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Procedia Environmental Sciences 15 (2012) 66 – 72

Procedia Environmental Sciences

The 1st Asia-Oceania Algae Innovation Summit (AOAIS-1)

On the bio-rearrangement into fully saturated fatty acids-containing triglyceride in *Aurantiochytrium* sp.

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Abstract

A strain of *Aurantiochytrium* sp. was grown in media with various concentrations of glucose to monitor triglyceride production as a potential source of oil for biodiesel. The fatty acid composition of triglyceride in the strain was unique, because the fatty acids consisted of only 6 molecular species, and the major species were myristic, pentadecanoic, palmitic, heptadecanoic, docosapentaenoic, and docosahexaenoic acids. When cells were cultured in glucose-rich (over 9%) medium for 4 days, the triglyceride yields were 0.5-1.0 g/L. After culture for 4 days, the fatty acid composition of triglyceride was nearly identical in all cells grown in media containing various concentrations of glucose. However, when cells were grown in medium containing 12% glucose for 12 days, unique triglyceride containing only saturated fatty acids accumulated. This bio-rearrangement into fully-saturated fatty acids-containing triglyceride may be utilized for the preparation of biodiesel oil.

Keywords: *Aurantiochytrium*; glucose-rich medium; triglycerides; fatty acid bio-rearrangement; biodiesel oil

1. Introduction

As energy demands continue to increase, the world is facing declining liquid fuel reserves. Many nations therefore developed alternative energy resources. Biofuels such as biodiesel and ethanol have been one of the most useful substances as new liquid energy resources. Fatty acid methyl esters have been

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doi:10.1016/j.proenv.2012.05.011

utilized as biodiesel oil, and oil quality is affected by fatty acid composition. At room temperature, long chain saturated and unsaturated fatty acids are solid and liquid, respectively, and the fluidity of unsaturated fatty acid methyl esters makes them suitable for use as biodiesel oil. However, polyunsaturated fatty acids (PUFAs) are easily oxidized, change to viscous polymers and/or form oxygen-containing fatty acids such as epoxide- and aldehyde-containing fatty acids, the radical oxygen produced from which corrodes the inside of diesel engines [1]. Therefore, controlling fatty acid composition is very important for the quality control of biodiesel oil.

Thraustochytrids including *Aurantiochytrium* are widespread in marine and brackish-water regions [2], and are assigned to the stramenopiles together with heterokont algae [3]. Many strains of thraustochytrids accumulate large amounts of triglyceride (50% to 80% of dry biomass) with a high proportion of long chain PUFA, particularly docosahexaenoic acid (DHA; 22:6n-3) and docosapentaenoic acid (DPA; 22:5n-6) [4-6]. For this reason, thraustochytrid oils are currently being developed as commercial sources of DHA [7], because thraustochytrids have interesting advantages for PUFAs production as compared with marine fishes: they preferably contain one specific PUFA, rather than a mixture of various PUFAs as contained in fish oils, which result in a lower purification cost; they are sustainable materials due to their high reproductive ability; and they have no typical fishy smell and unpleasant taste [8]. However, as mentioned above, thraustochytrid oils with a high content of PUFAs are not suitable for use as biodiesel oil.

Recently, we found that the per-saturated fatty acids–containing triglyceride was accumulated in the cells of *Aurantiochytrium* sp. under the specific culture condition. The finding supposed that the formation of the per-saturated fatty acids-containing triglyceride may be utilized for the quality control of biodiesel oil. In this paper, we report a culture condition of *Aurantiochytrium* sp. for the accumulation of fully-saturated fatty acids-containing triglyceride

2. Materials and Methods

2.1. Materials

Saturated and unsaturated fatty acid methyl esters were obtained from GL Science Co Ltd. (Tokyo, Japan), and 14% BF_3-methanol was purchased from Wako Chemicals (Osaka, Japan). HPTLC silica-gel 60 F254 plates were purchased from Merck KGaA (Darmstadt, Germany). All the other chemicals and solvents were of analytical grade.

2.2. Culture conditions

Aurantiochytrium sp. strain 18W-13a was grown in 50% seawater and GPY medium with various concentrations of glucose, 1% polypeptone and 0.5% yeast extract. During pre-culture, GPY medium containing 2% glucose was used. Cells were grown isothermally at 25°C in media containing various concentrations of glucose with continuous orbital shaking at 400 rpm, and were harvested at their early or late stationary phase. The culture batches were triplicate. The harvested cells were lyophilized and stored at -80 °C until use.

