Isolation of High Carotenoid-producing
*Aurantiochytrium* sp. Mutants and Improvement of Astaxanthin Productivity Using Metabolic Information

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Abstract: The marine eukaryotic microheterotroph thraustochytrid genus *Aurantiochytrium* is a known producer of polyunsaturated fatty acids, carotenoids, and squalene. We previously constructed a lipid fermentation system for *Aurantiochytrium* sp. strains using underutilized biomass, such as canned syrup and brown macroalgae. To improve the productivity, in this study, *Aurantiochytrium* sp. RH-7A and RH-7A-7 that produced high levels of carotenoids, such as astaxanthin and canthaxanthin, were isolated through chemical mutagenesis. Moreover, metabolomic analysis of the strain RH-7A revealed that oxidative stress impacts carotenoid accumulation. Accordingly, the addition of ferrous ion (Fe²⁺), as an oxidative stress compound, to the culture medium significantly enhanced the production of astaxanthin by the mutants. These approaches improved the productivity of astaxanthin up to 9.5 mg/L/day at the flask scale using not only glucose but also fructose which is the main carbon source in fermentation systems with syrup and brown algae as the raw materials.

Key words: *Aurantiochytrium* sp., carotenoid, polyunsaturated fatty acid, biomass, metabolomic analysis

1 INTRODUCTION

Carotenoids are red- or yellow-colored lipid-soluble pigments observed in various organisms, including plants, algae, fungi, and bacteria¹. They are important precursors of vitamin A, which is an essential component to maintain eye function and immunity², and play important roles in photosynthesis and removal of reactive oxygen species via the common conjugated double bonds³. Among carotenoids, xanthophylls containing oxygen atoms in their molecules, such as astaxanthin and canthaxanthin, reportedly eliminate single molecular oxygen and prevent lipid peroxidation more efficiently than carotenes, which lack oxygen atoms in their molecules⁴. Moreover, various physiological activities of xanthophylls⁵, including improvement of lipid metabolism⁶, anti-atherosclerosis⁷, antitumor⁸, and anti-inflammatory⁹, have been reported, and these activities are assumed to be derived from the antioxidative function described above. Therefore, xanthophylls are highly demanded in the fields of pharmaceutical, health food, chemical products, and cosmetics.

In order to address the massive demand, many researchers have explored supply sources of xanthophylls. In particular, various attempts, including extraction from crustaceans, chemical synthesis, and fermentative production¹⁰, have been performed for supplying astaxanthin. Currently, the major source of astaxanthin for health food and cosmetics is fermentative production using the green microalga *Haematococcus pluvialis*¹¹,¹². Additionally, *Xanthophyllomyces dendrorhous* is also used as an astaxanthin producer at the industrial scale. However, fermentative production of these microorganisms needs to be further improved in terms of productivity owing to the low prolif-
eration rate of these microorganisms. Although canthaxanthin is slightly inferior to astaxanthin in antioxidant activity, it exhibits various physiological activities similar to those of astaxanthin. Nevertheless, reports about fermentative production of canthaxanthin are limited compared with those of astaxanthin.

Previously, researchers have isolated the lipogenic and carotenogenic marine protist thaumastochytrid, genus *Aurantiochytrium*, and have examined its industrial utility. The genus *Aurantiochytrium* is a group of heterotrophic microorganisms found in the marine environment, oceans, and accumulates high value-added lipids, such as polyunsaturated fatty acids (PUFAs), as well as terpenoids, such as squalene and carotenoids. The lipid productivity of *Aurantiochytrium* sp. strains is significantly higher compared with that of other lipogenic microorganisms owing to their extremely high proliferation rate and capacity to accumulate lipid. Because microorganisms of this group are heterotrophic, stable supply of appropriate substrates are necessary for efficient fermentation. Monosaccharides, including glucose, galactose, and fructose; fatty acids; and heterotrophic, stable supply of appropriate substrates are necessary for efficient fermentation. Monosaccharides, including glucose, galactose, and fructose; fatty acids; and glycerol are able to support the growth of *Aurantiochytrium* strains. Biodiesel waste water and high-fructose corn syrup can also be utilized. We also reported the use of biomass, including *shochu* waste water, waste syrup of canned fruits, and brown macroalgae. In this research, we newly isolated two *Aurantiochytrium* sp. mutant strains exhibiting high carotenoid producing ability and improved their culture conditions based on the metabolome information for maximum astaxanthin production. We also applied the optimized culture condition for cultivation using fructose. Fructose is the main carbon source in waste syrup and is converted easily from mannitol, which is a major sugar for brown algae using a microbial catalyst.

