Metabolite Profile Analysis of *Aurantiochytrium limacinum* SR21 Grown on Acetate-based Medium for Lipid Fermentation

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Abstract: Thraustochytrids, a group of marine protists, are continuously gaining attention due to their capability in producing lipids for various biotechnological applications towards foods, medicines, chemicals, and biofuels. Although various substrates, predominantly glucose, have been used as carbon source for this microalga, it is desirable to adopt cheaper and more diversified substrate to expand their application range. In this study, we aimed to examine the ability of acetate, which can be easily generated from various resources by acetogenic microorganisms, as a substrate of *Aurantiochytrium limacinum* SR21. As a result of flask-scale analysis, specific growth rates ($\mu$) 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 while that in glucose medium was 6.8 g/L at 30 h, 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. 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 has an effect also in lipid productivity. The activity of the pentose phosphate pathway was found to be weak in acetate cultivation, thus NADPH was mainly produced in malate-pyruvate cycle. Lastly, mevalonate pathway was found to be activated in acetate cultivation which additionally competes with acetyl-CoA as starting material of fatty acid synthesis.

Key words: acetate, *Aurantiochytrium*, lipid production, metabolomics

1 Introductions

Thraustochytrids are a group of marine protists that have continuously gained attention due to their biotechnological importance in the production of lipids that can be used for fuel or high valued products. *Aurantiochytrium limacinum* SR21, formerly known as *Schizochytrium limacinum*, is a species that originated from the mangroves of Yap Island and is considered as a model organism for commercial production of DHA. Growth of this organism is heterotrophic which makes it ideal for production of a wide variety of microalgal metabolites at all scales, from bench experiments to industrial scale. The most commonly used carbon source for the heterotrophic cultures of microalga is glucose with far higher rates of growth and lipid productivity compared with other substrates.

However, problem begins to arise at large-scale point of view since use of glucose is expensive and may just be suitable for production of high-value products such as carotenoids and polyunsaturated fatty acids (PUFA). This problem will limit the range of application for these microalgae. Some researches explored the use of different substrates for strains of *Aurantiochytrium* sp. Our research group also indicated the way to use the food waste including *shochu* waste water and waste syrup of canned fruits as substrate for *Aurantiochytrium* sp. KH105. Additionally, we also focused on use of brown seaweeds that are abundantly present in coast area. However, *Aurantiochytrium* sp. KH105 cannot utilize the main saccharides of brown seaweeds directly, thus a two-stage fermentation system, including conversion of brown seaweed sugars to
simpler sugars by microbial catalyst, and lipid production by *Aurantiochytrium* sp. was developed\textsuperscript{12,19}.

In those research, we found the ability of particular strains of the genus *Aurantiochytrium* to assimilate some organic acids. Among such organic acids, an interest was developed in using acetate as a carbon source because of the ease of production from various substrates. As seen in methane fermentation systems\textsuperscript{13}, various biomass\textsuperscript{14,15} or syngas\textsuperscript{16} can be converted to acetate by acetogens, such as *Acetobacterium* sp.\textsuperscript{17}, *Moorella* sp.\textsuperscript{18,19}, and *Clostridium* sp.\textsuperscript{20}. Through this, the use of acetate by *A. limacinum* offers a more diversified range of starting materials as substrates. A study of Perez-Garcia et al.\textsuperscript{5} reviewed the metabolism of acetate by photosynthetic microalgaee under dark, aerobic conditions, however, few studies on the heterotrophic growth of non-photosynthetic microalgae with use of acetate as carbon source have been reported\textsuperscript{20}, and no studies so far about metabolism of acetate for lipid fermentation has ever been reported on thraustochytrids including *Aurantiochytrium* sp.

In this study, the growth and fatty acid productivity of *A. limacinum* SR21 in acetate-based medium was assessed for biotechnological application. Additionally, we analyzed the metabolic profile of strain SR21 assimilating acetate to comprehensively elucidate characteristic metabolic profile caused by acetate assimilation and explore the targets for improvement of lipid productivity of strain SR21 in acetate-based medium.

