Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

Selection of microalgae for high CO_2 fixation efficiency and lipid accumulation from ten *Chlorella* strains using municipal wastewater

Xia Hu, Jiti Zhou, Guangfei Liu, Bing Gui*

Key Laboratory of Industrial Ecology and Environmental Engineering, Ministry of Education, School of Environmental Science and Technology, Dalian University of Technology, Dalian 116024, China. E-mail: huxia.86@163.com

ARTICLE INFO

Article history:
Received 9 April 2015
Revised 15 August 2015
Accepted 27 August 2015
Available online 1 April 2016

Keywords:
Chlorella strains
CO_2 fixation
Lipid accumulation
Municipal wastewater influent

ABSTRACT

As significant differences in cellular physiology, metabolic potential and genetics occur among strains with morphological similarity, the screening of appropriate microalgae species for effective CO_2 fixation and biodiesel production is extremely critical. In this study, ten strains of *Chlorella* were cultivated in municipal wastewater influent (MWI) and their tolerance for MWI, CO_2 fixation efficiency and lipid productivity were assessed. The results showed that the biomass concentrations of four strains (*Chlorella vulgaris*, *Chlorella* 64.01, *Chlorella regularis* var. *minima* and *Chlorella* sp.) were significantly higher than other strains. When the cultivation systems were aerated with 10% CO_2, *Chlorella* sp. showed the highest CO_2 fixation efficiency (35.51%), while the highest lipid accumulation (58.48%) was observed with *C. vulgaris*. Scanning electron microscopy images revealed that the cells of both *Chlorella* sp. and *C. vulgaris* kept their normal morphologies after 15 day batch culture. These findings indicated that *Chlorella* sp. and *C. vulgaris* have fairly good tolerance for MWI, and moreover, *Chlorella* sp. was appropriate for CO_2 fixation while *C. vulgaris* represented the highest potential for producing biodiesel.

Introduction

As the major types of phytoplankton, microalgae possess the ability to fix carbon dioxide (CO_2) and can convert it into biomass with much higher efficiency than terrestrial plants (Khan et al., 2009; Lam and Lee, 2012), indicating that microalgae have an indispensable significance in maintaining the earth's ecosystems (Koller et al., 2014). Therefore, microalgae are considered to be one of the most promising species for CO_2 fixation and chemical energy production (Gao et al., 2008; Phukan et al., 2011; Raeesossadati et al., 2014), and have the potential for rapid growth rate and high carbon fixation capability (Cheng et al., 2013). As for microalgae selection, it has been proved that the *Chlorella* genus is a good option for microalgae cultivation to mitigate CO_2 and produce biodiesel (de Morais and Costa, 2007; Li et al., 2011b; Ramanan et al., 2010). Nevertheless, there are about ten species in the family of *Chlorella* which could grow photoautotrophically, mixotrophically and heterotrophically with high biomass accumulation (Petkov and Garcia, 2007). More importantly, Beacham et al. (2014) presented an interesting view that there were significant variations of cellular physiology, metabolic potential and genetics among strains that are morphologically similar. This means that differences in CO_2 fixation and lipid accumulation could exist among species from the family of *Chlorella*. But until now, studies to compare the capabilities

* *Corresponding author*. E-mail: jitizhou502@163.com (Jiti Zhou).

http://dx.doi.org/10.1016/j.jes.2015.08.030
1001-0742/

of CO_2 fixation and lipid accumulation among all of the *Chlorella* strains have been rare.

Additionally, there are several major challenges in the cultivation of microalgae, including high consumption of freshwater (Yang et al., 2011) and inorganic nutrients (Clarens et al., 2010; Lam and Lee, 2012) and abundant emissions of CO_2 (Lam et al., 2012; Rafiqul et al., 2005). One of the possible solutions to overcome these challenges is to replace freshwater with wastewater. It has been demonstrated that wastewater could be used as an economical resource for microalgae growth (Chen et al., 2014; Kong et al., 2010). It is well known that municipal wastewater contains carbon, nitrogen, phosphorus and other minerals, which are essential nutrients for algae growth and metabolism (Li et al., 2011a). Currently, secondary and tertiary wastewaters have been used for microalgae culturing, while some organic carbon was removed during the primary treatment process (Clarens et al., 2010; Lam and Lee, 2012). On the contrary, municipal wastewater influent (MWI) contains not only nitrogen and phosphorus but also organic carbon. In addition, it has been reported that MWI was also used for mixotrophic microalga cultivation (Min et al., 2011), which is the fastest way for biomass accumulation (Xu et al., 2006). Moreover, the nitrogen concentration is significantly lower in MWI compared with common culture medium, which is helpful for increasing lipid accumulation in microalgae. In view of all the advantages above, it is believed that the microalgae cultivated using MWI as the culture medium would increase lipid accumulation and remove the nutrients in MWI simultaneously. Meanwhile, it would also reduce energy consumption and save water resources.

Herein, MWI was used to cultivate ten significant strains of *Chlorella* for fixing CO_2 and producing lipid. In order to find the strains with high CO_2 fixation efficiency and lipid accumulation from the family of *Chlorella*, the survival capabilities of ten *Chlorella* strains were investigated and the robust strains toward MWI were found. Moreover, the strains with high CO_2 fixation efficiency and lipid accumulation were selected under the condition of 10% CO_2. It is believed that this study could provide some useful information about which *Chlorella* strains are suitable for culture in MWI systems for CO_2 fixation and biodiesel production.

