Producing carbohydrate-rich microalgal biomass grown under mixotrophic conditions as feedstock for biohydrogen production

Chun-Yen Chen^{1**}, Hung-Yu Chang¹ and Jo-Shu Chang^{2,3*}

¹*University Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan*

²*Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan*

³*Research Center for Energy Technology and Strategy, National Cheng Kung University, Tainan 701, Taiwan*

***Corresponding author:** Professor Jo-Shu Chang

E-mail: changjs@mail.ncku.edu.tw

Fax: +886-6-2357146

****Co-corresponding author:** Dr. Chun-Yen Chen

E-mail: ccy.ncku@gmail.com

Fax: + 886-6-2357146

ABSTRACT

Three indigenous microalgae strains (*Scenedesmus subspicatus* GY-16, *Chlorella vulgaris* FSP-E, and *Anistrodesmus gracilis* GY-09) were evaluated for their ability to accumulate carbohydrates to subsequently serve as feedstock for biohydrogen production. The results of photoautotrophic growth show that among the three strains examined, *C. vulgaris* FSP-E displayed the highest biomass productivity (825.6 mg/L/d) and carbohydrate productivity (365.8 mg/L/d). Mixotrophic growth of *C. vulgaris* FSP-E with the addition of 2.0 g/l of sodium acetate further increased the biomass and carbohydrate productivity to 1022.3 mg/L/d and 498.5 mg/L/d, respectively.. Moreover, operating photobioreactor on semi-batch mode enhanced the stability for prolonged incubation of the carbohydrate-rich *C. vulgaris* FSP-E and the biomass and carbohydrate productivity obtained were 1063.3 and 384.8 mg/L/d, respectively. The biomass of *C. vulgaris* FSP-E was then used as feedstock for biohydrogen production via separate hydrolysis and fermentation processes. The acidic hydrolysate (hydrolyzed with 1% H₂SO₄) was fermented with *Clostridium butyricum* CGS5, giving a maximum H₂ yield of 2.87 mmol H₂/g biomass and a H₂ production rate of 176.9 ml/h/l, which are higher than most reported values. The results obtained in this work indicate that carbohydrate-based microalgae feedstock shows good potential for biohydrogen production.

Keywords: carbohydrate, biohydrogen, microalgae, *Chlorella*, *Clostridium butyricum*, dark fermentation

1. INTRODUCTION

The rapid development of many emerging economies is increasing global energy consumption (Harun et al., 2010). Over 80% of current global energy demand is currently met by fossil fuels, but the use of these is associated with pollution, health problems and climate change (Hallenbeck and Benemann, 2002; Sivakumar et al., 2010). This has led to increasing efforts to develop alternative energy sources that are economic, sustainable, and environmentally benign (Mussatto et al., 2010). Microalgal biomass is one of the most common sources of renewable energy, which can be converted into several types of biofuels, such as biodiesel (Chen et al., 2011; Ho et al., 2011; Ho et al., 2010b), bioethanol (John et al., 2011) and biohydrogen (Hallenbeck and Benemann, 2002). Most biohydrogen production uses sugar or food crops as feedstock (Ghirardi et al., 2000), which has significant impacts on food supplies and competition for arable lands (Sun and Cheng, 2002). There is thus a pressing need to develop new feedstock (such as lignocellulosic materials or microalgal biomass) for commercial production of biohydrogen.

Some microalgal species have high carbohydrate contents (Chen et al., 2013). Their biomass is thus suitable to serve as the substrate for biohydrogen fermentation (Kapdan and Kargi, 2006; Melis, 2002). In microalgae, the carbohydrates are either accumulated in the plastids or become the major component of inner cell walls (Chen

et al., 2013). Due to the lack of lignin in microalgae-based cellulose, saccharification of the microalgal biomass becomes much easier than with lignocellulosic materials (Harun et al., 2013). In addition, microalgae grow faster than terrestrial plants, and also have a higher CO₂ fixation rate (Ho et al., 2013b). Microalgae are also able to grow on salt or wastewater streams (saline/coastal water, brackish seawater, domestic/municipal/industrial wastewater), thereby reducing freshwater use. Moreover, carbohydrate-rich microalgal feedstock has high compatibility with integrated production of fuels and co-products within biorefineries (Harun et al., 2010). These advantages indicate that carbohydrate-rich microalgal biomass is useful in the development of microalgae-based biofuels, such as biohydrogen.

This study evaluates the potential of three carbohydrates-producing microalgal strains to serve as the feedstock for biohydrogen production. The effects of the concentration of the organic carbon source (i.e., sodium acetate) on microalgal growth and carbohydrate production were explored. Semi-batch operation of a photobioreactor was carried out to investigate the stability of long-term operation on carbohydrate production and microalgae growth, in order to assess the feasibility for use in practical applications. Finally, the acidic hydrolysate of *Chlorella vulgaris* FSP-E biomass was used to produce biohydrogen via separate hydrolysis and fermentation (SHF) processes.

