



High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters

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Abstract

The aim of the study was to obtain high quality biodiesel production from a microalga *Chlorella protothecoides* through the technology of transesterification. The technique of metabolic controlling through heterotrophic growth of *C. protothecoides* was applied, and the heterotrophic *C. protothecoides* contained the crude lipid content of 55.2%. To increase the biomass and reduce the cost of alga, corn powder hydrolysate instead of glucose was used as organic carbon source in heterotrophic culture medium in fermenters. The result showed that cell density significantly increased under the heterotrophic condition, and the highest cell concentration reached 15.5 g L^{-1} . Large amount of microalgal oil was efficiently extracted from the heterotrophic cells by using *n*-hexane, and then transmuted into biodiesel by acidic transesterification. The biodiesel was characterized by a high heating value of 41 MJ kg^{-1} , a density of 0.864 kg L^{-1} , and a viscosity of $5.2 \times 10^{-4} \text{ Pa s}$ (at 40°C). The method has great potential in the industrial production of liquid fuel from microalga.

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Keywords: Biodiesel; Heterotrophic culture; *Chlorella protothecoides*; Fermenter; Corn powder hydrolysate

1. Introduction

As a biodegradable, renewable, and non-toxic fuel, biodiesel fuel has received considerable attention in recent years. It also contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than conventional diesel fuel (Lang et al., 2001; Antolin et al., 2002; Vicente et al., 2004). Biodiesel

fuel, which consists of the simple alkyl esters of fatty acids, is presently making the transition from a research topic and demonstration fuel to a marketed commodity. Annual US production in 2001 has been estimated at 57–76 million liters, with European production more than 10 times that size (Jon Van Gerpen, 2005). However, the economic aspect of biodiesel production limits its development and large-scale use. Biodiesel usually costs over $\text{US\$ } 0.5 \text{ L}^{-1}$, compared to $\text{US\$ } 0.35 \text{ L}^{-1}$ for conventional diesel fuel (Zhang et al., 2003).

Chlorella protothecoides is a microalga that can grow photoautotrophically or heterotrophically under

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different culture conditions. Heterotrophic growth of *C. protothecoides* supplied with acetate, glucose, or other organic compounds as carbon source, results in high biomass and high content of lipid in cells (Endo et al., 1977; Wu et al., 1994). With the addition of the organic carbon source (glucose) to the medium and the decrease of the inorganic nitrogen source in the medium, the heterotrophic *C. protothecoides* was cultivated with the crude lipid content up to 55.2%, which was about four times that in photoautotrophic *C. protothecoides* (Miao and Wu, 2004a). Therefore, *C. protothecoides* has not only become an important source of many products, such as aquaculture feeds, human food supplements, and pharmaceuticals (Kyle, 1992; Running et al., 1994; Borowitzka, 1995; Chen, 1996), but also been suggested as a very good candidate for fuel production (Wu et al., 1992; Wen et al., 2002; Miao and Wu, 2004a).

To increase the biomass and reduce the cost of alga, corn powder hydrolysate (CPH) as substrate in heterotrophic growth of *C. protothecoides* was used. *Chlorella protothecoides* was heterotrophically cultured in a 5 L stirred tank fermenter with CPH feeding, which gave significant improvement in cell density (15.5 g L^{-1}) and productivity. High quality biodiesel was obtained from heterotrophic microalgal oil by acidic transesterification. It was characterized by a high heating value of 41 MJ kg^{-1} , a density of 0.864 kg L^{-1} , and a viscosity of $5.2 \times 10^{-4} \text{ Pa s}$ (at 40°C).

2. Materials and methods

2.1. Microalga and medium

Chlorella protothecoides was provided by the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The culture medium and method were as described as Wu et al. (1992). The alga was grown autotrophically and axenically in batch cultures under $28 \pm 1^\circ \text{C}$ with continuous illumination at intensities of $40 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Aeration was provided by bubbling air at regular pressure. For the heterotrophic growth of *C. protothecoides*, 10 g L^{-1} glucose was added to the basal medium and the glycine was reduced to 0.1 g L^{-1} . The details of the culture of heterotrophic cells were reported in our previous research (Wu et al., 1994).