1. Materials and methods

1.1. Algae strain collection and culture media

Except for algae strain *Chlorella vulgaris* (ESP-6), which was obtained from National Cheng Kung University, the other nine *Chlorella* strains (Table 1) were purchased from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Before being transferred into MWI, all of *Chlorella* strains were cultured in BG-11 medium (Li et al., 2011b; Stanier et al., 1971) containing (mg/L) $NaNO_3$, 1500; K_2HPO_4, 40; Na_2CO_3, 20; $MgSO_4{\cdot}7H_2O$, 75; $CaCl_2{\cdot}2H_2O$, 36; citric acid, 6; ferric ammonium citrate, 6; $EDTANa_2$, 1; H_3BO_3, 2.86; $MnCl_2{\cdot}4H_2O$, 1.81; $ZnSO_4{\cdot}7H_2O$, 0.222; $CuSO_4{\cdot}5H_2O$, 0.079; $CoCl_2{\cdot}6H_2O$, 0.050; and $NaMoO_4{\cdot}2H_2O$, 0.391. The ten *Chlorella* strains were collected by centrifugation at 8000 r/min for 5 min at 25°C and re-suspended in sterilized MWI when the microalgae cells reached the late exponential phase.

Table 1 – Microalgae selected in this study.

Strain ID	Algae species
ESP-6	*Chlorella vulgaris*
FACHB-40	*Chlorella ellipsoidea*
FACHB-484	*Chlorella* sp.
FACHB-1220	*Chlorella pyrenoidosa*
FACHB-2	*Chlorella protothecoides*
FACHB-275	*Chlorella sorokiniana*
FACHB-4	*Chlorella saccharophila*
FACHB-1	*Chlorella luteorividis*
FACHB-752	*Chlorella* 64.01
FACHB-729	*Chlorella regularis* var. *minima*

1.2. Source and pretreatment of wastewater

The MWI was collected from Dalian Lingshui River Wastewater Treatment Plant. The characteristics are as follows: pH 7.8; COD 52.42 mg/L; TN 29.32 mg/L; NH^{4+}-N 26.13 mg/L; TP 3.62 mg/L; Ca^{2+} 33.34 mg/L; Mg^{2+} 12.33 mg/L; Mn^{2+} 0.1351 mg/L; and Fe^{2+} 0.2397 mg/L. At the beginning, the wastewater was filtered through a 0.45 μm membrane and then autoclaved at 121°C and cooled to room temperature before storing at 4°C. The filtered and autoclaved MWI was clear and colorless, and no potentially toxic components were detected in the water.

1.3. Supply of exogenous CO_2

The gases were bubbled continuously at the rate of 0.2 vvm (volume gas per volume media per min). The aeration system contained a CO_2 tank and an air compressor, and two flow meters were used to adjust the flow rates of ambient air or CO_2.

1.4. Experimental design

The experiments in the present study were carried out in two stages. The first stage aimed at choosing the top candidates for biomass accumulation from ten *Chlorella* strains to grow in the MWI. They were cultivated in 250 mL Erlenmeyer flasks containing 100 mL sterilized MWI in batch culture for 15 day. The culture conditions were as follows: light intensity 55 μmol photon/(m^2·sec), light/dark periods of 12 hr/12 hr, relative humidity 80%, and temperature 25°C. The second stage was targeted at determining the capabilities of CO_2 fixation and lipid accumulation of the top candidates chosen from the first stage, which was carried out in 1000 mL Erlenmeyer flasks containing 500 mL sterilized MWI in batch culture for 15 days, and the culture was aerated with filtered compressed gases (10% CO_2). The cultivation conditions were the same as the first stage.

1.5. Determination of algal biomass concentration

The biomass concentrations of microalgae were determined by the relationship between biomass concentration and absorbance (OD_{690}) (Ho et al., 2012; Jiang et al., 2011). The regression equations are presented in the Supporting Information (Table S1). Chlorophyll a content was determined according to a previous report (Porra et al., 1989) with some

modifications. In addition, the specific growth rate (k, day^{-1}) was calculated by Eq. (1).

$$k = \frac{\ln(X_t/X_0)}{t-t_0} \quad (1)$$

where X_t and X_0 indicate the biomass concentrations at the end and the beginning of the exponential phase, respectively, and t and t_0 are the time of end and beginning of the exponential phase, respectively.

1.6. Determination of CO_2 fixation efficiency

The CO_2 concentrations of influent and effluent were measured by a gas chromatograph (Techcomp, GC7900, China) equipped with a Thermal Conductivity Detector (TCD) detector and stainless steel packed column (4 mm × 2 m), with oven temperature of 100°C and carrier gas flow rate (nitrogen) of 40 mL/min. The TCD was kept at 100°C. CO_2 fixation efficiency (E) was calculated according to (Cheng et al., 2013) following Eq. (2).

$$CO_2 \text{ fixation efficiency } (\%) = \left(1 - \frac{CO_{2output}}{CO_{2input}} \times 100\%\right) \quad (2)$$

where $CO_{2output}$ = effluent CO_2 concentration × effluent flow rate; CO_{2input} = influent CO_2 concentration × influent flow rate.

1.7. Determination of lipid content

Confocal laser scanning microscopy (CLSM, Olympus FV1000) was used to visualize the oil droplet formation in microalgae cells. A lipophilic fluorescent dye, Bodipy 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a, 4a-diaza-sindacene; HEOWNS, Tianjin, China), was used to stain the oil-containing organelles of algal cells, with a final labeling solution of 1 mmol Bodipy 505/515 and 0.1% DMSO (v/v) (Cooper et al., 2010; Zhang et al., 2013). Bodipy fluorescence (green) was excited with an argon laser (488 nm) and detected at 505–515 nm. Auto-fluorescence (red) of algal chloroplasts was detected simultaneously at 650–700 nm. When the dye was added to the algal suspension, the intracellular oil-containing organelles were stained within 10 min, and the oil droplet formation was observed.

