Advances in Energy Research, *Vol. 1, No. 2 (2013) 107-116*
DOI: http://dx.doi.org/10.12989/eri.2013.1.2.107

Long-chain alcohols derived from the microalga Monoraphidium

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(Received April 7, 2013, Revised July 24, 2013, Accepted October 2, 2013)

Abstract. This study was to investigate the composition and characteristics of long-chained alcohols extracted from the algal strain *Monoraphidium* 3s35. The production of biomass was optimized using different cultivation methods. Under the aerated growth condition, this strain yielded up to 37.26% extracts of dry weight and 576 mgL^{-1} biomass. The major compounds of the extracts are mainly long-chained alcohols (89.24%), with carbon chain length ranging from 12 to 20. Interestingly, or the long-chained alcohols, 3-(2-Methoxyethyl)-1-nonanol, 3,7,11, 15-Tetramethyl-2-hexadecen-1-ol and oleyl alcohol accounted for 53.68%, 23.45%, and 12.11%, respectively. Because of their amphipathic nature, these long-chained alcohols have been widely used in bioenergy production and cosmetics industry. Furthermore, *Monoraphidium* 3s35 produced 9.73% of C_{17} and C_{20} alkanes, which can be used as an important supplement for the petrodiesel-like fuel.

Keywords: Microalgae; Monoraphidium sp.; long-chained alcohols; 1H-NMR; GC/MS

1. Introduction

Fossil fuels, which are the primary resources for energy and chemical products, are exhausted gradually (Bridgwater *et al*. 1999). Microalgae have been used as renewable resources to produce biofuels and valuable byproducts in various applications throughout the world (Singh and Gu 2010). Long chain alcohols, which are less corrosive, more gasoline-compatible fuels than bioethanol (Leathers *et al*. 2007), have been considered as interesting supplements for fossil fuels (Atsumi *et al*. 2008). Moreover, long-chained alcohols can also be transesterified to produce motor fuel for internal combustion engines (Vogel *et al*. 1991). Furthermore, long-chained alcohols (Rowland and Domergue 2012) especially fatty alcohol of a chain from 8 to 22, are widely used in industrial chemicals (cosmetics, food and solvents) to mainly produce the detergents and surfactants (Zheng *et al*. 2012). Due to their amphipathic nature (Rantamäki *et al*. 2011), long-chain alcohols can behave as nonionic surfactants, and thus can also be used as emulsifiers,

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 ISSN: 2287-6316 (Print), 2287-6324 (Online)

emollients (Savic *et al*. 2008) and thickeners in cosmetics and food industry.

Long chain alcohols were unavailable until the early 1900s. In the 1940s and 1950s, Karl Ziegler discovered the polymerization of ethylene obtained from petrochemicals for long chain alcohols (Ziegler 1966). Wax esters extracted from sperm oil of whales and jojoba, both of which are difficult to obtain (Buisson *et al*. 1982), are also the important source to produce long chain alcohols. Since the production and manufacture of fatty alcohols are still based on the synthesis of the extracts from fossil resources as well as animals and plants which are seldom available, the novel source and synthesis way for producing long chain alcohols (Metzger and Bornscheuer 2006) are becoming more and more important.

In the past decades, the production of fatty acid and esters from algae has been an area of considerable interest, because of several special features associated with algae (Scott *et al*. 2010). First, algae have higher productivities than land plants, with some species having doubling times of a few hours (Ryther 1959). Second, some algal species can accumulate very large amounts of fatty acids (Piorreck *et al*. 1984), esters (Marlowe *et al*. 1984) and carbohydrates (*Impellizzeri et al*. 1975). Lastly, algal cultivation does not compete for arable land. However, few efforts have been made to study the production of algal fatty alcohol, which is significant for the chemistry and food industry, as a promising supplement for the traditional fatty alcohol production.

This paper focused on the production of different fatty alcohols derived from the microalga *Monoraphidium* 3s35. Particularly, efforts have been made to quantitative and qualitative analyses of fatty alcohols using modern analytical tools.

