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Evaluation of the potential for possible bioethanol production by native Persian Gulf isolates, *Picochlorum* sp. D8 and *Chlorella* sp. S4, in different culture scales

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Abstract

In the wake of extensive fossil fuel use and CO_2 accumulation in the environment, biofuel production from microalgae has the potential to be more effective and leave less of an environmental footprint. Nutritional and environmental factors and their interactions affect the growth performance and biochemical constitution of different microalgae, as well as the behavior of microalgal cells in different culture scales. The present study evaluates the potential of two microalgae isolates, *Picochlorum* sp. D8, and *Chlorella* sp. S4, in different culture scales. Since high biomass and carbohydrate productivity were considered important factors in identifying these microalgae, an acid-thermal pretreatment was applied to measure the carbohydrate concentration. In addition, the carbohydrate composition of the selected microalgae was investigated using thin-layer chromatography (TLC). According to the observations, *Chlorella* sp. S4 exhibited the best dry biomass and carbohydrate productivity of 62 ± 6 mg/L/d and 19.16 ± 1.57 mg/L/d, respectively, in a 200 L indoor open raceway pond. *Picochlorum* sp. D8 achieved the highest biomass productivity of 26.24 ± 0.625 mg/L/d and carbohydrate productivity of 7.45 ± 0.53 mg/L/d in a 2 L Erlenmeyer flask. The TLC analysis detected glucose, galactose, and xylose as the main monosaccharides in *Chlorella* sp. S4 hydrolysate. The current study demonstrated *Chlorella* sp. S4's capacity to produce biomass on a large scale. The relatively high carbohydrate content of this microalga makes it a promising raw material for potentially producing bioethanol.

1. Introduction

Total energy demand has increased five times since 1950, and more than 80% of it is provided by fossil fuel compared to only 11% by biomass and 6.4% by nuclear energy (Jain 2019). The worldwide demand for biofuels as renewable alternative energy resources has increased due to the reduction of fossil fuels, climate change, and global warming (Kim et al. 2020, Lakatos et al. 2019). Bioethanol is considered an excellent substitute for petroleum oils (Khan et al. 2017). Since bioethanol is more efficient than conventional fuel and emits less CO_2 during production and usage than fossil fuel, it has a lower impact on the greenhouse effect and global warming (Kusmiyati et al. 2022). Different substrates such as edible sugar-based sources, lignocellulosic and algal biomass can produce first, second, and third-generation bioethanol (da

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Maia et al. 2020). In comparison to first and second–generation biofuels, microalgae exhibit promising potential for renewable fuel production due to several advantages (de Farias Silva and Bertucco 2016). They present great photosynthetic efficiency and growth rate, easy harvesting processes, and can be grown in extreme conditions (non-potable water and non-arable lands) in comparison with terrestrial plants (Chen et al. 2020, Choi et al. 2019). Algae require only three factors to grow: enough light, dissolved nutrients that are easily accessible, and CO_2 (Ganesan et al. 2020). The simple cell structure of microalgae, which is mostly composed of carbohydrates, proteins, and lipids is another unique characteristic of these organisms. The strain and environmental factors present during cultivation have a significant impact on the production of these different bioproducts (Sanghamitra et al. 2020). Some species of microalgae can store a lot of carbohydrates inside their cells. Carbohydrates are generally stored in the cell's inside (starch), inner (hemicellulose, cellulose), and outer layers of the cell wall (alginate, agar, pectin). To release the carbohydrate from the microalgae's cell walls, a pretreatment step (chemical, physical, biological, and their combination) is required to break down the cell wall (da Maia et al. 2020, Papachristou et al. 2020). Acids and alkalis are traditional chemicals that are easy, fast, and very effective. Acid offers higher yields (up to 100%) than alkali for the extraction of sugar from algal biomass (Choi et al. 2019). The maximum carbohydrate concentration of 252.84 mg/g has been reported for defatted biomass of *Nannochloropsis oculata* using 5.0% (v/v) H_2SO_4 at 121 $^{\circ}$C for 15 min (Fetyan et al. 2022).

