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Influence of medium components and pH on the production of odd-carbon fatty acids by *Aurantiochytrium* sp. SA-96

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Abstract

Odd-carbon fatty acids have been utilized for anaplerotic therapy for lifestyle-related diseases such as Alzheimer's disease and diabetes. We examined the culture condition of *Aurantiochytrium* for the production of pentadecanoic acid (C15) as an odd-carbon fatty acid, and demonstrated a produce method for odd-carbon fatty acid–enriched triglyceride. To determine the optimum conditions for C15 production, selection of effective components and their respective concentrations in the culture medium, choice of the suitable *Aurantiochytrium* strain, and determination of the optimum culture pH were performed.

The optimum conditions for the production of C15 were found to be as follows:

Strain: *Aurantiochytrium* sp. strain SA-96

Medium composition: 0.2% yeast extract 0.5% monosodium glutamate, 1.0% sea salt, 50 mM L-Val, 25 mM sodium propionate, 3.6% glucose, and 10% soy milk whey.

Culture system: Airlift culture vessels (5.0 L) and pH controller were used. Sodium hydroxide (1.0 M) solution was used for pH adjustment. Other parameters were as follows: medium volume, 3.0 L; air supply, 1.2 vvm; temperature, 23.5–26.6 °C; pH, 7.40–7.74; culture period, 72 h.

The yield of odd-carbon fatty acids (C15, 85% of odd FA) was 1.04 g L^{-1}.

Preparation of odd-carbon fatty acid–enriched triglyceride: Triglyceride was isolated from the extracted lipids from cultured cells and treated with ozone/hydrogen peroxide. By removing of carboxylic acid containing triglyceride, the odd-carbon fatty acid and C15 contents of the resultant triglyceride reached to 72.3 and 60.7%, respectively.

Keywords *Aurantiochytrium* · Odd carbon fatty acids · Pentadecanoic acid · L-valine · Propionate · Sodium propionate

Introduction

Aurantiochytrium, a marine thraustochyrid with a high growth rate, produces odd-carbon fatty acids (FAs) such as pentadecanoic acid (C15) (Song et al. 2013; Kaya et al. 2015) The hair glowing effect of C15-monoglyceride, which was due to propionyl-CoA derived from pentadecanoic acid (C15) by β-oxidation, has been demonstrated in hair follicles of rabbits (Adachi et al. 1993). Additionally, odd-carbon FAs have been used for anaplerotic therapy for Alzheimer's disease (Roe 2007; Bottiglieri and Roe 2010), cardiac disorder (Roe 2009; Rosiers et al. 2011), cancer (Baffy 2010), and diabetes (Miki et al. 1998; Forouhi et al. 2014). For anaplerotic therapy, synthesized triheptanoin has been used the food additive of medical foods (Roe 2007; Bottiglieri and Roe 2010). However, triglycerides consisted of odd-carbon FA from natural products are desired.

Odd-carbon FAs are metabolized into acetyl-CoA and propionyl-CoA by β-oxidation. The metabolic conversion from propionyl-CoA to methyl malonyl-CoA closely relates to anaplerotic reactions (Fig. 1). Previous studies concluded that propionate acts as a real anaplerotic

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substance (Owen et al. 2002; Kasumov et al. 2007). Propionyl-CoA is converted to methyl malonyl-CoA, which is subsequently converted to succinyl-CoA by the enzymatic activity of methyl malonyl-CoA mutase in a vitamin B_{12}-dependent manner. When succinyl-CoA, a member of the TCA cycle, is converted to succinic acid, GTP/ATP is produced (Johnson et al. 1998). GTP is a well-known signal transduction substance, which regulates multiple physiological functions is through the action of G-proteins (Wettschureck and Offermanns 2005). On the other hand, methyl malonyl-CoA, the key metabolite derived from propionyl-CoA, is an intermediate of branched-chain amino acid catabolism (Fig. 2) (Feller and Feist 1962; Murin et al. 2009). Considering that the odd-carbon FA, metabolism closely related to anaplerosis, the function is considerably different from that of even-carbon FAs as the common FAs.

Aurantiochytrium is a suitable microorganism for production of C15 as an odd-carbon FA (Shirasaka et al. 2005; Miyamoto et al. 2007; Song et al. 2013; Kaya et al. 2015). Propionic acid is a known precursor in the odd-carbon FA biosynthesis. However, the major metabolic pathway of propionic acid is not the production of odd-carbon FAs, but is anaplerosis (Kasumov et al. 2007) and the glycogenesis via pyruvate (Lehninger et al. 2008). Furthermore, propionic acid acts as an inhibitor of cell growth (Pennington and Appleton 1958). Therefore, it seems that propionic acid is not suitable to be as the precursor for odd-carbon FAs. In the cases of branched-chain amino acids, propionyl-CoA as a metabolite is formed from isoleucine, valine (Kinnory et al. 1955), and methionine (Harper et al. 1984), and is converted to methyl malonyl-CoA by methyl malonyl-CoA mutase with vitamin B_{12} as a coenzyme (Ludwig and Mattews 1997). When vitamin B_{12} is restricted in *Aurantiochytrium* cells, the concentration of propionyl CoA in cells ought to be increased, and the major pathway is expected to switch from the anaplerosis reaction to the synthesis pathway of odd-carbon FAs (Owen et al. 2002).

In this study, we examined the influence of medium components and pH condition on the production of C15 as the major odd-carbon FA by *Aurantiochytrium* sp. SA-96.