2 EXPERIMENTAL PROCEDURES

2.1 Microorganisms, culture media, and reagents

*Aurantiochytrium* sp. strain RH-7 was isolated from Seto Inland Sea in Japan using the pine pollen baiting method. *Aurantiochytrium* sp. strain RH-7 and its mutants isolated in this research were cultivated in GPY medium (3% glucose, 0.6% hipolypepton, 0.2% yeast extract, and 2% sea salts) or FPY medium (3% fructose, 0.6% hipolypepton, 0.2% yeast extract, and 2% sea salts) at 28°C with rotary shaking at 160 rpm. For improvement of astaxanthin productivity, 100 μM FeSO₄·7H₂O was added to the GPY and FPY medium. Reagents were purchased from Nacalai Tesque (Kyoto, Japan), Sigma-Aldrich (St. Louis, MO, USA), Toyobo (Osaka, Japan), Wako Chemical (Osaka, Japan), and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2 Isolation of mutant strains of *Aurantiochytrium* sp. RH-7

*Aurantiochytrium* sp. RH-7 cells from 50 mL of culture broth were washed twice with 50 mM phosphate buffer, pH 6.8, and suspended in the same buffer. Zoospores were recovered in the supernatant after low speed centrifugation at 300 × g for 15 min at 4°C and filtration through a 5 μm filter. Zoospores (6 × 10⁷) were incubated in 150 μg/mL 1-methyl-3-nitro-1-nitrosoguanidine (NTG) at 28°C for 30 min. Next, zoospores were recovered by centrifugation at 5,000×g for 10 min at 4°C, washed twice with phosphate buffer, spread on GPY agar medium, and incubated at 28°C. Strongly pigmented orange-to-red colonies were isolated and inoculated in GPY medium at 28°C with rotary shaking at 160 rpm for four days.

2.3 Carotenoid extraction

*Aurantiochytrium* cells from 1 mL broth were washed with distilled water and lyophilized. An equivalent volume of glass beads (0.5 mm in diameter) was added to the dried cells and vigorously mixed using bead crusher μT-12 (Taisei, Aichi, Japan). One milliliter of chloroform/methanol (2:1; v/v) was added and vigorously vortexed, and the chloroform phase containing total lipids was recovered by centrifugation at 2,340 × g for 10 min at 4°C. The solvent was then evaporated under nitrogen stream.

2.4 Carotenoid analysis by high-performance liquid chromatography

Total lipid was dissolved in 1 mL of acetone:methanol (7:3; v/v) and applied onto a high-performance liquid chromatography (HPLC) system (1,260 Infinity, Agilent Technology, Santa Clara, CA, USA) equipped with a reverse phase column (COSMOSIL 5C18-MS-II, 4.6 × 150 mm, Nacalai Tesque). Acetonitrile: dichloromethane: methanol (7:2:1; v/v) was used as the mobile phase at a flow rate of 1 mL/min. Carotenoids were detected and quantified by a diode array detector primarily at 450 nm and identified by comparing their retention times with those of authentic standards.

2.5 Fatty acid analysis by gas chromatography

For fatty acid analysis, methanolysis of total lipid was performed by adding 10% methanolic hydrochloric acid, followed by incubation at 60°C for 2 h. After evaporation of the solvents, fatty acid methyl esters (FAMEs) were extracted by hexane and dissolved in the same solvent. Fatty acid composition was analyzed by a gas chromatography (GC) system (GC-2014, Shimadzu, Kyoto, Japan) equipped with a capillary column (TC-70, 0.25 mm × 30 m, GL Sciences, Tokyo, Japan). The temperature of split injector, column oven, and flame ionization detector were maintained at 270°C, 180°C, and 270°C, respectively. FAMES were identified by comparing their retention times with those of the 37-Component FAME mix (Supelco, Bellefonte,
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2.6 Extraction of intracellular metabolites

Culture broth of Aurantiochytrium strains, cultured in GPY medium for 24 h, was mixed with 60% methanol and cooled at −40°C for quenching cellular metabolism. Cells were collected by centrifugation at 2,340 × g for 5 min at −20°C and suspended in methanol: chloroform: distilled water (5:2:2; v/v). An equivalent volume of glass beads and 50 µg ribitol, as internal standard, were added to the suspension and vortexed vigorously for 30 s. Intracellular metabolites were extracted by gentle shaking of the suspension at room temperature for 30 min, recovered in the aqeous phase by centrifugation at 15,000 × g for 10 min at 20°C, and lyophilized.

2.7 Metabolome analysis

The lyophilized metabolites were oximized and trimethylsilylated as previously described (23). Trimethylsilyl derivatives were separated by a DB-5HT capillary column (0.25 mm I.D. × 30 m, Agilent technology) equipped to a GC system (7,890 A, Agilent Technology) and coupled with a time-of-flight mass spectrometry (MS; JMS-T100CGV, JEOL, Tokyo, Japan). Each compound was identified by comparison of the retention time and MS/MS spectrum with those of the standard substance. The obtained data were analyzed by MassCenter (JEOL), Metalign (31), and Aloutput (30).

