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Effects of Nutrient Availability on the Biomass Production and CO_2 Fixation in a Flat Plate Photobioreactor

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ABSTRACT

A thermophilic cyanobacterium named *Thermosynechococcus* CL–1 (TCL–1) was cultivated in the flat plate photobioreactors under strong illumination with dense culture to examine the effects of nutrient concentrations on the biomass production, CO_2 fixation, and the potential as the feedstock for bioethanol production. The results show that concentrations of Na^+, NO_3^-, and $CaCl_2 \cdot 2H_2O$, should be decreased, but the $MgSO_4 \cdot 7H_2O$ concentrations should be enhanced to increase the biomass productivity or persistence of the growth. Most of the carbon contents in TCL–1 remain stable, about 40%, under various nutrient levels. In addition, the Su and Chu's medium was reorganized by picking up the nutrient concentrations resulted in the great biomass production. The adoption of the new medium for cultivating TCL–1 exhibits the great biomass productivity, CO_2 fixation rate, and glycogen productivity at 138.7, 221.5, and 75.9 mg L^{-1} h^{-1}, respectively, under 2,000 μE m^{-2} s^{-1} illumination in the 1.5 cm light path flat plate photobioreactors. The high biomass productivity, CO_2 fixation and glycogen productivity indicate the use of Su and Chu's medium exhibits high potential for applying TCL–1 in CO_2 fixation and bioethanol production potential in the flat plate phptobioreactors.

Keywords: Nutrient; Flat Plate Photobioreactor; *Thermosynechococcus*; CO_2 Fixation; Glycogen; Dense culture.

INTRODUCTION

The Climate Summit (COP21) of UN had been held in Paris of France on December 2015. The "Paris agreement" was achieved with voting unanimously by the representatives of 195 countries. It limited all countries to reduce greenhouse gas emissions and the target rising temperature is set no more 2°C than the temperature before the industrial revolution. Due to the accumulation of atmospheric CO_2 causes global warming in recent years, the techniques of CO_2 fixation by microalgae via photosynthesis has attracted the attention of researchers. The cultivation of microalgae to utilize CO_2 and to produce energy, food, chemicals and valuable nutrients has great potentials (Huang and Tan, 2014). With the advantage of not competing with corn corps, microalgae can be proposed as a sustainable fuel source (Mwangi *et al.*, 2015).

Cyanobacteria are prokaryotes that can fix inorganic carbon, harvest light energy, and store the carbon and light energy in the biomass. Scientists have been extracted by the high growth rate characteristic of cyanobacteria to examine applying cyanobacteria on CO_2 fixaiton, bioenergy production, and other applications (Parmar *et al.*, 2011; Sharma *et al.*, 2011; Klinthong *et al.*, 2015). No matter what the application technology is, the biomass production of the cyanobacteria is the fundamental technology (Parmar *et al.*, 2011). How to enhance the biomass productivity as well as target components under limited-resource such as nutrients, light, and production cost is worth to be investigated.

Basic design criteria of various type photobioreactors for bioenergy production are to enable the harvested light energy as large as possible (Kunjapur and Eldridge, 2010). Cultivation cyanobacteria with adequate biomass concentration in flat plate photobioreactors can enhance the light availability without the damage from strong illumination, and the improved biomass productivity can be obtained while the dense culture are used (Qiang and Richmond, 1996; Richmond, 2003). To prevent the growth are inhibited by the nutrient deficiency during illumination period, it is logistic to enhance the ingredient concentrations in the medium while the dense culture is selected for enhancing the biomass productivity. Unfortunately, little information is available to date that how the nutrients concentrations affect the cyanobacteria biomass production under dense culture in flat plate photobioreactors in a more

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economic concept, but actually this is the first and important step to ensure the biomass production or other application can be carried out successfully.

Bioethanol made from CO_2-fixed biomass can be considered partial carbon neutralization. Bioethanol can be obtained by the sugar in feedstocks after fermentation. The bioethanol production potential from feedstocks can be evaluated by the glucose and/or glycogen content in the biomass (Aikawa *et al.*, 2013). Glycogen, a branched polymer mainly composed of α-(1,4)-linked glucose with α-(1,6) branch linkage, can be synthesized as the major energy and carbon reservoir in cyanobacteria and can be degraded by α-amylase with glucoamylase (Yoo *et al.*, 2007). The glycogen synthesis in mesophilic cyanobacteria is regulated tightly by various environmental conditions, e.g., illumination intensities (Aikawa *et al.*, 2012), light period (Post, 1987), salinity (Guerra *et al.*, 2013), temperature ranges (Sakamoto and Bryant, 1998), and the nutrient concentrations such as carbon (Wu *et al.*, 2002), nitrogen (Hasunuma *et al.*, 2013; Xu *et al.*, 2013), and phosphorous (Sarma *et al.*, 2004). However, little is known about how the environmental factors affect the glycogen content in thermophilic cyanobacteria, especially for the fast-growing strain, *Thermosynechococcus* CL–1 (TCL–1). High carbohydrate productivities of TCL–1 have been examined in the previous study (Su *et al.*, 2013a, b); however, the glycogen content instead of the total carbohydrate should be carried out to exclude hard or non-fermentable pentose or hexose. In addition, the dense biomass concentration with optimum level about 3 g L^{-1} is the key to produce biomass, fix considerable quantities of carbon, and become great feedstock of bioethanol (Su *et al.*, 2013b).

The aim of this study is to evaluate the biomass production, CO_2 fixation rate and bioethanol production potential of the thermophilic cyanobacteria under dense culture. High illumination that for simulate the strong intensities outdoors was used to cultivate TCL–1, and the effects of nutrient concentrations on TCL–1 in flat plate photobioreactors were examined. In the end of this study, a new medium namely Su and Chu's medium for the thermophilic cyanobacteria culture is obtained via selecting the individual nutrient concentration that can obtain great biomass production, and the economic feasibility is also in the consideration.