Table 1
Design of 2^3 factorial experiments

Serial number	A	B	C
1	–	–	–
2	–	+	–
3	+	–	–
4	+	+	–
5	–	–	+
6	–	+	+
7	+	–	+
8	+	+	+

The three factors were alpha-amylase dosage (A), glucoamylase dosage (B), and reaction time (C). Levels were 0.005 g (–), 0.010 g (+) for A, 0.100 g (–), 0.200 g (+) for B and 1 h (–), 2 h (+) for C.

2.2. Production of CPH

The 2^3 factorial experiments were chosen to examine the optimum condition of corn powder hydrolyzed by alpha-amylase and glucoamylase (Table 1). The three factors were alpha-amylase dosage (A), glucoamylase dosage (B), and reaction time (C). Levels were 0.005 g (–), 0.010 g (+) for A; 0.100 g (–), 0.200 g (+) for B and 1 h (–), 2 h (+) for C.

Buffer solution was prepared with 0.2N of dipotassium hydrogen phosphate (103.00 mL) and 0.1N of citric acid (97.00 mL). Accurately 5.000 g corn powder, 10.00 mL buffer solution and distilled water were mixed, and the corn powder was hydrolyzed by alpha-amylase and glucoamylase at 60°C and pH 6.0.

2.3. Cultivation

Chlorella protothecoides in exponentially period was inoculated (10%, v/v) in a liquid medium. Heterotrophic cultivation of *C. protothecoides* was initially carried out in a 500-mL Erlenmeyer flask containing 300 mL medium at $28 \pm 1^\circ \text{C}$ under continuous shaking (180 rpm) and air flowing in the dark (Miao and Wu, 2004a). Further heterotrophic cultivation was performed in a 5-L fermenter (Biostat Q, B.BRAUN, Germany) containing 3.0 L medium, in which concentrated glucose solution was batch-fed. Dissolved oxygen concentration was controlled by increasing agitation speed and airflow. Aeration rate and the agitation speed were variable and initially set at 0.5 vvm and 300 rpm. Temperature was controlled $28 \pm 1^\circ \text{C}$.

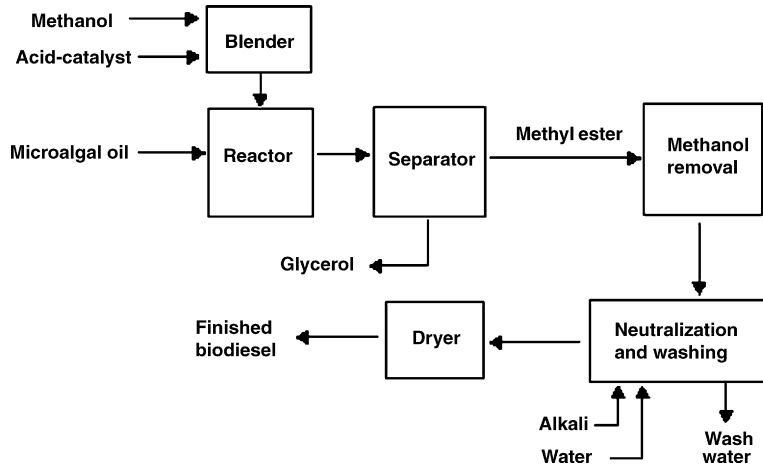


Fig. 1. Process flow schematic for biodiesel production.

2.4. Biodiesel preparation

Cells were harvested by centrifugation, washed with distilled water, and then dried by a freeze dryer. The main chemical components of heterotrophic *C. protothecoides* were measured as previous study (Miao et al., 2004b). Microalgal oil was prepared by pulverization of heterotrophic cell powder in a mortar and extraction with *n*-hexane.

Biodiesel was obtained from heterotrophic microalgal oil by acidic transesterification (Fig. 1). The optimum process combination was 100% catalyst quantity (based on oil weight) with 56:1 molar ratio of methanol to oil at temperature of 30 °C, which reduced product specific gravity from an initial value of 0.912 to a final value of 0.864 in about 4 h of reaction time.