In general, lipids are determined using the traditional gravimetric method or high performance liquid chromatography (Lin, 2007) and thin layer chromatography (Fan et al., 2012). These methods need not only a great amount of biomass to achieve accurate determination, but also some organic solvents such as chloroform/methanol in the extraction process (Bligh and Dyer, 1959; Lee et al., 2010), which could make the process environmentally unfriendly. The Fourier transform infrared (FT-IR) spectroscopic method was developed for quantitative analysis of biomacromolecular components in biomass like lipid, and the content of samples validated the accuracy of the FT-IR method (Meng et al., 2014; Pistorius et al., 2009). For the determination of lipid, egg phosphatidylcholine (egg-PC) was chosen as an external standard. The band around 3000–2800/cm^{-1} could be used to quantitatively determine lipid content (Pistorius et al., 2009). The absorbance spectra were collected between 4000 and 700 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans. An infrared spectrometer (Bruker VERTEX 70, Germany) was used to record the characteristic peak of egg-PC at 2800–3000 cm^{-1}, yielding the calibration Eq. (3).

$$A_L = 32.598\ T_L + 1.9709\ (R = 0.993) \quad (3)$$

where A_L is the characteristic peak areas of lipid, T_L (mg) is the weight of lipid.

The lipid content C_L (%) was calculated by Eq. (4).

$$C_L = \frac{T_L}{T_A} \times 100\% \quad (4)$$

where, T_L (mg) is the total weight of lipid, T_A (mg) is the total weight of dry microalgae powder.

1.8. The morphological observation of cells

Micaroalgae cells collected by centrifugation at 8000 r/min for 5 min at 4°C were fixed in 2.5% (v/v) glutaraldehyde for 24 hr and rinsed three times with sodium phosphate buffer (pH 7.4). The collected cells were dehydrated in ethanol for 10 min under concentrations ranging from 50% to 95% (v/v). Samples were dried at 60°C for 48 hr in a vacuum dryer (Estevez et al., 2001). The morphology of the cells was characterized using a field emission scanning electron microscope (FESEM, Hitachi SU8010, Japan) with an accelerating voltage of 5 kV.

1.9. Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as means of the replicates along with standard deviation (±SD). All statistical analysis was performed using SPSS 17.0. Data was assessed for normality and then subjected to ANOVA and 2-sample testing. The p values of less than 0.05 were considered to be significant.

2. Results and discussion

2.1. Screening the robust strains from ten *Chlorella* strains for MVI

To find the robust *Chlorella* strains with tolerance for the municipal wastewater system, ten significant *Chlorella* strains were cultivated in the MWI. After batch culture over 15 days, the growth properties of ten *Chlorella* strains are summarized in Fig. 1. For all strains, it was demonstrated that continuous growth occurred in the culture system, since both inorganic and organic carbon in the MWI could be absorbed and converted into biomass by the algal strains (Li et al., 2011b; Min et al., 2011). The results showed that the highest biomass concentration (0.81 g/L) was observed with *C. vulgaris*, ESP-6, achieving 44% higher biomass concentration than the least prolific strain *Chlorella saccharophila*, FACHB-4. Among the ten *Chlorella* strains, four strains achieved significantly higher biomass concentrations than other species assessed ($p \leq 0.05$), including ESP-6(0.81 g/L), FACHB-752 (0.75 g/L), FACHB-729 (0.71 g/L) and FACHB-484 (0.70 g/L).

The result revealed that the biomass accumulations of ten *Chlorella* strains were different under the same culture

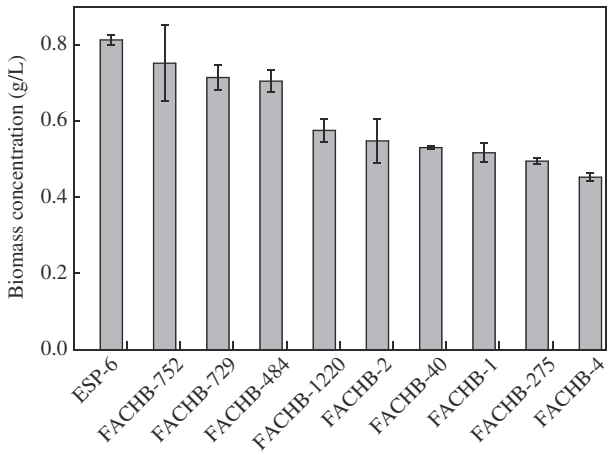

Fig. 1 – Biomass concentrations of ten *Chlorella* strains in municipal wastewater influent (MVI).

conditions. It was possible that cell sizes and growth rates were different among the ten species, and that environmental requirements varied among the ten *Chlorella* strains. As reported, the biomass accumulation could be influenced by size of cell, growth rate and the environmental requirements of a species (Burkhardt et al., 1999; Yang and Gao, 2003). Due to their better growth in the cultivation system, ESP-6, FACHB-752, FACHB-729 and FACHB-484 were chosen for further study.

2.2. Selecting the best strains for high CO_2 fixation efficiency and lipid accumulation of the four *Chlorella* strains

The CO_2 concentration of flue gas varies in the range 5%–20% (de Morais and Costa, 2007; Tang et al., 2011), and it was indicated that the 10% CO_2 condition had the best growth potential for algal growth (Ramanan et al., 2010; Tang et al., 2011). In our experiment, air with 10% CO_2 was bubbled into the culturing system. The four *Chlorella* strains chosen from the first stage were cultivated in MWI under 10% CO_2. As shown in Fig. 2, the biomass concentrations of FACHB-752 and ESP-6 were 0.74 and 0.71 g/L, and the specific growth rates were 0.127 and 0.392 day^{-1}, respectively; while the biomass concentrations and specific growth rates of FACHB-729 and FACHB-484 were significantly higher than those of FACHB-752 and ESP-6 (the highest biomass concentrations were 1.00 g/L and 0.98 g/L, and the growth rates were 0.153 and 0.152 day^{-1}, respectively), $p \leq 0.05$. After 6 days, ESP-6 and FACHB-752 grew slowly, so that the final biomass concentrations were lower than those of other two stains. The finding was not consistent with the results of the first stage. It was possible that FACHB-729 and FACHB-484 had better tolerance than FACHB-752 and ESP-6 to 10% CO_2 under nitrogen-insufficient conditions. It was also observed that the biomass concentrations of the four *Chlorella* strains increased steadily. The first reason was that biomass accumulation during nitrogen deficiency comes from newly fixed CO_2 (Yang and Gao, 2003). Next, the municipal wastewater contains other primary nutrients and micronutrients besides nitrogen, which were adequate for the cells growth. The last reason was that the chlorophyll of microalgae could be used as a nitrogen source to maintain cell growth (Li et al., 2010; Solovchenko et al., 2011). In addition, this finding was not consistent with previous research results indicating that high CO_2 concentrations (>5% CO_2) were harmful to the growth of microalgae (Chiu et al., 2008). This discrepancy may be due to differences in the inoculum concentration of microalgae cells. The tolerance capacity of microalgae for high concentrations of CO_2 can be heightened with an increase of the inoculated cell concentration (Ge et al., 2011). In previous reports, the initial cell density was low (about 0.01 g/L), whereas the initial inoculum biomass was 0.10 g/L in the present study. Thus it did not harm the microalgae cells when they were cultured under 10% CO_2.

