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ORIGINAL ARTICLE

Lipid production and composition of fatty acids in *Chlorella vulgaris* cultured using different methods: photoautotrophic, heterotrophic, and pure and mixed conditions

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Abstract This study investigated the biomass, lipid production, fatty acid content, and other nutrients present in microorganisms by using four culture methods: (1) photoautotrophic pure *Chlorella vulgaris* cultures (PP); (2) heterotrophic pure *C. vulgaris* cultures (PH); (3) mixed cultures of *Rhodotorula glutinis* and *C. vulgaris* under photoautotrophic conditions (MP); and (4) heterotrophic mixed cultures (MH). The microorganisms in MP culture showed the optimum growth condition and lipid production. Among the cultures, MP yielded the highest number of cells and biomass (5.9×10^5 cells/mL and 0.523 g/L, respectively). Furthermore, lipid production in MP culture was 114.22 mg/L, which is 136 % higher than that in MH culture (48.22 mg/L). Considering the higher contents of palmitic acid (C16:0) at 24.65 %, oleic acid (C18:1) at 56.34 %, and protein at 42.39 g/100 g in the MP culture than in other cultures, we proposed that MP could be used effectively to support the growth of microorganisms. This method could also be used as a potential approach for biodiesel production.

Keywords *Chlorella vulgaris* · Photoautotroph · Heterotroph · Mixed culture · Lipid production

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Introduction

With the exacerbating crisis of crude oil, studies have focused on the lipid production of oleaginous microorganisms (Cooksey et al. 1987; Crabbe et al. 2001). Various microorganisms, such as algae, bacteria, yeast, and molds, can be used as a raw material of biodiesel (Xue et al. 2010). Several characteristics, such as high growth rates, simple cell structures, and fatty acid compositions similar to those of vegetable lipid (Saenge et al. 2011), indicate that such microorganisms can be potentially used as feedstock in biodiesel production.

Metting and Pyne (1996) and Liu et al. (2008) suggested that autotrophic microalgae, among oleaginous microorganisms, can convert CO_2 to biofuels such as oil and biohydrogen by photosynthesis. Liang et al. (2009) reported that *Chlorella vulgaris* is one of the most promising feedstocks because of its lipid capabilities (30 % to 40 % of dry weight). In addition, microalgal cells can be cultured under heterotrophic and mixotrophic conditions. Heterotrophic cells utilize organic substrates, whereas mixotrophic cells absorb light and use inorganic and organic substrates, as energy and carbon sources, respectively (Borowitzka 1999).

By contrast, Zhu et al. (2008) found that yeast can produce high amounts of lipid contents. Li and Wang (1997) also revealed that yeast lipid provides many advantages. Meng et al. (2009) showed that *Rhodotorula glutinis* can produce a maximum lipid amount of 72 % of dry weight. The mixed cultivation of *Rhodotorula glutinis* and microalgae *Spirulina platensis* could boost biomass and lipid accumulation (Xue et al. 2010). In the mixed culture, the yeast provided CO_2 to the microalgae, whereas the microalgae could act as an oxygen generator for the yeast. Therefore, the red yeast *Rhodotorula glutinis* was considered as a part of the mixed culture.

Although lipid production has been improved, mixed cultures of different species have rarely been used for lipid production under photoautotrophic and heterotrophic

conditions. In this study, the biomass and lipid production of a mixed culture were compared with those of pure cultures. This study aimed to compare the lipid and fatty acid composition of *C. vulgaris* and *R. glutinis* that were cultivated under photoautotrophic and heterotrophic conditions, to provide scientific evidence for the biodiesel industry.

Materials and methods

Microorganisms and growth conditions

Yeast R. glutinis 2.704 (China General Microbiological Culture Collection Center) was used in this study. *R. glutinis* was grown in a malt extract agar slant (agar 20 g/L malt extract) at 26 °C for 48 h. The cells were transferred to a 1,000 mL flask containing 600 mL of liquid culture medium. Approximately 1 L of liquid culture medium contained 15 g of glucose, 2 g of $(NH_4)_2SO_4$, 1 g of yeast extract, 7 g of KH_2PO_4, 2 g of Na_2PO_4, and 1.5 g of $MgSO_4$. pH was adjusted to 6.0 by using 1 mol/L NaOH/HCl solution. The flasks containing the seed culture were incubated at 26 °C for 3 days.

