Isolation and characterization of *Aurantiochytrium* species useful for ω-3 fatty acids production

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CONTENTS

ABBREVIATIONS

14:0	Myristic acid
15:0	Pentadecanoic acid
16:0	Palmitic acid
16:1	Palmitoleic acid
17:0	Margaric acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
23:0	Tricosanoic acid
ASW	Artificial seawater
DCW	Dry cell weight
DHA	Docosahexaenoic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
ETA	Eicosatetraenoic acid
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FDS	Fermented defatted soybean
GC	Gas chromatography
GCMS	Gas chromatography-mass spectrometry
ω6-DPA	ω6-Docosapentaenoic acid
ω3-DPA	ω3-Docosapentaenoic acid
PKS	Polyketide synthase
PUFA	Polyunsaturated fatty acid
SDA	Stearidonic acid

GENERAL INTRODUCTION

ω3-Polyunsaturated fatty acid (ω3-PUFA) is defined as the fatty acid with the double bond presented at 3 atom away from the methyl terminal group. Abundant ω3-PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been reported to have several health benefits. For example, prevention or treatment of cardiovascular disease (1), visual and brain development (2), and anticancer (3). Therefore, they are extensively utilized in pharmaceuticals and functional foods.

In addition to abundant ω3-PUFA, rare ω3-PUFA such as ω3-docosapentaenoic acid (ω3-DPA), a few studies indicated it has beneficial bioactivities (4-6). Although rare ω3-PUFA exists in nature, its content is comparatively low and without commercial source. Thus, it was recognized to develop alterative resources, accumulate health claim evidence, and expand their applications as current issues.

On the other hand, EPA and DHA are essential nutrients for fish growth and development (7-9). Hence, they were also used as aquaculture feed ingredients (10). However, utilizing fishmeal and fish oil had some problem in terms of sustainability. Therefore, the approach replaced them by fish-free ingredients has been attempted as one of solutions to this problem. For instance, fishmeal and fish oil could be substituted by soybean meal (11, 12) and microbial oil (13, 14).

Recently, oleaginous microorganisms accumulated ω3-PUFA were attracted much attention because of easier to scale up and less affection by season and climate (15). Based on these advantages, microbial oil is recognized as alternative source for ω3-PUFA (16, 17).

In this study, several ω3-PUFAs-producing strains were isolated from brackish areas in Japan and evaluated the production of rare ω3-PUFAs. Additionally, DHA production in liquid and solid-state fermentation utilizing plant residues were also evaluated for developing aquaculture feed ingredients.

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CHAPTER 1

Isolation and characterization of the ω3-docosapentaenoic acid-producing microorganism *Aurantiochytrium* sp. T7

ABSTRACT

ω3-Docosapentaenoic acid (ω3-DPA), an ω3-polyunsaturated fatty acid (ω3-PUFA), is expected to have beneficial physiological functions to humans; however, because of its rarity in nature, it has not been fully analyzed. The author isolated an ω3-DPA producing microorganism strain T7 from brackish areas in Japan. Although most oleaginous microorganisms rarely accumulate ω3-DPA (<5% of total lipid), strain T7 accumulated ω3-DPA with more than 20% of total fatty acids. The strain T7 was identified as a related species of *Aurantiochytrium*. In *Aurantiochytrium* sp. T7, ω3-DPA production reached 164 mg/L culture broth, and the ω3-DPA content reached 23.5% of the total fatty acids when cultivated in a medium containing 2% glucose as the carbon source and 1% yeast extract as the nitrogen source, with a salinity equivalent to 50% of that of seawater and a pH in the acidic range (pH < 5.5). *Aurantiochytrium* sp. T7 is a promising producer of high-purity ω3-DPA containing-lipid for the functional analysis of ω3-DPA whose physiological function has hardly been elucidated, and a useful strain for investigating the novel metabolic pathway of fatty acids.

INTRODUCTION

ω3-Polyunsaturated fatty acids (ω3-PUFAs; Fig 1) are important dietary compounds because of their various physiological functions, such as brain development acceleration, allergy suppression, and circulatory disease risk reduction (1–3). ω3-PUFAs are also important precursors of biologically active substances in mammals (4). In recent years, the demand for ω3-PUFAs as pharmaceutical materials and food additives has rapidly increased. Among ω3-PUFAs, eicosapentaenoic acid (EPA; C20:5ω3), docosahexaenoic acid (DHA; C22:6ω3) and α-linolenic acid (ALA; C18:3ω3), which can be prepared from natural sources such as fish oils and vegetable oils, have been well studied because of their sufficient natural supply. For example, EPA and DHA, now typically derived from a natural marine source, have been reported to reduce the risk for sudden death caused by cardiac arrhythmia and all-cause mortality in patients with known coronary heart disease (5–7) and to prevent rheumatoid arthritis and asthma (8–10). ALA, contained in natural oils from higher plants such as linseed and soya, has been also reported to contribute to the prevention of several diseases, such as coronary heart disease and depressive illness (11, 12). As described, research on the physiological functions and practical applications of major ω3-PUFAs has been actively pursued.

On the other hand, there are only few reports on rare ω3-PUFAs, such as stearidonic acid (SDA; 18:4ω3), ω3-eicosatetraenoic acid (ETA, 20:4ω3) and ω3-docosapentaenoic acid (ω3-DPA; 22:5ω3) because of their limited supply to date. Recently, SDA has been reported to be accumulated in *Echium plantagineum*, and its purification method is now under way (13). ETA is hard to find in nature; thus, there are few reports on ETA production by gene manipulation of *E. plantagineum*, *Arabidopsis thaliana* and *Mortierella alpina* (14–17). ω3-DPA is present in several natural oils obtained from harp seal and so on, but its content is no more than 5% in total lipid (18, 19). These minor ω3-PUFAs have been also expected to possess beneficial function as precursors of bioactive substances as well as EPA and so on. Recently, it has been reported that ω3-DPA increases in compensation for DHA deficiency in animals (20–22), that ω3-DPA is specifically contained in human breast

milk as much as arachidonic acid (23) and that ω3-DPA derivatives suppress allergic diseases (24). Thus, a sufficient supply of ω3-DPA and its derivatives is required to elucidate their detailed physiological functions.

Thus, ω3-DPA production by alternative sources, such as oleaginous microorganisms, fungi, plants, and microalgae, has attracted the attention of researchers. Particular, the author focused on Labyrinthulea, a representative oleaginous microbe producing ω3-PUFAs, as the stable and large source of ω3-DPA. In this study, the author report the isolation of a new candidate strain for high ω3-DPA production, *Aurantiochytrium* sp. T7, and the evaluation of its ω3-DPA productivity and lipid profile.

MATERIALS AND METHODS

Strains, media and growth conditions

Marine microorganisms were isolated from fallen mangrove leaves collected from several brackish areas in Japan by using pine pollen as a bait (25). The collected zoospores were cultivated on GYA medium [20 g/L glucose, 10 g/L yeast extract (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and 18 g/L artificial sea salt (Daigo's artificial seawater SP; Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan)] containing 15 g/L agar and 200 mg/L chloramphenicol until colonies appeared. GYA medium was used for liquid cultivation, followed by the fatty acid composition analysis, unless otherwise noted.

For investigation of effects of nitrogen sources in medium, polypeptone, tryptone, tryptose, marine broth, neopeptone, and peptone were purchased from Nippon Becton Dickinson Company, Ltd. (Nippon BD, Tokyo, Japan). Corn steep liquor was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Analysis of the total fatty acid composition in lipid

For total fatty acid analysis, all strains were inoculated into a test tube containing 10 mL GYA medium and cultivated at 28°C with shaking (300 rpm) for 2–14 days. The cultured cells were harvested by centrifugation at 3,000 × *g* for 10 min and washed twice with distilled water. The cells were freeze-dried (Tokyo Rika Kikai Co., Ltd., Tokyo, Japan) for 12 h and then weighed to evaluate cell growth. The dried cells were disrupted by using a Multi-Beads Shocker (TOMY Seiko Co., Ltd., Tokyo, Japan) at 5,000 rpm for 30 s twice. Lipids were extracted from cells by the method of Bligh-Dyer (26). The extracted lipids were methyl-esterified in 10% methanolic HCl with tricosanoic acid (23:0) as an internal standard. The esterified samples were extracted with hexane and quantified by using a gas chromatograph (GC; Shimadzu, Kyoto, Japan) and a gas chromatograph-mass spectrometer (GC-MS; Shimadzu) equipped with a flame-ionization detector and a split injector using a TC-70 capillary column (GL Science, Inc., Tokyo, Japan), as previously

described (27). The fatty acid ester peaks were identified and calibrated with standard fatty acids. The data shown are the average values of at least three determinations.

Isolation of genomic DNA and identification of 18S rDNA sequences from strain T7

The cells were cultivated, harvested and freeze-dried. Then, the genomic DNA was extracted using a standard phenol/chloroform method (28). The 18S rDNA region was amplified by polymerase chain reaction (PCR) with the forward primer 18S1-F (5′-TACCTGGTTGATCCTGCCAG-3′) and the reverse primer 18S12-R ′(5′-CCTTCCGCAGGTTCACCTAC-3′) (29) with T7 genomic DNA as the template. The resultant PCR fragment was cloned into the pUC118 plasmid (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions, and the insert sequence was determined by the sequencing services of Eurofins Genomics, Inc. (Tokyo, Japan) with M13 primers. The sequence was compared with the 18S rDNA sequence of various microorganisms stored in the DNA Data Bank of Japan (DDBJ) by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Isolation and identification of high ω3-DPA producing strain T7

Approximately 300 strains were isolated, which indicated that certain level of salinity in brackish areas is optimal for the growth of oleaginous marine microorganisms. Next, all strains were sent to analyze their fatty acid compositions by GC. Comparing their gas chromatograms to that of ω3-DPA standard reagent, a high ω3-DPA producing strain T7 was found (Fig 2). This compound was confirmed to be ω3-DPA by GC-MS analysis (data not shown). Other than strain T7, 30 strains that accumulated ω3-PUFAs were found, but their ω3-DPA content was all less than 5% of total fatty acids but approximately 25% in T7 (Fig 3)

Species identification of strain T7

The almost complete length of 18S rDNA sequence (1778 bp) from strain T7 was amplified, cloned and sequenced. BLAST analysis against nucleotide sequences from various microorganisms in the DDBJ indicated that the 18S rDNA sequence of strain T7 exhibited 99.8% homology with *Aurantiochytrium limacinum* (Fig 4). Moreover, strain T7 showed typical *Aurantiochytrium* characteristics in morphological (yellowish-white, creamy, round colonies with raised ridges and wavy edges) and microscopic (spherical cells, thin cell walls and oil droplet vesicles) observations. Based on these results, strain T7 was identified as an *A. limacinum*-related species (Fig 5). The nucleotide sequence of strain T7 is available in the DDBJ database under the accession number LC651621.

Effects of cultivation conditions on the ω3-DPA productivity of *Aurantiochytrium* sp. T7

To evaluate the ω3-DPA productivity of *Aurantiochytrium* sp. T7, the effects of various cultivation conditions (carbon sources, nitrogen sources, salinity, temperature and initial pH of cultivation medium) were investigated. The cells of *Aurantiochytrium* sp. T7 were cultivated for 7 days, unless otherwise noted, because this strain showed the

maximum ω3-DPA production on day 7 when cultivated on the standard GYA medium (data not shown).

The effects of the carbon source on growth and ω3-DPA production are shown in Fig 6. D-Glucose, D-fructose, acetic acid and glycerol promoted cell growth, whereas other saccharides, organic acids and sugar alcohols did not. Glycerol elicited ω3-DPA productivity comparable to that seen for glucose.

The effects of the nitrogen source on growth and ω3-DPA production are shown in Fig 7. Polypeptone, tryptone, marine broth, corn steep liquor and yeast extract promoted cell growth and total lipid production. Yeast extract, which was used as the basic nitrogen source in GYA medium, was preferable for growth and ω3-DPA accumulation (day 7). Inorganic nitrogen sources, including $(NH_4)_2SO_4$, $NaNO_3$ and urea, did not support the sufficient growth of *Aurantiochytrium* sp. T7.

To investigate the effects of artificial seawater on growth and ω3-DPA production, different concentrations (0%–7.2% artificial sea salt, equivalent to a salinity of 0–2 times that of seawater) were studied (Fig 8). Seawater salinities of 0.9%, 1.8% and 3.6% were effective for growth and ω3-DPA production; especially, 1.8% sea salt was optimal. In contrast, growth and ω3-DPA production were suppressed in 0% and 7.2% seawater salinity.

To evaluate the effects of the cultivation temperature, strain T7 was cultivated at 16°C, 28°C and 37°C. The growth, fatty acid productivity and ω3-DPA production were all higher when cultivated at 28°C (data not shown).

The effects of the initial pH of the cultivation medium are shown in Fig 9. ω3-DPA production was approximately constant in the acidic pH range (pH 3.0–5.5) and decreased in the medium at pH > 5.5.

Time course of ω3-DPA accumulation in *Aurantiochytrium* sp. T7 and effects of glucose addition in culture

The time course of ω3-DPA accumulation in *Aurantiochytrium* sp. T7 was evaluated under optimized cultivation conditions for ω3-DPA production as follows: 2%

glucose, 1% yeast extract, 1.8% artificial sea salt, 28°C culture temperature and 5.0 initial pH (Fig 10). As a result, the accumulated amount of ω3-DPA increased in the later stage of the culture, while ω3-DPA was hardly detected in the early stage. ω3-DPA production reached 164 mg/L culture broth on day 4, and the ω3-DPA content reached 23.5% of total fatty acids on day 7. The glucose in the medium was completely consumed by day 2.

When glucose was added on day 3, the increase of ω3-DPA content in the later stage of culture was delayed and slightly restrained, and the decrease of other fatty acids was also suppressed (Fig 11).

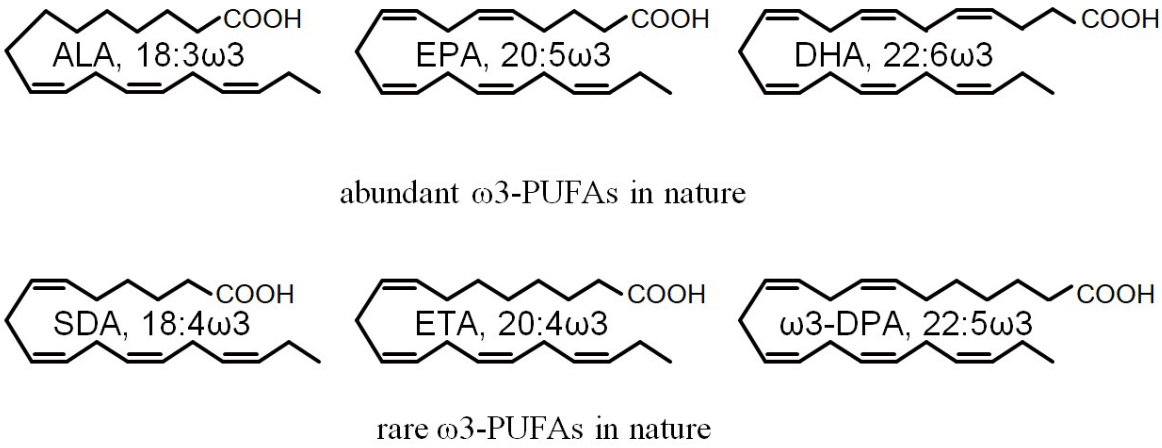

Fig 1. Structure of various ω3-PUFAs. ALA, α-linolenic acid; EPA, ω3-eicosapentaenoic acid; DHA, ω3-docosahexaenoic acid; SDA, stearidonic acid; ETA, ω3-eicosatetraenoic acid; ω3-DPA, ω3-docosapentaenoic acid.

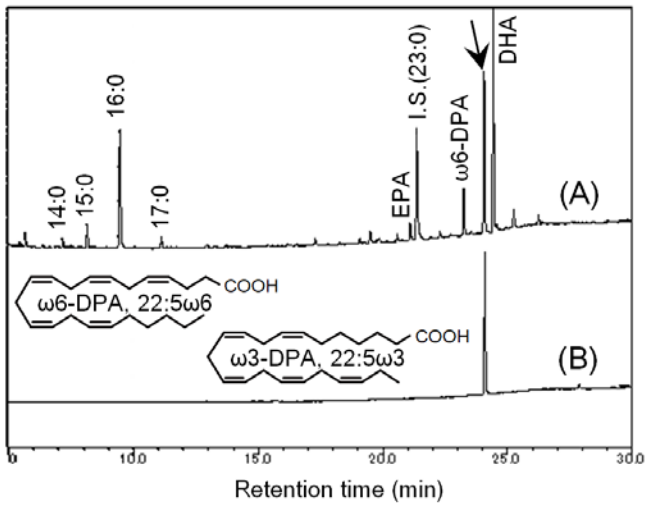

Fig 2. Gas chromatogram of fatty acids produced by (A) the isolated strain T7 and (B) ω3-DPA standard. Arrow indicates ω3-DPA. I.S., internal standard; other abbreviations, see Fig 1. All fatty acids were methyl ester-derivatized.

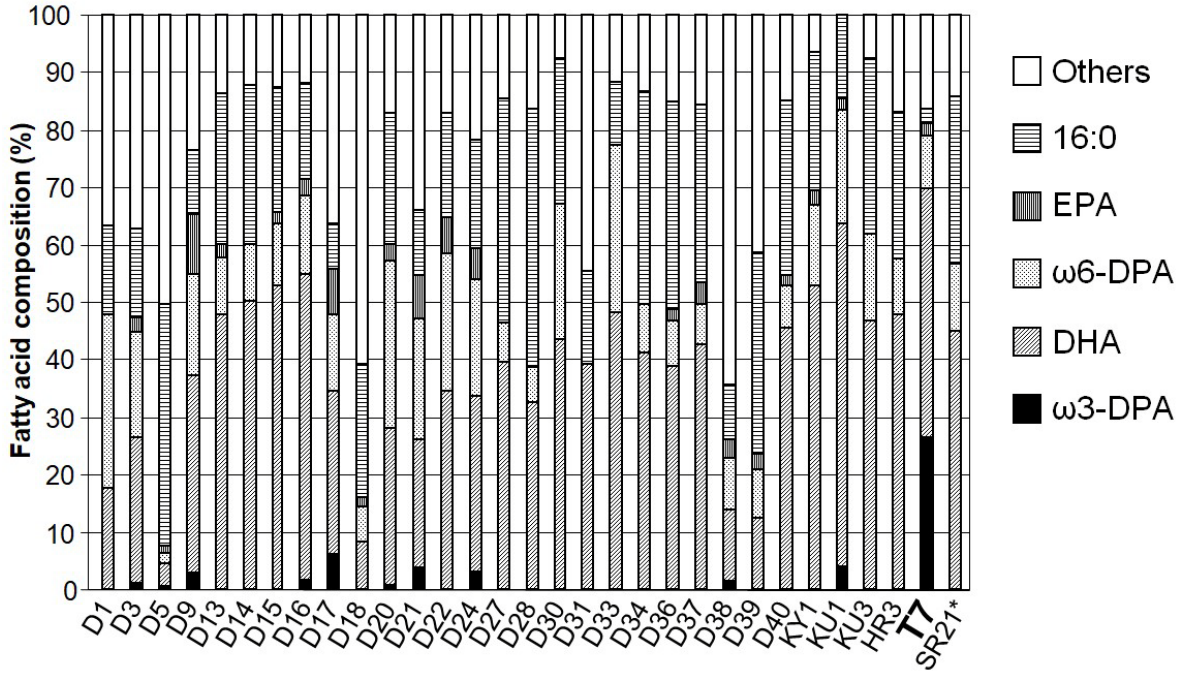

Fig 3. Comparison of fatty acid profiles produced by the isolates. All strains were cultivated for 7 days in GYA liquid medium. EPA, ω3-eicosapentaenoic acid; ω6-DPA, ω6-docosapentaenoic acid; DHA, ω3-docosahexaenoic acid; ω3-DPA, ω3-docosapentaenoic acid. *SR21, *Schizochytrium limacinum* SR21 (36) was used as a reference strain.

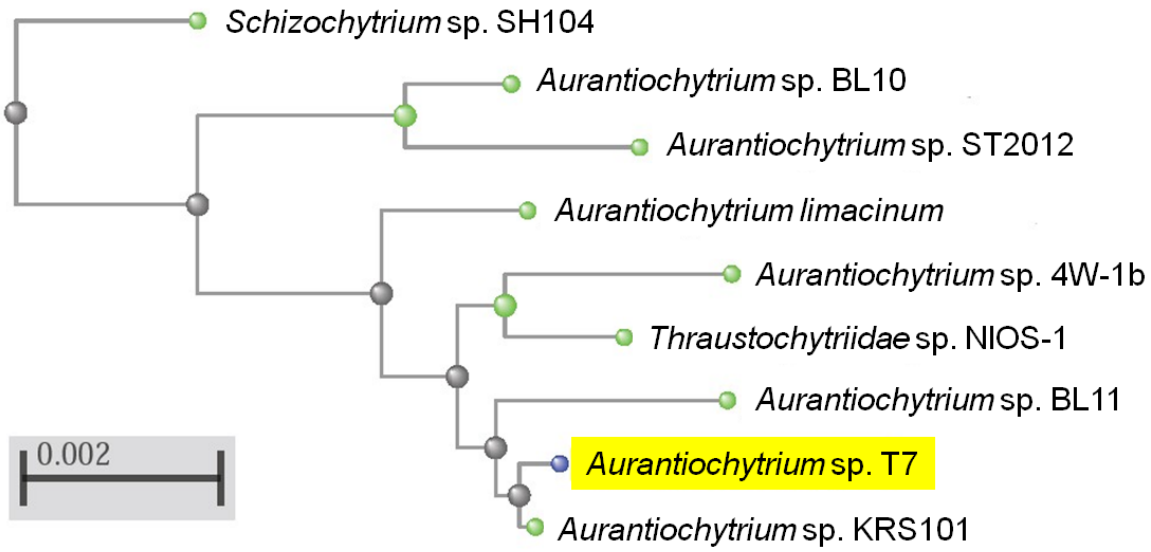

Fig 4. Phylogenetic tree of the strain T7 and close NCBI (BLASTn) strains based on the 18S rDNA gene sequences (fast minimum evolution method). The scale bar indicates 0.002 nucleotide. The highlight in the figure indicated the strain used in this study.

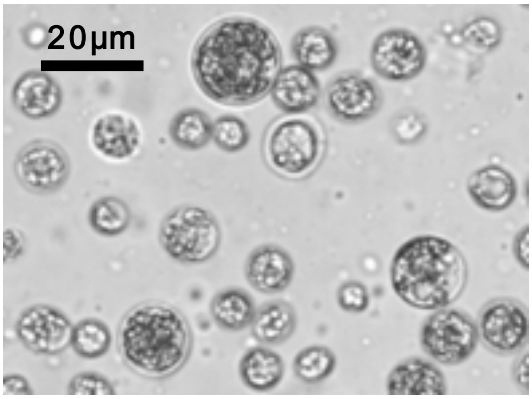

Fig 5. Microscopic image of *Aurantiochytrium* sp. T7. It was cultivated in GYA medium for 3 days.

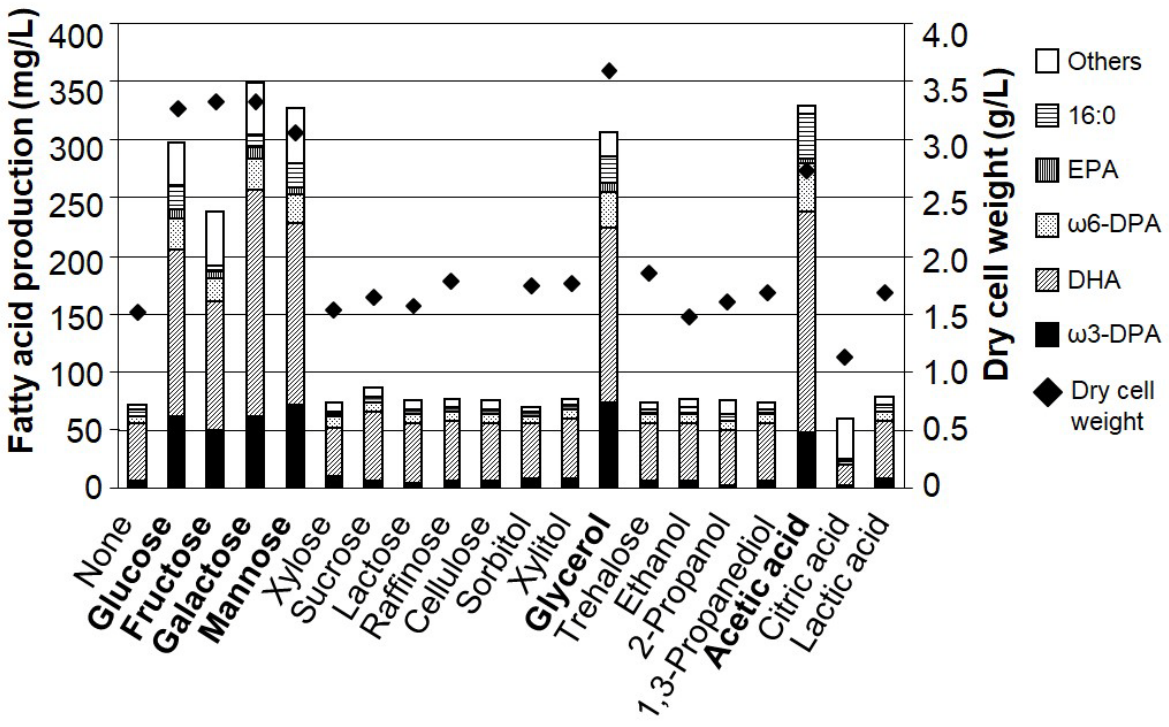

Fig 6. Effects of carbon sources on growth and fatty acid production in strain T7. They were cultivated in media containing 2% carbon source, 1% yeast extract and 1.8% artificial sea salt for 7 days. Because insoluble cellulose in the medium could not be separated from cultivated cells, dry cell weight in the cellulose medium was not measured. For all abbreviations, see Fig 2.

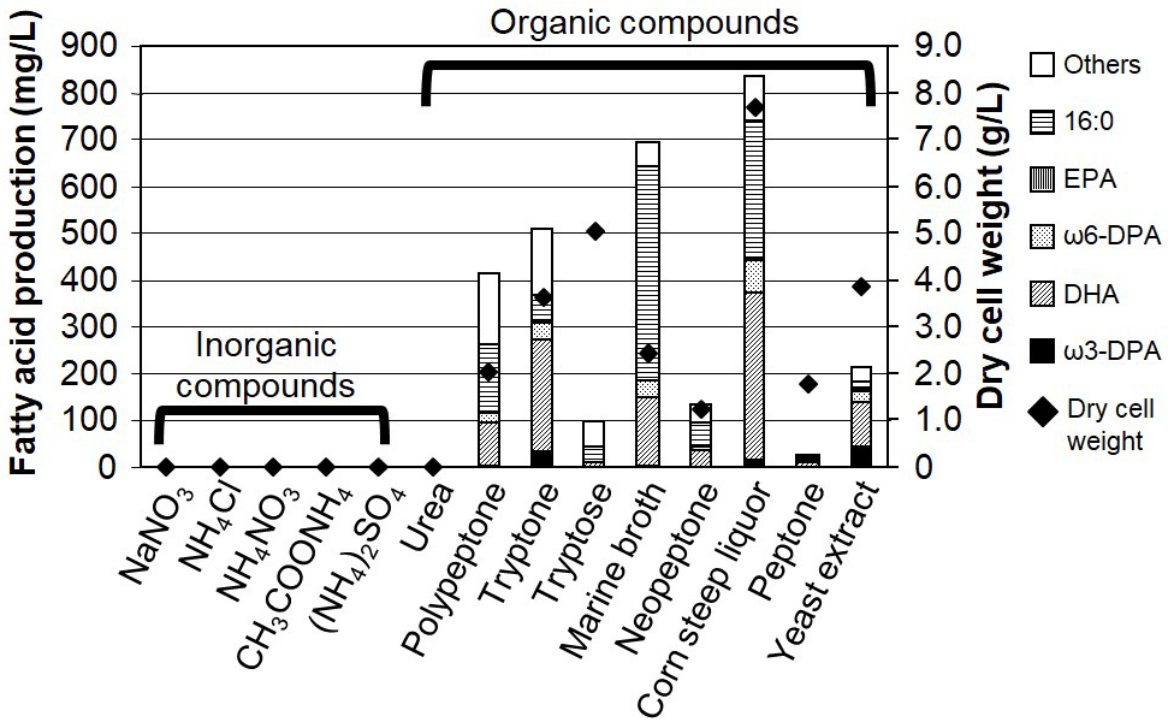

Fig 7. Effects of nitrogen sources on growth and fatty acid production in strain T7. They were cultivated in media containing 2% glucose, 1% nitrogen source and 1.8% artificial sea salt for 7 days. For all abbreviations, see Fig 2.

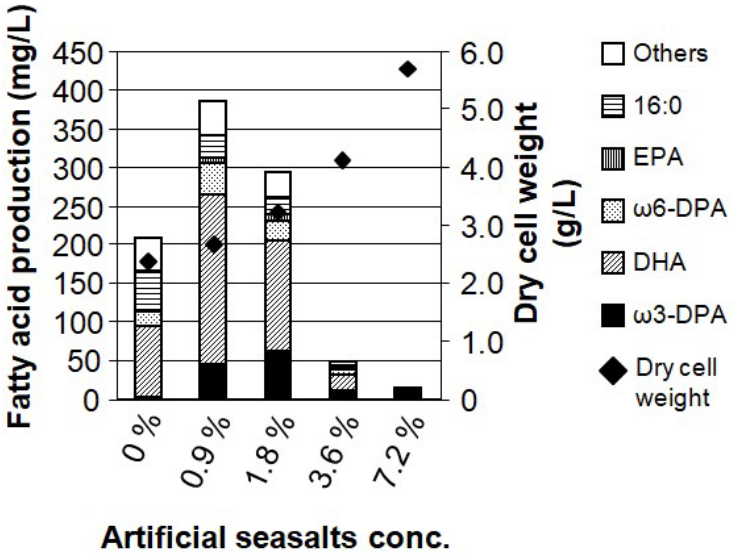

Fig 8. Effects of sea salt concentration on growth and fatty acid production in strain T7. They were cultivated in a medium containing 2% glucose, 1% yeast extract and 0%–7.2% artificial sea salt for 7 days. For all abbreviations, see Fig 2.

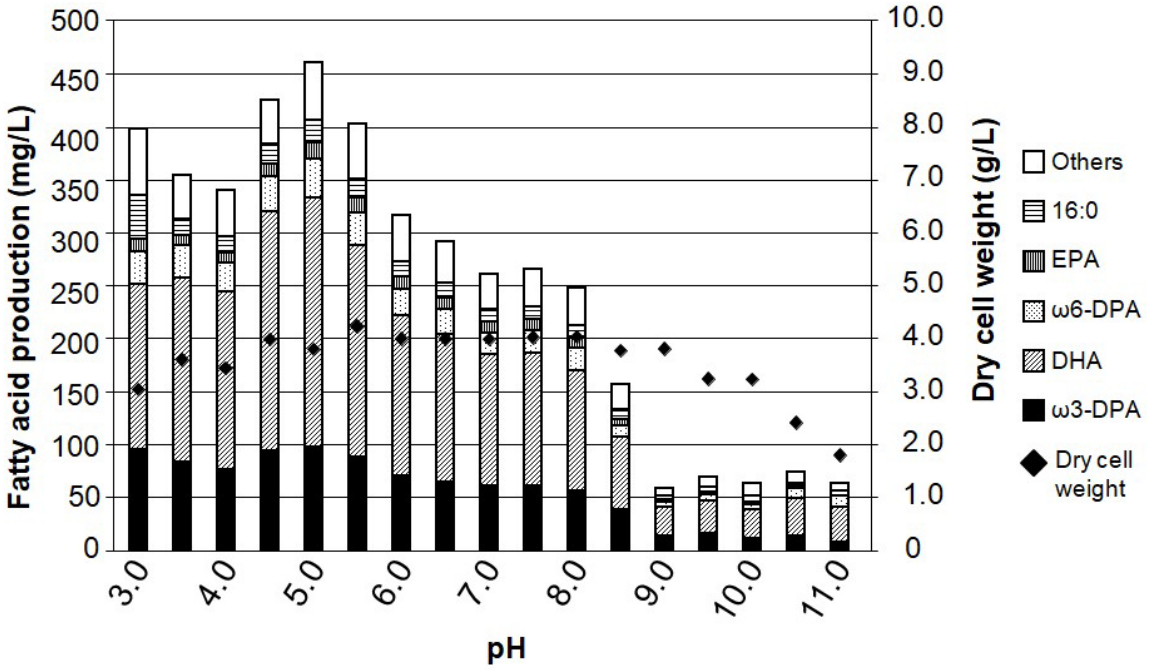

Fig 9. Effects of initial pH on growth and fatty acid production in strain T7. They were cultivated in GYA medium for 7 days with initial pH adjusted with HCl or NaOH. For all abbreviations, see Fig 2.

