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Production of Microalgal Lipids as Biodiesel Feedstock with Fixation of CO_2 by *Chlorella vulgaris*

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Summary

The global warming and shortage of energy are two critical problems for human social development. CO_2 mitigation and replacing conventional diesel with biodiesel are effective routes to reduce these problems. Production of microalgal lipids as biodiesel feedstock by a freshwater microalga, *Chlorella vulgaris*, with the ability to fixate CO_2 is studied in this work. The results show that nitrogen deficiency, CO_2 volume fraction and photoperiod are the key factors responsible for the lipid accumulation in *C. vulgaris*. With 5 % CO_2, 0.75 g/L of $NaNO_3$ and 18:6 h of light/dark cycle, the lipid content and overall lipid productivity reached 14.5 % and 33.2 mg/(L·day), respectively. Furthermore, we proposed a technique to enhance the microalgal lipid productivity by activating acetyl-CoA carboxylase (ACCase) with an enzyme activator. Citric acid and Mg^{2+} were found to be efficient enzyme activators of ACCase. With the addition of 150 mg/L of citric acid or 1.5 mmol/L of $MgCl_2$, the lipid productivity reached 39.1 and 38.0 mg/(L·day), respectively, which was almost twofold of the control. This work shows that it is practicable to produce lipids by freshwater microalgae that can fixate CO_2, and provides a potential route to solving the global warming and energy shortage problems.

Key words: biodiesel, biofuels, CO_2 mitigation, microalgae

Introduction

Global warming and shortage of energy are two critical issues to human social development in the 21st century. Worldwide shortage of fossil fuels is an urgent problem. It enforces scientists to accelerate the search for alternative fuels. In recent years, biodiesel has been receiving widespread attention due to its various favourable properties, such as non-toxic, biodegradable and renewable source of energy. In addition, it contributes no net carbon dioxide or sulphur to the atmosphere and emits less gaseous pollutants than the conventional fossil diesel (*1*).

In recent decades, biodiesel has become a hot research field. It has reached three generations (*2*). The first generation biodiesel applied edible vegetable oil as feedstock, which was produced from conventional oil crops such as rapeseed, soya bean, palm and sunflower. Because it competes for land with food crops, the use of these first generation biodiesel sources has generated many problems, especially the impact on global food markets and food security (*3*). The second generation biodiesel applied non-food oil as feedstock, such as jatropha, mahua, jojoba oil, tobacco seed and sea mango. In addition, waste cooking oil, restaurant grease and animal fats are also considered as a potential feedstock (*4*). Now, microalgal lipids are considered as the third generation biodiesel feedstock, which has been recognized as the most promising alternative feedstock source for biodiesel (*2,3*). The advantages are their high photosynthe-

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tic efficiency to produce biomass, higher growth rate (doubling times may be as short as 3.5 h) and higher lipid productivity (some species can accumulate up to 20–50 % (by mass) triacylglycerol) compared to the conventional terrestrial crops (*5*,*6*). Microalgae can grow in a closed bioreactor with high-density cultivation, thus high quality agricultural land is not required to cultivate the microalgal biomass. It is most significant that microalgae can capture carbon dioxide and convert it into the biomass and lipids. This can help solve both the problems of global warming and shortage of energy. Therefore, it is an ideal process of closed carbon cycle for sustainable development (*7*).

Even though microalgal lipids have shown a bright prospect, there are several obstacles to overcome for large-scale commercial and industrial applications (*8*). The current major technical challenges facing the microalgal fuel industry are technologies related to upstream cultivation and downstream processing (*9*). Specifically, upstream technologies are the key steps, which dominate the lipid content and lipid productivity. The upstream technologies include two areas: one is obtaining lipid-rich microalgal strains, the other is developing highly efficient cultivation techniques to enhance lipid productivity. Many novel lipid-rich microalgal strains have been bred after isolation from marine and freshwater environments (*10*,*11*) or by genetic manipulation (*12*). An economical process of algal culture for biodiesel production depends strongly on high lipid productivity, which relies on high lipid content and high biomass growth rate. Many factors, such as nitrogen deficiency (*13*,*14*) or supplementation (*15*), phosphate limitation (*14*), organic carbon supplementation (*16*), culture mode and culture time (*17*), can influence the lipid content and growth rate of microalgae. However, research regarding production of lipids associated with CO_2 fixation by freshwater microalgae is relatively scarce (*18*).