MATERIAL AND METHODS

Strain and the Inoculation System

The thermophilic cyanobacteria, TCL–1, was isolated from the Chin-Lun hot spring in eastern Taiwan as described previously (Hsueh *et al.*, 2007). The strain was cultivated continuously in the 25 L acrylic flat plate photobioreactor (2.5 cm light path) as an inoculation pool to support dense culture in the examined experiments. According to the stoichiometry and the results in the previous studies (Su *et al.*, 2013a, b), three folds modified Fitzgerald medium (abbreviated as MF medium) were used to fed into the inoculation pool under 2 mL min^{-1} flow rate by a peristaltic pump (Cole Parmer, 7524–50), and the ingredients of the 3 folds MF medium (in mg L^{-1}) were as follows: 7,920 $NaHCO_3$, 2,040 Na_2CO_3, 1,488 $NaNO_3$, 117 K_2HPO_4, 225 $MgSO_4 \cdot 7H_2O$, 108 $CaCl_2 \cdot 2H_2O$, 174 $Na_2SiO_3 \cdot 9H_2O$, 18 $FeC_6H_5O_7$, 18 citric acid, 3 Na_2EDTA, and 3 mL L^{-1} Gaffron solution (Takeuchi *et al.*, 1992). All the chemicals used in the culture medium were of analytical grades. Continuous illumination about 200 μE m^{-2} s^{-1} was accomplished by fluorescent lamps (Philips, TL5–21W).

Culture System

The used 1.5 cm light path photobioreactors are as described previously (Su *et al.*, 2013b). The inoculated biomass was taken from the 25 L inoculation pool, about 3.5 L, followed by centrifugation under 3,420 g (KUBOTA, 5930), and was re-suspended in the prepared 50°C medium. The inoculated TCL–1 biomass was about 3 g L^{-1} in the photobioreactors. The individual nutrient concentrations for six batch experiments are revealed in Table 1. Other than

Table 1. The examined nutrients concentrations for cultivating TCL–1 in the flat plate photobioreactors.

Nutrient	$NaNO_3$	K_2HPO_4	$MgSO_4 \cdot 7H_2O$	$CaCl_2 \cdot 2H_2O$	Ferric citrate and Na_2EDTA		CO_3^{-2}	
					Ferric citrate	Na_2EDTA	$NaHCO_3$	Na_2CO_3
Concentration (mM)	116.7	4.50	6.09	5.58	0.49	0.054	377.1	77.0
examined in the present	58.4	2.25	3.04	2.79	0.24	0.027	188.6	38.5
study	29.2[(6)]	1.13[(6)]	1.52[(6)]	1.40[(6)]	0.12[(6)]	0.013[(6)]	94.3[(6)]	19.2[(6)]
	14.6	0.56	0.76	0.70	0.06	0.007	47.1	9.6
	7.3	0.28	0.38	0.35	0.03	0.003	23.6	4.8
	2.9	0.11	0.15	0.14	0.01	0.001	9.4	1.9
1X modified Fitzgerald medium[(2)]	5.8	0.23	0.30	0.28	0.02	0.003	–	0.19
BG-11[(3)]	17.6	0.23	0.30	0.28	0.02	0.003[(4)]	–	0.19
Su and Chu's medium[(5)]	2.9	1.13	6.09	0.14	0.12	0.013	94.3	19.2

– represents without addition in the medium.
[(2)] Modified Fitzgerald medium was described previously (Takeuchi, *et al.*, 1992).
[(3)] BG-11 medium ingredients were described previously (Stanier, *et al.*, 1971).
[(4)] $Na_2MgEDTA$ (disodium magnesium salt) rather than Na_2EDTA.
[(5)] The great medium compositions with 3 mL L^{-1} Gaffron solution are defined in the present study (Takeuchi *et al.*, 1992).
[(6)] The controlled experiment is carried out under 5 folds MF medium with 3 mL L^{-1} Gaffron solution (Takeuchi *et al.*, 1992).

the ingredients in Table 1, 290 mg L^{-1} Na_2SiO_3•$9H_2O$, 30 mg L^{-1} citric acid were also added in the examined medium. Nutrients other than the examined nutrient were fixed at 5 folds modified Fitzgerald medium level. For example, the medium concentrations other than $NaNO_3$ were fixed at five folds Fitzgerald medium dosages while the effects of $NaNO_3$ were examined. The prepared medium was heated at 50°C incubator (HIPOINT, FH-130) before used. Five folds MF medium with 3 mL L^{-1} Gaffron solution was adopted as the controlled group based on the pre-experiments that this concentration could produce the biomass acceptably.

To prevent the water in the medium vaporizing quickly from the photobioreactor by aeration, the humid air, increasing the humidity by pumping the air into a deionized water-filled serum bottle before purged into the photobioreactor, was aerated through a high density polyethylene tube at the bottom of the photobioreactor under 0.5 vvm flow rate. The experiments were carried out in 12 hours, and the continuous illumination (2,000 μE m^{-2} s^{-1} in the PAR range) by cool white light emitting diodes (LEDs; EDISON, ET-5050x-3F1W) array was used to simulate strong illumination outdoors. The temperature of the liquid inside the flat plate photobioreactor was measured by thermometers and was controlled at 50 ± 1°C.

