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Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E

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HIGHLIGHTS

- An indigenous *C. vulgaris* FSP-E isolate exhibits high potential as sugar producer.
- Microalgal growth is improved by properly adjusting light intensity and inoculum size.
- Nitrogen starvation is very effective in promoting carbohydrate accumulation.
- The carbohydrate profile of the microalga is suitable for bioethanol fermentation.

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ABSTRACT

In this study, three indigenous microalgae isolates were examined for their ability to produce carbohydrates. Among them, *Chlorella vulgaris* FSP-E displayed relatively high cell growth rate and carbohydrate content. The carbohydrate productivity of *C. vulgaris* FSP-E was further improved by using engineering strategies. The results show that using an appropriate light intensity and inoculum size could effectively promote cell growth and carbohydrate productivity. Nitrogen starvation triggered the accumulation of carbohydrates in the microalga, achieving a carbohydrate content of 51.3% after 4-day starvation. Under the optimal conditions, the highest biomass and carbohydrate productivity were 1.437 and 0.631 g L^{-1} d^{-1}, respectively. This performance is better than that reported in most related studies. Since glucose accounted for nearly 93% of the carbohydrates accumulated in *C. vulgaris* FSP-E, the microalga is an excellent feedstock for bioethanol fermentation.

1. Introduction

The world is facing the threat of an energy crisis due to the depletion of limited resources of fossil fuel, and it has been predicted that the oil reserves may run out by 2050 (Harun et al., 2010). Therefore, the identification of sustainable energy resources is attracting increasing attention, and bioethanol is one such promising fuel, as it can be made from non-toxic, biodegradable, and renewable resources, and its use can lead to a decrease in the emissions of harmful air pollutants (Ho et al., 2012; John et al., 2011). So far, most commercialized bioethanol has been produced from food crops (e.g., sugarcane or cassava). However, producing enough bioethanol from these feedstocks to satisfy existing demand would have a serious impact on food supplies, as well as rainforests and arable land. Therefore, new feedstocks that are sustainable and suitable for commercial fuel ethanol production are still urgently needed (Mussatto et al., 2010).

Microalgal biomass, seen as a third generation feedstock for biofuels (Chen et al., 2011), is suitable for bioethanol production because some species have a high carbohydrate content located in their cell walls, which are cellulose-enriched with no lignin and hemicelluloses contents, and in their cytoplasm, with starch as the main carbohydrate source (Rosenberg et al., 2008; Subhadra and Edwards, 2010). Both starch and cell wall polysaccharides (mainly cellulose) can be easily converted to fermentable sugars to produce ethanol through microbial fermentation (Wang et al., 2011). In contrast to lignocellulosic materials, saccharification of microalgae-based carbohydrates is much easier, without the need for energy or chemical consuming pretreatment processes, since

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these carbohydrates consist mainly of starch and lignin-free cellulose. Moreover, since microalgae-based carbohydrates contain mainly hexose, the problems associated with pentose fermentation in bioethanol production can essentially be eliminated. In addition to their high carbohydrate content and low seasonal limitations (Siaut et al., 2011), microalgae have relatively higher photoconversion efficiency with regard to fixing CO_2. Therefore, they can grow much faster than terrestrial plants and are able to effectively accumulate large quantities of valuable end-products (Subhadra and Edwards, 2010). The other advantage is that microalgae can be cultivated on non-arable land and with non-potable water, thereby avoiding the issues of competition for land use and freshwater.

Since bioethanol is a low-value product, to enhance the economic feasibility of microalgal-based bioethanol production it is necessary to isolate a suitable microalgal strain with high carbohydrate content along with developing strategies to improve both biomass productivity and overall carbohydrate productivity. The ideal process is that the microalgae are able to produce bioethanol at the highest productivity with the highest carbohydrate content. Unfortunately, this is not always achievable, because accumulation of carbohydrates in microalgae usually occurs under conditions of environmental stress (typically nutrient deficiency), which are often associated with relatively low biomass productivity, and thus low overall carbohydrate productivity (Dragone et al., 2011). As a result, strategies that could achieve the best combination of carbohydrate content and biomass production rate should be applied to optimize overall carbohydrate production.

In this study, microalgal strains able to produce high levels of carbohydrates for use in bioethanol production were isolated, and the feasibility of using these for carbohydrate production was characterized using batch cultures in terms of their ability to accumulate carbohydrates and their biomass productivity. The best carbohydrate-producing microalgal strain was selected, and its carbohydrate productivity was further optimized by employing a statistical experimental design, as well as engineering strategies to select the key parameters with regard to the type of nitrogen source, light intensity, inoculum size, and length of nitrogen starvation.

2. Methods

2.1. Isolation and identification of microalgae

Microalgae strains were isolated from a freshwater area located in southern Taiwan. For microalgae isolation, the freshwater sample was inoculated into BG11 medium (Stanier et al., 1971) and grown at 25 °C in an artificial light cabinet under illumination with fluorescent lamps at 60 μmol m^{-2} s^{-1}on a light/dark cycle of 16 h/8 h. The culture was spread onto BG11 agar plates, and colonies were subcultured into the same medium until a culture dominated by the pure strain was obtained.

The identity of the microalgal isolates was determined through plastid 23S rRNA gene analysis. The DNA of the microalgae was extracted using the QiagenDNeasy Plant Mini Kit (Qiagen, Valencia, CA). The plastid 23S rRNA gene was amplified by polymerase chain reaction (PCR) using two universalalgal primers reported previously (Sherwood and Presting, 2007). The DNA sequences were acquired using a DNA sequencer (ABI Prism 310; Applied Biosystems), and these were then assembled using the Fragment Assembly System program from the Wisconsin package version 9.1(Madison, WI: Genetics Computer Group). The sequences of the microalgal strains were compared against the plastid 23S rRNA gene sequences available from the GeneBank database, and the strains was identified as *Chlorella vulgaris* ESP-6, *C. vulgaris* FSP-E, *Chlamydomonas orbicularis* Tai-04.