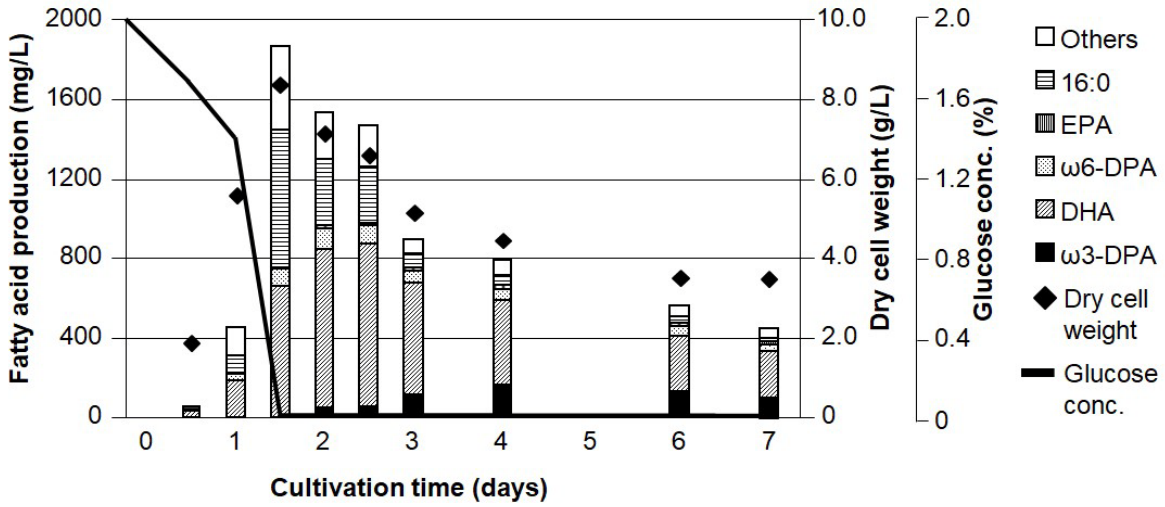

Fig 10. Time course of growth, fatty acid production and residual glucose concentration in culture of strain T7. It was cultivated in GYA medium. For all abbreviations, see Fig 2.

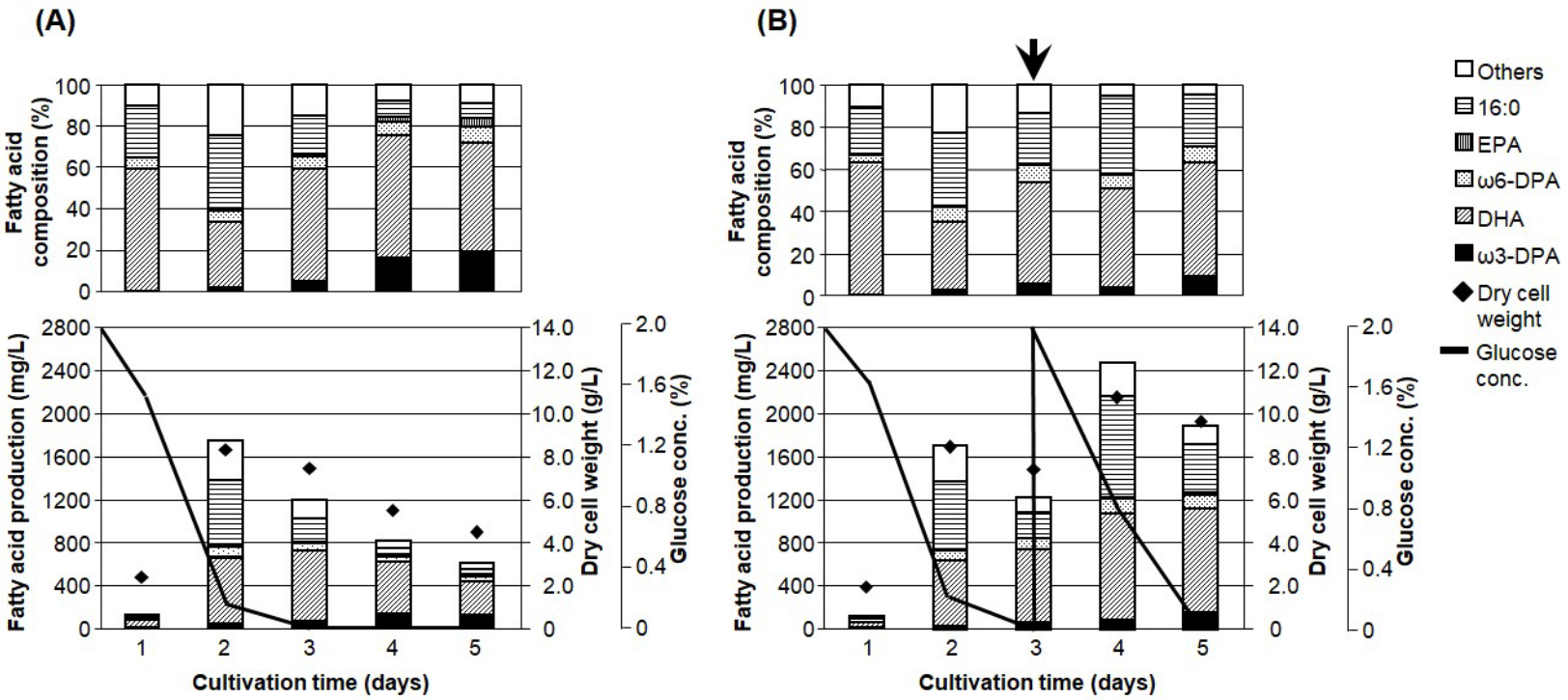

Fig 11. Effects of glucose addition on fatty acid composition in strain T7. (A) no glucose addition and (B) glucose addition to 2 % on day 3 (arrow). They were cultivated in GYA medium. For all abbreviations, see Fig 2.

DISCUSSION

The author screened 300 strains o f ω3-PUFA producing-microorganisms from brackish areas in Japan. These strains had various fatty acid compositions. This study focused on strain T7, which showed the highest ω3-DPA content among the isolated strains. The ω3-DPA content of strain T7 accounted for over 20% of the total fatty acids (Fig 2), which was higher than the previously reported ω3-DPA producing natural sources (<5% ω3-DPA). Using phylogenetic analysis, T7 was identified to belong to the heterotrophic *Aurantiochytrium* species. *Aurantiochytrium* sp. is known to be a DHA-producing microorganism, but its ω3-DPA content is limited to approximately 5% of the total fatty acids in all previous reports (30, 31). Actually, the author cultivated and analyzed several other microorganisms under the same conditions as that on the strain T7, but their ω3-DPA content was less than 5% (data not shown). These results suggested that an unknown mechanism for ω3-DPA production, not found in general *Aurantiochytrium* sp., is working in *Aurantiochytrium* sp. T7.

By evaluation of the effects of carbon sources in the cultivation medium on the growth and lipid production of *Aurantiochytrium* sp. T7, this strain was found to produce lipids well in the medium containing hexoses, acetic acid, or glycerol (Fig 6). These results suggested that these carbon sources are easily metabolized and utilized by the lipid biosynthetic pathways in this strain. Among them, glycerol is a by-product of biodiesel production (32), and interest on the processes for its utilization has been increasing. *Aurantiochytrium* sp. T7 is able to efficiently produce ω3-DPA from glycerol; therefore, it may be suitable for utilizing excess glycerol.

As shown in Fig 11, ω3-DPA accumulation in T7 was increased in the later stage of culture, whereas the other fatty acids decreased. Although glucose was added to the medium when glucose was completely depleted (day 3), increase of ω3-DPA content was delayed and slightly restrained. Meanwhile, the decrease of other fatty acids was suppressed. These results suggested that ω3-DPA is not directly biosynthesized from glucose in strain T7.

In general, ω3-PUFA producing-microorganisms have been known to synthesize ω3-PUFAs via the fatty acid synthase (FAS) pathway and/or polyketide synthase-like (PKS) pathway (33). In microorganisms with the FAS pathway, ω3-PUFA is synthesized via alternating steps of desaturation and elongation; therefore, various PUFAs, including precursors of ω3-PUFAs, are produced (34). In contrast, in microorganisms with the PKS pathway, the fatty acid profiles are simpler because DHA and/or EPA are formed through the reduction, dehydration and isomerization reactions of keto groups in cycles of polyketide-forming chain-elongation reaction (35). *Aurantiochytrium* sp. T7 is presumed to biosynthesize DHA and EPA through the PKS pathway because of its relatively simple PUFA composition. However, its ω3-DPA accumulation only in the later stage of culture in *Aurantiochytrium* sp. T7 suggested that ω3-DPA may be biosynthesized via an alternative pathway that does not involve FAS and PKS. By elucidating the accumulation mechanism of ω3-DPA in strain T7, it is expected that the new biosynthetic pathway of PUFA may be revealed, and the method of the specific production of unique fatty acids will be developed.

In recent years, ω3-DPA has attracted the attention of researchers because of several interesting reports about it; for example, ω3-DPA is contained in human breast milk as much as arachidonic acid (23). ω3-DPA is expected to possess beneficial function for human as with EPA and DHA and so on, but research on the bioactivity of ω3-DPA has not sufficiently progressed because of its low supply. *Aurantiochytrium* sp. T7 is a promising producer of ω3-DPA. Although ω3-DPA production by *Aurantiochytrium* sp. T7 remains lower (~150 mg/L broth) than other major ω3-PUFAs, such as DHA and EPA, industrially produced by oleaginous microorganisms (31), T7 has a useful potential to produce lipids containing a high composition of ω3-DPA, which are not found in existing natural resources, without relying on genetic recombination technology. In the future, *Aurantiochytrium* sp. T7 is expected to develop into new industrial uses in the fields of food materials and pharmaceuticals.

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CHAPTER 2

Utilization of fermented defatted soybean for polyunsaturated fatty acids production by a newly isolated strain, *Aurantiochytrium* sp. 6-2

ABSTRACT

ω3-Docosahexaenoic acid (DHA) is an important ω3-polyunsaturated fatty acid (ω3-PUFA) in fish oil and plays a key role for most saltwater fish growth and development. To support the continuous development of aquaculture industry, an alternative low cost source for DHA is essential. The author isolated a DHA-producing strain *Aurantiochytrium* sp. 6-2 and evaluated its DHA productivity by using fermented defatted soybean as the nitrogen source. *Aurantiochytrium* sp. 6-2 could be cultivated in a medium containing 15% glucose as the carbon source and 20% fermented defatted soybean as the nitrogen source and produce 47.7 g/L total fatty acids (12.2 g/L DHA) by optimizing aeration. Common hexose and organic nitrogen source could also be used as carbon and nitrogen source, respectively, for the cultivation of strain 6-2. Additionally, salinity equivalent to 25-200% of that of seawater and initial pH range of 6-9 is preferable for the growth and fatty acid production. Moreover, this strain could coculture with koji and *Bacillus* and grow on solid substrate with optimal moisture. Based on these results, *Aurantiochytrium* sp. 6-2 was revealed to be a promising strain for producing ω3-PUFAs from plant residues such as fermented defatted soybean for formulating a novel fish feed.

INTRODUCTION

Aquaculture is the fastest-growing and most diverse food production system in our world (1). However, production in the aquaculture industry such as fish and crustaceans has highly relied on imported feed ingredients (2).

Aquafeeds are traditionally composed of fishmeal and fish oil, which come from small prey fish species, such as herrings, sardines and anchovies. In addition, some parts are derived from fish wastes (3). Supplements of fishmeal and fish oil in fish feeds were aimed at providing protein and fatty acids at low prices (4). However, the cost has risen significantly due to insufficient wild-catch forage fish and high demand from the poultry, feed, and aquaculture industry (5-6). Also, the usage of baitfish for manufacturing fish feed has a negative effect on marine ecosystems (7-8) and competes for food with humankind (9). To conquer these difficulties, it is necessary to develop novel and low-cost fish feed without utilizing fish ingredients.

Various vegetable substitute materials, such as corn, canola, soybean, and wheat, have been used to decrease the portion of fishmeal and fish oil in fish feed (9). However, some studies indicated that fishmeal replacement in fish feed was less efficient. That is to say, fish needed more alternative feed to gain the same weight compared to the normal feed control group. And the existence of fiber and anti-nutritional factors in plant materials were possible reasons because they were interfere with the growth of aquatic species (9-12). In addition, fish oil was well-known to supply essential ω3-fatty acids to fishes and other aquatic species for growth and maintaining health (13). Unlike fishmeal replacement that affected feed conversion ratio (FCR), fish oil replacement has impacts on the fatty acid profiles of aquatic fishes. For example, to avoid notable reduction of omega-3 fatty acids, such as DHA and EPA, only part of fish oil could be replaced by plant oil. On the other hand, microalgae were able to substitute fish oil and without significant alteration of omega-3 fatty acids profile (9). Therefore, microalgae is regarded as one of the candidates to be utilized in fish oil replacement feed.

Several studies had shown that marine microalgae were capable of storing large amounts of PUFAs inside cells. Unicellular thraustochytrids, for example, *Aurantiochytrium*, *Schizochytrium* and *Thraustochytrium*, are well-known members for producing high levels of DHA (14). These species have two different fatty acid synthesis systems to produce various fatty acids. Standard fatty acid synthase (FAS) enzyme complex is mainly utilized for manufacturing C14:0 and C16:0, whereas C22:5 (DPA) and C22:6 (DHA) are synthesized via Polyketide synthases (PKS-enzyme complex) (15-16). Pioneered studies revealed that *Aurantiochytrium* sp. and *Schizochytrium* sp. could accumulate approximately 40-55% of total fatty acids inside their cells (17-23). To cultivate these species, glucose was commonly used as the carbon source because it promotes growth and could be metabolized into different products through respiration (24). For instance, high dry cell weight (171.5 g/L) and DHA concentration (35.33 g/L) were achieved by *Schizochytrium* sp. ATCC20888 (25). However, expensive ingredients in medium and the low conversion rate were caused high costs for microbial fatty acid production (24). Therefore, researchers began to focus on cheaper materials such as crude glycerol (24, 26), sugar refinery washing water (27), miso-processing wastewater (28), soybean curd wastewater (29) and orange peel extract (30) to produce fatty acids with low cost.

In Japan, there are many fermented foods made from plant materials such as miso, natto and soy sauce. Miso, a traditional fermented soybean paste, is produced from mixing steamed soybean with koji (*Aspergillus oryzae*) and salt for about 2 years fermentation (31). Several microorganisms, lactic acid bacteria, yeasts and molds, are known to participant in the fermentation process. In addition to fermentation, the flavor of miso also comes from other reactions such as the Maillard reaction (32). Natto with rich vitamins and several nutrients (33) is made by inoculating *Bacillus subtilis* on boiled soybean. Soy sauce is a liquid seasoning with light brown to black color. On account of its special taste (salty and umami taste), it is widespread from Asian countries to Western countries. Soy sauce also has a complex microbiota consisted of mold, yeast and bacteria during fermentation (34, 35).

In the present study, the author uses plant residues to develop a novel strategy to enhance fatty acid production by referring to Japanese traditional fermented food. The author screened and characterized of DHA-producing strain in section 1, cultivated strain 6-2 by using fermented defatted soybean as the nitrogen source in section 2, and evaluated DHA production in strain 6-2 by solid-state fermentation in section 3.

SECTION 1

Selection and characterization of strains for DHA production

In this section, the author isolated a high DHA-producing strain, *Aurantiochytrium* sp. 6-2, and evaluated the DHA productivity in various medium with different carbon sources, nitrogen sources, initial pH and artificial seasalts concentrations. As a result, common hexose, glucose, fructose, galactose and mannose, and organic nitrogen sources, yeast extract, polypeptone, tryptone, tryptose, marine broth and corn steep solid, could also be used by strain 6-2. Also, salinity and initial pH range of 6-9 was preferable for its growth and fatty acid production.

MATERIALS AND METHODS

Isolation of DHA-producing strains

Samples of seawater were collected in zipper storage bags from the shore near Seto Inland Sea (Setonaikai) in Japan. Pine pollen was added into samples as a bait (36) and samples were kept at 25°C for 4 days. Inoculation loop was used to pick up the pollen grains with fixed microorganisms and spread them onto agar plates. The formula of these agar plates was 1 g/L glucose, 1 g/L yeast extract (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan), 1 g/L polypeptone in 50% v/v seawater (18 g/L, Daigo's artificial seawater SP; Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan). Penicillin G (0.5 g/L) and Streptomycin sulfate (0.5 g/L) were supplemented to prevent the growth of bacteria. These plates were incubated at 28°C until colonies appeared. The colonies were subcultured by applying the same condition. Isolated strains then inoculated in GYA medium [20 g/L glucose, 10 g/L yeast extract and 18 g/L artificial sea salt] at 28°C for 4 days. Stock cultures were prepared by mixing the grown culture 0.7 mL with sterilized glycerol (100 g/L) and trehalose (50 g/L) reagent 0.7 mL into a 2 mL microtube, and stored at -80 °C.

Extraction of genomic DNA and identification based on 18S rDNA sequences from strain 6-2

After cultivated in GYA medium, the cells harvested by centrifugation were freeze dried. Next, the genomic DNA was extracted using a standard phenol/chloroform method (37). The 18S rDNA region was amplified by polymerase chain reaction (PCR) and PCR fragment was cloned into the pUC118 plasmid (TaKaRa Bio Inc, Shiga, Japan) according to the manufacture's instructions. The cloned sequence was determined by the sequencing services of Eurofins Genomics, Inc. (Tokyo, Japan). The sequence was compared with the 18S rDNA sequence of various microorganisms stored in the data bank by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cell observation with microscope

Microorganisms cultivated in GYA medium for 4 days were used for microscopic observation, examined the specimen and took pictures via microscope system (All-in-One Fluorescence Microscope BZ-X800, Keyence, Osaka, Japan).

Strains and culture conditions

Aurantiochytrium sp. 6-2 was cultured in GYA medium for pre-culture. For main culture, strain (with 1% inoculums from pre-culture) was cultivated in the medium [150 g/L glucose, 200 g/L defatted soybean or fermented defatted soybean, 3 g/L KH_2PO_4, 1 g/L Na_2SO_4, 1 g/L $MgCl_2$, 0.3 g/L $CaCl_2$].

Glucose, fructose, galactose, mannose, xylose, sucrose, maltose, lactose, raffinose and cellulose were purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). Yeast extract, polypeptone, peptone, tryptone, tryptose, malt extract, marine broth and neopeptone were purchased from Nippon Becton Dickinson Company, Ltd. (Nippon BD, Tokyo, Japan). Urea was purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). Corn steep solid was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Additionally, for evaluation of effects of salinity, artificial seasalt concentration in GYA

medium was adjusted with range of 0-200%. All cultivation were conducted at 28 °C with shaking (250 rpm), followed by the fatty acid composition analysis, unless otherwise noted.

Measurement of glucose concentration

Reagents required for this determination are available in the WAKO glucose II kit (Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan). 0.02 mL sample solution was mixed with 3 mL of reagent (contained phosphate buffer pH 7.1, phenol, mutarotase, glucose oxidase, peroxidase, ampyrone, L-ascorbate oxidase), and incubated at 37 ℃ for 5 minutes. The absorbance was measured at 505 nm with a spectrophotometer. Glucose concentration was calculated using a calibration curve prepared with a series of standard glucose solutions.

Dry cell weight measurements and analyses of fatty acid profile in strains

To analyze the composition of fatty acids that accumulated in the cell, strains were collected by centrifugation at 3000 rpm for 10 minutes and stored at -20 °C. After frozen dried overnight by using a freeze dryer (EYELA, Japan). Dry cell weight was determined by a mechanical scale.

For lipid extraction, frozen dried cells in a bead-beating tube were beaten with 1 mL deionized water with 0.2 - 0.3 g beads at 5000 rpm, 1 minute for 2 times. The lipids were extracted by the Bligh and Dyer method (38) as with 0.3 mg/mL of tricosanoic acid (C23:0) as an internal standard. Samples were mixed with 2 mL 10% methanolic HCl and 1 mL dichloromethane then incubated at 55 °C for 4 hours. After cooling down to room temperature, fatty acid methyl esters (FAMEs) were extracted with 4 mL n-hexane. Then samples were concentrated via a rotary evaporator and supplied to analyze fatty acids composition by gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a split injector using a TC-70 capillary column (GL Science, Inc., Tokyo, Japan). The initial temperature of the column was 180 °C. After 10 minutes, temperature raised to 236 °C at the speed of 2 °C/min and raised to 260 °C at the speed of 12 °C/min then held at 260 °C for 5 minutes (39). Peaks of fatty acids were identified by

referring to the retention times of standards (Sigma, USA). Calculation was conducted by comparing each peak area with internal standards. The data shown are the average values of at least three determinations.

RESULTS

Isolation and identification of high DHA producing strain 6-2

More than 1000 strains of microorganisms were isolated, and their fatty acid compositions were analyzed by GC. By comparing their fatty acid profiles, a high DHA producing strain 6-2 was found (Fig 1). Though several hundred strains that produced ω3-PUFAs were also found besides strain 6-2, their total fatty acid was lower than 600 mg/L. As a result, strain 6-2 produced approximately 1800 mg/L fatty acids contained 27% DHA (Fig 1). Furthermore, strain 6-2 grew faster than strain SR21 as the reference strain which was known as commercial strain for DHA production.

Simplified identification of strain 6-2

The part of 18S rDNA sequence (582 bp) cloned from strain 6-2 was sequenced. BLAST analysis against nucleotide sequences from various microorganisms in the database showed that this sequence had 97.6% homology with *Aurantiochytrium* species (Fig 2). In addition, strain 6-2 exhibited some classical *Aurantiochytrium* traits in morphological (round shape) and oil droplet vesicles in the cell (Fig 3) by microscopic observation. As a result, strain 6-2 was identified as *Aurantiochytrium* sp. 6-2.

Effect of various carbon and nitrogen sources on cell growth and fatty acid production

To study the growth and fatty acid productivity of *Aurantiochytrium* sp. 6-2, the effects of various carbon and nitrogen sources were evaluated. The effects of the carbon source on growth and fatty acid production were shown in Table 1. Strain 6-2 showed vigorous growth and high DHA production in medium contained hexose monosaccharides, such as glucose, fructose, galactose and mannose. In addition, there were no significant differences in dry cell weight, fatty acid and DHA production. However, xylose, sucrose, maltose, lactose, raffinose and cellulose were not available for the growth of this strain.

The usage of organic and inorganic nitrogen sources by *Aurantiochytrium* sp. 6-2 was also evaluated (Fig 4). Yeast extract, polypeptone, tryptone, tryptose, marine broth and corn steep solid promote cell growth and fatty acid production. Yeast extract was preferable for the growth and fatty acid production (day 2). And so does tryptone, tryptose and corn steep liquor. On the other hand, urea (inorganic nitrogen source) could not support the growth of *Aurantiochytrium* sp. 6-2.

Effects of salinity on the growth and fatty acid productivity

The effect of seasalt concentration on the growth and fatty acid production was examined over a range of 0 to 7.2%, which is equivalent to a salinity of 0 to 2 times that of seawater (Fig 5). Compared to 0% seawater salinity, other groups with the addition of artificial seasalt could promote the growth and fatty acid production. These results indicated that there was a wide tolerance towards salinity of strain 6-2. In contrast, the growth and fatty acids production were suppressed in 0% seawater salinity. In other words, it is essential to maintain a certain level of salinity to cultivate this strain.

Effects of initial pH on the cell growth and fatty acid production

The effects of the initial pH from 3.0 to 11.0 were tested on the growth and fatty acid production (Fig 6). Besides initial pH 3, strain 6-2 could consume glucose completely in just 2 days (Fig 6a). In contrast, with initial pH 3, more than 70% glucose was left even at day 4. This result showed that this strain could not grow well in this condition. Fig 6b indicated that a neutral or slightly alkaline initial pH (6-9) were appropriate for growth and fatty acid production by *Aurantiochytrium* sp. 6-2. In addition, the fatty acid production of this strain was slightly inhibited in the medium at pH 4,5,10 and 11.

Time course of strain 6-2 in GYA medium

The time course of *Aurantiochytrium* sp. 6-2 was conducted, which is cultivated in GYA medium for 4 days (Fig 7). In Fig 7a, glucose concentration was decreased to 0.4% at day 1 and totally consumed at day 2. Fatty acid production reached to the maximum at day

1 and started to decrease after that (Fig 6b). Myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), margaric acid (17:0) and stearic acid (18:0) were decreased with time while EPA, ω6-DPA and DHA were increased in terms of the fatty acid composition.

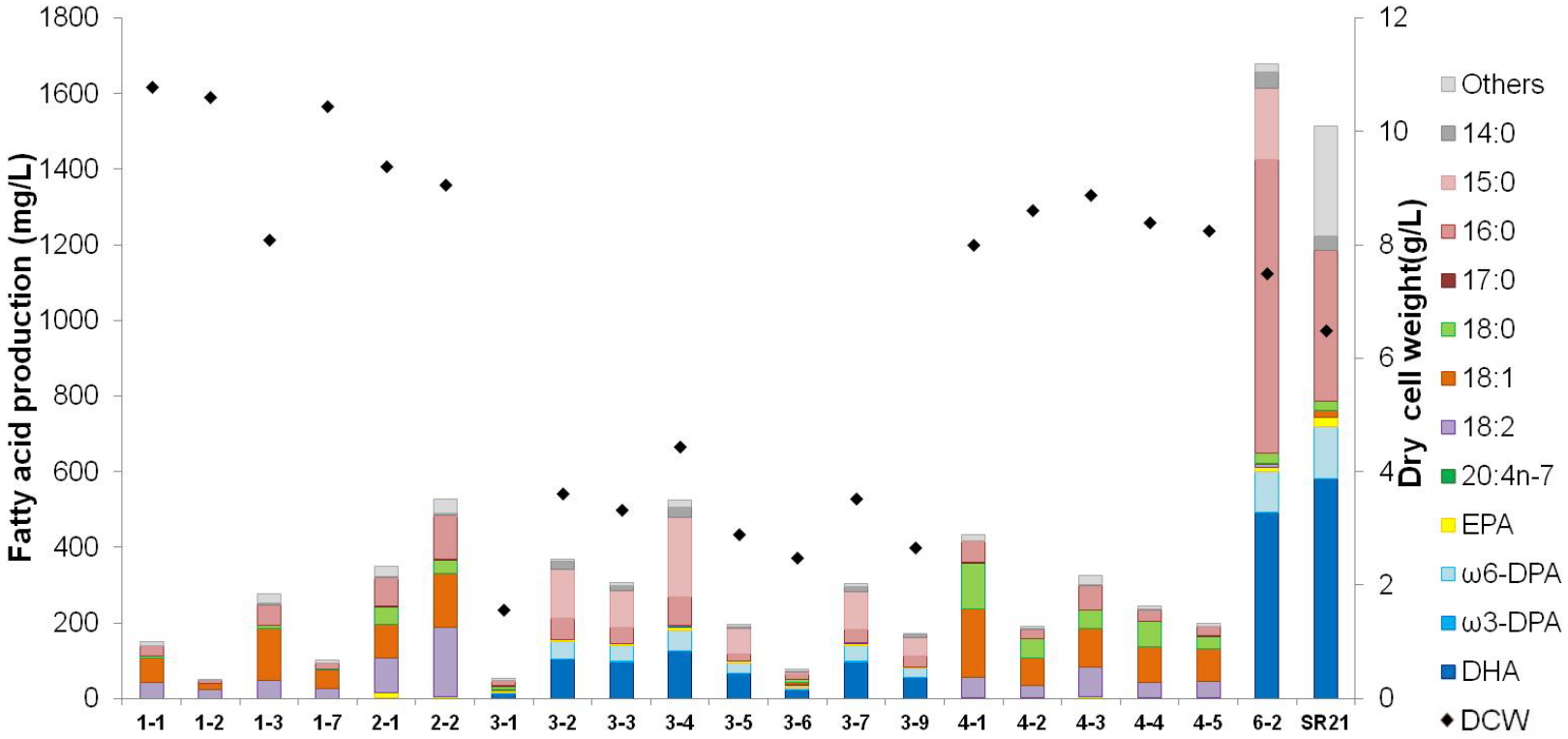


Fig 1. Comparison of fatty acid production by the isolates. All strains were cultivated at 28 ℃ with shaking (300 rpm) for 4 days in GYA medium [20 g/L glucose, 10 g/L yeast extract and 18 g/L artificial sea salt]. 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; EPA, ω3-eicosapentaenoic acid; ω6-DPA, ω6-docosapentaenoic acid; ω3-DPA, ω3-docosapentaenoic acid; DHA, ω3-docosahexaenoic acid; DCW, dry cell weight; SR21, *Schizochytrium limacinum* SR21 was used as a reference strain.

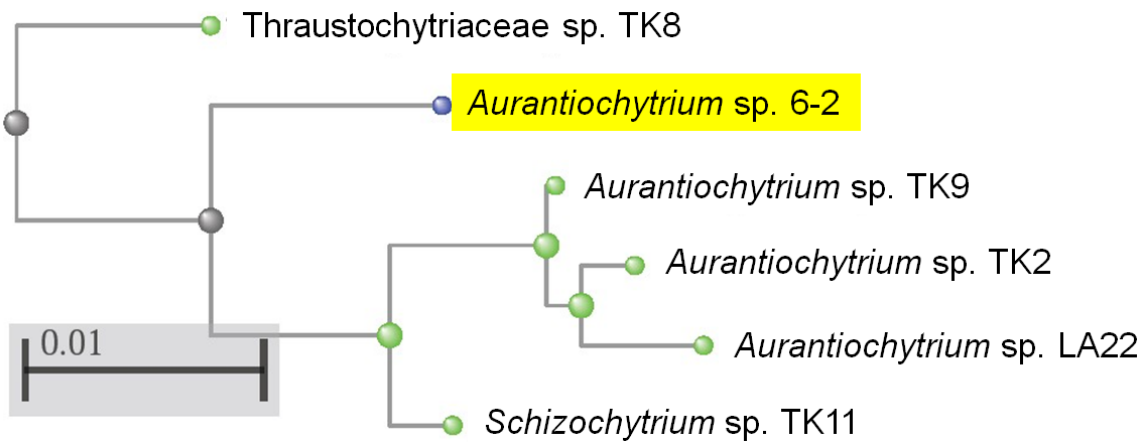


Fig 2. Phylogenetic tree of the strain 6-2 and close NCBI (BLASTn) strains based on the 18S rDNA gene sequences (fast minimum evolution method). The scale bar indicates 0.01 nucleotide. The highlight in the figure indicated the strain isolated in this study.

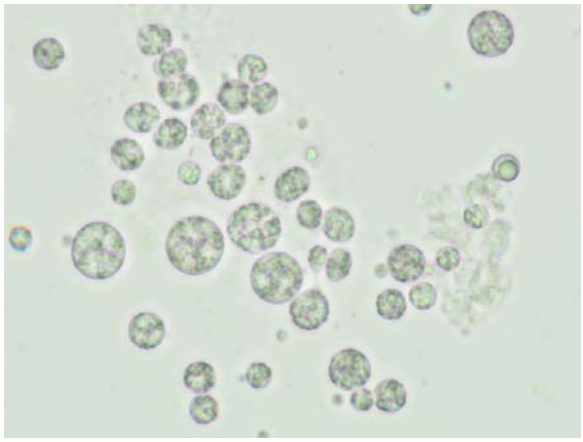


Fig 3. Microscopic image of strain 6-2. It was cultivated in GYA medium for 4 days.

Table 1. Effect of carbon sources on growth, fatty acids production and DHA production (mg/L) by *Aurantiochytrium* sp. 6-2. Cultivation was conducted in a medium containing 2 % different carbon sources, 1 % yeast extract and 1.8 % artificial seasalt at 28 ℃ with shaking (250 rpm) for 2 days and 4 days. Because insoluble cellulose in the medium could not be separated from cultivated cells, dry cell weight in the cellulose medium was not measured.