After the biomass productivities in flat plate photobioreactors were obtained under each individual nutrient concentrations, the Su and Chu's medium, designed based on the consideration of the greatest biomass productivity (Fig. 1), and the ingredients are as follows (in mg L^{-1}): 7,920 $NaHCO_3$, 2,040 Na_2CO_3, 248 $NaNO_3$, 195 K_2HPO_4, 1,500 $MgSO_4$•$7H_2O$, 18 $CaCl_2$•$2H_2O$, 290 Na_2SiO_3•$9H_2O$, 30 ferric citrate ($FeC_6H_5O_7$), 30 citric acid, 5 Na_2EDTA, and 3 mL L^{-1} Gaffron solution (Takeuchi *et al.*, 1992). The ingredients of the Gaffron solution are as follows (in mg L^{-1}): 3,100 H_3BO_4, 2230 $MnSO_4$•$4H_2O$, 283 $ZnSO_4$•$7H_2O$, 88 $(NH_4)_6Mo_7O_{24}$•$4H_2O$, 146 $Co(NO_3)_2$•$4H_2O$, 33 Na_2WO_4•$2H_2O$, 119 KBr, 83 KI, 154 $Cd(NO_3)_2$•$4H_2O$, 198 $NiSO_4(NH_4)_2SO_4$•$6H_2O$, 20 $VOSO_4$•$2H_2O$, 474 $Al_2(SO_4)_3K_2SO_4$•$24H_2O$, and added the H_2SO_4 to 0.05 M.

Biomass Productivity, CO_2 Fixation, and Glycogen Productivity Determination

Adequate samples were taken from the photobioreactor per 2 hours. Two milliliters of the samples were filtered (by 0.45 μm glass fiber filters; Advantec, GC-50), dried (by a 105°C oven for one day), and weighted by an analytical balance (METTLER TOLEDO, MS-105) to determine the biomass concentration in the aqueous phase. The biomass productivity can be obtained by the regression results of biomass concentration and the culture period within 8 hours.

For analyzing the CO_2 fixation rate and bioethanol production potential, 100 mL samples were taken from the photobioreactor within 8 hours after inoculation, followed by centrifuged under 2790 g (BOECO, U-32R), washed with deionized water three times, dried with a freeze dryer (EYELA, FDU-1200), and preserved in a –20°C refrigerator (SANYO, SCF-P6G) before analyzed. To determine the carbon content in TCL–1, about 3.5 mg of the dried biomass was analyzed with an elemental analyzer (Elementar, Vario EL III). The CO_2 fixation rate is the product of the yield of carbon content in the biomass times 3.67 (from 44/12).

To evaluate the TCL–1 bioethanol production potential, glycogen contents were determined by the methods as described previously with some modification (Aikawa *et al.*, 2013). About 30 mg dried biomass was measured and placed into Pyrex tube. After 1 mL 30% (w/w) KOH solution was added into the Pyrex tube (total volume of 8 mL), the tube was sealed by the cap with a Teflon spacer inside and was heated in a boiling water bath for 30 minutes. After cooling, about 1.55 mL 4 M HCl was added slowly into the Pyrex tube for the pH adjustment before shaking. 0.5 mL sample in the Pyrex tube was taken and diluted with 2 mL deionized water, and was adjusted the pH value within 3.6 to 4.2 by 30% KOH and 4M HCl by a pH meter (SUNTEX, SP-2200). After pH adjustment, adequate amyloglucosidase (Sigma, EC# 3.2.1.3) was added and heated in a 60°C water bath for 30 minutes to digest the glycogen. The liquid was filtered by 0.2 μm PVDF filters, and separated by sugar analyzed column (Sigma, C610H) in the high pressure liquid chromatography, HPLC, system (Thermo fisher, P1000) equipped with a refractive index detector (Shodex, RI-101). Phosphoric acid (0.1%) was the mobile phase under 0.5 mL min^{-1} flow rate in HPLC. The glycogen content in TCL–1 was evaluated by the glucose contents analyzed by HPLC (Howarth *et al.*, 2010). The glycogen productivity is the product of the biomass productivity and the glycogen content.

RESULTS AND DISCUSSION

Effects of Nutrient Concentrations on the Biomass Productivity of TCL–1

The examined nutrients, $NaHCO_3$ with Na_2CO_3, K_2HPO_4, and $FeC_6H_5O_7$ concentrations under the examined level show similar trends (Figs. 1(A), 1(C), and 1(F)), i.e., over-adding and the insufficient dosage cause the decrease in the biomass productivity. Among all the examined nutrient levels, the biomass productivity decreases sharply while the $NaHCO_3$ with Na_2CO_3 are greater than 94.3 with 19.2 mM, respectively (Fig. 1(A)). The sharp decrease may result in the high sodium cation concentration of 0.53 M (0.377 + 2 × 0.077) from sodium bicarbonate and sodium carbonate, about 88.7% Na^+ of the sea water, in the culture medium. Since TCL–1 exhibits high homolog to the freshwater strain *Thermosynechococcus* elongatus BP–1 (Hsueh *et al.*, 2007; Sugita *et al.*, 2007), the high dosage of Na^+ may harm the growth of TCL–1. The point of view can be supported by the fact that the growth can be inhibited by sodium ionic stress in cyanobacteria (Batterton and Van Baalen, 1971). In addition, the sodium chloride concentration higher than 20 mM (20 mM Na^+) can decrease the growth rate of *Thermosynechococcus elongatus* TA–1 (Leu *et al.*, 2013). Although the Na^+ tolerance characteristics of TCL–1 is lower than that of *Spirulina* (Vonshak *et al.*, 1988), a moderate salinity tolerance of TCL–1 can be achieved (0.13 M Na^+, with 113.5 mM CO_3^{-2}) with high biomass productivity. No matter what the system is adopted, the $NaHCO_3$ and Na_2CO_3 dosage for thermophilic freshwater strain are suggested to

