit the growth [44].
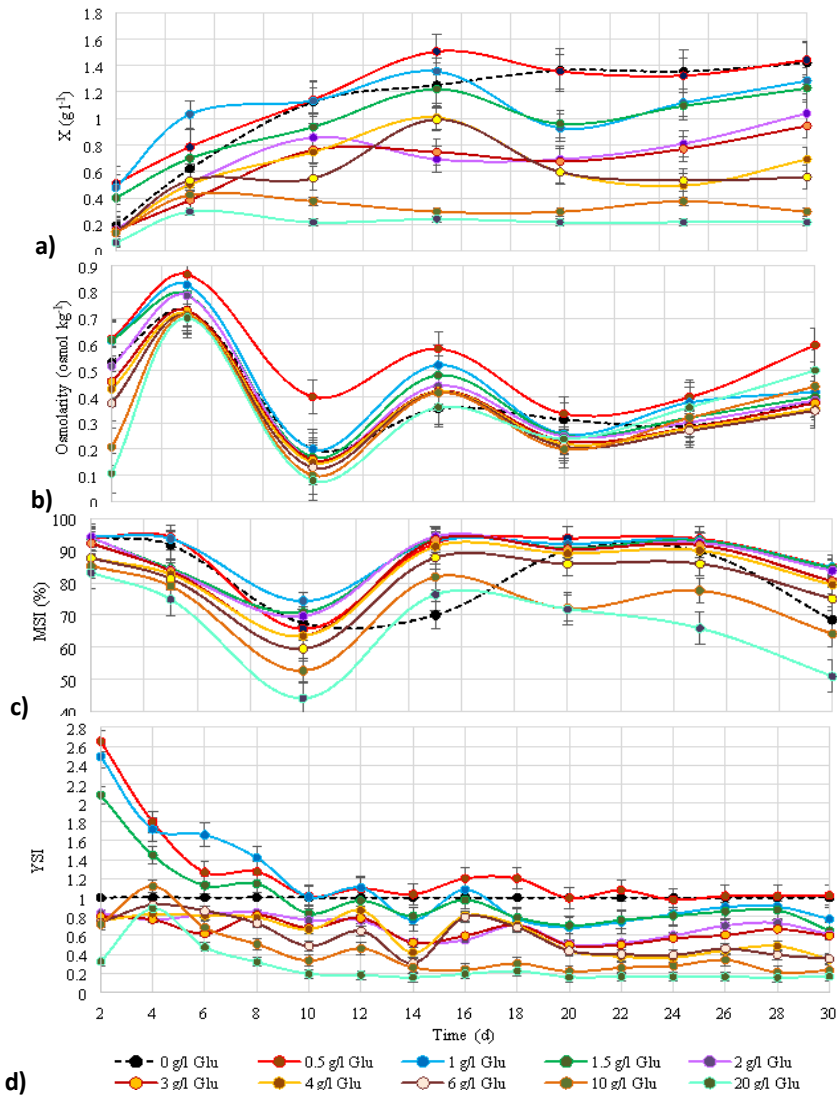

Fig. 2. Changes in growth and stress parameters of *S. platensis* under different glucose concentrations during the 30-day experiment: a) cellular concentration (X); b) osmolarity; c) membrane stability index (MSI); d) yield sustainability index (YSI)
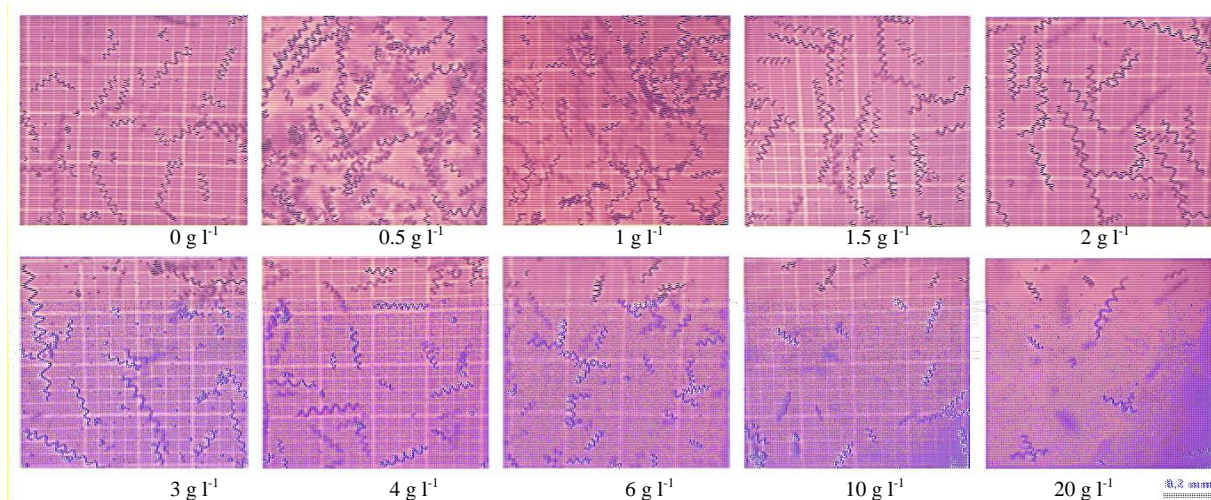

