EFFECTS OF SODIUM GLYOXYLATE AND LIGHT INTENSITY ON GROWTHS AND EXOPOLYSACCHARIDE PRODUCTIONS OF *Prasinococcus* sp. AND *Porphyridium cruentum*

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ABSTRACT

This work is a first attempt to investigate polysaccharide content of *Prasinococcus* sp. in comparison with the model strain of *Porphyridium cruentum*, as a potential polysaccharide producer, under two different light intensities with two different sodium glyoxylate monohydrate concentrations in F/2 medium. The microalgae strains were cultured in 1000 mL glass bottles containing 900 mL of F/2 medium at the air flow rate of 1 vvm under the temperature of 22±2 °C at two different light intensities (10 μE $m^{-2}s^{-1}$ and 50 μE $m^{-2}s^{-1}$) with two different concentrations of sodium glyoxylate monohydrate (control, 0.25, and 1.25 mM $C_2HNaO_3.H_2O$) for 14 days. The maximum specific growth rates of 0.236 day^{-1} and 0.298 day^{-1}, which corresponded to the doubling times of 2.945 day and 2.325 day, were obtained in F/2 control medium and in the low level glyoxylate treatment (0.25 mM) under 50 μE $m^{-2}s^{-1}$ for *Prasinococcus* sp. and *P. cruentum,* respectively. It is also important to underline that higher light intensity had a direct influence, whereas no effect was observed for glyoxylate treatments on the growth of *Prasinococcus* cells. Additionally, both high light intensity and mild level of glyoxylate stimulated faster growth for *P. cruentum*. On the other hand, total monosaccharide amount of exopolysaccharide fraction of *P. cruentum* increased with increasing the glyoxylate level, while the mild level of glyoxylate treatment (0.25 mM $C_2HNaO_3.H_2O$) stimulated the monosaccharide production of *Prasinococcus* cells.

KEYWORDS: light intensity; *Prasinococcus* sp.; *Porphyridium cruentum*; sodium glyoxylate; polysaccharide

1. INTRODUCTION

Cultivation of microalgae as a potential source of a variety of products, from fuels and animal feeds to vitamins and pharmaceuticals, has been investigated for 40 years. *Porphyridium* has made it a useful experimental system and an attractive organism for studies of its high content of polysaccharide, which has also drawn attention to its potential use as a source of valuable chemicals. Algal polysaccharides have aroused interest because of special properties that make them suitable for a variety of industrial purposes [1]. Algal polysaccharides can be used in industry as thickeners, stabilizers and emulsifiers [2]. *Porphyridium cruentum* and *Prasinococcus* sp. are good candidates for the production of valuable biochemical such as polysaccharides [3, 4].

P. purpureum is a unicellular red microalga from Rhodophyta class which has the potential to crop large amounts of proteins (28-39%), polysaccharides (40-57%) and lipids (9-14%) subsumed into dry algal mass [5]. *Prasinococcus* sp. is a non-motile green nanoalga from the class Prasinophyceae which has from a group of marine coccoid algae. The apical end of the cell wall region, named the ‘‘Golgi-decapore complex’’ (GDC), which plays an essential role in fabricating and releasing capsular fibrils from a unique sulfated and carboxylated polyanionic polysaccharide, here named “capsulan” [6].

The growth of algae is a function of many factors affect such as nutrients, pH, salinity, temperature and light [7]. Among these factors, light intensity that directly influences photosynthetic or respiratory mechanism is an important factor in defining optimal conditions for the culture [8]. The cells obtain their energy by metabolizing carbohydrate in light-dark cycle; light favors the accumulation of carbohydrate and in the absence of light [9]. Glyoxylate, a stimulator of carbon metabolism, was reported as a substance with the capability of inhibiting photorespiration and increasing photosynthesis in higher plants [10] and some cyanobacteria [11, 12]. An excess of carbon flux in algae, such as *Anabaena cylindrica* [13] and *Cyanospira capsulate* [14] occur in response to the addition of glyoxylate, which results in an intracellular accumulation of polysaccharide and a release of soluble extracellular polysaccharide [15].

This work is a first attempt to investigate sugar content of *Prasinococcus* sp. in comparison with the model strain of *P. cruentum*, as a potential polysaccharide producer, under two different light intensities with two different sodium

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glyoxylate monohydrate concentrations in F/2 medium and to determine the increase of exopolysaccharides (used as biopolymers) amount in the culture media. Furthermore, the locally isolated strain of *Prasinococcus* sp. was used in the experiments.