2.2. Microalgal strains and growth medium

Microalgae strains were isolated from freshwater area located in southern Taiwan. The medium used for microalgae cultivation was basal medium consisting of (g/L): KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4{\cdot}7H_2O$, 1.00; $CaCl_2$, 0.0835; H_3BO_3, 0.1142; $FeSO_4{\cdot}7H_2O$, 0.0498; $ZnSO_4{\cdot}7H_2O$, 0.0882; $MnCl_2{\cdot}4H_2O$, 0.0144; MoO_3, 0.0071; $CuSO_4{\cdot}5H_2O$, 0.0157; $Co(NO3)_2{\cdot}6H_2O$, 0.0049; EDTA·2Na, 0.50. The microalgal strains were grown under a light intensity of approximately 60–680 μmol m^{-2} s^{-1} (illuminated by TL5 tungsten filament lamps; Philips Co., Taipei, Taiwan). The light intensity was measured by a LI-250 Light Meter with a LI-190SA pyranometer sensor (LI-COR, Inc., Lincoln, Nebraska, USA).

2.3. Operation of photobioreactor

The photobioreactor (PBR) used to perform the microalgal growth and CO_2 fixation experiments was a 1-L glass-made vessel illuminated with an external light source (14 W TL5 tungsten filament lamps; Philips Co., Taipei, Taiwan) mounted on both sides of the PBR. The microalgal strains were pre-cultured and inoculated into the photobioreactor with an inoculum size of 0.02–0.20 g L^{-1}. The PBR was operated at 28 °C, pH 6.2, and an agitation rate of 300 rpm. Serving as the sole carbon source, 2.0% CO_2 with an aeration rate of 0.2 vvm was continuously provided for the microalgae culture during cultivation. The liquid sample was collected from the sealed glass vessel with respect to time to determine microalgae cell concentration, pH, lipid/carbohydrate content, and residual concentration of nitrogen source.

2.4. Determination of microalgal cell concentration

The cell concentration of the culture in the PBR was determined regularly by measuring optical density at a wavelength of 688 nm (denoted as OD_{688}) using a UV/VIS spectrophotometer (model U-2001, Hitachi, Tokyo, Japan) after dilution with deionized water to give an absorbance range of 0.05–0.90. The dry cell weight (DCW) of the microalgae biomass was estimated by filtering 50 ml aliquots of culture through a cellulose acetate membrane filter (0.45 μm pore size, 47 mm in diameter). Each loaded filter was dried at 105 °C until the weight was invariant. The dry weight of the blank filter was subtracted from that of the loaded filter to obtain the dry cell weight of the microalgae. The OD_{688} values were converted to biomass concentration via appropriate calibration between OD_{688} and dry cell weight, and the conversion factor was thus determined (i.e., 1.0 OD_{688} equals approximately 0.20–0.89 g DCW L^{-1} depending on different strains).

2.5. Determination of growth kinetic parameters

The time-course profile of the biomass concentration (X; g L^{-1}) was used to calculate the specific growth rate (d^{-1}) by plotting the dry cell weight on a logarithmic scale versus time and calculating the slope. The biomass productivity (P, mg L^{-1} d^{-1}) was calculated according to Eq. (1).

$$P = \frac{\Delta X}{\Delta t} \tag{1}$$

where ΔX is the variation of biomass concentration (mg L^{-1}) within a cultivation time of Δt (d).

2.6. Experimental design examining the effect of inoculum size and light supply

To investigate the interaction effects of inoculum size and light intensity on the biomass productivity of the microalga, an on-face central composite design was used for the experiment. Thirteen batch experiments were conducted, as indicated in Table 1. The range of inoculum size and light intensity was 0.08–0.20 g L^{-1} (central value = 0.14 g L^{-1}) and 270–630 μmol m^{-2} s^{-1} (central value = 450 μmol m^{-2} s^{-1}), respectively. The experimental design for X_1 (light intensity) and X_2 (inoculation) is described in Table 1. Stepwise regression analysis was conducted by generating the following second-order polynomial equation (Eq. (2)):

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_{11} X_1^2 + \alpha_{22} X_2^2 + \alpha_{12} X_1 X_2 \quad (2)$$

where Y is the expected response value predicted from RSM, and α_i, α_j and α_{ij} are the parameters estimated from the regression results. Two performance indexes (X_1: light intensity, and X_2: inoculum size) were used to assess their influences on biomass productivity.

2.7. Determination of residual concentration of nitrogen sources

Nitrate concentration was determined according to the modified method reported in Collos et al. (1999). A liquid sample collected from the photobioreactor was filtered using a 0.22 μm pore size filter and then diluted 20-fold with DI water for each sample. The sample was collected and residual nitrate content was determined according to optical density at a wavelength of 220 nm (i.e., OD_{220}) using a UV/VIS spectrophotometer (model U-2001, Hitachi, Tokyo, Japan).

The NH_4^+ concentration of the culture medium was determined with an ammonia photometer (Hanna Instruments, model HI 93715). A liquid sample collected from the photobioreactor was filtered using a 0.22 μm pore size filter and then 10 mL was taken with 4 drops of specific reagents for each sample. After setting for 3.5 min, the sample was examined with an ammonia photometer.

The urea concentration of the culture medium was analyzed with the manual urease procedure proposed in Butler et al. (1996). Samples (1.0 mL each) were taken from the microalgal culture, and then centrifuged at 10000 rpm for 5 min. The supernatant (100 μL) was taken and then added to 0.5 mL urease solution, which was incubated in a 37 °C water bath for 10 min. After that, the reaction mixture was removed from the water bath, and the reaction was stopped by the addition of 1.0 mL Phenol nitroprusside (enzyme terminator) and 1.0 mL Alkaline hypochlorite (chromogenic agent). After setting at room temperature for 30 min, optical density of the solution was measured at a wavelength of 570 nm (i.e., OD_{570}) using a spectrophotometer (model U-2001, Hitachi, Tokyo, Japan).