Materials and methods

Materials

Aurantiochytrium strains

Aurantiochytrium sp. SA-89 (NB6-3) isolated from seawater in Okinawa islands, Japan, was purchased from OP BIO Co. Ltd. (Okinawa, Japan). *Aurantiochytrium* sp. SA-96 (mutant) was induced from *Aurantiochytrium* sp. SA-89 by irradiation (150 Gy) of ^{12}C heavy ion beam. The heavy ion was accelerated using accelerators of RIKEN Nishina Center, Wako, Japan. The mutant (SA 96) was selected using indexes of high lipid content (weight % of lipid in dry cells) and increase in cell growth rate (biomass in a medium cultured for 72 h).

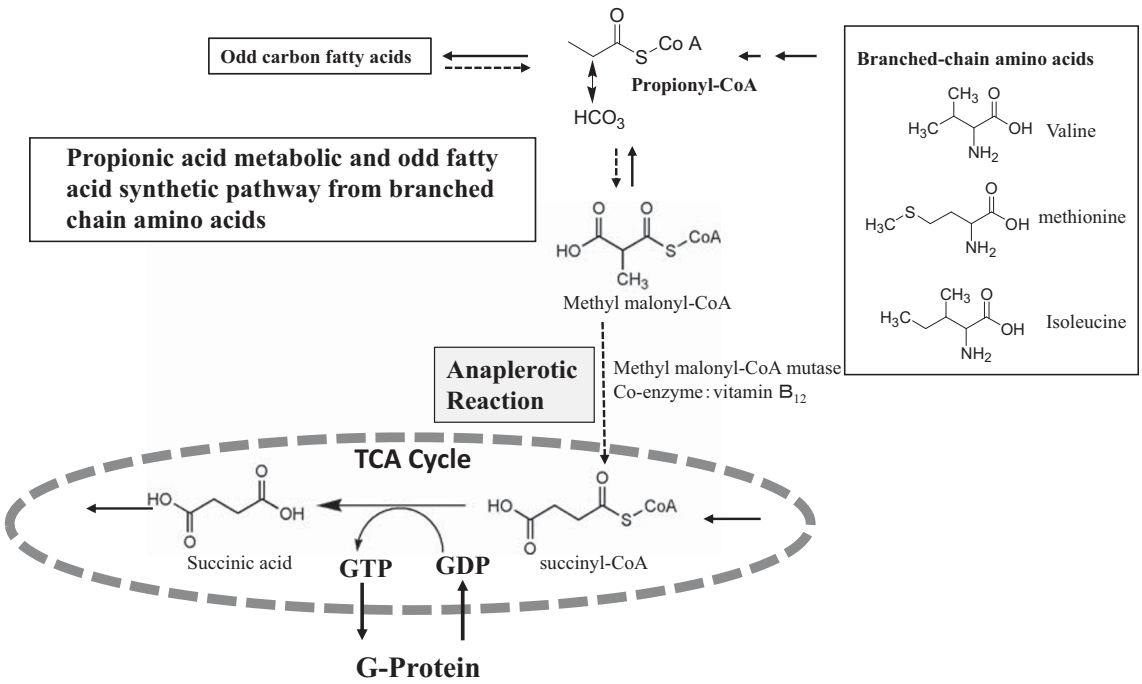

Fig. 1 Correlation between odd carbon fatty acids synthesis and propionyl-CoA related anaplerotic action