### 2 Experimental Procedures

#### 2.1 Strain, media, culture conditions, and reagents

*A. limacinum* SR21 was cultivated in GPY medium (3% glucose, 0.6% hipholyptene, 0.2% yeast extract, 50% artificial seawater) or APY medium (3% acetate, 0.6% hipholyptene, 0.2% yeast extract, 50% artificial seawater) at 28°C, with rotary shaking at 180 rpm. Reagents were purchased from Nacalai Tesque (Kyoto, Japan), Wako Chemical (Osaka, Japan), Sigma-Aldrich (St. Louis, MO, USA), and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

#### 2.2 Analysis of dry cell weight, fatty acids and residual substrate concentration

The biomass was quantified by measuring the dry cell weight. The cell was collected from culture by centrifugation (5 min, 12,000 g, 4°C) after which the cells were freeze-dried for 12 h. Fatty acid analysis was carried out according to method of Watanabe et al.\textsuperscript{21} with some modification. Total lipid was extracted by crushing the freeze-dried cells using glass beads (0.5 mm I.D.) and 1 mL chloroform/methanol (2:1, v/v) and mixed vigorously with beads crusher (μT-12, Taitec, Aichi, Japan). Arachidic acid was added as internal standard for quantification of fatty acids. The solution was transferred into a fresh tube, added with 0.5 mL distilled water, vortexed, and centrifuged. Chloroform layer was transferred into a fresh tube, and 10% methanolic hydrochloric acid was added for methanalysis of total lipid. After incubating at 60°C for 2 h, samples were dried under N\textsubscript{2} stream and fatty acid methyl esters were extracted with hexane. Fatty acid composition was analyzed using gas chromatography (GC-2014; Shimadzu, Kyoto, Japan) equipped with a capillary column (TC-70, 0.25 mm × 30 m, GL Science, Tokyo, Japan). The temperature of column oven, split injector (split ratio at 1:20), and flame ionization detector were 180°C, 210°C, and 270°C, respectively. The residual acetate concentration was analyzed using high performance liquid chromatography (HPLC) system (1260 Infinity, Agilent Technology, Santa Clara, CA, USA) equipped with an ion-exclusion column (RSpak KC-811, 6 μm, 8.0 × 300 mm, Showa Denko, Tokyo, Japan). 0.1% H\textsubscript{2}PO\textsubscript{4} was used as mobile phase at flow rate of 0.7 mL/min. For determining residual glucose concentration, Glucose (GO) assay kit (Sigma-Aldrich) was used.

#### 2.3 Cell quenching and extraction of intracellular metabolites

Sample was obtained as representative of the three phases of growth: early log, peak log, and stationary phases. The collected cells were quenched using chilled 60% methanol to stop all metabolic processes instantaneously and were centrifuged to separate from the supernatant. Extraction of intracellular metabolites was carried out in three extraction protocols using acetonitrile/methanol/water (2:2:1, v/v)\textsuperscript{22}, chloroform/methanol/water (5:2:2, v/v)\textsuperscript{23}, and pure methanol\textsuperscript{24}. Fifty micromgram of ribitol was added as internal standard for quantification of each metabolite. Samples were concentrated as needed by use of a solid-phase extraction cartridge (Strata-X-AW, Phenomenex, Torrance, CA, USA) or lyophilization.

#### 2.4 Metabolome analysis by mass spectrometry

Analysis of metabolites were performed using LC/ESI-MS and GC/El-MS. For LC/ESI-MS, separation was carried out on a SeQuant ZIC-HILIC column (150 mm × 4.6 mm, 5 μm, Merck) in HPLC connected to LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA). Mobile phases A and B, as well as the run gradient, were adapted from Li et al.\textsuperscript{24}, using a flow rate of 0.5 mL/min. The regular ESI ion source was operated in positive-ion mode within a mass range of 70-1200 m/z and negative-ion mode within a mass range of 65-700 m/z. Analytical parameters were as follows: source voltage, 2.6 kV; capillary voltage, +/− 30 V; tube lens voltage, +/− 80 V; capillary temperature, 380°C; desolvation temperature, 25°C; sheath gas flow rate, 70 arb; and aux gas flow rate, 20 arb. For GC/El-MS analysis, samples were first derivatized according to method of...
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Bando et al.\textsuperscript{24}. Separation was carried out by a DB-5HT capillary column (0.25 mm I.D. × 30 m, Agilent Technology) in GC system (7890A, Agilent Technology) connected to a mass spectrometry (JMS-T100CGV, JEOL, Tokyo, Japan). Helium was used as carrier gas at constant flow rate of 1.12 mL/min. The column temperature was held for 2 min delay at 80°C and then increased to 330°C at a rate of 15°C/min, where it was held for 6 min. Ionizing voltage was 70 V and ion chamber temperature was 280°C. For GC/EI-MS, selected ion chromatograms for specific metabolites were extracted by use of Xcalibur software (Thermo Fisher Scientific). For LC/ESI-MS, data obtained were analyzed by MassCenter (JEOL), Metalign\textsuperscript{25}, Aloutput\textsuperscript{26}, MetaboAnalyst 4.0\textsuperscript{27}, R 3.5.1\textsuperscript{28}, and R commander\textsuperscript{29}.