2. MATERIALS AND METHODS

2.1 Isolation, identification of *Prasinococcus* sp. and maintenance of microalgal strains.

Porphyridium cruentum EGEMACC 9 was obtained from Ege University Microalgae Culture collection (http://www.egemacc.com/), Izmir, Turkey. For *Prasinococcus* sp., seawater sample was collected by plankton net (20 µm mesh size) from the coast of Sıgacık-Teos in Turkey located geographically between 38°11'30.09" North latitude and 26°46'25.44" 29°42'25" East longitude. The sample was placed on ice in a cool container and arrived to the lab into 48 h. The isolation of the strain was done using serial dilution and the streaking plate method. The isolate was incubated at 25 °C at the light intensity of 30 µE $m^{-2}s^{-1}$ in 250 mL flasks for 14 days.

For light microscopy observations, the isolated strain of *Prasinococcus* sp. was examined for morphological features by using a Leica DMIL fluorescent microscope (Leica, Germany) light microscope with 63 x achromatic objective lens. Images were captured using Leica software (LAS). The morphological observations of *Prasinococcus sp.* and *P. cruentum* are shown in Figure 1.

The molecular identification of isolated strain *Prasinococcus* sp. was carried out by 18S rDNA sequencing. The culture was grown in a tube for 1-2 weeks and the cells were recovered by centrifugation at 3500 *g* for 5 min. DNA was isolated using a Zymo Research Fungal/Bacterial DNA MiniPrep™ (USA) and stored at -20 °C. The gene of interest was the nuclear small subunit (18S) rRNA. The primers (For; 5'-TGGTTGATCCTGCCAGTAG-3', Rev; 5'-TGATCCTTCCGCAGGTTCAC-3') were used for polymerase chain reaction (PCR) amplification and the PCR conditions was used as reported by Shoup and Lewis [16]. Analysis was performed in BioRad MyCycler thermal cycler in Helix Amp™ Hypersense DNA polymerase (Nannohelix) by the primer pairs following the manufacturer's instructions. Products of cycle sequencing were cleaned and dye terminator sequencing was done using the primers and analyzed on an Applied Biosystems 3130XL with16-capillary array.

Both of cultures were monoalgal (non-axenic) and cultivated in F/2 medium [17] at 22±2 °C under continuous illumination (100 µE $m^{-2}s^{-1}$) in 2-L sterile bottle for 15 days. For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation (1160 *g*, 3 min) and the supernatant was removed. The collected cells were transferred, incubated aseptically in 250 mL flasks containing 100 mL of F/2 medium under the light intensity of 40 µE $m^{-2}s^{-1}$with the agitation rate of 120 rpm at 22±2 °C for four days. Four-day-old culture of cells was used as inoculum at 10% volume for all experiments.

2.2 Growth conditions of microalgal strains.

The microalgae strains were cultured in 1000 mL glass bottles containing 900 mL of F/2 medium at the air flow rate of 1 vvm under the temperature of 22±2 °C at two different light intensities (10 µE $m^{-2}s^{-1}$ and 50 µE $m^{-2}s^{-1}$) with two different concentrations of sodium glyoxylate monohydrate (control, 0.25, and 1.25 mM $C_2HNaO_3.H_2O$) for 14 days. Illumination was provided both by LED downlight lamp (Cata 10 W CT-5254) and standard cool white fluorescent lamps (18 W) from the top and one side of the of bottles, respectively. Irradiance was measured in the center of the flask with a quantum meter (Lambda L1-185). The temperature was measured in the center of the bottle with a thermocouple (Dixell-XT115). Rotometers (Özgül-air Co. Izmir -Turkey) were used to provide the desired air flow rate.

F/2 medium was prepared and then autoclaved. Agar medium was prepared by the addition of 1.5 % agar powder to liquid media prior to autoclaving. All components (Sigma-Aldrich Co.) were used analytical grade.

The data were analyzed using one-way analysis of variance (ANOVA). A probability value of $p \leq 0.05$ was considered to denote a statistically significant difference, and $p \leq 0.01$ was also used to show the power of the significance. Results were reported as mean values with standard deviations (n=3) unless otherwise indicated.