3 RESULTS

3.1 Isolation of high carotenoid-producing Aurantiochytrium mutants

To improve carotenoid productivity, we attempted to isolate high carotenoid-producing Aurantiochytrium sp. strains from the highly proliferative Aurantiochytrium sp. RH-7 by chemical mutagenesis. By screening 1.8 × 10⁶ colonies treated with NTG, a strain, XS8, was found to produce carotenoids at a slightly higher content (data not shown). XS8 was further treated with NTG, and a mutant RH-7A, the colony of which was strongly orange in color, was isolated from 1.6 × 10⁵ colonies. The total carotenoid content of RH-7A was significantly higher than that of the parent strain. Canthaxanthin accounted for 44.1% of total carotenoid of RH-7A (Fig. 1A). We identified the strain RH-7A as Aurantiochytrium species by analyzing the 18 S ribosomal RNA sequence (data not shown). Furthermore, RH-7A was treated with NTG, thereby resulting in the isolation of another mutant RH-7A-7 with remarkably increased content (13.6 mg/L) of astaxanthin at the flask scale (Fig. 1A).

3.2 Metabolome analysis of Aurantiochytrium sp. RH-7 and RH-7A

To investigate the molecular mechanisms of the significant increase in carotenoids and astaxanthin productivity by the mutant strains, intracellular metabolome was analyzed by GC-MS and compared with that of the parent strain. By subjecting GC-MS data to principal component analysis (PCA), the metabolic profiles of RH-7 and RH-7A were clearly separated by the first principal component (PC1) (Fig. 2A). Substances showing strong correlation with PC1 (correlation co-efficiency of ≤ 0.065 or >0.075) were extracted by loading plot (Fig. 2B). Figure 2C shows 29 such substances that significantly decreased or increased in RH-7A. Among the substances increased in RH-7A, tricarboxylic acid (TCA) cycle-related substances, such as 2-oxoglutaric acid, malic acid, succinic acid, and citric acid, were detected. In addition, pentose phosphate pathway intermediates, such as ribulose-5-phosphate, ribose, and ribose-5-phosphate, also increased in RH-7A. Moreover, ribose-5-phosphate, myo-inositol, and sorbitol significantly increased in RH-7A. Ribose-5-phosphate is a biomarker for H. pluvialis cells exposed to oxidative stress (36), and previous reports have suggested that myo-inositol and sorbitol are defensive compounds against envi-
3.3 Effect of ferrous on carotenoid production by *Aurantiochytrium* sp. mutants

Because metabolome analysis in *Aurantiochytrium* sp. RH-7A revealed a significant increase of the biomarker to oxidative stress, the effect of ferrous ion, which induces oxidative stress by the Fenton reaction, on carotenoid production was examined. It was observed that supplementation of 100 μM FeSO₄·7H₂O to the medium did not significantly affect the amount of total carotenoid. However, astaxanthin contents in RH-7A and RH-7A-7 increased by 7.5- and 1.7-fold, respectively, whereas other carotenoids decreased (Fig. 3).

4 DISCUSSION

In this research, we selected *Aurantiochytrium* sp. RH-7 as the parent strain for isolation of high carotenoid-producing mutant strains. The proliferation rate of RH-7 was significantly higher than that of *Aurantiochytrium* sp. KH-105, which is a previously isolated carotenoid producer. Although strain RH-7 accumulated only traces of carotenoids, this strain was predicted to harbor inactivated carotenoid biosynthetic enzymes and was, therefore, expected to be activated by chemical mutagenesis. By mutagenesis of RH-7 using NTG, we isolated the high carotenoid-producing *Aurantiochytrium* sp. mutants RH-7A and RH-7A-7, producing carotenoids such as astaxanthin and canthaxanthin. The productivity of canthaxanthin by RH-7A (9.7 mg/L) and astaxanthin by RH-7A-7 was 37.9 mg/L at day 4, and the daily productivity was 9.48 mg/L/day. The highest formation rate of DHA, which is another major product of *Aurantiochytrium* sp. RH-7A-7, in the fructose medium was 105 mg/L/day at day 2.
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RH-7A-7 (13.6 mg/L) at day 4 of cultivation (Fig. 1A) were significantly higher than that by thraustochytrids reported previously (canthaxanthin, 6.5 mg/L; astaxanthin, 8.8 mg/L at day 6)\(^{40}\). Moreover, RH-7A accumulated canthaxanthin, an intermediate compound of astaxanthin biosynthesis, at a high rate (Fig. 1A). Because the productivity (2.44 mg/L/day) of canthaxanthin by RH-7A was also significantly higher than that of other microorganisms, such as Dietzia natronolimnaea (0.84 mg/L/day)\(^{41}\), Brevibacterium sp. (2.32 mg/L/day)\(^{42}\) and Gordonia jacobaea (1.91 mg/L/day)\(^{43}\), it was expected to have potential as an industrial canthaxanthin producer.