	Day 2			Day 4		
	DCW (g/L)	Fatty acid production (g/L)	DHA (g/L)	DCW (g/L)	Fatty acid production (g/L)	DHA (g/L)
None	1.83 ± 0.15	-*	-	2.93 ± 0.38	-	-
Glucose	8.10 ± 0.17	1.58 ± 0.21	0.59 ± 0.08	7.77 ± 0.21	1.11 ± 0.08	0.45 ± 0.08
Fructose	8.03 ± 0.60	1.74 ± 0.12	0.61 ± 0.04	7.60 ± 0.35	1.33 ± 0.10	0.47 ± 0.04
Glucose + Fructose	8.10 ± 0.17	1.70 ± 0.15	0.58 ± 0.04	7.27 ± 0.55	1.18 ± 0.15	0.45 ± 0.04
Galactose	8.13 ± 0.21	1.78 ± 0.14	0.60 ± 0.03	7.77 ± 0.21	1.40 ± 0.12	0.54 ± 0.05
Mannose	8.10 ± 0.89	1.79 ± 0.13	0.66 ± 0.06	7.37 ± 0.25	1.17 ± 0.04	0.50 ± 0.01
Xylose	2.87 ± 0.38	-	-	4.73 ± 0.25	-	-
Sucrose	2.93 ± 0.67	-	-	5.60 ± 0.10	-	-
Maltose	3.67 ± 0.47	-	-	5.47 ± 0.40	-	-
Lactose	2.47 ± 0.46	-	-	4.57 ± 0.21	-	-
Raffinose	2.87 ± 0.85	-	-	5.03 ± 1.18	-	-
Cellulose	-	-	-	-	-	-

* Not measurable

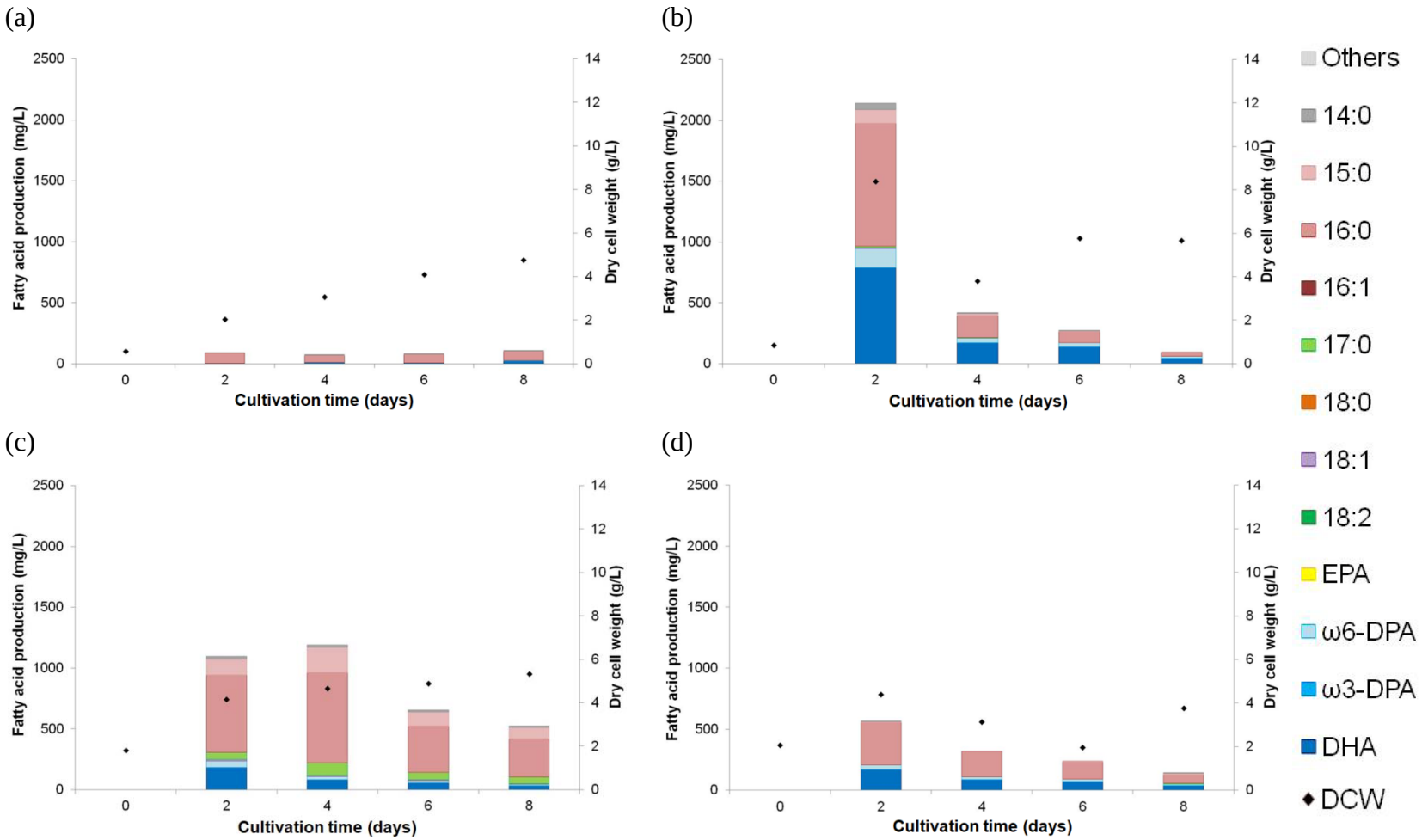


Fig 4. Effects of nitrogen sources on growth and fatty acid production in strain 6-2. Cultivation was conducted in a medium containing 2 % glucose, 1 % different nitrogen sources and 1.8 % artificial sea salt at 28 ℃ with shaking (250 rpm) for 0-8 days. (a) no nitrogen source (b) yeast extract (c) polypeptone (d) peptone. For all abbreviations, see Fig 1.

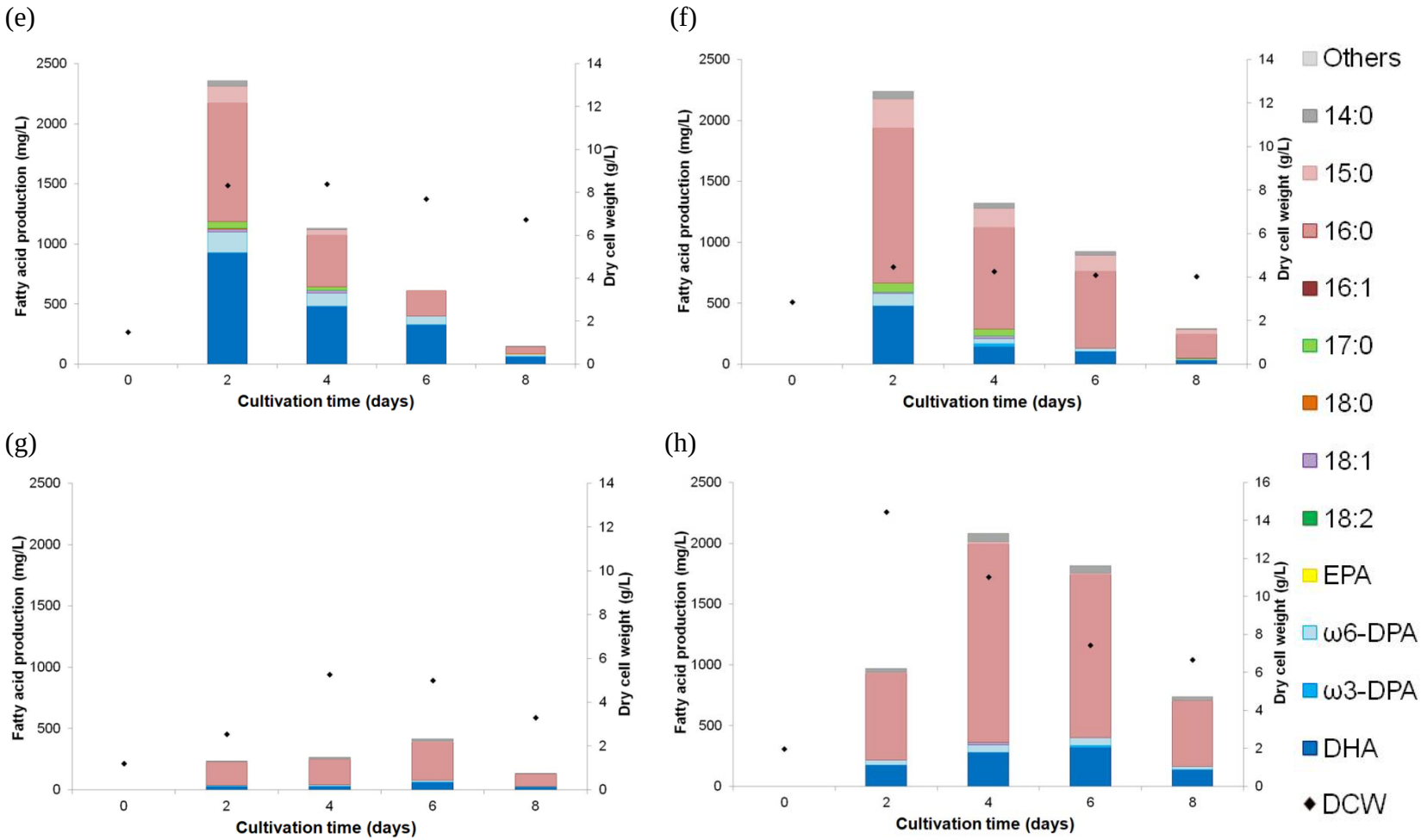


Fig 4 (cont.). Effects of nitrogen sources on growth and fatty acid production in strain 6-2. Cultivation was conducted in a medium containing 2 % glucose, 1 % different nitrogen sources and 1.8 % artificial sea salt at 28 ℃ with shaking (250 rpm) for 0-8 days. (e) tryptone (f) tryptose (g) malt extract (h) marine broth. For all abbreviations, see Fig 1.

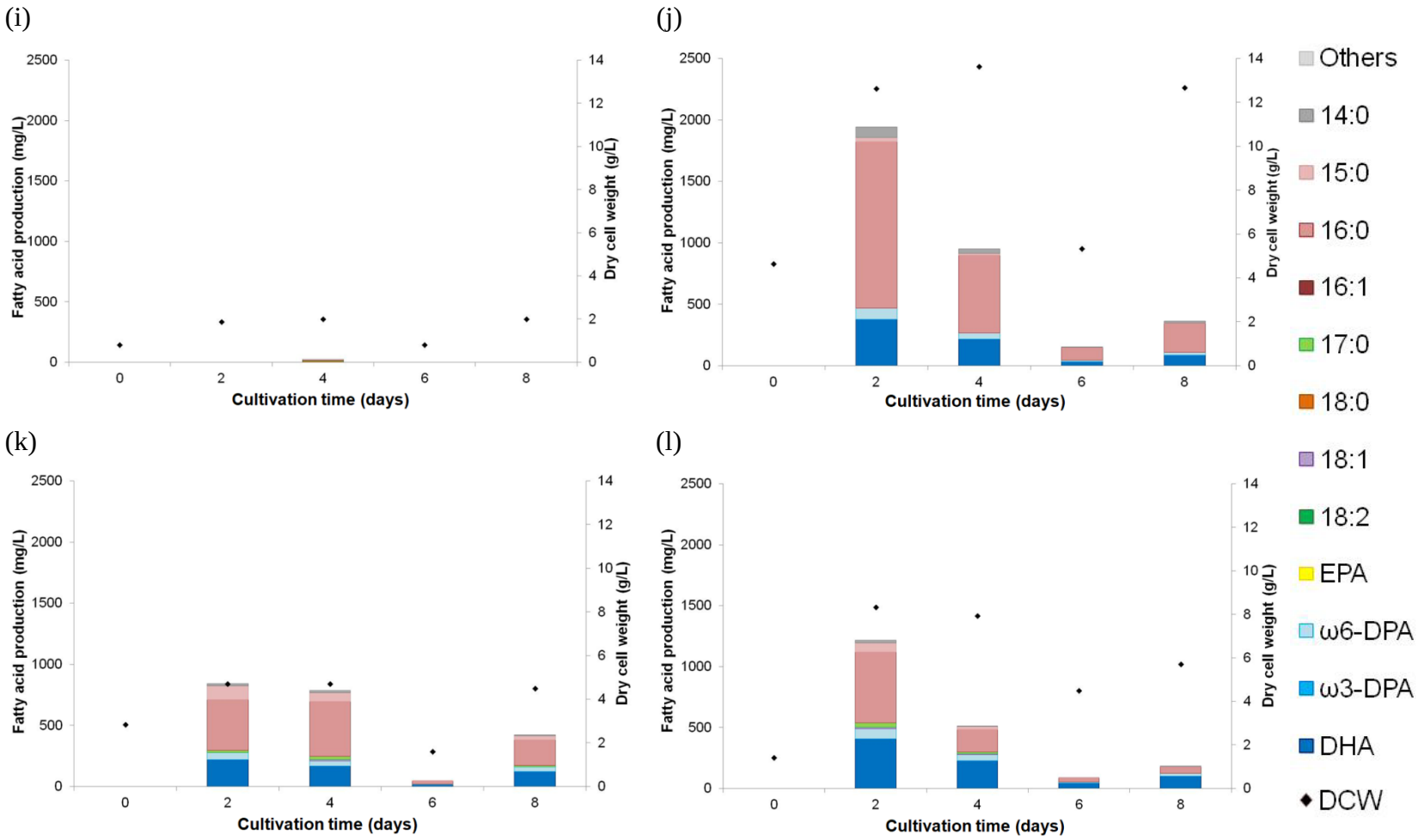


Fig 4 (cont.). Effects of nitrogen sources on growth and fatty acid production in strain 6-2. Cultivation was conducted in a medium containing 2 % glucose, 1 % different nitrogen sources and 1.8 % artificial sea salt at 28 ℃ with shaking (250 rpm) for 0-8 days. (i) urea (j) corn steep solid (k) neopeptone (l) 0.5% polypeptone + 0.5% yeast extract. For all abbreviations, see Fig 1.

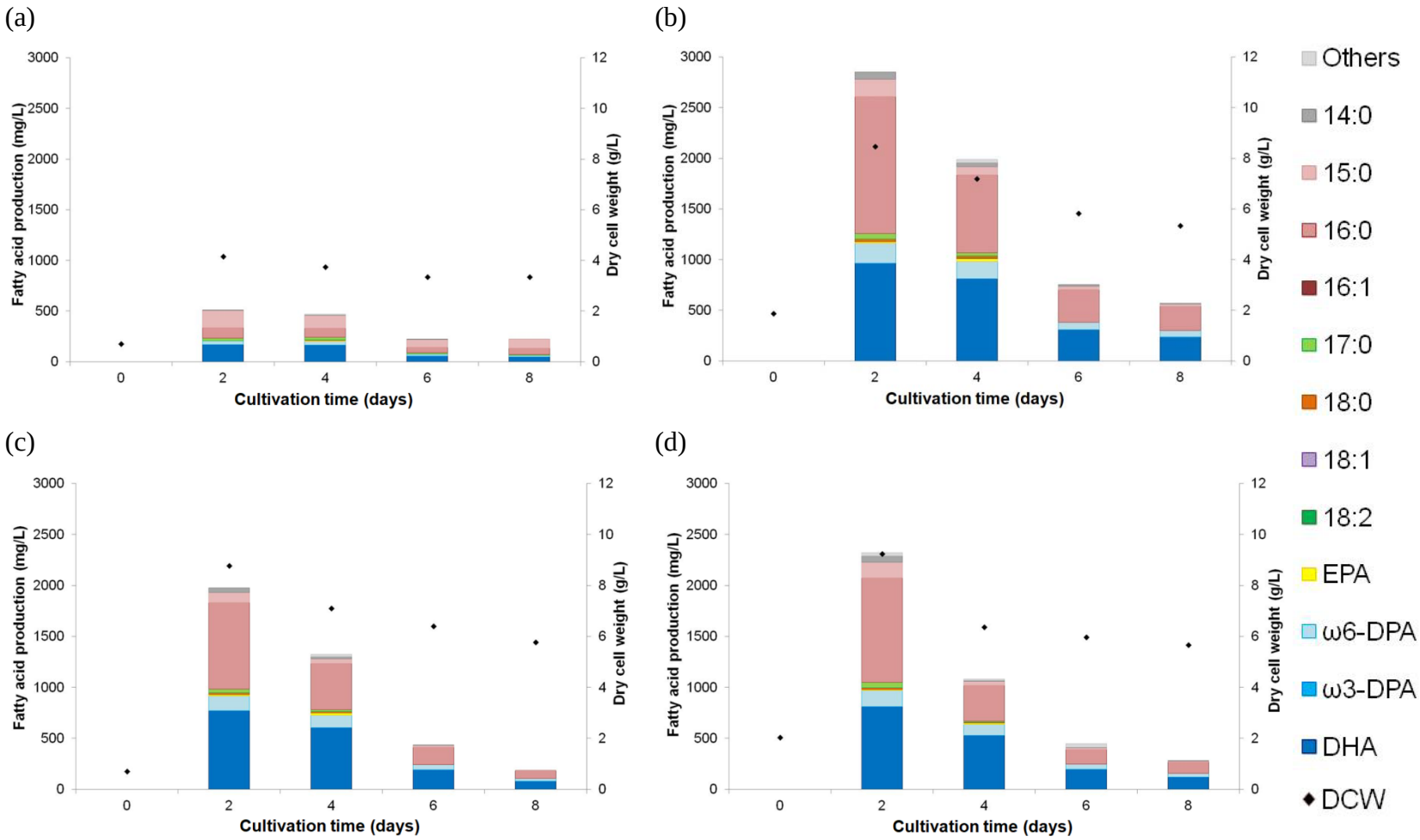
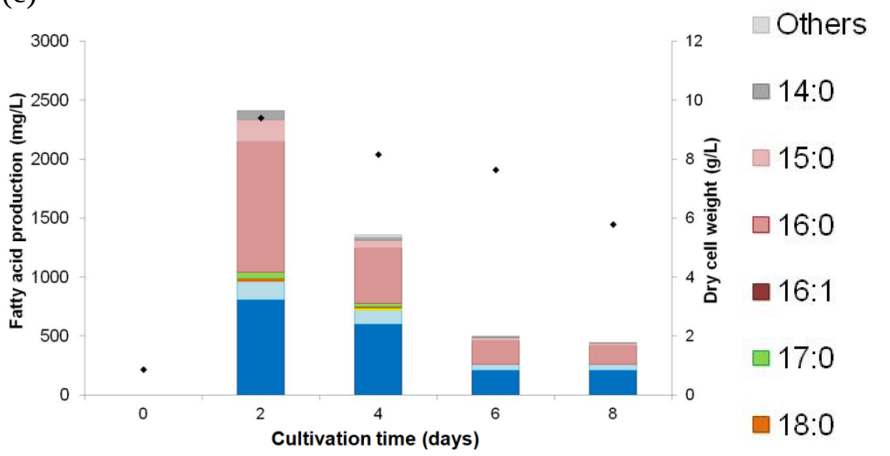


Fig 5. Effects of sea salt concentration on growth and fatty acid production in strain 6-2. Cultivation was conducted in a medium containing 2 % glucose, 1 % yeast extract and 0-7.2 % artificial sea salt at 28 ℃ with shaking (250 rpm) for 0-8 days. (e) 5.4% artificial sea salt (f) 7.2% artificial sea salt. For all abbreviations, see Fig 1.

(e)



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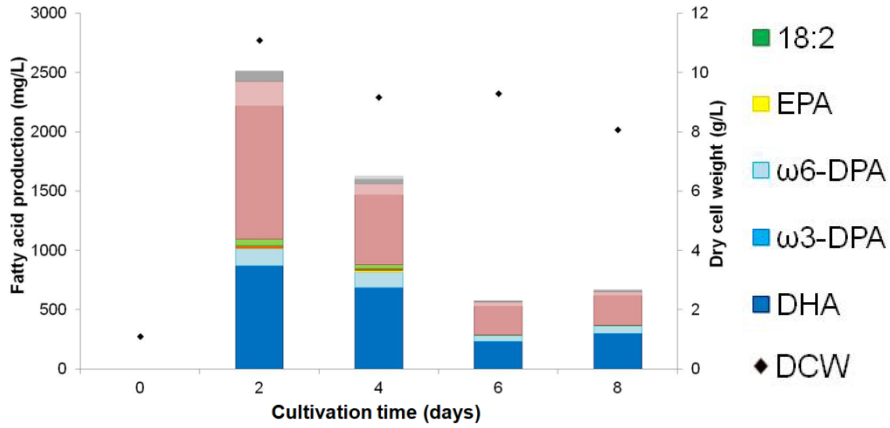


Fig 5 (cont.). Effects of sea salt concentration on growth and fatty acid production in strain 6-2. Cultivation was conducted in a medium containing 2 % glucose, 1 % yeast extract and 0-7.2 % artificial sea salt at 28 ℃ with shaking (250 rpm) for 0-8 days. (e) 5.4% artificial sea salt (f) 7.2% artificial sea salt. For all abbreviations, see Fig 1.

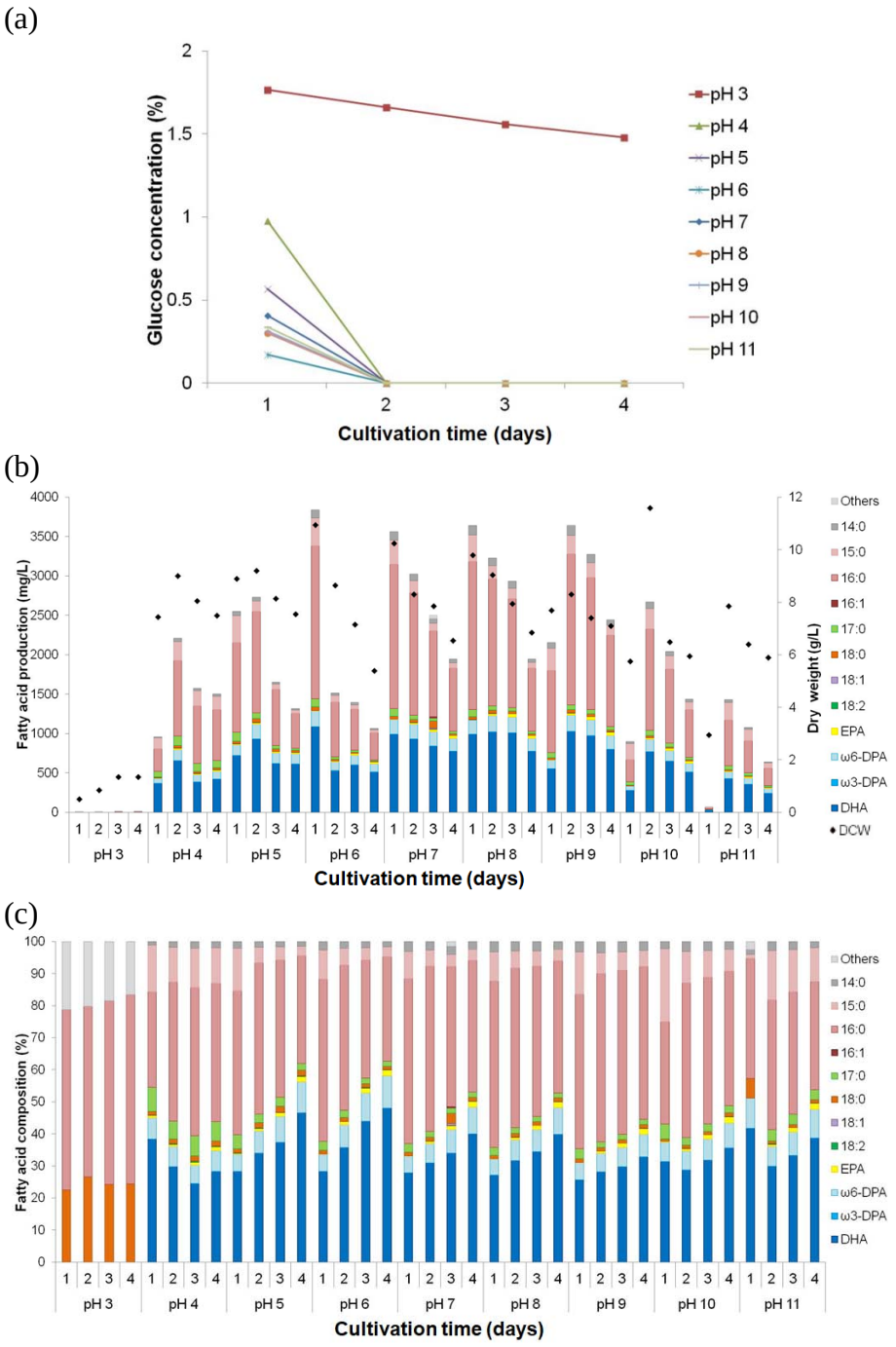


Fig 6. Effects of initial pH on (a) residual glucose concentration, (b) growth, fatty acid production and (c) fatty acid composition in strain 6-2. Cultivation was conducted in GYA medium at 28 ℃ with shaking (250 rpm) for 1-4 days. Initial pH adjusted with HCl or NaOH. For all abbreviations, see Fig 1.

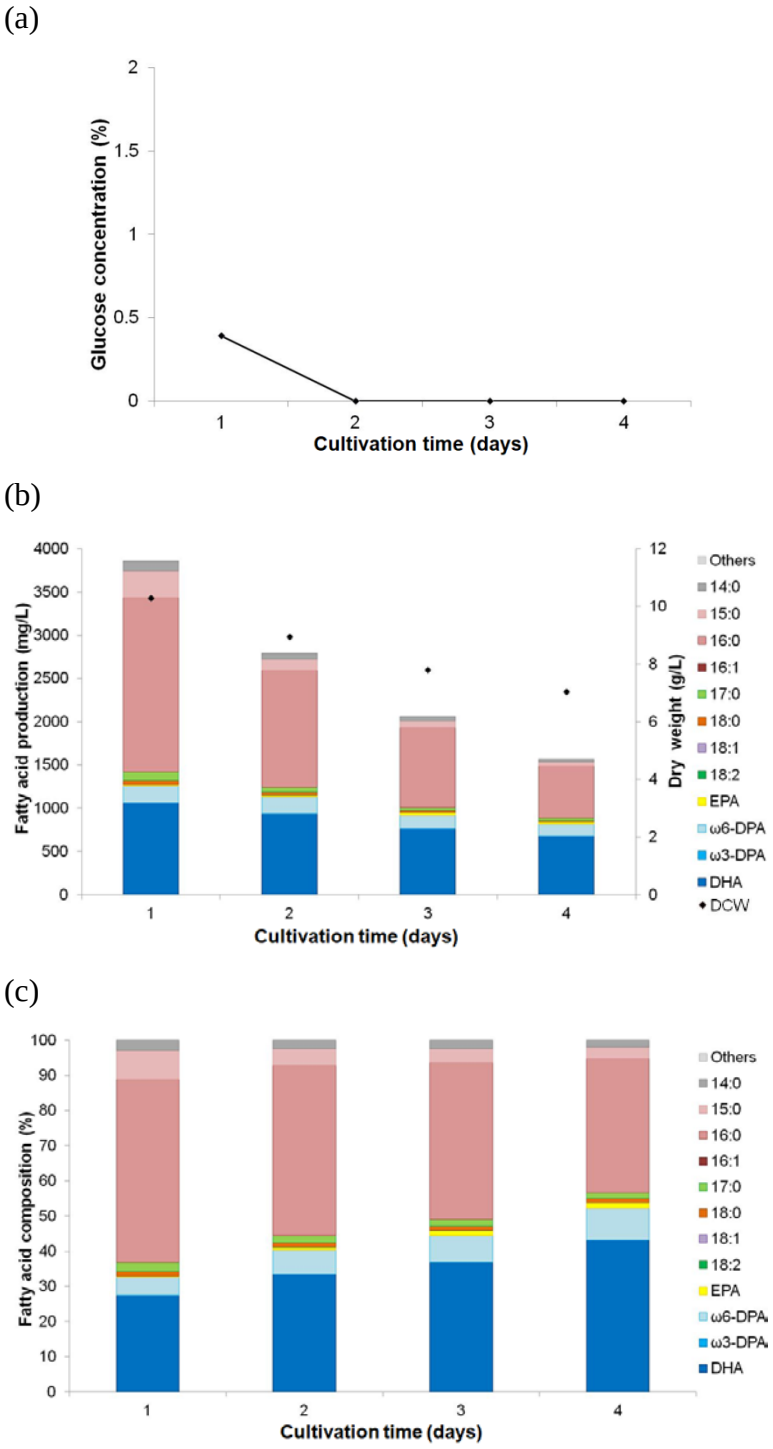


Fig 7. Time course of (a) residual glucose concentration, (b) growth, fatty acid production and (c) fatty acid composition in strain 6-2. Cultivation was conducted in GYA medium at 28 ℃ with shaking (250 rpm). For all abbreviations, see Fig 1.

DISCUSSION

In present section, the author screened more than 1000 strains of marine microorganisms from ocean in Japan. All strains had different fatty acid compositions. The author put emphasis on strain 6-2, which exhibited the highest fatty acid production among the isolated strains and grew faster than reference strain, *Schizochytrium limacinum* SR21 known commercial strain for DHA production. The DHA content of strain 6-2 occupied nearly 30% of the total fatty acids (Fig 1), which was higher than the *Aurantiochytrium* sp. S31 (*Schizochytrium* sp. S31) in previous study (40). By phylogenetic analysis, 6-2 was identified to belong to the heterotrophic *Aurantiochytrium* species (Fig 2). *Aurantiochytrium* sp. is famous as a DHA-producing microbe, and this specie stores DHA in lipid droplets as triacylglycerols form (41-43).

For the characterization, the author first evaluated the effects of carbon sources in the liquid medium on the growth, fatty acid and DHA production of *Aurantiochytrium* sp. 6-2. The author discovered that this strain grew well and produced large amounts of lipid by the medium containing glucose, fructose, galactose and mannose (Table 1). The results of using glucose and fructose are similar with previous study (40, 44). These carbon sources were considered easily metabolized and used by the lipid biosynthetic pathways in strain 6-2. Secondly, the influences of nitrogen sources on growth and lipid production were also studied. In Fig 4, strain 6-2 was found that yeast extract, tryptone, tryptose and corn steep solid could support sufficient growth and fatty acid production. In contrast, this strain could not assimilate urea as the nitrogen source. These results were not consistent with other studies (40, 44). Possible reason was the composition of medium that used in each research is different. Ingredients for preparing medium such as minerals definitely could affect the growth and lipid productivity.

Subsequently, different sea salt concentrations were evaluated. *Aurantiochytrium* sp. 6-2 could grow well and produce fatty acid as long as certain salinity concentration maintained in the medium (Fig 5). These results were similar to *Aurantiochytrium* sp. SD116 (44) *and Aurantiochytrium mangrovei* BL10 (45), but not totally accordant with

Aurantiochytrium sp. strain L3W which growth and fatty acid productivity were inhibited in high salinity (46). Though, no studies have reported the mechanisms of salinities works for *Aurantiochytrium* species, plausible explanation might be optimal osmotic stress and/or ionic stress are necessary (46). Last, to elucidate the effects of initial pH on growth and fatty acid productivity, the author prepared the medium with pH value ranged from 3 to 11 (Fig 6). In consequence, pH-value between 6 and 9 was optimal for strain 6-2. This result was slightly different from studies which revealed that *Aurantiochytrium* sp could not grow well in weak alkaline environment (44, 46).

SECTION 2

Evaluation of defatted soybean as nitrogen source for DHA production by the selected strain

In this section, the author evaluated defatted soybean as the nitrogen source in medium and optimized the cultivation condition by using fermented defatted soybean (FDS) to cultivate *Aurantiochytrium* sp. 6-2. According to the results, the most favorable concentration of fermented defatted soybean was 20%. In addition, enhanced aeration and mineral content (especially sulfate) could further increase the productivity. Moreover, strain 6-2 could grow well even under unsterilized condition by adding sodium acetate or coculturing with *Bacillus subtilis* MN15.

MATERIALS AND METHODS

Strains and culture conditions

Aurantiochytrium sp. 6-2 was cultured in GYA medium [20 g/L glucose, 10 g/L yeast extract and 18 g/L artificial sea salt (Daigo's artificial seawater SP; Daigo's artificial seawater SP; Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan)] (10 mL in a test tube) at 28 °C with shaking (250 rpm) for pre-culture. For main culture, strain 6-2 (with 1% inoculums from pre-culture) was cultivated in medium containing 150 g/L glucose, 200 g/L defatted soybean or fermented defatted soybean (FDS), 3 g/L KH_2PO_4, 1 g/L Na_2SO_4, 1 g/L $MgCl_2$, 0.3 g/L $CaCl_2$. Strain 6-2 was cultivated at 28 °C with shaking (250 rpm), followed by the fatty acid composition analysis, unless otherwise noted.

Procedure of making fermented defatted soybean (FDS)

37.5 g defatted soybean was soaked in deionized water for 12 hours at 4℃. After autoclaved and cooled to room temperature, defatted soybean was ground by tabletop mincer and mixed with 7.5 g starter (Momokawa miso. Co. Ltd., Niigata, Japan), 41.25 g

commercial raw rice koji (Banshu koujiya., Hyogo, Japan) and 10.13mL deionized water. Fermentation was conducted at 60 ℃ for 2-8 days and stored at -20 ℃ (Fig 1).

Measurement of glucose concentration

WAKO glucose II kit (Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan) was used for measurement of glucose concentration in the medium. 0.02 mL sample solution was mixed with 3 mL of reagent (contained phosphate buffer pH 7.1, phenol, mutarotase, glucose oxidase, peroxidase, ampyrone, L-ascorbate oxidase), and incubated at 37 ℃ for 5 minutes. The absorbance was measured at 505 nm with a spectrophotometer. Glucose concentration was calculated using a calibration curve prepared with a series of standard glucose solutions.

Aeration equipment in large scale flask

Two glass tubes (AS ONE. Co., Osaka, Japan; 3 cm long, outer diameter is 5 mm and inner diameter is 3.4 mm) were penetrated through the silicon plug (Shin-Etsu Polymer Co., Ltd, Tokyo, Japan; cat No: N-42, upper diameter is 44 mm, lower diameter is 30 mm, height is 78 mm). Both of them were equipped with filters (ADVANTEC Co. Ltd, Tokyo, Japan; cat No: 25JP020AN) which hole diameter is 0.2 μm. One of them is connected to tube 1 (AS ONE. Co., Osaka, Japan; 37 cm long, outer diameter is 7 mm, and inner diameter is 4 mm), tube 1’ (AS ONE. Co., Osaka, Japan; 5 cm long, outer diameter is 7 mm and inner diameter is 4 mm), tube 2 (AS ONE. Co., Osaka, Japan, 62 cm long, outer diameter is 5 mm and inner diameter is 3 mm) and Luer Fitting (PVDF, For Soft Tube, VRM308, AS ONE. Co., Osaka, Japan) was applicable tube with an inner diameter of 3 mm, and 60×100×15 mm. Pump that was used was Suisin SSPP-3S (Suisaku. Co., Tokyo, Japan), and air was supplied at 5.7 mL/s (minimum).

The cultivation was carried out using the equipment, 150 mL culture broth/500 mL Sakaguchi flask or 600 mL culture broth/2L Sakaguchi flask, at 28℃ and 120 rpm under aeration condition by the air pump.