2.3 Experimental analysis.

Samples were taken at indicated times, and the following growth parameters were measured immediately; the cell concentration was determined by counting duplicate samples in a Neubauer hemocytometer. Dry weight was determined in duplicate by filtering a 5-ml culture sample through preweighed Whatman GF/C filters and weighed after drying the cell mass at 60 °C for overnight.

For the chlorophyll-a measurement, cells were harvested at 3500 *g* for 3 min. Chlorophyll in the cells was extracted with 100 % (v/v) methanol until the powder color became gray. The amount of chlorophyll-a was determined spectrophotometrically by measuring the light absorption at different wavelengths (665 and 750 nm) [18]. The chlorophyll content was calculated by using the following equation: Chlorophyll-a (mg/L)= 13.9 (A_{665}-A_{750}), where A_{665} and A_{750} correspond to the absorbance of methanol extracted supernatant at 665 nm and 750 nm wavelength with 1 cm pathway cuvette, 13.9 is the extraction coefficient.

The total carbohydrate was determined using the phenol–sulfuric acid method at the absorbance value of 490 nm [19]. Monosaccharide standard solutions were prepared as reported by Harazono *et al*. [20]. Monosaccarides were measured by LC-MS/MS using an Agilent 1200 Capillary HPLC (Polo alto, USA) system with an ODS capillary column (ACE 5 C18 150 x 0.5 mm 5 µm) delivering 20

µL/min of the eluent. Elution was performed by isocratic mode using mixture of acetonitrile-water. The column of LC system was connected to an electrospray ion source (ESI positive). All mass spectrometric measurements were performed on a HCT Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Spectrometric conditions such as the ion optics voltages, nebulizer gas and dry gas flow rates, and the dry gas temperature were controlled by EsquireControl software 6.1. MS/MS spectra were carried out by collision-induced dissociation (CID). Data analysis was carried out using Data Analysis software (v.3.4, Bruker Daltonics).

The specific growth rate (µ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = (\ln C_2 - \ln C_1)/dt$, where C_2 is the final cell concentration, C_1 is the initial cell concentration and dt is the time required for the increase in concentration from C_1 to C_2. Doubling time (DT) was also calculated as $DT = \ln 2/\mu$.

The data were analyzed using one-way analysis of variance (ANOVA). A probability value of $p \leq 0.05$ was considered to denote a statistically significant difference, and $p \leq 0.01$ was also used to show the power of the significance. Results were reported as mean values with standard deviations (n=2) unless otherwise indicated.

3. RESULTS AND DISCUSSION

3.1 Morphological properties of microalgal strains and molecular identification of *Prasinococcus* sp..

Prasinococcus sp. cells are green color, unicellular, free-floating and sub-spherical in shape. The length of the cells varied from 8 µm to 12 µm and the diameter ranged