Evaluation of the potential of microalgae strains that can grow in brackish or seawater is necessary for sustainable microalgae cultivation (Guccione et al. 2014). Moreover, the amount of carbohydrates in algal biomass displays a remarkable role in the production of bioethanol (Choi et al. 2019). Although it is comfortable to culture microalgae in controlled laboratory conditions, increasing their cultivation scale is critical to evaluate their possibilities for bioethanol production (Tan et al. 2020). Unfortunately, there have been a few reports on the comparison of the biomass and carbohydrate productivity of microalgae in different culture scales.

Accordingly, the principal aim of the recent study was to investigate the biomass and carbohydrate productivity of two native microalgae isolates, *Picochlorum* sp. D8 (Gen Bank ID MT066402), and *Chlorella* sp. S4 (Gen Bank ID MK587688) (previously collected from the southern part of the Persian Gulf of Iran (Olia et al. 2020, Olia et al. 2019)) in different culture scales. The superior microalga with more biomass and carbohydrate productivity was cultured in a flat-plate photobioreactor and open raceway pond. Subsequently, the carbohydrate composition of selected microalga was determined using thin-layer chromatography. The hydrolysate of the superior isolate can be used as a feedstock for possible bioethanol production (Olia et al. 2022a).

2. Materials and methods

2.1 Microalgal strain and growth medium

Picochlorum sp. D8 and *Chlorella* sp. S4 were the two isolates of microalgae applied in recent research (Olia et al. 2020, Olia et al. 2019). Their genome sequences are available in GenBank with the accession numbers MT066402, and MK587688, respectively. Two Microalgae were grown in Rudic's (RM) medium, which has a salinity of 35 g/L. One liter of the medium contains: 300 mg $NaNO_3$, 80 mg K_2HPO_4, 58.5 mg $CaCl_2.2H_2O$, 20 mg KH_2PO_4, 20 mg NaCl, 10 mg $MgSO_4.7H_2O$, 1.5 mg $MnSO_4.H_2O$, 0.26 mg $Co(NO_3)_2.6H_2O$, 0.08 mg $CuSO_4.5H_2O$, 0.3 mg H_3BO_3, 0.3 mg $(NH_4O_6Mo_7O_{24})$. H_2O, 17 mg $FeCl_3.6H_2O$, 0.1 mg $ZnSO_4.7H_2O$, 7.5 mg EDTA (Moaddab et al. 2016, Moazami et al. 2011).

2.2. Cultivation of microalgae in different scales

2.2.1 Cultivation in an Erlenmeyer flask and plastic PET carboys

The pre-culture of each microalga (initial density of 10^6 cells/mL) was inoculated into a 2 L Erlenmeyer flask and a 20 L plastic PET carboys containing 1.5 L and 15 L of Rudic's (RM) medium, respectively. Each microalga was cultured with an aeration rate of 0.2 vvm and illuminated with white fluorescent tubes (a light intensity of 3200 lux), a photoperiod of 16 hours of light and 8 hours of darkness, temperature (25 ± 2 °C), and pH (6.8–7.2) for 16 days (Olia et al. 2022b).

The superior microalga with more biomass and carbohydrate productivity was cultured in a flat-plate photobioreactor and open raceway pond.

2.2.2 Cultivation in a flat-plate photobioreactor

In a 20 L indoor flat-plate photobioreactor (50 cm wide, 91 cm height, and 8 cm diameter) with a working volume of 15 L, *Chlorella* sp. S4 cells (as a superior microalga) were inoculated (initial density of 10^6 cells/mL). The columns were maintained under similar conditions as previously mentioned for 16 days (Lee et al. 2018).

2.2.3 Cultivation in an open raceway pond

For large-scale evaluation of biomass production, *Chlorella* sp. S4 cultivation (as a superior microalga) was done in a 200 L indoor open raceway pond with an operating volume of 150 L. The pond was 8 m long and 1.5 m wide, giving a culture area of 1 m^2. *Chlorella* sp. S4 cells (concentration of 10% (v/v)) were inoculated and allowed to grow for 16 days under conditions of photoperiod (16 h light: 8 h dark), a light intensity of 9000 lux, temperature (25 ± 2 °C), and pH (6.8–7.2). The paddle wheel was installed within the open raceway for mixing and was driven by a motor. It was able to circulate water continuously at the speed of 13 rpm.