2. MATERIALS AND METHODS

2.1 Microalgal strains and composition of growth medium

The three microalgae used in this study were *Scenedesmus subspicatus* GY-16, *Chlorella vulgaris* FSP-E, and *Anistrodesmus gracilis* GY-09 isolated from freshwater in southern Taiwan. The medium used to grow the microalgae consisted of (g/L): KH_2PO_4 , 1.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.00; urea, 0.56; $\text{EDTA} \cdot 2\text{Na}$, 0.50; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0498; H_3BO_3 , 0.1142; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0882; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0157; MoO_3 , 0.0071; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.0049; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0144; CaCl_2 , 0.0835. For the mixotrophic growth, CH_3COONa was added into the medium as the organic carbon source. The microalgal strains were grown at a light intensity of $450 \mu\text{mol}/\text{m}^2/\text{s}$ (illuminated by TL5 tungsten filament lamps), which was measured by a LI-250 Light Meter with a LI-190SA pyrometer sensor (LI-COR, Inc., Lincoln, Nebraska, USA).

2.2 Operation of photobioreactor

The photobioreactor (PBR) used in this study is a 1.0 L glass vessel (length = 15.2 cm; diameter = 9.4 cm) illuminated with light sources (14 watt TL5 tungsten filament lamps) placed on both sides of the PBR to achieve a light intensity of ca. $450 \mu\text{mol}/\text{m}^2/\text{s}$ (the average of light intensities measured at upper, center, and lower part of

the PBR). For the pre-culture of the microalgal strains, an inoculum size of 0.15 g/L was used and the PBR was operated at a temperature of $28 \pm 1^\circ\text{C}$ and an agitation rate of 150 rpm. 2.0% CO_2 (concentration was adjusted by mixing with air) was continuously fed into the microalgae culture medium at a rate of 0.1 vvm (volume gas per volume of the medium per min) during cultivation period. In some cases, sodium acetate was used as the organic carbon source. Samples were collected from the microalgae culture at the desired time intervals to determine pH, microalgae biomass concentration, residual nitrogen concentration and carbohydrate content.

2.3 Measurement of microalgae biomass concentration

The cell concentration of the culture in the PBR was determined by measuring the optical density at a wavelength of 685 nm (denoted as OD_{685}) using a UV/VIS spectrophotometer (model G10S UV-VIS, Thermo Fisher Scientific, Waltham, MA, USA). The dry cell weight concentration was converted from the value of OD_{685} after appropriate calibration.

2.4 Hydrolysis of microalgal biomass

Two hydrolysis methods were used to convert the microalgae-based carbohydrates into sugars. In the first method, 1% (w/w) H_2SO_4 was used to

hydrolyze the microalgae. The acidic hydrolysis was conducted at 121°C for 1 h. Next, the commercial cellulolytic enzyme “Accellerase 1500” (Genencor International, Rochester, New York, USA) was used to hydrolyze the microalgae. The enzymatic hydrolysis was carried out at 45°C and 200 rpm agitation for 3 days.

2.5 Hydrogen-producing bacterial strain and its growth conditions

The H₂-producing bacterial strain was isolated from a dark fermentation bioreactor using sucrose (20 g COD/l) as the carbon source for producing H₂ (Chang et al., 2002; Lee et al., 2004). The 16S rRNA gene sequence of *C. butyricum* CGS5 used in this study has been blasted in the NCBI nucleotide database under the accession number AY540109. The pure strains were pre-cultured under anaerobic conditions (Lo et al., 2008) consisting of (g/l): sucrose, 15; Na₂HPO₄, 5.0; NaCl, 2.0; KH₂PO₄, 1.0; Na₂S.9H₂O, 0.5; (NH₄)₂SO₄, 3.0; MgSO₄, 0.1. The H₂-producing medium consisted of (g/l): microalgae, 30; NH₄HCO₃, 6.72; K₂HPO₄, 0.125; NaHCO₃, 5.24; sodium thioglycolate, 0.5; L-cystein·HCl, 0.5; MgCl₂.6H₂O, 0.1; FeSO₄.7H₂O, 0.025; MnSO₄.6H₂O, 0.015; CuSO₄.5H₂O, 0.005; CoCl₂.5H₂O, 1.25×10⁻⁴. The biohydrogen fermentation was conducted at 35°C and initial pH of 7.5.

2.6 Determination of carbohydrate content of microalgae

Approximately 100 mg of the lyophilized dry microalgal biomass was put into a test tube. In the primary hydrolysis, 72% w/w sulfuric acid was added to the tubes, and the solid particles and acids were mixed for 1 h at 30°C. After the concentrated acid hydrolysis, the tubes were placed into an ice-cold water bath to terminate the reaction. The hydrolysate was diluted to 4% w/w sulfuric acid and then autoclaved for 1 h at 121°C as the secondary hydrolysis. The supernatant of the hydrolyzed microalgal biomass was neutralized with solid CaCO₃, and the composition of monosaccharides in the solution (mainly glucose, xylose and arabinose) was determined using a high-performance liquid chromatograph (HPLC) equipped with an RI detector. The column used for HPLC analysis was ICSep 156ICE-COREGEL 87H3 [19]. The concentration of the three monomeric sugars (glucose, xylose, and arabinose) was measured to estimate the degradation of the polysaccharides. The cellulose concentration was calculated based on the glucose concentration, while the hemicellulose concentration was calculated based on the xylose and arabinose concentrations.