2.5. Analytical methods

Cell growth was measured by means of the absorbance of the suspension at 540 nm as Becker (1994) showed. This value was transformed to biomass concentration, by a regression equation as

$$y = 0.2821x \quad (R^2 = 0.996, P < 0.05)$$

where y (g L⁻¹) is the cell concentration and x is the absorbance of the suspension at 540 nm.

Lipid in the algal cells was extracted according to Zhu et al. (2002). Glucose was analyzed with the method of Miller (1959).

The saponification (189.3 mg KOH g⁻¹) and acid value (8.97 mg KOH g⁻¹) of the microalgal oil were determined according to the method of Vicente et al. (2004). The molecular weight of the oil was calculated from saponification and acid value as

$$M = \frac{168300}{SV - AV},$$

where M is the molecular weight of the oil, SV the saponification value, and AV is the acid value. The average molecular weight of the oil was 933.

The properties of biodiesel such as density, viscosity, flash point, cold filter plugging point, solidifying point, and heating value were measured. The elemental compositions of biodiesel were determined by a CE-440 elemental analyzer (Peng et al., 2001).

The composition of the biodiesel was derivatized and analyzed by gas chromatographic–mass spectrometric analysis. Gas chromatography was performed on a 0.25 mm (i.d.) × 30 m fused silica column lined with a 0.25 μm film of polyethylene glycol (VF-5ms, from VARIAN, America). Samples (0.2 μL) were injected in split mode (split/column flow ratio 30:1). The column head pressure of the carrier gas (helium) was 3 kPa at the initial oven temperature, and its flow rate 1.0 mL min⁻¹. The injection temperature was 290 °C; the oven temperature was 100 °C for 2 min, rose to 300 °C over 20 min and was held at this temperature for 20 min (total run time 42 min). The GC–MS

Table 2
Influence of two enzymes and reaction time on DE value of hydrolysate

Alpha-amylase/ glucoamylase	Volume of solution (mL)	2 h							
		1 h			2 h				
		Absorbency	Glucose concentration ($\mu\text{g mg}^{-1}$)	Total glucose (g)	Dextrose equivalent (%)	Absorbency	Glucose concentration ($\mu\text{g mg}^{-1}$)	Total glucose (g)	Dextrose equivalent (%)
0.005/0.100	164	0.926	192.1	3.15	63.0	1.096	218.7	3.59	71.8
0.005/0.200	169	0.905	188.8	3.19	63.8	0.949	195.7	3.31	66.2
0.010/0.100	179	0.851	183.5	3.28	65.6	0.801	172.5	3.09	61.8
0.010/0.200	187	0.811	174.0	3.25	65.0	0.613	143.0	2.67	53.4

Temperature = 60 °C, pH 6.0.

apparatus was linked to a PC running software for data acquisition and processing.

3. Results and discussion

3.1. Hydrolysate from corn powder

Corn powder was hydrolyzed under 2^3 factorial experiments. Comparing the dextrose equivalents (DE) after 1 h, when the dosage of alpha-amylase fixed, the dosage of glucoamylase did not play a decisive role (Table 2). This meant that, starch was hydrolyzed but not saccharified with the reaction time of 1 h. After 2 h, all the reactions were determinately excessive under the conditions, except alpha-amylase and glucoamylase were 0.005 and 0.100 g. Because the reducing sugar produced in the excessive reactions was disproportioned. The optimum condition of the reaction in laboratory was alpha-amylase 0.005 g and glucoamylase 0.100 g per 5.000 g corn powder, at 60 °C and pH 6.0. After 2 h, the DE value reached 71.8%.

3.2. Cultivation of heterotrophic *Chlorella* in flasks

As shown in Fig. 2, the cell growth reached maximum value (3.92 g L^{-1}) after 144 h culture with the substrate of CPH, while the maximum value was 3.74 g L^{-1} with the substrate of glucose. It indicated that, it was feasible to use CPH as organic carbon

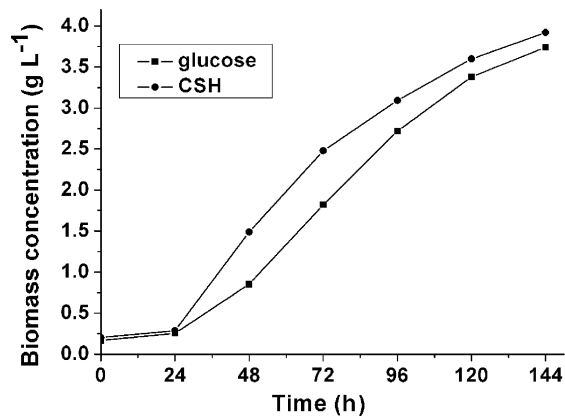


Fig. 2. Growth curve comparison of heterotrophic *Chlorella* between glucose and CPH medium in flasks.