Fig. 3. Effect of photoautotrophic and mixotrophic cultures with different glucose concentrations on the cell structure of *S. platensis*
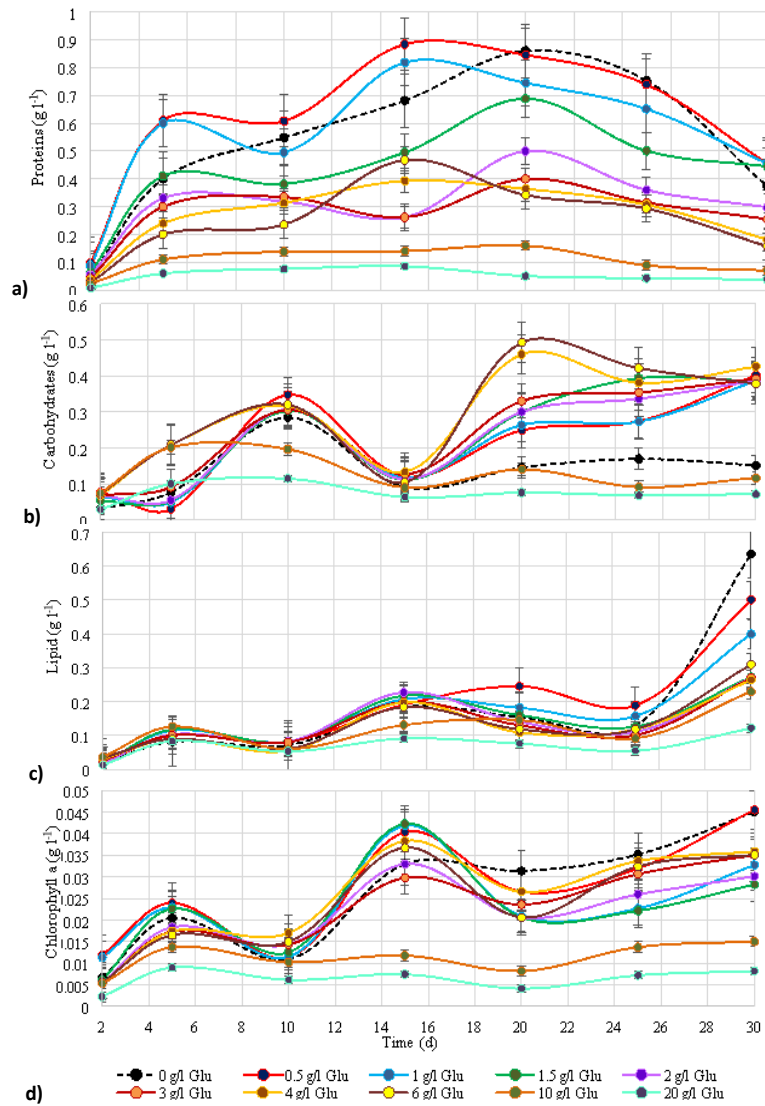
a)

b)

c)

d)

Fig. 4. Influence of glucose concentrations on a) protein, b) carbohydrates, c) lipid, and d) chlorophyll a contents (g l⁻¹) of *S. platensis* during the 30-day experiment. The values are means of three replications ± Std

