Enhanced biomass and lipid production by co-cultivation of *Chlorella vulgaris* with *Mesorhizobium sangaii* under nitrogen limitation

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Abstract

Microagal lipids have been considered as one of the most promising feedstocks for biodiesel production. In order to solve the contradiction of algae growth and lipid accumulation, *Chlorella vulgaris* was co-cultured with *Mesorhizobium sangaii* under nitrogen deficiency conditions. The biomass and lipid production of *C. vulgaris*–bacteria co-culture with initial ratio of algae/bacteria = 40:1 were significantly improved compared with the pure algae culture. The maximum biomass, lipid content and productivity of algae in the co-cultures at 40:1 ratio were 1.89 mg L\(^{-1}\), 51.2%, and 96.77 mg L\(^{-1}\) day\(^{-1}\), respectively, which were 1.5, 2.2, and 3.3 times higher than those of the pure algae culture. Furthermore, the proportion of unsaturated fatty acids and C18:1 fatty acid of the consortium system was also significantly increased. Our study clearly suggests that co-cultivation of algae–bacteria can effectively contribute to the quality and quantity of microalgal bio-oil and shows promising applications for production of algal biomass and biodiesel.

Keywords Co-culture · *Chlorella vulgaris* · Biomass · Lipid production · Fatty acids

Introduction

The demand for renewable and environment-friendly energy sources has increased in recent years due to the limited supply of petroleum reserves and the increasing emission of greenhouse gas from traditional fossil fuels (Zhou et al. 2011). Biodiesel has been deemed as a clean and renewable energy due to many obvious advantages (Sayre 2010).

As one of the third-generation biofuels, microalgae, especially, *Chlorella vulgaris*, has been considered to be one of the most promising candidates and widely studied as biodiesel feedstock because of explicit advantages, including higher growth rate and lipid content and no competition with agricultural food (Chisti 2007; Koller et al. 2014). Their lipid production capabilities have been reported as 14–60% of dry cell weight even under general growth conditions (Spolaore et al. 2006; Lv et al. 2010). Furthermore, the fatty acid composition of algal lipids is in accord with the properties of biodiesel (Takagi et al. 2006; Li et al. 2009), which mainly contains fatty esters derived from palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid (Pinzi et al. 2013). Moreover, through photosynthesis, algal cells can also absorb carbon dioxide and transform light energy into a variety of bio-actives, including lipids, proteins, carbohydrates, pigments, and antibiotics (Plaza et al. 2008), which brings more environmental and economic performances.

However, there are several serious constraints for biodiesel production using algae. One of the significant issues is the natural contradiction of lipid accumulation and biomass production (Li et al. 2011). For most algae, higher lipid contents are usually produced under certain stress conditions, such as nutrient limitation or high light intensity, which always induces with relatively low biomass production and finally results in lower total lipid production (Dash and Banerjee 2017). Therefore, to solve the contradiction of algal growth and lipid accumulation, it is necessary to develop a novel approach to simultaneously increase the biomass production and lipid accumulation of microalgae under certain conditions.
In the natural environment and large-scale open-pond algal cultivation systems, microalgae and microorganisms normally share a complex ecological relationship, which can be either parasitic, mutualistic, or commensal (Ashen and Goff 2000; Sapp et al. 2007; Guo and Tong 2014). Some bacteria can achieve high efficiency in removing pollutants and nutrients from wastewater and promote the biomass production of algae by metabolic complementarity (Ferro et al. 2019). Hernandez et al. (2006) reported that increased efficiency in removing phosphorus from wastewater could be achieved by cocultivation of algae with *Azospirillum brasilense*. The bacterium was firstly purified by spreading on 1.5% BG11 agar plates (Rippka et al. 1979) containing 100 mg L\(^{-1}\) Ampicillin, 50 mg L\(^{-1}\) Kanamycin, and 50 mg L\(^{-1}\) Spectinomycin. After culturing for 7–10 days, the algal colony was picked and spread on fresh agar plates with antibiotics. This procedure was repeated three times to obtain axenic microalgae, and the culture was finally confirmed under microscopy. Axenic *C. vulgaris* was grown at 25 °C under a 14 h light period per day at an irradiance of 120 μmol photons m\(^{-2}\) s\(^{-1}\).