2.3. Extraction of lipids

Lipids were extracted from lyophilized cells using a previously described procedure [9]. Aliquots of the extracted lipid and triolein as a standard were spotted on a silica gel 60 F254 HPTLC plate, and co-migrated using *n*-hexane/chloroform (2:1, v/v) as the solvent. After drying the plate, the spots were visualized under UV light or by heating after spraying with 20% sulfuric acid. Triglyceride on the developed plate showed R_F 0.3.The triglyceride fraction on the plates was scrapped off and extracted with chloroform-methanol (2:1, v/v).

2.4. Analysis of fatty acids

Fatty acids in the triglyceride fraction and total lipid were converted to methyl esters using 14% BF_3-methanol at 70 °C for 20min. Fatty acid methyl esters were analyzed using GC-FID (Shimadzu GC-2010), GC-EIMS (Shimadzu GC-2010 and QP-2010) and GC-CIMS (Agilent 7890A and JEOL Q-1000) with a fused silica DB-5MS (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific) and an InertCap 1MS (30 m × 0.25 mm i.d., film thickness 0.25 μm; GL Science Inc.) capillary columns. The GC operating conditions were as follows: column temperature, 50°C up to 230°C (9°C/min), held for 15 min; FID port temperature, 250°C; carrier gas (He) flow rate, 2.06 mL/min; FID H_2 flow rate, 40 mL/min; air flow rate, 400 mL/min.

2.5. Nuclear Magnetic Resonance (NMR) analysis

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded using a JEOL JNM-ECA500 spectrometer. $CDCl_3$ was used as a solvent and an internal standard (δH 7.26 or δC 77.0). Ten milligrams of lipid extract was fractionated using silica-gel (Merck, Kieselgel 60, 70–230 mesh) column chromatography with $CHCl_3$. Fluoranthene (1 mg) was added to the $CHCl_3$ fraction and was evaporated under reduced pressure. The sample was dissolved in $CDCl_3$ (0.8 mL) and the ^{1}H NMR was measured. The fluoranthene peak (δH=7.6 ppm) was integrated as 2H. The triglyceride peak (δH=4.3 ppm) was calculated by comparing it with the fluoranthene peak.

3. Results and Discussion

Aurantiochytrium sp. 18W-13a accumulates squalene [10] and triglyceride. Triglyceride was confirmed by ^{1}H, ^{13}C and 2D NMR. The downfield-shifted methine (δH=5.3 ppm, δC=69 ppm) and methylene (δH=4.3 and 4.1 ppm, δC=62 ppm) suggested the existence of an esterified glycerol. The triglyceride fractionated by TLC was identified by the COSY spectra.

As shown in Table 1, the biomass content was almost constant except that of cells grown in 12% glucose containing medium for 12 days culture, the mean was 4.18 ± 0.58 g/L, and the value of the exception was 6.91 ± 1.77 g/L. On the other hand, triglyceride contents of the cells grown in 3 and 6% glucose-containing media were rapidly decreased during 8 and 12 days culture, whereas the variation of the contents of the cells grown in 9 and 12 % glucose containing media was a few. As the result, only the biomass without triglyceride of cells grown in 12% glucose containing medium for 12 days culture was increased.

Fatty acid compositions of total lipid of cells grown in various conditions were examined. The fatty acid composition of triglyceride of total lipid was unique; the fatty acids consisted of 6 molecular species with the major acids of myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), heptadecanoic (C17:0), docosapentaenoic (DPA) (C22:5), and docosahexaenoic (DHA) (C22:6) (Table 2). It is very rare that one of the major fatty acids had an odd number of carbons in the acyl chain (C15:0) (Table 2).

Table 1. Effect of glucose concentration in GPY medium and culture period on biomass and triglyceride content in cells of *Aurantiochytrium* sp. strain 18W-13a

Culture condition		Whole-Biomass	Triglyceride production	*Biomass without triglyceride	Triglyceride % of whole biomass
Glucose conc. in medium (%)	Culture period (days)	(g/L)	(g/L)	(g/L)	
3	4	4.43 ± 0.60	0.50 ± 0.01	3.93	11.3
6	4	4.52 ± 1.66	1.08 ± 0.26	3.44	23.9
9	4	4.72 ± 1.62	0.98 ± 0.09	3.74	20.8
12	4	4.52 ± 0.99	0.69 ± 0.01	3.83	15.3
3	8	3.79 ± 0.64	0.20 ± 0.10	3.59	5.3
6	8	3.69 ± 1.11	0.30 ± 0.11	3.39	8.1
9	8	4.72 ± 1.68	0.63 ± 0.19	4.09	13.3
12	8	4.15 ± 1.70	0.52 ± 0.07	3.63	12.5
3	12	4.97 ± 0.09	0.30 ± 0.16	4.67	6.0
6	12	3.27 ± 0.80	0.09 ± 0.06	3.18	2.8
9	12	3.18 ± 0.41	0.71 ± 0.02	2.47	22.3
12	12	6.91 ± 1.77	0.80 ± 0.29	6.11	11.6

*Biomass without triglyceride (g/L) = Whole Biomass (g/L) −Triglyceride production (g/L). Cells were grown in GPY medium containing various concentration of glucose at 25°C with continuous orbital shaking at 400 rpm. Values are expressed as means± S.D of three replicate experiments.