2.8. Determination of the lipid composition

After appropriate cell growth, the microalgae cells were harvested from the culture broth by centrifugation (9000 rpm for 10 min). The cells were washed twice with deionized water, lyophilized, and weighed. The lipid composition was determined as fatty acid methyl esters (FAMEs) through the direct transesterification method proposed by Lepage and Roy (Su et al., 2007). The sample was analyzed using a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID). Samples were injected into a 30 m long capillary column (Type no. 260M143P, Thermo Fisher Scientific, Waltham, MA, USA) with an internal diameter of 0.32 mm. Helium was used as the carrier gas with a flow rate of 1.3 ml/min. The temperatures of the injector and detector were set at 250 and 280 °C, respectively. The oven temperature was initially set at 110 °C, increased from 150 to 180 °C at a rate of 10 °C/min, 180 to 220 °C at a rate of 1.5 °C/min, 220 to 260 °C at a rate of 30 °C/min, and held at 260 °C for 5 min.

2.9. Determination of the carbohydrate content and profile

The carbohydrate content and profile in the microalgae were determined using the modified quantitative saccharification (QS) method reported by the National Renewable Energy Laboratory (NREL), USA (Moxley and Zhang, 2007). A small amount of dry algal powder was added to 3 ml 72% (w/w) sulfuric acid and incubated for 30 min at 30ºC for the primary hydrolysis. The hydrolysate was then treated with 4% (w/w) sulfuric acid and incubated for 20 min at 121 °C as the secondary hydrolysis. The supernatant was neutralized and analyzed by high performance liquid chromatography equipped with a refraction index detector (RID-10A, Shimadzu, Japan) for sugar assays (Cheng and Chang, 2011).

2.10. Determination of crude protein content

For the estimation of crude protein, the total nitrogen content of microalgae was measured using an elemental analyzer (Elementarvario EL III). The crude protein concentration of the microalgae was estimated according to the correlation reported by Becker (Becker, 1994). The calibration between protein content and

Table 1
Experimental design for response surface analysis using light intensity and inoculation as the key operating parameters.

Run	Comment	Code value		Real value	
		X_1	X_2	X_1 Light intensity (μmol m^{-2} s^{-1})	X_2 Inoculation (g L^{-1})
1	FF	−1	−1	270	0.08
2	FF	−1	1	270	0.20
3	FF	1	−1	630	0.08
4	FF	1	1	630	0.20
5	Axial	−1	0	270	0.14
6	Axial	1	0	630	0.14
7	Axial	0	−1	450	0.08
8	Axial	0	1	450	0.20
9	Center-Ax	0	0	450	0.14
10	Center-Ax	0	0	450	0.14
11	Center-Ax	0	0	450	0.14
12	Center-Ax	0	0	450	0.14
13	Center-Ax	0	0	450	0.14

FF, fractional factorial design; Axial, axial points; Center-Ax, central points.

nitrogen content was estimated as "protein concentration = nitrogen content × 6.25".

3. Results and discussion

3.1. Isolation and identification of indigenous microalgae

Three microalgae strains were isolated from a freshwater area located in southern Taiwan. Two of the isolated strains (namely, ESP-6, and FSP-E) had the highest plastid 23S rRNA gene sequence relatedness with a green alga *Chlorellavulgaris* (GU939812) (99.95–99.99% sequence similarity) (data not shown). The genus *Chlorella* is placed in the member of the family *Chlorellaceae*, and is basically a genus of single-celled green algae, belonging to the phylum *Chlorophyta*, with a spherical shape and a cell size of about 2–10 μm, without flagella. Another strain (namely, Tai-04) had the highest plastid 23S rRNA gene sequence relatedness with a green alga *C. orbicularis* SAG 11–19 (AB411849) (99.95 sequence similarity) (data not shown). The genus *Chlamydomonas* is placed in the member of the family *Chlamydomonadaceae*, and is a genus of unicellular green algae with flagellates. Moreover, *C. orbicularis* has a spherical to short ellipsoidal shape, with a cell size of about 12–18 μm, and a smooth chloroplast surface.

3.2. Identification of biomass productivity, carbohydrate content, and carbohydrate productivity of the microalgal isolates

As third generation biofuels, microalgae have received considerable attention due to their high CO_2 fixation efficiency and high energy yields (Hirano et al., 1997; Ho et al., 2011). Selection of fast-growing and high-energy-content microalgal strains is of fundamental importance to the success of commercial applications of microalgae, in particular for low-value products like bioethanol (Mussatto et al., 2010). Screening of microalgal strains with high biomass productivity and carbohydrate content is thus crucial. The literature shows that the *Chlorella* sp. and *Chlamydomonas* sp. strains have been used as feedstock for bioethanol production (Hirano et al., 1997; Nguyen et al., 2009), and thus the indigenous isolates of *C. vulgaris* (ESP-E and FSP-E) and *C. orbicularis* (Tai-04) were examined for their biomass and carbohydrate productivity. Among the three indigenous isolates, *C. vulgaris* FSP-E displayed the highest biomass productivity (191.3 mg $L^{-1}d^{-1}$), while *C. orbicularis* Tai-04 gave the highest carbohydrate content (34.1%) and carbohydrate productivity (53.9 mg $L^{-1}d^{-1}$) (Table 2). These results show that before the employment of stress conditions to trigger carbohydrate accumulation, *C. orbicularis* Tai-04 seems to be a better carbohydrate producer in terms of both carbohydrate content and productivity. However, since accumulation of carbohydrates in microalgae usually requires stress conditions (Dragone et al., 2011), more investigations should be carried out (such as applying nitrogen starvation, and so on) to determine the best microalgal carbohydrate producer more accurately.