Dry weight measurements and analyses of fatty acid profile in strains

To analyze the composition of fatty acids that accumulated in the cell, strains were collected by centrifugation at 3000 rpm for 10 minutes and stored at -20 °C. After frozen dried overnight by using a freeze dryer (EYELA, Japan). Dry cell weight was determined by a mechanical scale.

For lipid extraction, frozen dried cells in a bead-beating tube were beaten with 1 mL deionized water and 0.2 - 0.3 g beads at 5000 rpm, 1 minute for 2 times. The lipids were extracted by the Bligh and Dyer method (38) as with 0.3 mg/mL of tricosanoic acid (C23:0) as an internal standard. Samples were mixed with 2 mL 10% methanolic HCl and 1 mL dichloromethane then incubated at 55 °C for 4 hours. After cooling down to room temperature, fatty acid methyl esters (FAMEs) were extracted with 4 mL n-hexane. Then concentrated via a rotary evaporator and send to analyze fatty acids composition by gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a split injector using a TC-70 capillary column (GL Science, Inc., Tokyo, Japan). The initial temperature of the column was 180 °C. After 10 minutes, temperature raised to 236 °C at the speed of 2 °C/min and raised to 260 °C at the speed of 12 °C/min then held at 260 °C for 5 minutes (39). Peaks of fatty acids were identified by referring to the retention times of standards (Sigma, USA). Calculation was conducted by comparing each peak area with internal standards. The data shown are the average values of at least three determinations.

RESULTS

Effects of defatted soybean and fermented defatted soybean on the fatty acid productivity of strain 6-2.

By comparing the usage of defatted soybean and fermented defatted soybean as the nitrogen source in the medium (Fig 2), strain 6-2 could not use defatted soybean as the nitrogen source directly because its fatty acid productivity is low (about 5 g/L total fatty acids). On the other hand, strain 6-2 can use defatted soybean that fermented by koji as described in Fig 1. Total fatty acids production could reach around 35g/L, which was 7 times higher than using defatted soybean. These results indicated that the fermentation process was crucial for fatty acid production by strain 6-2.

Effects of FDS concentration on fatty acid production

The effects of FDS addition on fatty acids production was studied over a range of 0 to 60% (w/v). Fig 3 was the result of cultivation strain 6-2 by using different concentrations of fermented defatted soybean in the FDS medium. In addition of 20% fermented defatted soybean into medium, fatty acid production could reach the maximum (around 30 g/L). This result was extremely higher than other concentrations. Also, it was found out that 20% was the most suitable concentration that could be used to culture strain 6-2.

Effects of different fermentation duration of FDS on fatty acid production

Fig 4 showed the results of adding 20% FDS with different fermentation duration. Even though fatty acid composition did not change significantly on 3 days and 7 days cultivation (Fig 4b), fatty acid production was relatively higher in groups with shorter fermentation days of FDS (within 5 days). In 8 days fermented defatted soybean medium, fatty acid production is slightly lower than others. In 3 days and 5 days fermented defatted soybean medium, fatty acid production at 3 days cultivation was higher than 5 days cultivation. In addition, DHA productivity in 5 days fermented FDS medium showed the

highest value at day 3 cultivation. Therefore, 5 days fermentation of defatted soybean was regarded as the optimal fermentation period for cultivation of strain 6-2.

Time course of strain 6-2 in 20% FDS medium

To evaluate the effect of 20% FDS medium on glucose consumption and fatty acid production, strain 6-2 was cultivated at 28℃, 250 rpm for 7 days. According to the result of time course in Fig 5, the fatty acid production was increased with glucose consumption. Glucose was depleted at day 6, and fatty acid production reached to the maximum (about 21 g/L). Although fatty acid content was raised during cultivation, the fatty acid composition did not change much.

Effects of inorganic nutrients on the growth and fatty acid productivity of strain 6-2

Effects of inorganic nutrients, magnesium chloride, calcium chloride, sodium sulfate, and potassium dihydrogen phosphate, on the growth, fatty acid production and fatty acid composition of strain 6-2 were shown in Fig 6, 7 and 8. In the FDS medium without magnesium chloride or sodium sulfate, fatty acid productivity of strain 6-2 decreased significantly (Fig 6). On the contrary, no obvious changes in fatty acid production could be observed in the medium without potassium dihydrogen phosphate or calcium chloride. Next, the effect of a single inorganic nutrient was also evaluated (Fig 6). According to the result, fatty acid production increased drastically (about 40 times higher than other groups) in the medium with 0.1% sodium sulfate addition. These results indicated that sodium sulfate was important nutrition for strain 6-2 growth in FDS medium.

Subsequently, magnesium chloride, sodium sulfate and sodium chloride were further studied in Fig 7. In the medium with sulfate ions, fatty acid production was 10-17 times higher than other condition. These results indicated that sulfate ion is essential when cultivated strain 6-2 in FDS medium. Last, the growth, fatty acid production and fatty acid composition were evaluated in FDS medium with 2 times mineral concentration (Fig 8). The result showed fatty acids productivity further increased at both 5 days and 8 days cultivation in FDS medium with 2 times mineral concentration.

Effects of aeration on fatty acid production in scale-up culture

In scale-up culture by 2 L Sakaguchi flask (AGC TECHNO GLASS. Co. Ltd., Sizuoka, Japan) using normal silicon plug, the inhibition of the growth was observed compared to normal test tube cultivation (data not shown). Since it could be insufficient aeration responsible for the growth inhibition, air supply equipment was prepared for cultivation (Fig 9a.). As a result, enhanced aeration improved the growth obviously (Fig 9b).

In Fig 10, strain 6-2 was cultivated in large-scale flasks and measured the fatty acid content on day 7 and 14. The fatty acid productivity with enhanced aeration showed 47.7 g/L total fatty acids (12.2 g/L DHA) production at day 7 and 32.4 g/L total fatty acids (6.3 g/L DHA) production at day 14. These results suggested that aeration was critical for the growth and fatty acid production.

Time course of fatty acid production by strain 6-2 in scale-up culture

Fig 11 was the time course of strain 6-2 in FDS medium with enhanced aeration. The fatty acid production increased with time and reached the maximum up to 31.6 g/L and DHA production reached up to 8.6 g/L. After 6 days cultivation, the amounts of fatty acids began to decrease but fatty acids composition did not change much.

Effects of sodium acetate addition in sterilized and unsterilized FDS medium on fatty acid productivity

To evaluate the growth of *Aurantiochytrium* sp. 6-2 in unsterilized medium, sterilized or unsterilized FDS medium with the addition of 2% sodium acetate (a well-known bacteriostatic agent) was prepared. Although strain 6-2 showed vigorous growth in in both condition, fatty acid productivity is slightly lower in unsterilized medium. On the other hand, fatty acid compositions in both conditions were similar. When sodium acetate was added into unsterilized FDS medium, production of fatty acid was improved. This

result suggested that sodium acetate could support strain 6-2 growth by suppressing other microorganisms under unsterilized condition.

Coculturing *Aurantiochytrium* sp. 6-2 and *Bacillus subtilis* MN15 in sterilized or unsterilized FDS medium

In unsterilized medium, contamination of *Bacillus* species could be expected to occur easily. Therefore, the coculture with *Aurantiochytrium* sp. 6-2 and *Bacillus subtilis* MN15 was conducted in both sterilized and unsterilized FDS medium to evaluate the effect on the growth and fatty acid production. The results showed the fatty acid production in unsterilized only inoculated strain 6-2 was lower than that in sterilized medium (Fig 13). On the other hand, the fatty acid production in cocultured medium was decreased to 60-80% compared to that of monoculture in sterilized or unsterilized medium, respectively. Although fatty acid with coculture in unsterilized condition was decreased, strain 6-2 was still able to produce significant amounts of fatty acids. It was considered that strain 6-2 could be available in unsterilized condition for DHA production.

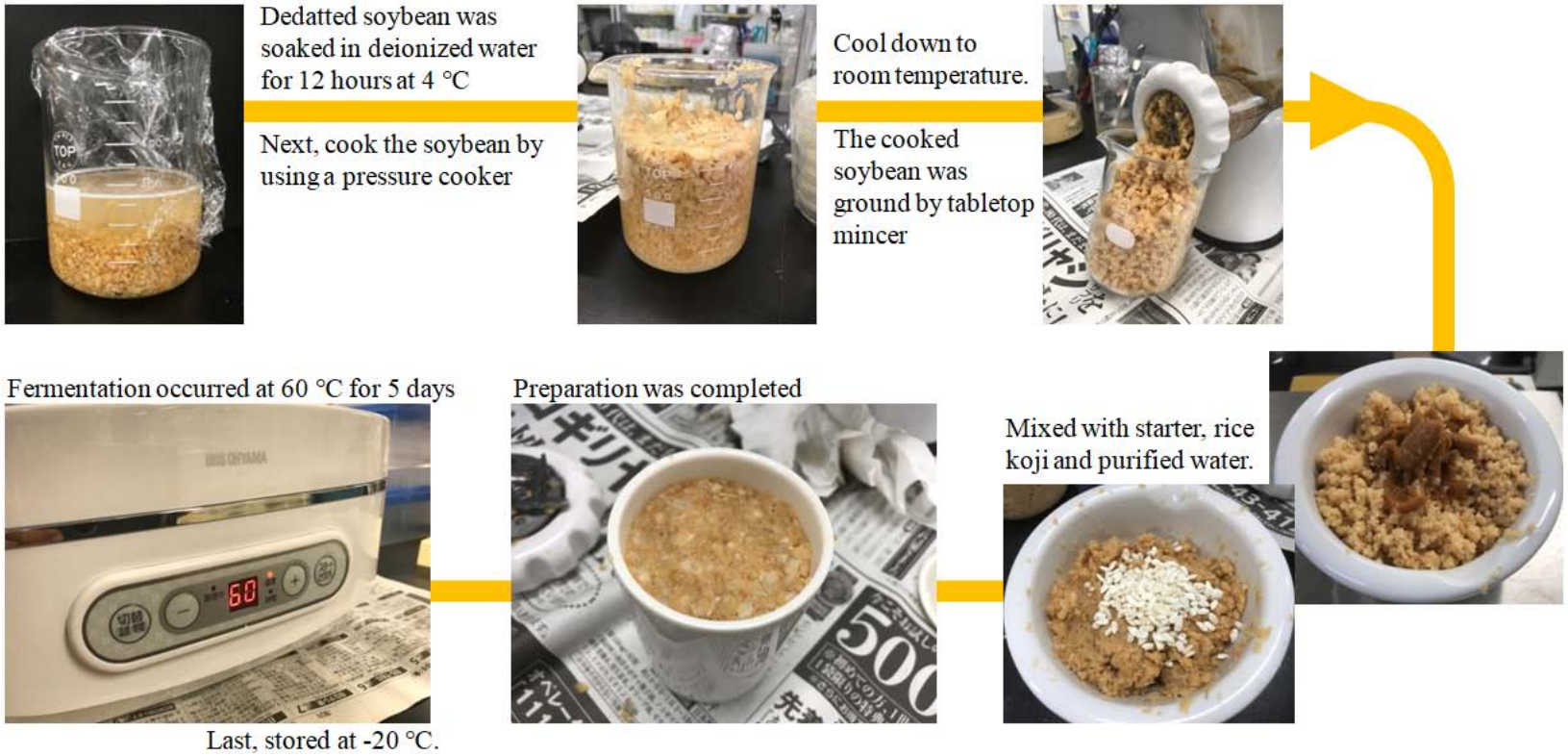


Fig 1. Procedure of preparing fermented defatted soybean (FDS).

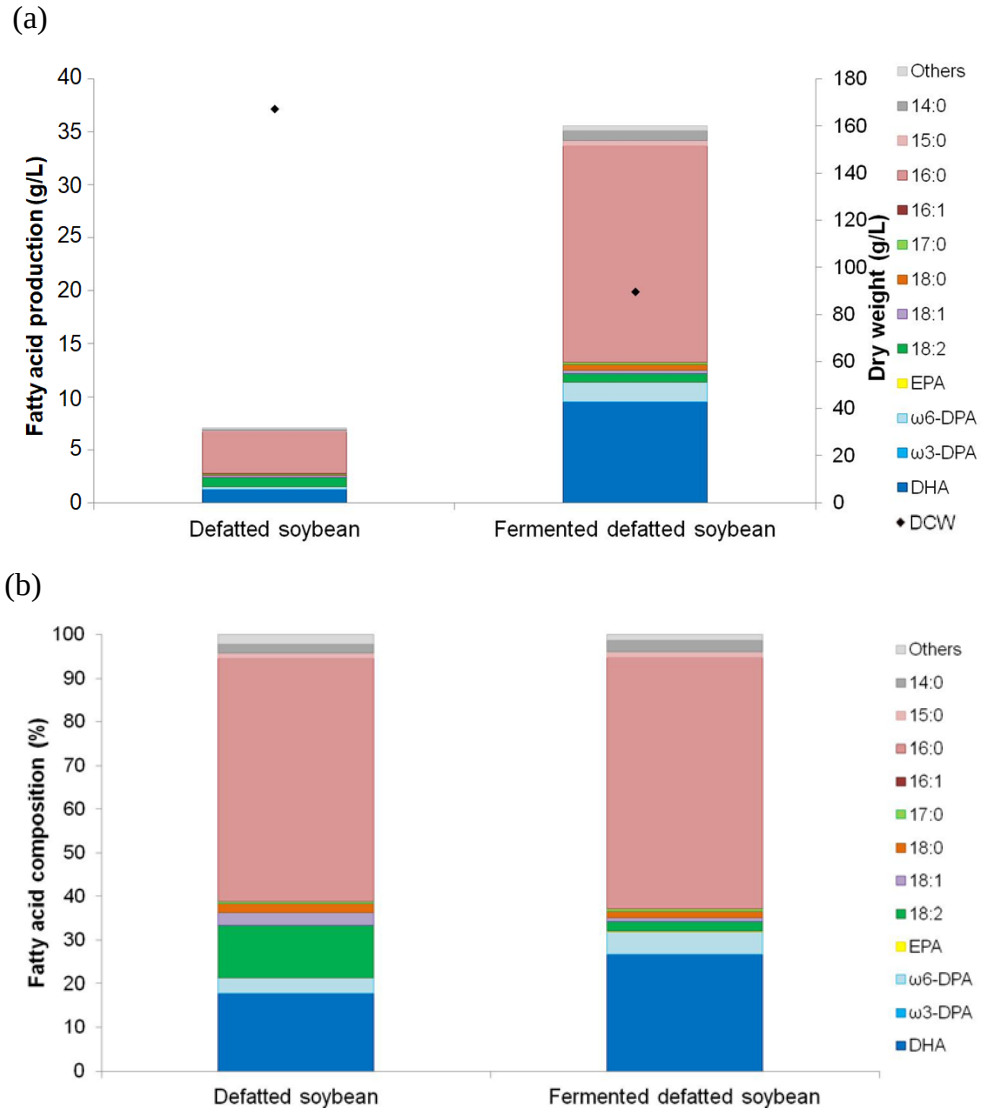
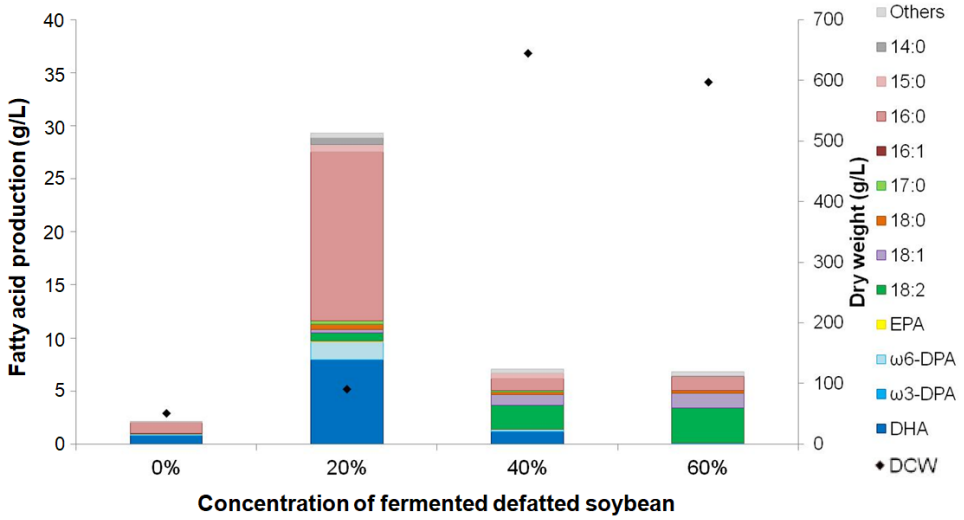


Fig 2. Effects of defatted soybean or fermented defatted soybean on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in liquid medium at 28 ℃ with shaking (250 rpm) for 4 days. Medium contained 150 g/L glucose, 200 g/L defatted soybean or fermented defatted soybean (FDS), 3 g/L KH_2PO_4, 1 g/L Na_2SO_4, 1 g/L $MgCl_2$, 0.3 g/L $CaCl_2$. 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; EPA, ω3-eicosapentaenoic acid; ω6-DPA, ω6-docosapentaenoic acid; ω3-DPA, ω3-docosapentaenoic acid; DHA, ω3-docosahexaenoic acid; DCW, dry cell weight.

(a)



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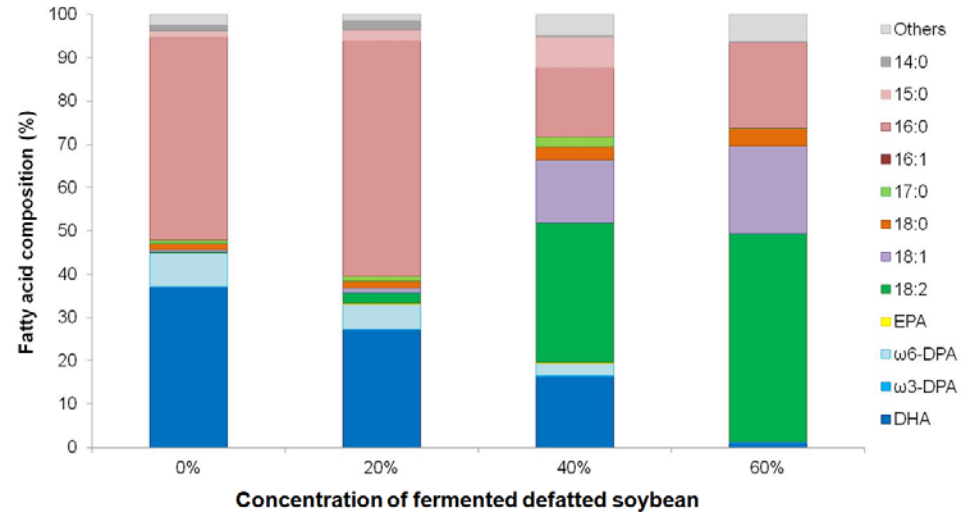
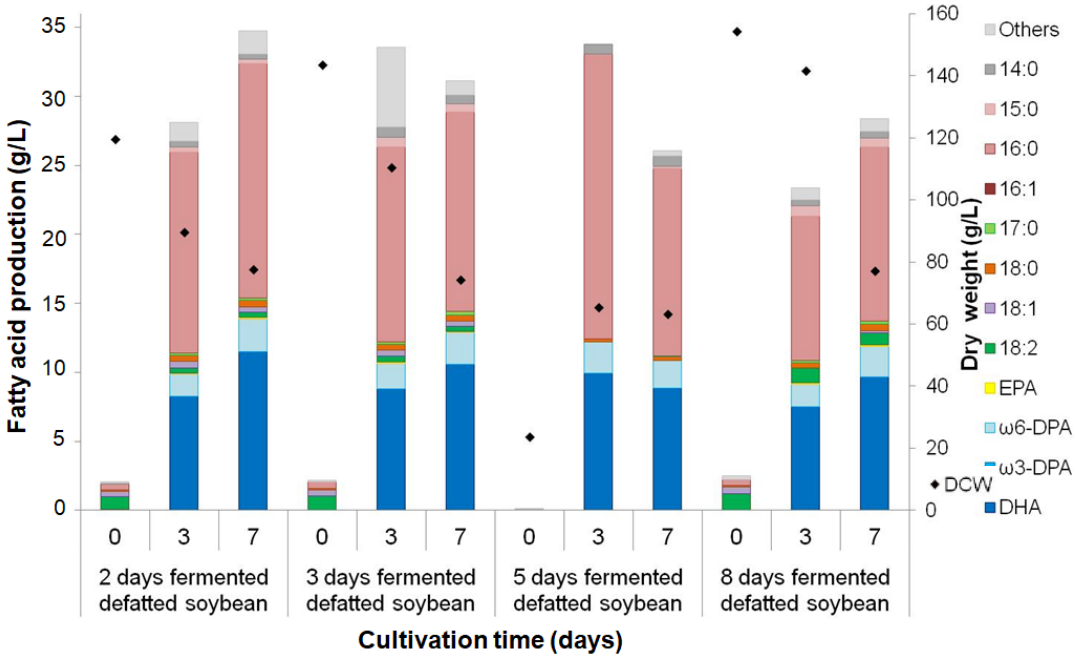


Fig 3. Effects of fermented defatted soybean concentrations on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium at 28 ℃ with shaking (250 rpm) for 4 days. For all abbreviations, see Fig 1.

(a)



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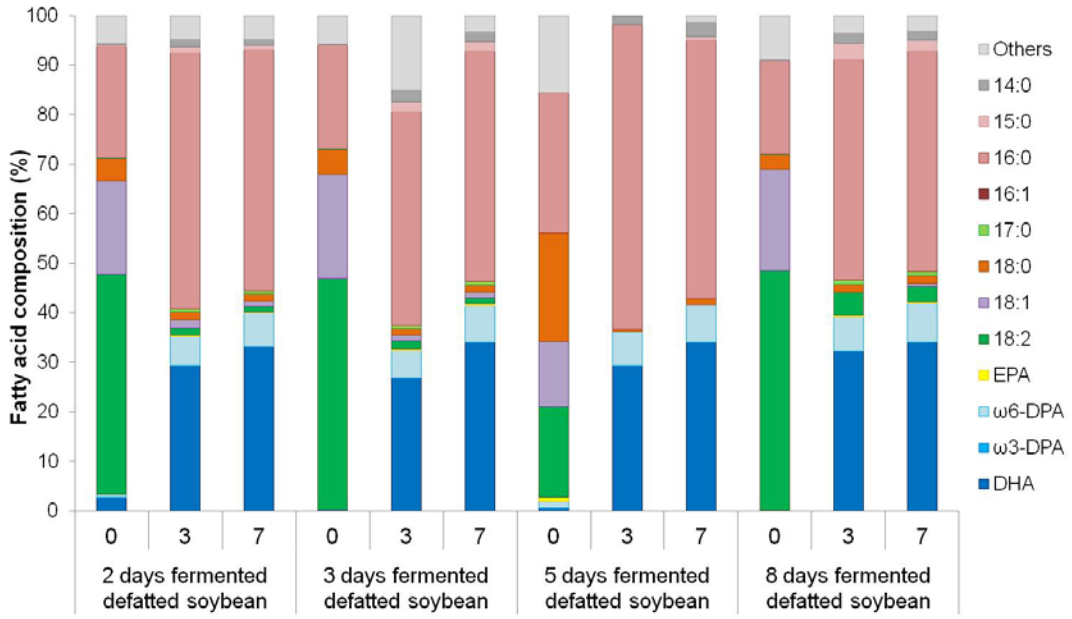
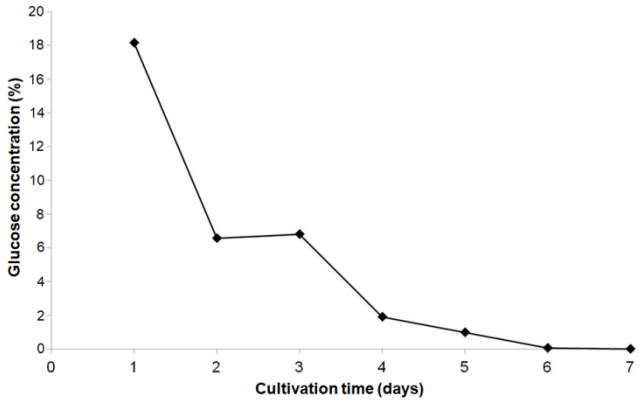


Fig 4. Effects of using defatted soybean with different fermentation time on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium at 28 ℃ with shaking (250 rpm). For all abbreviations, see Fig 1.

(a)



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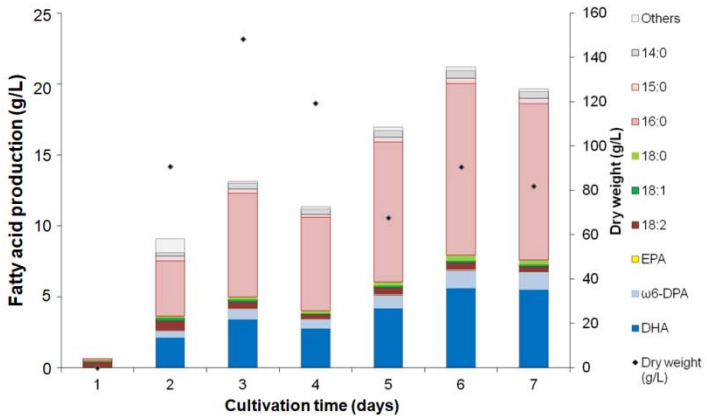

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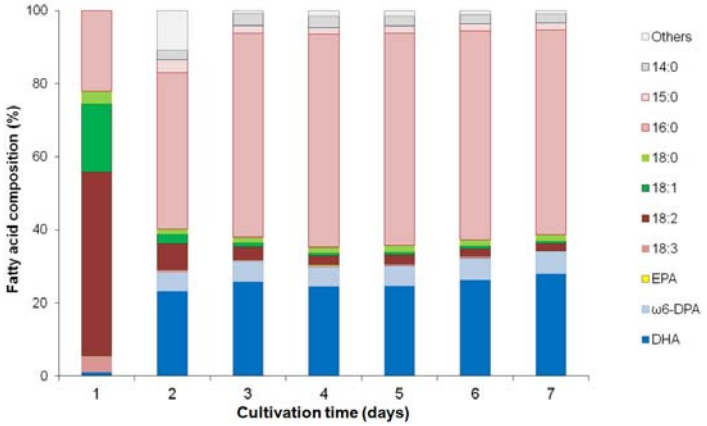
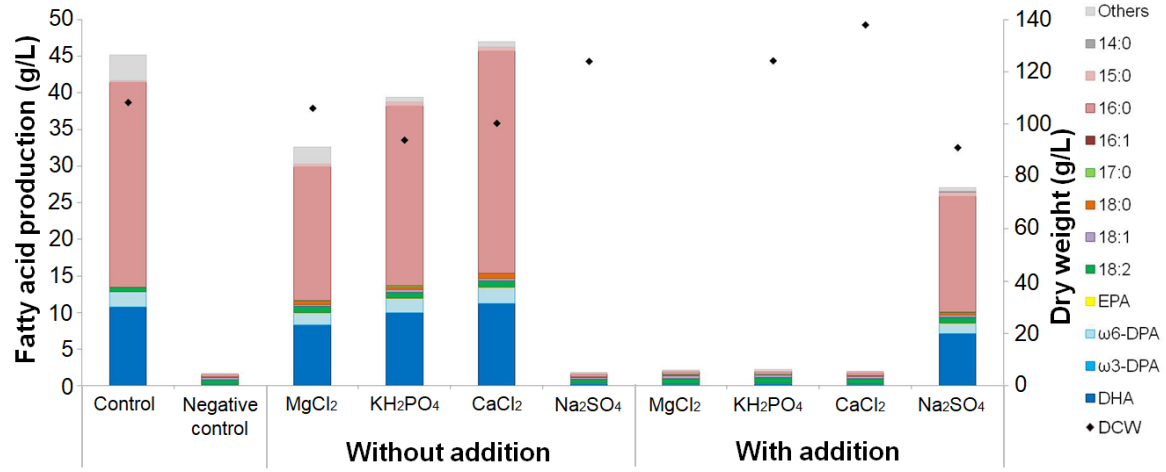


Fig 5. Time course of (a) residual glucose concentration, (b) growth, fatty acid production and (c) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium at 28 ℃ with shaking (250 rpm). For all abbreviations, see Fig 1.

(a)



(b)

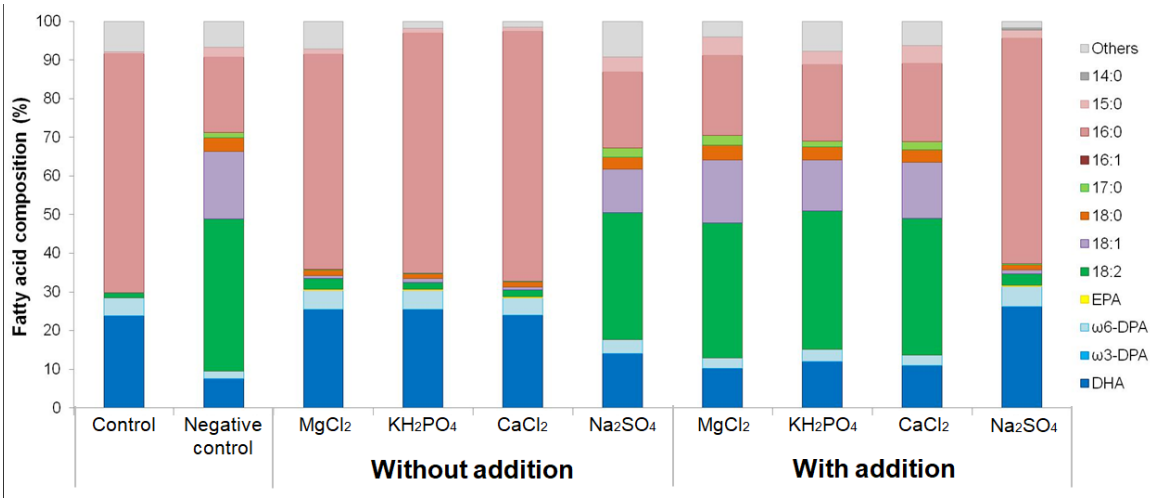
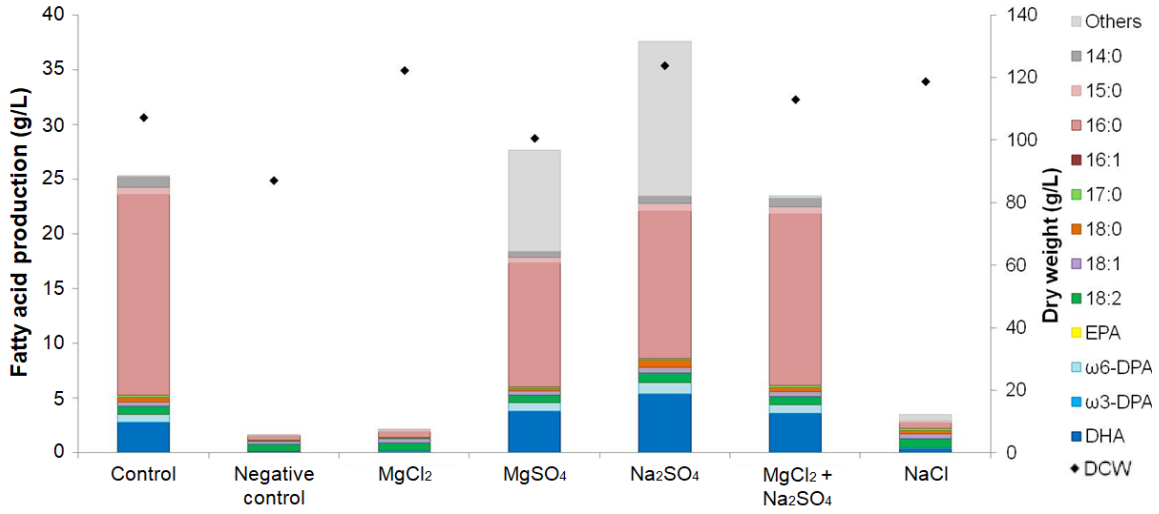


Fig 6. Effects of inorganic nutrients on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium at 28 ℃ with shaking (250 rpm) for 5 days. For all abbreviations, see Fig 1.

(a)



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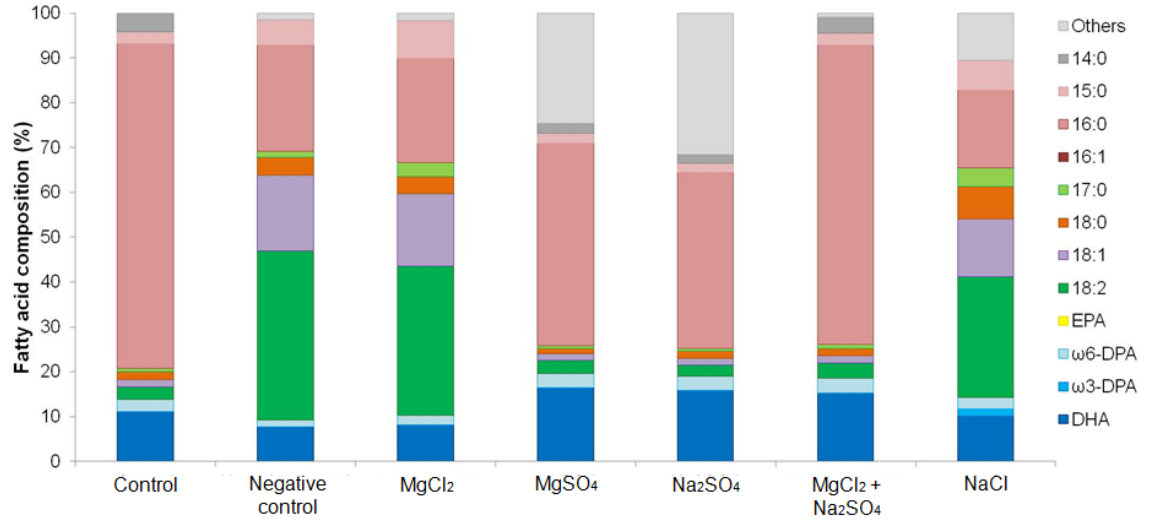


Fig 7. Effects of adding magnesium chloride, sodium sulfate and sodium chloride on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium at 28 ℃ with shaking (250 rpm) for 5 days. For all abbreviations, see Fig 1.