2. Method and materials

2.1. Experimental organisms

Microalgal strain *Monoraphidium* 3s35 used in this study was collected from the coastal waters of Shenzhen, Guangzhou Province, China. *Monoraphidium braunii* have been widely studied for the mechanism of nitrate reductase (Alfonso *et al*. 1991) and glutamine synthetase (Garcia-Fernandez *et al*. 1994). However, the production of fatty acid and alcohol production have seldom been studied for this green microalgal strain. The strain was maintained on BG-11 media with 1.5% agar supplemented. Individual experimental designs are carried out for 10 days with 3 replicates while each flask was considered a biological replicate. Slots on a shaker platform were randomly assigned and reassigned after each sampling to prevent bias and to balance off any variance in lighting. For biomass cultivation, 80 mL of axenic culture at exponential phase containing dry cell mass density of 50 mgL^{-1} (± 0.08 SE) was inoculated into 800 mL of sterile BG-11 medium in 1000 mL flasks stoppered with autoclavable foam, incubating at the air temperature of 26℃ with the initial pH 7.1. Three banks of cool white portable fluorescent lights (EIKO, PAR100, 220V) set on a timer provided an average photosynthetically active radiation of 100.5 μmol $m^{-2}s^{-1}$± 2.0 SE of light (Jorquera *et al*. 2010) with air bubbling. Light was on for the whole period with 12 h of dark-light period maintaining. The same condition was also applied to the rotary shaking cultivation without aeration.

2.2. Measurement of optical density (OD) and biomass

Optical density (OD) of the inoculated manure daily at 680 nm was measured daily as the algal

density indicator using a UV spectrophotometer (UV-1800, SHIMADZU), with 3 biological replicates. Biomass was collected filtering cultures through Gelman glass fiber filters (25 mm in diameter, Type A, 0.45 μm) and determined by wither after being dried at 65°C for 1 h. For composition analysis, aliquots of the mixed algal cultures were centrifuged, re-suspended in distilled water, and prepared for the instrumental analysis. A linear relationship between OD and dry weight (DW, gL^{-1}) was determined for this strain using the following equation (Wang *et al*. 2010)

$$\text{Dry weight } (gL^{-1}) = 0.1937 OD_{680} - 0.1116, \quad R^2 = 0.997 \tag{1}$$

The growth rate (GR, d^{-1}) (Donk and Hessen 1993) was calculated by fitting the OD for the first 5 days of culture to an exponential function

$$GR(d^{-1}) = \left(\ln OD_t - \ln OD_0\right) / t \tag{2}$$

where OD_0 is the optical density at initial day, OD_t is the optical density for day t and t is the time between the two measurements.

2.3 Extraction yield determination

Individual sample of 1.5 g cell biomass was extracted with chloroform/methanol (1/2, v/v) by Soxhlet extractor at 75°C for 12 h (Carter *et al*. 1961, Krohn *et al*. 2011, Torres-Durán *et al*. 1999). The resulting supernatants were, filtered with Whatman filter No. 1 (Whatman, USA), and washed with Milli-Q water. The lower organic phases were collected and evaporated to dryness using a vacuum rotary evaporator in a water bath at 50°C. Total lipid contents were determined gravimetrically. After weighing the extraction, 10ml of n-hexane is added to each flask for the subsequent characteristics analysis.

2.4 Characteristics analysis

^{1}H NMR Spectra were collected using a Bruker 500 ultra shield I100605 400-MHz spectrometer outfitted with a 5-mm broadband probe. Samples were prepared by dissolving 1ml of microalgae extraction. Samples were then filtered (0.45-μm PTFE) to remove any suspended particulates before loading into 5 mm diameter NMR tubes. 1H spectra were acquired with a 90o pulse angle, spinner frequency of 20 Hz, sweep width of 8000 Hz across 32 transients.