3.3. Effect of nitrogen starvation on carbohydrate accumulation of the indigenous microalgal isolates

To select the most suitable sugar-rich microalga as the feedstock for bioethanol production, nutrient limitation (in particular, nitrogen starvation) strategies were employed to stimulate the accumulation of carbohydrates in the isolated microalgal strains (Dragone et al., 2011). A number of studies (Ho et al., 2012; Siaut et al., 2011) have demonstrated that nitrogen-depletion leads to a sharp increase in the lipid or carbohydrate content of microalgae, because this forces them to transform protein or peptides to lipids or carbohydrates. As shown in Fig. 1, under the condition of nitrogen starvation for 11 days, the protein content of *C. vulgaris* FSP-E, *C. vulgaris* ESP-6, and *C. orbicularis* Tai-04 decreased from 60.38 to 21.09%, 47.78 to 22.84%, and 33.97 to 20.19%, respectively. In contrast, the carbohydrate content of *C. vulgaris* FSP-E, *C. vulgaris* ESP-6, and *C. orbicularis* Tai-04 increased from 12.16 to 54.13%, 18.16 to 48.59%, and 34.05 to 47.35%, respectively (Fig 1). Meanwhile, the lipid content also increased during nitrogen starvation, but the extent of this increase was less significant when compared to that for the carbohydrate (Fig. 1). These results indicate that the strategy of nitrogen starvation can dramatically promote the carbohydrate content of the three indigenous microalgal strains, especially for *C. vulgaris* FSP-E (increase by 4.4-fold).However, the nitrogen starvation strategy seems to give have less impact on carbohydrate accumulation in *C. orbicularis* Tai-04, which has the highest carbohydrate content before nitrogen starvation, leading to only a 39% increase. For *C. vulgaris* ESP-6, the increase in carbohydrate content triggered by nitrogen starvation was only 1.65-fold (Fig. 1). This suggests that the effectiveness of the nitrogen-starvation-driven carbohydrate increase in microalgae seems to be strain dependent.

Table 3 shows the effects of nitrogen starvation on biomass productivity, carbohydrate content, and carbohydrate productivity of the three indigenous strains. Unlike the results shown in Table 2, when under nitrogen starvation for 11 days, *C. vulgaris* FSP-E exhibits not only the highest biomass productivity (180 mg $L^{-1}d^{-1}$), but also the highest carbohydrate content (54.13%) and carbohydrate productivity (97.43 mg $L^{-1}d^{-1}$). The carbohydrate content of *C. vulgaris* FSP-E (54.13%; Table 3) is higher than most of the values reported in the literature (Ho et al., 2010; Sydney et al., 2010). Therefore, *C. vulgaris* FSP-E seems to have the highest potential for serving as a sugar feedstock for bioethanol fermentation, and was thus selected for further studies to improve its carbohydrate productivity.

3.4. Effects of nitrogen source on biomass productivity and cell composition of C. vulgaris FSP-E

Nitrogen is one of the most important nutrients for microorganisms to synthesize amino acids, proteins, and nucleic acids, and the type of nitrogen provided may have a major impact on the efficiency of cell growth and metabolite formation. It is known that microalgae can utilize nitrate, ammonium ions, and urea as nitrogen sources, but the metabolic processes of the nitrogen source could be different in each case. For instance, ammonium ions are readily utilized by microalgae, while urea is hydrolyzed to ammo-

Table 2
Biomass productivity, carbohydrate content, and carbohydrate productivity of indigenous microalgal isolates under batch growth with nearly 90% nitrogen source (i.e., nitrate) consumption. (Other conditions: light source, TL5 lamp; total light intensity, 60 μmol m^{-2} s^{-1}; CO_2 feeding concentration, 2.0%; CO_2 flow rate, 0.2 vvm.)

Microalgal strains	Cell size (μm)	Cultivation time (d)	Specific growth rate (d^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Carbohydrate content (%)	Carbohydrate productivity (mg L^{-1} d^{-1})
C. vulgaris FSP-E	3–5	10.5	0.99	191.3	12.16	23.26
C. vulgaris ESP-6	7–9	11.7	0.71	158.1	18.16	28.71
C. orbicularis Tai-04	15–20	15.8	0.41	158.2	34.05	53.87