In this study, we are focusing our efforts on producing microalgal lipids as biodiesel feedstock by a freshwater microalga, *Chlorella vulgaris*, with conversion of CO_2, which can contribute to CO_2 mitigation. Specifically, our aim is to evaluate the effect of culture parameters on microalgal lipid productivity. Furthermore, we will propose a practicable technique to enhance the microalgal lipid productivity by regulating the acetyl-CoA carboxylase (ACCase) activity, which is a key enzyme in fatty acid biosynthesis route in microalgae.

Materials and Methods

Microalgal strain and culture medium

Freshwater microalga *Chlorella vulgaris* FACHB-31 was used as the target species in this work. It was obtained from Institute of Hydrobiology (IHB), Chinese Academy of Sciences (CAS), Wuhan, PR China. *Chlorella vulgaris* was selected because of its high lipid content and stable growth rate at high CO_2 volume fraction level (*6*).

The basic medium for *Chlorella vulgaris* culture is modified BG-11 medium. A volume of 1 L of the medium contained (in g): $NaNO_3$ 1.5, $K_2HPO_4{\cdot}3H_2O$ 0.04, $MgSO_4{\cdot}7H_2O$ 0.075, $CaCl_2{\cdot}2H_2O$ 0.036, $C_6H_8O_7{\cdot}H_2O$ 0.006, $Fe(NH_4)_3(C_6H_5O_7)_2$ 0.006, EDTA-Na_2 0.001, Na_2CO_3 0.02, and 1 mL of trace metal solution. The trace metal solution contained (in g/L): H_3BO_3 2.86, $MnCl_2{\cdot}4H_2O$ 1.86, $ZnSO_4{\cdot}7H_2O$ 0.22, $Na_2MoO_4{\cdot}2H_2O$ 0.39, $CuSO_4{\cdot}5H_2O$ 0.08, and $Co(NO_3)_2{\cdot}6H_2O$ 0.05 (*19*,*20*).

Microalgal culture conditions

In autotrophic cultivation process, *C. vulgaris* can convert CO_2 into microalgal lipids. In general, a 4-day-old *C. vulgaris* culture was inoculated into a 500-mL bubble column photobioreactor with 200 mL of BG-11 medium to initial $A_{685\ nm}$=0.2 ($A_{685\ nm}$ is the absorbance at 685 nm used to indicate the microalgal biomass density based on turbidimetry). The CO_2 gas mix (up to 15 %, by volume, mixed with air) was used to continuously aerate the suspension from the bottom of the photobioreactor through a gas sparger loop. The photobioreactor was placed in an incubator at 28 °C. The illuminance at the photobioreactor surface was 6000 lux provided by a cool white fluorescent light source. The microalgae were illuminated in various photoperiod (light/dark) cycles. After 7 days of cultivation, the biomass and total lipid content were evaluated. All the experiments were carried out at least in duplicate.

Evaluation of biomass concentration

The microalgal biomass was expressed in g of dry cell per litre. The methodology of biomass assay evaluation was the same as in our previous paper about the capture of CO_2 by microalgae (*21*). Microalgal cells were collected by centrifugation at 20 000×*g* for 15 min (Avanti J-26XP, Beckman Coulter, Brea, CA, USA). The microalgal dry mass was evaluated by collecting the microalgal pellet and drying at 110 °C to constant mass.

Lipid extraction and lipid content evaluation assay

For lipid content analysis, cells were harvested by centrifugation at 20 000×*g* for 15 min, the cell pellet was lysed by repeated freezing and thawing cycles at –20 and 40 °C four times, and then it was freeze-dried at –50 °C. To enhance the microalgal cell lysis, the dried cells were ground using a mortar.

Petroleum ether/ethyl ether solution (2:1), analytical grade, was applied to extract the total lipid from the freeze-dried ground microalgae according to the modified method of Bligh and Dyer (*22*). In brief, 1 g of freeze-dried ground microalgae was extracted with 20 mL of petroleum ether/ethyl ether solution over 5 h. Then, isovolumetric mixture with 10 % KOH was added to the suspension to accelerate the cell debris precipitation. The suspension was centrifuged at 20 000×*g* for 15 min, and the upper phase was transferred into a pre-weighed glass vial. The petroleum ether/ethyl ether solution was evaporated at 60 °C under vacuum. The lipid content was measured gravimetrically, and calculated using the equation:

$$w(\text{lipid}) = \left(\frac{m_{\text{lipid}}}{m_{\text{DCM}}} \right) \cdot 100 \qquad /1/$$

where w(lipid) is the lipid content (in %), m_{lipid} is the mass of the extracted lipid (in g), and m_{DCM} is the dry microalgal biomass (in g).