As shown in Fig. 3, the chlorophyll a concentration of the four *Chlorella* strains increased steadily during the first 4 days, and the highest concentration was obtained with FACHB-729 (6.67 mg/L), followed by FACHB-484 (5.42 mg/L), FACHB-752 (4.20 mg/L) and ESP-6 (4.05 mg/L). However, the chlorophyll a concentration of the four *Chlorella* strains declined steadily after 4 days and the cells became yellow. This was due to the low nitrogen concentration (29.32 mg/L) in MWI, which was consumed gradually by algal growth, so that the algae grew under nitrogen deficiency. Some researchers considered that

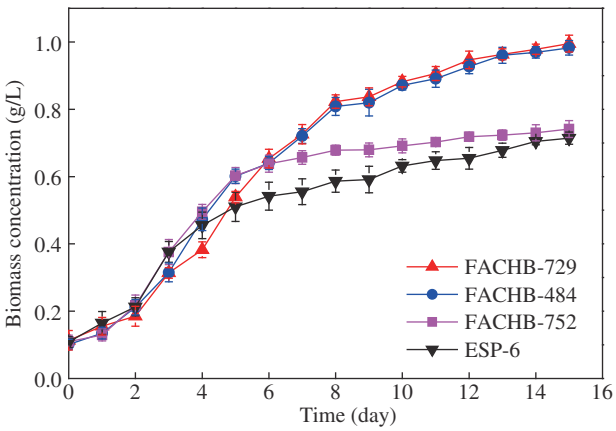

Fig. 2 – Biomass concentrations of four *Chlorella* strains in MVI with 10% CO_2.

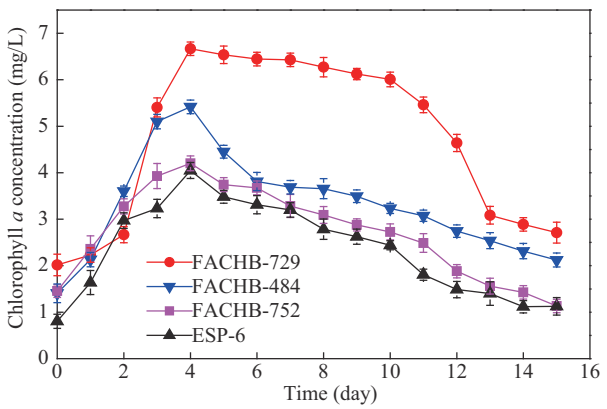

Fig. 3 – Chlorophyll a concentrations of four *Chlorella* strains in MVI with 10% CO_2.

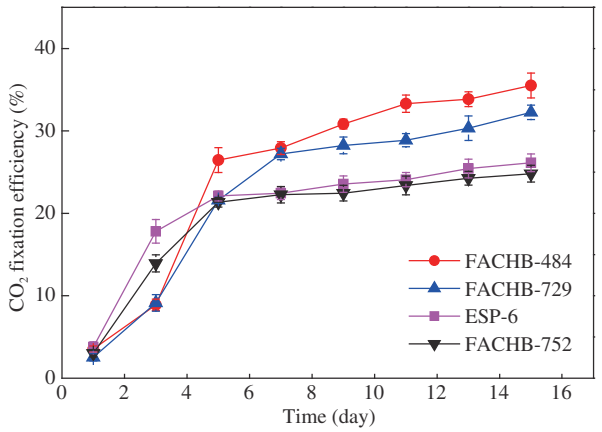

Fig. 4 – CO_2 fixation efficiencies of four *Chlorella* strains in MVI with 10% CO_2.

the decline of chlorophyll a concentration under stress in many algal species (Renaud et al., 1991; Sukenik et al., 1989) indicates that the photosynthetic phosphorylation activities of the chloroplasts of the four *Chlorella* strains are reduced (Demmig-Adams and Adams, 1996), leading to a decrease in the PSII photochemistry rate (Zhang et al., 2013).