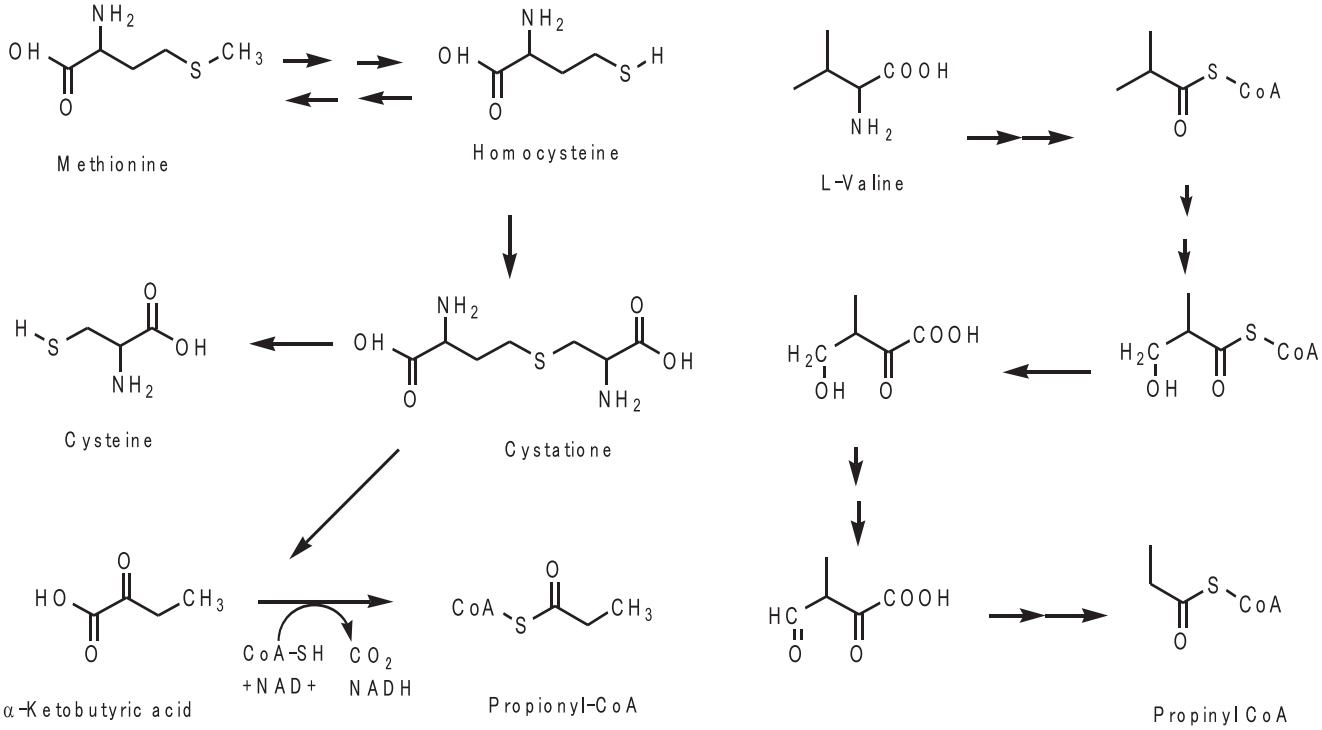

Fig. 2 Metabolic pathway of propionyl-CoA formation from methionine and valine

Chemicals

Amino acids [L-monosodium glutamate, L-valine (Val), D and L-methionine (Met), L-isoleucine (Ile), L-leucine (Leu), and L-threonine (Thr)] and D-glucose were from Wako Pure Chemicals, Osaka, Japan. Sodium propionate was from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Methyl esters of saturated and unsaturated fatty acids were from GL Science, Tokyo, Japan. Solvents and reagents were of analytical grade.

Basic culture medium

The basic culture medium consisted of 0.2% yeast extract (Yeastock S-Pd, Asahi Food and Health Care Co. Ltd., Tokyo, Japan), 0.5% monosodium glutamate, 1.0% sea salt (Red Sea salt, USA), 3.0% glucose and 10% soy milk whey.

Preparation of the soy milk whey was as follows: soy milk on the market (protein content, 3.5%) was heated at 80 °C, and added $MgCl_2 \cdot 7H_2O$ at the concentration of 0.7%. After cooling the $MgCl_2$ containing soy milk, aggregated protein was removed by centrifugation. The obtained soy milk whey was autoclaved at 115 °C for 30 min. The sterilized whey was stored at 5 °C until use. The medium was autoclaved at 121 °C for 20 min. Vitamin B_{12} contents in milk-whey and soy milk whey were determined according to the method of Matsumoto et al. (2012).

Methods

Culture condition

Small volume culture Each flask (500 mL) contained 200 mL medium, and set up in a reciprocal shaker (Thomas Kagaku Co Ltd., Japan). The shaker were maintained at 25 °C, 115 strokes/min for 72 h. After cultivation, cells were used for starter of mass culture, or were harvested by centrifugation at 3400 rpm for 30 min. The harvested cells were washed with saline and lyophilized.

Mass culture Airlift culture vessels (5.0 L) and pH controller (Automatic System Research, Saitama, Japan) were used (Fig. 3). NaOH (1.0 M) solution was used for the pH controller. The culture conditions were as follows; medium volume, 3.0 L; air supply, 1.2 vvm; temperature, 23.5–26.6 °C; pH, 7.40–7.74; culture period, 72 h. After cultivation, cells were harvested and lyophilized.

Lipid extraction and separation of triglyceride fraction

Lipid extraction Lipid was extracted from the lyophilized sample (200 mg) with 10 mL of chloroform /methanol (2:1, v/v). After centrifugation, 5.0 mL of the extracting solution was separated and added 1.0 mL of saline. The mixture was mixed well and centrifuged at 2800 rpm for 20 min. After centrifugation, the lower layer (chloroform

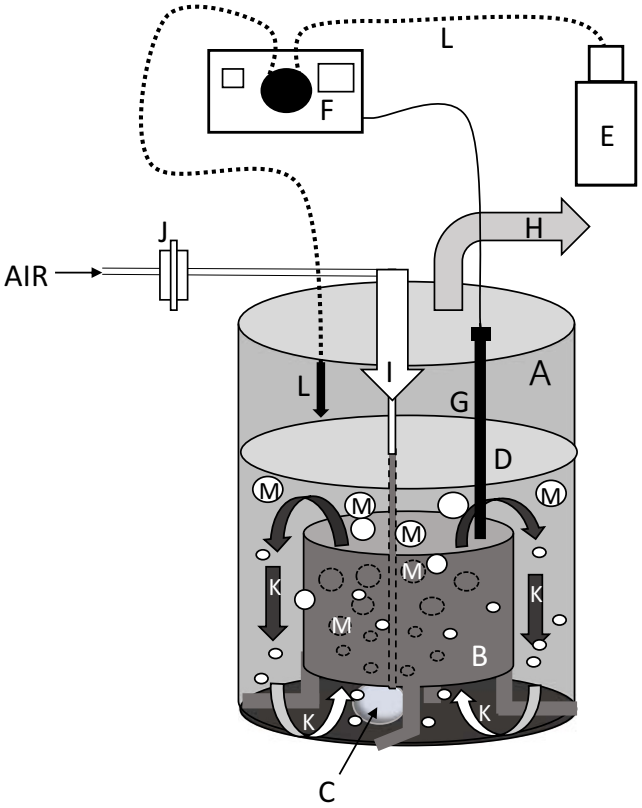

Fig. 3 Outline of the airlift culture vessel system. **a**, culture vessel; **b**, inner cylinder; **c**, ceramic sparger; **d**, medium surface; **e**, NaOH or HCl solution; **f**, pH controller; **g**, pH electrode; **h**, exhaust duct; **i**, air supply tube; **j**, air filter; **k**, medium and air bubbles streams; **l**, NaOH supply tube; **m**, air bubble

layer) was transferred to a test tube and dried under a nitrogen stream.

Isolation of triglyceride fraction Aliquot of extracted lipid was suspended with n-hexane, and applied to a silica-gel column (lipid 10 g per 20 mL bed volume) prepared with n-hexane. As the first, n-hexane (5 bed volumes) was passed through the column. After the solvent of n-hexane, chloroform (5 bed volumes) was applied to the column, and the eluted chloroform solvent was evaporated, and remaining residue was dissolved with n-hexane. The residue was confirmed as triglyceride by TLC (Kaya et al. 2015).