2.7 Enzyme activity assay

The enzyme (FPase) activity of cellulases was measured using Whatman No.1 filter paper. To determine FPase activity, 0.3 ml of supernatant from the sample was mixed with 0.3 ml of 1% (w/v) Whatman filter paper in acetate buffer (pH 5.0) and incubated at 50°C for 60 min. The amount of reducing sugar generated was determined by measuring the absorbance at 540 nm (OD_{540}) with a UV/VIS spectrophotometer using DNS agent as a color indicator. One unit of FPase activity (FPU) was defined as the amount of enzyme which produced 1.0 μ g of reducing sugar from the substrate per minute.

2.8 Gas products analysis

The gas products (mainly H_2 and CO_2) were analyzed by gas chromatography (Model 9800, China Chromatography, Taipei, Taiwan) equipped with a thermal conductivity detector. Argon was used as the carrier gas (Lo et al., 2010). The concentration of volatile fatty acids and ethanol in the filtered supernatant of the culture medium were also detected by HPLC equipped with a refraction index detector and ICSep ICE-COREGEL 87H3 column, which was also used to analyze the monosugars, as mentioned above. The mobile phase was 0.008 N H_2SO_4 with a flow rate of 0.4 ml/min. The injection sample volume was 20 μ l, and the column temperature was controlled at 70°C. The dinitrosalicylic acid (DNS) method was used

to determine the reducing sugar concentration (Miller, 1959).

3. RESULTS AND DISCUSSION

3.1 Microalgae growth and carbohydrate accumulation of the three microalgae isolates

In selecting the most suitable strains for use as microalgae-based feedstock for biohydrogen production, the two main criteria for commercial applications are fast growth and a high-carbohydrate content. In addition, since a nitrogen-deficient condition is usually the most effective form of environmental stress with regard to stimulating carbohydrate accumulation in microalgae (Ho et al., 2013a), it is also necessary to observe carbohydrate productivity during nitrogen starvation. In this study, three isolated microalgal strains, namely *Scenedesmus subspicatus* GY-16, *Chlorella vulgaris* FSP-E, and *Anistrodesmus gracilis* GY-09, were grown on basal medium in a 1 L PBR under 2% CO₂ aeration at a rate of 0.2 vvm, and illumination at a light intensity of 450 $\mu\text{mol}/\text{m}^2/\text{s}$. The inoculum size was 0.15 g/L. **Figure 1** shows the time course of the microalgae growth, carbohydrate productivity and urea concentration for the GY-16, FSP-E and GY-09. The maximum biomass production of GY-16, FSP-E and GY-09 was 5.53, 5.66 and 4.67 g/L, respectively (**Fig. 1a**). During the nitrogen starvation period, the maximum carbohydrate contents of GY-16, FSP-E,

and GY-09 can reach 52.82%, 54.84, and 38.99%, respectively (Fig. 1b and Table 1).

As seen in Table 1, among the three microalgal strains examined in this work, *C. vulgaris* FSP-E had the best performance with regard to the production of microalgae biomass and carbohydrate, and was thus selected as the feedstock for biohydrogen production.

3.2 Effects of sodium acetate concentration on carbohydrate production and microalgae biomass of *Chlorella vulgaris* FSP-E

The organic carbon source is generally the key factor affecting the heterotrophic growth of microalgae (Berberogu et al., 2008; Chen et al., 2008; Mojaat et al., 2008), and the addition of sodium acetate usually can significantly increase the growth rate and biomass production of microalgae. It is thus of interest to examine the effects of different sodium acetate concentrations (1000–3000 mg/l) on mixotrophic growth and carbohydrate production. The photobioreactor and operating conditions in this experiment were the same as those used for autotrophic cultivation. Table 2 and Fig. 2 show the effects of three different sodium acetate concentrations on the performance of biomass and carbohydrate production. The results show that when using acetate concentrations of 1000 mg/l, 2000 mg/l and 3000 mg/l, the maximum biomass production obtained was 5.66, 5.12 and 4.93 g/L, respectively (Fig. 2a). The

biomass productivity increased significantly from 866.5 to 1002.4 mg/L/d when the sodium acetate concentration increased from 1000 to 2000 mg/L. However, a further increase in sodium acetate concentration to 3,000 mg/L caused a decrease in biomass productivity to 712.9 mg/L/d (Table 2). The results in Fig. 2a and Table 2, show that adding an excessive amount of sodium acetate seems to have negative effects on the growth of *C. vulgaris* FSP-E, likely due to substrate inhibition, as observed in an earlier study (de Moraes and Costa, 2007).