3 Results

3.1 Growth and fatty acid production of Aurantiochytrium limacinum SR21 on acetate-based media

To test if acetate can support growth and lipid productivity, A. limacinum SR21 was cultivated in media containing different concentrations (1-6%) of acetate (APY) in comparison to glucose (GPY) for 72 h. As shown in Fig. 1, the strain SR21 grown in GPY media in a glucose concentration-dependent manner while cells grown in APY media showed an optimum range of acetate concentration at 3 and 4% with dry cell weights of 11.2 g/L and 13.4 g/L, respectively. Those are comparable to the cell growth in GPY media at the equivalent concentrations of glucose giving 10.7 g/L and 13.8 g/L, respectively. In terms of fatty acid production, total fatty acid (TFA) was highest at 4% APY with 3.2 g/L whereas that of 4% GPY was 6.3 g/L. To avoid the cell growth inhibition at higher concentration of acetate, media containing 3% acetate or glucose were used for further analysis.

The growth pattern with four stages, namely lag, log, stationary, and death phases, in APY and GPY media were similar but time points of those stages were shifted as shown in Fig. 2. In 3% APY medium (Fig. 2A), a lag phase was observed at 0-12 h, followed by a log phase until 42 h after which the organism entered a stationary phase up to 78 h. Production of fatty acid was evident as early as 18 h but reached maximum production around 48 h with 4.8 g/L, however, after 72 h, the amounts of fatty acid decreased to 3.3 g/L. The amount of residual acetate from the medium corresponded with the growth. Upon reaching maximum growth at 42 h, acetate was also completely depleted. The strain SR21 grew significantly faster in 3% GPY medium as seen in Fig. 2B. Lag phase was observed at 0-6 h, followed by log phase until 24 h after which the organism entered the stationary phase up to 78 h. The residual glucose was totally depleted at 24 h. The highest TFA production in GPY medium was at 30 h with 6.8 g/L which is higher than that (4.8 g/L) from 3% APY at 48 h. The values of A. limacinum growth kinetics in both media were computed, and it was found that in glucose, the organism has a growth yield of 1.48 g-cell/g-glucose, specific growth rate of 0.98 h\textsuperscript{-1}, and TFA yield of 0.06 g/g-glucose while in acetate, the organism has a growth yield of 0.23 g-cell/g-acetate, specific growth rate of 0.55 h\textsuperscript{-1}, and TFA yield of 0.01 g/g-acetate.

3.2 Principal component analysis of metabolite

A total of 1,737 and 1,663 peaks were detected in GC/EI-MS. To investigate the clues explaining the variations in growth rates and fatty acid production of strain SR21 as-

![Fig. 1 Comparison on the growth and fatty acid production of A. limacinum SR21 in different concentration of APY and GPY media at 28°C for 72 h. DCW, dry cell weight.](image)