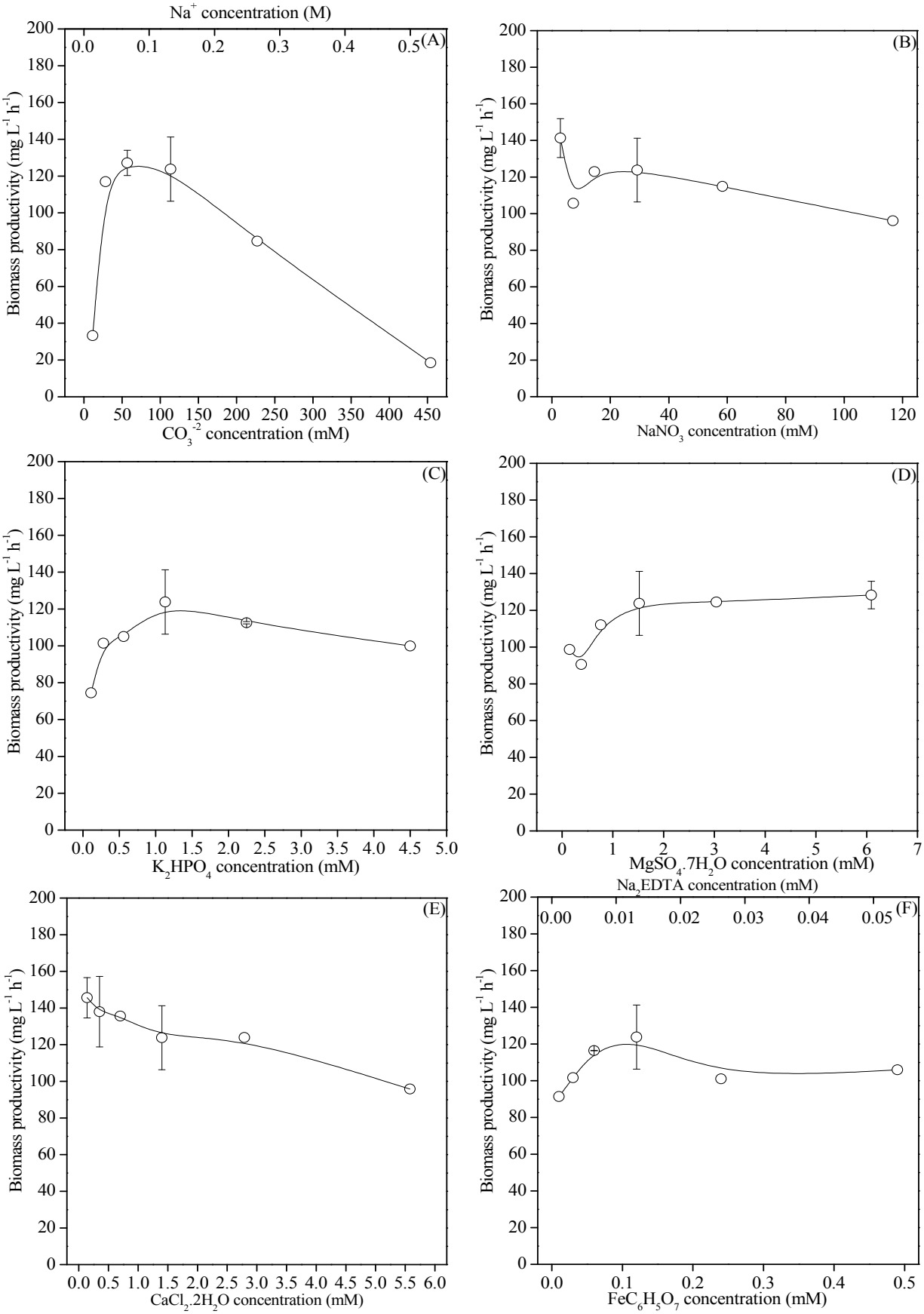

Fig. 1. Effects of (A) $NaHCO_3$ and Na_2CO_3, (B) $NaNO_3$, (C) K_2HPO_4, (D) $MgSO_4 \cdot 7H_2O$, (E) $CaCl_2 \cdot 2H_2O$, and (F) $FeC_6H_5O_7$ with Na_2EDTA on the biomass productivity.

maintain the sodium anion concentration lower than 0.15 M (25% Na^+ of the sea water) to prevent the reduction of photosynthetic rate under high salinity (Vonshak *et al.*, 1988).

The biomass productivity exhibits an increasing trend under lower NO_3^- level, especially under 2.9 mM NO_3^-, even the initial biomass density is 3 g L^{-1} (Fig. 1(B)). This indicates the NO_3^- affinity of TCL–1 can be high even under strong light intensity in the flat plate photobioreactor. The high NO_3^- affinity of TCL–1 is also discovered in the previous study (Su *et al.*, 2013a). The NO_3^- concentration in the tubular photobioreactor was only about 0.1 mM, but the biomass productivity of 1.5 g L^{-1} d^{-1} could be achieved with nitrogen content of 5.2% in the biomass under 4.18 mM NO_3^- d^{-1} fluxes. According to the result from the elemental analyzer, the nitrogen contents in TCL–1 drop from 6.99% to 5.18% under 29.2 mM to 2.9 mM NO_3^-, respectively, in the present study (data not shown). The increased biomass productivity under lower NO_3^- level may result from the degradation of the light–harvesting complex, chlorophyll or phycobilisome, and thus increase the light penetration in flat plate photobioreactor under dense culture. Besides, the addition of sodium nitrate is a heavy burden in the biomass production cost. For example, the Zarrouk or Schlösser medium that contain about 29 mM NO_3^- is often used to cultivate *Spirulina* (Zarrouk, 1966; Vonshak, 1982; Arata, 2013). Another culture medium, BG-11 medium, is a widely accepted medium for *Chroococcales* cultivation that also contains 17.6 mM NO_3^- (Stanier *et al.*, 1971). Although the modified BG-11 medium has been presented, the NO_3^- concentration only has decreased to 9.4 mM (Shukla and Kashyap, 2003). Besides, various NO_3^- concentrations have been examined in *Spirulina* and have been found that 3 mM NO_3^- can be adopted to cultivate *Spirulina* without the sacrifice of the biomass productivity under strong light illumination (700 μE m^{-2} s^{-1} illumination intensities) (Aikawa *et al.*, 2012). The lowest NO_3^- concentration, 2.9 mM NO_3^-, in this study is also similar in Aikawa's study (Aikawa *et al.*, 2012), and indicates the sodium nitrate concentration of 2.9 mM can be a great concentration for thermophilic cyanobacteria culture. The biomass productivity reaches to 141.3 mg L^{-1} h^{-1} under 2.9 mM NO_3^- in this study.

