Doctoral Dissertation

Isolation of a novel thraustochytrid strain and its application for treatment of food processing wastewater and production of polyunsaturated fatty acids

(新規スラウストキトリッド株の単離とその食品製造排水処理や多価不飽和脂肪酸生産への応用)

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Abstract

Certain thraustochytrids are known to produce valuable polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (DHA) for human consumption and fish aquaculture. PUFAs production from thraustochytrids is largely limited by the need to prepare sophisticated artificial growth medium. Because thraustochytrid is heterotrophic, it can be used to remove organic compounds and to simultaneously produce PUFAs. Thus, food-procesing wastewater is proposed as growth medium to replace artificially-prepared growth medium given its available nutrient and lack of harmful compounds.

Four strains of *Aurantiochytrium* sp. (L2R, L2Y, L3W and L4Y) were isolated from the mangrove leaf samples, and *Aurantiochytrium* sp. L3W showed the highest specific growth rate (0.27 h^{-1}) among the four. Gene sequence analysis (18S rRNA) gene sequence analysis revealed the novelty of this strain. *Aurantiochytrium* sp. L3W grew at the ranges of temperature from 15°C to 35°C, pH from 3 to 9, and salinity from 0.3 to 70 PSU. Under the optimal condition (25°C, 30 PSU, pH7) *Aurantiochytrium* sp. L3W produced 270 mg/g of FAs including 135 mg/g of DHA.

Usability of salinity-adjusted miso-processing (MP) and bean boiling (BB) wastewater for cultivation of *Aurantiochytrium* sp. L3W was investigated. *Aurantiochytrium* sp. L3W could utilize 52% of the DOC and 37% of the DN from sterilized BB and produced biomass that contained 137 mg/g of FAs, including 96.2 mg/g of DHA. Cultivation in sterilized MP resulted in the production of biomass containing 147.6 mg/g of FAs, including 97.8 mg/g of DHA, and removal of 47% of the DOC and 55% of the DN from the wastewater.

Given that the wastewater may be diluted to meet standards of sewerage discharge, Aurantiochytrium sp. L3W was cultivated on unsterilized diluted BB and MP. DHA production by *Aurantiochytrium* sp. L3W was reduced by dilution factor due to lower concentration of available nutrients: 10.7 mg-DHA/g-biomass in the 10x diluted BB and 16.8 mg-DHA/g-biomass in the 20x diluted MP.

Unsterilized condition resulted in lower concentration of DHA and EPA compared to the sterilized condition. Undiluted and unsterilized BB resulted in of 61.2 mg-DHA/g-biomass and 1.19 mg-EPA/g-biomass, comparable to fivefold-diluted and sterilized BB. However, unsterilized MP suffered from contamination. These results confirmed the

potential usage of BB and MP wastewater as growth media for *Aurantiochytrium* sp. L3W with simultenous DOC and DN removal.

Digestibility of the resultant biomass of *Aurantiochytrium* sp. L3W by the enzymes extracted from the stomach of rainbow trout was investigated. Gut-stomach from rainbow trout (*Onchorhynchus mykiss*) was extracted in freezing temperature after 3 days starvation. 20 g-wet of the gut-stomach sample was homogenized at 4 °C in a mixture of 100 ml of 100 mM Tris-HCl buffer and 50 ml of 20 mM $CaCl_2$ (pH 8.0) to extract the enzymes. The biomass of *Aurantiochytrium* sp. L3W was found to be digestible by the enzymes. This suggest that the produced biomass can readily be used as a fish feed additive.

Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1. Usefulness of Polyunsaturated Fatty Acids

Animal cells utilize three main types of fatty acids (FAs): saturated FAs (no double bond), monounsaturated FAs (single double bond), and polyunsaturated FAs (multiple double bonds). Fatty acid is identified by its number of carbons and number of desaturation bonds, e.g., docosahexaenoic acid (DHA) which contains 22 carbons and 6 double bonds is noted as 22:6. Hence, polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. PUFAs are further classified into two main groups: ω -6 and ω -3 series, which are essential for humans and other animals to ingest from consumption because the body cannot synthesize them.

Various polyunsaturated fatty acids (PUFAs) have been known to physiologically support bodily and neural health at cellular level (Marszalek and Lodish, 2005; Smith et al., 2015). Among them, eicosapentaenoic acid (EPA; ω-3 20:5) and docosahexaenoic acid (DHA; ω-3 22:6) have been termed as "essential fatty acids" due to their core function in human body which is obtained from food (Kaur et al., 2014).

The deficiencies in PUFA can be associated with defects in cellular function, which may lead to diseases, as PUFAs are important constituents of cell membranes and cell signaling systems (Simopoulos et al., 2002). Many studies have further demonstrated that PUFAs are essential dietary components for humans (Spector and Kim, 2015). The ω-3 PUFAs affect many cardiovascular and blood anticoagulation, and are also known to decrease the incidence of coronary heart disease, stroke, and rheumatoid arthritis (Bernstein et al., 2012). Evidence on the benefits and risks of ω-3 PUFAs for human health has also been previously reviewed by Tapiero et al. (2002). As the importance of the presence and proportions of various PUFAs is

better understood, the value and demand for these dietary components also increases.

PUFAs for human consumption have been derived mainly from commercial fish sources in the form of fish oil. Fish oil is extracted using physical pressure (Taati et al., 2018) or enzymatic process (Ramakrishnan et al., 2013). The extraction is subsequently followed by multiple refining steps to further purify the PUFAs and remove the undesirable odor in order to make the crude fish oil safe and edible (Gupta et al., 2012). PUFAs, especially DHA, are also important for aquaculture of fish to attain satisfactory growth of fish. Therefore, PUFAs have been spiked to fish feed used in marine fish aquaculture, and fish oil has been extracted from other less valuable fish commodities and used as a source of PUFAs (Torrecillas et al., 2017).

Because of its beneficial use, fish oil commands such a high price at 1800–3000 USD/ton (Fish Oil and Meal World, 2019) which varies according to its ω-3 purity and profile. The decline of catch and limited supply of commercial fish, issue regarding fishery industry sustainability, seasonal availability, contamination of mercury in the body of water, along with issues with purity of the PUFAs have prompted researched into possible alternative sources of PUFA (Hooper et al., 2006). Several biotechnology applications are potential as alternative to PUFA production (Lopes da Silva et al., 2019), as continuously using small or low-value fishes for PUFAs production will lead to over exploitation, competition with food, and disrupt sustainability (Dineshbabu et al., 2019).

1.2. Thraustochytrids as An Alternative PUFAs source

Thraustochytrids such as *Aurantiochytrium, Thraustochytrium,* and *Schizochytrium* are heterotrophic stramenopilan protists found in the marine and coastal environment (Yokoyama and Honda, 2007; Jaseera et al., 2019). Thraustochytrids accumulate lipids, containing PUFAs, faster than any other oleaginous microorganisms (Ratledge, 2004). These microorganisms are known to produce PUFAs, prominently DHA and EPA (Ryu et al., 2013; Ward and Singh, 2005). The production of these ω-3 and ω-6 PUFAs by thraustochytrids may provide an easier and less expensive means of producing PUFAs-rich biomass and oils. This has brought considerable interest from the biotechnology industry as a potential alternative to traditional commercial sources of PUFA.

PUFAs content in thraustochytrids was reported to vary with different strains and culture conditions in several preceding studies (Patel et al., 2019, Wang et al., 2019). For example, the amount of DHA produced is reported to be:

- 10 mg-DHA/g (*Aurantiochytrium* sp. KH105; Yamasaki et al. 2006),
- 15.0 mg-DHA/g (*Aurantiochytrium* sp. KRS101, Ryu et al. 2013),
- 43.6 mg-DHA/g (*Schizochytrium limacinum* PA-968, Humhal et al. 2019),
- 88.5 mg-DHA/g (*Aurantiochytrium* sp. T66, Patel et al., 2019).