2.3. CO_2 fixation efficiency of four *Chlorella* strains

For the study of CO_2 fixation, the CO_2 concentrations were detected at both influent and effluent every 2 days. As shown in Fig. 4, the amount of CO_2 fixation showed a linear increase with cultivation time accompanied with biomass accumulation, during the 15-day period. On day 15, the peak CO_2 fixation efficiencies of FACHB-484, FACHB-729, FACHB-752 and ESP-6 were 35.51%, 32.26%, 26.14% and 26.14%, respectively. During the first 5 days, the CO_2 fixation efficiencies of the four strains increased rapidly, and then slowed down subsequently. FACHB-484 and FACHB-729 showed higher CO_2 fixation efficiency with higher growth than those of the other two strains in the subsequent 10 days of experimental operation ($p \leq 0.05$). But no significant difference in CO_2 fixation was observed between FACHB-484 and FACHB-729 ($p \geq 0.05$), and their CO_2 fixation efficiencies were also higher than those in previous reports, in which CO_2 fixation efficiency was only 17% for *Spirulina platensis* and 32% for *Chlorella sp.* under 10% CO_2 aeration (Ramanan et al., 2010). This could be due to the efficiency of CO_2 removal or fixation being dependent on the physiological conditions of microalgal species, such as potential for cell growth and ability for CO_2 metabolism in a closed culture system (Cheng et al., 2006; Chiu et al., 2008; de Morais and Costa, 2007). Although the biomass concentrations of FACHB-729 and FACHB-752 were higher than those of FACHB-484 and ESP-6, the CO_2 fixation efficiencies were lower than those of FACHB-484 and ESP-6, respectively. This did not agree with a previous study, which showed higher growth and subsequently higher CO_2 fixation efficiency (Ramanan et al., 2010). It is possible that FACHB-729 and FACHB-752 cells were damaged due to the stress of nitrogen deficiency (Fig. 7), which indicated that FACHB-729 and FACHB-752 could not tolerate nitrogen deficiency, compared with the other two *Chlorella* strains. Therefore, by comparing FACHB-484 with the other strains, we infer that it was the most appropriate strain to capture CO_2.

2.4. Lipid accumulation of four *Chlorella* strains

To visualize the oil-droplet formation of the four *Chlorella* strains, the cells stained with Bodipy 505/515 were observed by CLSM. Bodipy 505/515 fluorescence (green) of oil droplets was detected in the four *Chlorella* strains, and the green fluorescence intensities of ESP-6 and FACHB-752 were stronger than those of FACHB-729 and FACHB-484 (Fig. 5). On the contrary, Chl fluorescence (red) intensities of FACHB-729 and FACHB-484 cells were stronger than those of ESP-6 and

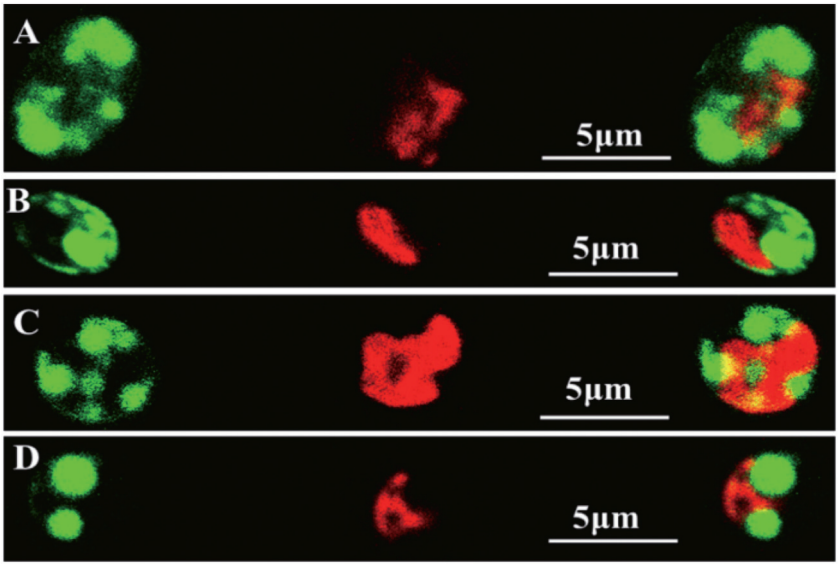

Fig. 5 – CLSM images of four *Chlorella* strains labeled *in vivo* with Bodipy 505/515. A: ESP-6, B: FACHB-752, C: FACHB-729, D: FACHB-484.

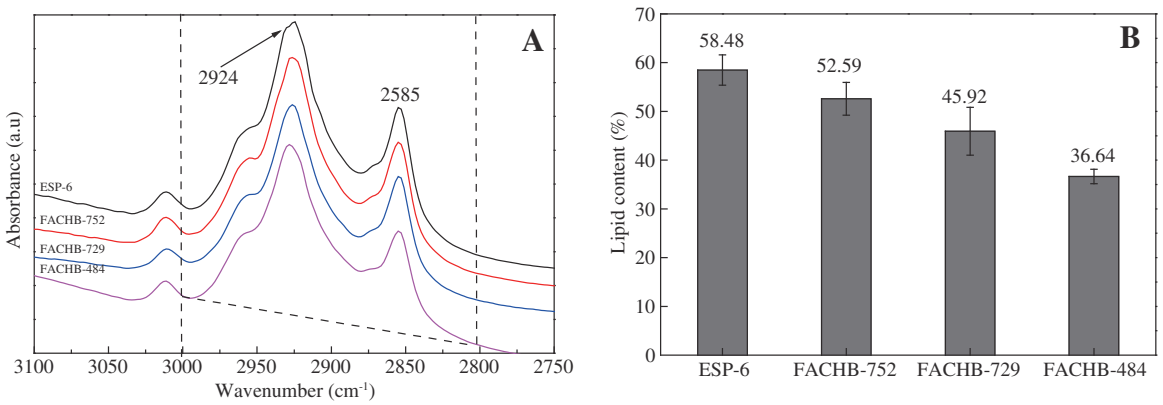

Fig. 6 – (A) Expanded section of infrared spectra showing the band around 3000–2800 cm^{-1} of four *Chlorella* strains, (B) lipid content of four *Chlorella* strains in MVI with 10% CO_2.

FACHB-752, and the strongest Chl fluorescence intensity was detected in the FACHB-484 cell. This indicated that the lipid contents of ESP-6 and FACHB-752 were higher than those of FACHB-729 and FACHB-484. However, it was difficult to compare the green fluorescence intensity between ESP-6 and FACHB-752.