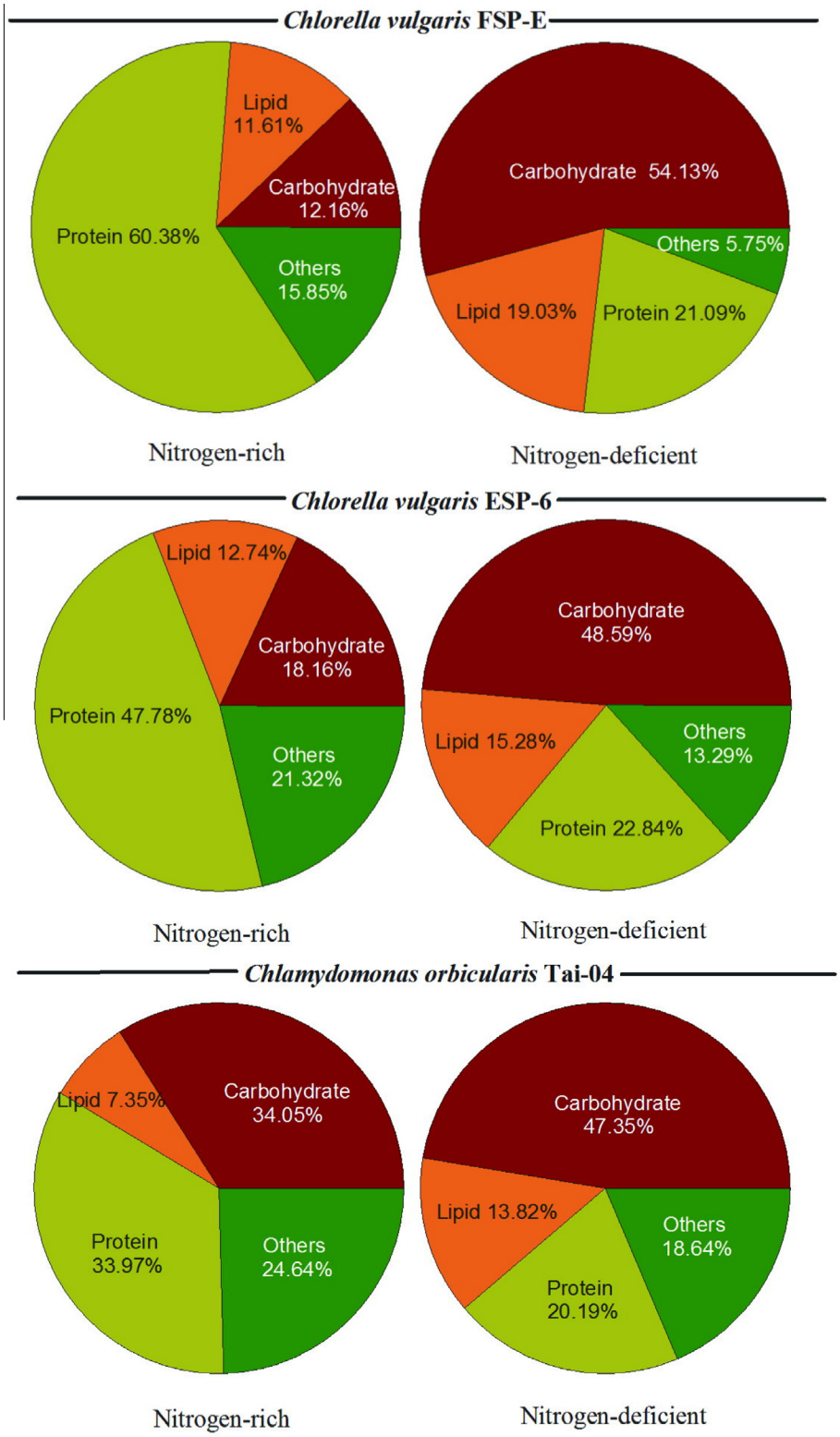

Fig. 1. Biochemical compositions of indigenous microalgal isolates cultivated in the nitrogen-rich and nitrogen-deficient condition. (Light source: TL5; total light intensity = 60 μmol m^{-2} s^{-1}; inoculation = 0.02 g L^{-1}; CO_2 aeration = 2.0%; CO_2 flow rate = 0.2 vvm.)

nium ions prior to being utilized by algae (Sze, 1998). Likewise, nitrate is also converted to ammonium ions by nitrate reductase and nitrite reductase before being utilized by algae (Sze, 1998). Therefore, when the culture medium contains both ammonium ions and nitrate, the former would be preferentially assimilated by microalgae, whereas the latter would often not be utilized until all the ammonium salts have been consumed (Becker, 1994; Sze, 1998). To reveal the effects of different nitrogen sources on microalgae growth and carbohydrate production, four commonly used nitrogen sources (i.e., KNO_3, NH_4Cl, NH_4NO_3, and urea) were used to cultivate *C. vulgaris* FSP-E. As shown in Table 4, different nitrogen sources seemed to have minimal effects on the chemical composi-

Table 3
Effects of nitrogen starvation on biomass productivity, carbohydrate content, and carbohydrate productivity of indigenous microalgal isolates under batch growth. Culture conditions: nitrogen (i.e., nitrate) starvation period, 11 days; light source, TL5 lamp; total light intensity, 60 μmol m^{-2} s^{-1}; CO_2 feeding concentration, 2.0%; CO_2 flow rate, 0.2 vvm.

Microalgal strains	Cultivation time (d)	Nitrogen starvation time (d)	Biomass productivity (mg L^{-1} d^{-1})	Carbohydrate content (%)	Carbohydrate productivity (mg L^{-1} d^{-1})
C. vulgaris FSP-E	24	11	180	54.13	97.43
C. vulgaris ESP-6	24	11	153	48.59	74.34
C. orbicularis Tai-04	28	11	163	47.35	77.18

Table 4
Biomass productivity, specific growth rate, biochemical composition, and price of indigenous *C. vulgaris* FSP-E cultivated under batch growth with different nitrogen sources. (Other conditions: light source, TL5 lamp; total light intensity, 60 μmol m^{-2} s^{-1}; CO_2 feeding concentration, 2.0%; CO_2 flow rate, 0.2 vvm.)

Nitrogen source	Cultivation time (d)	Nitrogen starvation time (d)	Specific growth rate (d^{-1})	Biomass production (g L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Carbohydrate content (%)	Glucose content (%)	Lipid content (%)	Protein content (%)	Others (%)	Price (dollar/kg N-source)	Price (dollar/kg DCW)
KNO_3	24	11	0.938	3.97	165.42	49.8 ± 0.2	45.5 ± 0.2	10.8 ± 0.3	24.8 ± 0.7	14.6 ± 0.2	36	11.5
NH_4Cl	24	11	0.983	3.82	159.12	43.9 ± 0.2	38.9 ± 0.1	11.7 ± 0.9	28.8 ± 0.9	15.6 ± 0.1	31	5.5
NH_4NO_3	24	11	1.149	3.88	161.67	44.9 ± 0.1	40.0 ± 0.4	9.9 ± 0.5	28.4 ± 0.1	16.7 ± 0.1	31	4.0
Urea	21	11	1.206	4.42	210.48	51.0 ± 0.7	46.6 ± 0.6	11.9 ± 0.7	24.3 ± 0.3	12.8 ± 1.1	23	2.0

DCW: dry cell weight.

tion of the microalgal strain after nitrogen depletion, whereas using urea as the nitrogen source resulted in the highest total carbohydrate and glucose content, with values of 51.0 ± 0.7% and 46.6 ± 0.6%, respectively. It has also been noted that urea is the most favorable nitrogen source for algal growth, because it leads to a minor change in the pH of the medium (Becker, 1994), which was also observed in this study. Therefore, the highest specific growth rate (1.206 d^{-1}), biomass production (4.42 g L^{-1}), and biomass productivity (210.48 mg $L^{-1}d^{-1}$) were obtained when using urea as the nitrogen source for the growth of *C. vulgaris* FSP-E. In addition, the cost of urea is the lowest among the four nitrogen sources examined, as shown in Table 4, being approximately $2.0 per kilogram of biomass, which is two-to five-fold lower than the other three nitrogen sources. For all these reasons, urea seems to be the best nitrogen source for our purposes, and was thus used for the growth of *C. vulgaris* FSP-E in the rest of the experiments in this study.

It is also noted in Table 4that glucose is the dominant sugar component in the microalgae-based carbohydrates, accounting for 88.6–91.4% of total carbohydrates. The rest of the sugar components (all less than 10% out of the total carbohydrates) were mainly xylose, galactose, arabinose, and rhamnose (data not shown). This result again highlights the advantage of using the microalgae-based carbohydrates as feedstock for bioethanol fermentation, due to the abundance of glucose.