2.3. Growth measurement

A spectrophotometer (Milton Roy-20D) was applied to check daily microalgae growth in various culture scales at 620 nm. After cultivation, microalgal cells were separated by centrifugation (2794×g, 10 °C for 15 min). The pellets of two microalgae were washed three times in 100 mL of distilled water. After drying at 60 °C for 24 h, the dry cell weight of each microalga (g/L) was measured. pH changes were investigated during the period of cultivation of each microalga. The calculation of specific growth rate (μ) and the doubling time (day) (t_d) of microalgae was performed according to Eq. (1) and Eq. (2), respectively (Omori and Ikeda 1984, Tillich et al. 2014):

$$\mu = \frac{\ln\left(\frac{X_t}{X_0}\right)}{t - t_0} \quad (1)$$

$$t_d = \frac{\ln 2}{\mu} \quad (2)$$

With X_t and X_0, as the optical density at culture time t and t_0, respectively.

The biomass productivity, P (g/L/d) was measured by the following equation Eq. (3) (Liu et al. 2019):

$$P_{\text{biomass}} = \frac{DW_t - DW_0}{t} \quad (3)$$

Where t is the cultivation time, DW_t and DW_0, are dry biomass of microalgae (g/L) at time t and t_0, respectively.

2.4. Pretreatment of biomass

Approximately 0.5 g of dried powder from two isolates was mixed with 25 mL of 1.5% (v/v) H_2SO_4 and stirred for 10 min. Each sample underwent a 20 min autoclave at 121 °C. With 4 M NaOH, the pretreated samples were neutralized. The samples were then centrifuged (6654 ×g for 15 min at 4 °C) to obtain the supernatant for total carbohydrate content measurement (Harun and Danquah 2011, Miranda et al. 2012).

2.5. Determination of total carbohydrate and carbohydrate productivity

Anthrone colorimetric assay was applied to measure the extracted carbohydrate content of two microalgal biomass. Briefly, each sample (1 mL) was added to anthrone reagent (4 mL). Anthrone reagent was prepared by dissolving 2 g of anthrone powder in 1 liter of 72% (v/v) H_2SO_4. Then, the mixture incubation was done at 100 °C for 8 min, and the absorbance was recorded at 630 nm after cooling. The calibration curve was created using different concentrations of D (+) glucose (Hodge and Hofreiter 1962). The carbohydrate productivity, *P* (g/L/d) was measured by the following equation Eq. (4):

$$P_{\text{carbohydrate}}=\frac{C_t-C_0}{t} \quad (4)$$

Where *t* is the cultivation time, C_t and C_0, are the carbohydrate concentration (g/L) at time *t* and t_0, respectively.

2.6. Thin-layer chromatography

The carbohydrate composition of *Chlorella* sp. S4 hydrolysate was evaluated using thin-layer chromatography. For analysis, 20 × 20 cm silica gel plate (TLC Silica gel 60 F254) were dried at 70 °C for 3 h. The microalgal hydrolysate was diluted with deionized water at a dilution ratio of 1:5, and 1:10 (v/v). Then, 2 µL of each dilution was spotted on the silica gel plate. Solvent systems applied for separating the carbohydrate composition were a mixture of acetonitrile: water: acetic acid (91:12:6 (v/v/v)). The plate was then dried in a fume hood, and detection was done using 0.5 mL of Anisaldehyde in 9 mL of 95% (v/v) ethanol, 0.5 mL of 98% H_2SO_4(v/v), and 0.1 mL of acetic acid and heated at 100 °C for 3 h. Calibration was done using saccharides such as glucose, galactose, arabinose, mannose, rhamnose, xylose, and ribose with a concentration of 0.5% (w/v) (Schulze et al. 2017).

2.7. Statistical analysis

In the present study, all the experiments were done in triplicate and the results were shown as mean ± standard deviation (SD). GraphPad Prism version 5.0 (Graph-Pad Software, Inc., USA) was applied to evaluate the statistical analyses. *p*-value <0.05 was considered significant.