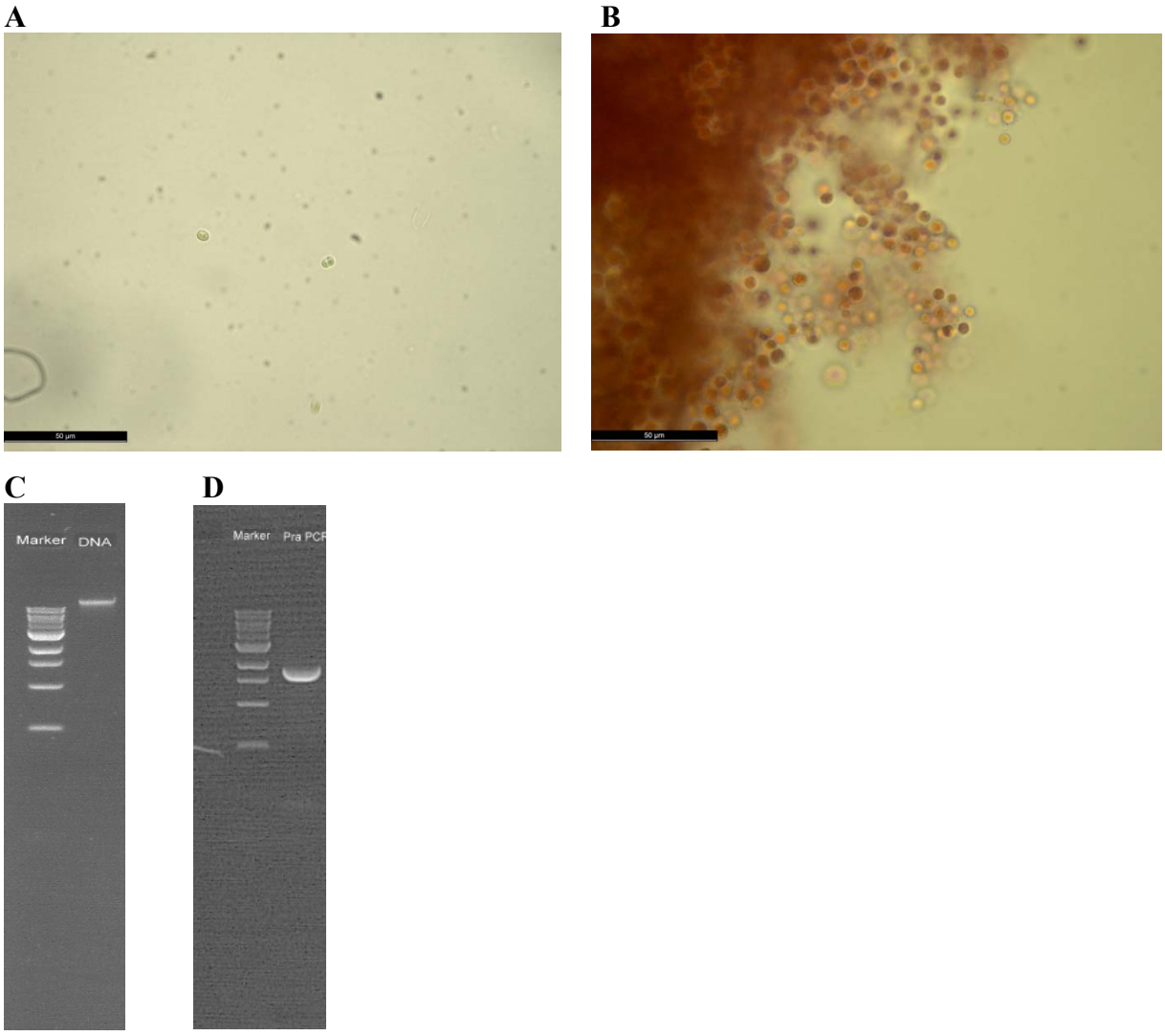

FIGURE 1 - Identification of *Prasinococcus* sp. EGEMACC 50 and *Porphyridium cruentum* EGEMACC 9: (A) light microscopy observation (63 x magnification) for *Prasinococcus* sp.; (B) light microscopy observation (63 x magnification) for *P. cruentum*; (C) DNA amplification of the 18s rDNA of *Prasinococcus* sp.. (D) PCR amplification of the 18s rDNA of *Prasinococcus* sp..