2.5 Composition analysis

Extraction compositions were analyzed (Vijayaraghavan and Hemanathan 2009) using an Agilent 5973 series high-temperature gas chromatography-mass spectrometer (GC-MS), fitted with an auto injector. GC-MS was equipped with a capillary column (DB-5 ms, 30 m x 0.25 mm x 0.25 μm film thickness) (J and W Scientific, USA) for the identification of alkane and fatty alcohol. The injector and detector temperatures were set at 280℃ while the initial column temperature was set at 80℃. One microliter of sample was injected into the column and analyzed using a 100:1 split ratio. After 3 min, the oven temperature was raised to 315℃ at a ramp rate of 5℃ min-1 and finally maintained at this temperature for 12 min. The helium carrier gas was

programmed to maintain a constant flow rate of 2 mL min-1. Mass spectra were acquired and processed using both Agilent ChemStation (Agilent, USA) and AMDIS32 software (Interpretation from NIST).

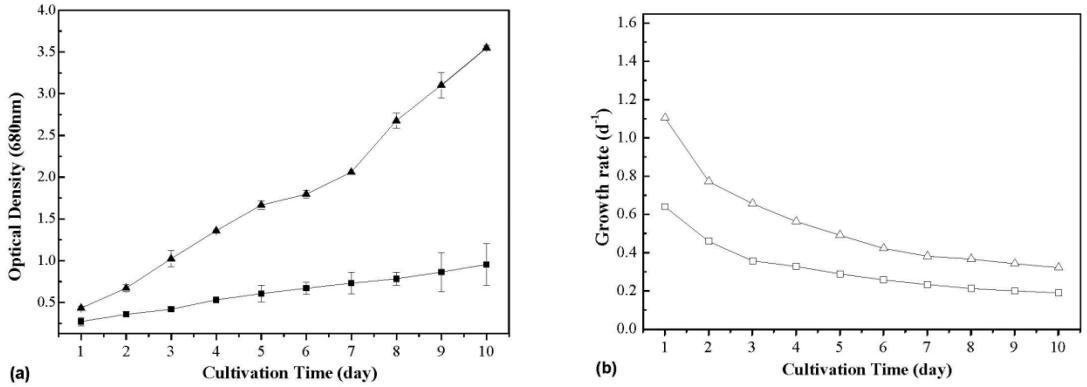
3. Results and discussion

3.1 Biomass production

Optical density of algal growth under different conditions was measured at 680 nm. The growth curves are shown in Figs. 1(a)-1(d). Comparing with cultivating on the rotary shaker, the optical density of the microalga *Monoraphidium* 3s35 was much higher under the aeration condition than that under the shaking condition. After 10-day cultivation, the optical density of *Monoraphidium* 3s35 reached the value of 3.548±0.004, which was about 3.46 times higher than that under the shaking condition. Thus, results of this study support the previous report that the bountiful supply of air improves the biomass accumulation reported in other microalgal species.

Both of cultivation conditions yielded the similar growth curve trade with the much faster growth rate under the aeration condition. It was noticed that that the initial growth rates under bubbling (1.11 d-1) and shaking (0.64 d-1) conditions were 3.44 and 3.37 times faster than their corresponding growth rates at the end of the cultivation period. The availability of nutrients (Gerloff and Krombholz 1966) and the stability of pH (Kratz and Myers 1955) have been reported to be the main factors affecting algal growth. For *Monoraphidium* 3s35, the value of pH observed after 10-day cultivation under aeration can reach up to 11.48, which is much higher than the suitable cultivation pH (7.0) of microalga. Thus, the low growth rates were likely ascribed to the nutrient limitation and the pH change in the liquid medium.

Moreover, the final biomass of *Monoraphidium* 3s35 under air bubbling condition reached up to 576 mgL after 10-day cultivation, which is 7.89 times more than that under shaking cultivation condition.