In general, DHA is always available from all thraustochytrids, but the presence of EPA largely depends on the strain being cultured and the culture conditions.

Further researches for application of thraustochytrids for PUFAs production are still continuing to address several shortcomings in the process:

- artificially prepared growth medium is expensive, reuse of effluent from food industry for cultivation may suggest cheaper price and more sustainable practice (Leong et al., 2019),
- obtaining higher proportion of important PUFAs such as DHA and EPA to help the refining process to obtain higher quality PUFAs (Kwak et al., 2019),
- more efficient production from the strains with higher specific growth rate to obtain more quantity of PUFAs-enriched biomass (Park et al., 2018)

These studies suggest that thraustochytrids could form an important part in the supply of such products by further optimization on the strain, cultivation media, and culture conditions.

1.3. Challenges for Sustainable PUFAs Production using Thraustocytrids on Food-processing Wastewater

Although production of PUFAs from the heterothropic thraustocytrids has been demonstrated (Kothri et al., 2020), there are still several shortcomings in the production process. The production is largely limited by the need to prepare highly-sophisticated artificial growth medium. Because of its high content of organic matter, food-processing wastewater may be a cost-effective alternative medium to culture the heterotrophic thraustocytrids, although sterilization may still be required as a pretreatment (Yamasaki et al., 2006; Unagul et al., 2007; Ryu et al., 2013; Humhal et al., 2019; Rashid et al., 2020). However, the requirement for sterilization may reduce the cost-effectiveness, despite the use of the organics in wastewater to grow the thraustocytrids (Lopez da Silva et al., 2019). Therefore, pretreatment of the wastewater should be minimized for sustainable production of PUFAs

Addition of salt to food-processing wastewater may be advantageous because the increased salinity may adversely impact contaminating microorganisms present in the wastewater. A previous study on microalgal cultivation demonstrated that the increased salinity helped by inactivating the cilliates in a mixture of secondary-treated wastewater used for microalgal cultivation (Nakai et al., 2020). Also, because thraustochytrids are halophilic, adjustment of salinity may result in improvement of biomass production (Chen et al., 2016).

Biomass yield and PUFAs production by some thraustochytrids can be varied due to changes in physical and chemical conditions of the cultivation, salinity, pH, and temperature. For example:

- increase of salinity from 7.5 ‰ to 30 ‰ may slightly increase biomass yield from the cultivation (Leaño et al., 2003),
- variation pH of the culture media from 4.0 to 9.0 may affect the cell growth of thraustochytrids (Gao et al., 2013),
- lower temperature condition (15 °C, compared to the optimal 25 °C) may reduce the biomass and change the PUFAs profile (Ma et al., 2017).

Therefore, the culture condition should be optimized in consideration of growth and PUFAs production characteristics of the target strains of thraustochytrids. The other way around is also true, several thraustochytrids stains could be developed or adapted to fit with the available wastewater samples.

Evaluation on adaptability of the thraustochytrids strains will need to consider various characteristics and required pre-treatment of the food-processing wastewater for the growth media: salinity, pH, sterilization, and dilution. Subsequently, several performance criteria, such as total biomass and lipid production, PUFA yield and profile, specific growth rate of the strain, are to be evaluated.

A food processing wastewater for cultivation is to be chosen considering the underlying characteristics of the target strain. Additionally, from the perspective of wastewater treatment, performance of the thraustochytrids strain to utilize dissolved organic carbon and nitrogen from the growth media is also to be evaluated. Several studies have utilized various food-processing wastewater as the growth medium, such as whey (Humhal et al. 2019), liquor distillery wastewater (Yamasaki et al., 2006), coconut water (Unagul et al., 2007), waste syrup (Iwasaka et al., 2013), and corn steep liquor (Wang et al., 2019). But so far, no studies has addressed the utilization of carbon and nitrogen from the wastewater samples.

1.4. Application of Thraustocytrids Biomass for Fish Feed

If successful cultivation of thraustochytrids using food processing wastewater is achieved, then how to most effectively use the harvested biomass is to be considered. Researches on application of thraustochytrids for fish feed supplement or substitute have been carried out to some extent (Brignol et al., 2018; Nobrega et al., 2019; Fernandes et al., 2019). In general, inclusion of thraustochytrids biomass doesn't always increase the weight of fish but will likely to increase DHA concentration in the flesh. This performance varies based on the type of fish and feeding strategy (Fossier-Marchan et al., 2018).

Fish meal and oil have the traditional source for aquaculture feed due to high protein content, excellent amino acid profile, high nutrient digestibility, general lack of anti-nutrients (Maita et al., 2007). The stagnation of fisheries and climate change consequently adversely affects the availability of fish meal and fish oil. This in turn creates a demand and motivates the study for alternative fish meal and fish oil.

Supplement or substitute of fish meal from plant sources (Krogdahl et al., 2010) or insect (Barragan-Fonseca et al., 2017) has come short with varying degree of success related to fish growth for different species of fish. For example, using the same 12-week feeding period with plant protein ingredients, sea bream showed remarkable decrease of feed intake (Gómez-Requeni et al., 2004) while such decrease has not been observed in sea bass (Kaushik et al., 2004). Substituting fish oil with vegetable oil also showed very poor acceptance of DHA and EPA in the flesh of sea bream (Fountoulaki et al., 2009).

There are several feeding strategies that have been investigated to incorporate thraustochytrids biomass in fish food: enriching larval feed with live thraustochytrid cells prior, directly feeding dried thraustochytrids in the form of pellets or powder, and formulating a fishmeal that includes thraustochytrid-derived oil as an ingredient (Fossier Marchan et al.,2018). Although less pretreatment processes may be preferable in terms of process simplification, usability of raw thraustochytrid biomass in fish aquaculture has not been aregued. That might be because no study has fed raw thraustochytrid biomass to fish.

1.5. Objectives of This Study

In the present study, we explore the potential of for cultivation in sterilized and unsterilized food processing wastewater. At first, isolation of thraustochytrids was conducted to find the strains durable to wide range of pH, salinity and temperature. Then, food processing wastewater was applied for cultivation of the isolated strain under an unsterile condition to obtain the raw thraustochytrid biomass containing PUFAs. Because confirmation of the digestibility of raw thraustochytrid biomass by fish enzymes would be the first step to exploring the possibility of its use as a fish feed component, the raw biomass of the isolated strain was tested for digestibility by enzymes extracted from fish stomach. Finally, culture conditions of the isolated

thraustocytrid to sustain removal of carbon and nitrogen and production of PUFAs in food processing wastewater were explored.

1.6. Research Flow

This dissertation is divided into the following chapters as shown in **Fig. 1**.

Chapter 1 introduced usefulness of PUFAs for human consumption and fish aquaculture, fish oil as a conventional PUFAs and explained the necessity of its alternation. Then challenges for sustainable production of PUFAs using thraustochytrids were addressed.

Chapter 2 describes isolation of thraustochytrids, identification of the four isolated four strains: *Aurantiochytrium* sp. L2R, L2Y, L3W and L4Y based on 18S rRNA gene sequence and investigation of the growth and lipid production characteristics including PUFAs.

Chapter 3 investigated effects of dilution of the miso-processing and bean-boiling wastewater discharged from a miso factory on the removal efficiency of dissolved organic carbon and dissolved nitrogen from the wastewaters and production of biomass and PUFAs by *Aurantiochytrium* sp. L3W for the compatibility.

Chapter 4 investigated usability of wastewater discharged from a miso factory for cultivation of *Aurantiochytrium* sp. L3W and established a culture condition without sterilization. Finally, digestibility of its raw biomass by the enzymes extracted from the stomach of rainbow trout was investigated to confirm the feasibility of using raw thraustochytrid biomass as an additive of PUFAs source for fish feed.