3.5. Effects of combinations of inoculum size and light intensity on the growth of C. vulgaris FSP-E

A high biomass productivity is required for microalgal strains that can serve as the feedstock for bioethanol fermentation. The biomass productivity of microalgae is highly related to the growth rate, which is very sensitive to the culture conditions, such as irradiance, temperature, nutrients, CO_2 supply, and inoculum size. In particular, the irradiance supply strongly influences the cell growth for the phototrophic growth of microalgae (Ho et al., 2012). Therefore, an appropriate light intensity is of great importance to ensure a high growth rate of algae. The effects of light intensity on microalgal growth can be classified as taking place in several phases as the irradiance increases, such as during light limitation, light saturation, and light inhibition conditions (Xue et al., 2011). Moreover, Lopez-Elias et al., (2011) found that using a higher inoculum size could lead to higher biomass concentration when the microalgae *Tetraselmis chuii* was cultured on F/2 medium. Therefore, an optimal combination of the inoculum size and light intensity to enhance biomass production and productivity

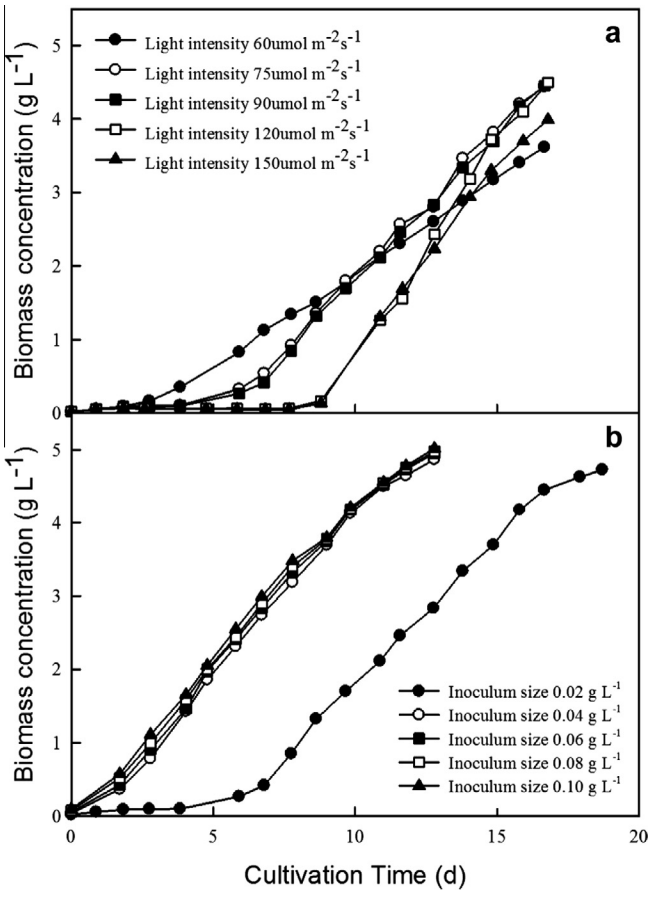

Fig. 2. The relationship between light intensity and inoculum size and its effects on biomass production of *C. vulgaris* FSP-E. (Light source: TL5;CO_2 aeration = 2.0%; CO_2 flow rate = 0.2 vvm.)

may need to be identified. For this purpose, *C. vulgaris* FSP-E was grown at different combinations of inoculum size and light intensity (other condition: urea as the nitrogen source in basal medium, aerated with 2% CO_2 at 0.2 vvm) to investigate the interacting effects of the two factors on microalgal growth.

Fig. 2a shows the cell growth exhibited a clear lag phase when the inoculum size was small (0.02 g L^{-1}) and the light intensity was over 75 μmol m^{-2} s^{-1}, resulting in lower biomass productivity. Moreover, higher light intensity was positively correlated to the higher growth rate during the exponential phase, but caused the extension of lag phase. On the other hand, when the inoculum size increased from 0.02 g L^{-1} to 0.04–0.10 g L^{-1} (under a fixed light intensity of 90 μmol m^{-2} s^{-1}), the lag phase shown in Fig. 2a disappeared, and the biomass productivity sharply increased from 252.8 to around 390 mg L^{-1} d^{-1} (Fig. 2b). Therefore, it is clear that appropriate combinations of light intensity and inoculum size could enhance biomass productivity, and thus the carbohydrate productivity can be improved when the carbohydrate content remains at a similar value.

To further determine the optimal combination of light intensity and inoculum size to promote cell growth, a response surface methodology (RSM) experimental design was used, taking biomass productivity as the target response. Based on the results of the thirteen experiments conducted according to the RSM design (Table 1), the estimated response surface model (in the form of a second-order regression equation) for biomass productivity is shown in Eq. (3).

$$\text{Biomass productivity}\,(\text{mg L}^{-1}\,\text{d}^{-1}) = -1.6557 + 0.0156X_1 - 1.5946X_2 + 0.0037X_1X_2 - 2.0008\times10^{-5}X_1^2 + 1.6820X_2^2 \quad (3)$$

where X_1 and X_2 indicate the coded value of light intensity and inoculum size, respectively. Simulations of the experimental data were quite successful with a regression coefficient (R^2) of up to 0.9985 (Eq. (3)). The high R^2 value means that the quadratic model is able to represent values in the experimental region in an accurate manner. However, the inoculum size did not present a statistically significant effect on biomass productivity, whereas a sufficiently high inoculum size was required to eliminate a long lag phase when using a high light intensity (Fig. 2a and b). Since the parameters of α_2, α_{12}, and α_{22} (see Eqs. (2) and (3)) have large p values (0.1876, 0.0071, and 0.6484, respectively), these parameters were eliminated in Eq. (3) to form a simplified equation (Eq. (4)).

$$\text{Biomass productivity}\,(\text{mg L}^{-1}\,\text{d}^{-1}) = -1.8299 + 0.0160X_1 - 1.9936\times10^{-5}X_1^2 \quad (4)$$