The overall lipid productivity, r_P(lipid) in mg/(L·day), was calculated using the following equation:

$$r_P(\text{lipid}) = \frac{\Delta m_{\text{lipid}}}{V \cdot \Delta t} \qquad /2/$$

where Δm_{lipid} is the variation of lipid mass within cultivation time of Δt (in day), and V is the culture volume.

Fatty acid composition analysis

The fatty acid profile of the total lipids extracted from *C. vulgaris* was analyzed using a gas chromatography--mass spectrometry (GC-MS Agilent 5975C, Agilent Technologies, Santa Clara, CA, USA). Fatty acid methyl esters (FAMEs) were prepared by transesterification using KOH/methanol catalysis process (*16*). FAMEs were analytically quantified by GC-MS (Agilent Technologies). The conditions for the GC were: capillary column HP-5MS (30 m×0.25 mm, i.d.=0.25 μm), N_2 as carrier gas at 1.2 mL/min flow rate, injector temperature 250 °C, initial oven temperature of 80 °C, then linearly ramped to 180 °C at 15 °C/min, and then to 250 °C at 2 °C/min, and finally held at 250 °C for 16 min, 1-μL injection with 10:1 split ratio. Conditions for MS were electron ionization mode, electron energy 7 eV, ion source temperature 230 °C, quadrupole temperature 150 °C, mass scan range 50–550 atomic mass units (amu) to remove the solvent, using NIST08 database (National Institute of Standards and Technology, Gaithersburg, MD, USA) as the source of libraries to identify the peaks (*23*).

Results and Discussion

Characterization of the lipid production by Chlorella vulgaris

C. vulgaris, a freshwater microalga, was chosen to convert CO_2 into microalgal lipids as biodiesel feedstock due to its easy culture, fast growth and significant lipid content (*16,21*). *C. vulgaris* growth, lipid accumulation and lipid productivity are shown in Fig. 1 under basic culture conditions, *i.e.* 5 % CO_2, 1.5 g/L of $NaNO_3$ as a nitrogen source and 12 h light:12 h dark photoperiod.

There was about one-day lag phase during *C. vulgaris* growth. It took seven days to reach the stationary phase under the basic culture conditions. Lipid accumulation started from the early exponential growth phase. The highest lipid content was reached at the stationary phase. Considering the biomass and the lipid content, the maximum overall lipid productivity was observed in the stationary phase, respectively on the 8th and 7th day.

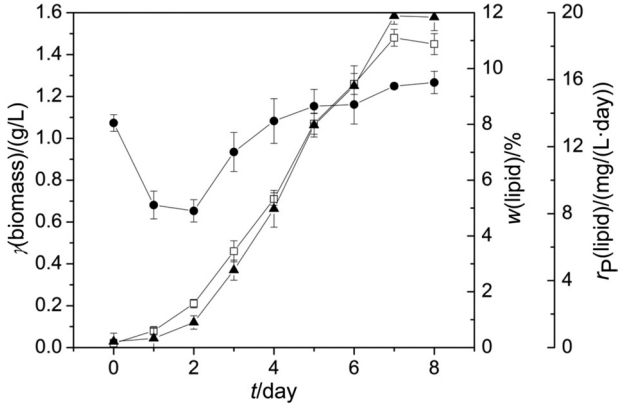

Fig. 1. Biomass and lipid production by *C. vulgaris* with time □ biomass, ● lipid content, ▲ lipid productivity (r_P)

The fatty acid composition of the microalgal lipids from *C. vulgaris* was analyzed using GC-MS, after transesterification of the total lipids to FAME. The chromatogram and the identification of corresponding peaks are presented in Fig. 2 and Table 1.