A remarkable change of fatty acid composition of total lipid was not observed, but the contents of unsaturated fatty acids gradually decreased with the increasing of glucose concentration in the medium. On the other hand, the fatty acid composition of triglyceride showed a significant variation when the glucose concentration in the medium was increased from 3% to 12% (Table 3). The decrease in PUFA was particularly remarkable. In particular, triglycerides containing PUFA completely disappeared from the cells grown in medium containing 12% glucose for 12 days, and the constituent fatty acids in the triglyceride were rearranged to only three saturated fatty acids (Table 3), pentadecanoic acid (C15:0), palmitic acid (C16:0) and heptadecanoic acid (C17:0). Palmitic acid accounted for over 50% of the constituent fatty acids in the triglycerides.

The lipid metabolism of the strain was altered by glucose concentration in medium and culture age of cells. Usually, glucose was utilized as a carbon source for cell growth. Table 1 shows the biomass without triglyceride (g/L), which was estimated from the data of the whole biomass and the triglyceride content.

The biomass without triglyceride in the medium containing 3% glucose for 4 days culture was 3.93 g/L, also, those in the media containing 6%, 9% and 12% glucose were 3.44, 3.74 and 3.83 g/L, respectively.

The biomass without triglyceride includes proteins, nucleotides, carbohydrates and phospholipids which are important cell components and these amounts must correlate with cell number. In fact, cell number of this strain increased with increasing 1% to 3% of glucose (data not shown), suggesting that glucose was mostly utilized for cell reproduction. From the present results, the glucose-rich was probably not suitable for cell growth. However, some metabolic changes occurred in the 12%

Table 2. Changes in fatty acid compositions of total lipid of *Aurantiochytrium* sp. strain 18W-13a grown in medium with various concentrations of glucose

Culture condition		Fatty acid					
Glucose conc. in medium (%)	Culture period (days)	C14:0	C15:0	C16:0	C17:0	C22:5	C22:6
3	4	1.1 ± 0.8	26.8 ± 1.9	11.2 ± 1.3	8.8 ± 0.4	17.7 ± 0.5	34.4 ± 1.1
6	4	2.0 ± 1.4	32.1 ± 2.1	13.2 ± 1.5	11.4 ± 0.5	13.7 ± 1.7	27.7 ± 2.8
9	4	6.7 ± 0.5	31.6 ± 0.2	21.2 ± 0.8	7.9 ± 0.2	10.3 ± 0.4	22.3 ± 0.7
12	4	6.9 ± 1.3	28.5 ± 1.8	23.8 ± 0.9	7.4 ± 0.1	10.0 ± 0.8	23.4 ± 1.7
3	8	2.1 ± 0.3	29.3 ± 0.9	11.1 ± 1.1	8.6 ± 0.1	17.6 ± 0.4	31.3 ± 0.1
6	8	3.2 ± 0.2	35.3 ± 1.9	14.1 ± 1.3	11.0 ± 0.4	12.8 ± 0.2	23.5 ± 0.3
9	8	4.2 ± 1.1	37.6 ± 4.8	19.3 ± 1.5	11.5 ± 0.7	8.9 ± 1.2	18.5 ± 2.6
12	8	4.4 ± 1.7	31.5 ± 3.6	23.6 ± 1.1	10.7 ± 0.8	9.2 ± 1.3	20.6 ± 2.5
3	12	1.3 ± 0.9	26.2 ± 3.1	11.1 ± 1.2	8.5 ± 0.5	18.9 ± 0.6	34.0 ± 1.7
6	12	1.1 ± 1.5	25.3 ± 2.0	13.4 ± 0.7	11.3 ± 0.7	18.0 ± 1.3	30.9 ± 1.4
9	12	4.0 ± 1.5	33.6 ± 4.0	19.4 ± 0.4	9.3 ± 0.4	11.2 ± 1.2	22.6 ± 3.3
12	12	7.6 ± 0.3	36.2 ± 5.0	24.8 ± 2.0	9.7 ± 1.5	7.2 ± 0.9	14.5 ± 3.5

Cells were grown in GPY medium containing various concentrations isothermally at 25°C with continuous orbital shaking at 400 rpm for 4 days and 12 days. Fatty acid composition is expressed as the mean ± SD (n=3). C14:0, myristic acid; C15:0, pentadecanoic acid; C16:0, palmitic acid; C17:0, heptadecanoic acid, C22:5, docosapentaenoic acid; C22:6, docosahexaenoic acid.