Chapter 5 summarizes the results of this study.

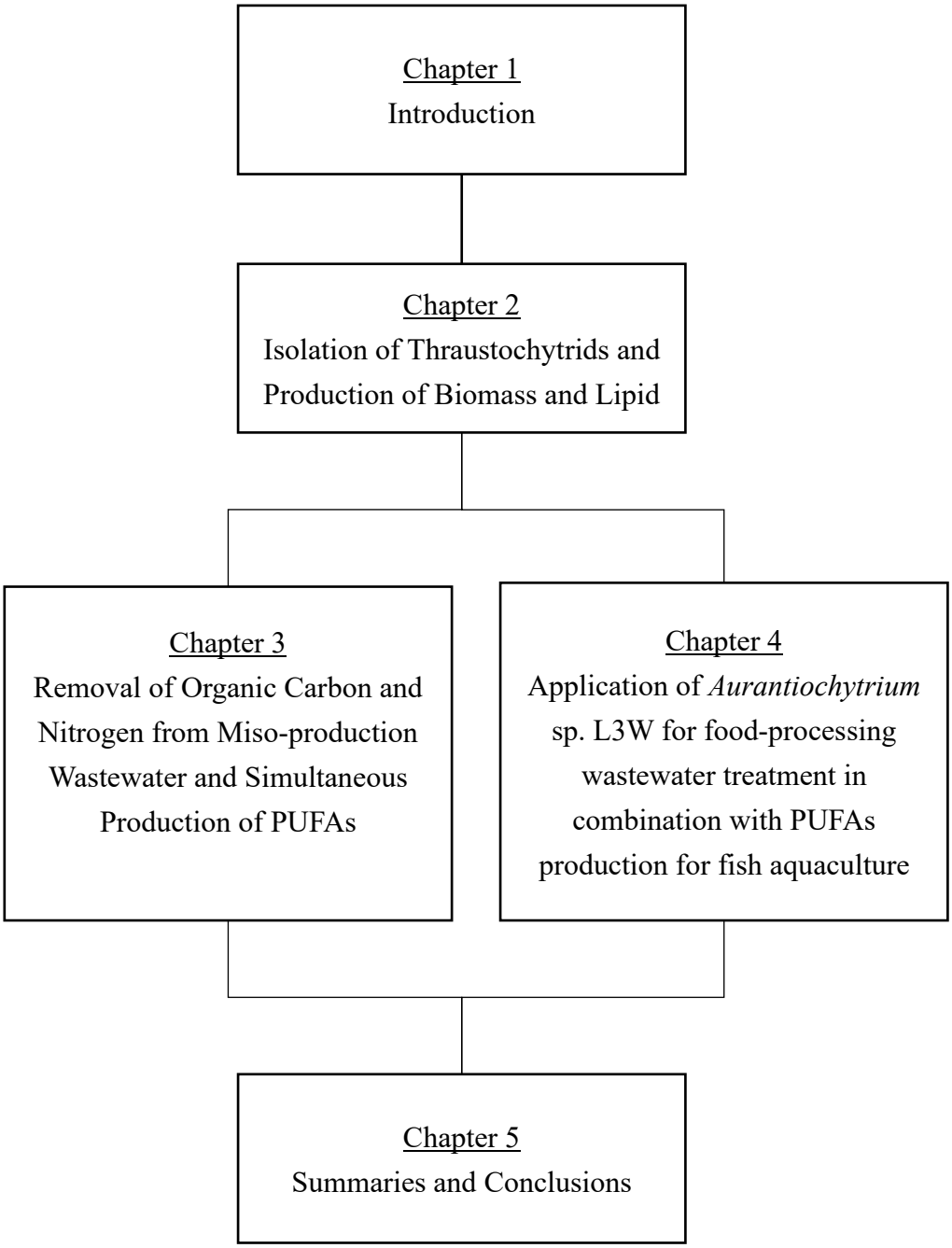

Figure 1.1 Logical flow of the framework of this study.

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Chapter 2: Isolation of Thraustochytrids and Production of Biomass and Lipid

2.1. Introduction

Thraustochytrids, such as *Aurantiochytrium* and *Schizochytrium,* are heterotrophic stramenopilan protists belonging to kingdom Chromista (Fossier-Marchan et al., 2018) and known to produce lipid consisting essential ω -3 PUFAs such as docosahexaenoic acid (DHA) other PUFAs (Taoka et al., 2009; Gao et al., 2013; Raghukumar et al., 2008). ω-3 PUFAs have received worldwide attention because of the beneficial effects on human health, and nowadays DHA supplements are manufactured (Zhang et al., 2020).

As thraustochytrids are heterotrophic in nature, organic compounds such as glycerol, glucose, fructose and yeast extract, peptone, urea have been used as the substrates (Unagul et al., 2007; Raghukumar et al., 2008; Hong et al., 2011; Gao et al., 2013; Fossier-Marchan et al., 2018) while sodium nitrate and ammonium sulfate may be used as nitrogen sources (Gao et al., 2013; Humhal et al., 2019).

Due to expensive nature of commercial substrates, use of food processing wastewater (Unagul et al., 2007; Yamasaki et al., 2006) and liquid waste (Iwasaka et al., 2013) may be a good option for culturing from the economical viewpoint; however, sterilization may be required to avoid negative impacts by contaminating microorganisms. Since involvement of a sterilization process may impair the cost effectiveness, pretreatment of wastewater for its use should be minimized but ensure growth of the target thraustochytrids. To conquer this dilemma, use of thraustochytrids exhibiting durable growth may be one of the options.

Thraustochytrids were once reported to have a broad pH tolerance of 5 to 8 for growth and DHA production, and a later study reported *Aurantiochytrium* sp. SD116 capable of growing at pH4 (Cristóvão et al., 2015). These results suggest that strains with higher tolerance for an acidic condition need to be found to realize a cultivation process under competition with other microorganisms using unsterile wastewater. In addition, high salt tolerance makes strains more competitive. Thraustochytrids prefers a salinity range from 20‰ to 34‰ (Fossier-Marchan et al., 2018; Raghukumar et al., 2002); however, others reported higher tolerance of *Schizochytrium limacicinum* strain SR21 towards salinity at 0-200% range (Raghukumar et al., 2008; Yokochi et al., 1998). As for temperature, 25-30°C is generally favor optimal growth (Fossier-Marchan et al., 2018; Raghukumar et al., 2008).

In addition to natural strains with high pH and salt tolerances utilization of genetically modified strains may be one of the options to realize durable growth and FAs production in unsterile wastewater. As strains of thraustochytrids with high Ω-3 FAs productivity have been designed by genetic engineering approaches, environmental tolerance might also be genetically modified (Fossier-Marchan et al., 2018).

The present study aimed at finding strains of thraustochytrids to tolerant for wide pH, salinity and/or temperature ranges. We conducted isolation and identification of strains of thraustochytrids. After investigating growth and lipid contents, tolerance for temperature, pH and salinity and FAs production were further tested.

2.2. Materials and Methods

2.2.1. Isolation of thraustochytrids

As isolation sources of thraustochytrids, mangrove leaves were collected from the coastal area of the main island of Okinawa, Japan, while sand filtered seawater (about 30 PSU) was obtained from the Takehara Fisheries Research Station, Hiroshima University, Hiroshima, Japan. Placing each leaf sample on the agar plates of American Type Culture Collection's (ATCC) 2673 thraustochytrid medium (10.0 g of agar, 5.0 g of D-(+)-glucose, 15.0 g of peptone, 1.0 g of yeast extract and 1 L of seawater) to which both penicillin and streptomycin were added at 100 mg/L, colonies of thraustochytrids were obtained by incubation at 25°C in dark. Each colony was taken and inoculated into 20 ml of the ATCC's 790 By+ medium (5.0 g of D-(+)-glucose, 1.0 g of peptone, 1.0 g of yeast extract and 1 L of seawater) in a 50 ml Erlenmeyer flask and cultivated at a rotation rate of 70 rpm and 25°C in dark. After dilution using the 790 By+ medium, 100 µl of the diluted culture solution was again inoculated onto the ATCC 2673 agar plate for further separation. Repeating the cultivation and separation processes, the four strains were finally isolated.