Unlike a sharp decrease of biomass productivity can be observed under high $NaHCO_3$ and Na_2CO_3 level, the biomass productivity under higher K_2HPO_4 does not change apparently (Fig. 1(C)). However, an obvious decrease of the biomass productivity under lower K_2HPO_4 concentrations (< 1.13 mM) is found. As the K_2HPO_4 concentration is lower than 0.11 mM, the biomass productivity is only 64.4% of that under 1.13 mM K_2HPO_4. This indicates the K_2HPO_4 dosage in the original MF medium, 0.23 mM, is insufficient to support 3 g L^{-1} TCL–1 biomass growth. Due to the important role of phosphorous in cell division, membrane construction, and energy transportation, the phosphorous deficiency leads to the failure of producing nucleic acids and proteins (Ji and Sherrell, 2008). Although the saturated PO_4^- concentration is extremely low that only about 0.4 μM in *Synechococcus* sp., the higher PO_4^- concentration results in the better specific growth rate can also be discovered (Timmermans *et al.*, 2005). The dramatic divergence may come from the different inoculation biomass concentration, and indicates that the concentration should be increased to a higher level, 1.13 mM, to support the dense culture of cyanobacteria.

Magnesium ion is a critical element in chlorophyll, and also affects the RNA synthesis, function enzymes, and influences bacterial permeability (Utkilen, 1982; Scanlan *et al.*, 1989). Magnesium deficiency can thus inhibit the cell division, and the cell becomes enlarge (Utkilen, 1982). The requirement of magnesium sulfate to maintain the TCL–1 growth in flat plate photobiorector is larger than the suggested dosage in MF medium and BG-11 medium (Fig. 1(D) and Supplement data 1). Insufficient of the magnesium sulfate also decrease the biomass productivity about 20% (under 6.09 and 0.15 mM magnesium sulfate). In addition, high magnesium sulfate concentration can ensure the biomass producing successfully during cultivation under strong illumination in flat plate photobioreactors. A distinguished divergence between the highest and the lowest magnesium sulfate concentration (a biomass concentration gap up to 0.7 g L^{-1} in the end of cultivation) can be observed, and suggests that the magnesium sulfate under dense culture with flat plate system should be increased to 3.04 mM at least. This requirement is three orders higher than that in Utkilen's study (Utkilen, 1982). Until the magnesium concentration was decreased to 5 μM that the growth of *Anacystis nidulans* started to be inhibited by the magnesium concentration (Utkilen, 1982). The divergence may come from the magnesium affinity of TCL–1 is low or from the dense biomass concentrations. While most of the cyanobacteria cultivation technology focuses on the lower biomass inoculation concentration, the deficient of the magnesium may occur easily accompanied with the increased biomass concentration, especially for high density biomass cultivation technology. In the present study, 6.09 mM $MgSO_4 \cdot 7H_2O$ are suggested to support the growth under 3 to 4.5 g L^{-1} biomass concentration of cyanobacteria in the flat plate photobioreactor.

Although the calcium is in the core (Mn_4O_5Ca) of Photosystem II to catalyze the light-driven oxidation of water, and the chloride is required for O_2 evolution activity in photosynthesis (Yocum, 2008; Cox *et al.*, 2013), the requirement of the calcium chloride is low for TCL–1 (Fig. 1(E)). In contrary, the lower the concentration of calcium chloride, the higher is the biomass productivity. Actually, calcium chloride dissolved in water can make the medium opaque, especially under higher calcium chloride concentration such as 5.58 mM. Therefore reducing the available light by adding too much calcium chloride under dense culture may strengthen the light shading effect followed by the reduction of the biomass productivity. Another possible reason may come from the higher chloride concentration. The salt-stress signal can be induced by Na^+ salt only or by both of Na^+ and Cl^- through different mechanism (Marin *et al.*, 2003). All of the examined conditions are under high Na^+ stress (about 0.15 M Na^+), especially for freshwater cyanobacteria strain, that the stress induced by both of Na^+ and Cl^- leads to the excessive stress in TCL–1. In a word, to prevent shading of light or the inhibition of chloride, the

addition of calcium chloride should be carefully, and the concentration are suggested to lower than 0.14 mM for freshwater strains.

Although Fe is abundant in nature, the Fe deficiency is easily discovered due to the poor solubility (Singh *et al.*, 2003). Iron deficiency results in the dramatic change in cellular physiology and ultrastructure, and also reducing the growth rate of cyanobacteria (Scanlan *et al.*, 1989; Singh *et al.*, 2003). In this study, the biomass productivities decrease as the ferric citrate concentrations are lower than 0.12 mM (Fig. 1(F)). Therefore, the ferric citrate concentration is suggested to maintain at least 0.12 mM.