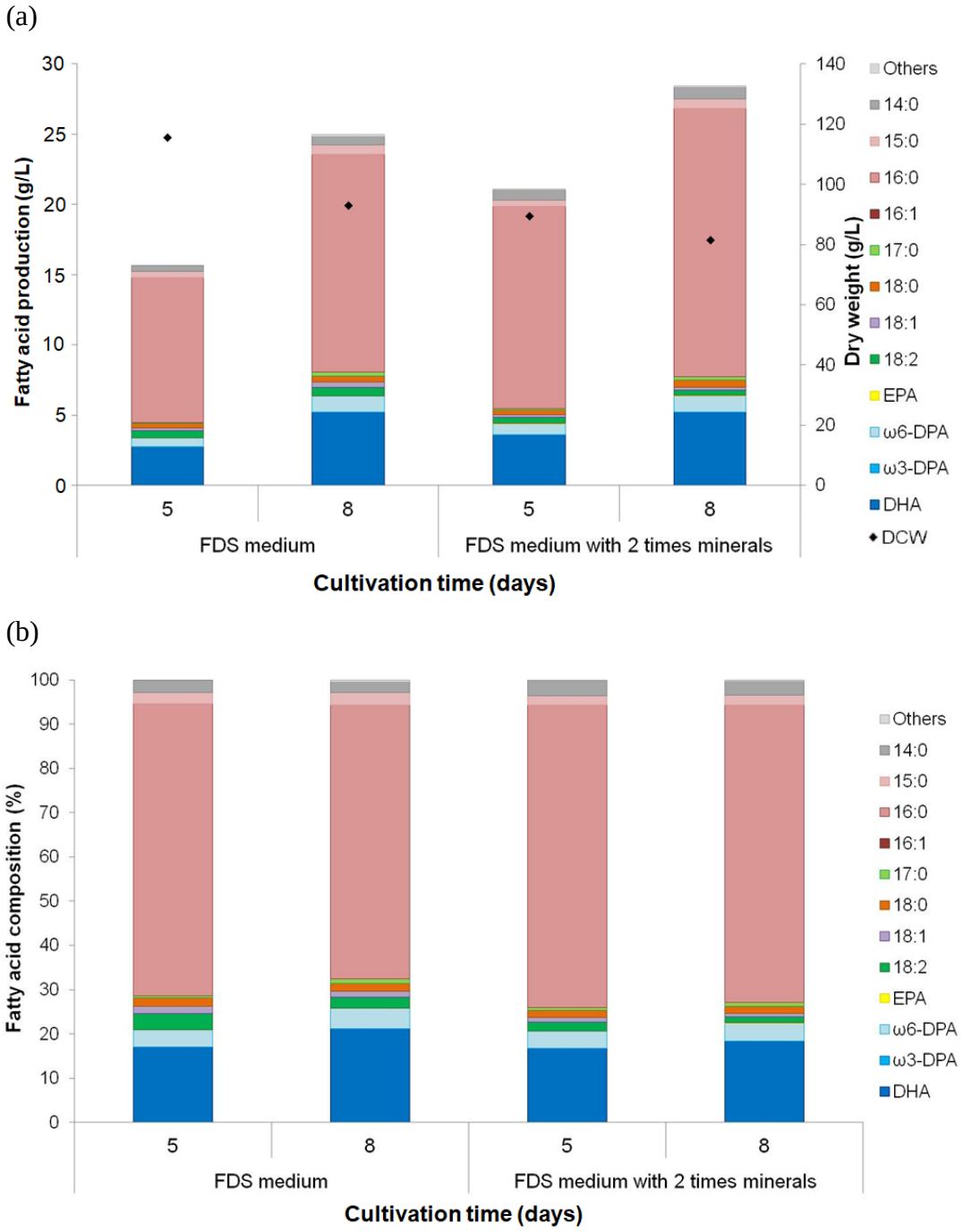
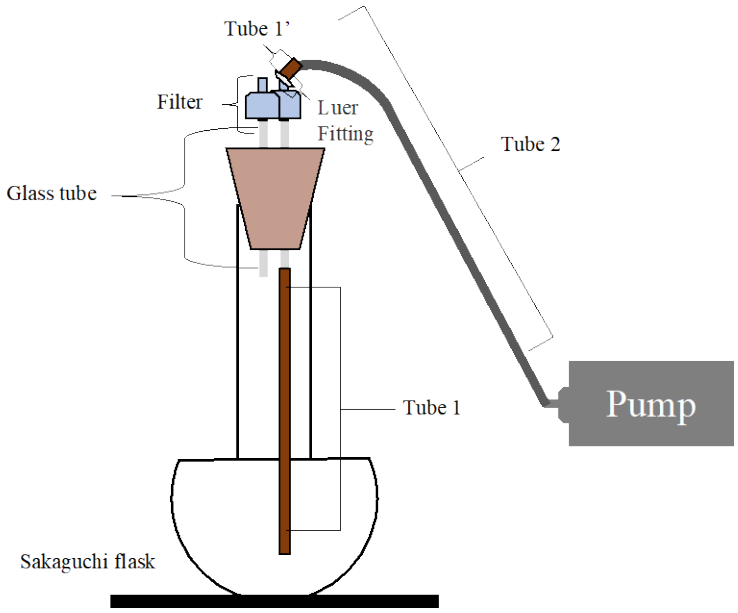


Fig 8. Effects of 2 times inorganic nutrients on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium at 28 ℃ with shaking (250 rpm) for 5 and 8 days. For all abbreviations, see Fig 1.

(a)



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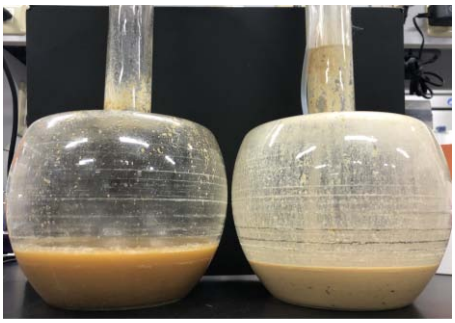
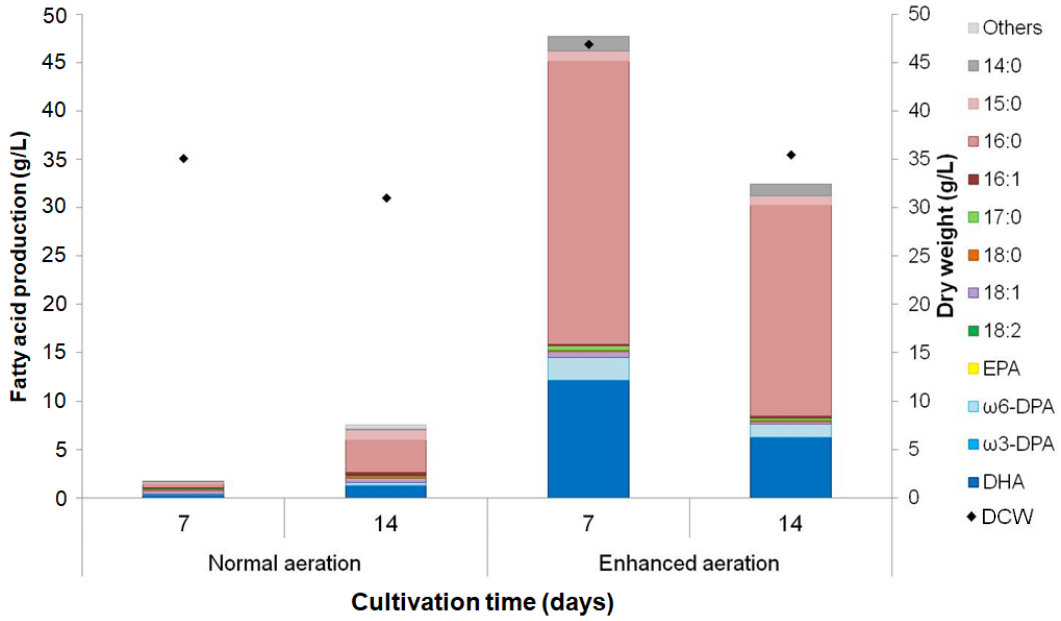


Normal aeration | Enhanced aeration

Fig 9. (a) Modified Sakaguchi flask for sufficient aeration. (b) Growth of strain 6-2 grow in a large-scale flask with intact aeration and enhanced aeration.

(a)



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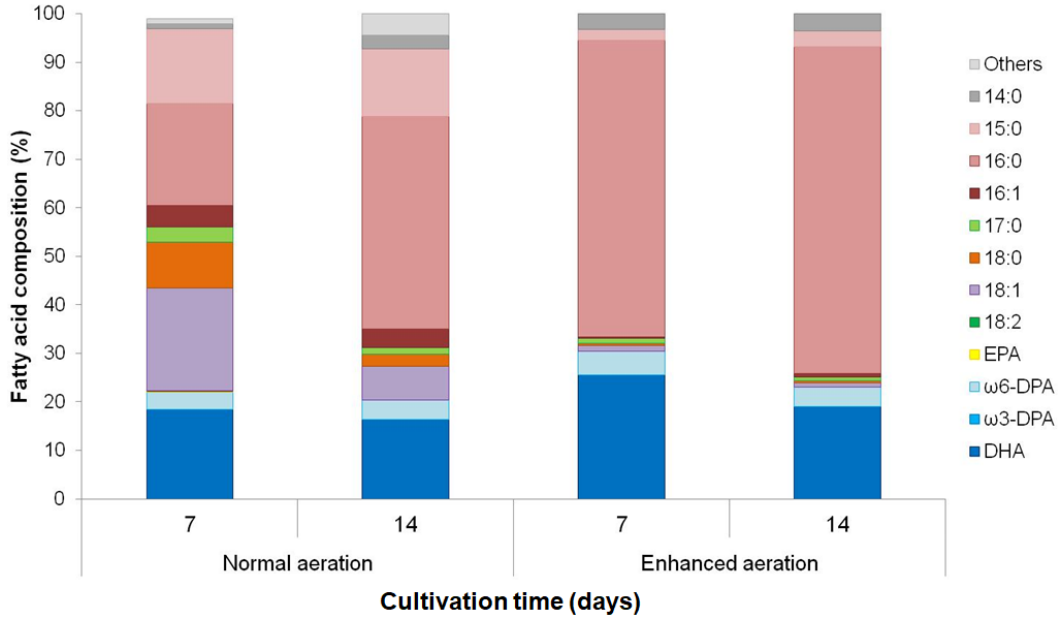


Fig 10. Effects of aeration on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in FDS medium in large-scale flask at 28 ℃ with shaking (120 rpm) for 7 days and 14 days. For all abbreviations, see Fig.1.

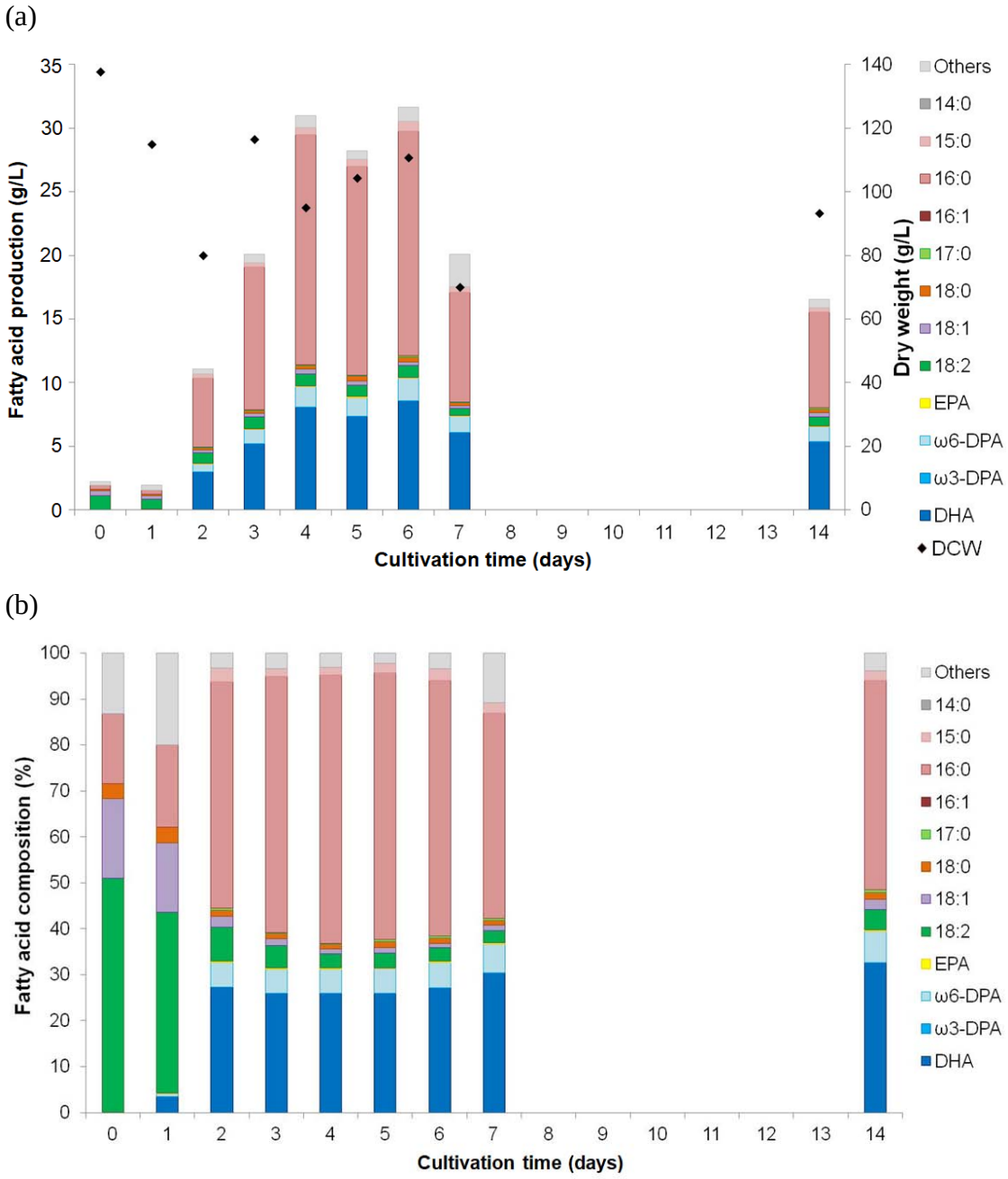


Fig. 11. Time course of (a) fatty acid production and (b) fatty acid composition in strain 6-2 in scale-up culture. Cultivation was conducted in FDS medium with enhanced aeration at 28 ℃ with shaking (120 rpm). For all abbreviations, see Fig. 1.

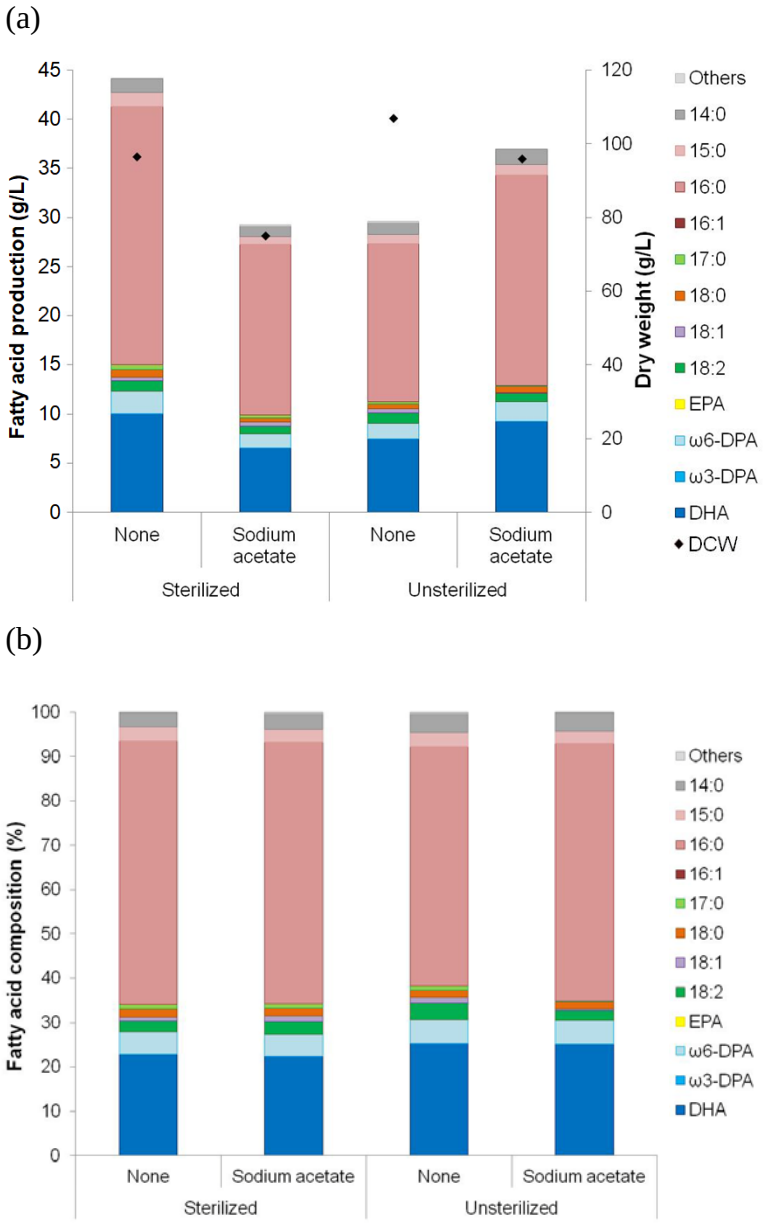


Fig 12. Effects of adding sodium acetate on (a) growth, fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted in sterilized or unsterilized FDS medium at 28 ℃ with shaking (250 rpm) for 6 days. For all abbreviations, see Fig 1.

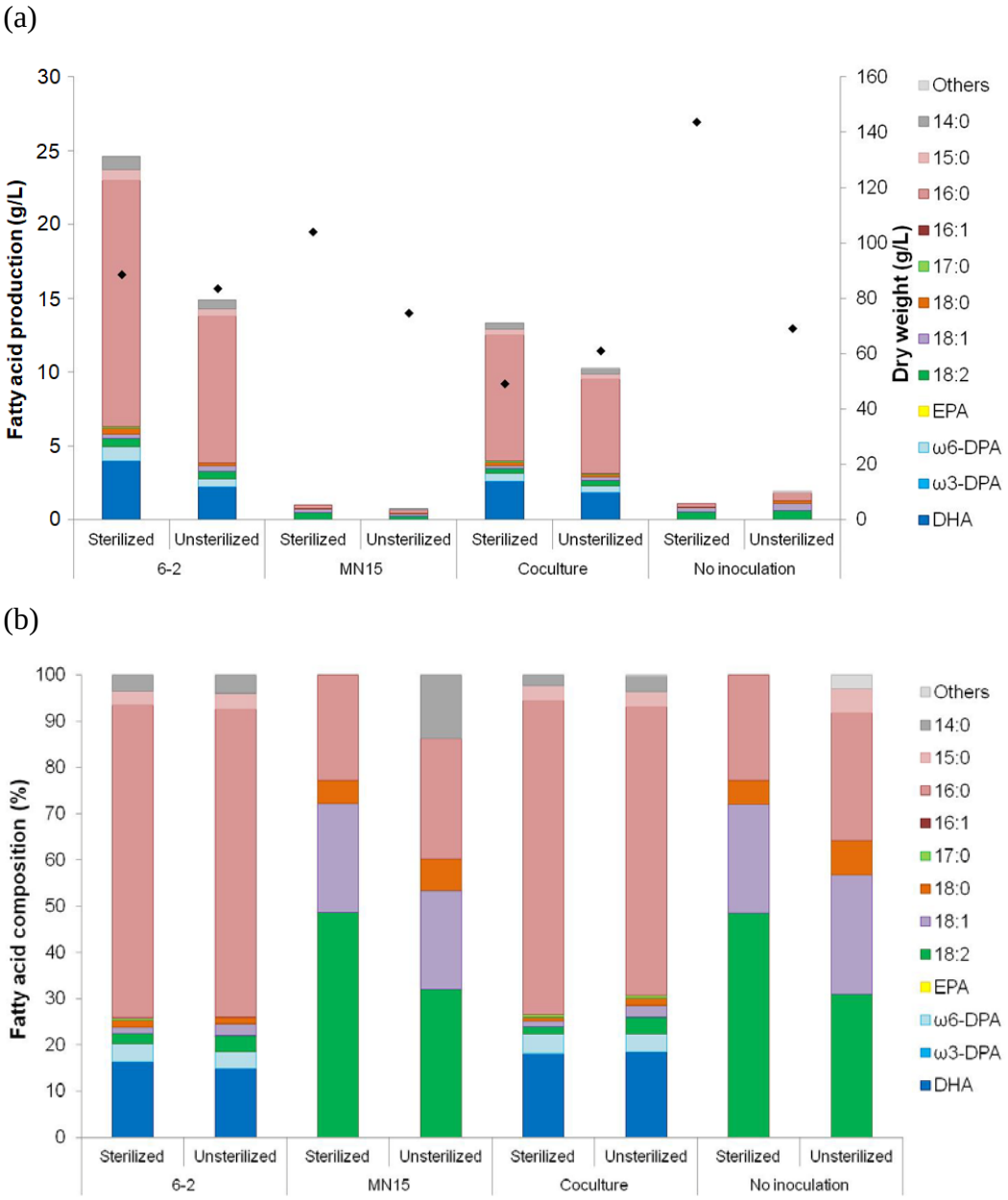


Fig 13. Effects of coculturing *Aurantiochytrium* sp. 6-2 and *Bacillus subtilis* MN15 on (a) growth, fatty acid production and (b) fatty acid composition. Cultivation was conducted in sterilized or unsterilized FDS medium at 28 ℃ with shaking (250 rpm) for 6 days. For all abbreviations, see Fig 1.

DISCUSSION

Firstly, the author compared the usage of defatted soybean and fermented defatted soybean (FDS) in the liquid medium. As a result, *Aurantiochytrium* sp. 6-2 could utilize FDS more efficiently. In the medium of 20% FDS addition, total fatty acid production reached more than 35 g/L with nearly 30% of DHA production. This result was 7 times higher fatty acid production than that in the medium used the defatted soybean. The plausible reason was that enzymes which secreted by koji hydrolyzed the defatted soybean and the released nutrients supported the growth of strain 6-2 for DHA production.

Koji, *Aspergillus oryzae*, is a filamentous fungus, as it is extensively used in the making various fermented foods in Japan. For example, sake, miso, soy sauce and mirin in Japan was prepared by using koji, which secreted protease, amylase and lipase could breakdown proteins and polysaccharides in soybeans or rice into amino acids and monosaccharides. By hydrolysis during the fermentation, various aroma, special taste, functional components were also formed. Moreover, glucoamylase, 3 types of endopeptidases (acidic endopeptidases, neutral endopeptidases and alkaline endopeptidases) and exopeptidases also joined the reactions. In addition, yeasts (*Zygosaccharomyces rouxii*) and lactic acid bacteria (*Tetragenococcus halophilus*) also participated in fermentation and responsible for forming aroma, alcohol and lactic acid (47). In consequence, fermented defatted soybean contains high nutritional value.

The author found that the medium added 20% (Fig 4) of the 5 days fermented defatted soybean (Fig 3) was optimal for strain 6-2 growth. In the medium feeding more FDS (40-60%), fatty acid productivity drastically declined (Fig 3). One possible reason was high titer of FDS would made the medium become slurry and influence the aeration.

Next, the influences of mineral on fatty acid productivity were evaluated (Fig 6-8). Sodium sulfate was recognized as the key factor for strain 6-2 in FDS medium. In Fig 7, both sodium sulfate and magnesium sulfate had significant effects on promoting fatty acid production. In particularly, sulfate was an important nutrient for strain 6-2 growth. In recent year, many papers were reported C/N ratio enhanced fatty acid production (48-53).

Recently, plant residue and industrial waste and so on were attracted much attention as nitrogen source in terms of economical point. On the other hand, minerals balances of these resources were often not sufficient for microbial cultivation. Therefore, minerals (especially for sulfur) should also take into consideration.

In Fig 10, the fatty acid productivity of strain 6-2 cultivated with enhanced aeration further increased compared to that of intact condition. This result suggested that dissolved oxygen in the medium deeply affect the growth of this strain. Other papers also pointed out that low oxygen level would have a negative impact on growth of *Aurantiochytrium* SW1 (18) and alter the fatty acid profile of *Aurantiochytrium limacinum* SR21 (49) and Thraustochytrid strain ACEM 6063 (54).

Last, the growth and fatty acid production of *Aurantiochytrium* sp. 6-2 in the unsterilized medium was evaluated (Fig 12-13). Although fatty acid productivity in strain 6-2 slightly decreased in the medium without autoclave, strain could still have the ability of significant amounts of fatty acid production. The reason why strain 6-2 kept the yield of fatty acids because other bacteria could be inhibited in the medium with addition of sodium acetate. Organic sodium salts, for instance, citric, lactic and acetic sodium salt have been utilized to inhibit the growth of microorganisms and prolong the shelf life of food products (55-59). They were also widely used based on antibacterial activities toward pathogens (60-62) and generally recognized as safe (GRAS) (61). Thus, it would be safe to use in aquaculture feed as well.

SECTION 3

Evaluation of DHA production by solid-state fermentation

In present section, the author cultivated *Aurantiochytrium* sp. 6-2 on different solid substrates and evaluated fatty acid production. As a result, high fatty acid productivity was achieved by using defatted soybean as the substrate. It was found that glucose feed and moisture content was essential for solid-state fermentation. Furthermore, the author found that mixing koji or *Bacillus* species could help strain 6-2 to use starch as carbon source

MATERIALS AND METHODS

Solid-state fermentation on fermented defatted soybean

Aurantiochytrium sp. 6-2 was pre-cultured in GYA medium [20 g/L glucose, 10 g/L yeast extract and 18 g/L artificial sea salt (Daigo's artificial seawater SP; Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan)] (10 mL in a test tube) and cultivated at 28 °C with shaking (250 rpm). Next, inoculate 2% of strain 6-2 directly on 5 g fermented defatted soybean and mixed by using sterilized spatula in the petri dish. After sealed by parafilm, the petri dish was cultivated at 28 °C for 28 days.

Solid-state fermentation on plant residues

Aurantiochytrium sp. 6-2 was first cultured in FDS medium [150 g/L glucose, 200 g/L fermented defatted soybean, 3 g/L KH_2PO_4, 1 g/L Na_2SO_4, 1 g/L $MgCl_2$, 0.3 g/L $CaCl_2$] and cultivated at 28 °C with shaking (250 rpm). And then, the culture mixed with plant residues, rice koji, defatted rice bran, and defatted soybean, with ratio 1:1 was cultivated at 28 °C under 100% humidity.

Analyses of fatty acid profile in strains

To analyze the composition of fatty acids that accumulated in the solid substrates, samples were collected by using sterilized spatula and stored at -20 °C. After frozen, samples were dried overnight by using a freeze dryer (EYELA, Japan).

For lipid extraction, frozen dried cells in a bead-beating tube were beaten with 1 mL deionized water and 0.2 - 0.3 g beads at 5000 rpm, 1 minute for 2 times. The lipids were extracted by the Bligh and Dyer method (38) as with 0.3 mg/mL of tricosanoic acid (C23:0) as an internal standard. Samples were mixed with 2 mL 10% methanolic HCl and 1 mL dichloromethane then incubated at 55 °C for 4 hours. After cooling down to room temperature, fatty acid methyl esters (FAMEs) were extracted with 4 mL n-hexane. Then concentrated via a rotary evaporator and send to analyze fatty acids composition by gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a split injector using a TC-70 capillary column (GL Science, Inc., Tokyo, Japan). The initial temperature of the column was 180 °C. After 10 minutes, temperature raised to 236 °C at the speed of 2 °C/min and raised to 260 °C at the speed of 12 °C/min then held at 260 °C for 5 minutes (39). Peaks of fatty acids were identified by referring to the retention times of standards (Sigma, USA). Calculation was conducted by comparing each peak area with internal standards. The data shown are the average values of at least three determinations.

RESULTS

Effects of fermented defatted soybean on solid-state fermentation

To evaluate the fatty acid productivity of *Aurantiochytrium* sp. 6-2, strain 6-2 was inoculated directly on fermented defatted soybean at 28℃ for 28 days. Fig 1 is the time course of fatty acid production and composition in culture of strain 6-2 on fermented defatted soybean by solid-state fermentation. Fatty acid content was increased with fermentation time and reached the maximum on day 7 (approximately 46.2 mg/g dry weight). Meanwhile, DHA production reached to the highest value of 2.89 mg/g dry weight. After that, though the content of DHA and ω6-DPA did not change obviously, other fatty acids began to decrease with fermentation days.

Effects of various solid substrates on solid-state fermentation

To evaluate various solid substrates for solid-state fermentation, strain 6-2 was cultivated on rice koji, defatted rice bran, defatted soybean and the mixture of these ingredients with the ratio of 1:1:1 for 6 days at 28 ℃. DHA accumulation was observed in all conditions. Especially the solid-state fermentation with defatted soybean showed the highest value in both fatty acid and DHA production, which were 27.36 mg/g and 3.1 mg/g dry weight at day 6, respectively (Fig 2c). These results indicated that defatted soybean was optimal substrate in solid-state fermentation by strain 6-2.

Effects of moisture content for solid-state fermentation

To evaluate different moisture contents in solid-state fermentation, *Aurantiochytrium* sp. 6-2 were cultivated in 0-100% moisture conditions for 6 days at 28 ℃. In this case, 100% moisture content was defined as the condition defatted soybeans absorbed the maximum amount of water. As the results, DHA accumulation was only observed under 75% and 100% moisture conditions (Fig 3a). DHA production was approximately 6 times higher under 100% moisture condition than that of 75% moisture

condition. This result indicated that higher moisture content is preferable for strain 6-2 growth in solid-state fermentation.

Time course of cultivating *Aurantiochytrium* sp. 6-2 with defatted soybean by solid-state fermentation

Fig 4 showed the time course of *Aurantiochytrium* sp. 6-2, which was cultivated on defatted soybean for 10 days. From day 1 to day 7, DHA and total fatty acid production were increased. After that, both of them started to decrease. The maximum DHA and total fatty acid content were 12.57 mg/g and 60.38 mg/g dry weight respectively. Compared to the initial amounts at day 0, they showed approximately 94% and 20% (w/v) increasing, respectively.

Effects of glucose addition to solid-state fermentation on fatty acid and DHA production

The effects of glucose addition were evaluated on solid-state fermentation (Fig 5). After 5 days fermentation, both DHA and total fatty acid production were increasing in the condition with 5% glucose addition. This result indicated that glucose feeding improved fatty acids production with solid-state fermentation by strain 6-2.

Effects of mixing different solid substrates on fatty acid productivity

To investigate the effects of using starch, defatted soybean, 2 types of koji (soybean koji and rice koji) and the mixture of these 3 materials as ingredients for solid-state fermentation, strain 6-2 was inoculated on these substrates and fermented for 5 days (Fig. 6). When starch was used as the single substrate, fatty acid accumulation was hard to detect. This result indicated that this strain could not utilize starch directly. The fermentation in using soybean koji as the substrate showed apparently the highest amount of fatty acid production (Fig. 6b), because soybean koji was contained significant amounts of lipids. In fact, only 2% DHA (w/w) was observed in this case, suggested that strain 6-2 did not grow well. In the case of using mixing ingredients as the substrates, significant amounts of DHA

were observed (Fig. 6b). This result suggested that koji could help decompose the plant materials such as starch and protein into glucose and amino acids, resulting in supporting the growth of strain 6-2.

Effects of different defatted rice bran sizes on fatty acid production

Fig 7 was the result of using different particle sizes of defatted rice bran as the substrate for solid-state fermentation. After 5 days of fermentation, the group that used defatted rice bran powder had higher DHA accumulation and fatty acid production. Therefore, it was suitable to grind defatted rice bran into powder before inoculation.

Effects of mixing defatted soybean (grain and powder) with rice koji on fatty acid production

To evaluate different particle sizes of defatted soybean and rice koji on fatty acid productivity, *Aurantiochytrium* sp. 6-2 was inoculated on several different substrates for solid-state fermentation. As a result, there were no significant differences between defatted soybean grain and powder. However, when soybean was mixed with rice koji in different ratios, fatty acid production increased. The fermentation combined with 75% rice koji and 25% defatted soybean powder showed the maximum fatty acid and DHA production, 58.27 mg/g and 5.77 mg/g, respectively.

Effects of carbon sources and sodium acetate by coculturing strain 6-2 and *Bacillus subtilis* MN15 on the fatty acid production

At chapter 2.2, the co-culture with *Aurantiochytrium* sp. 6-2 and *Bacillus subtilis* MN15 was evaluated in both sterilized and unsterilized liquid FDS. Also, to evaluate the effects in solid-state fermentation, *Aurantiochytrium* sp. and *B. subtilis* MN15 were both inoculated on defatted soybean. There were no differences between fermentation with and without sodium acetate in using glucose as carbon source. DHA was hard to detect in using starch as the carbon source, because strain 6-2 could compete with other microorganisms.