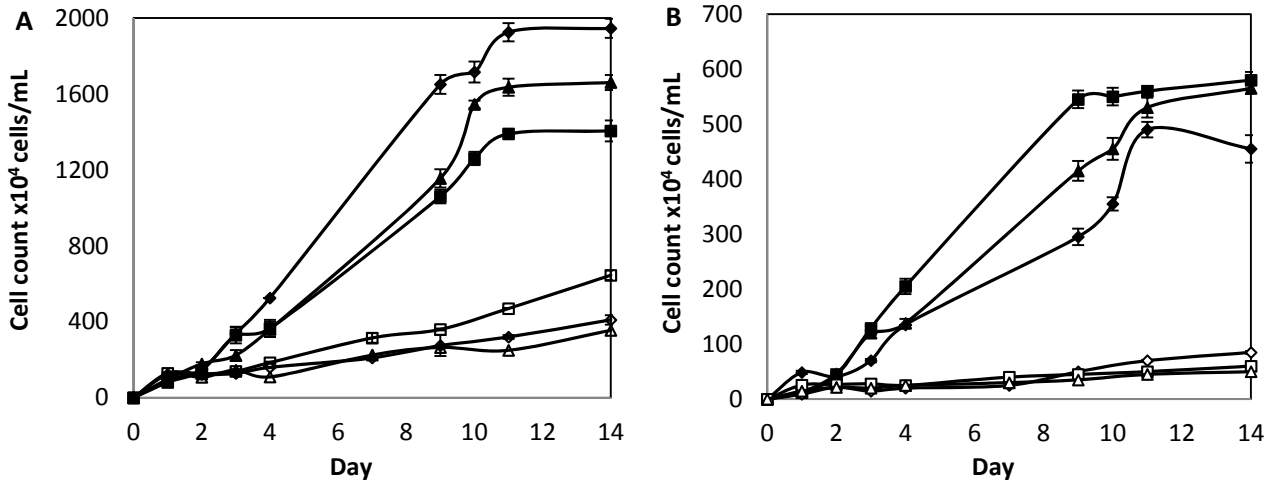

FIGURE 2 - Cell counts (x10^4 cells/mL) of microalgal strains under different light intensities with different concentrations of sodium glyoxylate monohydrate: (A) *Prasinococcus* sp., (B) *Porphyridium cruentum*. (♦) control under the light intensity of 50 µE m^{-2} s^{-1}, (■) 0.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 50 µE m^{-2} s^{-1}, (▲) 1.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 50 µE m^{-2} s^{-1}, (◊) control under the light intensity of 10 µE m^{-2} s^{-1}, (□) 0.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 10 µE m^{-2} s^{-1}, (Δ) 1.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 10 µE m^{-2} s^{-1}.

from 5 µm to 8 µm (Figure 1A). The separation of the Prasinophyceae from the rest of the green algae was first based on studies carried out with the light microscopy [21]. The unique structure features of *Prasinococcus sp.* are single parietal nucleus and single stellate chloroplast with prominent pyrenoid surrounded by small starch grains. Asexual reproduction by fragmentation of cell masses or by aplanospores (endospores) formed in sporangia differentiated from vegetative cells [22].

Porphyridium cells are spherical to obovoid red unicellular with stellate chloroplast and prominent central pyrenoid. The length of the cells varied from 5 µm to 10 µm and the diameter ranged from 7 µm to 10 µm in stationary phase. Cells solitary, but often grouped into irregular colonies with ill-defined mucilaginous matrix. Species distinguished by chloroplast color (Figure 1B).

For molecular identification, DNA and PCR products of *Prasinococcus* sp. were isolated and separated on 1 % agarose gel at 5 V/cm stained with SYBR safe and visualized with 312 nm UV light (Figure 1C and 1D).

The sequence data were submitted to the National Center for Biotechnology Information (NCBI) BLAST database for verification. BLAST search on NCBI-nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST) resulted in the highest similarity to *Prasinococcus* sp. (GenBank Acc. No.: JQ726705). The isolated & identified strain of *Prasinococcus* sp. was joined to Ege University Microalgae Culture Collection (EGEMACC) and coded with EGEMACC 50.

3.2 Evaluation of the effects of sodium glyoxylate and light intensity for *Prasinococcus* sp..

As seen in Figure 2A, the cell count of *Prasinococcus* sp. under high light intensity increased about 3 times compared to that of *Prasinococcus* cells under low light intensity. The maximum cell count, 1.945±0.05 x 10^7 cells/mL, was obtained in F/2 control medium under the light intensity of 50 µE m^{-2}s^{-1} and the lowest (3.55±0.15 x 10^6 cells/mL) was obtained with the addition of 1.25 mM $C_2HNaO_3.H_2O$ in F/2 medium under the light intensity of 10 µE m^{-2}s^{-1} ($p > 0.05$). Parallel results for the chlorophyll-a concentrations were monitored (Figure 3A). Chlorophyll-a concentration increased as the light intensity increased, while the higher level of glyoxylate treatments showed a declining trend on chlorophyll-a concentration for *Prasinococcus* cells.

It is known that the total polysaccharide is important when considering the engineering yield, whereas the reducing sugar is related to the microbial yield [23]. The total polysaccharide concentration was increased with increasing the glyoxylate level under the low light intensity, but no glyoxylate effect was observed on the total polysaccharide concentration under the high light intensity (Figure 4A). This might be due to the metabolic changes. Colony formation was also observed for *Prasinococcus* cells. It is noteworthy to mention that morphological state affects the metabolic change and *vice versa* [24]. The maximum increase of total polysaccharide was obtained with the value of 1.11 ng/cell in F/2 control medium under the light intensity of 50 µE m^{-2}s^{-1} for *Prasinococcus* cells. As reported by Yang *et al*. [27], the influence of glyoxylate on the production of total polysaccharide for *Chlorella pyrenoidosa* showed a rectangular hyperbolic response and the maximum increase of total polysaccharide increased from 0.28 to 1.07 pg/cell at 40 µE m^{-2}s^{-1}. When monosaccharide composition of exopolysaccharide fraction for *Prasinococcus* sp. was taken into the consideration under the light intensity of 50 µE m^{-2}s^{-1}, the mild level of glyoxylate treatment (0.25 mM $C_2HNaO_3.H_2O$) stimulated the monosaccharide production, whereas high level (1.25 mM $C_2HNaO_3.H_2O$)