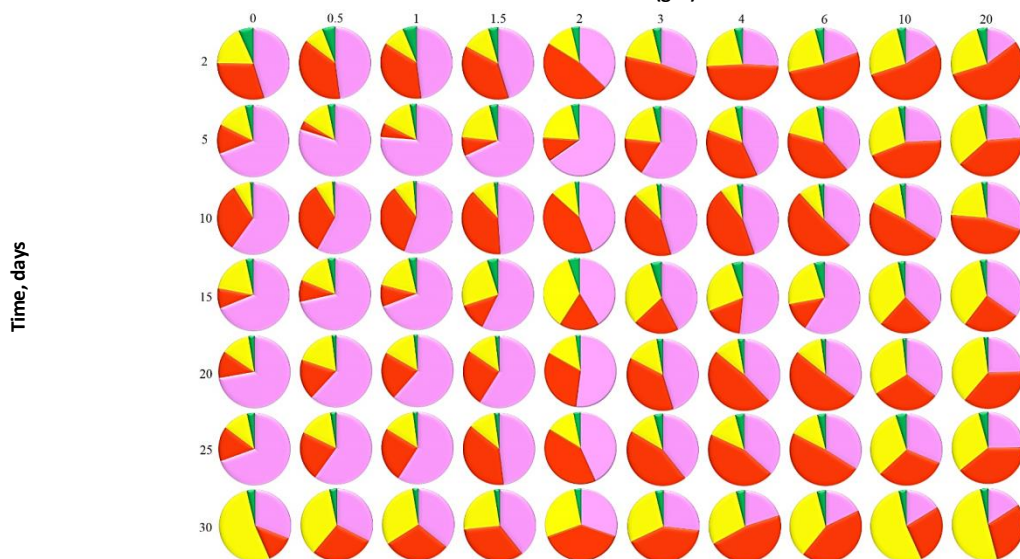**Glucose concentrations (g l⁻¹)**

Fig. 5. Comparison of the performance of protein (pink), carbohydrates (red), lipid (yellow), and chlorophyll a (green) harvest indices (%) of *S. platensis* under phototrophic and mixotrophic cultivation

The yield curves of carbohydrates showed completely opposite trends to the one observed in proteins and were maximized at 10 and 20 days (Fig. 4b). The production of carbohydrates was higher at the second growth cycle except for G0, G10 and G20. The highest carbohydrates concentration occurred on the 20th day in G6 and G4, respectively, which seemed to accumulate carbohydrates in a stress condition. This mechanism can support protein synthesis until the nitrogen supply in the medium is restored [45]. The same results were obtained with diatoms species like *Achnanthes brevipes* and *Tetraselmis* spp. under nitrogen starvation [46]. These results were consistent with the work of Miao and Wu [47], who reported 45% more of total carbohydrates in heterotrophically grown *Chlorella* sp. However, Kaplan *et al.* [37] indicated that the carbohydrate amount is not a major factor contributing to the stress in *Klebsormidium* species.

Many reports showed that glucose increased the net lipid productivity in heterotrophic cultures of *Auxenochlorella protothecoides* (30 g l^{-1}) [31], *Chlorella vulgaris*, *Botryococcus braunii* and *Scenedesmus* sp. (2%) [48] and mixotrophic cultures of *Chlorella* sp. (20 g l^{-1}) [49], *C. vulgaris* (20 g l^{-1}) [32] and *C. sorokiniana* [50]. It was also previously reported that the net lipid productivity enhances in microalgal species with increased glucose concentration as long as light is not a limiting factor [9,51], but the ability of *Spirulina* species of lipid production in such high concentrations was not measured before. The initial concentrations of lipid in all cultures were lower than those on the 30th day of continuous cultivation, indicating that the lipid production is time-consuming (Fig. 4c). This suggests that the optimal harvesting time of lipid begins from the 30th day after algal cultivation. This may be because the proteins and carbohydrates break down into lipids under stress condition, which may result in a strengthened respiration action and cause an inhibitory effect of high glucose concentration on the growth (Fig. 1) [4], whereas at the beginning of the growth phase, a strengthened photosynthesis action required a higher glucose concentration in order to meet the demands of growth and make more chlorophyll, proteins and carbohydrates. Thus, cultures with more nutrients (G0, G0.5 and G1) made more lipids, too. It is worth mentioning that although the HI of lipids was the highest in G10 and G20, their lower weights resulted in a significant reduction in the lipid yield. In fact, the lower carbon to nitrogen ratio in recent treatments was related to the lipid accumulation.