Pure C. vulgaris cultures under photoautotrophic conditions (PP) The microalgae *C. vulgaris* FACHB-31 (Freshwater Algae Culture Collection of the Institute of Hydrobiology) was incubated in blue-green medium (BG11 Medium) at 26 °C at a light intensity of 4.0 klux by using GXZ intellectualized illumination incubator (Jiangnan Instrument Factory, Ningbo, China) for 72 h, with a 12 h:12 h light: dark cycle for a seed culture. Approximately 1 L of BG11 medium contained 1.5 g of $NaNO_3$, 0.04 g of K_2HPO_4, 0.075 g of $MgSO_4 \cdot 7H_2O$, 0.036 g of $CaCl_2 \cdot 2H_2O$, 0.006 g of citric acid, 0.006 g of ferric ammonium citrate, 0.001 g of $EDTANa_2$, 0.02 g of Na_2CO_3, and 1 ml/L of A5 solution. Each liter of A5 solution contained 2.86 g of H_3BO_3, 1.86 g of $MnCl_2 \cdot 4H_2O$, 0.22 g of $ZnSO_4 \cdot 7H_2O$, 0.39 g of $Na_2MoO_4 \cdot 2H_2O$, 0.08 g of $CuSO_4 \cdot 5H_2O$, and 0.05 g of $Co(NO_3)_2 \cdot 6H_2O$. pH was adjusted to 7.0 with 1 mol/L of NaOH/HCl solutions.

Pure C. vulgaris cultures under heterotrophic conditions (PH) For heterotrophic growth, the seed cells were incubated in BG11 medium containing 30 g/L of glucose. The flasks containing 700 mL of medium sterilized in an autoclave were inoculated with 10 % seed culture of microalgae. The initial cell count of microalgae was 2.3×10^5 cells/mL. The cultures were cultivated for 10 days at 26 °C in the dark. All of the experiments were repeated at least twice.

Mixed cultures under photoautotrophic conditions (MP) For mixed cultures, the flasks containing 700 mL of BG11 medium sterilized in an autoclave were inoculated with 10 % seed culture mixture of yeast and microalgae. The initial cell counts of yeast and microalgae were 2.9×10^5 and 2.3×10^5 cells/mL, respectively. The cultures were cultivated for 10 days at 26 °C at a light intensity of 4.0 klux with a 12 h:12 h light:dark cycle. All of the experiments were repeated in duplicate.

Mixed cultures under heterotrophic conditions (MH) For heterotrophic growth, the seed cells were inoculated in a BG11 medium containing 30 g/L of glucose. The flasks containing 700 mL of medium sterilized in an autoclave were inoculated with 10 % seed culture mixture of yeast and microalgae. The initial cell counts of yeast and microalgae were 2.9×10^5 and 2.3×10^5 cells/mL, respectively. The cultures were cultivated for 10 days at 26 °C in the dark. All of the experiments were repeated at least twice.

Microorganism cell amount and dry cell weight

The individual cell counts of yeast and microalgae were determined using a hemocytometer (Cai et al. 2007). The cells were centrifuged at 9,000 rpm for 5 min. The cells were washed with distilled water thrice and dried in a freezing vacuum until a constant weight was obtained. The powdered microorganism was subsequently cooled to room temperature in a desiccator before the weight was obtained (Kavadia et al. 2001).

Lipid extraction and analysis

The cells were harvested and lyophilized for lipid extraction and analysis. Total lipid was extracted from 300 mg of lyophilized biomass with a solvent mixture of chloroform, methanol, and water (2:1:0.75 by vol.) according to the modified Folch procedure (Folch et al. 1957). The extract was dried in a rotary evaporator, weighed, re-suspended in chloroform, and stored at 20 °C in nitrogen gas to prevent lipid oxidation.

Fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by acid transesterification (Jham et al. 1982). In brief, the lyophilized cells were incubated overnight with a solvent mixture of toluene and 1 % sulfuric acid in methanol (1:2, v/v) at 50 °C to produce FAMEs that were then extracted with hexane. FAMEs were analyzed using an Agilent 6,890 N capillary gas chromatograph equipped with a flame ionization detector (FID) and an Agilent 19091S-433 HP-5MS capillary column (30 m×0.25 mm). Helium was used as carrier gas. Initial column temperature was set at 60 °C, which was progressively raised to 280 °C at 10 °C/min. The injector was retained at 250 °C with an injection volume of 2 μL in a splitless mode. FAMEs were identified by chromatographically comparing with authentic standards (Sigma). The quantities of individual FAMEs were estimated from the peak areas on

the chromatogram by using heptadecanoic acid (Sigma) as an internal standard.

Crude protein analysis

Protein content was estimated by the Kjeldahl method using a KDY-9820 Kjeldahl apparatus (Beijing, China). After digestion, distillation, and titration were performed, the crude protein content was calculated by multiplying the nitrogen content by a factor of 6.25 (GB 5009.5-2010).

Total carbohydrate

The water-soluble sugar and water-insoluble polysaccharides in the sample were hydrolyzed with hydrochloric acid to form reducing sugar. The hydrolyzate was rapidly dehydrated with sulfuric acid to yield a furfural derivative and synthesized an orange solution containing phenol. Total carbohydrate was determined by using the external standard method (GB/T 15672-2009) at an absorbance of 490 nm.

Chlorophyll

Chlorophyll was extracted from the sample with acetone and then layered with diethyl ether. After the sample was washed with sodium sulfate solution, the purified chlorophyll sample was quantified at 642 and 660 nm wavelength by colorimetry (SN/T 1113-2002).

Phycocyanin

The sample was dissolved in phosphate buffer solution, frozen, and thawed to separate the pigment-protein from the cells. Phycocyanin content was measured by spectrophotometry (SN/T 1113-2002).

Statistical analysis

Data were analyzed in triplicate, presented as mean ± S.D., and analyzed by t-test ($P<0.05$) to detect significant differences in various culture conditions of microorganisms.

Results and discussion

Growth conditions

Microalgae can grow under autotrophic conditions by utilizing radiant energy from the sun or heterotrophic conditions by transferring carbohydrate to carbon and energy sources (Orosa et al. 2000; Ip and Chen 2005; Sun et al. 2008). The growth parameters of microorganisms grown under four different culture conditions were measured in batch cultures. The microorganisms were then grown in the same culture conditions and basal medium, except carbon sources and light conditions, to ensure that the microorganisms were all subjected to ideal growth conditions (data not shown). The growth and biomass of microalgae and yeast cells at 8 days of cultivation were compared among the four culture methods (Figs. 1, 2 and 3).

The number of cells continuously increased until the end of cultivation. For the first 3 days of cultivation, the difference in the number of microalgae among the four culture methods was not significant. The biomass of microorganisms in the MP culture was higher than that in other cultures from 3 days (Fig. 3) as a result of the significant growth rate of yeasts in MP culture (Fig. 2). The stationary phase of the yeast growth curve was observed at an earlier period than that of the microalgae growth curve, indicating that the yeast may have dominated the mixed culture in terms of the number of cells; therefore, postulating that the yeast better favored the mutualism (Cheirsilp et al. 2012) is reasonable. The growth condition in MP culture was more favorable than that in the pure yeast culture, because the presence of microalgae promoted the growth of yeast.

After 7 days, the biomass of microorganisms decreased because of depleted nutrients in the culture medium used. However, the growth rates in PH and MP cultures were higher than those in the other two cultures. The biomass of microorganisms in MP and PH cultures increased faster than in the other two cultures. After 5 days of cultivation, the MP culture produced the largest number of cells and highest biomass among the cultures.