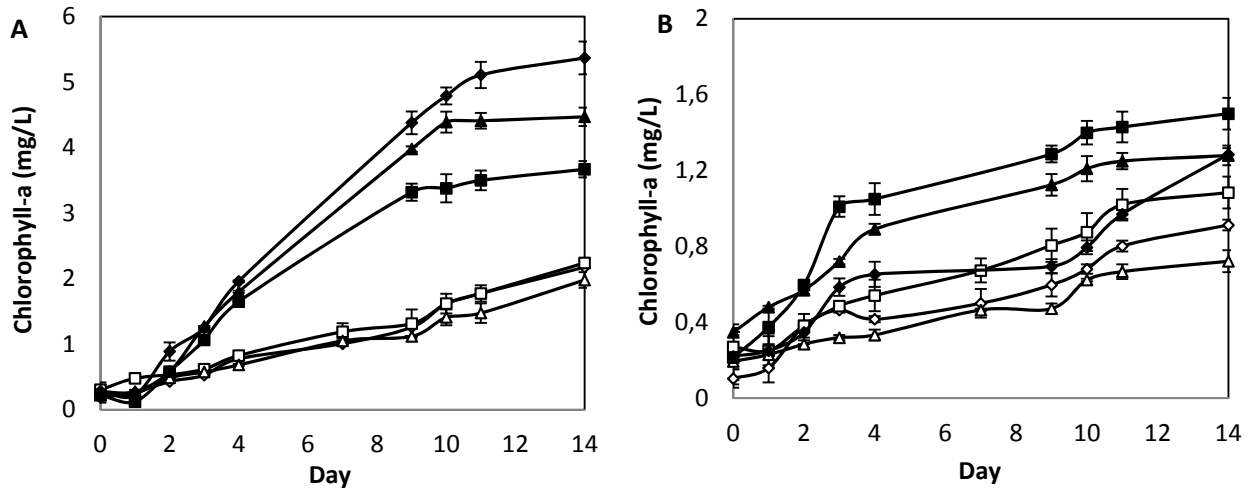

FIGURE 3 - Chloropyll-a concentrations (mg/L) of microalgal strains under different light intensities with different concentrations of sodium glyoxylate monohydrate: (A) *Prasinococcus* sp., (B) *Porphyridium cruentum*. (♦) control under the light intensity of 50 µE m^{-2} s^{-1}, (■) 0.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 50 µE m^{-2} s^{-1}, (▲) 1.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 50 µE m^{-2} s^{-1}, (◊) control under the light intensity of 10 µE m^{-2} s^{-1}, (□) 0.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 10 µE m^{-2} s^{-1}, (Δ) 1.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 10 µE m^{-2} s^{-1}.