Fig. 4d indicates that when the initial glucose concentration was more than 6 g l^{-1}, the inhibitory action at higher glucose concentrations impeded chlorophyll production. Consequently, the interaction of chlorophyll and other nutrients production corresponded to the harmonious relationship among these metabolic processes and the decrease in photosynthetic pigments led to a decrease in the growth rate [52]. Hence, as mentioned before, the net photosynthesis under osmotic stress decreased to about 20–35% of the initial value [38], most likely owing to the darker medium color. Whereas both autotrophic and mixotrophic cultivations promoted an increase in chlorophyll content on days 5, 15 and 30, mixotrophic cultivations produced more pigments only at the first cycle and the photoautotrophic culture contained more chlorophyll at the second growth cycle, probably because of the lower concentration of nutrients in the growth media at the first cycle of autotrophic growth, and higher turbidity and SI at the second cycle of mixotrophic cultures. These observations go along with findings by Sun *et al.* [30], who demonstrated the pigment yield increases with increasing glucose concentration.

Conclusion

The objectives of this work were to analyze the feasibility of the use of organic carbon source as a supplement in a modified culture medium and its influence on the mixotrophic growth, metabolites amount and composition of *S. platensis*. Then, the algae were cultured in a photobioreactor using a mineral growth medium supplemented with 10 different concentrations of glucose. Based on the experimental results from this study, cultivation of microalgae in growth medium amended with glucose in the presence of light have shown higher growth rates, biomass, chlorophyll, proteins, carbohydrates and lipids percentages and yields. In fact, glucose stimulates the metabolic pathways involved in proteins, lipids and carbohydrates synthesis than those generated by the autotrophic culture with light as the only energy source. This result may be obtained for some reasons, which include: A. Significantly higher algal growth rates during the exponential phase enhance performance, growth characteristics and protein concentrations. B. Manipulation with *S. platensis* growth conditions alters both the chemical composition of algal cells and the growth medium due to reorientation of carbon allocation into proteins, carbohydrates and lipids. C. Composition of nutrients in the growth media is as important as their concentrations. D. Secretion of soluble materials such as polysaccharides, free amino acids, hormones and other organic compounds from the cells is higher in the presence of a carbon source [28]. E. No photoinhibition was noticed in the mixotrophic culture compared to the autotrophic culture [15]. F. Metabolism of glucose is performed by means of the pentose phosphate and Embden–Meyerhof pathways under heterotrophic and autotrophic conditions, respectively [53]. G. ATP and NADPH are produced faster by the algal cells under mixotrophic conditions [42]. H. Fermentation products (ethanol, acetate and lactate) were not detected during mixotrophic growth in *S. platensis*, and respiratory activities even in the light do not let such products act as an inhibitor [26]. I. The overall consumption of carbon is ultimately determined by how efficiently it is metabolized and transported through the cell membrane [54]. This process and transport of ions across the cell membrane is proton dependent [55] which utilized glucose from the medium. J. *S. platensis* achieves homeostasis by physiological and structural parameters to proliferate over a wide range of

environmental conditions. K. There exists the optimum carbon concentration at which the highest specific growth rate is obtained, due to an inhibitory effect of high glucose concentration.

It is, therefore, concluded that mixotrophic cultivation is preferred for enhancing the biomass and metabolites productivities in *S. platensis*. To support low cost production in commercial success and aquaculture applications in particular, we suggest adding 0.5 g l^{-1} of glucose to the growth medium formulation. The diversity in the biochemical composition among different treatments indicates that the purpose of cultivation can change the proper concentration and 6 g l^{-1} of glucose could be successfully implemented to increase the carbohydrates yield. It is also suggested that the cultivation time should be long enough to support the growth and commercial biomass production. Although different aspects of growth and stress parameters identified in this study provided insights into processes that occur in the photobioreactor, further research about the metabolism of *S. platensis* should be carried out to elicit the individual contributions of each environmental stress and strain.

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