Preparation of odd-carbon FA-enriched triglyceride

Ozone/hydrogen peroxide treatment of triglyceride Isolated triglyceride (about 10 g) was suspended with 100 mL of n-hexane. The hexane solution was transferred to a 300 mL round-bottom flask containing 50 mL of 30% hydrogen peroxide. Ozone gas was babbled into the flask at 20 mL min^{-1} for 90 min. The oxidation reaction was performed at 20 °C in a draft chamber.

Purification of odd-carbon FA-enriched triglyceride After the oxidation reaction, the n-hexane layer was evaporated and dissolved with 10% water containing mixture of ethanol/ethyl acetate (1:1, v/v). The mixture was applied to a DEAE column (formate form). Triglyceride was eluted with the same solvent (4 bed volumes), and was obtained as a white solid after evaporation of the solvent.

GC analysis of fatty acids

FAs in lipid were converted to methyl esters using 14% BF3-methnol at 70 °C for 20 min. FA methyl esters (Injection: 1 μL/1.0 mL n-hexane) were analyzed using GC-FID (Shimadzu GC-2025) with a DB-23 (length, 60 m; internal diameter 0.25 mm; film thickness 0.15 μm; J&W Scientific). The GC operating conditions were as follows: column temperature, 50 °C held for 1 min, up to 175 °C (25 °C min^{-1}), and then up to 230 °C (4 °C min^{-1}), held for 5 min; FID port temperature, 250 °C; carrier gas (He) flow rate, 2.06 mL min^{-1}; FID H2 flow rate, 40 mL min^{-1}; and air flow rate, 450 mL min^{-1}. Fatty acid methyl esters of lipid samples were identified using authentic FA methyl esters (GL Science, Japan).

Results

Effect of C15 production on addition of cheese whey or soy milk whey to the culture medium

Cheese whey contains vitamin B_{12} about 3.0 μg L^{-1}, whereas soy milk whey does not. When *Aurantiochytrium* sp. SA-96 was cultured in 10% cheese whey or 10% soy milk whey, the C15 content in cells cultured using the soy milk whey containing medium was about 2.6 times higher than that using the cheese whey containing medium (Table 1).

Selection of the meriting strain of *Aurantiochytrium* sp. for C15 production

In this experiment, two strains were selected. One is the higher growth rate strain (SA-89) (increasing in biomass, g L^{-1} per 72 h), and the other is the higher fatty acid content strain (SA-96) (increasing in fatty acid content in biomass, mg g^{-1} dry cells). The strains were cultured in D- or L-Met containing medium for comparison of C15 production ability. The results are shown in Table 2. Strain SA-89 could metabolized L-Met, while could not D-Met. In the case of SA-96, Both D- and L-Met were converted to C15. When Strain SA-89 was being grown with L-Met, the biomass production was reduced to 36% of the control (10 mM Gly containing medium), but odd-carbon FA synthesis was increased to 150% of the control. The same phenomenon was also observed in strain SA-

Table 1 Comparison of pentadecanoic acid (C15) production of *Aurantiochytrium* sp. SA-96 by cheese whey or soy milk whey containing medium

Whey containing medium	Biomass (g L^{-1})	Total FA (%) in DCW	C15 in Total FA (%)	C15 (mg g^{-1} DCW)	C15 production (mg L^{-1})
Cheese whey	3.4	13.5	7.2	9.7	33.0
Soy milk whey	4.2	15.2	13.3	20.2	84.8

Medium: 1.2% sea salt, 0.2% yeast extract, 3.0% glucose, 10 mM L-Met, and 10% cheese whey or 10% soy milk whey. Culture condition: medium, 200 mL; temp. 25 °C; aeration, 115 strokes min^{-1}; period, 7 2 h. *DCW* dry cell weight. Values are averages of two independent experiments

96. C15 production of strain SA-96 was about 85 mg L^{-1} (L-Met containing medium). This value was 2.6 times higher than that of strain SA-89. From the results, we selected Strain SA-96 for odd-carbon FA production.

Effect of propionyl-CoA producing amino acids in the culture medium on C15 production

In the amino acid metabolism it is well known that Met, Val, Ile, and Thr are converted to propionyl-CoA, which is the first taken in odd-carbon FA. In order to examine the contribution of C15 production, L-Met, Val, Ile, or Thr containing medium was prepared. After culture, lipids were extracted from each flask, and C15 was determined using GC. C15 (%) in total FA from the Met, Val, Ile, Thr, or Leu containing medium were 13.3, 15.7, 14.8, 13.8, and 5.5%, respectively. Leu containing medium was used as a non-propionyl-CoA-producing amino acid. The results showed that L-valine containing medium was the effective medium for C15 production (Table 3).

Effect of pH during culture on cell growth

Aurantiochytrium can grow in conditions with pH values between pH 3.0 and 9.0. Correlation of pH and the cell growth was examined. pH was shifted from 4.0 to 8.0 using the pH controller with a pH electrode. Cell growth was drastically changed by the pH shift; increasing as pH raised, cell growth increased more. The results were shown in Fig. 4. After 72 h of inoculation, at pH 4.0, the biomass concentration was 4.2 g L^{-1}. On the other hand, when pH was set up at 7.4, the biomass was increased to 12.4 g L^{-1}. These results suggest that the most suitable pH for the odd-carbon FA production in culture medium was around 7.4. The fatty acid compositions of cells cultured in various pH were almost unchanged.

Effect of L-valine concentration in the culture medium on C15 production using an airlift culture vessel

These above-obtained results were applied to an airlift mass culture vessel for C15 production, that is, (1) *Aurantiochytrium* sp. SA-96 strain was selected; (2) soy milk whey and L-valine were used as culture components; and (3) pH of the culturing medium was maintained 7.4.