(a) OD_{680} value of *Monoraphidium* 3s35 (-▲- with air bubbling, -■- with illumination rotary shaker)

(b) Growth rate of *Monoraphidium* 3s35 (-△- with air bubbling, -□- with illumination rotary shaker)

Fig. 1 Growth curves of *Monoraphidium* 3s35 in BG-11 medium with different cultivation ways

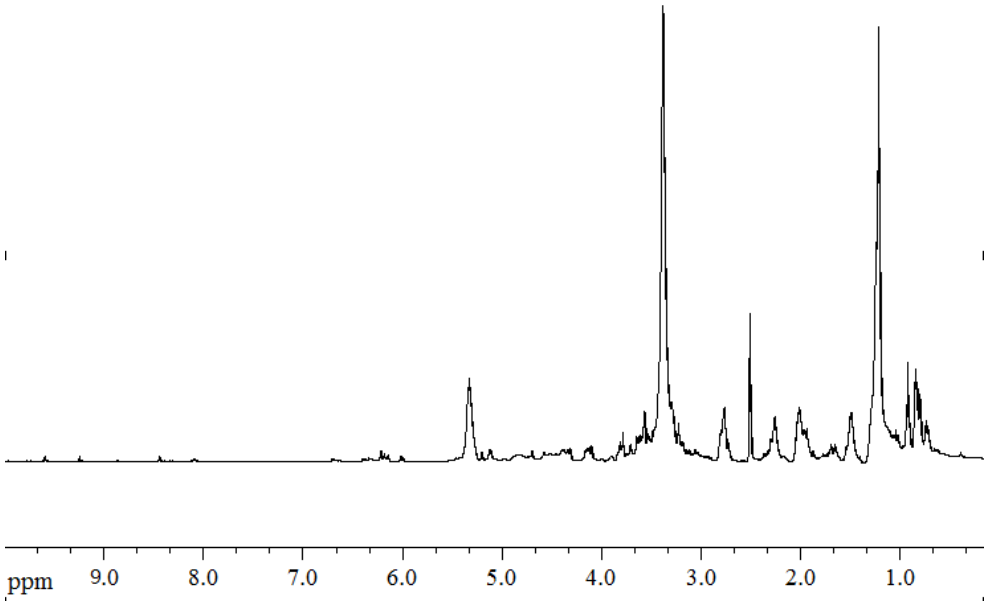


Fig. 2 ^{1}H NMR spectrum analysis of the extraction from microalgae *Monoraphidium* 3s35

3.2 Extraction yields and characteristics analysis

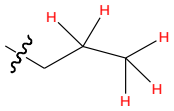
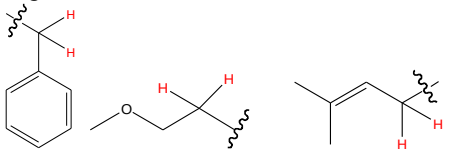
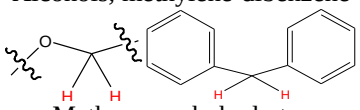
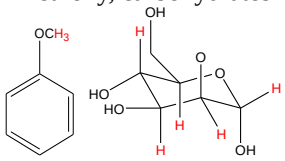
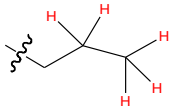
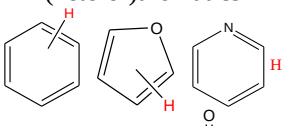
The yield and characteristics analysis of the resulting biomass of *Monoraphidium* 3s35 were extracted using Soxhlet extraction method (Ahmed *et al*. 2012). The functional structures of the organic fractions were characterized using ^{1}H NMR. The resulting spectra are shown in Fig. 2. The integral percentage values of selected regions of the spectra are summarized in Table 1. The extraction yield reached up to 37.26% of *Monoraphidium* 3s35 cell biomass. ^{1}H NMR spectra revealed 2 major distinct components in the extracts derived from microalgae *Monoraphidium* 3s35.