Although the biomass and lipid content of the heterotrophic microalgae are approximately four times higher than those in photoautotrophic microalgae (Miao and Wu 2004; Xu et al. 2006), the mixed cultures containing yeast under photoautotrophic conditions were more beneficial for biomass growth under a more economical culture condition. This higher productivity is attributed to the mutualistic relationship between

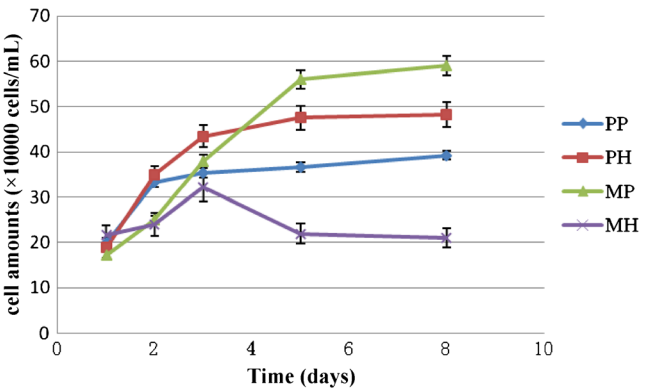

Fig. 1 Microalgae cell amounts in pure *Chlorella vulgaris* cultures under photoautotrophic conditions (PP), pure *Chlorella vulgaris* cultures under heterotrophic conditions (PH), mixed cultures under photoautotrophic conditions (MP), and mixed cultures under heterotrophic conditions (MH)

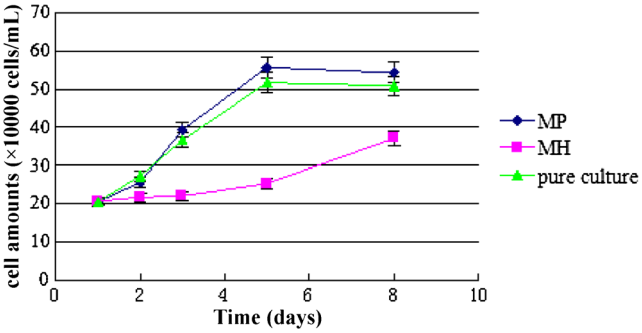

Fig. 2 Yeast cell amounts in pure yeast cultures, mixed cultures under photoautotrophic conditions (MP), and mixed cultures under heterotrophic conditions (MH)

the two species in the mixed culture. Xue et al. (2010) monitored the dissolved oxygen in *R. glutinis* culture after *S. platensis* was added to the culture and found that microalgae can provide additional oxygen concentration for yeasts to enhance aerobic metabolism. CO_2 produced during yeast metabolism can be used by microalgae in photosynthesis (Cheirsilp et al. 2012). In addition, the mixed culture was adjusted to the appropriate pH level to maintain a stable acidic environment favorable for yeast growth (Fig. 4).

Lipid class composition

Lipid was extracted from microalgal cells grown in PP, PH, MP, and MH cultures, and observed for 10 days to investigate the lipid production of microorganisms (Fig. 5). The highest biomass concentration was observed in the MP culture; thus, lipid production was higher in the MP culture than in other cultures. The microorganisms grown in the MP culture could accumulate a maximum of 114.22 mg of lipids in 1 L of microorganism culture liquid; the lipid content was approximately 100 % higher than that in the PP culture (64 mg/L) at 7 days. By contrast, the lipid production of the pure yeast culture under the same culture conditions is 86.31 mg/L. In addition, the initial cell amounts of the yeast and microalgae in

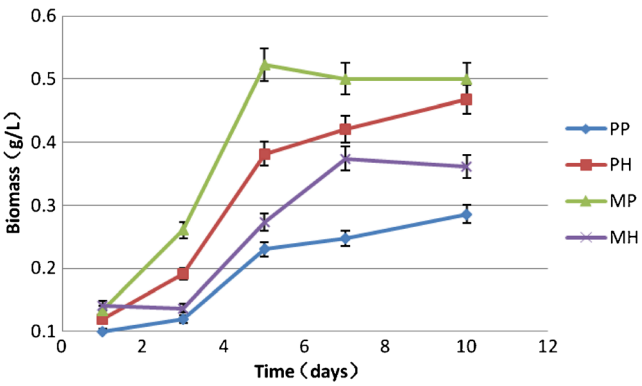

Fig. 3 Microorganism biomass in pure *Chlorella vulgaris* cultures under photoautotrophic conditions (PP), pure *Chlorella vulgaris* cultures under heterotrophic conditions (PH), mixed cultures under photoautotrophic conditions (MP), and mixed cultures under heterotrophic conditions (MH)