The bacterium *Mesorhizobium sangaii* 13218 was purchased from the China General Microbiological Culture Collection Center (CGMCC) and stored on GYM medium containing 0.4% (w/v) glucose, 0.4% (w/v) yeast extract, and 1.0% (w/v) maltose. All liquid cultures of 13218 were incubated at 30 °C in an orbital shaker at 200 rpm until the OD\(_{600}\) reached 1.0 and then used as the inoculum for the following co-culture experiments.

**Co-culture of *C. vulgaris* and 13218 bacteria**

The axenic *C. vulgaris* and 13218 bacteria were pre-cultivated to exponential phase separately. After centrifugation at 8000 × g for 15 min, the cell pellets were washed twice with BG-11 medium without any N nutrient (BG11-N medium) and re-suspended in fresh BG11-N medium. The initial ratios of algae (OD\(_{750}\) = 1.0) and bacteria (OD\(_{600}\) = 1.0) set in the co-culture system were 10:1, 40:1, 70:1, 100:1, and 300:1 (v/v), respectively. To increase the cell growth of algae, the cultures were bubbled with sterile air (0.95 L min\(^{-1}\)) and CO\(_2\) (0.05 L min\(^{-1}\)) using a gas distributor at the bottom of each shaker. All cultures were at 25 °C under a 14 h light with light intensity of 130 μmol photons m\(^{-2}\) s\(^{-1}\) and 10 h dark cycle for 10 days. The symbiotic morphology of co-cultures was observed using a transmission electron microscope (JEM-1400plus, JEOL, Japan) (Damiani et al. 2010).

**Determination of cell growth and biomass**

The biomass production of *C. vulgaris* was determined by measuring the content of chlorophyll *a* (Chla) in monocultures and co-cultures (Dash and Banerjee 2017). Briefly, after centrifuging at 8000 × g for 15 min, the cell pellets were washed twice with distilled water, and then 96% methanol was added, followed by a sonication bath for 20 min. The Chla concentration of microagal cells was measured spectrophotometrically at 653 and 666 nm (Porra et al. 1989).

The biomass and Chla concentration of *C. vulgaris* were correlated as per the following equation:

\[
\text{Microalgae dry biomass (g)} = 1.249 \times \text{OD}_{653}, R^2 = 0.9985 \quad (1)
\]
The specific growth rate \((\mu, \text{day}^{-1})\) was obtained according to the following equation:

\[
\mu = \frac{(\ln N_f - \ln N_i)}{(t_f - t_i)}
\]

where \(N\) is the biomass concentration \((\text{g L}^{-1})\) at final \((f)\) or initial \((i)\) time \((t)\).

**Lipid extraction**

Total lipid extraction from microalgal cells was according to the procedure of Bligh and Dyer (1959). The lyophilized cells \((W_1)\) were suspended in 5 mL of chloroform/methanol \((2/1, \text{v/v})\) and sonicated for 30 min in an ultrasonic cleaner (KQ-100KDE, Kunshan, China) at 25 °C. After the agitation, the mixture was centrifuged at 8000 \(\times g\) for 15 min at 4 °C and the supernatant was transferred to another pre-weighed tube \((W_2)\). The residues were further extracted twice, and the chloroform phases were combined. After evaporating and drying to constant weight under nitrogen atmosphere, the lipids were gravimetrically quantified \((W_3)\). The lipid content was expressed as % dcw (dry cell weight) as according to the following formula:

\[
\text{lipid content (\%dcw)} = \frac{(W_3 - W_2) \times 100}{W_1}
\]

Neutral lipid contents of each culture were measured by fluorometric straining with Nile Red (NR) (Chen et al. 2009). Briefly, the microalgal cells were first collected by centrifuging at 8000 \(\times g\) for 15 min and re-suspended in 20% DMSO with the absorbance at 540 nm as 1.0. NR was then added with the final concentration of NR as 0.24 μg mL\(^{-1}\). The mixture was kept in dark for 5 min, and the fluorescence intensity was measured using a spectrofluorometer (FluoroMax-4, Horiba, France) at the selected excitation \((520 \text{ nm})\) and emission \((574 \text{ nm})\) wavelengths. As an excellent stain for lipid bodies, NR-stained microalgal cells were determined according to the reported procedures (Genicot et al. 2005). The stained cells were observed and photographed using a fluorescence microscope (Dmi8, Leica, Germany) at 520 nm excitation and 574 nm emission. Lipid bodies fluoresced yellow and chloroplasts red in the microalgae.