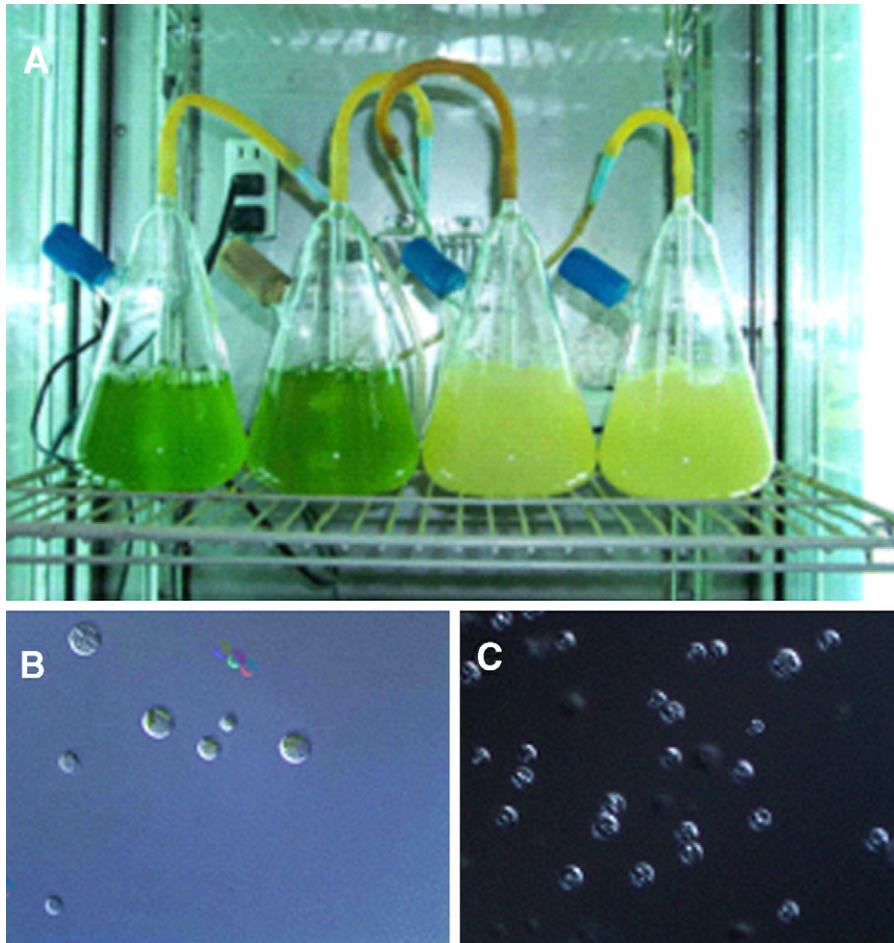


Fig. 3. (A) Growth of the cells of *C. protothecoides* under autotrophic (left, green) and heterotrophic (right, yellow) culture conditions. (B and C) Cells of autotrophic and heterotrophic *C. protothecoides* under differential interference microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

to cultivate *Chlorella*. During most of the cultivation time, CPH resulted superior to glucose solution, which due to CPH contained some beneficial components to *Chlorella*. Lipid content in the algal cells was 54.7% with glucose feeding, and 55.3% with CPH feeding, which was not significantly different.