To further determine the lipid content of the four *Chlorella* strains accurately, the FT-IR spectroscopic method was chosen for the quantitative analysis of the lipid content in this study. The FT-IR spectra of the four *Chlorella* strains at 2800–3000 cm^{-1} are shown in Fig. 6A. The lipid weights of the four *Chlorella* strains were calculated according to the calibration equation Eq. (3), then the lipid contents (%) were calculated by Eq. (4), as shown in Fig. 6B. The highest lipid content was obtained with ESP-6 (58.48%), followed by FACHB-752 (52.59%), FACHB-729 (45.92%) and FACHB-484 (36.64%). No significant difference in lipid content was observed among ESP-6, FACHB-752 and FACHB-729 ($p \geq 0.05$), but FACHB-484 was distinct from the other three strains ($p \leq 0.05$). Because the cells of FACHB-729 and FACHB-752 were damaged under the nitrogen deficiency conditions (Fig. 7), ESP-6 was the optimal strain to produce biodiesel compared with the other strains. This finding confirmed again that nitrogen deficiency is an effective environmental stress for lipid accumulation (Rodolfi et al., 2009). When microalgae are cultured under nitrogen deficiency conditions, there is insufficient nitrogen for protein synthesis, and the excess carbon from photosynthesis is converted into storage molecules such as triglyceride or starch (Scott et al., 2010). Another study demonstrated that the main channeling of

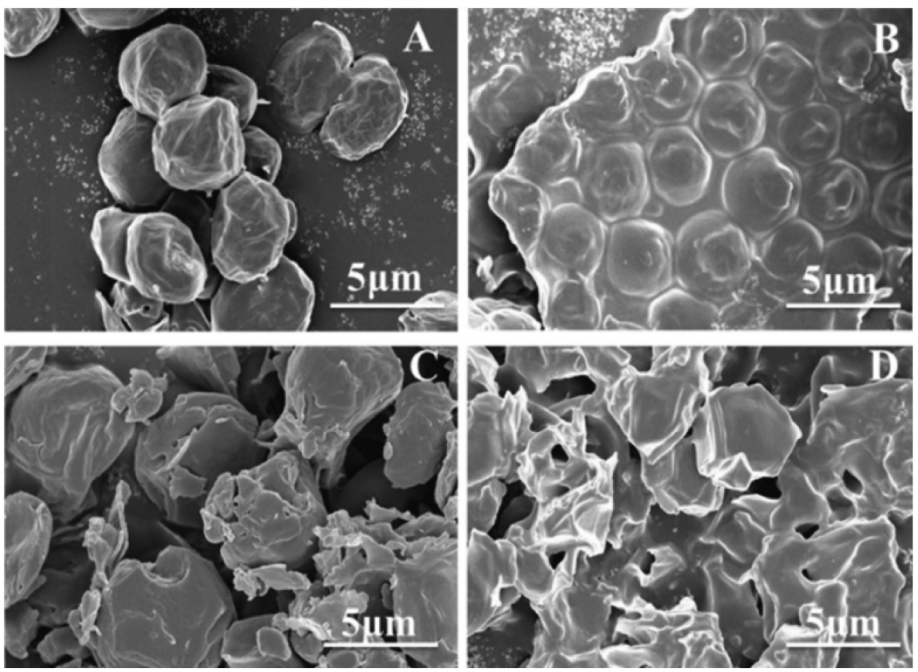

Fig. 7 – SEM images of four *Chlorella* strains in municipal wastewater with 10% CO_2. A: ESP-6, B: FACHB-484, C: FACHB-729, D: FACHB-752.

photosynthates to lipids played a protective role in nitrogen starvation and led to a decrease in chlorophyll a concentration and increase in lipid content in microalgae cells (Solovchenko et al., 2011).

2.5. The morphology changes of four Chlorella cells

After 15 day cultivation, the SEM images of the four *Chlorella* strains were observed (Fig. 7). The cells of ESP-6 and FACHB-484 kept their normal morphologies and the cells of ESP-6 had morphologies with the most integrity, while the cells of FACHB-729 were partially broken and the cells of FACHB-752 were severely broken. It is apparent that these strains were cultivated under nitrogen-insufficient conditions for a long time and they continued to grow under nitrogen starvation. It was reported that nitrogen starvation pressure led to an increase in cell morphological complexity. In addition, increased damage to PSII and level of membrane peroxidation were associated with lipid accumulation under nitrogen starvation (Zhang et al., 2013). It is indicated that FACHB-729 and FACHB-752 could not tolerate nitrogen deficiency, while ESP-6 and FACHB-484 have fairly good tolerance for MWI nitrogen levels. Therefore, FACHB-729 and FACHB-752 could not be used to fix CO_2 and produce biodiesel. In contrast, FACHB-484 and ESP-6 were appropriate for CO_2 fixation and biodiesel production, not only due to their high CO_2 fixation ability and lipid content, respectively, but also for their fairly good tolerance for MWI with nitrogen deficiency. To prove the conclusion concerning the nitrogen deficiency tolerance of the four *Chlorella* strains, the strains were cultivated in BG-11 medium in which the nitrogen concentration was identical to that of municipal wastewater (BG-11 medium with nitrogen deficiency). After 15 day cultivation, the SEM images of the four *Chlorella* strains were observed (Fig. 8). The cells of FACHB-729 were partially broken and the cells of FACHB-752 were severely broken, while the cells of ESP-6 and FACHB-484 kept their normal morphologies. This finding was consistent with the results that four *Chlorella* strains cultivated in municipal wastewater and proved the conclusion about nitrogen deficiency tolerance.

3. Conclusions

The selection of *Chlorella* strains for high CO_2 fixation efficiency and lipid accumulation was investigated using municipal wastewater influent (MWI), and the selected ten *Chlorella* strains were capable of growing in MWI. In this study, *Chlorella* sp. showed the highest CO_2 fixation efficiency and *C. vulgaris* exhibited the highest lipid accumulation capability. Under a nitrogen deficient environment, *Chlorella* sp. and *C. vulgaris* maintained their morphologies after 15 day batch culture, indicating that they had fairly good tolerance to MWI. Therefore, *Chlorella* sp. could be appropriate for CO_2 fixation, while *C. vulgaris* could produce biodiesel.