3. Results and Discussion

3.1 The growth performance of two microalgae

Some of the important factors that can affect microalgal biomass production are strain selection, cultivation conditions (e.g., light intensity, temperature, pH, etc.), and cultivation systems (open pond system or closed system) (Chisti 2016). For algal biotechnology applications, such as algal biofuel systems, dry biomass productivity, cell concentration, and composition are fundamental variables (Chioccioli et al. 2014). Since each algal cell is capable of photosynthesis, nutrients and CO_2 can be taken up directly from the environment and used in photosynthesis to create new cells and increase biomass (Bialon and Rath 2018). Algae are a good choice for CO_2 assimilation because of their fast growth rates, which can result in substantial carbon sequestration and help in mitigating global warming. According to scientists, 1 kg of dry biomass absorbs 1.88 kg of carbon dioxide (Iglina et al. 2022). The growth performance of *Chlorella* sp. S4 and *Picochlorum* sp. D8 in various culture scales is shown in Figure 1. No statistical differences in biomass productivity, dry biomass, and optical density were observed between two different culture scales (Erlenmeyer flask, and Plastic PET carboys) for *Picochlorum* sp. D8. The highest dry biomass of 0.42 ± 0.01 g/L, biomass productivity of 26.24 ± 0.625 mg/L/d, and optical density of 0.76 ± 0.03 were obtained in an Erlenmeyer flask (Fig. (1-1) and (1-3)). According to Figure (1-5), the pH variations ranged from 7.53 ± 0.104 to 8.2 ± 0.01.

Comparable observations were reported for *Picochlorum atomus* with dry biomass of 0.56 g/L and productivity of 27 mg/L/d in small-scale cultivation (von Alvensleben et al. 2013).