As indicated in Fig. 2b and Fig. 2c, the maximum carbohydrate contents of *C. vulgaris* FSP-E when grown on medium with the addition of 1000, 2000, and 3000 mg/l of sodium acetate were 54.0, 51.3 and 52.7%, respectively, during the nitrogen starvation period. The urea consumption rate was almost the same for all three sodium acetate concentrations during cultivation in the first 5 days. In addition, the maximum carbohydrate productivity (416.5 mg/L/d) was obtained at a sodium acetate concentration of 2000 mg/l, whereas using sodium acetate concentrations of 1,000 mg/L and 3,000 mg/L led to lower carbohydrate productivities of 365.8 mg/L/d and 349.3 mg/L/d, respectively (Table 2). 2,000 mg/l thus seems to be the optimal sodium acetate concentration for microalgae growth and carbohydrate production.

3.3 Effects of replacement ratio on carbohydrate production and microalgae

biomass of *Chlorella vulgaris* FSP-E with semi-batch operations

In an attempt to further increase carbohydrate productivity, microalgae cultivation was also carried out in semi-batch mode under the conditions described above (i.e., medium, basal medium; nitrogen source, urea at 560 mg/l; light intensity, 450 $\mu\text{mol}/\text{m}^2/\text{s}$; CO_2 concentration, 2%; CO_2 aeration rate, 0.2 vvm; acetate concentration, 2.0 g/l). The semi-batch operation was used because it allows easier control of the carbon and nitrogen source concentration that is fed into the microalgae culture. The semi-batch operation comprises repetitive feeding and discharging cycles for microalgae growth and carbohydrate production. Semi-batch operation is also more suitable for large-scale microalgae cultivation due to the lower costs and lower energy consumption.

The semi-batch operation started with a batch operation mode. When the cell density reached approximately 5.0 g/L (at which the maximum carbohydrate accumulation was observed (data not shown)), different volumes of microalgae culture medium were discharged from the PBR and replaced with fresh medium at a replacement ratio of 10%, 25%, 50% and 75%. The urea consumption rate was almost the same during the first 6 days. **Figure 3a** shows that the cumulative microalgae growth increased as the medium replacement ratio was raised from 10% to 25%. The highest microalgae growth (6.22 g/L) was observed when using a replacement ratio of

25%, whereas it started to decrease when this ratio was further increased from 25% to 75%, as the biomass production dropped to 5.46 g/L with 75% replacement. The maximum biomass productivity (1173.6 mg/L/d) was obtained at a 10% replacement ratio, while it started to decrease as the replacement ratio was further increased from 10% to 75%, giving a productivity of 759-1063 mg/L/d. The best microalgae biomass productivity obtained from semi-batch operations is much higher than that from the batch operations (1002 mg/L/d).

In addition to biomass productivity, the carbohydrate accumulation ability is also very important for the different operation modes. **Figure 3b** shows that the maximum carbohydrate content and productivity obtained were 49.5% and 371.4 mg/L/d, respectively, when the replacement ratio was 10%. The carbohydrate productivity decreased with an increase in the replacement ratio from 10% to 75%. This phenomenon could be attributed to the slower cell growth when a higher replacement ratio was used (i.e., the culture medium was more diluted), resulting in a lower biomass concentration and carbohydrate productivity (**Fig. 3b**). Consequently, **Fig. 3** clearly demonstrates that using the semi-batch operation mode could not only enhance biomass concentration, but also lead to a significant increase in carbohydrate productivity. Furthermore, the highest carbohydrate production performance of *C. vulgaris* FSP-E (371.4 mg/L/d) obtained with the semi-batch operation at a

replacement ratio of 10% is higher than that obtained in related studies (from 21 to 304 mg/L/d) (Table 3). This also indicates that using biomass of *C. vulgaris* FSP-E for carbohydrate production has considerable potential for commercial applications. Analysis of the carbohydrate composition of *C. vulgaris* FSP-E shows that the main component was glucose (about 92.4% of total carbohydrates). A small amount of xylose (0.82% of total carbohydrates) was also detected in the microalgae-based carbohydrates. These results suggest that when considering the biomass concentration, the best medium replacement ratio in the semi-batch operation is 25%. However, when considering both microalgal biomass productivity and carbohydrate production, a 10% replacement ratio is better. The results also show that using semi-batch operation can effectively enhance the carbohydrate productivity of *C. vulgaris* FSP-E, and so has good potential for producing high-carbohydrate-content microalgae for use in the production of biofuels or bio-based chemicals.

3.4 Biohydrogen production using hydrolyzed microalgae biomass as the feedstock

A hydrogen-producing bacterium *C. butyricum* CGS5 was used to convert hydrolyzed microalgal biomass to biohydrogen with separate hydrolysis and fermentation (SHF) processes. The *C. vulgaris* FSP-E biomass (carbohydrate content

= 45.6%) was used as the feedstock for biohydrogen production. **Table 4** shows the reducing sugar concentration obtained from microalgae by using different microalgae hydrolysis methods. Using the acidic hydrolysis method resulted in higher reducing sugar production (12.1 g/L) and higher sugar recovery (94%).