Acknowledgments

The authors wish to thank Professor Jo-Shu Chang in the Department of Chemical Engineering, National Cheng Kung University and Professor Xin-Qing Zhao in the school of life science and biotechnology, Dalian University of Technology, who provided the *C. vulgaris*, ESP-6 for this study. The authors also wish to extend their appreciation to Hao Zhou and

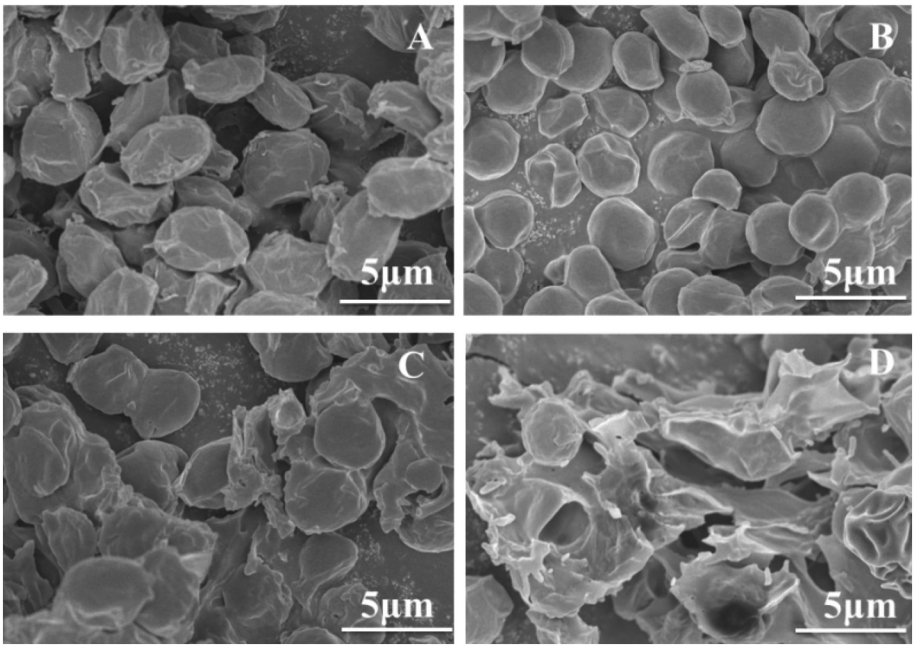

Fig. 8 – SEM images of four *Chlorella* strains cultured in nitrogen deficient BG-11 medium with 10% CO_2. A: ESP-6, B: FACHB-484, C: FACHB-729, D: FACHB-752.

Pancras Ndokoye in the School of Environmental Science and Technology, Dalian University of Technology, for proofreading the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.08.030.

REFERENCES

Beacham, T.A., Bradley, C., White, D.A., Bond, P., Ali, S.T., 2014. Lipid productivity and cell wall ultrastructure of six strains of *Nannochloropsis*: implications for biofuel production and downstream processing. Algal Res. 6, 64–69.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (8), 911–917.

Burkhardt, S., Zondervan, I., Riebesell, U., 1999. Effects of CO_2 concentration on C:N:P ratio in marine phytoplankton: a species comparison. Limnol. Oceanogr. 44 (3), 683–690.

Chen, M., Zhang, L., Li, S.Z., Chang, S., Wang, W.R., Zhang, Z.Y., et al., 2014. Characterization of cell growth and photobiological H_2 production of *Chlamydomonas reinhardtii* in ASSF industry wastewater. Int. J. Hydrog. Energy 39 (25), 13462–13467.

Cheng, J., Huang, Y., Feng, J., Sun, J., Zhou, J.H., Cen, K.F., 2013. Mutate *Chlorella* sp. by nuclear irradiation to fix high concentrations of CO_2. Bioresour. Technol. 136, 496–501.

Cheng, L.H., Zhang, L., Chen, H.L., Gao, C.J., 2006. Carbon dioxide removal from air by microalgae cultured in a membrane-photobioreactor. Sep. Purif. Technol. 50 (3), 324–329.

Chiu, S.Y., Kao, C.Y., Chen, C.H., Kuan, T.C., Ong, S.C., Lin, C.S., 2008. Reduction of CO_2 by a high-density culture of *Chlorella* sp. in a semicontinuous photobioreactor. Bioresour. Technol. 99 (9), 3389–3396.

Clarens, A.F., Resurreccion, E.P., White, M.A., Colosi, L.M., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ. Sci. Technol. 44 (5), 1813–1819.

Cooper, M.S., Hardin, W.R., Petersen, T.W., Cattolico, R.A., 2010. Visualizing "green oil" in live algal cells. J. Biosci. Bioeng. 109 (2), 198–201.

de Morais, M.G., Costa, J.A.V., 2007. Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of carbon dioxide. Energy Convers. Manag. 48 (7), 2169–2173.

Demmig-Adams, B., Adams, W.W.I., 1996. Chlorophyll and carotenoid composition in leaves of *Euonymus kiautschovicus* acclimated to different degrees of light stress in the field functional. Aust. J. Plant Physiol. 23 (5), 649–659.

Estevez, M.S., Malanga, G., Puntarulo, S., 2001. Iron-dependent oxidative stress in *Chlorella vulgaris*. Plant Sci. 161 (1), 9–17.

Fan, J.L., Yan, C.S., Andre, C., Shanklin, J., Schwender, J., Xu, C.C., 2012. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii*. Plant Cell Physiol. 53 (8), 1380–1390.

Gao, C.F., Zhai, Y., Ding, Y., Wu, Q.Y., 2008. Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl. Energy 87 (3), 756–761.

Ge, Y., Liu, J., Tian, G., 2011. Growth characteristics of *Botryococcus braunii* 765 under high CO_2 concentration in photobioreactor. Bioresour. Technol. 102 (1), 130–134.

Ho, S.H., Chen, W.M., Chang, J.S., 2012. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour. Technol. 113, 244–252.