It is really hard to exclude the effect of nutrient concentration on the light availability while the nutrient effects are discussed under high dosage for supporting dense culture of cyanobacteria. The nutrients in the medium not only contribute the essential elements for cyanobacteria growth but also participate in the absorption, scattering, and shading the light at specific light wavelength. Although many studies have concerned in the optical properties of how the light is absorbed or scattered by cyanobacteria or microalgae in photobioreactors as well as how the photic volume is influenced by the biomass concentration (Gitelson *et al.*, 1996; Merzlyak *et al.*, 2008), fewer researches are concern about how the nutrient concentrations affect the light availability. Even the scattered and absorbed light from individual cell dominates the optical characteristic in flat plate photobioreactors under ultrahigh biomass densities (i.e., the biomass concentration greater than 10 g L^{-1}), it is unusual to cultivate cyanobacteria strain other than *Spirulina* under such a high biomass inoculation system (Gitelson *et al.*, 1996; Hu *et al.*, 1996). Most of the cultivated biomass concentration is less than 5 g L^{-1}, and thus the optical properties of the culture medium may become a hidden factor to influence the light availability. There should be a balance between the nutrient supplement for sufficient element and the reduction of light waste in the medium composition.

Before the start of high dense culture experiments of thermophilic cyanobacteria in flat plate photobioreactors, we were suffered from the selection of nutrient concentration to maintain the growth of cyanobacteria in such a system as the high biomass productivity in a more economic concept were considered. Before the use of 5 folds MF medium in a previous study (Su *et al.*, 2013b), we have tried to cultivate TCL–1 under some medium folds, e.g., 1, 5, and 10 folds MF medium, but only 5 folds MF mediumcan ensure the biomass productivity to be acceptable (data not shown). However, there was still a significant drawback that the costly medium may restrict the post–application. After the comparison of the medium concentration with BG-11 medium (Stanier *et al.*, 1971), we discovered that most of the nutrient concentrations in MF and BG-11 were similar, but the one fold MF medium couldn't result in acceptable biomass productivity. These reasons enforce us to construct a new medium, Su and Chu's medium, to overcome the nutrient insufficient dosages and lower the medium cost by decreasing the sodium nitrate and calcium chloride concentrations dramatically (the cost assessment of inorganic carbon is not included since the HCO_3^- can be obtained by coal–fired plant). The medium concentrations can support the biomass production of TCL–1 efficiently with lower price (Table 2). The biomass productivity is 138.7 mg L^{-1} h^{-1} while the Su and Chu's medium is adopted, and the biomass productivity is 123.7 mg L^{-1} h^{-1} while 5 folds MF medium is used (the controlled group). These results indicate the high potential for applying the Su and Chu's medium for biomass production, and the medium is worth to be examined for cultivating thermopilic cyanobacteria and/or mesophilic cyanobacteria is feasible in the future.

Effects of Nutrient Concentrations on the CO_2 Fixation by TCL–1

The amount of CO_2 fixed in the biomass relates to two decisive parameters, the carbon content in the biomass and the biomass productivity. Among all the examined nutrients (Figs. 2(A)–2(F)), the concentration of $NaHCO_3$ with Na_2CO_3 shows significant effect on the drop of the carbon content under high feeding concentration (454.1 mM CO_3^{-2} with 0.53 M Na^+, Fig. 2(A)). The carbon contents decrease from 41.6 to 36.5% under the minimum to maximum CO_3^{-2} concentration. By and large, the varied carbon contents of TCL–1 are within ± 1% under each examined nutrient concentrations except the one under highest $NaHCO_3$ plus Na_2CO_3 feeding level. Besides, most of the carbon contents are about 40%. These results indicate most of the treated nutrients affect the CO_2 fixation rate by the biomass production, rather than the carbon content in the biomass in the system. The amount of CO_2 fixed should be determined by the biomass produced in the system. The more the biomass produce, the more the CO_2 can be fixed.

It is not surprised that the CO_2 fixation rate is affected obviously by the biomass productivity (Figs. 2(A)–2(F)). Higher variations, less than 100 mg CO_2 L^{-1} h^{-1}, can be observed under various $NaNO_3$ and $CaCl_2$•$2H_2O$ concentrations. However, the enormous divergences of CO_2 fixation rates are discovered under various $NaHCO_3$ with Na_2CO_3 concentrations. To prevent the decrease of the CO_2 fixation rates and the biomass concentrations, lower Na^+ stress is essential. Factors that can affect biomass production in the system should be removed for high CO_2 fixation rate.

As the CO_2 fixation rates of TCL–1 are compared with

Table 2. The biomass productivity, carbon and glycogen content in the biomass, the CO_2 fixation rate, and the glycogen productivity of TCL–1 in flat plate photobioreactors while the Su and Chu's medium is selected as the culture medium.

Utilized medium	Biomass productivity (mg L^{-1} h^{-1})	Carbon content (%)	CO_2 fixation rate (mg L^{-1} h^{-1})	Glycogen content (%)	Glycogen productivity (mg L^{-1} h^{-1})
Su and Chu's medium	138.7 ± 4.7	43.6 ± 0.1	221.5 ± 6.9	52 ± 5.2	75.9 ± 12.8