However, DHA and fatty acid production with addition of sodium acetate were almost the same as using glucose.

Effects of different temperatures on fatty acid production and composition

In Fig 10, stain 6-2 was cocultured with *Bacillus subtilis* MN15 on defatted soybean for 5 days at 28℃ and 37℃. The fermentation condition at 28 ℃ showed higher DHA and fatty acid production than that at 37 ℃. These results suggested strain 6-2 preferred 28 ℃ for solid-state fermentation.

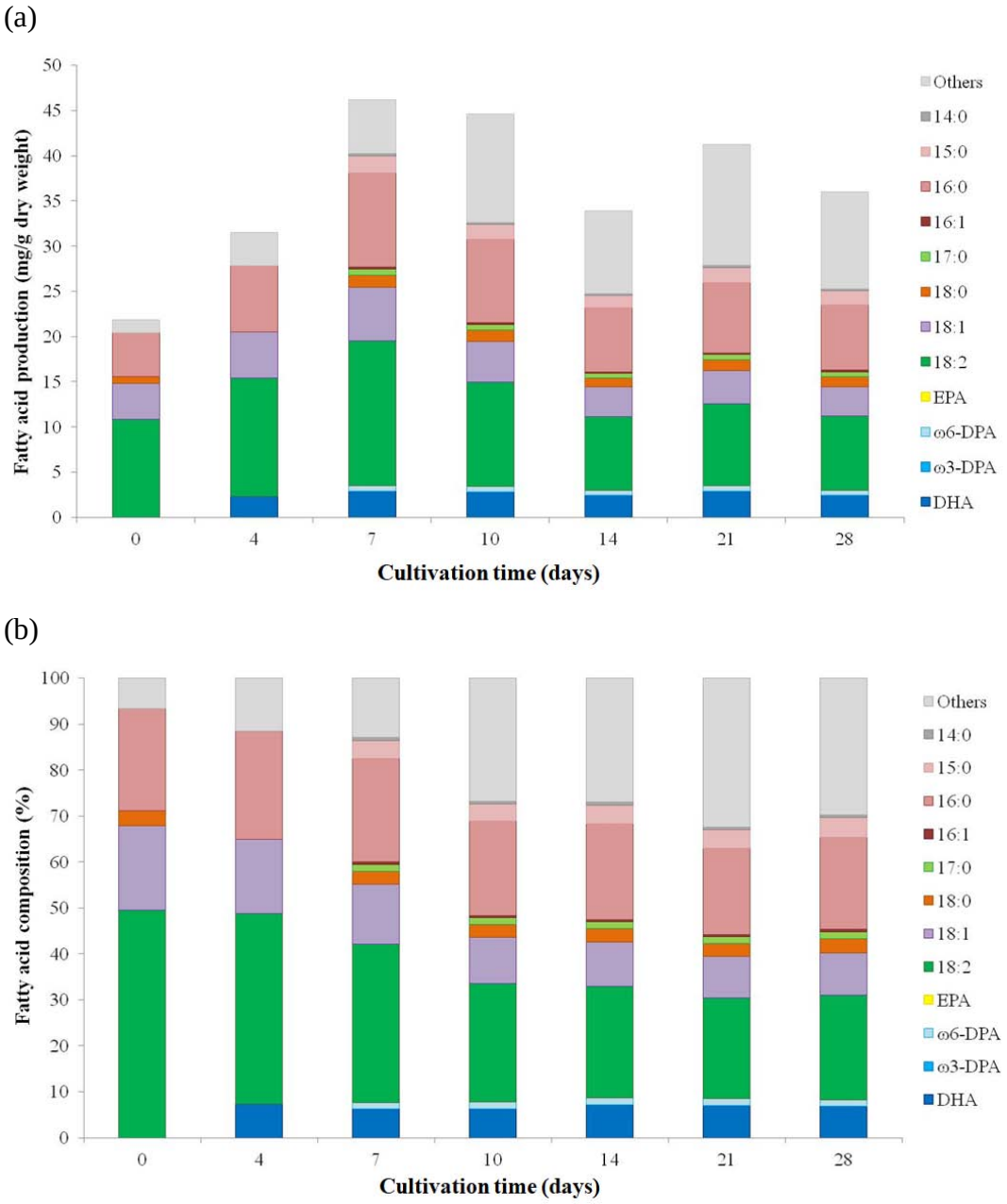


Fig 1. Time course of (a) fatty acid production and (b) fatty acid composition in strain 6-2 in solid-state fermentation. Cultivation was conducted on fermented defatted soybean at 28 ℃ for 28 days. 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; EPA, ω3-eicosapentaenoic acid; ω6-DPA, ω6-docosapentaenoic acid; ω3-DPA, ω3-docosapentaenoic acid; DHA, ω3-docosahexaenoic acid.

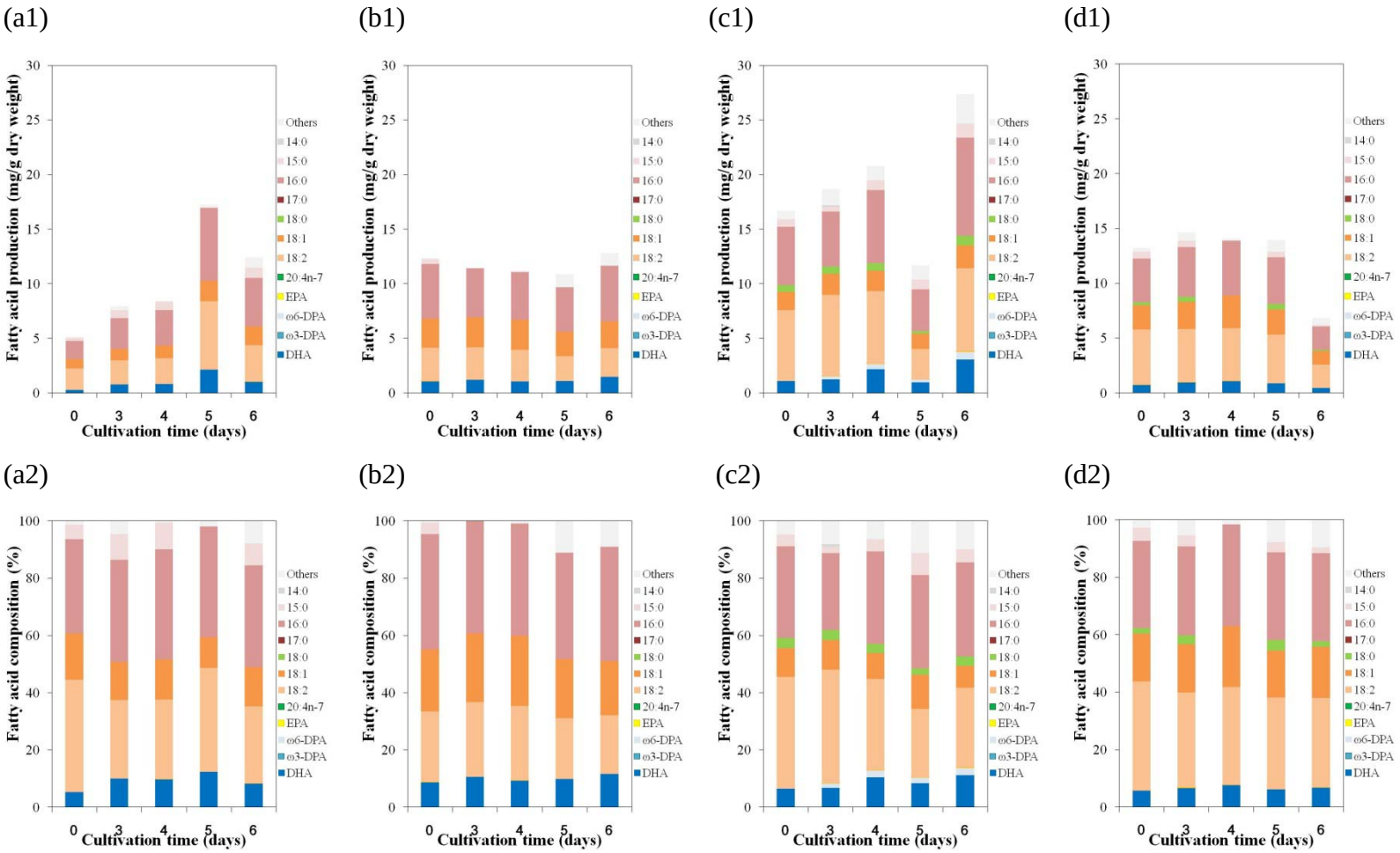


Fig 2. Effects of various solid substrates on (1) fatty acid production and (2) fatty acid composition in strain 6-2 in solid-state fermentation. Cultivation was conducted on various solids at 28 ℃ for 6 days. (a) rice koji (b) defatted rice bran (c) defatted soybean (d) mix. For all abbreviations, see Fig 1.

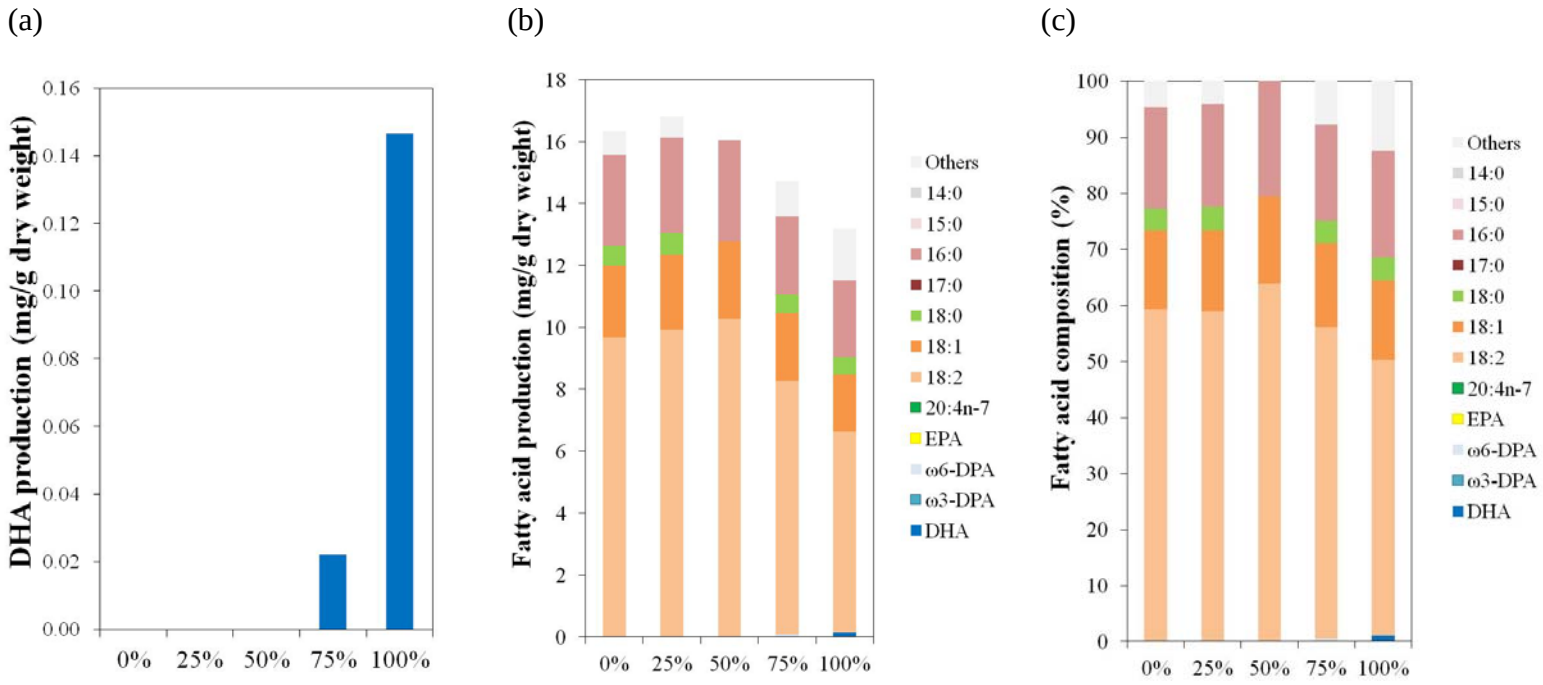
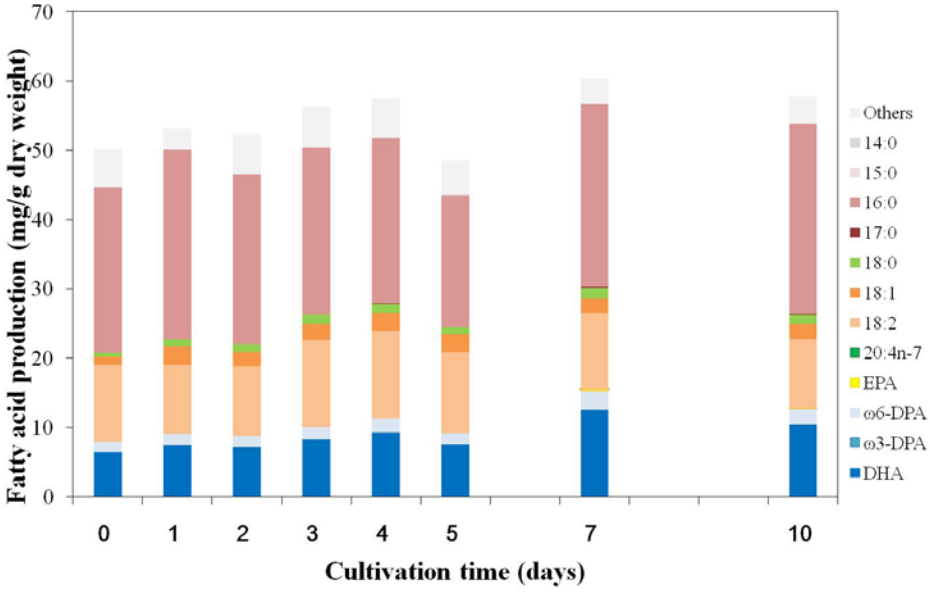


Fig 3. Effects of moisture content on (a) DHA, (b) fatty acid production and (c) fatty acid composition in strain 6-2 in solid-state fermentation. Cultivation was conducted on defatted soybean under various moisture conditions at 28 ℃ for 6 days. For all abbreviations, see Fig 1.

(a)



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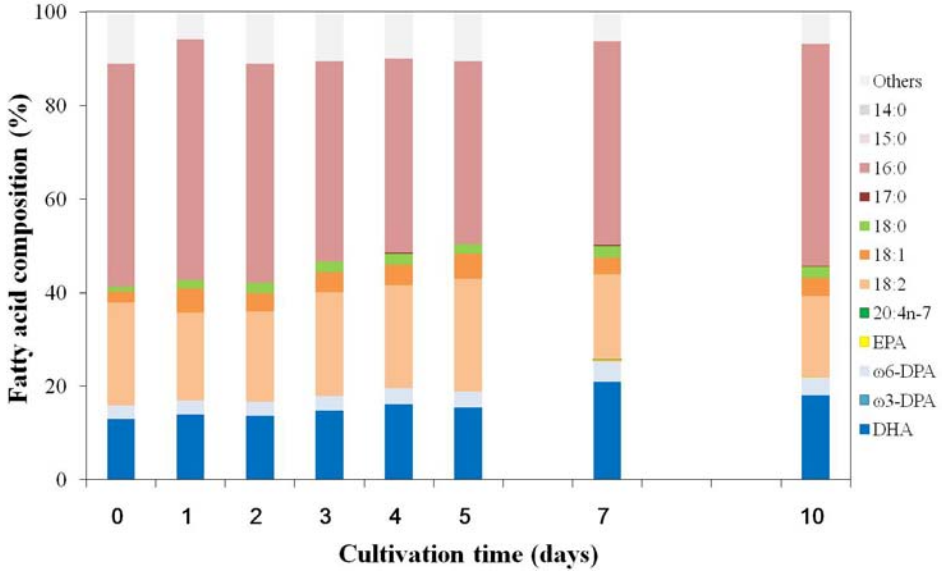


Fig 4. Time course of (a) fatty acid production and (b) fatty acid composition in strain 6-2 in solid-state fermentation. Cultivation was conducted on defatted soybean at 28 ℃ for 10 days. For all abbreviations, see Fig 1.

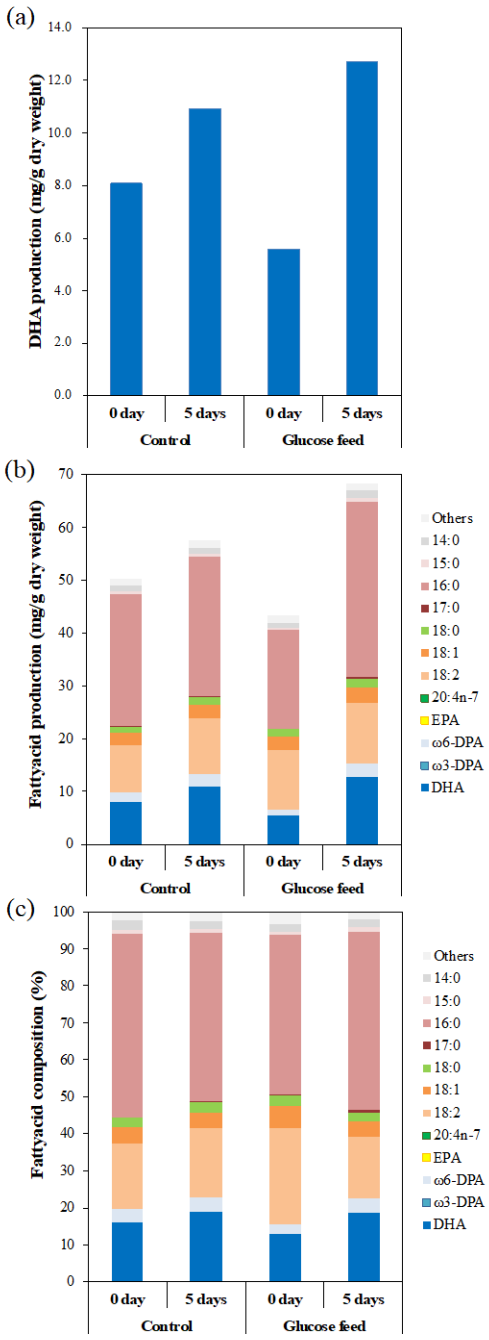
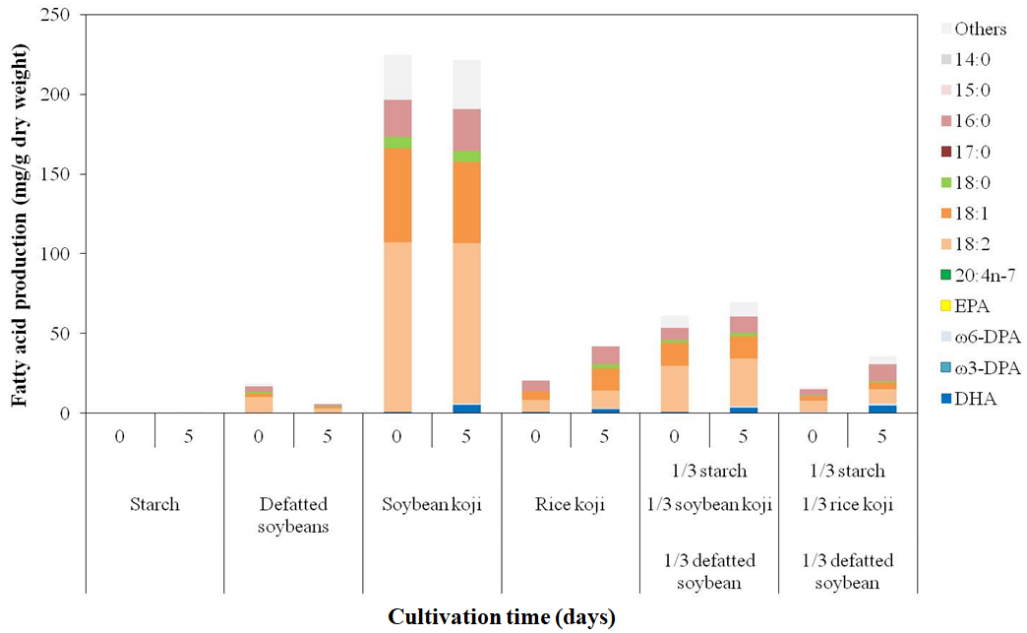


Fig 5. Effects of glucose addition on (a) DHA, (b) fatty acid production and (c) fatty acid composition in strain 6-2 by solid-state fermentation. Cultivation was conducted on defatted soybean under various moisture conditions at 28 ℃ for 5 days. For all abbreviations, see Fig 1.

(a)



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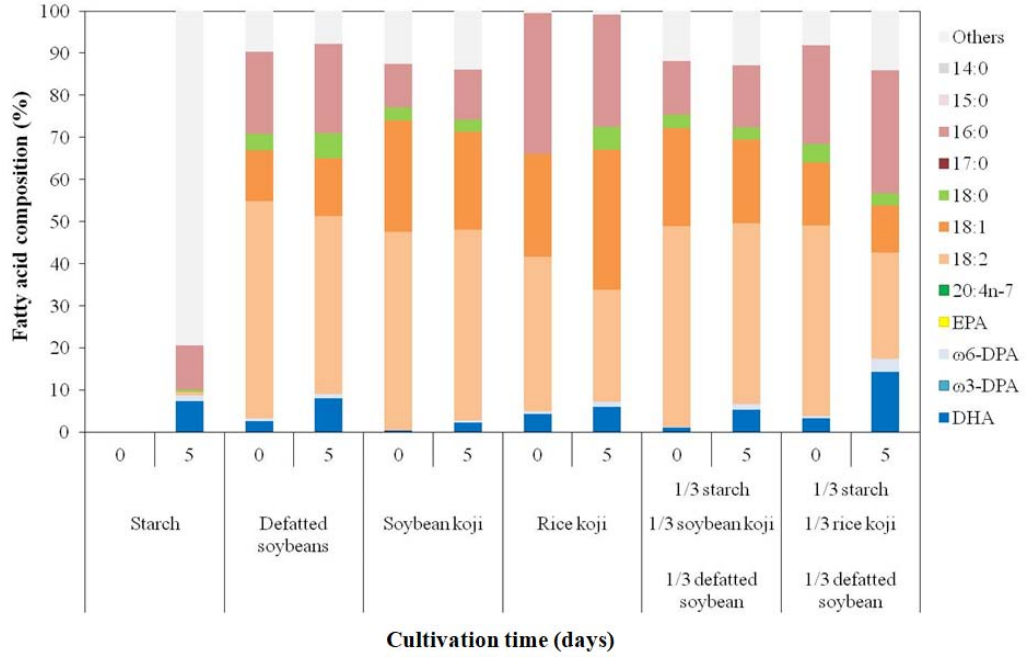


Fig 6. Effects of different solid substrates on (a) fatty acid production and (b) fatty acid composition in strain 6-2 in solid-state fermentation. Cultivation was conducted at 28 ℃ on different solid substrates for 5 days. For all abbreviations, see Fig 1.

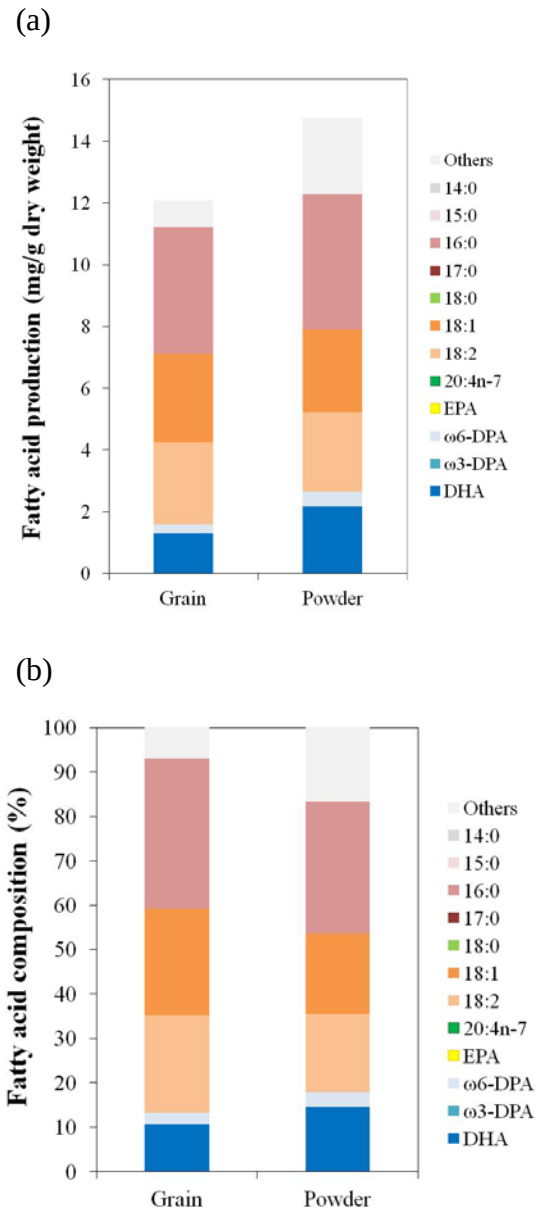
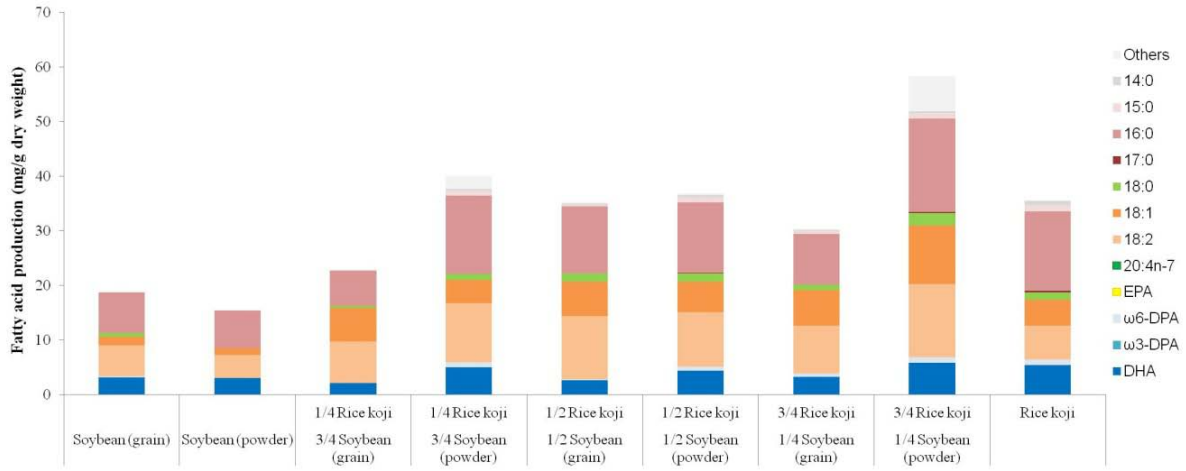


Fig 7. Effects of different particle sizes on (a) fatty acid production and (b) fatty acid composition in strain 6-2 in solid-state fermentation. Cultivation was conducted on defatted rice bran grain or powder at 28 ℃ for 5 days. For all abbreviations, see Fig 1.

(a)



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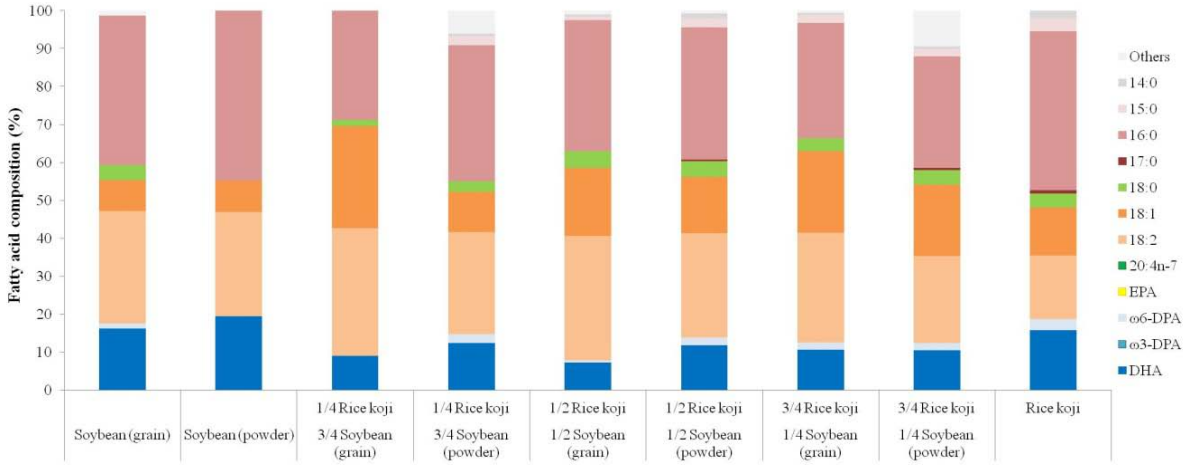
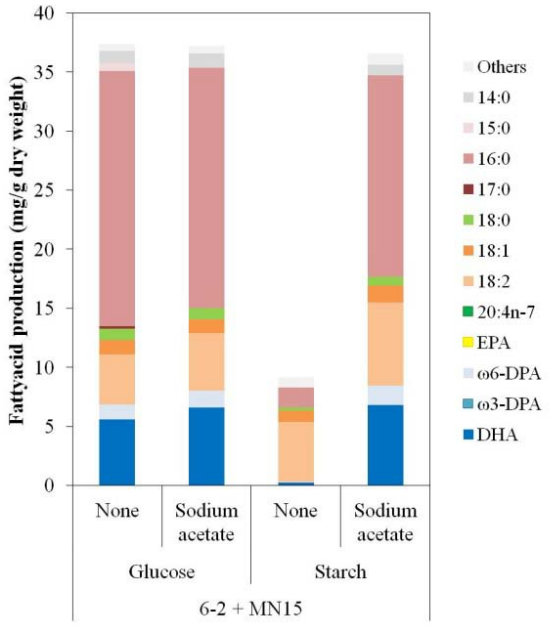


Fig 8. Effects of mixing defatted soybean and koji on (a) fatty acid production and (b) fatty acid composition in strain 6-2. Cultivation was conducted at 28 ℃ for 5 days in solid-state fermentation. For all abbreviations, see Fig 1.

(a)



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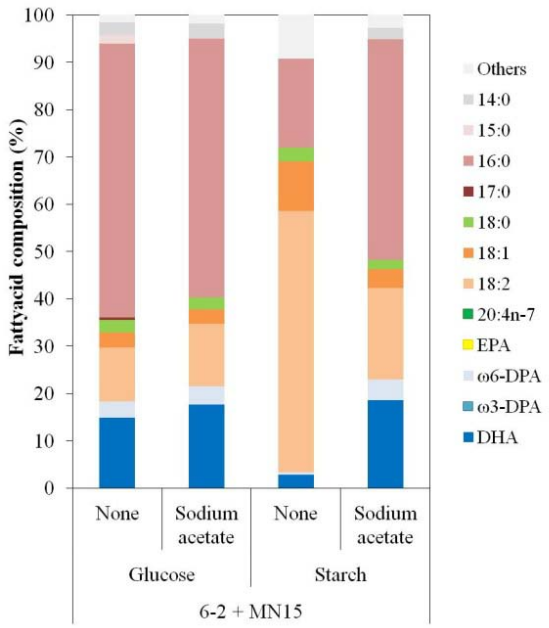
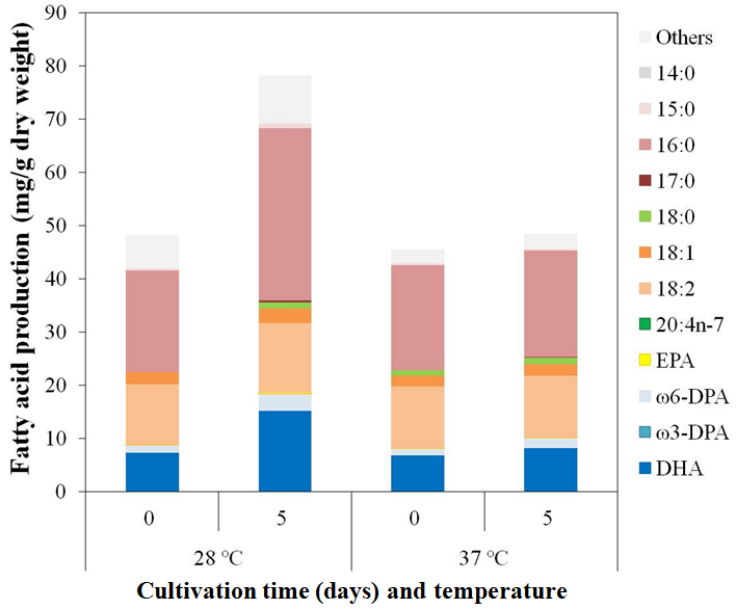


Fig 9. Effects of carbon sources and sodium acetate on (a) fatty acid production and (b) fatty acid composition when coculturing strain 6-2 and MN15 on defatted soybean at 28 ℃ in solid-state fermentation. For all abbreviations, see Fig 1.