**FAME (fatty acid methyl ester) analysis**

Fatty acids in algae were analyzed according to the previously reported method (Wang et al. 2018). Briefly, 20 mg lyophilized cells were dispersed in 0.4 mL of 50% H\(_2\)SO\(_4\) and 2 mL CH\(_3\)OH, and the mixture was incubated at 60 °C for 30 min. After heating, 1 mL of n-hexane was added to the mixture and the organic phase was collected. Heptadecanoic acid methyl ester \((\text{C}_{17}\text{H}_{37}\text{COOCH}_3)\) was used as the standard. The fatty acid composition of algae was analyzed using a GC-Agilent 7890B and HP-5 MS capillary column (Agilent, USA). The temperatures of the injector and detector were 280 and 300 °C, respectively. The initial column temperature was set at 50 °C for 2 min, increased to 280 °C at a rate of 10 °C min\(^{-1}\), and held for 10 min. Nitrogen was used as the carrier gas. The components were identified by comparing their retention times and fragmentation patterns with that of FAME standards.

**EPS analysis**

The protein and polysaccharide concentrations in the extracellular polymeric substances of \(C.\ vulgaris\) were measured according to reported procedures (Wang et al. 2015). Algal cells \((2 \text{ mL})\) were centrifuged at 8000 \(\times g\) for 15 min at 4 °C. The supernatant was filtered through a 0.45 μm membrane to extract the soluble substances. After washing twice with ddH\(_2\)O, the cell pellet was re-suspended and incubated in a water bath at 80 °C for 20 min. The mixture was centrifuged at 8000 \(\times g\) for 15 min at 4 °C and filtered through a 0.45 μm membrane to collect the bound substances. The protein content was measured by the Bradford’s protein assay (Bradford 1976), and bovine serum albumin was used as a standard. The polysaccharide content was detected by the phenol–sulfuric assay using glucose as the standard (Dubois et al. 1956).

**Statistical analysis**

Statistical analysis was performed using SPSS 17.0. All experiments were repeated three times independently. Data were shown as the mean ± standard deviation. Statistical significance was determined at \(P < 0.05\).

**Results**

**Effect of the initial ratios of \(C. vulgaris\) to 13218 on biomass production of co-culture systems**

To improve the biomass and lipid production of microalgae, \(C. vulgaris\) and bacteria 13218 were co-cultured under nitrogen-deprived conditions with continuous bubbling of 5% CO\(_2\). The morphology of co-cultures was observed using a transmission electron microscope. As shown in Fig. 1, the bacteria attached onto the surface of microalgae and the mature consortium was gradually formed with prolonged culture time, which could enhance substance exchange between the algae and bacteria. The initial ratio of algae/bacteria would influence the growth of algae (Shu et al. 2013). Therefore, five different initial ratios of microalgae and bacteria were tested and illustrated in Fig. 2. As a result, the biomass of co-cultures at the ratio of 10:1 or 300:1 showed no significant difference with that of the pure algal culture \((P > 0.05)\). However, the biomass and cell growth of \(C. vulgaris\) in co-cultures with the ratio of 40:1, 70:1, or 100:1 were significantly higher than those in axenic cultures during the cultivation.
(P < 0.05). The maximum biomass production (1.89 mg L\(^{-1}\)) and specific growth rate (0.037 day\(^{-1}\)) of co-culture were observed when the algae/bacteria ratio was 40:1 after 10 days of co-cultivation, which were 1.52 times higher than that of pure algal culture (1.24 mg L\(^{-1}\) and 0.024 day\(^{-1}\), respectively). These results indicated that co-cultivation with 13218 bacteria can significantly enhance the production of algal biomass.