Comparison of intracellular metabolites between RH-7 and RH-7A revealed significant changes in compounds related to TCA cycle and pentose phosphate pathway (Fig. 2C). In animals, citric acid accumulated in the TCA cycle is exported to the cytosol and converted to acetyl-CoA\(^{44}\). Moreover, NADPH is produced by the conversion of malic acid to pyruvic acid by the malic enzyme\(^{45}\). Acetyl-CoA and NADPH are the starting material and coenzyme of the mevalonate pathway, respectively\(^{46}\). In the cells of Aurantiochytrium sp., isopentenyl diphosphate, a precursor of carotenoids, can be synthesized through the mevalonate pathway. Because the pentose phosphate pathway is also a major supply source of NADPH, activation of this pathway facilitates flow of the mevalonate pathway. Therefore, it is speculated that activation of the TCA cycle and pentose phosphate pathway induces activation of the mevalonate pathway and subsequent carotenoid synthesis.

Metabolome analysis of RH-7A also suggested a relationship between oxidative stress and carotenoid production. Myo-inositol and sorbitol are accumulated in response to various environmental stress factors that induce oxidative stress in the cells\(^{37, 38}\). Ribose-5-phosphate has been reported as a biomarker in the astaxanthin-producing microalgae H. pluvialis, which is exposed to oxidative stress induced by strong light, ferrous \((\text{Fe}^{2+})\) ions, acetate, and hydrogen peroxide\(^{36}\). Therefore, we examined the effect of ferrous ion on carotenoid production and confirmed that astaxanthin production by Aurantiochytrium sp. mutants increased significantly (Fig. 3). The active conversion of precursors to astaxanthin with high antioxidant activity may be due to a protective response against the oxidative stress induced by ferrous ions.

We previously constructed a lipid fermentation system for Aurantiochytrium cells using waste syrup\(^{27}\) and brown algae\(^{25}\) as culture substrates. The main component of waste syrup is fructose, which is a good carbon source for Aurantiochytrium cells. With respect to brown algae, one of the main sugar components, mannitol, was converted to fructose by the acetic acid bacterium Gluconobacter oxydans. Therefore, we investigated the ability of Aurantiochytrium sp. RH-7A-7 to assimilate fructose and produce high value-added lipids. RH-7A-7 proliferated considerably and produced astaxanthin and PUFA in both glucose- and fructose-based medium (Fig. 4). The highest astaxanthin productivity reached at 9.48 mg/L/day. This value is significantly higher than 3.60 mg/L/day reported in our previous...
research\textsuperscript{[25]} and 5.36 mg/L/day obtained from the astaxanthin-producing yeast \textit{X. dendrorhous}\textsuperscript{[47]}.

Astaxanthin is accumulated as a fatty acid ester in \textit{H. pluvialis}, whereas \textit{Aurantiochytrium} sp. and \textit{X. dendrorhous} accumulate the free form of astaxanthin\textsuperscript{[19, 48]}. A recent report indicated that the activity of free astaxanthin is comparable to that of the physiological activity of esterified astaxanthin in mammals with dyslipidemia\textsuperscript{[49]}. Therefore, the strain RH-7A-7 has a potential to be a new source of astaxanthin. In addition, the highest DHA productivity (105 mg/L/day at day 2) suggests that \textit{Aurantiochytrium} sp. RH-7A-7 can be utilized as producer of PUFA and astaxanthin in a fructose-based medium using waste syrup and brown macroalgae as sustainable raw materials. The strain RH-7A-7 also contains odd-chain fatty acids such as C15:0 and C17:0 (Fig. 4C). Previous reports have indicated accumulation of odd-chain fatty acids in \textit{A. limacinum} SR21 because of the weak catabolic activity of propionyl-CoA, which is expected to be the starting material of these fatty acids\textsuperscript{[50]}. Because the parent strain RH-7 was closely related to \textit{A. limacinum} SR21, according to 18 S ribosomal RNA sequence comparison (data not shown), it was presumed that strain RH-7A-7 accumulated odd chain fatty acids owing to the same reason (Fig. 4C).

5 CONCLUSION

We isolated high carotenoid-producing \textit{Aurantiochytrium} sp. mutant strains, producing carotenoids such as astaxanthin and canthaxanthin. Metabolome analysis suggested a relationship between oxidative stress and carotenoid production. Supplementation of ferrous ions as oxidative stress inducer significantly increased astaxanthin production. The mutant strain RH-7A-7 proliferated considerably and produced astaxanthin and DHA in both glucose- and fructose-based medium. The productivities of carotenoids were significantly higher than those reported in our previous research and other studies.

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