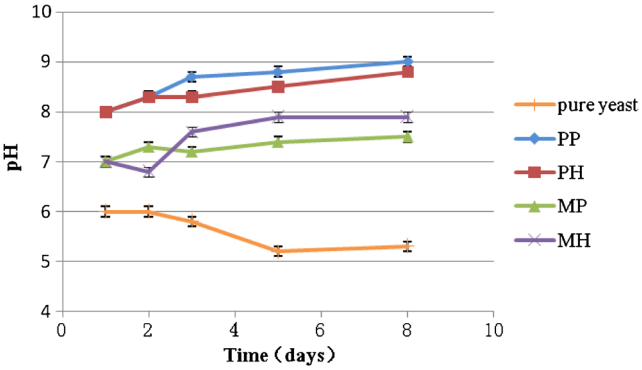

Fig. 4 Microorganism culture pH in pure yeast cultures, pure *Chlorella vulgaris* cultures under photoautotrophic conditions (PP), pure *Chlorella vulgaris* cultures under heterotrophic conditions (PH), mixed cultures under photoautotrophic conditions (MP), and mixed cultures under heterotrophic conditions (MH)

the MP and MH mode are similar, but the lipid production under the MP mode is higher than that under the MH mode (52.47 mg/L). Therefore, the reasons for increased lipid production might include the extra lipid from the yeast, as well as the increase in biomass concentration in the mixed culture compared with the pure cultures, and the interaction between the two species.

At 10 days, a slight decrease in lipid production was observed. This result could be attributed to the cellular degradation of storage lipids during microorganism metabolism or the depletion of carbon source. Mujtaba et al. (2012) found that total lipid productivity and content tend to increase as CO_2 supply increases. Culture termination of the yeast at 10 days (Fig. 2) caused a decrease in CO_2 supply. Lipid production decreased with carbon source consumption (Papanikolaou et al. 2004; Fakas et al. 2007). Moreover, Pruvost et al. (2011), emphasized by the marked effects of nitrogen starvation, which triggers triacylglycerol accumulation while affecting sugar and protein contents (Li et al. 2005; Taha et al. 2010). In addition, lipid and other storage material

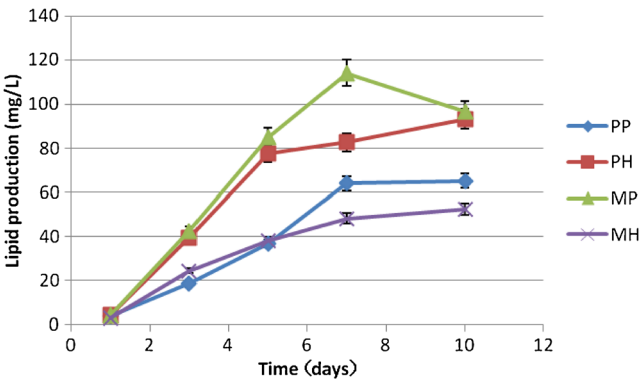

Fig. 5 Lipid production of microorganism in pure *Chlorella vulgaris* cultures under photoautotrophic conditions (PP), pure *Chlorella vulgaris* cultures under heterotrophic conditions (PH), mixed cultures under photoautotrophic conditions (MP), and mixed cultures under heterotrophic conditions (MH)

Table 1 Percentage composition of fatty acids in microorganism with different culture methods (%)

	Pure cultures		Mixed cultures	
	Photoautotrophic conditions	Heterotrophic conditions	Photoautotrophic conditions	Heterotrophic conditions
C15:0	0.11±0.02[a]	3.87±0.11[c]	1.27±0.01[b]	6.25±0.03[d]
C16:0	44.99±0.21[d]	28.01±0.77[c]	24.65±0.29[b]	29.89±0.34[a]
C16:1	5.86±0.03[b]	8.42±0.06[d]	4.44±0.24[a]	7.12±0.31[c]
C16:2	ND	1.06±0.01	1.28±0.00	ND
C17:0	5.72±0.12	ND	ND	1.9±0.07
C17:1	0.29±0.00	3.87±0.03	ND	ND
C18:0	1.09±0.01[a]	10.76±0.01[d]	9.15±0.01[c]	6.97±0.02[b]
C18:1	1.67±0.01[a]	32.89±0.10[b]	56.34±0.22[d]	42.67±0.01[c]
C18:2	25.4±0.29[c]	5.71±0.01[b]	ND	5.19±0.01[a]
C18:3	12.49±0.37	ND	ND	ND
C19:1	2.6±0.22[b]	5.02±0.15[c]	2.12±0.01[a]	ND