Figure 4 shows H₂ production from the hydrolysate of *C. vulgaris* FSP-E by different microalgae hydrolysis methods, and it can be seen that the use of acidic hydrolysate can increase H₂ production. The H₂ production rate and H₂ yield were 176.9 ml/h/l and 1.42 mol H₂/mol reducing sugar (or 2.87 mmol H₂/g biomass), respectively. Using enzymatic hydrolysate as a substrate to produce biohydrogen, the H₂ production rate and H₂ yield were 135.2 ml/h/l and 1.35 mol H₂/mol reducing (or 2.87 mmol H₂/g biomass) sugar, respectively. Although the non-hydrolyzed microalgal biomass (mixed with water) contained a very small amount of sugar (1.4 g/L), it could still be converted into H₂ by dark fermentation, since it is known that *C. butyricum* CGS5 can produce amylase enzyme to hydrolyze starch (Lo et al., 2011), and thus can utilize the starch that accumulates in the microalgal biomass to produce biohydrogen. The H₂ production rate and yield obtained from using non-hydrolyzed microalgae were 122.4 ml/h/l and 1.92 mmol H₂/g biomass, respectively.

The biohydrogen production from hydrolyzed microalgae obtained in this study is quite good when compared with the results from previous works (**Table 5**). The

maximum H₂ yield (2.87 mmol hydrogen/g microalgal biomass) and H₂ productivity (176.9 ml/h/l) obtained in this work are higher than the previously reported values. Therefore, using the acidic hydrolysate of *C. vulgaris* FSP-E for hydrogen production via the SHF process might be feasible for commercial biohydrogen production using microalgae as the feedstock.

4. CONCLUSIONS

This study evaluated the feasibility of producing biohydrogen from microalgal feedstock. The results show that the type of microalgal strain, addition of organic carbon source (i.e., sodium acetate) and medium replacement ratio in semi-batch operations all significantly affect the cell growth and carbohydrate production of the microalgal isolates. *Chlorella vulgaris* FSP-E exhibited the best carbohydrate production performance when it grew on basal medium supplemented with 2.0 mg/l sodium acetate under semi-batch operation with a replacement ratio of 25%. The highest biomass accumulation and carbohydrate productivity were 6.22 and 361 mg/L/d, respectively. Using acidic hydrolyzed microalgal biomass as the feedstock, *Clostridium butyricum* CGS5 could produce biohydrogen efficiently via the SHF process at a maximum H₂ production rate of 176.9 ml/h/l and a H₂ yield of 2.87 mmol hydrogen/g microalgae biomass.

338

339 **ACKNOWLEDGEMENTS**

340 The authors gratefully acknowledge the financial support provided for this study
341 by the Core Research for Evolutional Science and Technology (CREST) of the
342 Promoting Globalization on Strategic Basic Research Programs of the Japan Science
343 and Technology Agency (JST). The authors also gratefully acknowledge the support
344 from the Top University Project of NCKU, Energy Technology Program for Academia
345 of Bureau of Energy of Ministry of Economic Affairs, and from Taiwan's Ministry of
346 Science and Technology under grant numbers MOST 104-3113-E-006 -003, MOST
347 103-2221-E-006-190-MY3, and NSC 102-3113-P-110-011.

348

349 **REFERENCES**

350 Berberogu, H., Jay, J., Pilon, L., 2008. Effect of nutrient media on photobiological
351 hydrogen production by *Anabaena variabilis* ATCC 29413. Int J Hydrogen
352 Energy 33, 1172-1184.
353 Chang, J.S., Lee, K.S., Lin, P.J., 2002. Biohydrogen production with fixed-bed
354 bioreactors. Int. J. Hydrogen Energy 27, 1167-1174.
355 Chen, C.Y., Yang, M.H., Yeh, K.L., Liu, C.H., Chang, J.S., 2008. Biohydrogen
356 production using sequential dark and photo fermentation processes. Int. J.
357 Hydrogen Energy 33, 4755-4762.
358 Chen, C.Y., Yeh, K.L., Aisyah, R., Lee, D.J., Chang, J.S., 2011. Cultivation,

359 photobioreactor design and harvesting of microalgae for biodiesel production: A
360 critical review. *Bioresour. Technol.* 102, 71-81.

361 Chen, C.Y., Zhao, X.Q., Yen, H.W., Ho, S.H., Cheng, C.L., Lee, D.J., Bai, F.W.,
362 Chang, J.S., 2013. Microalgae-based carbohydrates for biofuel production.
363 *Biochem. Eng. J.* 78, 1-10.