The first major component (48.07%) was alcohols (3.0-4.4 ppm), with alkane functionality accounting for 34.73% as the second major component (Vardon *et al*. 2011). Furthermore, *Monoraphidium* 3s35 can produce 12.30% of α-to-heteroatom/unsaturated functionality (1.5-3.00 ppm), which is possibly due to the unsaturated aliphatic chains (Mullen *et al*. 2009). Moreover, 7.81% of carbohydrates and 0.52% of aromatic functionality were also found in the extracts of *Monoraphidium* 3s35. It is noticed that alcohols are the predominant compounds extracted from microalgae *Monoraphidium* 3s35, which has seldom been reported, indicating that microalgae might be able to serve as an important potential source of fatty alcohols. Thus, further research for the specific composition qualification and quantification have been undergone by GC/MS.

3.3 Extraction composition analysis by GC-MS

The GC-MS chromatogram of the extracts derived from the microalga *Monoraphidium* 3s35 extraction revealed 6 peaks (Fig. 3), suggesting the presence of six compounds. The chemical compounds identified based on the GC-MS analysis are summarized in Table 2.

Table 1 ^{1}H NMR spectral distribution analysis of functional groups present in the extracts from microalgae *Monoraphidium* 3s35 based on integrated peak areas assigned to characteristic spectral regions and chemical shift range

Shifts (ppm)	Proton Assignment	Sample (%) *Monoraphidium* 3s35
	Alkanes	
0.5-1.5		34.73
	Aliphatics α-to heteroatom or unsaturation	
1.5-3.0		12.30
	Alcohols, methylene-dibenzene	
3.0-4.4		48.07
	Methoxy, carbohydrates	
4.4-6.0		4.37
	Alkanes	
0.5-1.5		34.73
	(hetero-)aromatics	
6.0-8.5		0.52
9.5-10.1	 Aldehydes	0.10

The results of GC-MS analysis further confirmed that the alcohols were the predominant component of the extracts derived from the microalga *Monoraphidium* 3s35. Interestingly, the extracts contained up to 53.68% of 3-(2-Methoxyethyl)-1-nonanol, which is equivalent to 20.00% of total dry biomass. Furthermore, fatty alcohols such as oleyl alcohol and 3,7,11,15-Tetramethyl-2 -hexadecen-1-ol accounted for up to 12.11% and 23.45%, respectively, in the extracts. Overall, results of GC-MS analyses revealed that the extracts of the microalga *Monoraphidium* 3s35 contained 89.24% of alcohols, which had carbon chain length ranging from C12 to C20 with C12

alcohol (53.68%) as the predominant component. Long chain alcohols have been reported to be less corrosive, more gasoline-compatible fuels than bioethanol (Leathers *et al*. 2007) and are superior renewable and dense fuel (Atsumi *et al*. 2008). Moreover, long-chained alcohols can also be used to produce motor fuel for internal combustion engines through transesterification (Vogel *et al*. 1991). At this point, there were no efficient "long chain" alcohol synthesis processes available. Thus, the long-chained alcohol from microalgae cam be a potential renewable source of "long-chain" alcohols, which can be used for bioenergy market, as well as the cosmetics and food industry because of their amphipathic nature.

Table 2 Major compounds representing >1% of the GC/MS total ion chromatogram areas from extractions of microalgae *Monoraphidium* 3s35

No.	Compound	Molecular Formula	Area (%) *Monoraphidium* 3s35
1	Heptadecane	$C_{17}H_{36}$	240.28
2	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	$C_{20}H_{40}$	280.31
3	3-(2-Methoxyethyl)-1-nonanol	$C_{12}H_{26}O_2$	202.19
4	Oleyl Alcohol	$C_{18}H_{36}O$	268.28
5	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.31
6	Dodecanoic acid, 2-hexen-1-yl ester	$C_{18}H_{34}O_2$	282.26