2.2.2. Identification of the strains

Identification was conducted by analyzing the sequences of 18s rDNA. The culture solution of each strain was centrifuged at 3000 rpm for 20 min, and the resultant pellet was lyophilized. Extracting DNA from the lyophilized biomass according to the modified Marmur's procedure (Marmur, 1961), the polymerase chain reaction (PCR) was performed using Euk A/Euk B primers (Medlin et al., 1988), PrimeStar HS DNA Polymerase (Takara Bio Inc., Kusatsu, Japan) and BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Foster City, USA). Finally, the PCR products were subjected to a DNA sequencer (ABI PRISM 3130 x1 Genetic Analyzer System, Thermo Fisher Scientific Inc., Foster City, USA),

and the resultant electropherogram was analyzed using Chromas Pro 1.7 (Technelysium Pty Ltd., South Brisbane, Australia).

Based on only single peaks of assembly sequences, we finally determined 18S rDNA sequences consisting of 1018-1474 bps for each strain. The 18S rDNA sequence was then searched on the nucleotide BLAST (Basic Local Alignment Search Tool) using the blastn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A neighbor joining phylogenetic tree was produced by using ClustalW algorithm (MEGA X software). As phylogenetic references, we used the 18S rRNA gene sequence data of the 30 closely related species retrieved from the NCBI GenBank database. The gene sequences of *Bacillaria paxillifer* (M87325) and *Ochromonas danica* (M32704) were used as outgroups (Nakazawa et al., 2012).

2.2.3. Growth characteristics

Each strain was pre-cultured in 20 ml of the 790 By+ medium in a 50 ml Erlenmeyer flask at a rotation rate of 70 rpm and 25°C in dark for 3 days, and then the pre-culture solution was inoculated into 100ml of the 790 By+ medium in a 500 ml Erlenmeyer flask at the initial cell density of 5×10^3—10^4 cells/ml. The culture flasks were incubated in triplicate at a rotation rate of 70 rpm and 25°C in dark condition

In the experiments to understand effects of temperature on growth of an isolated strain, the incubation temperature was changed to 15°C—35°C. When effects of salinity on its growth was investigated, dilution of seawater with MilliQ water or addition of NaCl to seawater were carried out in preparation of the 790 By+ medium to attain the preset salinities of 0.3 PSU—70 PSU, whereas pH of the 790 By+ medium was adjusted using 50 mM of phosphate buffer (H_3PO_4, NaH_2PO_4, NaH_2PO_4) to attain pH3 and pH6 or 50 mM of tris(hydroxymethyl)aminomethane to attain pH7, pH8, and pH9 together with 1M HCl.

2.2.4. Analysis

During the cultivation period, the cell number was periodically measured by microscopic observation using a Thoma hemocytometer (0.1-mm depth, Matsuyoshi, Tokyo, Japan). For determination of the biomass production, 20 ml of the culture solution at the stationary phase was centrifuged twice at 7000 rpm for 15 min, and the cells pellet was washed with MilliQ water to remove salt at the interval of centrifugation operations. Finally, the sample was filtered through a glass fiber filter (GF/A, Whatman, Buckinghamshire, UK) and dried at 100°C for 3 h, followed by weighing. For determination of lipid content, the salt-removed cell pellet was obtained from 50 ml of the culture solution and extracted using 2:1 chloroform and methanol. After filtration through a GF/A filter, the filtrate was dried at 35°C for evaporation of the solvents, followed by weighing.

Because *Aurantiochytrium* sp. L3W was novel and showed the excellent growth characteristic, it was cultured at 25°C, pH7, and 30 PSU, and the resultant biomass was analyzed for FAs. In addition, we also investigated the effect of pH on the FAs production by analyzing the biomass of *Aurantiochytrium* sp. L3W cultivated at pH3, 6, 7, 8, and 9. Briefly, the culture solution of *Aurantiochytrium* sp. L3W was centrifuged at 3000 rpm for 20 min according to modified Miller's procedure (Miller, 1982), and the biomass pellet was washed with Milli-Q water. As the internal standard, undecanoic acid (U0004, Tokyo Chemical Industry, Tokyo, Japan) dissolved in n-hexane at 1000 mg/L was used. After lyophilization of the biomass pellet overnight, 10 μl of the internal standard solution was added, and then the biomass pellet was methylated and purified using the FAs methylation-purification kit (06482-04, Nacalai-tesque, Kyoto, Japan). Finally, the sample was subjected to a gas chromatograph equipped with a flame ionization detector (GC/FID, Agilent 7820A, Agilent, Santa Clara, USA), where the

Supelco 37-component FAME mix (CRM47885, Sigma-Aldrich, St. Louis, USA) was used as the standards of methylated FAs.

For statistical analysis of biomass and lipid productions, one-way analysis of variance (ANOVA) and Turkey test were conducted. The data were assumed to be accepted with confidence interval $p < 0.05$

2.3. Results and Discussion

2.3.1. Identification of the isolated strains

By the isolation campaign, the four strains L2R, L2Y, L3W, and L4Y were isolated, and the sequence data of theses strains are available in the DDBJ/EMBL/GenBank databases under the accession numbers; L2R, LC586066; L2Y, LC586067; L3W, LC586065; L4Y, LC586068. The 18S rDNA sequence of the strain L3W was close to that of *Aurantiochytrium acetophilum* (accession number: MH319325), *Aurantiochytrium limacinum* (accession number: JN986842), and *Aurantiochytrium* sp. 4W-1b (accession number: AB810947.1), and homology was 98.9% (**Table 2.1**). However, the neighbor joining phylogenetic tree showed that the strain L3W was not included in the clades of the previously reported strains (**Fig. 2.1**). This indicates that the strain L3W is a novel strain of *Aurantiochytrium* sp. As for the other 3 strains L2R, L2Y and L4Y respectively showed 99.9%, 99.8% and 99.9% homology with the previously identified strains of *Aurantiochytrium* sp.

Table 2.1 BLAST library search results with the highest homology for the strain L3W (a), L2R (b), L2Y (c), and L4Y (d) based on the 18S rDNA sequence.

(a) L3W (1018 bp)

Registered name	Strain	Accession No.	Homology
Aurantiochytrium sp.	HS399	MH319325.1	98.9%
Aurantiochytrium limacinum	SL1101	JN986842.1	
Aurantiochytrium sp.	4W-1b	AB810947.1	

(b) L2R (1450 bp)

Registered name	Strain	Accession No.	Homology
Aurantiochytrium sp.	15A-6a	AB811001.1	99.9%
Aurantiochytrium sp.	18W-13a	AB810942.1	

(c) L2Y (1229 bp)

Registered name	Strain	Accession No.	Homology
Aurantiochytrium sp.	SEK218	AB290573.1	99.9%

(d) L4Y (1455 bp)

Registered name	Strain	Accession No.	Homology
Aurantiochytrium sp.	9W-3a	AB810991.1	99.9%
Aurantiochytrium sp.	15A-6a	AB811001.1	
Aurantiochytrium sp.	18W-16a	AB811030.1	
Aurantiochytrium sp.	16A-4a	AB811020.1	

Figure 2.1 Neighbor joining phylogenetic tree based on 18S rRNA gene sequence of the strains L2R, L2Y, L3W, and L4Y.