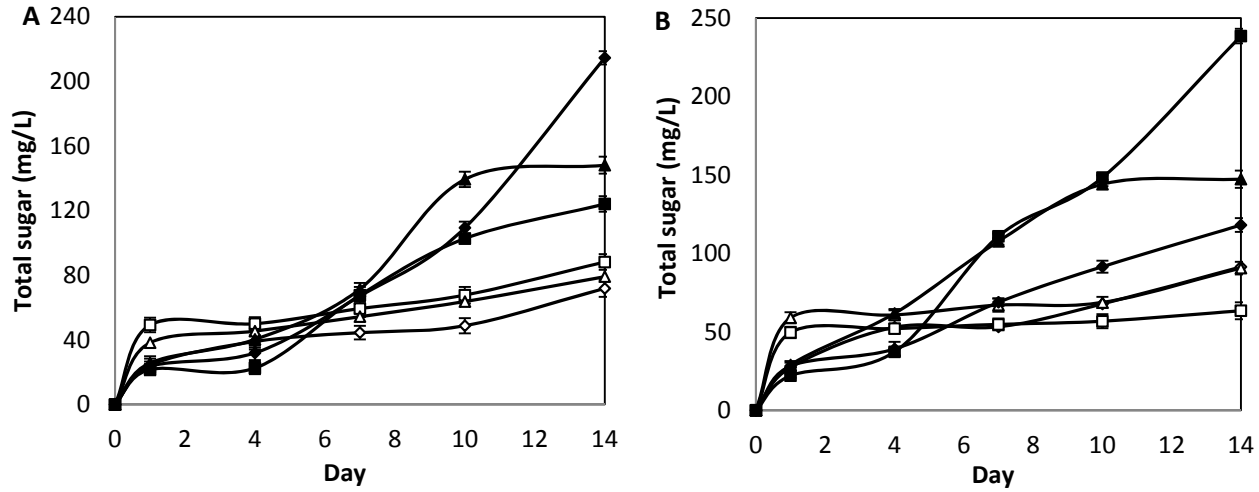

FIGURE 4 - Total sugar concentrations (mg/L) of microalgal strains under different light intensities with different concentrations of sodium glyoxylate monohydrate: (A) *Prasinococcus* sp., (B) *Porphyridium cruentum*. (♦) control under the light intensity of 50 µE m^{-2} s^{-1}, (■) 0.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 50 µE m^{-2} s^{-1}, (▲) 1.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 50 µE m^{-2} s^{-1}, (◊) control under the light intensity of 10 µE m^{-2} s^{-1}, (□) 0.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 10 µE m^{-2} s^{-1}, (Δ) 1.25 mM $C_2HNaO_3.H_2O$ under the light intensity of 10 µE m^{-2} s^{-1}.

TABLE 1 - Monosaccharide compositions of exopolysaccharides fractions for *Prasinococcus* sp. and *P. cruentum* under the light intensity of 50 µE m^{-2} s^{-1}.

	Prasinococcus sp.			*Porphyridium cruentum*		
Monosaccarides (mg/L)	Control	0.25 mM $C_2HNaO_3.H_2O$	1.25 mM $C_2HNaO_3.H_2O$	Control	0.25 mM $C_2HNaO_3.H_2O$	1.25 mM $C_2HNaO_3.H_2O$
Arabinose + Xylose	1.587	2.571	0.654	2.336	3.717	3.402
Galactose	0.721	1.461	0.474	2.470	5.126	5.601
Glucose	0.138	0.444	0.075	0.613	5.996	8.047
N-acetylgalactosamine	0.001	0.002	N/A	0.001	0.018	0.019
N-acetylglucosamine	0.001	0.017	N/A	0.002	0.056	0.018
Mannose	1.905	1.635	1.729	2.074	3.244	6.041
Rhamnose	0.685	1.467	0.449	0.694	1.224	1.489

TABLE 2 - Results of obtaining kinetic parameters for *Prasinococcus* sp. and *P. cruentum* under the light intensity of 50 µE m^{-2} s^{-1}.

	Prasinococcus sp.			*Porphyridium cruentum*		
Kinetic parameters	Control	0.25 mM $C_2HNaO_3.H_2O$	1.25 mM $C_2HNaO_3.H_2O$	Control	0.25 mM $C_2HNaO_3.H_2O$	1.25 mM $C_2HNaO_3.H_2O$
Cell Count (x10^4 cells/mL)	1945±50	1405±55	1660±40	455±25	580±15	565±10
Dry weight (mg/L)	0.40±0.05	0.38±0.03	0.12±0.06	0.15±0.04	0.39±0.08	0.39±0.07
Chlorophyll-a (mg/L)	5.37±0.25	3.67±0.125	4.47±0.14	1.29±0.03	1.5±0.08	1.28±0.05
Total sugar (mg/L)	214.6±4.4	124.09±4.6	148.12±5.5	118.1±4.4	238.53±5.4	147.3±4.0
Specific growth rate (µ; day^{-1})	0.236	0.220	0.218	0.172	0.298	0.287
Doubling time (day)	2.945	3.161	3.181	4.028	2.325	2.415
Cell Productivity (mg/L/day)	0.384	0.262	0.319	0.092	0.107	0.091

inhibited the production sharply, as shown in Table 1. Arabinose and xylose were the dominant monosaccharides for *Prasinococcus* sp.