The modified regression model (Eq. (4)) also fitted the experimental data quite well, with a high R^2 value of 0.9925. This indicates that an inoculum size between 0.08 and 0.20 g/L did not have a statistically significant effect on biomass productivity when the light intensity applied was in the range of 270–630 μmol m^{-2} s^{-1}. The three-dimensional response surface is illustrated in Fig. 3 based on Eq. (4). The highest point on the response surface indicates the optimal operating conditions attaining the highest biomass productivity. According to Fig. 3, the biomass productivity tended to increase along with the light intensity, until it reached the highest point at around 450 μmol m^{-2} s^{-1}. However, further increasing illumination intensity to 630 μmol m^{-2} s^{-1}, resulted in a sharply drop in the response of biomass productivity, suggesting that excessive illumination would inhibit the cell growth, which is commonly recognized as the photo-inhibition effect (Melis, 1999). Therefore, the RSM analysis shows that the optimal environmental factors leading to a predicted optimum biomass productivity of 1.387 g L^{-1} d^{-1} was at a light intensity of 450 μmol m^{-2} s^{-1} and an inoculum size of 0.14 g L^{-1}. A confirmation experiment using the optimal light intensity and inoculum size identified by RSM analysis shows an actual biomass productivity of 1.408 g L^{-1} d^{-1}, which is within 5% of the predicted value. These results clearly indicate that illumination is a very important parameter for the enhancement of biomass productivity, while the inoculum size needs to be controlled at a sufficiently high value in response to the light intensity used to avoid the presence of a prolonged lag phase during microalgae growth.

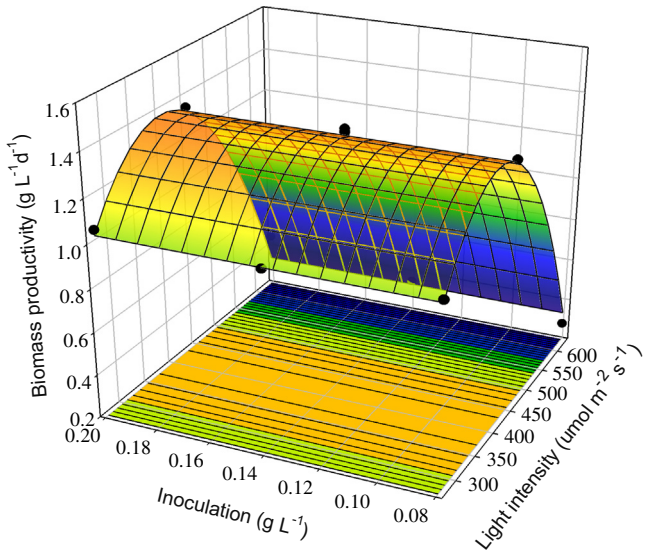

Fig. 3. Response surfaces showing the effects of light intensity and inoculation on biomass productivity of *C. vulgaris* FSP-E. (Light source: TL5; CO_2 aeration = 2.0%; CO_2 flow rate = 0.2 vvm.)

3.6. The effect of nitrogen starvation period on carbohydrate accumulation of C. vulgaris FSP-E

The results presented above indicate that the nitrogen starvation strategy is very effective in stimulating carbohydrate accumulation in *C. vulgaris* FSP-E. As demonstrated in Fig. 1, cultivation under nitrogen starvation leads to a sharp decrease in protein, along with significant increases in the lipid and carbohydrate contents. However, it is necessary to explore how long the microalgal cells should be cultivated under the nitrogen-starvation conditions in order to obtain the optimal carbohydrate production performance. In this work, *C. vulgaris* FSP-E was cultivated under the optimal conditions determined by the RSM design for around 6 days, during which the changes in urea concentration, carbohydrate content, and carbohydrate productivity were monitored as a function of time. As shown in Fig. 4, after urea concentration was nearly completely consumed (at approximately day 2), the carbohydrate and carbohydrate productivity increased from 14.76 to 51.32% and 0.193 to 0.631 g L^{-1} d^{-1}, respectively. In contrast, the protein content decreased from 59.09 to 19.63% (data not shown) during the nitrogen starvation period. Ho et al. (2012) and Siaut et al. (2011)noted that the condition of nitrogen starvation causes a reduction in protein content, along with an enhancement of energy-rich products, such as lipids and carbohydrates, and this is exactly what was observed in this study.

Fig. 4 shows that the maximum carbohydrate content of 51.32% occurred after four days cultivation (representing a nitrogen-starvation period of two days), and further increases in the nitrogen-starvation period did not lead to higher carbohydrate accumulation. Therefore, in this study, we observed a highly correlative relationship between the amount of storage material (carbohydrates) and the duration of the nitrogen starvation period. This finding suggests that the duration of nitrogen starvation could be an important

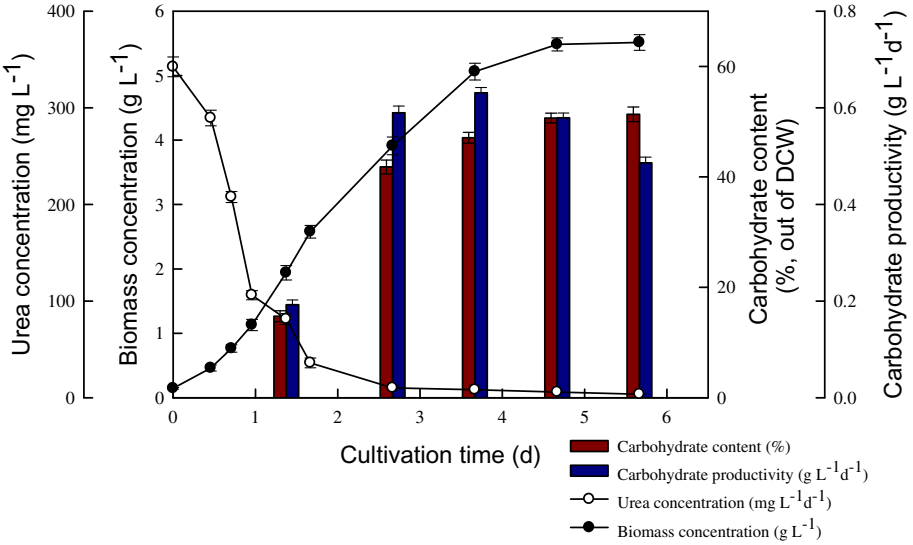

Fig. 4. Time-course profiles of urea concentration, carbohydrate content, and carbohydrate productivity during the growth of *C. vulgaris* FSP-E. (Light source: TL5; total light intensity = 450 μmol m^{-2} s^{-1}; inoculation = 0.14 g L^{-1}; CO_2 aeration = 2.0%; CO_2 flow rate = 0.2 vvm.)