(a)



(b)

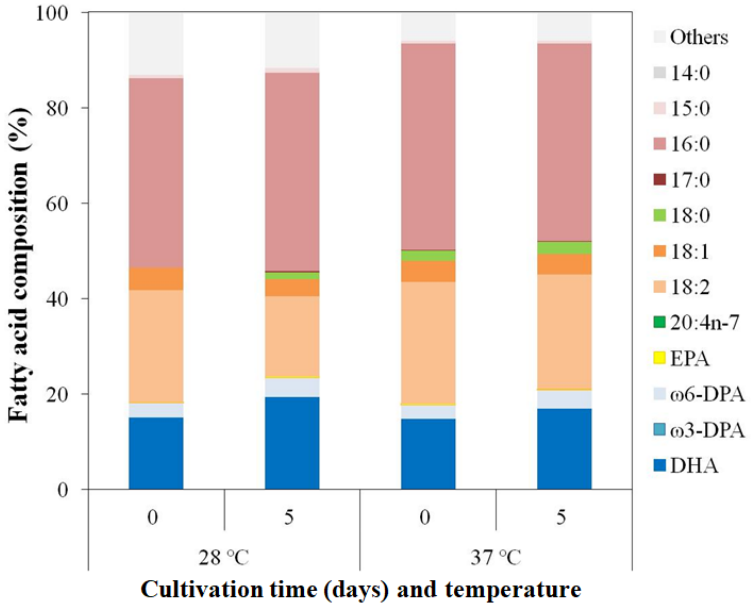


Fig 10. Effects of different cultivation temperatures on (a) fatty acid production and (b) fatty acid composition when coculturing strain 6-2 and MN15 on defatted soybean in solid-state fermentation. Cultivation was conducted on defatted soybean for 5 days. For all abbreviations, see Fig 1.

DISCUSSION

Solid-state fermentation, short for SSF, is a fermentation process that occurs by using solid substrate as the source of nutrients and in absence or near absence of water (63). In present section, the author addressed on cultivating *Aurantiochytrium* sp. 6-2 in solid-state fermentation.

First of all, several solid substrates and moisture were examined. In Fig 2, strain 6-2 was inoculated on four different materials (rice koji, defatted rice bran, defatted soybean and mixture). As a result, the fermentation in using defatted soybean showed higher fatty acid production. For adjusting of the moisture content, various amounts of deionized water were added to defatted soybean. As shown in Fig 3, when moisture content is 100%, DHA accumulation could reach maximum. This result indicated that water content was critical to support the growth and metabolism of microbes (64-65).

Secondly, koji was used to promote the utilization of solid substrate. Recently, fungi were used as the model organisms to yield enzymes, metabolites and spores on solid substrate such as agriculture waste (66). Due to its potential, koji was considered as the candidate to coculture with strain 6-2 in solid-state fermentation. In Fig 6, both commercial rice koji and soybean koji were evaluated. Consequently, koji could assist in hydrolyzing solid substrates and turn them into the form that could be used by strain 6-2. Although the group with soybean koji had a higher fatty acid productivity, it was considered that these fatty acids came from the soybean (from soybean koji). Because DHA content is almost equal with others that utilized rice koji. In Fig 8, total fatty acid content was increased when solid substrates were ground into powder. Koji were easier to grow on powder size substrate might be plausible reason.

Finally, *Bacillus subtilis* was used to coculture with *Aurantiochytrium* sp. 6-2 on defatted soybean. *B. subtilis* was found in many environments, for example, plant and feces (67). *B. subtilis* was also known as producer of alpha amylase (68) and for making natto (69). Since it could not only hydrolyze starch and plant materials, but also regarded as safe, it was considered as one of promising strain to co-culture with strain 6-2. As shown in Fig 9,

starch was also available as carbon source for fatty acid production in co-cultured fermentation with *B. subtilis* MN15 and *Aurantiochytrium* sp. 6-2 by addition of sodium acetate which could suppress other bacteria. In Fig 10, 28 °C fermentation condition showed higher fatty acids and DHA production than that of 37 °C. This result suggested that strain 6-2 preferred 28 °C as well as liquid cultivation for solid-state fermentation.

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CHAPTER 3

Evaluation of polyunsaturated fatty acids production in solid-state cultivation system

ABSTRACT

In recent years, microalgae has been received attention from researchers because of their potential for producing ω3-polyunsaturated fatty acid (ω3-PUFA) with sustainability. In this chapter, the author evaluated the solid-state cultivation system for fermentation of defatted soybean and PUFA production by DHA producing strains to reduce production cost and increase productivity. As a result, optimal process for preparing fermented defatted soybean (FDS) was as follows. Mixing defatted soybean (without heat treatment), rice koji and deionized water at a ratio of 1.0 : 1.2 : 2.4. Fermentation was carried out at 40-45°C for 7 days. Maximum production can be achieved by strain D36, which is 60.7 g/L (20.8 g/L) of total fatty acids (DHA) in 4 days in unsterilized medium with 20% optimized FDS. Furthermore, fatty acid productivity of strain D13 increased approximately 36% in 3 days in solid-state fermentation by mixing FDS medium with defatted soybean at the ratio of 2 : 1 (in weight). Owing to large quantities of ω3-PUFA were produced during fermentation, this strategy can be applicable to develop a novel sustainable ω3-polyunsaturated fatty acid (ω3-PUFA) production substituted fish oil.

INTRODUCTION

Recently, marine algae obtained much attention due to their potential in producing omega-3 lipid and used in nutraceuticals and functional foods (1). Microalgae could accumulate 20-70% fatty acids in cells, and it was higher than oil-producing plants. Therefore, microalgae were considered as a renewable resource to yield functional lipids as nutritional supplements (2). Besides, the fatty acid profile could be modified by manipulating culture conditions (3-4). In addition, unlike plants that need land for production, many microalgae could grow in seawater and save the usage of fresh water (2). As a result, microalgal oil was regarded as sustainable resource with less water usage, high yield and low carbon footprint (5).

Intake of ω3-docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are known as essential nutrition for fish growth and development. That is to say, large amounts DHA production substituted fish oil is essential for the sustainable development of aquaculture industry (6). In recent years, it has been pointed out that DHA supplied from fish oil acquired from wild catch fish were not environmentally friendly and lack sustainability due to overfishing (7). Therefore, using microalgal oil to substitute fish oil has become one of strategies to conquer the difficulties. For example, microbial oil was involved with fishmeal as the feed for salmon, catfish and yellowtail (8-12). All studies reported that normal growth and development of target animals have been observed by feeding microalgal oil. In brief, microbial oil was confirmed to be the alternative resource to replace fish oil.

To cultivate microorganisms generally, carbon source (glucose) and nitrogen source (yeast extract) are used in laboratory. In addition, sterilized medium is essential as well. The most common and reliable way to sterilize the medium is heat sterilization. However, it would also accelerate the Maillard reaction between reducing sugars and amino acids during heat treatment. One major product from the Maillard reaction (13) called Hydroxymethylfurfural (HMF) had been reported that it could inhibit the growth of fungi, yeast, and bacteria (14).

On the other hand, solid-state fermentation (SSF) is a prospective technology for producing microbial products. For instance, animal feeds, biofuel, foods, chemicals and pharmaceuticals (15). It has developed in Asian countries over decades and had wide application (16) such as miso paste and natto in Japan. Moreover, SSF had several advantages, for example high productivity, low cost (16) and high level of aeration (17). Furthermore, SSF might make possible to avoid collection of cells, drying and extraction of fatty acids in preparation of aquaculture feed ingredients. Additionally, fatty acids productivity might be expected to be higher than liquid fermentation.

Therefore, this chapter focused on optimization of fermented condition for defatted soybean (FDS) preparation based on DHA production by liquid culture. And then, the author also evaluated solid-state fermentation for DHA production using optimized fermented defatted soybean.

MATERIALS AND METHODS

Strains and culture conditions

Heterotrophic microalgae strains were cultured in GYA medium [20 g/L glucose, 10 g/L yeast extract (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and 18 g/L artificial sea salt (Daigo's artificial seawater SP; Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan)] was prepared via filter sterilization for pre-culture. For main culture, strains (with 1% inoculums from pre-culture) were cultivated in GYA(G15Y2A) medium [150 g/L glucose, 20 g/L yeast extract and 18 g/L artificial sea salt] or FDS medium [150 g/L glucose, 200 g/L fermented defatted soybean, 3 g/L KH_2PO_4, 1 g/L Na_2SO_4, 1 g/L $MgCl_2$, 0.3 g/L $CaCl_2$ and chloramphenicol 100 μg/mL]. All medium were used for liquid cultivation (5 mL in a test tube) and cultivated at 28 °C with shaking (250 rpm), followed by the fatty acid composition analysis, unless otherwise noted.

Measurement of growth of DHA producing strain

Strains were cultured in GYA medium. Then the absorbance at 600 nm at 6, 12, 18, 24, 30, 36, 42 and 48 hours were measured by a spectrophotometer (Molecular Devices, Tokyo, Japan).

Heat treatment of FDS

Weight 1-20% fermented defatted soybean (from chapter 2) accurately in the test tube. After sealing the test tube by the silicon plug, heat treatment with an autoclave machine (121 ℃ for 15 minutes) was conducted. After, cooling down to room temperature, autoclaved fermented defatted soybean was used for preparing FDS liquid medium. Three strains, *Schizochytrium limacinum* SR21, *Aurantiochytrium* sp. 6-2 and strain D36, were used for evaluation the heat treated FDS based on glucose consumption at day 2 to day 6.

Measurement of glucose concentration

WAKO glucose II kit (Fujifilm Wako Pure Chemical Co., Ltd., Osaka, Japan) was used for measurement of glucose in the medium. 0.02 mL sample solution was mixed with 3 mL of reagent (contained phosphate buffer pH 7.1, phenol, mutarotase, glucose oxidase, peroxidase, ampyrone, L-ascorbate oxidase), and incubated at 37 ℃ for 5 minutes. The absorbance was measured at 505 nm with a spectrophotometer. Glucose concentration was calculated using a calibration curve prepared with a series of standard glucose solutions.

Procedure of preparing FDS with different amount of deionized water and under different temperatures

500 g defatted soybean, 600 g dry rice koji (Marukome, Japan) and 1000 - 1600 ml of deionized water were mixed at room temperature and waited for 3 hours. Then the grinder (Yazaki, japan) was used to crush the mixture. The crushed mixture was put in a gas barrier bag and sealed with two packs of oxygen absorbers. Fermentation carried out at 35-50 ℃ for 7 days. After that, pasteurized at 65 ℃ for 1 hour and stored at -20 ℃.

Measurement of water content in FDS

Twenty grams of fermented defatted soybean were weighted accurately. After frozen dried for 48 hours by using a freeze dryer (EYELA, Japan). Water content was determined by a mechanical scale.

Determination of pH value in FDS

One gram of fermented defatted soybean was diluted with distilled water to 10 mL and measured the pH value by a pH meter (pH meter F-52, HORIBA, Kyoto, Japan).

Total nitrogen (TN) analysis

To analyze the total nitrogen of fermented defatted soybean (FDS), FDS samples were analyzed by the persulfate digestion-UV absorption method (18) with modification. First, 3 g of fermented defatted soybean was ground well by using a mortar and pestle. Next,

1,000-fold dilution was performed and 20 mL of diluted sample was added in a 50 mL-sealable container and mixed with 4 mL of persulfate solution (3 w/v% $K_2S_2O_8$, 4 w/v% NaOH), and was autoclaved at 121 ℃ for 30 minutes. After cooling down, 2.5 mL of the digested sample was transferred to a test tube and was mixed with 0.5 mL of HCl (12 M HCl: water = 1:12 (v/v)) to adjust pH to about 3. The solution was measured at the wavelength of 220 nm with a spectrophotometer by using a quartz cuvette. Total nitrogen content was calculated using a calibration curve prepared with a series of standard KNO_3 solutions.

Determination of glucose concentration in FDS

One gram of fermented defatted soybean was weighted accurately. Next, dilute with distilled water to 5 mL. Glucose concentration was measured by using WAKO glucose II kit as described previously.

Determination of starch content

The Starch assay kit from Boehringer Mannheim (Ingelheim, Germany, cat. no. 10 207 748 035) was used for measurement of starch. Samples of fermented defatted soybean were weighed into 50 mL centrifuge tubes (0.3~0.5 g weighed accurately) and recorded the weight. 10 mL of 40% ethanol was added into the centrifuge tube and centrifuged at 3000 rpm for 10 minutes after mixing. Use Nylon filters (30 μm) to filter the supernatant and the filtrate was discarded. This washing procedure was conducted 3 times. Both precipitate in the centrifuge tubes and on the filter was collected and transfer with 4 portions of 5 mL dimethylsulfoxide (DMSO, total 20 mL) into a flask (100 mL), add 5 mL of 8M HCl and seal the flask with parafilm. Flasks were Incubated at 60 °C, 120 rpm for 60 minutes. Then cooled down immediately to room temperature, added 50 mL distilled water and adjusted the pH value between 4 and 5 with sodium hydroxide. After that, transferred to a volumetric flask (100 mL), and filled up to 100 mL with distilled water. Wait for a few minutes and take 0.1 mL sample solution from the top to avoid precipitate. 0.1 mL sample solution was mixed with 0.2 mL amyloglucosidase solution (14 U/mL), and incubated at

60°C for 15 minutes. Add 1 mL triethanolamine buffer (pH 7.6 with approximately 2.78 mg NADP and 7.04 mg ATP) and 1 mL distilled water. After mixing, wait for 3 minutes and measure OD_{340} of the solution (A_1). Then 0.020 mL of enzyme reagent (contained hexokinase 285.71U, and glucose-6-phosphate dehydrogenase 142.86 U) was added, OD_{340} was measured (A_2) after 15 minutes. The difference ($A_2 - A_1$) for both sample and reagent blank were determined and the absorbance difference of the reagent blank was subtracted from the absorbance difference of the sample, thereby obtaining ΔA.

$$\Delta A = (A_2\text{-}A_1)_{\text{sample}} - (A_2\text{-}A_1)_{\text{reagent blank}}$$

The concentration of starch (g/L) was calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ (g/L)}$$

where V = final volume (mL); MW = molecular weight of the target (g/mol), for starch: $MW_{glucose} - MW_{water} = 162.1$; ε = extinction coefficient of NADPH at 340 nm = 6.3 ($L \times mmol^{-1} \times cm^{-1}$); d = light path (cm); v = sample volume (mL).
In addition, the result of starch content is calculated from the amount weighted:

$$\text{Starch content} = \frac{\text{c starch (g/L sample solution)}}{\text{weight sample in g/L sample solution}} \times 100 \text{ (g/100 g)}$$

Cultivation of microalgae in solid-state fermentation

Microalgae strains were cultivated in GYA(G15Y2A) or FDS medium for 4 days. After that, mixed with defatted soybean at a ratio of 2:1 and with supplementation of 15% glucose, 0.3% KH_2PO_4, 0.3% Na_2SO_4, 0.1% $MgCl_2$, 0.03% $CaCl_2$ in the petri dish. And

then, petri dish was sealed by parafilm and cultivated at 28 °C under 100% humidity for 3 days.

Dry weight measurements and analyses of fatty acid profile in strains

To analyze the composition of fatty acids that accumulated in the cell, strains were collected by centrifugation at 3000 rpm for 10 minutes and stored at -20 °C. After frozen dried overnight by using a freeze dryer (EYELA, Japan). Dry cell weight was determined by a mechanical scale.

For lipid extraction, frozen dried cells in a bead-beating tube were beaten with 1 mL distilled water and 0.2 - 0.3 g beads at 5000 rpm, 1 minute for 2 times. The lipids were extracted by the Bligh and Dyer method (19) as with 0.3 mg/mL of tricosanoic acid (C23:0) as an internal standard. Samples were mixed with 2 mL 10% methanolic HCl and 1 mL dichloromethane then incubated at 55 °C for 4 hours. After cooling down to room temperature, fatty acid methyl esters (FAMEs) were extracted with 4 mL n-hexane. Then concentrated via a rotary evaporator and send to analyze fatty acid composition by gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a split injector using a TC-70 capillary column (GL Science, Inc., Tokyo, Japan). The initial temperature of the column was 180 °C. After 10 minutes, temperature raised to 236 °C at the speed of 2 °C/min and raised to 260 °C at the speed of 12 °C/min then held at 260 °C for 5 minutes (20). Peaks of fatty acids were identified by referring to the retention times of standards (Sigma-Aldrich Japan, Tokyo, Japan). Calculation was conducted by comparing each peak area with internal standards. The data shown are the average values of at least three determinations.

RESULTS

Screening results of DHA-producing strains

Approximately 40 strains were cultured in GYA medium to evaluate their fatty acid productivity. As shown in Fig 1, 4 strains (D13, D36, D37 and D40) could produce large amounts of fatty acids (>1000 mg/L). Additionally, their DHA contents were also up to than 500 mg/L over. Thus, these 4 strains and *Schizochytrium limacinum* SR21 (reference strain), *Aurantiochytrium* sp. T7 and 6-2 were selected for further studies.

Effects of glycerol on fatty acid production

To evaluate the effects of glycerol as the carbon source in GYA medium, 7 strains were cultivated in the liquid medium containing 2% glucose or glycerol. As shown in Fig 2a, all strains could use glycerol as the carbon source. For strain SR21, T7, D13, D37 and D40, they showed higher growth and fatty acid productivity in using glycerol rather than that in using glucose. In addition, only strain T7 could accumulate ω3-DPA among all strains. Fatty acid profiles in each strain were similar.

Growth curve of selected 7 strains

The growth curves of the 7 strains were shown in Fig 3. By comparing with each strain, strain T7 grew slower than other strains. In addition, pre-culture for 12-24 hours could shorten lag phase. The suitable pre-cultivation time to subculture was considered around 12 hours for various comparative experiment by 7 strains, because the growth of each strain was almost the same based on the absorbance at 600 nm at 12 hour pre-cultivation.

The effects of heat treatment on the growth of strain SR21, 6-2 and D36

To confirm whether the medium with heat treatment would have a negative impact on growth of strains, the growth of strain SR21, 6-2 and D36 were evaluated based on glucose consumptions. As shown in Fig 4, glucose was consumed with cultivation time.

However, higher remaining glucose was observed in the medium with increasing the composition of heat treatment FDS. These results indicated that medium with heat treatment definitely affect the growth of microalgae. As a result, medium without heat treatment was used in further experiments. Also, heat treatment in the process of preparing FDS would be skipped.

Compositional analysis of fermented defatted soybean

Seven different fermented defatted soybeans (A-G) were prepared by mixing with different amounts of deionized water in a range of 2.0-3.2 compared to defatted soybean. The results of their compositional analysis were described in Table 1. The pH value and total nitrogen content decreased with the increasing of water content (%). The glucose concentration in fermented defatted soybean was ranged of 9-14% and starch content was around 0.4-0.5%. Strain 6-2 was inoculated in these fermented defatted soybeans (A-G) liquid mediums and elevated fatty acid production. In consequence, strain 6-2 had higher fatty acid productivity in the medium with fermented defatted soybean C (Fig 5). Therefore, fermented defatted soybean C was used for further studies.

Effects of different fermentation temperatures on fermented defatted soybean

Defatted soybeans were fermented under different temperatures (35-50 °C). Then they were used to prepare FDS liquid medium for cultivation of strain 6-2 and D40 (Fig 6). According to the result, FDS which fermented at these 40-45 °C were optimal for lipid fermentation by strain 6-2 and D40.

Evaluation of DHA production in optimized FDS liquid medium

Seven strains were inoculated in GYA (G15Y2A) and FDS medium, and measured glucose concentration at day 2 to 4 and analyzed their fatty acid profiles at day 4 (Fig 7 and 8). In 4 days, only strain D40 can nearly consume all the glucose in FDS medium (Fig 7g). However, at least more than 2% glucose was still left in GYA medium for all strains. For fatty acid production, all strains produced higher amounts of fatty acids in FDS medium

than that in GYA medium. Among them, strain 6-2, D36 and D40 could produce 46.7 g/L (12.8 g/L), 60.7 g/L (20.8 g/L) and 55.1 g/L (16.1 g/L) of total fatty acids (DHA) in 4 days even in unsterilized FDS medium. These results suggested that fermented defatted soybean could be used as nitrogen source effectively, especially for strain 6-2, D36 and D40. It also indicated that fermentation process could indeed help microalgae strain to utilize plant materials such as defatted soybean.

Evaluation of DHA solid-state fermentation by using pre-cultured via liquid FDS

To evaluate lipid fermentation on solid substrates by 7 strains, pre-cultivated in GYA (G15Y2A) and FDS medium for 4 days were mixed with defatted soybean with a ratio of 2:1. As shown in Fig 9, fatty acid production by strain D13, D37and D40 could be further increased (14-36%) in solid-state fermentation. Especially, fatty acids production of strain D13 could be increased from 42 mg/g to 57 mg/g (approximately 36%) in 3 days in solid-state fermentation. These strains were considered as good strains for solid-state fermentation.

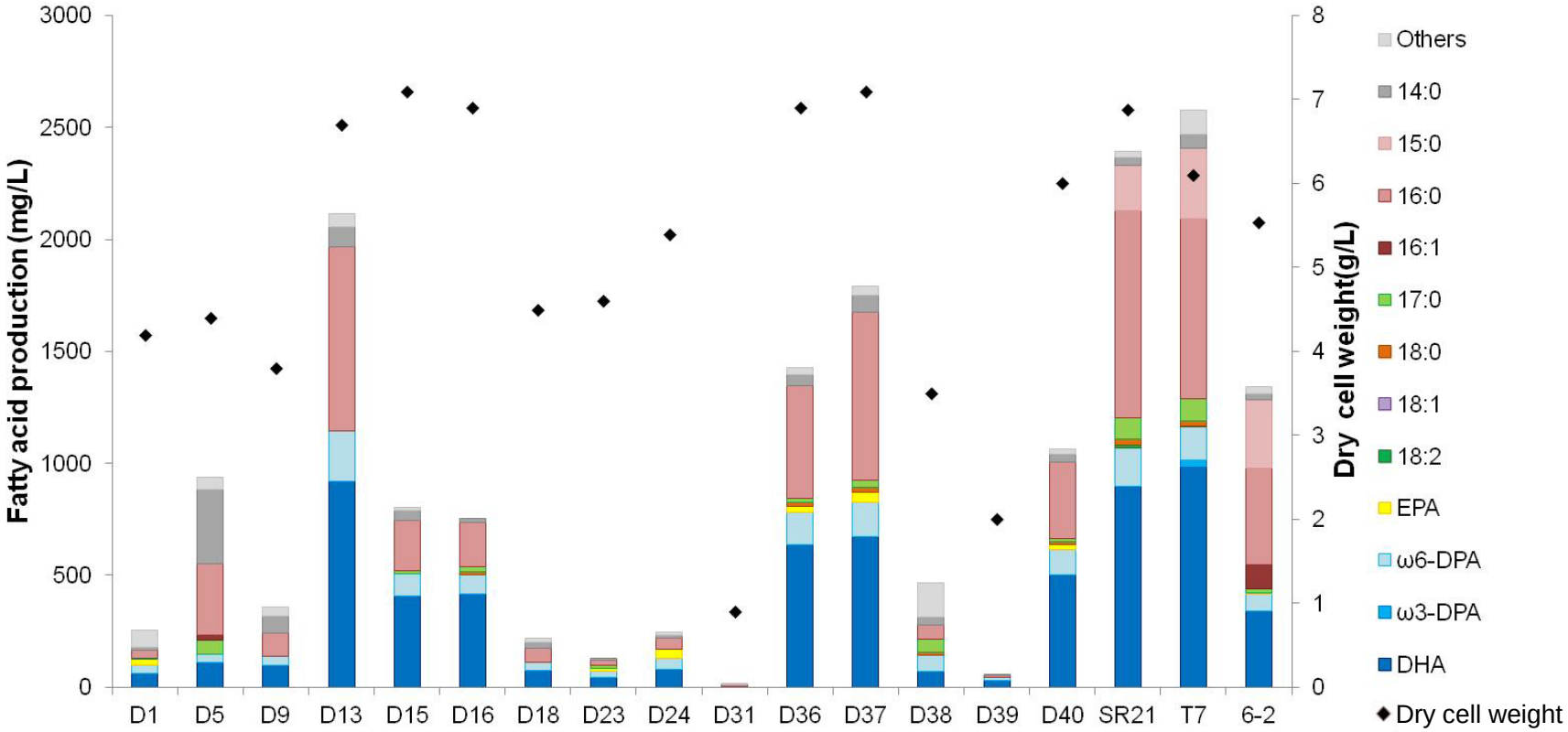


Fig 1. Comparison of fatty acid production by laboratory stocked strains. Cultivation was conducted at 28 °C with shaking (250 rpm) for 4 days in GYA medium [2% glucose or glycerol, 1% yeast extract, 1.8% artificial sea salts]. 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; EPA, ω3-eicosapentaenoic acid; ω6-DPA, ω6-docosapentaenoic acid; ω3-DPA, ω3-docosapentaenoic acid; DHA, ω3-docosahexaenoic acid; SR21, *Schizochytrium limacinum* SR21 was used as a reference strain.

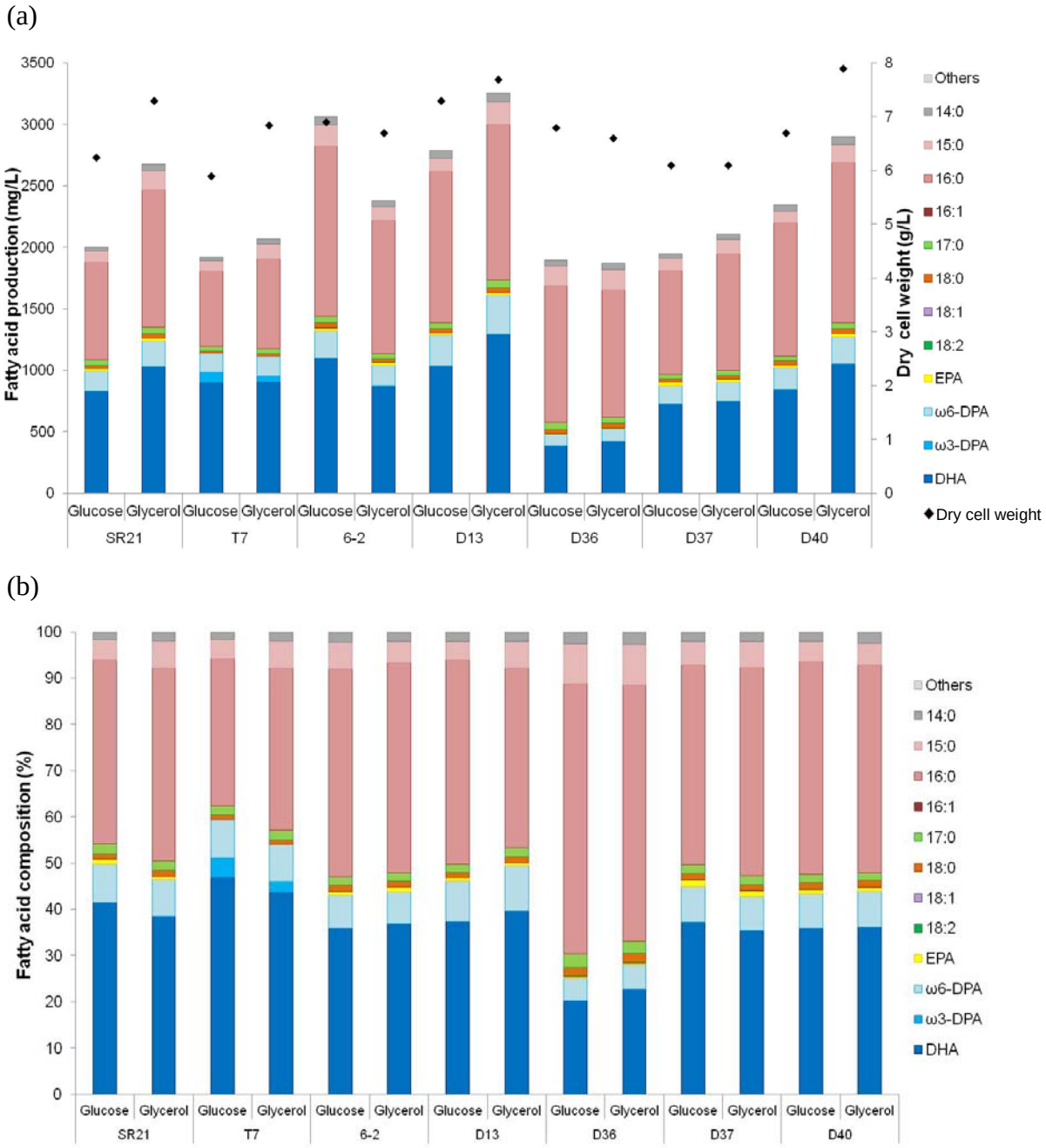


Fig 2. Comparison of (a) growth, fatty acid production and (b) fatty acid composition in strain SR21, T7, 6-2, D13, D36, D37, D40. Cultivation was conducted at 28 °C with shaking (250 rpm) for 2 days in liquid medium [2% glucose or glycerol, 1% yeast extract, 1.8% artificial sea salts]. For all abbreviations, see Fig. 1.

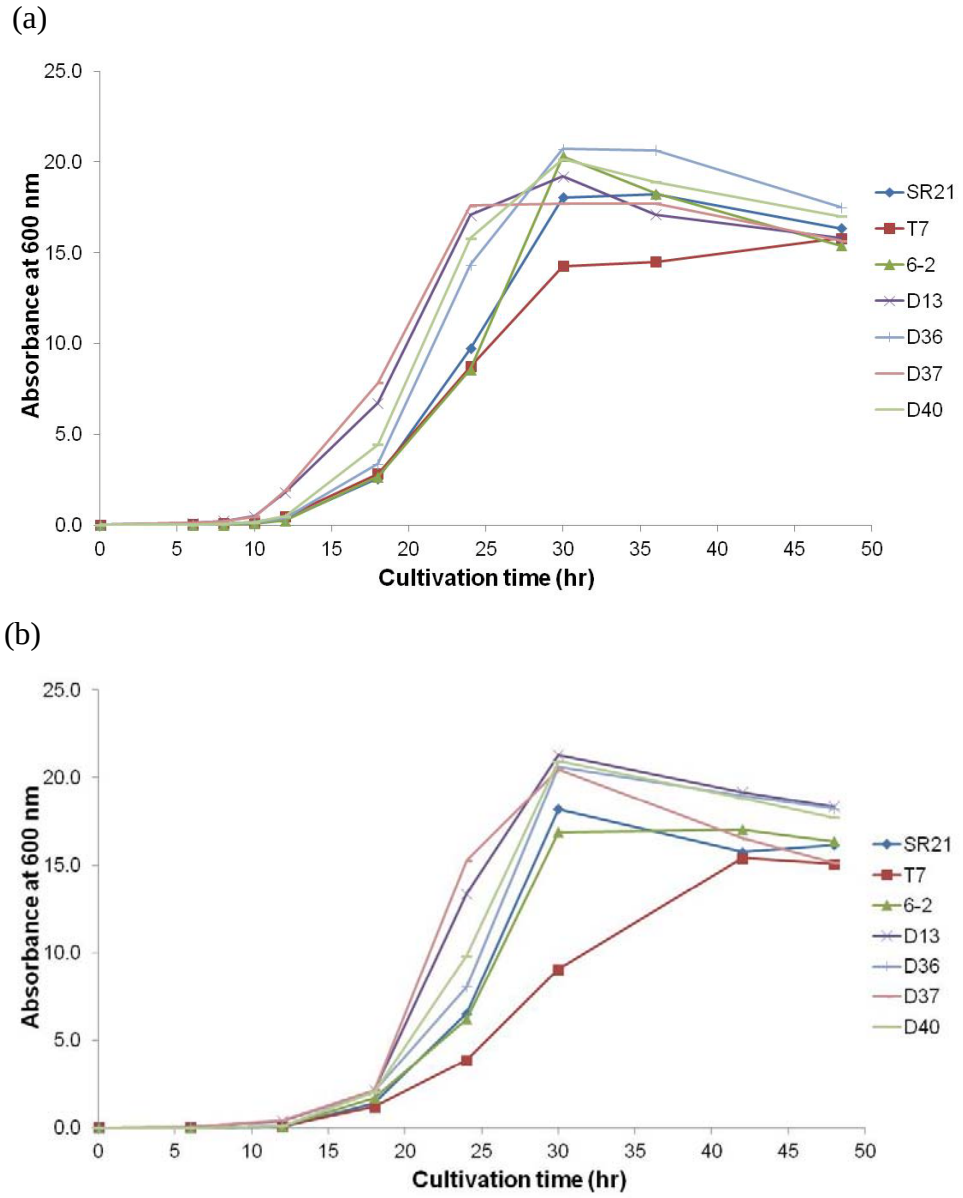
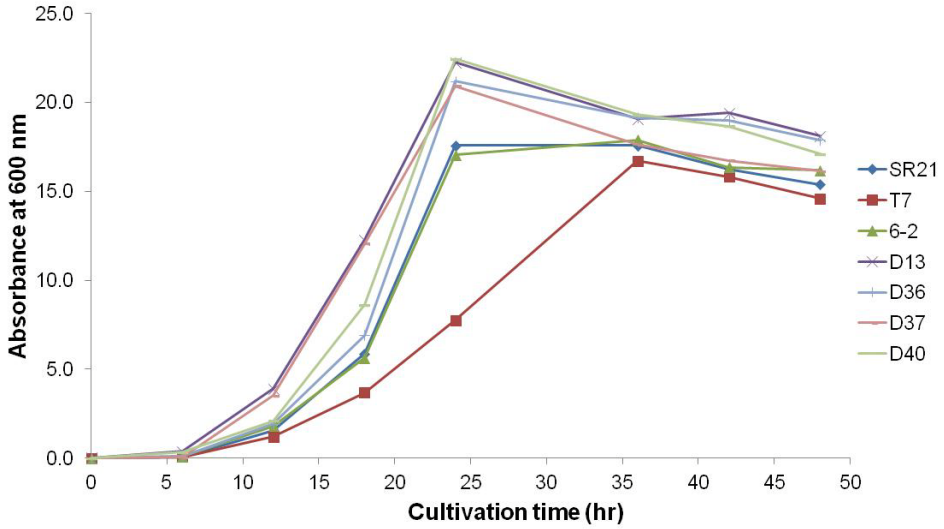


Fig 3. Growth curve of strain SR21, T7, 6-2, D13, D36, D37 and D40. Cultivation was conducted in GYA medium [2% glucose or glycerol, 1% yeast extract, 1.8% artificial sea salts] at 28 °C with shaking (250 rpm). (a) 7 strains were inoculated directly from glycerol stocked culture and (b) 7 strains were inoculated from 6 hours preculture.