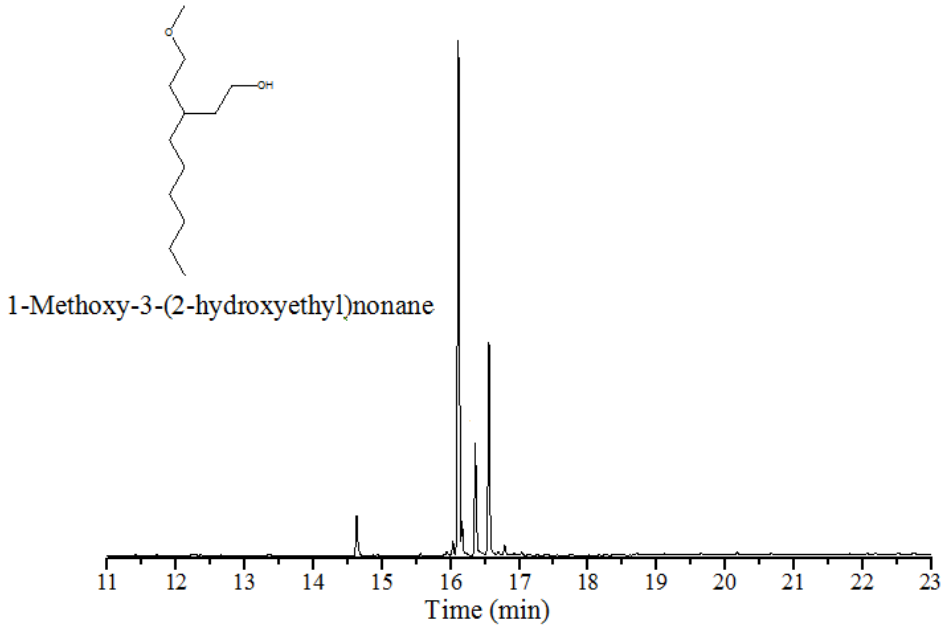


Fig. 3 GC-MS spectrum analysis of the extractied composition analysis from microalgae *Monoraphidium* 3s35

The microalga *Monoraphidium* 3s35 produced 9.73% alkanes and only 1.04% esters in the extracts, with their carbon chain ranging from 17 to 20, which are heptadecane (4.76%), 3,7,11,15-tetramethyl-2-Hexadecene (4.97%) and 1.03% of Dodecanoic acid, 2-hexen-1-yl ester (1.03%). Alkanes, as the main composition of petroleum-derived diesel fuel (Šimáček *et al*. 2011), possesse much better quality, stability and combustion efficientcy than biodiesel with the main components of esters (Knothe 2010). Thus, the microalga *Monoraphidium* 3s35 can certainly used as renewabel source for alkanes, which can be used as a supplement for petrodiesel-like fuel with the resembling compositions petroleum-derived diesel.

4. Conclusions

The extracts derived from the microalga *Monoraphidium* 3s35 has been studied in the manuscript. Results showed that the yield of extracts was up to 37.26% of dry biomass for *Monoraphidium* 3s35

- For the extracts, analyzed by GC/MS, alcohols with carbon chain length ranging from C12 to C20 accounted for 89.24%, with 53.68% of C12 alcohol (3-(2-Methoxyethyl)-1-nonanol) as the predominant component. It indicated that microalgae could be a potential renewable source of long chain alcohols.
- Due to the unique chemical features, these alcohols can be potentially used as less corrosive and more gasoline-compatible fuels, as well as detergents and surfactants in cosmetic and food industry.
- Moreover, long-chain alkanes (9.73%) are also one of the main products obtained from the extracts of *Monoraphidium* 3s35 and can be used as an interesting supplement for the petrodiesel-like fuel.

Acknowledgements

This project was partially supported by Shenzhen Development and Reform Commission grant 835 (2011)(GYW), National Natural Science Foundation of China (Grant No. 31170109)(GYW), National Natural Science Foundation of China for Distinguished Young Scholars of China (Grant No. 21206047)(XWY), and General Financial Grant from the China Postdoctoral Science Foundation (Grant No. 2012M520106)(XWY).

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