When L-Val concentration in the culture medium was changed to 10, 20, 50, and 100 mM, C15 ratio in total FA increased in a manner dependent on the L-Val concentration increase (Table 4). However, total FA content in lyophilized cells decreased when the concentration of L-Val was 100 mM, whereas biomass amount did not vary with the varying concentration of L-Val. As the results, the most effective concentration of L-Val for C15 and odd-carbon FA production was 50 mM (Table 5).

The conversion efficiency from L-Val to odd-carbon FA was obtained as follows:

Table 2 Comparison of pentadecanoic acid (C15) production ability of *Aurantiochytrium* sp. SA-89 and SA 96 cultured in D-Met or L-Met containing medium

Strains	Amino acid	Biomass (g L^{-1})	Total FA (mg g^{-1} DCM)	C15 in total FA (%)	C15 (mg L^{-1})
SA-89	control	12.6	63	3.3	26.2
SA-89	D-Met	13.1	62	3.7	30.1
SA-89	L-Met	4.6	63	13.2	38.3
SA-96	control	9.9	158	4.5	70.4
SA-96	D-Met	4.1	159	12.8	83.4
SA-96	L-Met	4.2	152	13.3	84.8

Medium: 1.2% sea salt, 0.2% yeast extract, 3.0% glucose, 10 mM D- or L-Met, and 10% soy milk whey

Culture condition: medium, 200 mL; temp. 25 °C; aeration, 115 strokes min^{-1}, period, 72 h. *DCW* dry cell weight. Values are averages of two independent experiments; As control, 10 mM Gly containing medium was used instead of D- or L-Met

Table 3 Effect of propionyl-CoA producing amino acids on C15 production

Addition of amino acid (10 mM)	Biomass (g L^{-1})	Total FA (mg g^{-1} DCW)	C15 in total FA (%)	C15 (mg L^{-1})
control	9.9	158	4.5	70.4
L-Met	4.2	152	13.3	84.9
L-Val	6.8	117	15.7	124.9
L-Ile	7.0	104	14.8	107.7
L-Thr	7.4	112	13.8	114.4
L-Leu	9.8	152	5.5	81.9

Medium: 1.2% sea salt, 0.2% yeast extract, 3.0% Glucose, and 10% soy milk whey. 10 mM of L-Met, Val, Ile, Thr, or Leu was added into the medium. Culture condition: medium, 200 mL; temp. 25 °C; aeration, 115 strokes min^{-1}; period, 72 h. *DCW* dry cell weight. Values are averages of two independent experiments; As control, 10 mM Gly containing medium was used instead of propionyl-CoA producing amino acids

A: Area dot counts of total FA by GC-FID (GC injection weight,1.0 μg (1.0 μg/1.0 μL), 265,000 (average of 7 experiments); **B**: Area dot counts of C15 (85% of odd FA) by GC-FID (GC injection weight, 1.0 μg), 263,000 (average of quadruple experiments); **C**: Sampling weight for lipid extraction, 200 mg; **D**: Total FA (mg g^{-1} of lyophilized cells) = Area dot counts/A × 1/0.2; **E**: Total FA (g L^{-1}) = D × Biomass (g L^{-1})/1000; **F**: Odd FA (g L^{-1}) = E × Odd FA (%) × 265,000/263,000; **G**: Odd FA (mM L^{-1}) = F/257 (mw of $C_{15}H_{30}O_2 \cdot CH_3$); **H**: Conversion efficiency = [(G 0.62 mM L^{-1}*)/L-Val (mM L^{-1})] × 100. (*0.62 mM L^{-1} is the odd-carbon FA concentration in biomass cultured in non L-Val medium).

As shown in Table 5, in the L-Val-to-odd-carbon FA conversion, the most efficient concentration of L-valine was 20 mM, while the maximum odd-carbon FA production (1.04 g L^{-1}) was observed for an L-Val concentration of 50 mM. When L-Val concentration in the culture medium was 100 mM, only FA production decreased (to 64% of that obtained with 50 mM L-Val).

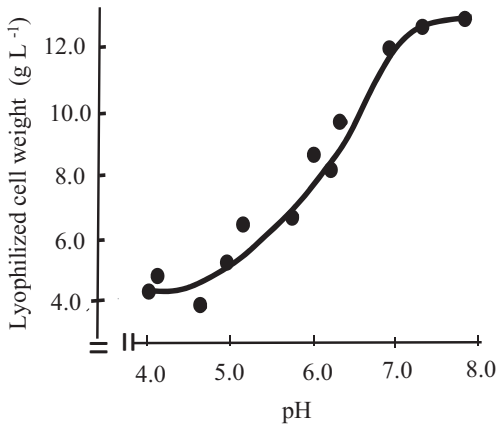

Fig. 4 Relationship between medium pH and cell growth. Medium: 1.2% sea salt, 0.2% yeast extract, 3.0% glucose, 10 mM L-Val, and 10% soy milk whey. Culture condition: medium, 200 mL; temp. 25 °C; aeration, 115 strokes min^{-1}, period,72 h. Values in the figure are averages of duplicate experiments. pH was controlled using pH controller and 1.0 M NaOH or 1.0 M HCl

Effect of glucose concentration on odd-carbon FA production

Glucose is an important energy source, and acetyl-CoA is an important metabolite. Carbon of glucose is an essential element of the cell machinery, including FAs. In the microorganism cultivation, an increase in glucose concentration leads to growth of biomass growth. The effect of glucose concentration on C15 production in the L-Val containing medium was examined.

In the airlift culture, about 3% glucose in the medium was consumed over 72 h. In the case of 1.8% glucose in the medium, the glucose was completely consumed for 72 h after inoculation. When cells were grown with 3.6% glucose containing the medium for 72 h, the remaining glucose in the medium was only 0.48%. In the case of 7.2% glucose, the remaining glucose was 3.91%. The results show that glucose consumption in the culture system was unaltered by of the amount of glucose available.