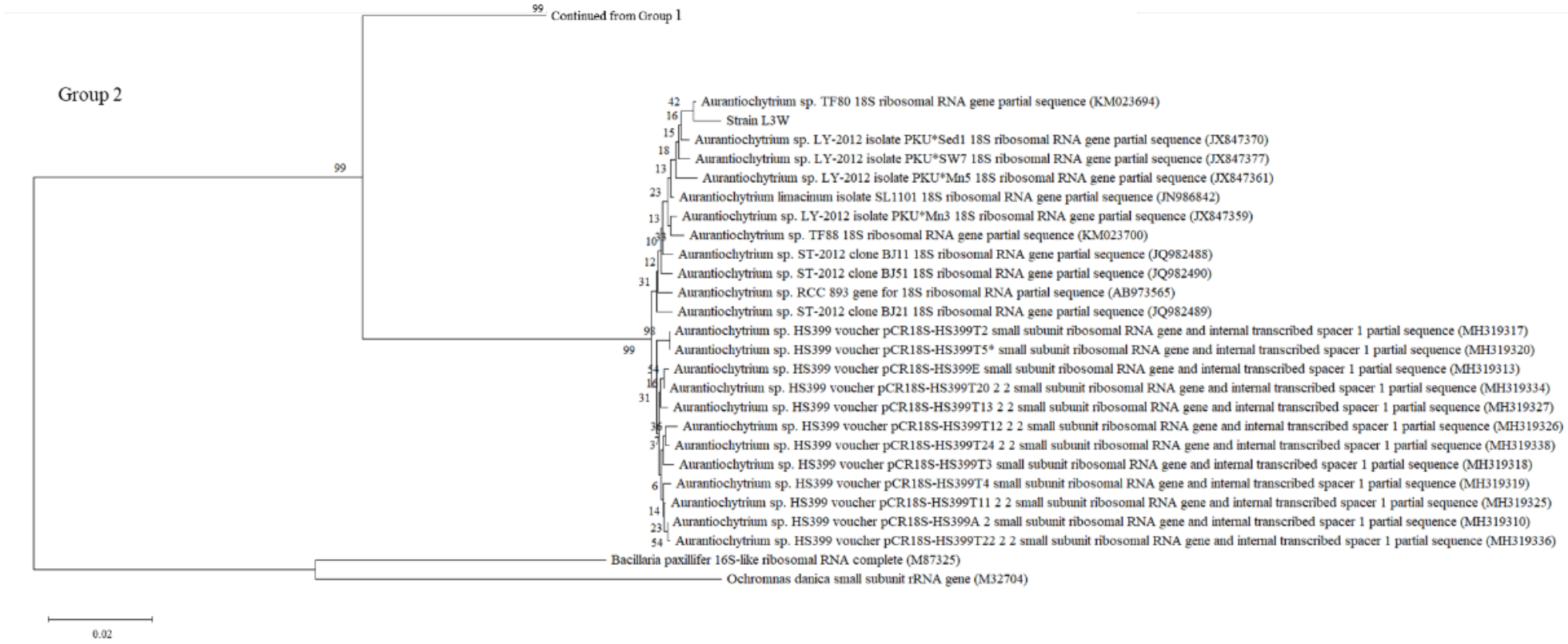

Figure 2.1. (Continued)

2.3.2. Growth and lipid production of the four strains

Figure 2.2 shows the growth curve of *Aurantiochytrium* sp. L3W that demonstrated the fast growth, where the stationary phase appeared after 30 h. Analyzing the growth during the first 30 h of cultivation, *Aurantiochytrium* sp. L3W showed a high specific growth rate of 0.27 h^{-1}. Specific growth rates of the strains L2R, L2Y, and L4Y were estimated by the growth test too; however, the values did not exceed that of the strain L3W (**Fig. 2.3**).

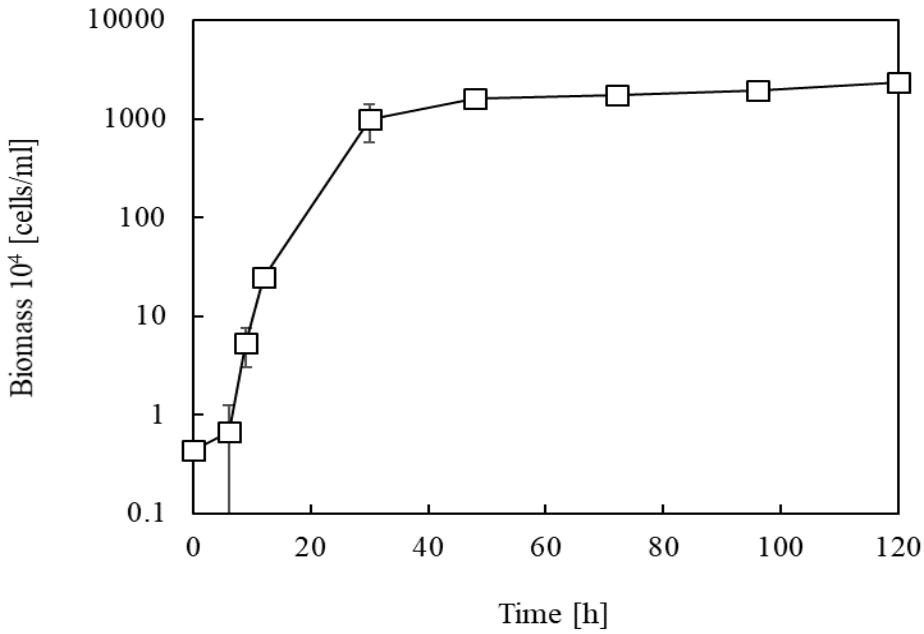

Figure 2.2. Growth curve of *Aurantiochytrium* sp. L3W in the 790 By+ medium at 25°C. Bars indicate standard deviation (n=3).

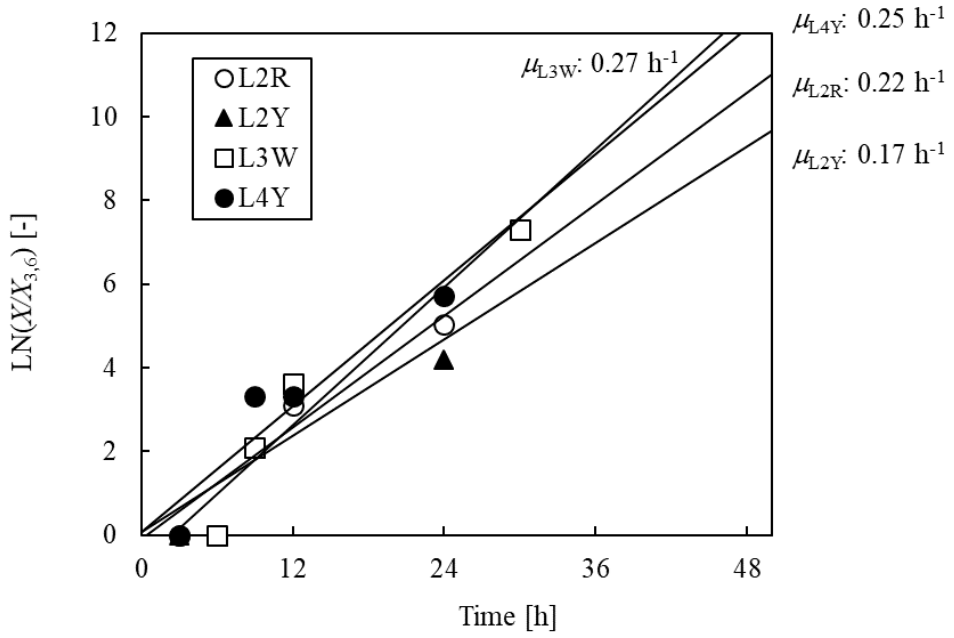

Figure 2.3. Comparison of the growth of the four strains during the first 30 h of the cultivation. The specific growth rates were determined based on the cell number after 3 h with an exception for the strain L3W of which value was calculated on the basis of cell number after 6 h.