3.3. The main chemical components of heterotrophic *Chlorella* cells

As shown in Fig. 3, heterotrophic growth of *Chlorella protothecoides* results in not only the disappearance of chlorophyll in cells (Fig. 3A) but also accumulation of high lipid content in cells. Lipid con-

tent in heterotrophic cells reached as high as 55.2%, which was about four times that in autotrophic cells (Table 3). The heterotrophic cells were full of lipid vesicles, which can be easily observed under differ-

Table 3

Contents of the main chemical components of autotrophic (AC) and heterotrophic (HC) *Chlorella protothecoides* cells

Component (%)	AC	HC
Protein	52.64 ± 0.26	10.28 ± 0.10
Lipid	14.57 ± 0.16	55.20 ± 0.28
Carbohydrate	10.62 ± 0.14	15.43 ± 0.17
Ash	6.36 ± 0.05	5.93 ± 0.04
Moisture	5.39 ± 0.04	1.96 ± 0.02
Others	10.42 ± 0.65	11.20 ± 0.61

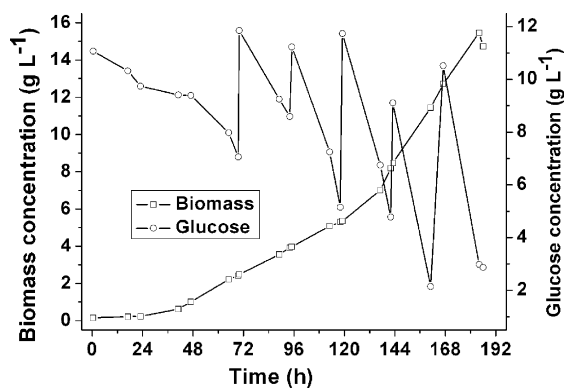


Fig. 4. Growth and glucose consumption curve of heterotrophic *Chlorella* in 5 L fermenter.

ential interference microscopy (Fig. 3C). The lipid-soluble compounds from the autotrophic cells appeared in a blackish green with chlorophyll and carotenoid as the major components, whereas the lipid-soluble compounds from the heterotrophic cells appeared in a state of light yellow grease, which were mainly lipid compounds (referred as oil). The fatty acid composition of the oil has been demonstrated to be mainly composed of oleic acid, linoleic acid, cetane acid, etc. by hydrolysis, esterification, and gas chromatographical analysis as reported in our previous work (Wu et al., 1992).

3.4. Cultivation of heterotrophic *Chlorella* in the fermenter

As shown in Fig. 4, the cell growth reached 15.5 g L^{-1} after 184 h culture in 5 L bioreactor, then decreased to 14.3 g L^{-1} in the subsequent 2 h culture. A high density heterotrophic culture of *C. protothe-*

coides with CPH feeding was established in the 5 L stirred tank fermenter. Lipid content in the algal cells cultivated in the fermenter was 46.1%, which was a little lower than that in the Erlenmeyer flasks.

3.5. Biodiesel produced from heterotrophic *Chlorella*

To assess the potential of biodiesel as a substitute of diesel fuel, the properties of biodiesel such as density, viscosity, flash point, cold filter plugging point, solidifying point, and heating value were determined. A comparison of these properties of diesel fuel (Ma and Hanna, 1999; Lang et al., 2001; Al-Widyan and Al-Shyoukh, 2002; Antolin et al., 2002; Vicente et al., 2004), biodiesel from microalgal oil and ASTM biodiesel standard is shown in Table 4. Most of these parameters comply with the limits established by ASTM related to biodiesel quality (Antolin et al., 2002).

The physical and fuel properties of biodiesel from microalgal oil in general were comparable to those of diesel fuel. The biodiesel from microalgal oil showed much lower cold filter plugging point of $-11 \text{ }^{\circ}\text{C}$ in comparison with the diesel fuel (Table 4).

The gas chromatograph of biodiesel is shown in Fig. 5. The fatty acid methyl esters (FAMES) of the biodiesel are presented in Table 5. There were nine FAMES derivatized in the biodiesel, and the most abundant composition was oleic acid methyl ester with the content of 60.84%. Oleic acid methyl ester, octadecadienoic acid methyl ester, and octadecanoic acid methyl ester are 18 carbon acid methyl esters, and the total content of these three FAMES was over 80%. This resulted in the high quality of the biodiesel.