Total FA content obtained when using 3.6% glucose containing medium showed the maximum of was maxed out at 23.3% of the dry cells. The total FA content obtained from 7.2% glucose containing medium was drastically decreased to 10.8%. This decrease was most likely due to the unbalanced molecular ratio of propionyl-CoA (propionic acid) from L-Val and acetyl-CoA (acetic acid) from glucose (Table 6).

Effect of propionate on odd-carbon FA production

Propionic acid is well known as a cell growth inhibitor (Pennington and Appleton 1958). Alternatively, the acid is a precursor of odd-carbon FA. When cells were grown in the L-Val and propionate-containing medium, the odd-carbon FA production was expected to be affected. To confirm the effect on the odd-carbon FA production, cells of *Aurantiochytrium* sp. SA-96 were grown with the 50 mM L-Val and various concentrations of sodium propionate-containing medium. As the results, the FA composition of total lipid in the cells was changed

Table 4 Effect of various concentrations of L-Val in culture medium on fatty acid composition

Fatty acid	Fatty acid composition (%)				
	0 mM L-Val	10 mM L-Val	20 mM L-Val	50 mM L-Val	100 mM L-Val
C12	0.5 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
C13	0.3 ± 0.01	1.0 ± 0.01	1.0 ± 0.01	0.9 ± 0.01	2.2 ± 0.02
C14	13.1 ± 0.17	10.4 ± 0.18	9.9 ± 0.09	7.3 ± 0.07	8.5 ± 0.09
C15	4.3 ± 0.07	13.4 ± 0.26	16.2 ± 0.33	17.4 ± 0.34	20.8 ± 0.22
C16	31.0 ± 0.49	21.9 ± 0.41	21.0 ± 0.42	20.9 ± 0.41	20.6 ± 0.39
C17	0.8 ± 0.01	2.0 ± 0.01	2.1 ± 0.01	2.1 ± 0.01	3.2 ± 0.02
C20:4	2.6 ± 0.06	1.0 ± 0.05	1.2 ± 0.03	1.0 ± 0.02	1.2 ± 0.01
C20:5	8.3 ± 0.21	14.1 ± 0.21	13.2 ± 0.27	12.7 ± 0.23	11.2 ± 0.31
C22:5	33.2 ± 081	32.4 ± 0.91	29.1 ± 0.83	26.9 ± 0.81	27.1 ± 0.73
C22:6					
others	5.9 ± 0.06	3.5 ± 0.20	6.0 ± 0.08	10.6 ± 0.09	5.0 ± 0.06
Odd FA (%)	5.4	16.4	19.3	20.3	24.2

Medium: 1.0% sea salt, 0.2% yeast extract, 0.5% monosodium glutamate, 3.6% glucose, L-Val (10, 20, 50, or 100 mM) and 10% soy milk- whey. Culture system, airlift culture vessel system; Culture condition: strain, SA-96; medium volume, 3.0 L; temp., 24.5–27.5 °C; pH, 7.4–7.7; aeration volume, 1.2–1.3 vvm; culture period, 72 h. Values are averages with standard deviations (±SD) of three independent experiments

altered significantly. When the concentration of sodium propionate in the medium was altered from 10 to 50 mM, As increase in the concentration of sodium propionate, the ratio of C15 in the total FA was increased, and that of palmitic acid (C16) was decreased remarkably, whereas that of myristic acid (C14) as an even fatty acid was stayed at the same values. The results seem to indicate that the elongation reaction from C14-CoA to C16 was hindered by the synthesis pathway of C15. Ratio of poly unsaturated FA such as docosahenxaenoic acid (C22:6) in the total FA almost unaltered (Table 7). Probably, the reason of the unchanged phenomenon is due to the fact that polyunsaturated FAs are synthesized by polyketide pathway (Metz et al. 2001), which the pathway is considerably different from that of FA. The parameters on the odd-carbon FA production are summarized in Table 8. Biomass (g L^{-1}), lipid in dry cells (g L^{-1}) and total FA (g L^{-1}) were gradually decreased as increase in the concentration of propionate. The odd carbon FA (odd-carbon FA, g L^{-1}) showed an intricate locus. At 25 mM propionate, production of odd carbon FA was reached its maximum as 1.33 g L^{-1}.

Preparation of odd-carbon FA-enriched triglyceride

The triglyceride isolated from the lipids of cells cultured in the medium contained 50 and 25 mM sodium propionate was consisted of 42.2% odd FA, 20.7% even carbon saturated FA, and 37.1% unsaturated FA. When the triglyceride was treated with ozone/hydrogen peroxide, double bonds of unsaturated FA were cleaved, and these were converted to carboxylic acid. Mono- and di-carboxylic acids containing triglycerides ought to be formed. These triglycerides were removed using DEAE ion exchange resin of formate type. As the results, the yield of the odd-carbon FA-enriched triglyceride from the original triglyceride, which was isolated from cells cultured in the medium contained L-Val and sodium propionate, was 48%. The FA composition of the odd-carbon FA-

Table 5 Relationship between L-Val concentration and odd carbon fatty acid (Odd FA) production