364 Choi, J.A., Hwang, J.H., Dempsey, B.A., Abou-Shanab, R.A.I., Min, B., Song, H.,
365 Lee, D.S., Kim, J.R., Cho, Y., Hong, S., Jeon, B.H., 2011. Enhancement of
366 fermentative bioenergy (ethanol/hydrogen) production using ultrasonication of
367 *Scenedesmus obliquus* YSW15 cultivated in swine wastewater effluent. *Energy*
368 *Environ Sci* 4, 3513-3520.

369 de Morais, M.G., Costa, J.A.V., 2007. Isolation and selection of microalgae from coal
370 fired thermoelectric power plant for biofixation of carbon dioxide. *Energy Convers*
371 *Manage* 48, 2169-2173.

372 Dragone, G., Fernandes, B.D., Abreu, A.P., Vicente, A.A., Teixeira, J.A., 2011.
373 Nutrient limitation as a strategy for increasing starch accumulation in microalgae.
374 *Appl. Energy* 88, 3331-3335.

375 Ghirardi, M.L., Zhang, J.P., Lee, J.W., Flynn, T., Seibert, M., Greenbaum, E., Melis,
376 A., 2000. Microalgae: a green source of renewable H₂. *Trends Biotechnol.* 18,
377 506-511.

378 Hallenbeck, P.C., Benemann, J.R., 2002. Biological hydrogen production;
379 fundamentals and limiting processes. *Int. J. Hydrogen Energy* 27, 1185-1193.

380 Harun, R., Danquah, M.K., Forde, G.M., 2010. Microalgal biomass as a fermentation
381 feedstock for bioethanol production. *J. Chem. Technol. Biotechnol.* 85, 199-203.

382 Harun, S., Balan, V., Takriff, M.S., Hassan, O., Jahim, J., Dale, B.E., 2013.
383 Performance of AFEX (TM) pretreated rice straw as source of fermentable
384 sugars: the influence of particle size. *Biotechnol Biofuels* 6.

385 Ho, S.-H., Chen, C.-Y., Yeh, K.-L., Chen, W.-M., Lin, C.-Y., Chang, J.-S., 2010a.
386 Characterization of photosynthetic carbon dioxide fixation ability of indigenous
387 *Scenedesmus obliquus* isolates. Biochem. Eng. J. 53, 57-62.

388 Ho, S.H., Chen, C.Y., Lee, D.J., Chang, J.S., 2011. Perspectives on microalgal
389 CO₂-emission mitigation systems - A review. Biotechnol. Adv. 29, 189-198.

390 Ho, S.H., Chen, C.Y., Yeh, K.L., Chen, W.M., Lin, C.Y., Chang, J.S., 2010b.
391 Characterization of photosynthetic carbon dioxide fixation ability of indigenous
392 *Scenedesmus obliquus* isolates. Biochem. Eng. J. 53, 57-62.

393 Ho, S.H., Huang, S.W., Chen, C.Y., Hasunuma, T., Kondo, A., Chang, J.S., 2013a.
394 Characterization and optimization of carbohydrate production from an indigenous
395 microalga *Chlorella vulgaris* FSP-E. Bioresour. Technol. 135, 157-165.

396 Ho, S.H., Kondo, A., Hasunuma, T., Chang, J.S., 2013b. Engineering strategies for
397 improving the CO₂ fixation and carbohydrate productivity of *Scenedesmus*
398 *obliquus* CNW-N used for bioethanol fermentation. Bioresour. Technol. 143,
399 163-171.

400 John, R.P., Anisha, G.S., Nampoothiri, K.M., Pandey, A., 2011. Micro and macroalgal
401 biomass: A renewable source for bioethanol. Bioresour. Technol. 102, 186-193.

402 Kapdan, I.K., Kargi, F., 2006. Bio-hydrogen production from waste materials Enzyme
403 Microb. Technol. 38, 569-582

404 Kim, M.S., Baek, J.S., Yun, Y.S., Sim, S.J., Park, S., Kim, S.C., 2006. Hydrogen
405 production from *Chlamydomonas reinhardtii* biomass using a two-step
406 conversion process: Anaerobic conversion and photosynthetic fermentation. Int. J.
407 Hydrogen Energy 31, 812-816.

408 Lee, K.S., Wu, J.F., Lo, Y.S., Lo, Y.C., Lin, P.J., Chang, J.S., 2004. Anaerobic
409 hydrogen production with an efficient carrier-induced granular sludge bed
410 bioreactor. Biotechnol. Bioeng. 87, 648-657.