![Fig. 2 Time-course analysis of cell growth and fatty acid production of A. limacinum SR21 in APY (A) and GPY (B) media. DCW, dry cell weight; ACE CONC, residual acetate concentration; GLU CONC, residual glucose concentration.](image)
simulating different substrates, a dataset of intracellular metabolites obtained by GC/EI-MS was subjected to the principal component analysis (PCA). Two synthesized variables, the principal component 1 (PC1) and the principal component 2 (PC2) were generated from multivariate data of metabolites, and PC1 and PC2 covered 34.4% and 18.2% of the total detected metabolites pools variance, respectively. As shown in Fig. 3A, clusters of different time points in GPY medium are clearly separated, while clusters in APY medium are gathered nearby. Therefore, the fluctu-
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The variations of measured metabolites were further viewed in a heat map of clustering analysis. As shown in Fig. 4, these metabolites could be classified into three clusters. Cluster 1 included the metabolites that exhibited higher levels, especially during the log phase of growth, in APY medium. These were tricarboxylic acid (TCA) cycle or glyoxylate cycle metabolites such as isocitrate, citrate, oxaloacetate, malate, and fumarate. Cluster 2 showed the metabolites that exhibited higher levels during the early log phase of growth in GPY medium which included coenzymes and some amino acids that may play a role in energy production and have implications in the growth rate of SR21. Cluster 3 showed the metabolites that exhibited higher levels in all stages of growth in GPY medium, and in turn exhibited low levels in all stages of growth in APY medium. These included metabolites from the pentose phosphate pathway and glycolytic pathway, as well as majority of the amino acids detected.

3.4 Pathway analysis

Apart from performing multivariate data analysis to examine the global variations between different experimental conditions, the change of individual compound was also analyzed in greater detail using data from LC/ESI-MS. The amounts of the metabolites are indicated with relative peak intensities computed over peak intensity of internal standard. Figure 5 shows the metabolic fluctuations in the central pathways of strain SR21 assimilating glucose and acetate for the three stages of growth. Overall, compounds related to glycolysis and pentose phosphate pathway were higher in glucose assimilation while compounds related to TCA cycle were higher in acetate assimilation. The level of acetyl-CoA, which is the start substrate of fatty acid biosynthesis, did not differ between assimilation of different carbon sources, on the other hand, the level of NADPH, which is an essential coenzyme of fatty acid biosynthesis, was significantly lower in acetate assimilation.

4 Discussion

In this research, we focused on the potential of acetate as carbon source for fatty acid production by A. limacinum SR21. The growth of the strain was observed in acetate-based media at particular concentrations of acetate and certain pH range, supporting the fact that acetate can be used as alternative carbon source to glucose. However, under the conditions tested, specific growth rates and fatty acid productivity were lower in acetate assimilation compared to glucose assimilation. These results suggested the necessity of optimization of culture condition and molecular breeding of the strain to improve lipid productivity from acetate. The variations in growth and fatty acid production indicated that there were significant differences in cellular behaviors under the two substrate cultivations, however there has been no report of the metabolic profile of this microorganism assimilating acetate as far as we know. Therefore, metabolome analysis was carried out to reveal the intracellular metabolism of strain SR21 and to find targets in improving fatty acid productivity from a specific substrate.

When a multivariate analysis, PCA, was applied to the metabolites analyzed by GC-MS, score plots showed the different metabolic profiles of the strain SR21 assimilating different carbon sources (Fig. 3A). Moreover, the loading
plots indicated that steroid metabolites were increased in SR21 cells assimilating acetate (Fig. 3B; Table 1). These metabolites could be synthesized through the mevalonate pathway, and this pathway uses acetyl-CoA as a starting material. Since saturated and polunsaturated fatty acids were synthesized also from acetyl-CoA by fatty acid synthase and polunsaturated fatty acid synthase, respectively, in *Aurantiochytrium* sp., the mevalonate pathway activated by acetate assimilation might compete with fatty acid synthesis. Thus, the suppression of mevalonate pathway might be leads to the improvement of fatty acid productivity.

Heat map and pathway analyses showed the significant differences of the central metabolism of strain SR21 assimilating different carbon sources (Fig. 4). Compounds related to TCA cycle except α-ketoglutarate were significantly higher in acetate assimilation. This phenomenon is presumed to be the consumption of acetate by activated TCA cycle to relieve the acid stress. On the other hand, absence of α-ketoglutarate in acetate assimilation may imply the use of an alternate pathway, the glyoxylate cycle. The presence of the glyoxylate cycle has been widely known for the assimilation of non-fermentable carbon sources like acetate and ethanol in a wide variety of organisms including *Chlamydomonas* sp. and *Chlorella* sp. When acetate enters into the cell, it gets converted to acetyl-CoA and, unlike acetyl-CoA from glucose assimilation, it does not go through the TCA cycle but the glyoxylate cycle. The glyoxylate cycle has reactions in common with the TCA cycle, but the combined action of its two key enzymes, isocitrate lyase and malate synthase, replaces the steps from isocitrate to malate to avoid loss of carbon as carbon.
dioxide. As seen in Fig. 5, the presence of glyoxylate and the absence of \( \alpha \)-ketoglutarate was observed in acetate cultures. When this happens, not only was loss of carbon bypassed, but the release of energy molecules such as NADH and GTP are also bypassed\(^{36}\). This may explain the lower production of coenzymes in acetate assimilation that could affect growth and lipid production.