In the previous studies, specific growth rates of other strains were reported to be 0.15 h^{-1} for *Aurantiochytrium mangrovei* SK-02 (Chodchoey and Verduyn, 2012); 0.12 h^{-1} for *A. limacinum* (Abad et al., 2015); 0.077 h^{-1} for *A. mangrovei* FB3 (Fan et al., 2010); and 0.60 d^{-1} (0.025 h^{-1}) for *Aurantiochytrium* sp. AF0043 (Trovão et al., 2020).

In addition, specific growth rates of *Aurantiochytrium* sp. mh0186 (later identified as *A. limacinum* mh0186) (0.2 h^{-1}), *Aurantiochytrium* sp. KRS101 (0.08 h^{-1}; Hong et al., 2011), *Aurantiochytrium* sp. JMVL1 (0.2 h^{-1}; Jaseera et al., 2019), and *Aurantiochytrium* sp. SW1 (0.05 h^{-1}; Nazir et al., 2018) were estimated on the basis of the presented growth curves. Although the culture condition of *Aurantiochytrium* sp. L3W was different from that in the previous studies (Hong et al., 2011; Taoka et al., 2011; Chodchoey and Verduyn, 2012; Nazir et al., 2018) in terms of the temperature and composition of culture media, a comparison of the specific growth rates suggests that *Aurantiochytrium* sp. L3W might demonstrate relatively fast growth as compared to the strains reported before.

Figure 2.4 compares the biomass and lipid productions by the strains L2R, L2Y, L3W, and L4Y, where, among the four strains, the strain L3W showed the highest biomass and lipid production on average. The averaged lipid content to the biomass was 42% for the strain L3W, and this was comparable to *Aurantiochytrium limacinum* mh0186 (33%; Taoka et al., 2009) and (45%; Taoka et al., 2011), *Aurantiochytrium* sp. KRS101 (43.4%; Hong et al., 2011), *Aurantiochytrium* sp. AF0043 (31%; Trovão el al., 2020), and *Aurantiochytrium* sp. SW1 (48%; Nazir et al., 2018). Since the strain L3W was a novel strain showing the high specific growth rate (**Figs. 2.1** and **2.3**) and biomass and lipid productions (**Fig. 2.4**), we further tested its growth characteristics.

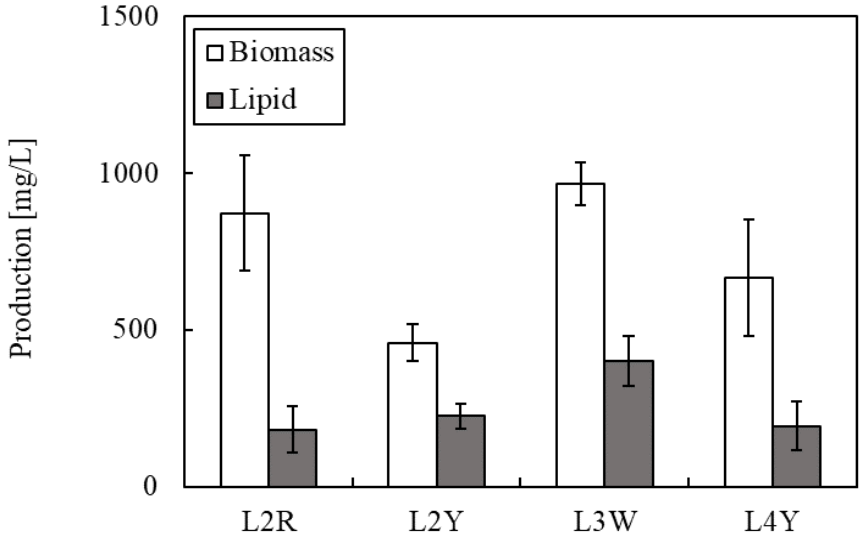

Figure 2.4. Biomass and lipid productions by the strains L2R, L2Y, L3W and L4Y. Bars indicate standard deviation (n=3) with an exception for the lipid production by L3W of which bars indicate the difference between the averaged and measured

2.3.3. Growth characteristics of the strain L3W

2.3.3.1. Effect of temperature

Figure 2.5 shows the effect of temperature on the growth of *Aurantiochytrium* sp. L3W, where its biomass and lipid productions changed. Among the tested temperatures, the biomass production at 25°C was highest ($p < 0.016$). In addition, the lipid production at 25°C was significantly higher than that at 30°C and 35°C ($p < 0.0068$); however, a significant difference was not found between 15°C and 25°C. The results collectively showed that 25°C was optimal for *Aurantiochytrium* sp. L3W. This is reasonable because the optimal temperature is within the range of monthly average atmospheric temperature (17.0°C—28.9°C, from 1981 to 2010) in Okinawa Pref. (Japan Meteorological Agency, 2020) from which we collected the isolation source for *Aurantiochytrium* sp. L3W.

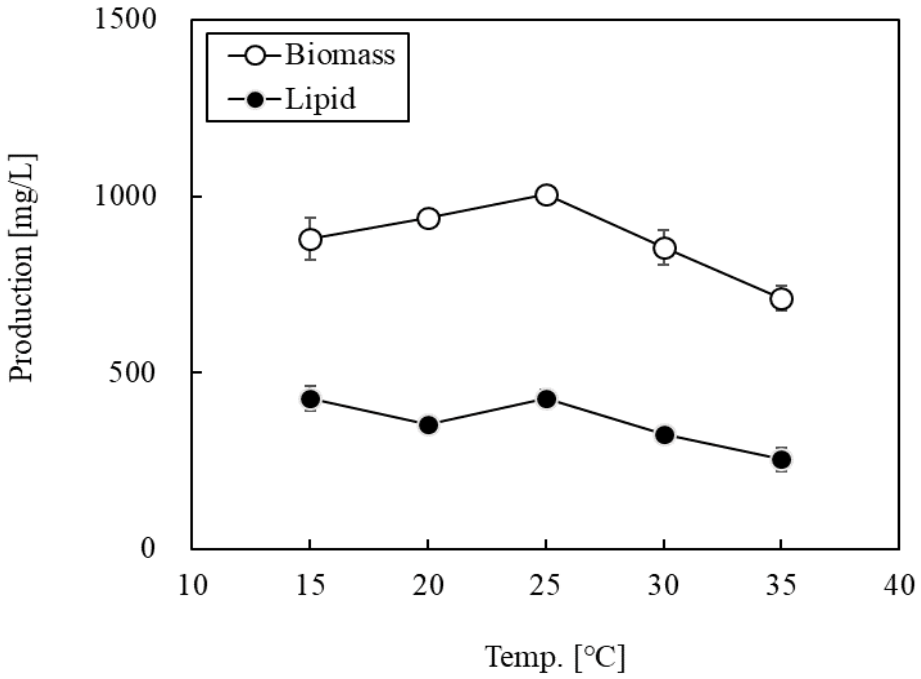

Figure 2.5. Effect of temperature on the biomass and lipid productions by *Aurantiochytrium* sp. L3W at 30 PSU and pH7. Bars indicate standard deviation (n=3). Biomass production was highest at 25°C ($p < 0.016$), and lipid production at 25°C was significantly higher than that at 30°C and 35°C ($p < 0.0068$)

Temperatures of 25°C—30°C is generally favor for growth of thraustochytrids (Raghukumar et al., 2008). In the previous studies, similar optimal temperatures were reported for *Aurantiochytrium* sp. SD116 (25°C; Gao et al., 2013) and *Aurantiochytrium* sp. KRS101 (28°C; Hong et al., 2011), while no significant change was observed in growth of *A. limacinum* mh0186 at a range from 15°C to 35°C (Taoka et al., 2009).