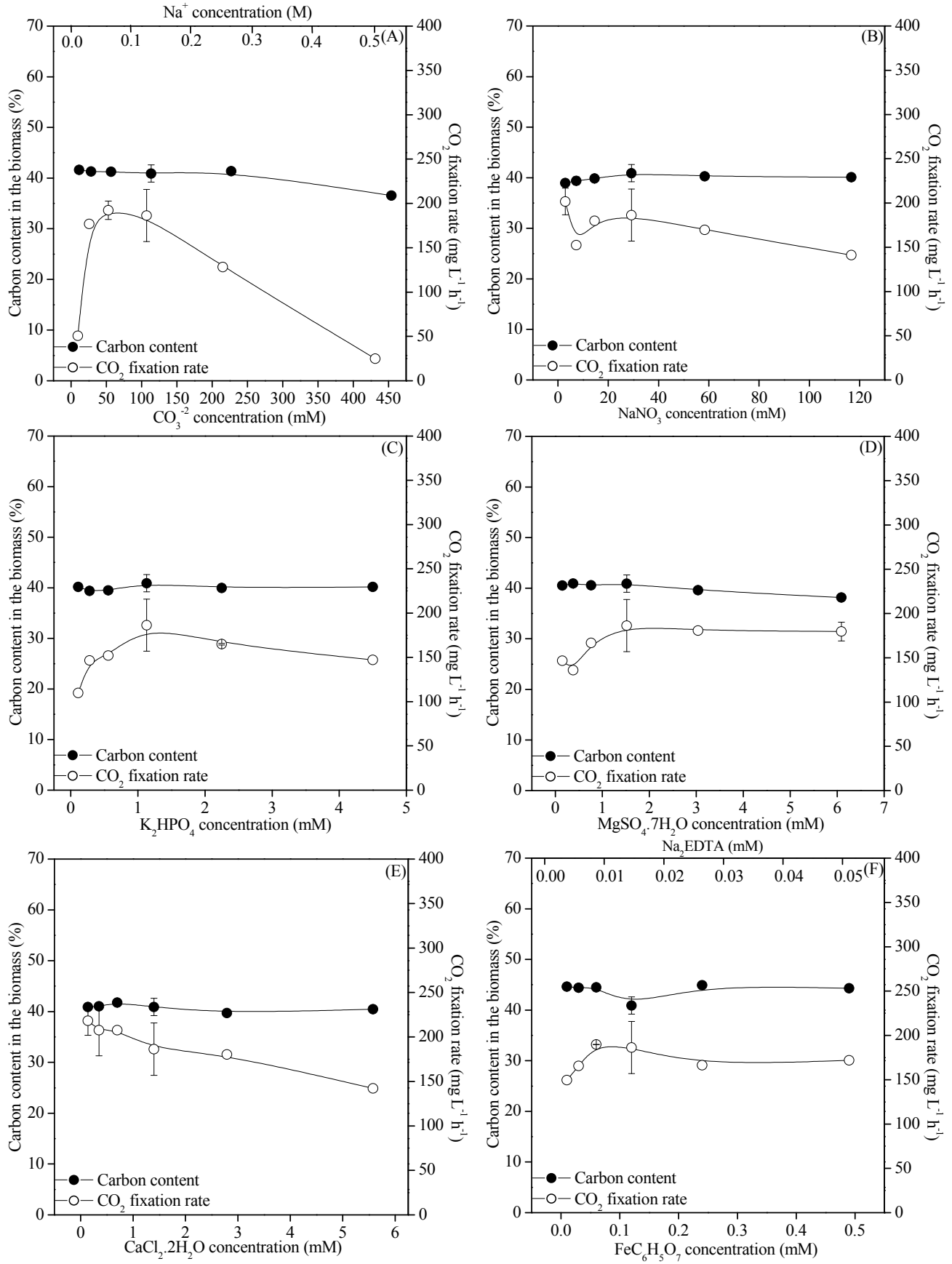

Fig. 2. Effects of (A) $NaHCO_3$ and Na_2CO_3, (B) $NaNO_3$, (C) K_2HPO_4, (D) $MgSO_4 \bullet 7H_2O$, (E) $CaCl_2 \bullet 2H_2O$, and (F) $FeC_6H_5O_7$ with Na_2EDTA on the carbon content and CO_2 fixation rate.

that of other cyanobacteria or microalgae strain, one can find that TCL–1 exhibits great potential while applying the strain in CO_2 fixation technology. The great CO_2 fixation rate can be achieved at 221.5 mg L^{-1} h^{-1} (about 0.22 g L^{-1} h^{-1}) under the use of Su and Chu's medium in this study (Table 2). On the other hand, the CO_2 fixation rates of other cyanobacteria or microalgae cultivated in flat plate photobioreactors are only 0.21 g L^{-1} h^{-1} of *Synechocystis aquatilis* SI–2 (0.13 g L^{-1} h^{-1} biomass productivity with assumed 45% carbon, cultivated under 40 ± 1°C) (Zhang *et al.*, 2002), 0.09 g L^{-1} h^{-1} of *Chlorella* sp. HA–1 (0.06 g L^{-1} h^{-1} biomass productivity with 46.1% carbon) (Morita *et al.*, 2000; López *et al.*, 2013), and of 0.47 *Anabaena* sp. PCC 7120#11 (0.02 g L^{-1} h^{-1} biomass productivity with assumed 45% carbon) (Ketseoglou and Bouwer, 2013). However, the biomass production or CO_2 fixation can't be improved further more by enhancing the biomass concentration, e.g., 5 g biomass L^{-1}, even after the use of the Su and Chu's medium (data not shown). This result indicates the limitation may be reached in the system. The limitation may come from the limited aeration rate (0.5 vvm in the present study), the element requirement other than the examined nutrient, or other restricted metabolites (Hu *et al.*, 1996; Richmond *et al.*, 2003).

It should be noted that most of the cultivated strain for CO_2 fixation purpose were mesospheric. That means cooling system should be participated in the cultivation system to prevent microalgae died from the high temperature, especially the flat plate system were adopted (Klinthong *et al.*, 2015). Due to high CO_2 fixation rate (0.22 g L^{-1} h^{-1}, c.a. 1.76 g L^{-1} $8h^{-1}$) and thermophilic characteristic, TCL-1 exhibits great potential for CO_2 fixation during outdoor cultivation.