Among compounds related to TCA cycle and glyoxylate cycle, citrate plays a major role in fatty acid synthesis. Citrate highly accumulated in mitochondria is transported to cytosol and converted to acetyl-CoA which is substrate for fatty acid synthesis\(^{30}\). However, as seen in Fig. 5, acetyl-CoA levels observed in acetate assimilation was not significantly different from that in glucose assimilation. In addition, malate and oxaloacetate, which are sources for anabolic reactions such as gluconeogenesis or amino acid synthesis, tend to accumulate rather than being utilized for cell growth in acetate assimilation. There seem to be a high amount of malate, oxaloacetate, and pyruvate at early stages of growth in acetate assimilation, however the levels of amino acids synthesized from those tricarboxylates were significantly lower in acetate assimilation. From these results, it is presumed that acetate assimilation inhibits the transportation or conversion of TCA cycle related-compounds.

While glyoxylate cycle was activated, the levels of compounds related to pentose phosphate pathway were significantly lower in acetate assimilation (Fig. 5). It is possible that reduced pentose phosphate pathway by acetate assimilation affects fatty acid synthesis. This is because that NADPH is essential cofactor used in the biosynthesis of lipids such as fatty acids and steroids\(^{27}\). NADPH could be produced in two pathways, one is in the pentose phosphate pathway which uses glucose-6-phosphate as the starting material, and the other is in malate-pyruvate cycle\(^{35}\), from the conversion of malate to pyruvate by malic enzyme. It can be observed that NADPH was significantly higher in glucose than in acetate and this was due to the combined activity of the two pathways, while cells grown in acetate must only synthesize NADPH from malate-pyruvate conversion.

Metabolome analysis of the strain SR21 showed some clues to improve the fatty acid productivity using acetate as substrate. Use of glyoxylate cycle is essential for utilizing acetate as a solo carbon source, however simultaneous activation of TCA cycle and glyoxylate cycle seems to be desirable to facilitate the production of reduced cofactor NADH thereby improving energy production for the strain’s growth. Since acetate assimilation may inhibits the utilization of compounds of TCA cycle for anabolic reaction, it is presumed that overexpression of enzymes to transport or convert them is effective. As pentose phosphate pathway is reduced because of the shortage of start substrate, the reaction by malic enzyme, which is another major reaction to produce NADPH, should be enhanced to improve the fatty acid productivity. Suppression of mevalonate pathway is also considered important because active consumption of acetyl-CoA by activated mevalonate pathway may inhibit the fatty acid production.

Conclusion

Acetate showed its potential as alternative carbon source to glucose for Aurentiochytrium limacinum SR21. It was able to support maximum growth at 3% and 4% APY which is not significantly different from growth in GPY, however, lipid productivity was significantly lower in APY compared to that in GPY. We analyzed intracellular metabolites to explain the difference in growth rate and fatty acid productivity in assimilating different carbon sources. Metabolome analysis indicated that cells grown in GPY showed significant activity in glycolysis, pentose phosphate pathway and amino acids metabolism while cells grown in APY showed significant activity in glyoxylate cycle, malate-pyruvate cycle and mevalonate pathway. These results also indicated the possible targets to improve the fatty acid productivity from acetate: 1) activation of the TCA cycle to improve growth rate; 2) activation of enzymes for transporting or converting TCA metabolites to be use for anabolic reactions; 3) overexpression of malic enzyme for increased NADPH production; and 4) suppression of the mevalonate pathway to prevent further consumption of acetyl-CoA. This is the first report showing the metabolic profile of Aurentiochytrium sp. assimilating acetate.

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