Table 3. Changes in fatty acid compositions of triglyceride of *Aurantiochytrium* sp. strain 18W-13a grown in medium with various concentrations of glucose

Culture condition		Fatty acid					
Glucose conc. in medium (%)	Culture period (days)	C14:0	C15:0	C16:0	C17:0	C22:5	C22:6
3	4	2.6 ± 1.9	19.9 ± 1.7	15.9 ± 6.3	5.3 ± 3.8	20.0 ± 1.4	36.5 ± 2.9
6	4	1.9 ± 1.3	10.9 ± 2.1	9.5 ± 1.0	4.0 ± 2.9	28.0 ± 1.1	45.7 ± 2.6
9	4	5.7 ± 0.5	24.7 ± 1.7	18.3 ± 2.9	7.9 ± 1.6	15.7 ± 2.4	27.7 ± 4.1
12	4	1.9 ± 2.6	14.1 ± 6.0	24.0 ± 1.2	11.6 ± 3.2	15.3 ± 2.3	33.2 ± 4.7
3	8	0.9 ± 0.7	14.2 ± 3.0	15.7 ± 3.7	9.5 ± 3.0	25.1 ± 0.4	34.5 ± 3.0
6	8	0.9 ± 0.9	7.1 ± 1.2	7.2 ± 1.3	9.7 ± 4.5	27.4 ± 2.5	47.6 ± 5.3
9	8	3.8 ± 0.5	15.8 ± 0.3	16.7 ± 4.0	8.9 ± 3.5	17.9 ± 2.3	36.9 ± 3.1
12	8	6.2 ± 0.9	23.0 ± 3.2	16.4 ± 2.3	8.8 ± 0.7	16.5 ± 1.9	29.2 ± 3.8
3	12	n.d.	13.6 ± 7.8	10.2 ± 3.4	9.8 ± 3.7	27.4 ± 5.0	39.0 ± 9.4
6	12	2.4 ± 0.5	21.7 ± 1.4	11.0 ± 0.7	8.7 ± 0.4	20.8 ± 1.5	35.3 ± 0.7
9	12	4.2 ± 3.1	19.8 ± 6.4	16.9 ± 4.9	7.9 ± 2.8	19.2 ± 5.4	31.9 ± 10.7
12	12	n.d.	27.9 ± 4.6	52.8 ± 4.2	19.3 ± 3.0	n.d.	n.d.

The culture condition and abbreviations are same as Table 2. n.d, not detected.

glucose medium for 12 days, because the biomass without triglyceride was significantly increased (6.11 g/L), and per-saturated fatty acids-containing triglyceride was formed. The metabolic changes and the rearrangement into per-saturated fatty acids-containing triglyceride were correctly reproduced under the experimental conditions described above, but the mechanism is still unclear.

Remarkable decreases of PUFAs were observed when the glucose concentrations in the medium were increased from 3% to 6%, 9% and 12%. In particular, in the cells grown in medium containing 12% glucose for 12 days, desaturase activity related to PUFA synthesis and elongation from C15:0 to C17:0 was completely suppressed associated with decrease of triglyceride to normal content. Probably, the metabolic changes and the rearrangement into per-saturated fatty acids–containing triglyceride were related closely.

As mentioned in Introduction Section, PUFAs and long chain saturated fatty acids cause serious problems when we use algal oils as biodiesel fuels (BDFs). The findings suppose that it is possible to rearrange the fatty acid composition of triglyceride including decrease of PUFAs and log chain saturated fatty acid by change of culture conditions such as glucose concentration, culture age, etc.

A strain of *Aurantiochytrium* sp. accumulates triglyceride consisting of only saturated fatty acids when grown in medium containing 12% glucose for 12 days. Cells of this *Aurantiochytrium* strain are able to rearrange the fatty acid composition of the triglyceride according to different culture conditions. This bio-rearrangement should be more intensively investigated for the preparation of a high quality of biodiesel oil.

Acknowledgement

We would like to thank Mr. Ryoji Kose at University of Tsukuba for his help on fatty acid analyses using GC and GC/MS. This work was supported by the Japan Science and Technology Agency (JST)-CREST funding and the Special Funds for Next Generation Energy Development Project, MEXT, Japan.

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Received 18 June 2011; accepted 7 August 2011