Table 4

Comparison of properties of biodiesel from microalgal oil and diesel fuel and ASTM biodiesel standard

Properties	Biodiesel from microalgal oil	Diesel fuel ^a	ASTM biodiesel standard
Density (kg L^{-1})	0.864	0.838	0.86–0.90
Viscosity ($\text{mm}^2 \text{ s}^{-1}$, cSt at $40 \text{ }^{\circ}\text{C}$)	5.2	1.9–4.1	3.5–5.0
Flash point ($^{\circ}\text{C}$)	115	75	Min 100
Solidifying point ($^{\circ}\text{C}$)	-12	-50–10	-
Cold filter plugging point ($^{\circ}\text{C}$)	-11	-3.0 (max -6.7)	Summer max 0 Winter max < -15
Acid value (mg KOH g^{-1})	0.374	Max 0.5	Max 0.5
Heating value (MJ kg^{-1})	41	40–45	-
H/C ratio	1.81	1.81	-

^a The data about diesel fuel was taken from published literature as indicated in the text.

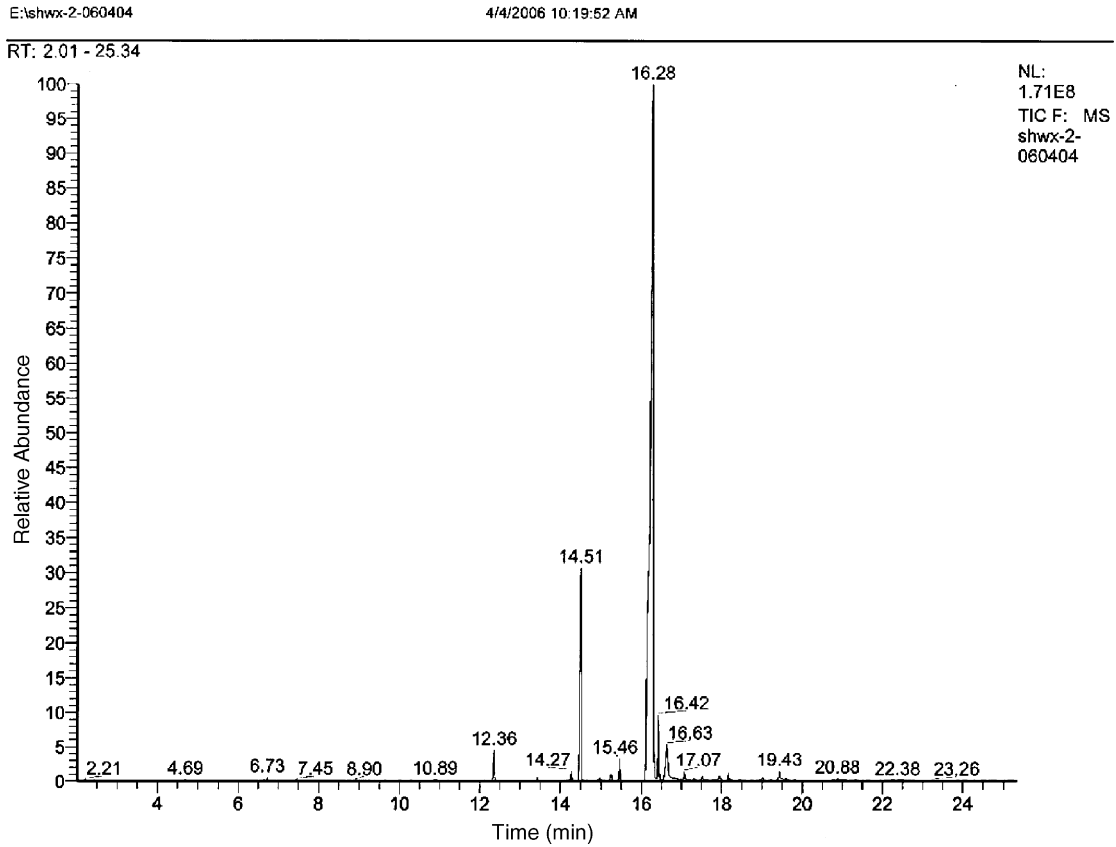


Fig. 5. Gas chromatogram of the fatty acid methyl ester in biodiesel.