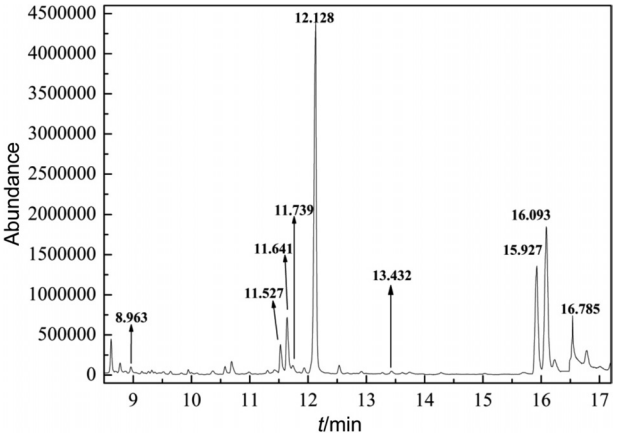

Fig. 2. GC-MS chromatogram of fatty acid methyl esters (FAMEs) prepared from *C. vulgaris* lipids

Table 1. Peak identification from GC-MS spectra of fatty acid methyl esters (FAMEs) of *C. vulgaris*

FAME*	Retention time/min	Composition/%
C14:0	8.963	1.01
C16:0	12.128	40.3
C16:1	11.739	1.15
C16:2	11.527	3.16
C16:3	11.641	6.35
C17:0	13.432	0.47
C18:0	16.785	6.36
C18:1	16.093	25.7
C18:2	15.927	15.5

*FAME identification was done in Cn:m format, where n is the number of carbon atoms in the carbon chain, and m is the number of double bonds

The major components of FAME from *C. vulgaris* are fatty acids C16:0 and C18:1. The ratio of saturated fatty acids in the total FAME is 48.14 %. The profiles of the FAME from other green microalgae, such as *Chlorococcum humicola* (*24*), *Scenedesmus obliquus* CNW-N (*25*) and *Chlorococcum* sp. (*16*) have also been reported. In these green microalgae, the major components are also fatty acids C16:0, C18:0 and C18:1.

The cetane number (CN) is the most significant property to assess the quality of biodiesel (*26*). It has an inverse relationship with the ignition delay time in the

combustion chamber. The minimum value of CN for biodiesel is 47 or 51 according to the USA and European standards (American Society for Testing and Materials D6751-07a fuel standard, and European standard EN 14214:2012) (*27*). The CN of the FAMEs from *C. vulgaris* was estimated using a combination model from the work of Stansell *et al.* (*27*). According to the referred model, the CN value for the FAMEs from *C. vulgaris* is 57.3. This suggests that the lipids produced by the freshwater microalgae *C. vulgaris* meet the requirement for biodiesel, which makes them a practicable feedstock for biodiesel.

Effect of nitrogen scarcity on the lipid production of C. vulgaris

In marine microalgae research, nitrogen source is a key factor to control the lipid accumulation. Nitrogen starvation will enhance lipid accumulation in most marine microalgae (*28*). It has similar effect in freshwater microalgae (*13*,*14*). The effects of nitrogen scarcity on the *C. vulgaris* biomass and lipid accumulation were investigated by reducing $NaNO_3$ concentration from 1.5 to 0 g/L (at concentration decrements of 1.5, 1.125, 0.75, 0.375 and 0 g/L of $NaNO_3$).

The results in Fig. 3 show that nitrogen starvation significantly enhanced the lipid accumulation in *C. vulgaris*. When supplying 0.75 g/L of $NaNO_3$, the maximum lipid fraction of 14.5 % was obtained. On the other hand, nitrogen deprivation inhibited the microalgal growth. The biomass growth remarkably decreased along with the nitrogen limitation, and with 0.375 g/L of $NaNO_3$, there was an even bigger decrease in the cell growth. The lipid content and biomass concentration taken together, the maximum overall lipid productivity was achieved at 0.75 g/L of $NaNO_3$.

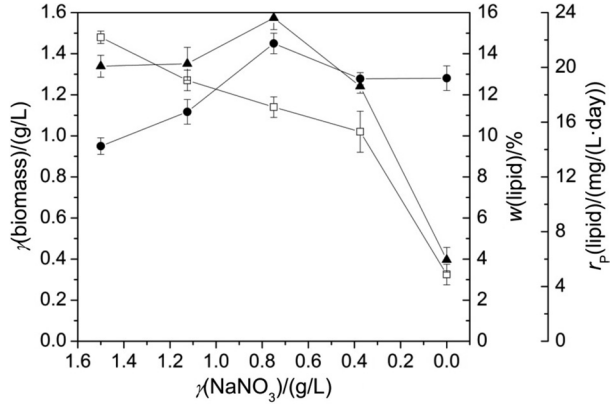

Fig. 3. Biomass and lipid production by *C. vulgaris* under different nitrogen supply conditions
□ biomass, ● lipid content, ▲ lipid productivity (r_P)