Table 5
Comparison of the performance of biomass production, biomass productivity, and carbohydrate productivity of *C. vulgaris* FSP-E with the that obtained from other microalgal strains reported in the literature.

Strains	Operation mode	Cultivation time (d)	Biomass production (g L^{-1})	Biomass productivity (g $L^{-1}d^{-1}$)	Carbohydrate content (%)[a]	Carbohydrate productivity (g $L^{-1}d^{-1}$)[a]	References
C. vulgaris (CCAP 211/11B)	Batch	14	0.52	0.037	55.0	0.021	Illman et al. (2000)
C. vulgaris (P12)	Batch	N.D.	N.D.	0.485	41.0[b]	0.199[b]	Dragone et al. (2011)
C. reinhardtii UTEX 90	Fed-batch	4	2.40	0.507	60.0 (35.0[b])	0.304	Nguyen et al. (2009)
C. reinhardtii	Batch	3	1.45	0.484	53.1	0.257	Kim et al. (2006)
Tetraselmis subcordiformis	semi-CSTR	N.D.	N.D.	N.D.	N.D.	0.255[b]	Zheng et al. (2011)
Nannochloropsis sp.(MN41)	Batch	3.33	0.32	0.096	72.6	0.070	Cheng et al. (2008)
S. obliquus	Batch	12	2.63	0.280	40.0	0.112	Ho et al. (2010)
C. vulgaris	Batch	6	1.70	0.254	44.0	0.112	Liang et al. (2009)
C. vulgaris FSP-E	Batch	5.67	5.51	1.437[c]	51.3[c]	0.631[c]	(This study)

N.D.: not determined.
[a] Total carbohydrate.
[b] Starch only.
[c] Maximum value.

indicator and operating parameter to manipulate the accumulation of carbohydrates. As indicated in Table 5, the carbohydrate production performance in this study is better than that obtained in related reports. In particular, the carbohydrate productivity obtained in this study (a maximum value of 0.631 g L^{-1} d^{-1} for a two-day starvation period, or around four-day total cultivation; Fig. 4) is the highest among the reported values.

Under the environmental stresses, some microalgae may accumulate a large amount of carbohydrates, which are either located in the cell wall (mainly in the form of cellulose and soluble polysaccharides) (Domozych et al., 2012) and in the plastids (mainly in the form of starch) (Rismani-Yazdi et al., 2011). These glucose polymers produced via cellulose/starch are the predominant components in the cell walls and stored products of microalgae. Microalgae are considered a promising feedstock for bioethanol production because they have cellulose-based cell walls, with accumulated starch as the main carbohydrate source. Both starch and cell wall polysaccharides can be easily converted into fermentable sugars for subsequent bioethanol production via microbial fermentation (Wang et al., 2011). Fig. 5 provides detailed information regarding time-dependent variations of carbohydrate profiles during the period of nitrogen starvation. It shows that only the glucose content increased from 11.2% to 48.4%, but the content of the other monosaccharides remained nearly the same. This result is quite positive from the perspective of using the microalgal biomass as feedstock for bioethanol fermentation, since the main carbohydrate component of *C. vulgaris* FSP-E is glucose (up to 93% of the total carbohydrate). Due to its relatively high carbohydrate productivity and glucose content, *C. vulgaris* FSP-E seems to be a good candidate as a feedstock for bioethanol production. Earlier research (John et al., 2011) proposed that some specific microalgal species are very promising candidates as the feedstock for bioethanol. This work demonstrated that the indigenous microalgal isolate *C. vulgaris* FSP-E is apparently a promising one for this purpose, thereby deserving further development for commercial applications.

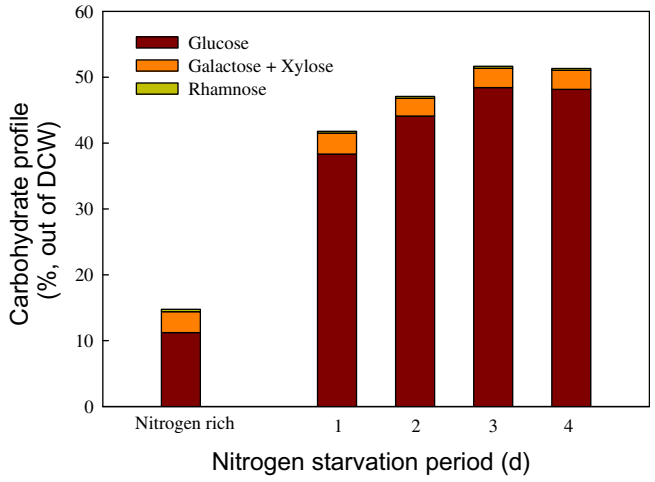

Fig. 5. The performance of carbohydrate profiles of *C. vulgaris* FSP-E cultivated under nitrogen starvation conditions for different periods. (Light source: TL5; total light intensity = 450 μmol m^{-2} s^{-1}; inoculation = 0.14 g L^{-1}; CO_2 aeration = 2.0%; CO_2 flow rate = 0.2 vvm.)

4. Conclusions

This study demonstrated an effective strategy to enhance both the carbohydrate content and biomass productivity of an indigenous microalga *C. vulgaris* FSP-E to serve as feedstock for bioethanol production. The carbohydrate productivity of *C. vulgaris* FSP-E was improved via selecting an appropriate nitrogen source (i.e., urea), light intensity, inoculum size, and nitrogen starvation periods. The highest biomass and carbohydrate productivity obtained in this work were 1.437 and 0.631 g L^{-1} d^{-1}, respectively, which are better than most of the reported values. The carbohydrate content reached 51.3%, and 93% of this was glucose, indicating a carbohydrate composition that is very suitable for bioethanol production.

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