**Lipid accumulation of *C. vulgaris*–13218 bacteria co-cultures**

Different initial ratio of microalgae and bacteria would result in different final lipid contents and biomass productivity of *C. vulgaris*. As shown in Fig. 3a, the lipid content of *C. vulgaris* co-cultured with different ratios of 13218 (initial ratio of algae/bacteria = 40:1, 70:1, and 100:1) ranged from 40.6 to 51.2%, which was significantly higher than that of pure algal culture (34.9%) (P < 0.05). The maximum lipid productivity was 96.77 mg L\(^{-1}\) day\(^{-1}\) with initial ratio of algae/bacteria = 40:1, which was about 2.2 times that of the pure algae culture (43.28 mg L\(^{-1}\) day\(^{-1}\)). Conversely, the final lipid content and productivity under initial ratio of algae/bacteria = 10:1 or 300:1 showed a minor differences from the control culture (P > 0.05). Similar results have been reported that the optimal initial ratio of algae/bacteria existed and had an important effect on the biomass production of microalgae (Shu and Tsai 2016).
The neutral lipid accumulation of *C. vulgaris* was also characterized when co-cultured with bacteria 13218 strain. Within the initial algae/bacteria ratio ranging of 40:1 to 100:1, a significantly increasing trend of neutral lipid contents and productivity was observed (Fig. 3b). Maximum neutral lipid content and productivity were 32.2% and 60.86 mg L$^{-1}$ day$^{-1}$ of *C. vulgaris*, respectively, at the algae/bacteria ratio of 40:1, which were 2.2 and 3.3 times that of the pure algae culture (14.7% and 18.23 mg L$^{-1}$ day$^{-1}$, respectively). Oil droplets in co-cultures of 40:1 (algae/bacteria) showed a significant increase compared to the control group (Fig. 4) ($P < 0.05$). Meanwhile, a significant difference was observed between neutral lipid proportion (% total lipid) of microalgae in algae–bacteria co-cultures under the ratio of 40:1, 70:1, or 100:1 and the pure algae culture ($P < 0.05$). Minor differences of overall maximum neutral lipid content and productivity were observed with co-cultures with initial ratio of 10:1 (17.1% and 23.60 mg L$^{-1}$ day$^{-1}$, respectively) and 300:1 (16.4% and 21.16 mg L$^{-1}$ day$^{-1}$, respectively). These results indicated that the co-cultivation system of *C. vulgaris* and 13218 bacteria can increase the lipid accumulation of algae and the ratio of 40:1 (*C. vulgaris* and 13218 bacteria) was optimum for the growth and lipid accumulation of *C. vulgaris*–13128 co-culture.

**Fatty acid composition of algal oils produced from the co-culture and mono-culture**

The C16 and C18 fatty acids were the main components of *C. vulgaris* (Fig. 5a), which are the ideal sources for biodiesel production (Tang et al. 2011), contributing between 79 and 85% of the total fatty acids. The fatty acid profiles obtained in this study were consistent with previously reported data.

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**Fig. 3** Variation of total lipid (a) and neutral lipid accumulation (b) under different ratios of *C. vulgaris*/13218 after the 10-day co-cultivation
In addition, the content of C16/C18 in cocultures at the initial ratio of 40:1, 70:1, or 100:1 was higher than the pure algae culture. Oleic acid (C18:1), a key metric of biodiesel quality (Ho et al. 2010), accounted for 39.86, 37.19, and 36.73%, respectively, of the total fatty acids in the coculture system within the range 40:1 to 100:1, which were higher than that in pure algae culture (31.15%). As shown in Fig. 5b, the monounsaturated fatty acid (MUFA) content of algae–bacteria co-cultures ranged from 50.74 to 52.13%, and the polyunsaturated fatty acid (PUFA) content ranged from 14.23 to 14.95%, which were both higher than that in the pure algae (48.39 and 11.99%, respectively). The content of saturated fatty acid (SFA) showed a consistent decrease in cocultures compared to the control group.