Table 5

Fatty acid methyl esters in the biodiesel

No.	Molecular formula	Relative molecular mass	Fatty acid methyl ester	Relative content (%)
1	$C_{15}H_{30}O_2$	242	Methyl tetradecanoate	1.31
2	$C_{17}H_{34}O_2$	270	Hexadecanoic acid methyl ester	12.94
3	$C_{18}H_{36}O_2$	284	Heptadecanoic acid methyl ester	0.89
4	$C_{19}H_{34}O_2$	294	9,12-Octadecadienoic acid methyl ester	17.28
5	$C_{19}H_{36}O_2$	296	9-Octadecenoic acid methyl ester	60.84
6	$C_{19}H_{38}O_2$	298	Octadecanoic acid methyl ester	2.76
7	$C_{20}H_{38}O_2$	310	10-Nonadecenoic acid methyl ester	0.36
8	$C_{21}H_{40}O_2$	324	11-Eicosenoic acid methyl ester	0.42
9	$C_{21}H_{42}O_2$	326	Eicosanoic acid methyl ester	0.35

The results suggest that the new process, which combines bioengineering and transesterification, was a feasible and effective method for the production of high quality biodiesel from heterotrophic microalgal oil. The biodiesel from heterotrophic microalgal oil could be a competitive alternative to conventional diesel fuel.

4. Conclusion

The research of liquid fuel produced from microalga was begun at middle 1980s in 20 centuries. Transesterification and catalytic cracking were usually adopted to convert fat in the cell of microalga as gasoline and

diesel at that time. This kind of method was limited by low temperature, and the outcome function was highly influenced by the fat constitute. What was more, the fat content in the microalga was required to be very high, otherwise the economic performance would be hard to acquire.

Because it was hard to obtain microalga with high content of fat, scientists turned to develop a new method pyrogenation. The method of pyrolyzing microalga to produce liquid fuel was put forward by Doctor Bayer in Germany in 1986. In 1993, high quality oil of low nitrogen and low sulphur was got successfully by Professor Ben-Zion Ginzburg in Israel, using *Dunaliella salina* as material of pyrogenation. Liquefaction of *Botryococcus braunii* was performed with sodium carbonate as a catalyst for conversion into liquid fuel and recovery of hydrocarbons at high pressure (10 MPa N₂ press) under 300 °C. The income liquid oil reached 57–64 wt%, and the quality was comparable to petroleum (Dote et al., 1994). *Dunaliella tertiolecta* with a moisture content of 78.4 wt% was converted directly into oil by thermochemical liquefaction at 340 °C in 60 min holding time. The oil yield was about 37% on an organic basis, and had a viscosity of 150–330 mm² s⁻¹ and a calorific value of 36 MJ kg⁻¹ (Minowa et al., 1995).

To increase the fat content in microalga, the technique of metabolic controlling through heterotrophic growth of *C. protothecoides* was applied, which resulted in the crude lipid content of 55.2% (Wu et al., 1994). The microalgal oil was efficiently extracted from the heterotrophic cells, and then transmuted into biodiesel by acidic transesterification.

The present study introduced an integrated method for the production of biodiesel from heterotrophic *C. protothecoides*. CPH was got from corn powder by the co-hydrolyzation of alpha-amylase and glucoamylase with the dosages of 0.005 and 0.100 g per 5.000 g corn powder, at 60 °C and pH 6.0 after 2 h reaction. The DE value reached 71.8%. CPH was used as the substrate of heterotrophic growth of *C. protothecoides* which was cultivated in Erlenmeyer flasks and 5 L fermenter. In Erlenmeyer flasks, the cell had the concentration of 3.92 g L⁻¹ and the lipid content of 55.3% after 144 h culture with CPH feeding, which was superior to glucose feeding. In 5 L fermenter, the cell growth reached 15.5 g L⁻¹ after 184 h culture, and then microalgal oil was efficiently extracted from the heterotrophic cells. Biodiesel which was obtained from heterotrophic

microalgal oil by acidic transesterification was characterized by a high heating value of 41 MJ kg⁻¹, a density of 0.864 kg L⁻¹, and a viscosity of 5.2 × 10⁻⁴ Pa s (at 40 °C). The results suggest that the new process was a low-cost, feasible, and effective method for the production of high quality biodiesel from microalga.

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