- Liang, Y.N., Sarkany, N., Cui, Y., 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol. Lett.* 31, 1043-1049.
- Lo, Y.C., Chen, C.Y., Lee, C.M., Chang, J.S., 2010. Sequential dark-photo fermentation and autotrophic microalgal growth for high-yield and CO₂-free biohydrogen production. *Int. J. Hydrogen Energy* 35, 10944-10953.
- Lo, Y.C., Chen, W.M., Hung, C.H., Chen, S.D., Chang, J.S., 2008. Dark H₂ fermentation from sucrose and xylose using H₂-producing indigenous bacteria: Feasibility and kinetic studies. *Water Res* 42, 827-842.
- Lo, Y.C., Huang, L.F., Cheng, C.L., Chen, J., Chang, J.S., 2011. Using a starch-rich mutant of *Arabidopsis thaliana* as feedstock for fermentative hydrogen production. *Bioresource Technol* 102, 8543-8546.
- Melis, A., 2002. Green alga hydrogen production: progress, challenges and prospects. *Int J Hydrogen Energy* 27, 1217-1228.
- Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *AnaCh* 31, 426-428.
- Mojaat, M., Pruvost, J., Foucault, A., Legrand, J., 2008. Effect of organic carbon sources and Fe²⁺ ions on growth and beta-carotene accumulation by *Dunaliella salina*. *Biochem. Eng. J.* 39, 177-184.
- Mussatto, S.I., Dragone, G., Guimaraes, P.M.R., Silva, J.P.A., Carneiro, L.M., Roberto, I.C., Vicente, A., Domingues, L., Teixeira, J.A., 2010. Technological trends, global market, and challenges of bio-ethanol production. *Biotechnol. Adv.* 28, 817-830.
- Park, J.H., Yoon, J.J., Park, H.D., Kim, Y.J., Lim, D.J., Kim, S.H., 2011. Feasibility of biohydrogen production from *Gelidium amansii*. *Int J Hydrogen Energ* 36, 13997-14003.

437 Park, J.I., Lee, J., Sim, S.J., Lee, J.H., 2009. Production of hydrogen from marine
 438 macro-algae biomass using anaerobic sewage sludge microflora. *Biotechnol*
 439 *Bioproc E* 14, 307-315.
 440 Sivakumar, G., Vail, D.R., Xu, J.F., Burner, D.M., Lay, J.O., Ge, X.M., Weathers, P.J.,
 441 2010. Bioethanol and biodiesel: Alternative liquid fuels for future generations.
 442 *Eng. Life Sci.* 10, 8-18.
 443 Sun, Y., Cheng, J.Y., 2002. Hydrolysis of lignocellulosic materials for ethanol
 444 production: a review. *Bioresour. Technol.* 83, 1-11.
 445 Yang, Z.M., Guo, R.B., Xu, X.H., Fan, X.L., Li, X.P., 2010. Enhanced hydrogen
 446 production from lipid-extracted microalgal biomass residues through
 447 pretreatment. *Int. J. Hydrogen Energy* 35, 9618-9623.
 448 Zheng, Y., Chen, Z.A., Lu, H.B., Zhang, W., 2011. Optimization of carbon dioxide
 449 fixation and starch accumulation by *Tetraselmis subcordiformis* in a rectangular
 450 airlift photobioreactor. *African Journal of Biotechnology* 10, 1888-1901.

FIGURE LEGENDS

- Figure 1 Time-course profiles of the (a) biomass concentration, (b) urea concentration and (c) carbohydrate content of *Scenedesmus subspicatus* GY-16, *Chlorella vulgaris* FSP-E, and *Anistrodesmus gracilis* GY-09 grown phototrophically on basal medium (urea concentration = 0.565 g/L; light intensity = 450 $\mu\text{mol}/\text{m}^2/\text{s}$; CO_2 aeration = 2% and 0.05 vvm).
- Figure 2 Time-course profiles of (a) biomass concentration, (b) urea concentration and (c) carbohydrate content of *C. vulgaris* FSP-E grown mixotrophically on basal medium with the addition of different sodium acetate concentrations (urea concentration: 0.565 g/L; light source: TL5; light intensity: 450 $\mu\text{mol}/\text{m}^2/\text{s}$; CO_2 aeration: 2% and 0.05 vvm).
- Figure 3 The effects of medium replacement ratio on (a) the biomass concentration and the biomass productivity; (b) the carbohydrate content and carbohydrate productivity of *C. vulgaris* FSP-E in semi-batch cultivation with basal medium (urea concentration: 0.565 g/L; acetate concentration: 2 g/L; light source: TL5; light intensity: 450 $\mu\text{mol}/\text{m}^2/\text{s}$; CO_2 aeration: 2% and 0.05 vvm).
- Figure 4 Performance of biohydrogen fermentation with *Clostridium butyricum* CGS5 using biomass of *C. vulgaris* FSP-E hydrolyzed with different methods. (a) Cumulative H_2 production and H_2 production rate; (b) H_2 yield (operating conditions: temperature: 35°C, initial pH: 7.5).

Figure 1

Figure 2

Figure 3

Figure 4

Table 1 Biomass productivity, carbohydrate content, carbohydrate productivity and final pH of *Scenedesmus subspicatus* GY-16, *Chlorella vulgaris* FSP-E, and *Anistrodesmus gracilis* GY-09 grown phototrophically on basal medium (CO₂ supply: 2%, 0.2 vvm; light source: TL5; light intensity: 450 $\mu\text{mol/m}^2/\text{s}$; working volume: 1L).