L-Val Conc mM	Biomass (g L^{-1})	Total FA (g g^{-1} DCW)	Total FA (g L^{-1})	Odd FA in total FA (%) (C15%)	Odd FA (g L^{-1})	Odd FA (mM L^{-1})	Conversion efficiency (%)
0	18.6 ± 0.18	0.208 ± 0.009	3.869 ± 0.204	5.4 ± 0.09	0.209 ± 0.015	0.81 ± 0.05	–
10	20.1 ± 0.19	0.165 ± 0.008	3.317 ± 0.192	16.4 ± 0.28	0.545 ± 0.041	2.12 ± 0.15	21.2
20	20.9 ± 0.20	0.215 ± 0.011	4.478 ± 0.267	19.3 ± 0.35	0.856 ± 0.067	3.33 ± 0.26	16.7
50	21.1 ± 0.22	0.233 ± 0.012	4.948 ± 0.283	20.3 ± 0.36	1.048 ± 0.075	4.08 ± 0.29	8.2
100	21.4 ± 0.25	0.144 ± 0.011	3.090 ± 0.248	24.2 ± 0.24	0.756 ± 0.067	2.94 ± 0.26	2.9

Total FA area dot count/265000 /100 = total FA (g g^{-1} DCW); Total FA (g g^{-1} DCW) × Biomass (g L^{-1}) = Total FA(g L^{-1}). Total FA (g L^{-1}) × Odd FA (%) × 265,000/263000/100 = Odd FA (g L^{-1}); Odd FA g L^{-1} / 257 (mw of $C_{15}H_{30}O_2 \cdot CH_3$) × 1000 = Odd FA mM L^{-1}; [(Odd FA mM − 0.81 mM (0 mM L-Val)/ L)/L-Val addition (mM)] × 100 = Conversion efficiency (%). Values are averages with standard deviations (±SD) of three independent experiments

Table 6 Effect of glucose concentration on C15 production

Glucose concentration (%)		Biomass (dry weight) (g L^{-1})	Total FA (g g^{-1} DCW)	Total FA (g L^{-1})	Odd FA in total FA (%)	Odd FA g L^{-1}
Before inoculation	72 or144 h after inoculation					
1.8%	0.01 (72 h)	12.3	0.061	0.75	12.3	0.092 Av. $n = 2$
3.6%	0.48 ± 0.07 (72 h)	21.1 ± 0.22	0.233 ± 0.012	4.916 ± 0.283	20.3 ± 0.36	1.048 ± 0.075 SD, $n = 3$
7.2%	3.91 (72 h)	25.7	0.108	2.776	26.4	0.733 Av. $n = 2$
	1.19 (144 h)	26.8	0.116	3.109	11.0	0.342 Av, $n = 2$

Medium: 1.0% sea salt, 0.2% yeast extract, 0.5% monosodium glutamate, 1.8, 3.6 or 7.2% glucose, L-Val, 50 mM and 10% soy milk- whey. Culture system, airlift culture vessel system; Culture condition: strain, SA-96; medium volume, 3.0 L; temp., 24.5–27.5 °C; pH, 7.4–7.7; aeration volume, 1.2 vvm; culture period, 72 or 144 h. Values in the table are averages of two independent experiments(AV), or are averages with standard deviations (SD) of four independent experiments. *DCW*, dry cell weight; *Odd FA*, Odd carbon fatty acid; *FA*, fatty acid. Glucose concentration was determined using glucose determination kit (Miwa et al. 1972)

enriched triglyceride constituted 72.3% odd-carbon FA and 27.7% saturated even-carbon FA. Interestingly, the C15 content of the triglyceride reached to 60.7% (Table 9). The obtained odd-carbon FA-enriched triglycerides can be used in anaplerotic therapy for lifestyle-related diseases such as Alzheimer's disease and diabetes.

Table 7 Effect of sodium propionate (NaC_3) on fatty acid composition of total lipid from cells

Fatty acid	Fatty acid composition (%)			
	0 mM NaC_3	10 mM NaC_3	25 mM NaC_3	50 mM NaC_3
C11		0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.01
C12	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	0.2 ± 0.01
C13	0.9 ± 0.04	2.2 ± 0.13	3.4 ± 0.21	4.2 ± 0.22
C14	7.3 ± 0.35	10.0 ± 0.61	10.6 ± 0.65	7.7 ± 0.41
C15	17.4 ± 0.92	29.3 ± 1.81	35.7 ± 2.21	37.1 ± 2.00
C16	20.9 ± 1.04	13.7 ± 0.85	10.3 ± 0.66	8,7 ± 0.46
C17	2.1 ± 0.10	3.9 ± 0.23	4.2 ± 0.26	4.4 ± 0.23
C20:4	1.0 ± 0.04	1.2 ± 0.07	1.1 ± 0.06	1.1 ± 0.05
C20:5	12.7 ± 0.63	10.6 ± 0.65	9.5 ± 0.59	9.1 ± 0.49
C22:5	26.9 ± 1.34	27.7 ± 1.71	24.2 ± 1.55	25.8 ± 1.39
C22:6				
others	10.6 ± 0.53	1.0 ± 0.05	0.5 ± 0.03	1.4 ± 0.07
Odd FA	20.4	35.4	43.3	45.7

Medium: 1.0% sea salt, 0.2% yeast extract, 0.5% monosodium glutamate, 3.6% glucose, 50 mM L-Val, 10% soy milk- whey and various concentration of sodium propionate (NaC_3). Culture system, airlift culture system; Culture condition: strain, SA-96; medium, 3.0 L; temp., 24.5–27.5 °C; pH, 7.4–7.7; aeration volume, 1.2–1.3 vvm; culture period, 72 h. Values are averages with standard deviations (±SD) of three independent experiments

Discussion

To examine the effect of vitamin B_{12} on production of odd-carbon FA, cheese whey (containing B_{12}) and soy milk whey (not containing B_{12}) were used. As shown in Table 1, the difference of C15 content is reflected in the difference of vitamin B_{12} content in the medium. L-Met in medium is converted to propionyl-CoA, and further converted to methyl malonyl-CoA. The methyl malonyl-CoA is formed succinyl-CoA by methyl malonyl CoA mutase with vitamin B_{12} as the coenzyme. In the case of vitamin B_{12} restricted medium (soy milk whey containing medium), the enzyme activity of methyl malonyl-CoA mutase is ought to decrease. As the results, propionyl-CoA content is increased. The increase in propionyl-CoA content is connected to induce the synthesis of odd-carbon FAs.