Glyoxylate is thought to be the main precursor and can be converted to oxalate by glycolate oxidase (GO), a peroxisomal enzyme, and by lactate dehydrogenase (LDH), a predominantly cytoplasmic enzyme [25, 26]. In this study, higher light intensity had a direct influence, whereas no effect was observed for glyoxylate treatments on the growth of *Prasinococcus* cells. Furthermore, high levels of glyoxylate in culture medium could inhibit growth directly, while mild levels could stimulate the exopolysaccharide production of *Prasinococcus* cells. The probable cause on the decrease of the formation of monosaccharide of exopolysaccharide fraction when increasing the level of glyoxylate is the saturation of the glycolate pathway.

Evaluation of the effects of sodium glyoxylate and light intensity for *P. cruentum*.

P. purpureum 337 cells produce three types of sulphated polysaccharides, namely intracellular polysaccharides, pericellular polysaccharides and hydro-solubilized polysaccharides with a molecular weight of 3-5 x 10^6 Da. This indicates that the raw material might be processed to excellent green biolubricants [28]. The hydro-solubilized polysaccharides are dissolved in culture medium and are called exopolysaccharides [30, 31].

As seen in Figure 2B, the maximum cell count (5.80±0.15 x 10^6 cells/mL) was obtained with the low level glyoxylate treatment (0.25 mM) under the light intensity of 50 µE $m^{-2}s^{-1}$ for *P. cruentum.* The cell concentration decreased by only 2.6% for high level glyoxylate treatment (1.25 mM) in comparison with the low level glyoxylate treatment (0.25 mM) under the light intensity of 50 µE $m^{-2}s^{-1}$. Similar light intensity effects were found for each microalgal strain. The maximum chlorophyll-a concentration of 1.50±0.08 mg/L was found with the low level glyoxylate treatment (0.25 mM) under the light intensity of 50 µE $m^{-2}s^{-1}$ for *P. cruentum*, which indicated that cells could adjust well to the growth conditions (Figure 3B). The maximum specific growth rates of 0.298 day^{-1}, which corresponded to the doubling time of 2.325 day, was obtained with the low level glyoxylate treatment (0.25 mM) under the light intensity of 50 µE $m^{-2}s^{-1}$ for *P. cruentum* (Table 2). Both high light intensity and mild level of glyoxylate stimulated faster growth for *P. cruentum.* Light quality was a key factor for controlling the growth and polysaccharide production. The growth rate of *P. cruentum* increased with enhanced of light intensity; however a light level beyond the saturation point inhibited the growth of microalgae [2].

As seen in Figure 4B, the total sugar concentration of 238.53±1.5 mg/L in the low level glyoxylate treatment (0.25 mM) increased about 2.1 times compared to the total polysaccharide concentration in the high level glyoxylate treatment (1.25 mM) under the light intensity of 50 µE $m^{-2}s^{-1}$ for *P. cruentum*. The maximum increase of total sugar was obtained with the value of 4.11 ng/cell under these conditions. On the other hand, total monosaccharide amount of exopolysaccharide fraction of *P. cruentum* increased with increasing the glyoxylate level. Glucose was the dominant monosaccharide, followed by mannose for *P. cruentum* (Table 1). In the present study, there was a strong indication that glyoxylate induced exopolysaccharide production for *P. cruentum*.

4. CONCLUSIONS

The maximum specific growth rates of 0.236 day^{-1} and 0.298 day^{-1}, which corresponded to the doubling times of 2.945 day and 2.325 day, were obtained in F/2 control medium and in the low level glyoxylate treatment (0.25 mM) under 50 µE $m^{-2}s^{-1}$ for *Prasinococcus* sp. and *P. cruentum,* respectively. When considering the notable differences between *Prasinococcus* sp. and *P. cruentum*, total monosaccharide amount (24.617 mg/L) of exopolysaccharide fraction of *P. cruentum* was 3.24 times higher than the total monosaccharide amount of *Prasinococcus* sp.. This might be due to the existing more polysaccharide in the cell wall of *Prasinococcus* sp.. It is also important to underline that higher light intensity had a direct influence, whereas no effect was observed for glyoxylate treatments on the growth of *Prasinococcus* cells. Additionally, both high light intensity and mild level of glyoxylate stimulated faster growth for *P. cruentum*.

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The authors have declared no conflict of interest.

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