Microalgal strain	Biomass productivity (mg/L/d)	Carbohydrate content (%)	Carbohydrate productivity (mg/L/d)	Final pH
GY-16	760.2 $\pm$ 56.7	52.8 $\pm$ 4.4	256.0 $\pm$ 17.6	8.37 $\pm$ 0.72
FSP-E	825.5 $\pm$ 78.4	54.0 $\pm$ 4.5	365.8 $\pm$ 27.4	8.40 $\pm$ 0.57
GY-09	709.2 $\pm$ 53.6	39.0 $\pm$ 3.2	255.5 $\pm$ 20.8	8.08 $\pm$ 0.53

Table 2 The effects of different sodium acetate concentrations on the biomass productivity, carbohydrate content, carbohydrate productivity and final pH. (CO₂ supply: 2%, 0.2 vvm; light source: TL5; light intensity: 450 $\mu\text{mol/m}^2/\text{s}$; working volume: 1 L).

Sodium acetate concentration (mg/l)	Biomass productivity (mg/L/d)	Carbohydrate content (%)	Carbohydrate productivity (mg/L/d)	Final pH
1000	796.2 $\pm$ 61.8	46.2 $\pm$ 3.8	312.9 $\pm$ 24.2	7.67 $\pm$ 0.53
2000	1002.4 $\pm$ 57.8	51.3 $\pm$ 4.2	416.5 $\pm$ 29.6	6.51 $\pm$ 0.64
3000	712.9 $\pm$ 45.1	52.7 $\pm$ 4.55	349.3 $\pm$ 27.7	7.76 $\pm$ 0.68

Table 3 Comparison of the biomass productivity and carbohydrate productivity from different microalgal strains reported in the literature.

Microalgal strains	Operation mode	Biomass concentration (g L ⁻¹)	Biomass productivity (g L ⁻¹ d ⁻¹)	Carbohydrate content (%) ^a	Carbohydrate productivity (g L ⁻¹ d ⁻¹) ^a	References
<i>C. vulgaris</i> (P12)	Batch	N.D. ^c	0.485	41.0 ^b	0.199 ^b	(Dragone et al., 2011)
<i>C. reinhardtii</i>	Batch	1.45	0.484	53.1	0.257	(Kim et al., 2006)
<i>Tetraselmis subcordiformis</i>	Semi-continuous	N.D. ^c	N.D. ^c	N.D. ^c	0.255 ^b	(Zheng et al., 2011)
<i>S. obliquus</i>	Batch	2.63	0.280	40.0	0.112	(Ho et al., 2010a)
<i>C. vulgaris</i>	Batch	1.70	0.254	44.0	0.112	(Liang et al., 2009)
<i>C. vulgaris</i> FSP-E	Batch	5.12±0.29 ^b	1002.4±57.8 ^b	51.3±4.2 ^b	416.5±29.6 ^b	(This study)
<i>C. vulgaris</i> FSP-E	Semi-batch	5.71±0.48 ^b	1173.6±83.5 ^b	49.5±3.78 ^b	371.4±30.1 ^b	(This study)

^aTotal carbohydrate

^bMaximum value

^cN.D.: not determined

Table 4 The reducing sugar concentration obtained from *Chlorella vulgaris* FSP-E by using different microalgae hydrolysis methods.

Methods	Biomass concentration (g/L)	Carbohydrate content (%)	Reducing sugar obtained (g/L)	Sugar recovery (%) ^a
Suspended in water	30.0	45.6 ± 0.89	1.4± 0.01	11± 0.01
Acidic hydrolysis	30.0	45.6 ± 0.89	12.12± 0.03	94± 0.03
Enzymatic hydrolysis	30.0	45.6 ± 0.89	11.27± 0.02	88± 0.02

^aSugar recovery (%) = (reducing sugar obtained/total carbohydrate concentration in the microalgal biomass) x 100%

Table 5 Comparison of the performance of microalgae-based biohydrogen production obtained in this work and related studies.

H ₂ producer	Microalgae feedstock	Culture type	H ₂ production rate (ml h ⁻¹ l ⁻¹)	H ₂ production yield	References
<i>Clostridium</i> sp.	<i>Laminaria japonica</i>	Batch	70	1.14 mmol H ₂ /g dry algae	(Park et al., 2009)
Anaerobic sludge	<i>Scenedesmus</i> sp.	Batch	33	1.86 mmol H ₂ /g-volatile suspended solid	(Yang et al., 2010)
Anaerobic sludge	<i>Gelidium amansii</i>	Batch	106	2.18 mmol H ₂ /g dry algae	(Park et al., 2011)
Anaerobic sludge	<i>Scenedesmus obliquus</i> YSW15	Batch	120	1.9 mol H ₂ / mol glucose	(Choi et al., 2011)
<i>Clostridium butyricum</i> CGS5	<i>Chlorella vulgaris</i> FSP-E	Semi-batch	176.9	2.87 mmol H ₂ /g dry algae 1.42 mol H ₂ /mol reducing sugar	This study