accumulation processes can be attributed to several factors, including solar radiation, water temperature, and phosphate content (Crowe et al. 2012). Sukenik et al. (1989) indicated that low light penetration results in low lipid and carbohydrate synthesis. Therefore, the degradation of lipid, as shown in Fig. 5, is probably related to low light penetration in increased biomass and cell density (Bellou and Aggelis 2012).

Although this culture technique exhibited a slight improvement as a result of cost-effective large-scale biodiesel production, high operational costs of cultures hamper the development of lipid production by microorganisms. Nevertheless, biodiesel production based on the autotrophic growth of microalgae can be a technically feasible and economical solution. However, a high density of microalgal biomass is difficult to obtain in a PP culture because light penetration is inversely proportional to the number of cells (Chen and Johns 1991, 1995). To overcome this challenge, many researchers increased biomass and lipid production by using cultured microalgae under heterotrophic conditions with organic carbon sources (Miao and Wu 2004; Xu et al. 2006; Li et al. 2007). Furthermore, the amount of produced lipid is significantly higher under heterotrophic conditions than under autotrophic conditions (65.1 and 93.333 mg/L in PP and PH cultures; Fig. 5); however, organic carbon sources are high in cost. Previous studies showed that the MP culture is more beneficial and cost effective, without the need for additional organic carbon sources. For the same microorganism strain, mixed cultures under photoautotrophic conditions can be used as more feasible and economical feedstock to produce biodiesel compared with those under photoautotrophic or heterotrophic conditions.

Fatty acid composition of individual lipid classes

As a feedstock for biodiesel production, microorganisms should exhibit characteristics similar to the standard, such as lipid composition and fatty acid ester structure, including carbon chain length, branching of the chain, and degree of unsaturation (Knothe 2005). To determine the fatty acid composition of biodiesel, we performed esterification with methanol by potassium hydroxide catalysis after hydrolysis. Table 1 presents the fatty acid composition of lipids extracted from microorganisms grown under the four culture conditions. The length of fatty acid chains ranged from C15:0 to C19:1. The main fatty acids comprised long-chain fatty acids with 16 and 18 carbon atoms, particularly C16:0 (palmitic acid) and C18:1 (oleic acid). The lipids obtained from the pure microalgal culture, or PP culture, contained mostly palmitic acid (44.99 %) and linoleic acid (25.4 %). The fatty acid composition and content of the other three cultures were similar.

The composition and structure of fatty acid esters determine the properties of a biodiesel fuel, such as cetane number, heat of combustion, and viscosity. These properties are directly

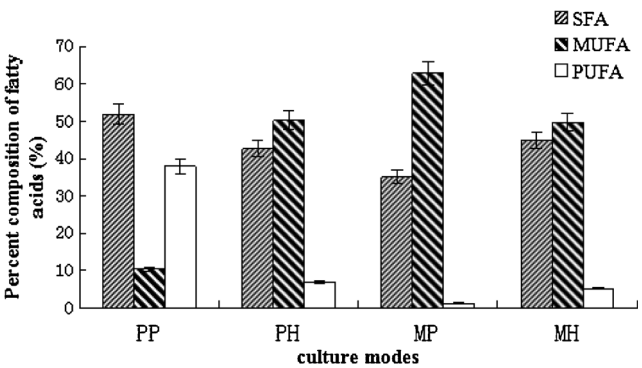

Fig. 6 Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acid (PUFA) content comparison of microorganisms in pure *Chlorella vulgaris* cultures under photoautotrophic conditions (PP), pure *Chlorella vulgaris* cultures under heterotrophic conditions (PH), mixed cultures under photoautotrophic conditions (MP), and mixed cultures under heterotrophic conditions (MH)