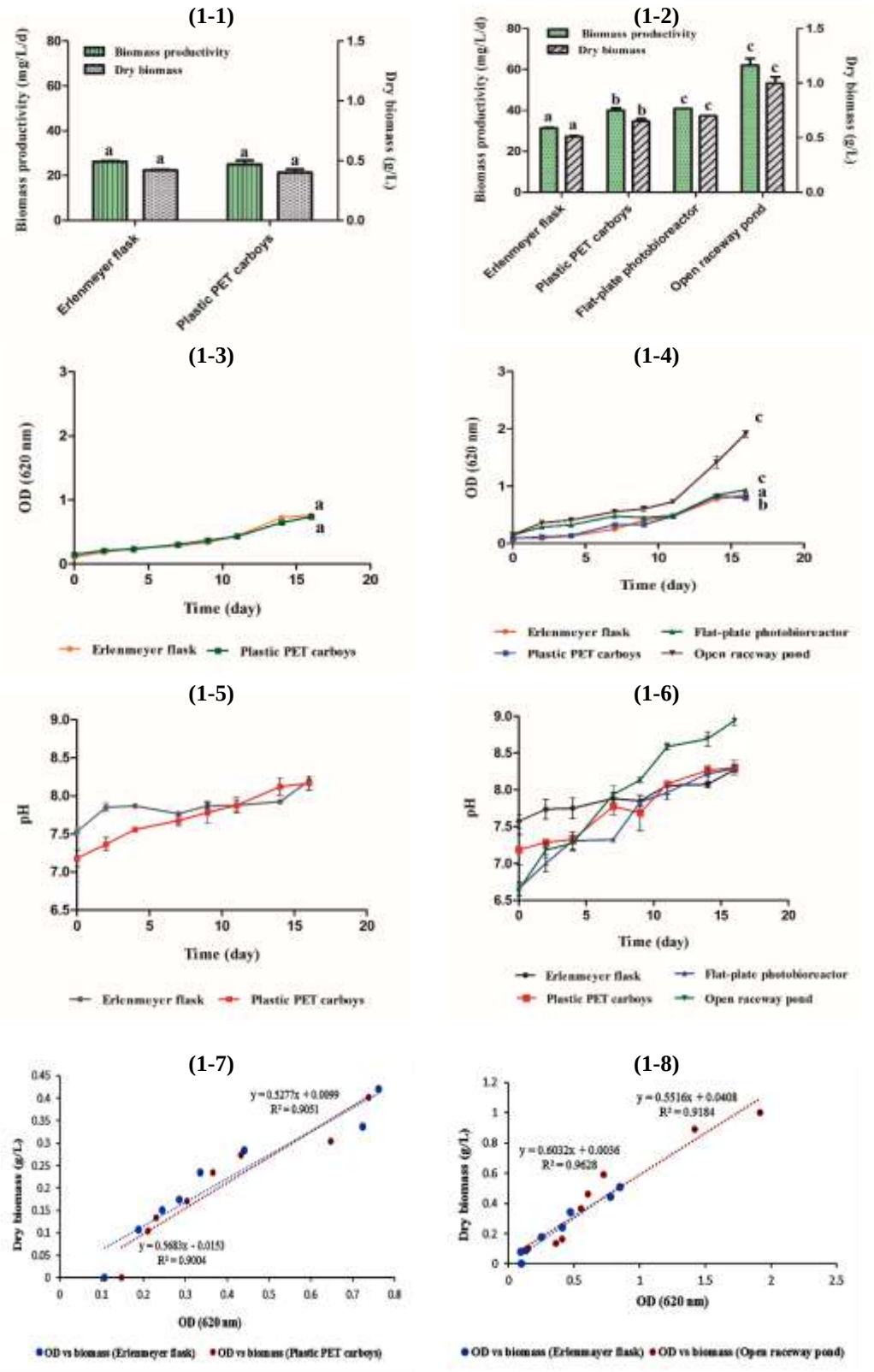

Figure 1: The growth performance according to pH changes in media during the algal growth phase in different culture scales. (1-1) and (1-2) show dry biomass and biomass productivity, (1-3) and (1-4) show optical density, [(1-5) and (1-6)] and [(1-7) and (1-8)] show the relationship between optical density and dry biomass weight for *Picochlorum* sp. D8 and *Chlorella* sp. S4, respectively. Different letters (a, b, and c) indicate significant differences between growth performance in various culture scales ($p < 0.05$). Data are shown as mean ± SD.

According to Fig. (1-2) and (1-4), *Chlorella* sp. S4 was able to reach its highest optical density of 1.91 ± 0.104, dry biomass of 1 ± 0.1 g/L, and biomass productivity of 62 ± 6 mg/L/d in an open raceway pond. Statistical differences were assumed significant at $p < 0.05$ ($p = 0.0001$). The changes in pH in an open raceway pond were in the range of 6.64 ± 0.14 - 8.94 ± 0.121, as presented in Figure (1-6).

One study reported a high biomass productivity of 16 mg/L/g and a biomass concentration of 0.23 g/L for *Chlorella vulgaris* in a plastic bag photobioreactor (Yousif et al. 2022). Sharma et al. (2016) reported *Chlorella minutissima* had a biomass concentration of 1.08 g/L and biomass productivity of 60.24 mg/L/d in a bubble column photobioreactor. They found *Chlorella* sp.1's highest biomass productivity was 53.16 mg/L/d and biomass concentration of 0.95 g/L (Sharma et al. 2016).

Results in this study showed a linear relationship between optical density and dry biomass weight (g/L) in the Erlenmeyer flask (R^2= 0.90) and plastic PET carboys (R^2= 0.90) for *Picochlorum* sp. D8, and in the Erlenmeyer flask (R^2= 0.96) and open raceways pond (R^2= 0.91) for *Chlorella* sp. S4, (Figs. (1-7) and (1-8)).

Table 1 illustrates the specific growth rate and doubling time of the two microalgae isolates in various culture scales. According to the results, *Chlorella* sp. S4 presented the maximum growth rate of 0.225 ± 0.039 (μ day^{-1}), and the lowest doubling time of 3.14 ± 0.61 (day) in an open raceway pond. The maximum growth rate of 0.164 ± 0.018 (μ day^{-1}) and lowest doubling time of 4.25 ± 0.51 (day) was obtained for *Picochlorum* sp. D8 in an Erlenmeyer flask.

Table 1. The specific growth rate (μ day^{-1}), and doubling time (day) of two microalgae isolates in different culture scales.

Isolates	Culture scales	Specific growth rate (μ day^{-1})	Doubling time (day)
Chlorella sp. S4	Erlenmeyer flask	0.166 ± 0.011	4.17 ± 0.3
	Plastic PET carboys	0.183 ± 0.007	3.40 ± 0.53
	Flat-plate photobioreactor	0.184 ± 0.008	4.09 ± 0.25
	Open raceway pond	0.225 ± 0.039	3.14 ± 0.61
Picochlorum sp. D8	Erlenmeyer flask	0.164 ± 0.018	4.25 ± 0.51
	Plastic PET carboys	0.133 ± 0.017	5.24 ± 0.64

Compared to *Chlorella* sp. S4, *Picochlorum* sp. D8 presented lower biomass productivity in an Erlenmeyer flask and Plastic PET carboys under the same conditions. The low biomass productivity might be due to photoinhibition, which indicates that *Picochlorum* sp. D8 does not receive enough light because of shading phenomena. Also, the photon flux density in different culture scales can directly affect microalgal growth (Richmond 2008). The low biomass concentration of microalga and the small size of the cells can affect biomass harvesting and make the process expensive and energy intensive. Higher biomass productivity and final dry biomass of *Chlorella* sp. S4 may be attributed to more compatibility of the isolate to culture conditions. The comparison of specific growth rate, and doubling time between two isolates showed faster growth of *Chlorella* sp. S4 than *Picochlorum* sp. D8. At $p < 0.05$ (p= 0.0001), statistical differences were considered significant.