(c)



(d)

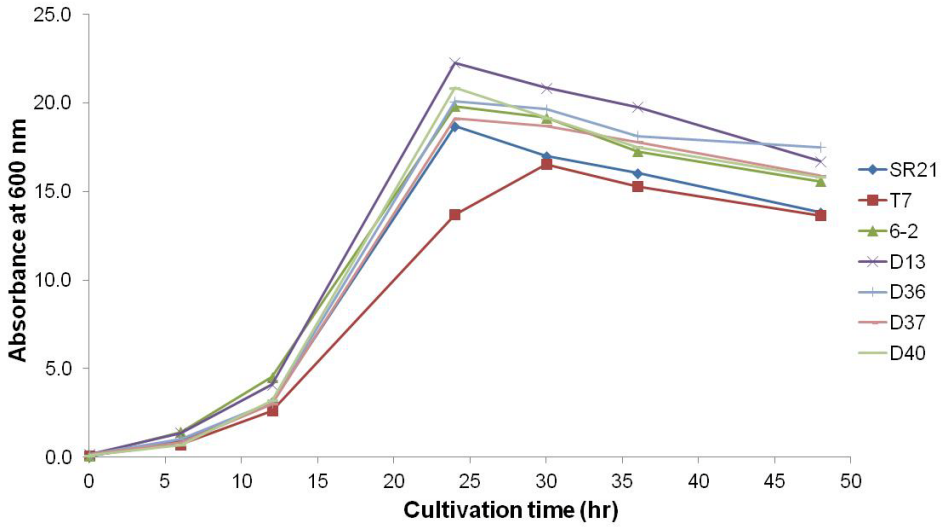


Fig 3 (cont.). Growth curve of strain SR21, T7, 6-2, D13, D36, D37 and D40. Cultivation was conducted in GYA medium [2% glucose or glycerol, 1% yeast extract, 1.8% artificial sea salts] at 28 °C with shaking (250 rpm). (c) 7 strains were inoculated from 12 hours preculture and (d) 7 strains were inoculated from 24 hours preculture.

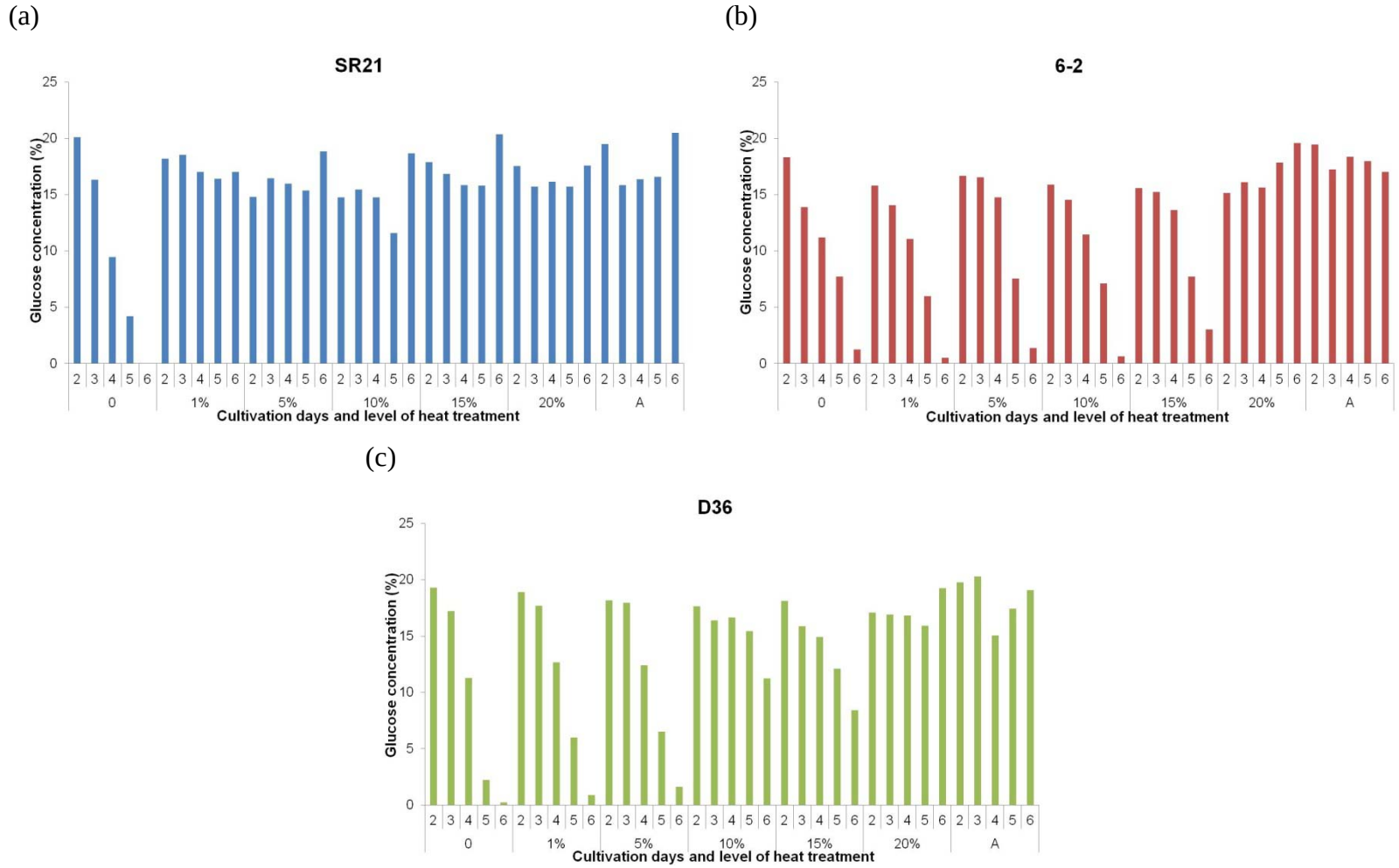


Fig 4. Residual glucose concentration (%) in FDS medium with different content of heat treatment FDS. (a) strain SR21, (b) strain 6-2 and (c) strain D36. 0, no heat treatment; A, the medium is sterilized in an autoclave at 121°C for 15 minutes.

Table 1. Compositional analysis of fermented defatted soybeans.

Type	Moisture content (%)	pH	Total nitrogen content (%)	Free glucose concentration (%)	Starch content (%)
A	48.57%	4.563	2.49 ± 0.03%	14.16 ± 0.43%	1.58 ± 0.06%
B	50.82%	4.824	2.45 ± 0.06%	9.57 ± 0.12%	1.10 ± 0.01%
C	53.45%	4.809	2.29 ± 0.10%	12.48 ± 1.59%	0.92 ± 0.03%
D	55.47%	4.493	2.28 ± 0.16%	9.97 ± 0.77%	0.80 ± 0.03%
E	57.63%	4.671	2.46 ± 0.05%	10.71 ± 0.96%	0.70 ± 0.00%
F	58.40%	4.598	2.51 ± 0.18%	10.00 ± 0.08%	0.73 ± 0.01%
G	60.03%	4.282	2.50 ± 0.13%	9.87 ± 0.26%	0.43 ± 0.02%

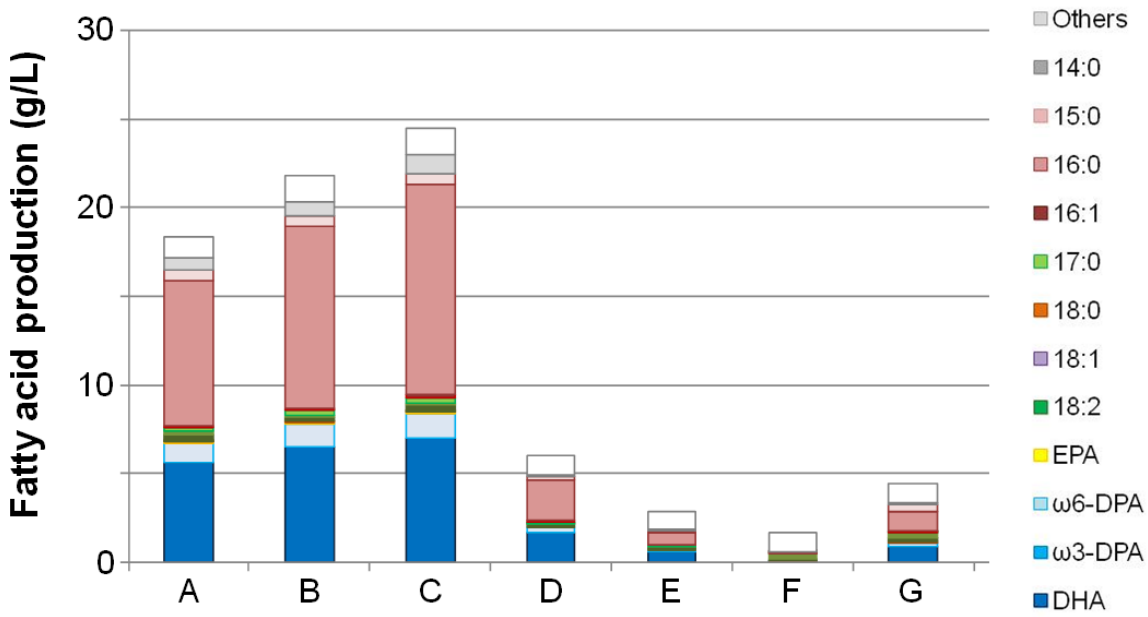


Fig 5. Fatty acid production in strain 6-2 by using fermented defatted soybeans with different moisture content in FDS medium. Cultivation was conducted at 28 °C, 250 rpm for 3 days.. Moisture content of fermented defatted soybean (A-G) were listed in Table 1. For all abbreviations, see Fig 1.

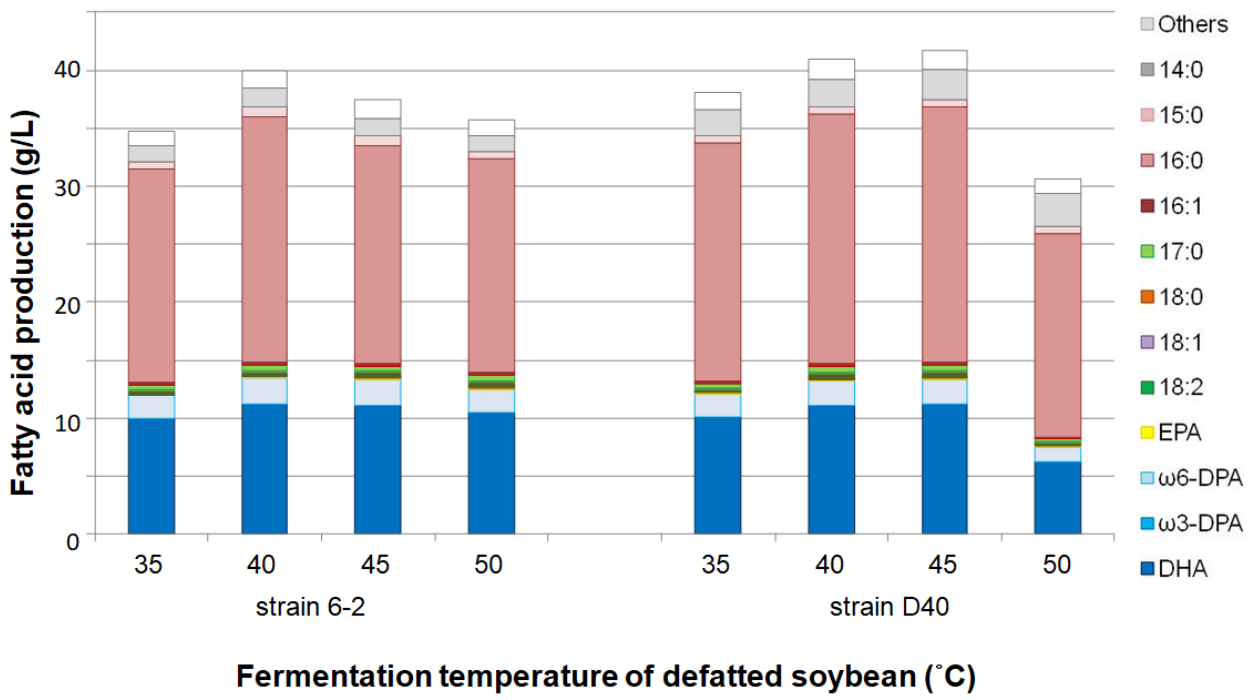


Fig 6. Fatty acid production by strain 6-2 and D40 in using FDS with different fermentation temperature.. Cultivation was conducted at 28 °C with shaking (250 rpm) for 3 days. For all abbreviations, see Fig 1.

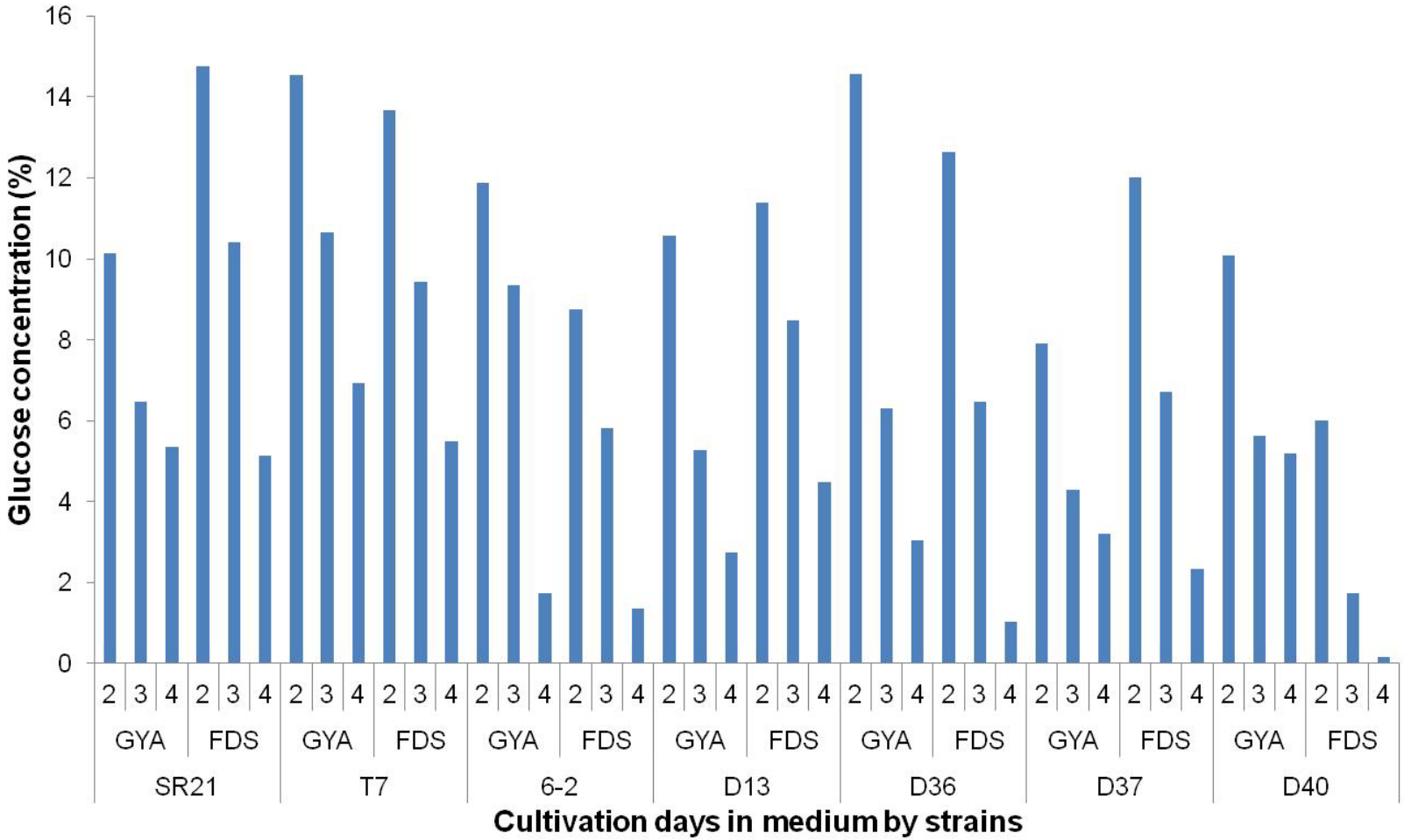
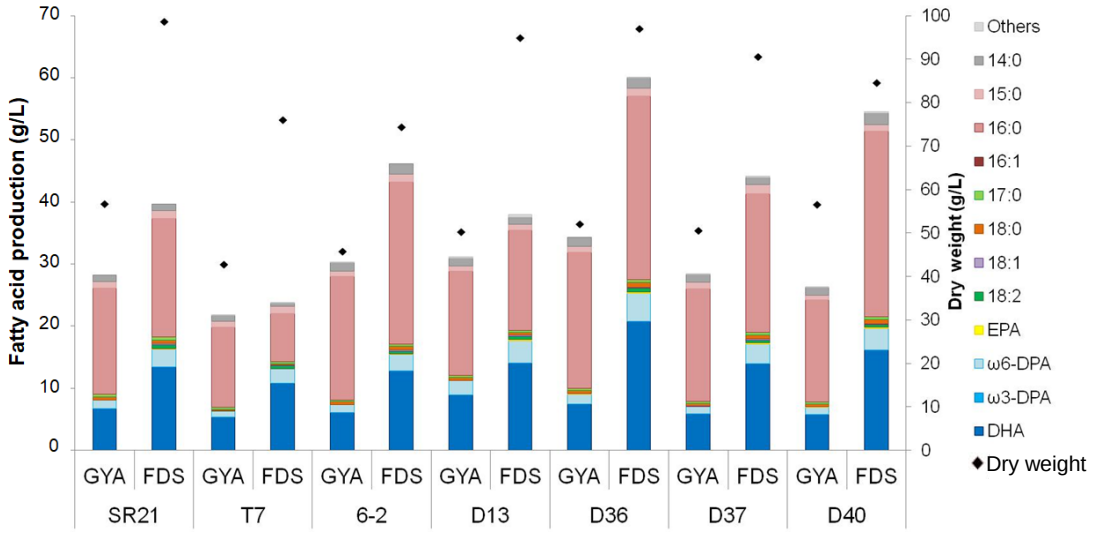


Fig 7. Residual glucose concentration (%) in GYA and FDS medium when cultivating *Schizochytrium limacinum* SR21, *Aurantiochytrium* sp. T7, *Aurantiochytrium* sp. 6-2, strain D13, D36, D37 and D40 at 28 °C with shaking (250 rpm) for 4 days. GYA medium contained 150 g/L glucose, 20 g/L yeast and 18 g/L artificial sea salt. FDS medium contained 150 g/L glucose, 200 g/L fermented defatted soybean, 3 g/L KH_2PO_4, 3 g/L Na_2SO_4, 1 g/L $MgCl_2$ and 0.3 g/L $CaCl_2$.

(a)



(b)

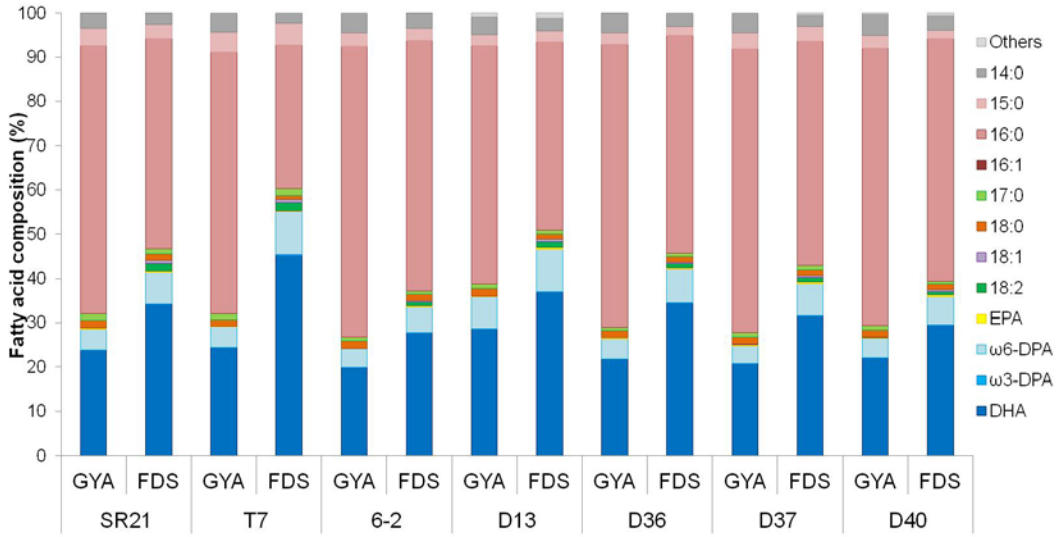


Fig 8. (a) Fatty acid production and (b) fatty acid composition when cultivating *Schizochytrium limacinum* SR21, *Aurantiochytrium* sp. T7, *Aurantiochytrium* sp. 6-2, strain D13, D36, D37 and D40 in GYA and FDS medium at 28 °C with shaking (250 rpm) for 4 days. GYA medium contained 150 g/L glucose, 20 g/L yeast and 18 g/L artificial sea salt. FDS medium contained 150 g/L glucose, 200 g/L fermented defatted soybean, 3 g/L KH_2PO_4, 3 g/L Na_2SO_4, 1 g/L $MgCl_2$ and 0.3 g/L $CaCl_2$. For all abbreviations, see Fig 1.

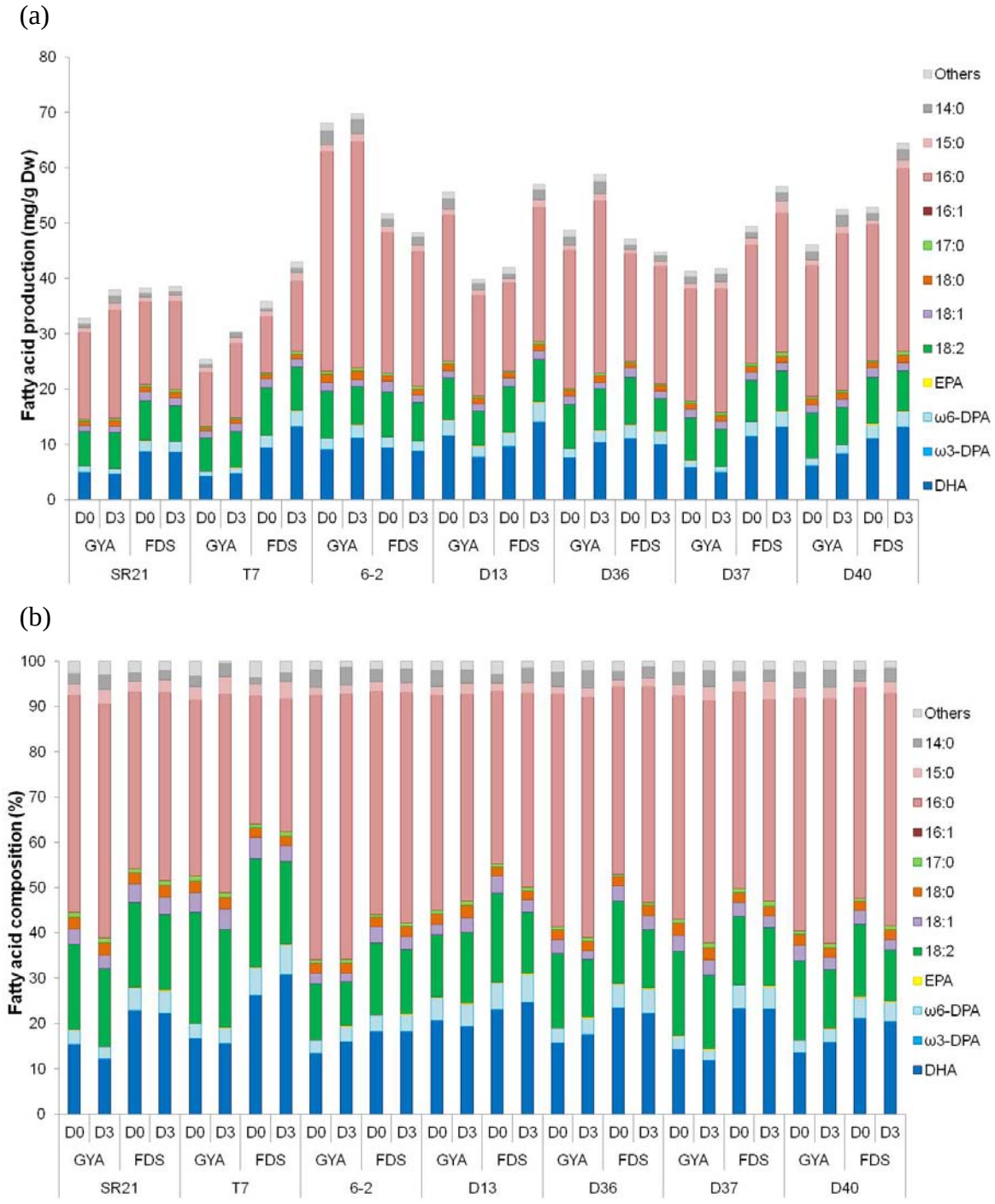


Fig 9. (a) Fatty acid production and (b) fatty acid composition in solid-state fermentation by 7 different strains. Cultivation was conducted at 28 °C under 100% humidity for 3 days. For all abbreviations, see Fig. 1.

DISCUSSION

In this study, the author screened the microalgae strains that stored in laboratory and found out that *Schizochytrium limacinum* SR21, *Aurantiochytrium* sp. T7, 6-2, strain D13, D36, D37 and D40 could produce large amounts of fatty acids (Fig 1). Many researches had revealed that these species could yield considerable amounts of polyunsaturated fatty acids (PUFA) (21-25).

As shown in Fig 2, all strains were able to use glycerol as the carbon source to yield PUFA. Glycerol is a cheaper ingredient (26), and fatty acid productivity by glycerol was competitive with that by glucose (27-30). Also, glycerol is a byproduct derived from biodiesel production (31); therefore, it would be environmentally friendly to use excess glycerol.

Microalgae strains utilize carbon sources such as glucose to synthesize PUFA through the biosynthetic pathway. In other words, by measuring the glucose consumption in the medium, fatty acid content that accumulated in cells could be inferred. Therefore, FDS medium was prepared with different consumption of heat treated FDS. The effects of heat treatment of FDS on the growth of microalgae were evaluated based on glucose consumption. As shown in Fig 4, the growth of strain SR21, 6-2 and D36 were suppressed with increasing content of heat treated FDS. Plausible reason was the products from Maillard reaction inhibited the growth of strains. The Maillard reaction was a chemical reaction between reducing sugars and amino acids, and generally, it was responsible for producing aromas compounds and distinct tastes during heat treatment of foods. One major product, hydroxymethylfurfural (HMF), derived from the Maillard reaction was known to related to antimicrobial activity in honey (32-33) and may leave a negative effect on the growth of microalgae.

Seven different moisture contents of fermented defatted soybeans were prepared in this chapter (Table 1). When the moisture content was higher in the FDS sample, the pH value became lower. The plausible reason was that higher water activity promoted the growth of microorganisms and these strains might produce organic acids to lower the pH

value. In addition, the higher titer of glucose and starch were left in sample A, which also indicated that low water activity inhibit the growth of microbes and thus limit the hydrolyzation of ingredients. The effects of fermentation temperature on preparing fermented defatted soybean were also studied. As shown in Fig 6, though FDS prepared at 35-50°C are all optimal for lipid fermentation by strain 6-2 and D40, the most suitable temperature for fermentation is considered around 40-45 °C. As the previous study suggested, amylolytic and proteolytic activity from koji decreased significantly when the temperature is lower than 40 °C, while half of the enzyme activity would be lost within 2 hours at 55 °C (34).

In Fig 7 and 8, 7 strains were inoculated to optimized FDS liquid medium and measured both glucose concentration and fatty acid production. Consequently, strain 6-2, D36 and D40 could produce 46.7 g/L (12.8 g/L), 60.7 g/L (20.8 g/L) and 55.1 g/L (16.1 g/L) of total fatty acids (DHA) in 4 days even in unsterilized FDS medium. Finally, solid-state fermentation by mixing GYA or FDS cultured medium with defatted soybean was conducted. As the result shown in Fig 9, fatty acid production further increased (approximately 14-36%) in strain D13, D37 and D40. After optimizing the culture conditions in solid-state fermentation, it might be able to develop a novel sustainable ω3-PUFA production substituted fish oil.

In conclusion, A*urantiochytrium* sp. 6-2, strain D36 and D40 exhibited the potential in using optimized fermented defatted soybean to produce PUFA including DHA even in unsterilized medium. Also, fatty acid productivity of strain D13 can further increase 36% in solid-state fermentation by mixing FDS culture medium with defatted soybean. In summarize, large amounts of PUFA produced by these strains could be applicable to develop a novel type of low cost ω3-PUFA production for fish feed.

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CONCLUSIONS

The author isolated an ω3-DPA producing microorganism strain T7 from brackish areas in Japan. Although most oleaginous microorganisms rarely accumulate ω3-DPA (<5% of total fatty acids), ω3-DPA production in *Aurantiochytrium* sp. T7 reached 164 mg/L culture broth, and the ω3-DPA content reached 23.5% of the total fatty acids when cultivated in a medium containing 2% glucose as the carbon source and 1% yeast extract as the nitrogen source, with a salinity equivalent to 50% of that of seawater and a pH in the acidic range (pH < 5.5). In addition, the accumulated amount of ω3-DPA increased in the later stage of the culture, while ω3-DPA was hardly detected in the early stage. ω3-DPA production was observed after glucose consumption, indicating that ω3-DPA is not directly biosynthesized from glucose in strain T7. In summary, *Aurantiochytrium* sp. T7 is a promising producer of ω3-DPA highly containing-lipid useful for the functional analysis of ω3-DPA whose physiological function has hardly been elucidated, and a useful strain for investigating the novel metabolic pathway of fatty acid biosynthesis.

In chapter 2, *Aurantiochytrium* sp. 6-2 was isolated from marine areas in Japan and evaluated its DHA productivity by using fermented defatted soybean as the nitrogen source. Despite *Aurantiochytrium* sp. 6-2 failed to use defatted soybean, it was cultivated in a medium containing 15% glucose as the carbon source and 20% defatted soybean fermented by koji as the nitrogen source. Common hexose and organic nitrogen source could also be used as carbon and nitrogen source, respectively, for the cultivation of strain 6-2. Also, salinity equivalent to 25-200% of that of seawater and initial pH range of 6-9 was preferable for biomass and fatty acid production. By optimizing aeration, the maximum fatty acid production could reach 47.7 g/L (DHA 12.2 g/L). In addition, fatty acid productivity could be influenced significantly by sulfate concentrations. Based on these results, *Aurantiochytrium* sp. 6-2 was revealed to be a promising strain for producing ω3-PUFAs from plant residues such as fermented defatted soybean for formulating a novel fish feed.

In chapter 3, the author evaluated the solid-state cultivation system for defatted soybean fermentation and polyunsaturated fatty acid production by DHA producing strains to reduce production cost and increase productivity. The optimal process for preparing fermented defatted soybean (FDS) was as follows. After mixing defatted soybean without heat treatment, dry rice koji and ultrapure water at a ratio of 1.0 : 1.2 : 2.4 (in weight), fermentation was carried out at 40°C for 7 days. Strain 6-2, D36 and D40 could produce 46.7 g/L (12.8 g/L), 60.7 g/L (20.8 g/L) and 55.1 g/L (16.1 g/L) of total fatty acids (DHA) in 4 days in unsterilized optimized FDS medium. Moreover, fatty acid productivity of strain D13, D37 and D40 could be further increased approximately 14-36% in 3 days in solid-state fermentation using FDS medium mixed with defatted soybean. The optimal conditions were as follows. After mixing FDS medium with defatted soybean at the ratio of 2 : 1 (volume to weight), it was cultivated at 28°C under 100% humidity for 3 days.

Owing to rare ω3-PUFA and large quantities of DHA at low cost were produced by solid-state fermentation, *Aurantiochytrium* species were useful for ω-3 fatty acids production, and this could be applicable to solve the problems in pharmaceuticals, foods and aquaculture industries.

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2) Chang-Yu Wu, Shohei Katsuya, Tomoyo Okuda, Akinori Ando, and Jun Ogawa. Utilization of fermented defatted soybean for polyunsaturated fatty acids production by a newly isolated strain, *Aurantiochytrium* sp. 6-2. In preparation.
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