The results showed that L-valine containing medium was the effective medium for C15 production (Table 3), and the most effective concentration of L-Val for C15 and odd-carbon FA production was 50 mM. L-Val is converted to propionyl-CoA, which is the primary substrate in odd-carbon FA synthesis. However, a part of propionyl-CoA is converted to propionic acid by hydrolysis. The formation of propionic acid is the cause of the pH drop in the culture medium. Furthermore, propionic acid inhibits acetyl-CoA metabolism. The results obtained with the 100 mM L-Val medium indicated that high concentrations of L-Val in the medium inhibited FA synthesis.

The glucose consumption during the culture in the medium is interesting. When the culture using 7.2% glucose medium was extended up to 144 h, glucose concentration in the medium decreased to 1.19%. This value showed that glucose consumption was 3% per 3 days. In the 144 h culture, total FA content in the dry cells was constant, whereas odd-carbon FA

Table 8 Effect of propionate (NaC_3) on the parameters of odd FA production

	0 mM NaC_3	10 mM NaC_3	25 mM NaC_3	50 mM NaC_3
Biomass (dry cells) g L^{-1}	21.1 ± 0.22	18.3 ± 0.17	17.4 ± 0.16	16.4 ± 0.15
Total Lipid fraction g L^{-1}	9.17 ± 0.50	6.81 ± 0.37	4.87 ± 0.26	2.10 ± 0.12
Total FA g L^{-1}	4.95 ± 0.28	2.03 ± 0.15	3.05 ± 0.23	1.54 ± 0.11
Odd FA g L^{-1}	1.044 ± 0.08	0.723 ± 0.05	1.325 ± 0.10	0.707 ± 0.05
Total FA/total lipid fraction	0.54	0.30	0.63	0.73

Parameters were obtained from the experiments of Table 7

content in the total FA rapidly decreased to 26.4% of that for a 72-h culture to 11.0%. One hundred forty-four-hour culture after inoculation is just the death phase. At the death phase, anaplerotic reaction related to propionyl-CoA and consumption of odd-carbon FA may be stimulated.

Propionic acid is a cell growth inhibitor. For understanding the inhibition mechanism, we focused the weight ratio of total FA/total lipid. The ratio obtained from the medium without propionate was 0.54. At 10 mM propionate, the ratio was decreased to 0.30. This result suggests that FA synthesis was suppressed, and non-FA compounds, such as carotenoids and terpenoids, were not suppressed by propionate. When the concentration was beyond 10 mM propionate, the ratios were increased to 0.63 at 25 mM and 0.73 at 50 mM propionate. At these concentrations, FA and non-FA compounds productions were decreased. The results suggest that the decrease in the production of non-FA compounds was more drastic than that of FA.

The matter of the cost on the odd-carbon triglyceride production is an important primary factor. To suppress the cost of raw materials, soy milk whey was utilized instead of the Bacto Tryptone as a component of the standard culture medium. In a tofu manufacturing process, soy milk whey is usually discarded. However, the whey is a valuable unused resource that is consisted of carbohydrate, protein, ash as major components and minor components such as phytochemicals. The soy milk whey as the unused resources contributed the lowering cost of raw materials of the culture medium.

As a next step, we shall search for unused carbohydrate resources that can replace glucose for the culture, and aim to increase yield of odd-carbon FA-containing triglyceride.

Table 9 Condensation of odd FA in triglyceride by the oxidation

Fatty acid	Fatty acid composition (%)	
	None treated Triglyceride	Ozone / H_2O_2 treated Triglyceride
C11	0.2	0.2
C12	0.2	0.2
C13	3.2	4.6
C14	9.5	11.4
C15	34.5	60.7
C16	11.0	16.1
C17	4.3	6.8
C20:4	1.1	0.0
C20:5	9.8	0.0
C22:5	26.2	0.0
C22:6		
Odd FA	42.2	72.3
Even Saturated FA	20.7	27.7
Unsaturated FA	37.1	0.0
Treated triglyceride	5.0 g	
Odd FA enriched triglyceride		2.4 g

n-Hexane solution of triglyceride was treated with ozone and hydrogen peroxide

The reaction condition was described in the Methods Section

Conclusion

Considering the above results obtained, the optimum conditions for production of C15 (odd-carbon FA) are as follows:

Strain: *Aurantiochytrium* sp. strain SA-96.

Medium composition: 0.2% yeast extract 0.5% monosodium glutamate, 1.0% sea salt, 50 mM L-Val, 25 mM sodium propionate, 3.6% glucose, and 10% soy milk whey.

Culture system: Airlift culture vessels (5.0 L) and the pH controller attached with a pH electrode were used. NaOH (1.0 M) solution was used for the pH adjustment. Medium volume, 3.0 L; aeration volume, 1.2 vvm; temperature, 23.5–26.6 °C; pH, 7.40–7.74; culture period, 72 h.

This resulted in production of 1.04 g L^{-1} odd-carbon FA (C15, 85% of odd-carbon FA).

Preparation of odd carbon fatty acid enriched triglyceride: Triglycerides were isolated from the extracted lipids from cultured cells, and treated with ozone/hydrogen peroxide. By removing the carboxylic-acid-containing triglycerides, the C15 content of the triglycerides reached to 60.7%.

Author contribution statement K.K. conceived this study. Experiments on culture mediums were performed by F.S. To obtain mutants strains, the heavy ion (^{12}C) irradiation to a wild strain of *Aurantiochytrium* were

performed by Y.K and T.A. K.K analyzed the results, designed figures, and wrote the manuscript.

Compliance with ethical standards

Competing interests The authors declare no competing interests.

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