Effects of Nutrient Concentrations on the Bioethanol Production Potential of TCL–1

Glycogen is polymer of glucose, and the estimation of the bioethanol production potential from cyanobacteria biomass can be evaluated from the glycogen or the glucose content in the biomass. The enhancement of the glycogen content can mainly be observed by the reduction of the NO_3^- and $CaCl_2$•$2H_2O$ level (Table 3) while other treatment shows no obvious enhancement as compared to the controlled group. High glycogen content of TCL–1 can be achieved, 58.6%, under the lowest initial NO_3^- concentration, 2.9 mM. Similar enhancement can be observed in *Spirulina maxima* that the glycogen content can be enhanced to 70 % 163 h after the transfer to nitrogen–free medium (De Philippis *et al.*, 1992). Moreover, the total carbohydrate content can be increased to 45% in TCL–1 under N–deprived level under continuous operation (Su *et al.*, 2013a). However, the glycogen contents in the present study are much higher than the content in the previous study (about 1.4%) although the cultivated cyanobacteria strains are similar (*Thermosynechococcus elongatus* BP–1) (Eberly and Ely, 2012). The distinguished divergence may come from the strong illumination intensity (< 180 μE m^{-2} s^{-1} in Eberly and Ely's study and 2,000 μE m^{-2} s^{-1} in this study) and the different types of photobioreactor used for cultivation (Eberly and Ely, 2012), and maybe the different cyanobacteria strains that from different isolation places (Hsueh *et al.*, 2007; Eberly and Ely, 2012). Moreover, the biomass concentrations may also be another important factor to influence the glycogen content. It has been present that the accumulation of carbohydrate is proportional to the increase in biomass density in flat plate photobioreactors (Qiang *et al.*, 1996). The explanation is that under strong light illumination, the excess energy and carbon are turned to carbohydrate biosynthesis in flat plate photobioreactors (Qiang *et al.*, 1996). A shift from low to high illumination (50 to 440 μE m^{-2} s^{-1}) can enhance the glycogen content quickly and obviously (De Philippis *et al.*, 1992). The increased glycogen content can also be observed in *Anabaena variabilis* while the light intensity is increased (Ernst and Böger, 1985) The similar enhancement of glycogen content (about 1.7 folds) can be observed in *Spirulina platensis* while higher photo fluxes (500 μE m^{-2} s^{-1}) are selected (Nomsawai *et al.*, 1999). Recently, the glycogen content in *Spirulina platensis* is discovered that can be enhanced to 60.9% under scarce $NaNO_3$ level (3 mM) and strong illumination (500 μE m^{-2} s^{-1}) (Arata *et al.*, 2013). Since the illumination intensity is set at 2,000 μE m^{-2} s^{-1}, and the cyanobacteria are cultivated in flat plate photobioreactors, the high glycogen content in the study is reasonable, especially the $CaCl_2$•$2H_2O$ concentration is decreased.

Actually, the accumulation of glycogen and its production is possible to be carried out under appropriate nitrogen and energy supplement without the reduction of the biomass productivity or the glycogen productivity (Aikawa *et al.*, 2012; Su *et al.*, 2013a, b). These results intensify the use of flat plate photobioreactors, or short light path photbioreactors, to cultivate cyanobacteria for the bioethanol production. Under appropriate distribution of the period in light and dark zone with adequate nutrient supplement, the high glycogen production can be achieved. While the Su and Chu's medium are used, the great glycogen productivity, 75.9 mg L^{-1} h^{-1}, can be achieved in Su and Chu's medium (Table 2). This value is comparable with the high glycogen

Table 3. Effects of $NaNO_3$ and $CaCl_2$•$2H_2O$ concentrations on the glycogen content and productivity to examine the bioethanol production potential by using TCL–1 biomass as feedstocks. Except the described nutrients, the nutrient dosage of 5 folds MF medium was used.

Nutrient status	Glycogen content (%)	Glycogen productivity (mg L^{-1} h^{-1})
29.2 mM $NaNO_3$ with 1.4 mM $CaCl_2$•$2H_2O$ (controlled group)	54.3 ± 5.3	71.4 ± 12.9
2.9 mM $NaNO_3$ with 1.4 mM $CaCl_2$•$2H_2O$	58.6 ± 0.8	82.7 ± 6.2
29.2 mM $NaNO_3$ with 0.14 mM $CaCl_2$•$2H_2O$	64.5 ± 1.0	93.9 ± 7.1

productivity achieved in *Spirulina,* 0.29 g L^{-1} d^{-1} (Aikawa *et al.*, 2012). If TCL–1 can be digested directly by the lysozyme with combined use of amylase–expressing yeast to produce bioethanol, the yield (in Aikawa *et al*'s study, 0.44 L kg^{-1} dry–biomass, about 60% glycogen in the biomass) can be similar with that *Spirulina* was used as feedstock (Arata *et al.*, 2013), and the bioethanol production rate can be 0.4 mL L^{-1} in a flat plate photobioreactor under 8 hours illumination.

CONCLUSIONS

This study demonstrates the effects of supplying various nutrient concentrations on the biomass, CO_2 fixation, and glycogen production in flat plate photobioreactors with dense culture of thermophilic cyanomacteria, TCL–1. After the use of Su and Chu's medium, the biomass productivity, CO_2 fixation and glycogen productivity are 138.7, 221.5, and 75.9 mg L^{-1} h^{-1}, respectively. The high biomass productivity, CO_2 fixation and glycogen productivity indicate the use of Su and Chu's medium exhibits high potential for applying TCL–1 in CO_2 fixation and bioethanol production potential in the flat plate phptobioreactors.

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SUPPLEMENTARY MATERIAL

Supplementary data associated with this article can be found in the online version at http://www.aaqr.org.

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