The phenomenon that nitrogen starvation could enhance the lipid content was also found in other microalgae, *Chlorella vulgaris* ESP-31 (*13*), *Chlamydomonas reinhardtii*, *Scenedesmus subspicatus* (*29*), *Chlorophyta* (*30*), *Botryococcus* sp. (*14*). There have been several proposals to explain that nitrogen scarcity enhances lipid accumulation in microalgae. Botham and Ratledge (*31*) proposed that the conversion of glucose into lipids is triggered when nitrogen is exhausted, due to the presence of high-energy charge (ratio of adenosine triphosphate to adenosine diphosphate, ATP/AMP). Sheehan *et al.* (*32*) stated that nitrogen deficiency led to the inhibition of cell division and growth without immediately reducing lipid production, thus leading to an accumulation of lipids in the microalgal cells. In our experiment, the relationship of nitrogen to biomass and lipid content provided a direct experimental support to Sheehan's hypothesis.

Effect of CO_2 on the lipid production of C. vulgaris

The carbon in microalgal biomass came from CO_2, which is why the CO_2 content in the aeration gas is a key factor to microalgal mitigation of CO_2 (*21*). Similarly, this is the key factor that affects the conversion of CO_2 into microalgal lipid. The effects of CO_2 volume fraction in the inlet gas on the biomass and lipid production are shown in Fig. 4 with CO_2 fraction increasing from 0 to 15 %.

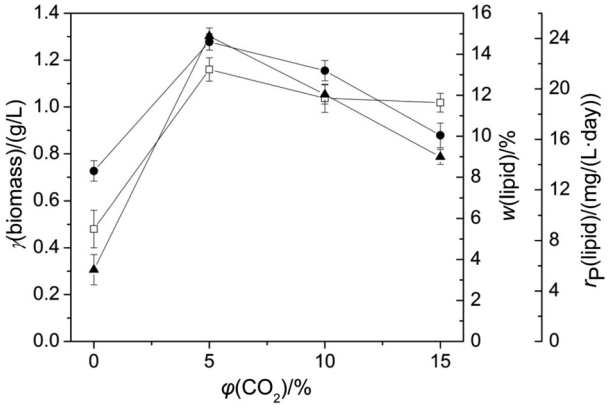

Fig. 4. Effect of CO_2 volume fraction on the biomass and lipid production by *C. vulgaris*
□ biomass, ● lipid content, ▲ lipid productivity (r_P)

The results show that CO_2 fraction affected both growth and lipid content of *C. vulgaris*. An increasing CO_2 fraction from 0 to 5 % enhanced the biomass growth and lipid content. However, further increase of CO_2 fraction from 5 to 15 % inhibited *C. vulgaris* growth and lipid accumulation. With about 5 % CO_2, the biomass, lipid content and overall lipid productivity were acceptable. These results are similar to the data for *Chlorococcum* sp. obtained by Harwati *et al.* (*16*).

Effect of photoperiod on the lipid production of C. vulgaris

For photosynthetic microorganisms, the biomass productivity and CO_2 capture ability highly relate to the amount of light energy received (*33*). In addition, the photoperiod (light cycle) is also important for the microalgal growth and lipid accumulation. Five different photoperiods (24:0, 18:6, 12:12, 6:18 and 0:24 h light/dark cycle) were employed in the culture of *C. vulgaris* at 5 % CO_2 and 0.75 g/L of $NaNO_3$ (relative to the standard BG-11 medium). The results of biomass, lipid con-

tent and overall lipid productivity after a 7-day cultivation period are shown in Table 2.