In addition, *A. limacinum* mh0186 could grow at 10°C, however, the growth was significantly lower than that at 15°C and 28°C (Taoka et al., 2011). The growth characteristics of *Aurantiochytrium* sp. L3W in terms of temperature (**Fig. 2.5**) is similar to these strains of *Aurantiochytrium* sp., though the growth test was not performed at 10°C. Since 25°C was optimal for *Aurantiochytrium* sp. L3W, further testing was performed at this temperature.

2.3.3.2. Effect of salinity

Figure 2.6 shows effects of salinity on the biomass and lipid productions by *Aurantiochytrium* sp.L3W. *Aurantiochytrium* sp. L3W could grow at a range of salinity from 0.3 to 70 PSU; however, the biomass production below 3.0 PSU was less than 10% of that at 15 PSU. At 15 and 30 PSU the highest biomass production was attained ($p < 0.029$), and the higher salinities over 30 PSU resulted in lowering biomass production. Similarly to the biomass production, the highest lipid production was observed at 15 and 30 PSU ($p < 0.001$). These results confirmed that the optimal salinity for *Aurantiochytrium* sp. L3W was in a range from 15 to 30 PSU. As for suppression of growth of thraustochytrids at low and high salinities, no paper has reported its mechanisms; however, osmotic stress and/or ionic stress might be the most plausible explanation.

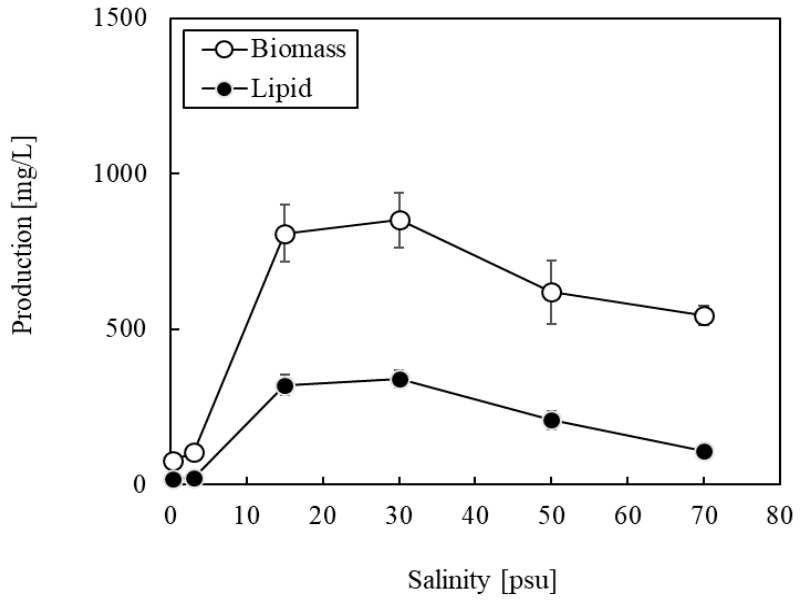

Figure 2.6. Effects of salinity on the biomass and lipid productions by *Aurantiochytrium* sp. L3W at 25°C and pH7. Bars indicate standard deviation (n=3). Biomass and lipid production were highest at 15 and 30 PSU at $p < 0.029$ and $p < 0.001$, respectively.

Thraustochytrids have an absolute requirement for sodium which cannot be replaced by potassium, and their preferable salinity range is reported to be from 20‰ to 34‰ (Raghukumar et al., 2002). Later, Fossier-Marchan et al. (2018) reported that optimal salinity in thraustochytrids generally corresponds to 50–100% seawater, though salinity optima and tolerance levels for growth vary among strains. For example, the previous study showed that the optimal salinity for *Aurantiochytrium* sp. KRS101 was 15 g-sea salt/L at a range of salinity from 2 to 50 g-sea salt/L (Hong et al., 2011). As for *Aurantiochytrium* sp. SD116, it could grow at a range of salinity from 0 to 60 g-sea salt/L, and the optimal salinity was 15 g-sea salt/L (Gao et al., 2013). In this study, we used the sand filtered seawater with salinity of about 30 PSU, and salinity optimal for growth of *Aurantiochytrium* sp. L3W was 15 and 30 PSU (**Fig. 2.6**). This agree with the previous studies reported by and Raghukumar et al. (2008), Gao et al. (2013), Fossier-Marchan et al. (2018).

In addition, *Aurantiochytrium* sp. L3W could grow at 70 PSU which was about 200% of the sand filtered seawater, and its salinity tolerance to high salinity was same as that of *Schizochytrium limacinum* SR21 (Yokochi et al., 1998). This growth characteristic might be suitable for utilization of saline food processing wastewater and liquid waste such as fish canning wastewater (Cristóvão et al., 2015).

2.3.3.3. Effect of pH

Effects of pH on the biomass and lipid productions by *Aurantiochytrium* sp. L3W are shown in **Fig. 2.7**, where the biomass production was highest at pH6, 7 and 8 ($p < 0.0125$). During the experimental period, the pH value was maintained at the set value ± 0.2. As for the lipid production, a significant difference was not observed at the tested pH range. The result indicates that the optimal pH for *Aurantiochytrium* sp. L3W exists in a range from pH6 to 8. In

addition, the averaged lipid content at pH3 (67%) and pH9 (49%) was higher than pH7 (38%), though the biomass production was suppressed under these pH conditions. Plausible explanation might be that some of carbon sources were used to accumulate lipid rather than cell growth (Gao et al., 2013).

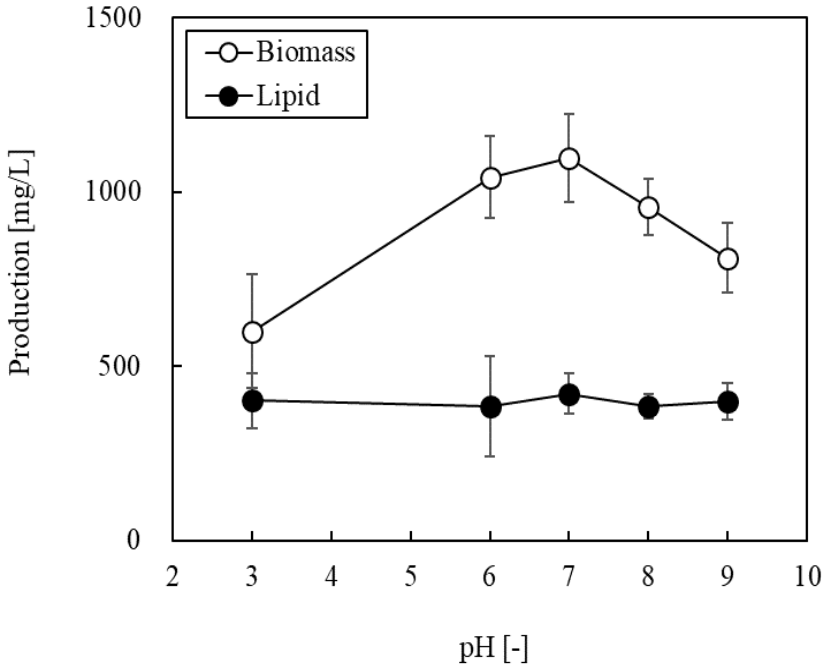

Figure 2.7. Effects of pH on the biomass and lipid productions by *Aurantiochytrium* sp. L3W at 25°C and 30 PSU. Bars indicate standard deviation (n=5) with exceptions for pH6 and pH8 (n=4). Biomass production was highest at pH6, 7, and 8 ($p < 0.001$).

Thraustochytrids are known to have a broad pH tolerance at a range from 5 to 8 for growth (Raghukumar et al., 2008). The optimal pH value for *Aurantiochytrium* sp. L3W was similar to what previously reported for *Aurantiochytrium* sp. KRS101 (pH7; Hong et al., 2011) and pH6 for *Aurantiochytrium* sp. SD116 (pH6; Gao et al., 2013). However, *Aurantiochytrium* sp. L3W demonstrated the interesting growth characteristics in terms of pH tolerance. *Aurantiochytrium* sp. L3W could grow at pH3 and pH9. The previous study showed that *Aurantiochytrium* sp.