Contents of protein and polysaccharide in the co-culture and mono-culture

It has been reported that the EPS plays a key role in the stability of activities and structures in co-cultures (Munoz et al. 2009). The contents of protein and polysaccharide in the supernatant and bound were determined and shown in Fig. 6. The protein concentration of EPS in bound of pure C. vulgaris culture was increased from 5.27 to 30.92 mg L$^{-1}$ after 10 days of cultivation (Fig. 6a). In the co-culture system with initial ratio of algae/bacteria = 40:1, the bound protein concentration was increased in the range of 5.15 to 24.68 mg L$^{-1}$ after 10 days, which was lower than that in pure algae culture. In contrast, the supernatant protein concentration in the co-cultures ranged from 2.04 to 11.94 mg L$^{-1}$, which was higher than that in the control with 7.65 mg L$^{-1}$ after 10 days of cultivation.

As shown in Fig. 6, the polysaccharide concentration in bound of pure C. vulgaris culture increased from 5.09 to 30.01 mg L$^{-1}$ after cultivation for 6 days, which reached approximately 35.87 mg L$^{-1}$ after 10 days of cultivation. In addition, the bound polysaccharide concentration in cocultures at the ratio of 40:1 (algae/bacteria) was higher than that in the control after 10 days culture and the changes of the polysaccharide concentration in supernatant were contrary to
those of polysaccharide concentration in bound. The supernatant polysaccharide concentration of *C. vulgaris* in the pure algae culture increased from 3.16 mg L$^{-1}$ on day 2 to 23.46 mg L$^{-1}$ on day 10, which was higher than that obtained in the co-cultures.

**Discussion**

Microalgae have been considered to be one of the most promising candidates for biodiesel production. In order to solve the contradiction of algae growth and lipid accumulation, *C. vulgaris* was co-cultured with five different bacteria (Fig. S1). As a result, *C. vulgaris* in co-cultures with *Mesorhizobium sangaii* (13218) shows maximum biomass concentration and lipid content than that in monocultures and other co-cultures during the cultivation. Therefore, the 13218 strain was chosen as the optimal condition for the studies on co-culture systems. As a nitrogen-fixing aerobic bacteria species, 13218 strain is thought to absorb nitrogen from the environment and then may supply some nitrogen sources within the algae–bacteria symbioses system. Then, the N and C budget of the samples was measured by elemental analyzers (Vario EL III, Germany). As shown in Table S1, the N
and C budget of co-cultures after the 10-day cultivation was higher than that of pure algae culture. These results indicated that the bacterial component of the consortium could absorb nitrogen and CO₂ from the environment, which could benefit and enhance the biomass and lipid production of algae.

Not only the selected 13218 strain but some other bacteria also have been reported to increase microalgal biomass after co-culture with *C. vulgaris*. For example, *Rhizobium* has been reported to stimulate the growth of several species of algae, such as *C. vulgaris* and *Scenedesmus obliquus* (Kim et al. 2014; Cho et al. 2015). Similarly, *Pseudomonas diminuta*, a strain isolated from microalgal cultures, can also play a key role in mutualistic interactions with *Scenedesmus bicuscularis* (Mouget et al. 1995). The reason for this enhancement might be that the algae–bacteria co-cultures can form a symbiotic system and exchange more resources under the nutrient limited conditions compared to the monocultures, resulting in increased algal growth and lipid accumulation (Weis et al. 2008). In the bacteria–algae system, bacteria have been reported to release a lot of extracellular metabolites, such as amino acids, enzymes, vitamins, carbohydrates, and lipids, as a byproduct of its respiratory metabolism for the growth of algae (Moroney and Somanchi 1999). Meanwhile, algal cells can secrete nutrients produced by photosynthesis to support bacteria growth (Sayre 2010). Algal and bacterial growth is also promoted through metabolic regulations and material exchange. In this study, *C. vulgaris* and 13218 bacteria could cooperate with each other in the utilization of limited resources under nutrition-deprived conditions, benefitting growth and biomass accumulation in the co-culture system. In addition, the synergistic effects were more likely to have an O₂/CO₂ balance effect in co-cultures (Cheirsilp et al. 2012). Bacteria release CO₂ as a byproduct of respiration (Moroney and Somanchi 1999), and algal cells can then capture the CO₂ by photosynthesis and convert this into algal biomass (Sayre 2010).