Jiang, L.L., Luo, S.J., Fan, X.L., Yang, Z.M., Guo, R.B., 2011. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO_2. Appl. Energy 88 (10), 3336–3341.

Khan, S.A., Rashmi, Hussain, M.Z., Prasad, S., Banerjee, U.C., 2009. Prospects of biodiesel production from microalgae in India. Renew. Sust. Energ. Rev. 13 (9), 2361–2372.

Koller, M., Muhr, A., Braunegg, G., 2014. Microalgae as versatile cellular factories for valued products. Algal Res. 6, 52–63.

Kong, Q.X., Li, L., Martinez, B., Chen, P., Ruan, R., 2010. Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. Appl. Biochem. Biotechnol. 160 (1), 9–18.

Lam, M.K., Lee, K.T., 2012. Microalgae biofuels: a critical review of issues, problems and the way forward. Biotechnol. Adv. 30 (3), 673–690.

Lam, M.K., Lee, K.T., Mohamed, A.R., 2012. Current status and challenges on microalgae-based carbon capture. Int. J. Greenhouse Gas Control 10, 456–469.

Lee, J.Y., Yoo, C., Jun, S.Y., Ahn, C.Y., Oh, H.M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. 101, S75–S77.

Li, Y.C., Chen, Y.F., Chen, P., Min, M., Zhou, W.G., Martinez, B., et al., 2011a. Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour. Technol. 102 (8), 5138–5144.

Li, Y.T., Han, D.X., Hu, G.R., Sommerfeld, M., Hu, Q., 2010. Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. Biotechnol. Bioeng. 107 (2), 258–268.

Li, Y.C., Zhou, W.G., Hu, B., Min, M., Chen, P., Ruan, R.R., 2011b. Integration of algae cultivation as biodiesel production feedstock with municipal wastewater treatment: strains screening and significance evaluation of environmental factors. Bioresour. Technol. 102 (23), 10861–10867.

Lin, J.T., 2007. HPLC separation of acyl lipid classes. J. Liq. Chromatogr. Relat. Technol. 30 (14), 2005–2020.

Meng, Y.Y., Yao, C.H., Xue, S., Yang, H.B., 2014. Application of Fourier transform infrared (FT-IR) spectroscopy in determination of microalgal compositions. Bioresour. Technol. 151, 347–354.

Min, M., Hu, B., Zhou, W.G., Li, Y.C., Chen, P., Ruan, R., 2011. Mutual influence of light and CO_2 on carbon sequestration via cultivating mixotrophic alga *Auxenochlorella protothecoides* UMN280 in an organic carbon-rich wastewater. J. Appl. Phycol. 24 (5), 1099–1105.

Petkov, G., Garcia, G., 2007. Which are fatty acids of the green alga *Chlorella*? Biochem. Syst. Ecol. 35 (5), 281–285.

Phukan, M.M., Chutia, R.S., Konwar, B.K., Kataki, R., 2011. Microalgae *Chlorella* as a potential bio-energy feedstock. Appl. Energy 88 (10), 3307–3312.

Pistorius, A.M.A., DeGrip, W.J., Egorova-Zachernyuk, T.A., 2009. Monitoring of biomass composition from microbiological sources by means of FT-IR spectroscopy. Biotechnol. Bioeng. 103 (1), 123–129.

Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta Bioenerg. 975 (3), 384–394.

Raeesossadati, M.J., Ahmadzadeh, H., McHenry, M.P., Moheimani, N.R., 2014. CO_2 bioremediation by microalgae in photobioreactors: impacts of biomass and CO_2 concentrations, light, and temperature. Algal Res. 6, 78–85.

Rafiqul, I., Weber, C., Lehmann, B., Voss, A., 2005. Energy efficiency improvements in ammonia production—perspectives and uncertainties. Energy 30 (13), 2487–2504.

Ramanan, R., Kannan, K., Deshkar, A., Yadav, R., Chakrabarti, T., 2010. Enhanced algal CO_2 sequestration through calcite deposition by *Chlorella* sp. and *Spirulina platensis* in a mini-raceway pond. Bioresour. Technol. 101 (8), 2616–2622.

Renaud, S.M., Parry, D.L., Thinh, L.V., Kuo, C., Padovan, A., Sammy, N., 1991. Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. J. Appl. Phycol. 3 (1), 43–53.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., et al., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102 (1), 100–112.

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., et al., 2010. Biodiesel from algae: challenges and prospects. Curr. Opin. Biotechnol. 21 (3), 277–286.

Solovchenko, A., Khozin-Goldberg, I., Recht, L., Boussiba, S., 2011. Stress-induced changes in optical properties, pigment and fatty acid content of *Nannochloropsis* sp.: implications for non-destructive assay of total fatty acids. Mar. Biotechnol. 13 (3), 527–535.

Stanier, R.V., Kunisawa, R., Mandel, M., Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (*Order Chrococcales*). Bacteriol. Rev. 35 (2), 171–205.

Sukenik, A., Carmeli, Y., Berner, T., 1989. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. J. Phycol. 25 (4), 686–692.

Tang, D.H., Han, W., Li, P.L., Miao, X.L., Zhong, J.J., 2011. CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresour. Technol. 102 (3), 3071–3076.

Xu, H., Miao, X.L., Wu, Q.Y., 2006. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J. Biotechnol. 126 (4), 499–507.

Yang, Y., Gao, K.S., 2003. Effects of CO_2 concentrations on the freshwater microalgae, *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Chlorophyta). J. Appl. Phycol. 15 (5), 379–389.

Yang, J., Xu, M., Zhang, X.Z., Hu, Q., Sommerfeld, M., Chen, Y.S., 2011. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour. Technol. 102 (1), 159–165.

Zhang, Y.M., Chen, H., He, C.L., Wang, Q., 2013. Nitrogen starvation induced oxidative stress in an oil-producing green alga *Chlorella sorokiniana* C3. PLoS One 8 (7), e69225.