The cost of biomass production is a major barrier at the moment. To address this problem, researchers must find species, particularly those with high biomass productivity, high density, and high resistance to a variety of severe conditions (Je and Yamaoka 2022). In the present study, although both microalgae isolates can grow in media with a high salt concentration (35 g/L), *Chlorella* sp. S4 demonstrated adequate growth characteristics that would make biomass production economically feasible and selected for further scaling.

3.2. Total carbohydrate content, and productivity of two microalgae isolates

During the photosynthesis process, microalgae can convert the absorbed carbon into carbohydrates that can be further applied for bioethanol production (Ighalo et al. 2022). The transition from nutrient sufficiency to limitation affects the composition of microalgae cells, which are predominantly composed of protein, carbohydrates, and lipids (Hanifzadeh et al. 2018). The comparison of carbohydrate content, and productivity of two microalgae in different culture scales is shown in Figure 2. The statistically significant differences ($p < 0.05$) in carbohydrate content and productivity were observed in different culture scales. The maximum carbohydrate productivity of 19.16 ± 1.57 mg/L/d and carbohydrate concentration of 0.3 ± 0.025 g/L were obtained for *Chlorella* sp. S4 in an open raceway pond (Fig. (2-1)). No statistically significant differences in carbohydrate content and productivity were found between the two various culture scales (Erlenmeyer flask and Plastic PET carboys) for *Picochlorum* sp. D8. In an Erlenmeyer flask, *Picochlorum* sp. D8 exhibited the most increased carbohydrate productivity of 7.45 ± 0.53 mg/L/d and carbohydrate content of 0.12 ± 0.008 g/L (Fig (2-2)).

The results revealed that the carbohydrate content and productivity of *Picochlorum* sp. D8 were lower than those obtained for *Chlorella* sp. S4 using acid-thermal treatment. The differences may be attributed to various physiological responses of microalgae to extrinsic and intrinsic factors during cultivation in different culture scales (Richmond 1999, 2000). It has been reported that carbohydrate productivity of 18.30 mg/L/d was obtained for *Halochlorella rubescens* in small-scale cultivation (Tan and Kassim 2020). Carbohydrate productivity of 22 mg/L/d was achieved for *Chlorella vulgaris* LEB-104 using a batch operation strategy (Wang et al. 2016). Another study reported that *Nannochloropsis gaditana* obtained maximum carbohydrate productivity of 25 mg/L/d in small-scale cultivation (Onay 2020).