The initial ratios of algae/bacteria are important factors for construction of this bacteria/algae symbiosis system and have significant effects on the lipid accumulation of the algae. Our results are consistent with previous reports (Wang et al. 2016; Xie et al. 2016), which demonstrated that an optimal ratio of algae/bacteria existed for the growth and the lipid accumulation of algal cells in co-cultivation systems and had a marked effect on the total lipid content and production (Dash and Banerjee 2017). At a higher algae–bacteria ratio, the bacteria cannot effectively consume the O₂ from algal photosynthesis and provide enough nitrogen nutrients for algal growth, resulting in lower biomass and lipid accumulation. Similarly, a lower algae–bacteria ratio would result in a lower cell density of algal cells thus decreasing lipid accumulation.

In this study, the lipid production of bacteria was quite low in the co-cultivation system, and, therefore, lipid accumulation was primarily supported by *C. vulgaris*. According to our GC results, the C16 and C18 fatty acids were the two main components of *C. vulgaris* oil, consistent with the previously reported data (Shekh et al. 2013), indicating the co-culture used in this study could be used as a potential biodiesel feedstock (Gui et al. 2008). Meanwhile, the high proportion of unsaturated fatty acids in the co-culture oils could be instrumental in ameliorating the cold temperature performance and kinematic viscosity of the microbial oil (Knothe 2011). The changes of the fatty acid proportion in co-cultures might be due to the metabolic regulation and nutrient exchange between microalgae and bacteria (Gyurjan et al. 1984).

EPS, containing mainly of proteins and polysaccharides (Watanabe et al. 2006), plays a major role in the symbiosis of algae and bacteria (Viret et al. 1994), and the variations of EPS concentrations have the potential to influence the algae and bacteria system (Imase et al. 2008). In our co-cultivation system, with initial ratio of algae/bacteria = 40:1, the bound protein concentration was lower than that in pure algae culture. In contrast, the supernatant protein concentration in the co-cultures was higher, which was similar with the previous study (Wang et al. 2015). This is thought to be mainly because the bacteria in co-cultures can decompose the protein in the bound state and release them to the supernatant, which could be used for the growth of algae. Consequently, the growth of algal cells in the co-culture system could increase under nitrogen-deprived conditions. Meanwhile, the accumulation of polysaccharide in supernatant has been reported to inhibit the growth of algae (Subashchandrabose et al. 2011). In our study, the supernatant polysaccharide concentration in co-cultures was reduced compared to the control. These results indicated that the bacteria in co-cultures might use polysaccharide as a source of carbon and energy, which could reduce the rate of polysaccharide accumulation. In addition, the bound polysaccharide concentration in co-cultures was higher than that of the pure algal culture and the increase of bound polysaccharide can strengthen the symbiosis between algae and bacteria, which could be a benefit for the consortium stability of the co-cultivation system.

**Conclusions**

The co-cultivation of *C. vulgaris* and bacterium *M. sangaii* under nitrogen limitation significantly improved the biomass and lipid production of microalgae. The initial ratio of algae/bacteria had a significant effect on the growth and lipid accumulation of algae. The maximum biomass, lipid content, and productivity of algae were 1.89 mg L⁻¹, 51.2%, and 96.77 mg L⁻¹ day⁻¹, respectively, at the initial ratio of algae/bacteria = 40:1, which were 1.5, 2.2, and 3.3 times higher than the pure algae culture. Furthermore, C16:0 and C18:1 fatty acids were the major fatty acids of the co-culture oils indicating the co-culture oils could be used for potential biodiesel
production. The concentrations of protein and polysaccharide in the supernatant and bound were significantly changed by the addition of *M. sangaii* 13218 bacteria, accounting for the improvement of algal biomass and lipid production. This study provides a promising method for increasing the biomass and lipid production of microalgae.

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**Author contributions** Conceived and designed the experiments: BG, LX, and ZW. Performed the experiments: ZW, HW, XL, HL, QZ, and YY. Analyzed the data: BG, ZW, and LX. Contributed reagents/materials/analysis tools: BG and ZW. Wrote the paper: BG, ZW, and SQ.

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