論文の要旨
（Thesis Summary）

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論文題目 Physiological characterization of thraustochytrids in acetate assimilation and development of molecular breeding platforms for efficient lipid production
(効率的脂質生産に向けた Thraustochytrids の酢酸代謝特性の解析及び分子育種基盤の開発)

Chapter 1. Background, objectives, and significance of the study

Marine biomass has attracted much attention in the fields of renewable bioenergy and value-added industrial and biological materials due to the promised sustainability of its use. Specifically, thraustochytrids, a group of marine protists, are capable of producing lipids, such as carotenoids, polyunsaturated fatty acids (PUFA), and hydrocarbons, for various biotechnological applications towards food, medicines, chemicals, and biofuels.

Thraustochytrids is heterotrophic which makes it ideal for production of a wide variety of microalgal metabolites at all scales. The most commonly used carbon source is glucose, with far higher rates of growth and lipid productivity compared with other substrates; however, problems arise at a large-scale point of view since the use of glucose is expensive and may only be suitable for production of high-value products such as carotenoids and PUFA. Hence it is important to find a cheaper sustainable option for large-scale cultivation to expand the application range. In search for a cheaper alternative substrate, an interest was developed in using acetate as a carbon source because of the ease of production from various biomass or waste gas containing CO_2 by the conversion of acetogens.

In this study, the capability of *Aurantiochytrium* strains of thraustochyrids to assimilate acetate by characterizing its growth, lipid productivity, and metabolism was investigated. The concept of metabolic engineering through molecular breeding with the final objective of producing lipids efficiently using this substrate was also tested.

Chapter 2. Metabolite profile analysis of *Aurantiochytrium limacinum* SR21 grown on acetate-based medium for lipid fermentation

Different species differ in their ability to assimilate nutrients; hence, it is important to explore the use of different substrates for strains of *Aurantiochytrium* sp. The laboratory had studied the use of food waste including *shochu* wastewater and waste syrup of canned fruits as substrate for *Aurantiochytrium* sp. KH105, as well as the use of macroalgae where the ability of particular strains to assimilate some organic acids such as acetate was found.

In this study, the ability of acetate, which can be easily generated from various resources by acetogenic microorganisms, as a substrate of *A. limacinum* SR21 was examined. Flask-scale analysis indicated that specific growth rates (μ) of the strain SR21 grown in 3% acetate- or glucose-based medium were 0.55 and 0.98 h^{-1}, respectively. The maximum yield of total fatty acid in acetate medium was 4.8 g/L at 48 h, and 6.8 g/L at 30 h in glucose medium, indicating that acetate has potential as substrate.

Metabolome analysis was performed to comprehensively elucidate characteristic metabolic fluctuations caused by acetate assimilation and identify targets to improve the fatty acid productivity from acetate. Mevalonate pathway was found to be activated in acetate cultivation which additionally competes with acetyl-CoA as starting material of fatty acid synthesis. It was found that the use of glyoxylate cycle, which bypasses

release of energy molecules such as NADH and GTP, and the inhibition of utilization of compounds from TCA cycle for anabolic reactions, may cause the slow growth in acetate which also affects lipid productivity. The activity of the pentose phosphate pathway was found to be weak in acetate cultivation, thus NADPH, which is essential coenzyme for fatty acid production, was mainly produced in malate-pyruvate cycle, and the activation of this cycle was predicted to be the target for improvement of fatty acid productivity.

Chapter 3. Characterization of genes related to fatty acid degradation in *A. limacinum* SR21 for subsequent improvement of triglyceride productivity by genome editing

Over the past few decades, there has been a great deal of effort to manipulate strains in order to produce novel lipids, especially for industrial applications. In addition to expanding the applications of lipids produced by genus *Aurantiochytrium*, new strains that will have the ability to produce lipids using acetate as carbon source by employing a genome editing technique as a molecular breeding tool were isolated.

First, the improvement of the insertion efficiency of donor DNA containing antibiotic resistance gene and homologous arm at target locus by using CRISPR-Cas9 system was considered. A donor DNA was introduced into the *crtIBY* gene administering carotenoid production of a carotenoid-producing strain *Aurantiochytrium* sp. RH-7A, and a strain showing both antibiotic resistance and a carotenoid-less white colony was selected as a strain with site-specific knock-in. As a result, the ratio of the number of white colonies to the total number of antibiotic-resistant colonies was significantly improved by the presence of CRISPR-Cas9 targeting *crtIBY*.

From previous metabolomic studies, assimilation of acetate activated the mevalonate pathway and caused shortage in NADPH, all these leading to decrease in fatty acid production. Additionally, reports from animal cells indicate the activation of β-oxidation in acetate assimilation condition by activation of AMP kinase. Thus, the functions of enzymes associated with β-oxidation, such as acyl-CoA oxidases (*ALAcox1, ALAcox2,* and *ALAcox3)*, 3-hydroxyacyl-CoA dehydrogenase (*ALHadh1*), and carbon catabolite-depressing protein kinase (*ALSnf1*) were studied by CRISPR-Cas9 mediated gene disruption and evaluation of the effect on fatty acid productivity in the mutant strains. As the results, the disruption of *ALAcox1* and *ALAcox2* which were related to peroxisomal β-oxidation caused significant increase in cellular total fatty acid content, with values of 43% and 50%, respectively. In terms of acetate assimilation, the disruption of *ALSnf1* caused drastic reduction of cell growth in acetate media, whereas same proliferative properties as the wild type strain was observed for the other mutants. These findings contribute to the improvement of lipid productivity of *Aurantiochytrium* sp.

Chapter 4. Conclusion and Broader Applications

In this study, the use of acetate as a cheaper renewable source for lipid production by thraustochytrids was proposed and investigated. The physiological characterization of this strain has led to identification of key enzymes that may increase the conversion efficiency from acetate to lipid. With the advancement of metabolic engineering, using genetic engineering, new strains could be developed to get the desired product, whether increasing lipid productivity or targeting other compounds of interest.

By selecting the type of acetogenic microorganism with different assimilability, extremely diverse biomass including lignocellulose and CO_2 gas can be used as a raw material for lipid production. As a result, not only other applications aside from high-value products can be pursued, this study also contributed to the development of multiple streams of process (gas-to-lipids and biomass-to-lipids) that utilize renewable substrates for energy and wide ranged-value products.