Table 2. Biomass and lipid production by *C. vulgaris* in different photoperiod (evaluated after a 7-day cultivation period)

t(light)/t(dark)	γ(biomass)	w(lipid)	r_P(lipid)
h/h	g/L	%	mg/(L·day)
24:0	1.28±0.03	13.3±0.40	24.4±1.2
18:6	1.60±0.05	14.5±0.45	33.2±0.5
12:12	1.12±0.03	14.9±0.55	23.8±1.1
6:18	0.38±0.05	13.9±0.60	7.6±1.2
0:24	0.04±0.04	9.4±0.45	0.6±0.6

r_P=productivity

In general, lengthening light photoperiod remarkably increased *C. vulgaris* biomass concentration and lipid content, and overall lipid productivity. This is similar to the results reported by Tang *et al.* (*34*) in their study of *Dunaliella tertiolecta*, where they concluded that growing these microalgae under the same light intensity but using different photoperiod led to much faster cell growth for a longer illumination period. With an 18:6 h light/dark cycle, the maximum biomass (1.6 g/L), lipid content (14.5 %), and overall lipid productivity (33.2 mg/(L·day)) were obtained. Photosynthetic organisms are usually exposed to a light/dark cycle in nature, which has made them build a mechanism able to balance and consort the photosynthesis and other anabolism for example through lipid synthesis. That is why the highest lipid content and lipid productivity can be obtained under a 18:6 h light/dark cycle.

Improvement of the lipid production in C. vulgaris by regulating acetyl-CoA carboxylase (ACCase)

Triglycerides are the main components of microalgal lipids, which are the main feedstock for biodiesel. The triglyceride synthesis route in microalgae consists of the following three phases: (*i*) formation of acetyl coenzyme A (acetyl-CoA) in the cytoplasm, (*ii*) elongation and desaturation of carbon chain of fatty acids, and (*iii*) biosynthesis of triglycerides (*35*). In this route, acetyl-CoA carboxylase (ACCase) is a key regulatory enzyme of the fatty acid biosynthesis (*36*). The most important function of ACCase is to provide the malonyl-CoA substrate for the biosynthesis of fatty acids (*37*). In this work, we propose to use an enzyme activator, such as citric acid and Mg^{2+}, to activate the ACCase, and thus to enhance the lipid biosynthesis.

Citric acid is a highly effective activator of ACCase. Thampy and Wakil (*38*) found that an addition of 10 mmol/L of citric acid could activate the ACCase activity 10-fold *in vitro*. Based on this, we chose citric acid as the ACCase activator, and added it into the culture medium to enhance the growth and lipid production of *C. vulgaris*. The concentrations of citric acid used in this test were 29, 60, 90, 120 and 150 mg/L. The CO_2 fraction in the aeration gas was 5 %.

The biomass and oil production by *C. vulgaris* with added citric acid are shown in Fig. 5. The results demonstrate that the lipid content increased from 9.6 (control) to 15.9 % with the addition of a small amount of citric acid (30 mg/L). However, further increase of citric acid lowered the lipid content in *C. vulgaris*. This indicates that a low concentration of citric acid will enhance lipid accumulation, and a high concentration of citric acid will inhibit lipid synthesis in *C. vulgaris*. The reason is that the high concentration of citric acid not only regulates the ACCase activity in fatty acid route, but also interferes with other metabolisms in microalgae. However, adding citric acid could always enhance the growth of *C. vulgaris*. Similarly, adding citric acid could increase the overall lipid productivity of *C. vulgaris*. The maximum overall lipid productivity, 39.1 mg/(L·day), was achieved when 150 mg/L of citric acid were added. The overall lipid productivity was enhanced almost onefold compared to the control (19.9 mg/(L·day)). Besides the ability of citric acid to activate the ACCase, another reason for improving lipid productivity with the addition of citric acid is that it can also be utilized as exogenous organic carbon source by *C. vulgaris*. The addition of exogenous organic carbon source increases the microalgal biomass and lipid content, as has been widely reported (*39–41*). When studying the exogenous glucose supplementation to enhance the biomass and lipid yield of *S. obliquus*, Mandal and Mallick (*39*) found that glucose addition could improve the biomass productivity. In Mandal's report, adding a small amount of glucose could enhance the lipid content from 9.4 to 11.8 %. However, adding more glucose decreased the lipid content from 11.8 to 6.6 %. Our results about citric acid show a similar trend to Mandal's results about glucose.