Table 2 Comparison of microorganism nutrients under different culture conditions

	Pure cultures		Mixed cultures	
	Photoautotrophic conditions	Heterotrophic conditions	Photoautotrophic conditions	Heterotrophic conditions
Total carbohydrate (%)	40.83±1.09[a]	53.75±3.24[b]	38.63±1.17[a]	60.25±4.34[c]
Protein (g/100 g)	23.48±4.17[b]	11.3±1.22[a]	42.39±7.54[c]	23.35±4.34[b]
Chlorophyll (g/100 g)	0.97±0.36[b]	0.25±0.12[a]	0.98±0.64[b]	0.07±0.06[a]
Phycocyanin (g/100 g)	1.20±0.99[a]	1.60±1.33[a]	1.29±0.72[a]	1.79±1.49[b]

proportional to chain length and inversely proportional to unsaturation degree (Knothe 2009). For example, the cetane number of palmitic methyl ester (16:0) is 74.5, which is higher than that of palmitoleic methyl ester (16:1) at 51.0, and lower than that of stearic methyl ester (18:0) at 86.9. Without reducing the cetane number to an unacceptably low level, the improvement of oleic acid (C18:1) in the fatty acid profile has become a compromise between oxidative stability and cold flow. In Table 1, the highest oleic acid content was observed in the MP culture among the four cultures. This result indicated that the mixed cultures under photoautotrophic conditions can potentially be used as biodiesel feedstock.

Large-scale unsaturated fatty acid production from microorganisms is of great interest among researchers. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) have a significant function in growth conditions. Unsaturated fatty acids (UFA) and MUFA in the MP culture were higher than those in other cultures; PUFA in the PP culture was higher than that in other cultures (Fig. 6). The changes in fatty acid composition can be achieved under different culture conditions. The MP culture showed an increased UFA and MUFA accumulation; by comparison, the PP culture exhibited an increased PUFA accumulation.

Nutrient analysis

The nutritional value of microorganisms is influenced by their shape, size, biochemical composition, and digestibility (Webb and Chu 1983). The variations in nutritional content were investigated because of the different culture conditions presented in Table 2. The intracellular total carbohydrate under heterotrophic conditions was higher than that under photoautotrophic conditions. However, the protein content of microorganisms was unrelated to nutritional type; the MP culture yielded the highest protein content among the cultures. The chlorophyll content under photoautotrophic conditions was higher than that under heterotrophic conditions because of light conditions. Considering that the difference between MP and PP cultures was not significant, we found that the chlorophyll content may seldom be influenced by a mixed culture. The phycocyanin content, which was higher than the three other cultures, did not exhibit any significant difference, as in the PH culture.

Naidu et al. (1999) conducted acute and subacute studies on both sexes of albino rats to demonstrate the safety of *Rhodotorula* as a source of nutritional additive. Its use as a feed additive for laying hens has also been reported (Eugenia et al. 1997). In accordance with previous reports (Buzzini and Martini 2000; Buzzini 2000), *Rhodotorula glutinis* strain can be a suitable candidate for biotechnological applications, particularly for carotenoid and ergosterol production. Such product can be an additional natural source of nutrition factors in the feed and food industries (Marova et al. 2010).

Microalgae are rich in protein, carbohydrates, pigments, and essential fatty acids, which make them a potentially healthy food (Benevides et al. 1998). Significant differences were observed in the pigment and biochemical composition of microalgae cultured under different photoperiods and irradiances (Seyfabadi et al. 2011). For example, the high protein content in an MP culture may be dependent on growth habitats and environmental conditions compared with that in other culture conditions. Similar results attributed to seasonal alterations have been demonstrated for numerous macrophytic algae (Durako and Dawes 1980).

Conclusion

The nutritional type (autotrophs and heterotrophs) and the mixed culture of *C. vulgaris* and *R. glutinis* have important functions in microorganism growth, lipid production, fatty acid profile, and nutritional value. In general, the mixed culture of *C. vulgaris* and *R. glutinis* under photoautotrophic conditions demonstrated higher biomass, total lipid yield, and oleic acid content than photoautotrophic cells. Therefore, a mixed culture containing yeast under photoautotrophic conditions is more beneficial for microorganism growth than other cultures; with more economical culture conditions, mixed cultures may be used as a raw material for profitable biodiesel production.

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