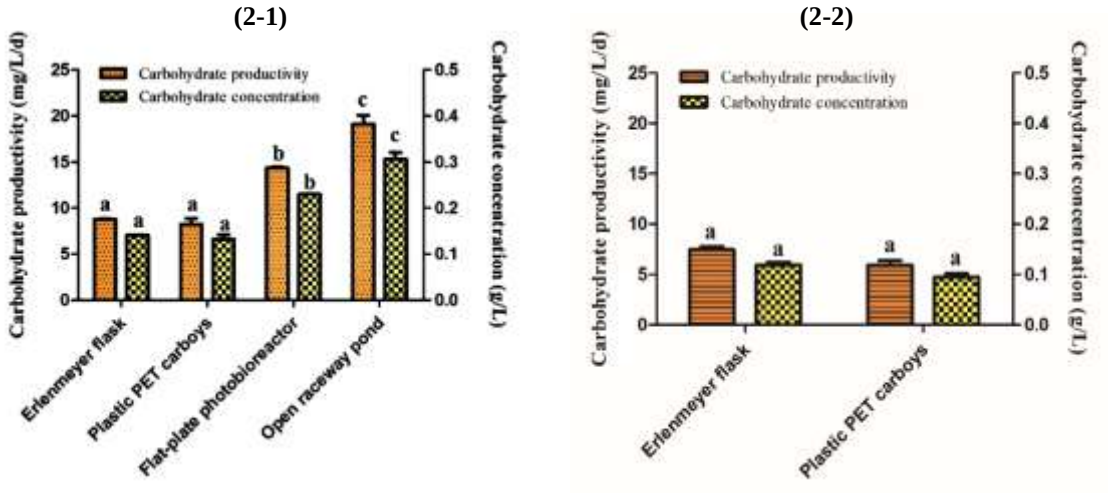

Figure 2: The carbohydrate concentration, and productivity of (2-1) *Chlorella* sp. S4, and (2-2) *Picochlorum* sp. D8 in various culture scales. Different letters (a, b, and c) indicate significant differences between carbohydrate concentration, and productivity in various culture scales ($p < 0.05$). Data are shown as mean ± SD.

3.3. Carbohydrate composition of *Chlorella* sp. S4

Separation of carbohydrate composition of *Chlorella* sp. S4 hydrolysate through the TLC technique revealed the presence of three different monosaccharides (Fig. 3). Comparison of R_f values of standards with those of monosaccharides in *Chlorella* sp. S4 hydrolysate (1:10 (v/v) dilution) exhibited the presence of glucose, galactose, and xylose with retention time (Rf) values of 0.27, 0.28, and 0.45, respectively. In another study, xylose rhamnose, galactose, and mannose were reported as the main monosaccharides of *Parachlorella kessleri*. Rhamnose, galactose, and mannose were detected as the main monosaccharides in the EPS of *Chlorella vulgaris* (Ciempiel et al. 2022). Glucose and rhamnose were reported as the dominant monosaccharides in *Chlorella vulgaris* (El-Naggar et al. 2020). The differences in the monosaccharides compositions of microalgal hydrolysate may be attributed to different nutritional and environmental conditions that might affect growth and carbohydrate composition

Figure 3: Thin layer chromatogram of *Chlorella* sp. S4 hydrolysate. Letters a, and i represent microalgal hydrolysate (a dilution of 1:5, and 1:10 (v/v)), and letters b to h represent standard monosaccharides such as galactose, arabinose, glucose, xylose, mannose, rhamnose, and ribose, respectively.

Based on the obtained results of the present study, an open raceway pond was a suitable system for *Chlorella* sp. S4 cultivation and carbohydrate production. High biomass and carbohydrate productivities were observed for the microalga in an open raceway pond. Based on TLC analysis, the presence of fermentable sugars such as glucose, and galactose in *Chlorella* sp. S4 hydrolysate strengthens the potential of this isolate for bioethanol production. In our other study, HPLC analysis confirmed the presence of glucose and galactose in amounts of 3.19% and 1.61% (w/w) of total carbohydrates in *Chlorella* sp. S4 hydrolysate. The fermentation parameters for bioethanol production from *Chlorella* sp. S4 hydrolysate was summarized in Table 2 (Olia et al. 2022a).

Table 2. Fermentation parameters for bioethanol production from *Chlorella* sp. S4 hydrolysate by *Saccharomyces cerevisiae* (Thermo-Tolerant) (Olia et al. 2022a).

Fermentation parameters	Amounts
Initial total carbohydrate concentration (% (w/v))	7.33
Consumed carbohydrate concentration ((% (w/v))	2.22
Initial glucose concentration ((% (w/v))	0.83
Fermentation time (h)	48
Ethanol concentration (% (v/v)	1.087
Ethanol yield (g/g consumed carbohydrate)	0.4
Ethanol productivity (g/L/h)	0.172

4. Conclusion

The comparison of biomass and carbohydrate productivity of two microalgae isolates in different culture scales suggests that an open raceway pond is suitable for *Chlorella* sp. S4 cultivation. The biomass and carbohydrate productivity were enhanced approximately two times using an open raceway pond compared to small-scale cultivation. The results indicated this isolate could be used for more biomass production on a larger cultivation scale. The presence of fermentable sugars (glucose and galactose) in *Chlorella* sp. S4 hydrolysate through TLC analysis can reveal the potential of this isolate for bioethanol production (published data). However, more studies are necessary to introduce *Chlorella* sp. S4 as a third-generation raw material for possible pilot-scale production of bioethanol.

Conflict of interest

All authors declare that they have no competing interests.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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