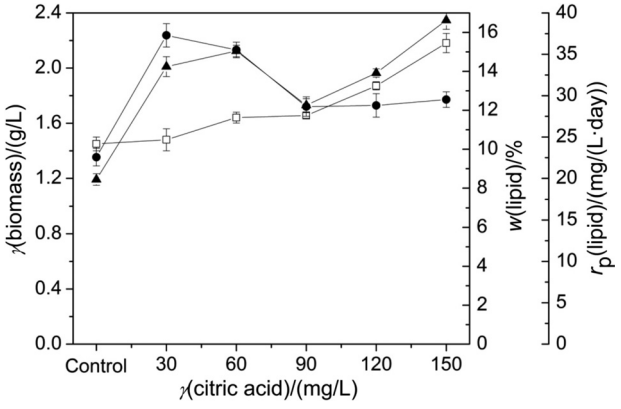

Fig. 5. Improving the biomass and lipid production by *C. vulgaris* with the addition of citric acid
□ biomass, ● lipid content, ▲ lipid productivity (r_P)

Mg^{2+} is a general activator of many enzymes. The published papers show that it is also a valid activator of ACCase. Nikolau and Hawke (*42*) investigated the kinetic properties of the purified ACCase from maize leaf, and found that Mg^{2+} could activate the ACCase from it. Sasaki *et al.* (*43*) studied the ACCase from pea plant (*Pisum sativum* cv. Alaska) chloroplasts, and found that the enzyme showed maximal catalytic activity with 2.5–5 mmol/L of Mg^{2+}. In this work, we investigated the ad-

dition of extra Mg^{2+} (as $MgCl_2$, at 0.6, 0.9, 1.2, 1.5, 1.8 mmol/L) to the basic BG-11 medium to enhance the lipid production of *C. vulgaris*.

The results in Fig. 6 evidenced that adding extra Mg^{2+} could regulate *C. vulgaris* biomass growth and lipid production. The maximum lipid content (16.5 %) and overall lipid productivity (38.0 mg/(L·day)) were obtained at 1.5 mmol/L of Mg^{2+}. The lipid productivity was enhanced almost onefold compared to the control (19.8 mg/(L·day)). These results are consistent with the results of the activation of ACCase by Mg^{2+} *in vitro* reported by Sasaki *et al.* (*43*). This provides a possible technique to enhance lipid productivity by microalgae. $MgCl_2$ is a common and cheap salt (about $110/t). With the addition of just a low concentration of Mg^{2+}, the lipid productivity of *C. vulgaris* can be doubled, which confirms good economical feasibility.

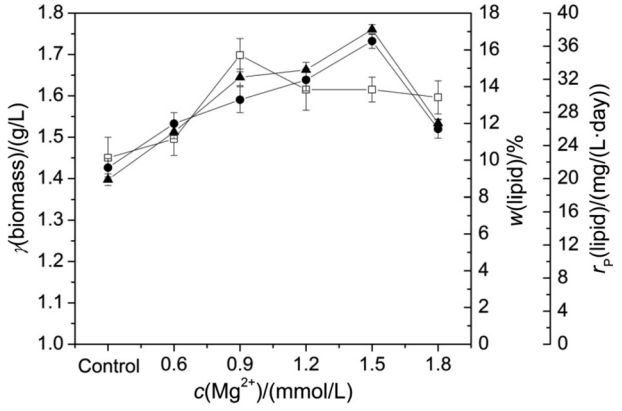

Fig. 6. Improving the biomass and lipid production by *C. vulgaris* with adding extra Mg^{2+}
□ biomass, ● lipid content, ▲ lipid productivity (r_P)

Conclusions

In this work, the conversion of CO_2 into microalgal lipids as biodiesel feedstock by a freshwater microalga *Chlorella vulgaris* was investigated. We determined the parameters that affect lipid accumulation in *C. vulgaris*. Nitrogen source starvation, photoperiod and CO_2 volume fraction are the key factors responsible for the lipid productivity by the freshwater microalga *C. vulgaris*. With 5 % CO_2, 0.75 g/L of $NaNO_3$ and light/dark cycle of 18:6 h, the lipid content and overall lipid productivity reached 14.5 % and 33.2 mg/(L·day), respectively. In addition, we proposed a practicable technique to enhance the lipid productivity based on activating ACCase, which is the key enzyme for fatty acid biosynthesis in microalgae. With the addition of 150 mg/L of citric acid or 1.5 mmol/L of $MgCl_2$ as ACCase accelerant, the lipid productivity reached 39.1 and 38.0 mg/(L·day), respectively, which was almost twofold compared to the control. This work shows the applicability of converting CO_2 into microalgal lipids and provides a potential route to solving the global warming and energy shortage issues.

Acknowledgements

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