SD116 could grow at pH4 and pH9 (Gao et al., 2013). However, to the best of our knowledge, no paper has reported a strain of thraustochytrid capable of growing at pH3. The result suggests good potential of the strain L3W for utilization of acidic food processing wastewater and liquid waste such as pineapple cannery waste (Suwannasing et al., 2015).

2.4. Potential of the strain L3W for FAs production

Based on the effects of temperature, salinity and pH on the biomass and lipid productions by *Aurantiochytrium* sp. L3W (**Figs. 2.5-2.7**), *Aurantiochytrium* sp. L3W was cultivated under the optimal condition of 25°C, 30 PSU, and pH7, and resultant biomass was analyzed for FAs. As shown in **Fig. 2.8,** the biomass of *Aurantiochytrium* sp. L3W contained FAs at 270 mg/g, and DHA accounted for a half of its FAs content. The second abundant fatty acid was palmitic acid, and together with DHA it accounted for the total fatty acid content at 83%. The DHA concentration of 135 mg/g was within the range of that in other strains of *Aurantiochytrium* spp. reported before (**Table 2.2**). Although the DHA content of *Aurantiochytrium* sp. L3W is not high, optimization of culture condition may allow this strain to produce more DHA. For example, about four times increase in the DHA production was observed for *A. limacinum* mh0186 (Nagano et al., 2009; Taoka et al. 2009). In addition, a fed-batch or continuous fermentation strategy may be used to improve the DHA productivity (Fossier-Marchan et al., 2018).

Table 2.2 Comparison of the DHA content introduced in this study.

Strain	DHA (mg/g-dry cell)	Medium	Ref.
Aurantiochytrium limacinum mh0186*	121	Artificial	Taoka, 2009
Aurantiochytrium sp. SD116	247	Artificial	Gao, 2013
Aurantiochytrium sp. KRS101	169	Glucose, corn steep	Hong, 2011
Aurantiochytrium mangrovei Sk-02**	214	Coconut water	Unagul, 2007
Aurantiochytrium sp. KH105**	115	Shochu wastewater	Yamasaki, 2006
Aurantiochytrium sp. KH105	32	Waste syrup	Iwasaka, 2013
Aurantiochytrium limacinum mh0186	111	Artificial	Yokochi, 1998
Aurantiochytrium mangrovei Sk-02	126	Artificial	Chodchoey, 2012
Aurantiochytrium limacinum ATCC MYA-1381	150	Artificial	Abad, 2015
Aurantiochytrium sp. SW1	250	Artificial***	Nazir, 2018
Aurantiochytrium sp. T66	124	Forest biomass hydrolysates	Patel, 2019
Aurantiochytrium limacinum mh0186	467	Artificial***	Nagano, 2009
Aurantiochytrium sp. L3W	135	Artificial	This study

* The strain was once reported as *Aurantiochytrium* sp. mh0186.

** The strain was once reported as *Schizochytrium mangrovei*.

*** Optimization of the culture condition was carried out.

Aurantiochytrium sp. L3W grew at the wide ranges of temperature from 15°C to 35°C, salinity from 0.3 PSU to 70 PSU, and pH from 3 to 9, though the biomass production by *Aurantiochytrium* sp. L3W was suppressed out of the optimal condition (**Figs. 2.5-2.7**). Since the pH tolerance of the strain L3W is a unique property, we investigated an effect of pH on production of FAs by *Aurantiochytrium* sp. L3W. In the previous paper, a range of pH from 5 to 8 was reported as one of essential criteria for production of DHA by thraustochytrids (Raghukumar et al., 2008); however, we confirmed production of DHA and pentadecanoic and margaric acids by *Aurantiochytrium* sp. L3W at pH3 and 9.

As the pH value decreased or increased from 7, the abundance of FAs in the biomass of *Aurantiochytrium* sp. L3W became lower. On the other hand, the lipid content was not affected by pH at the tested range (**Fig. 2.8**). The previous studies confirmed production of squalene in addition to DHA by *A. mangrovei* (Fan et al., 2010) and *Aurantiochytrium* sp. T66 (Patel et al., 2019) and xanthophylls by *Aurantiochytrium* sp. KH105 (Yamasaki et al., 2006; Iwasaka et al., 2013). Based on these finding, alteration of the lipid composition in the biomass might be the plausible explanation for reduction of the FAs content by the change of pH. Because squalene and xanthophylls are valuable lipid (Patel et al., 2019; Yamasaki et al., 2006), our future study will address effects of pH on lipid composition.

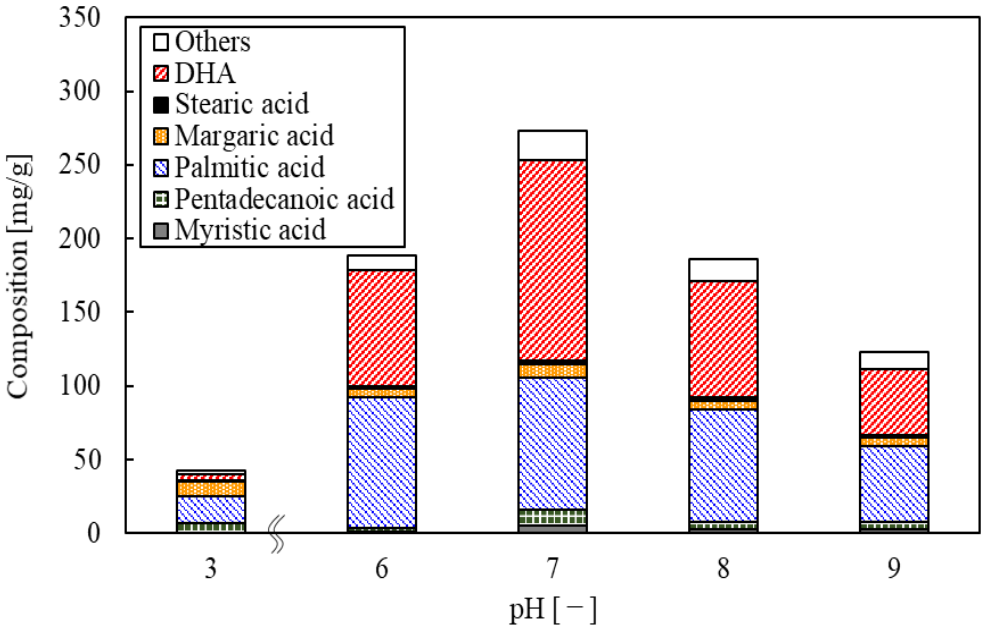

Figure 2.8. Effects of pH on the total and composition of PUFAs production

The growth characteristics of *Aurantiochytrium* sp. L3W in terms of pH and salinity tolerance may be advantageous in removal of organic compounds in acidic and saline wastewater for production of FAs. As shown in **Figs. 2.9 and 2.10**, adjustment of pH and salinity suppressed the growth of microorganisms originated in the bean boiling and miso-processing wastewater samples, indicating the importance of pH and salinity tolerance for use of unsterile wastewater as a culture medium.

This suggests combinational use of pH and salinity tolerance may result in successful growth and DHA production, though *Aurantiochytrium* sp. L3W could grow at pH3. In addition, certain food industries discharge acidic and/or saline liquid waste. For example, a pickled cruciferous vegetables (*hiroshimana*) production process generated acidic and saline seasoning liquid waste (35 PSU, pH4) (unpublished). Because of acidity and salinity, treatment and utilization of pickle production waste remain challenging. Applicability of *Aurantiochytrium* sp. L3W for various kinds of unsterilized food processing wastewater will be further tested in near future.

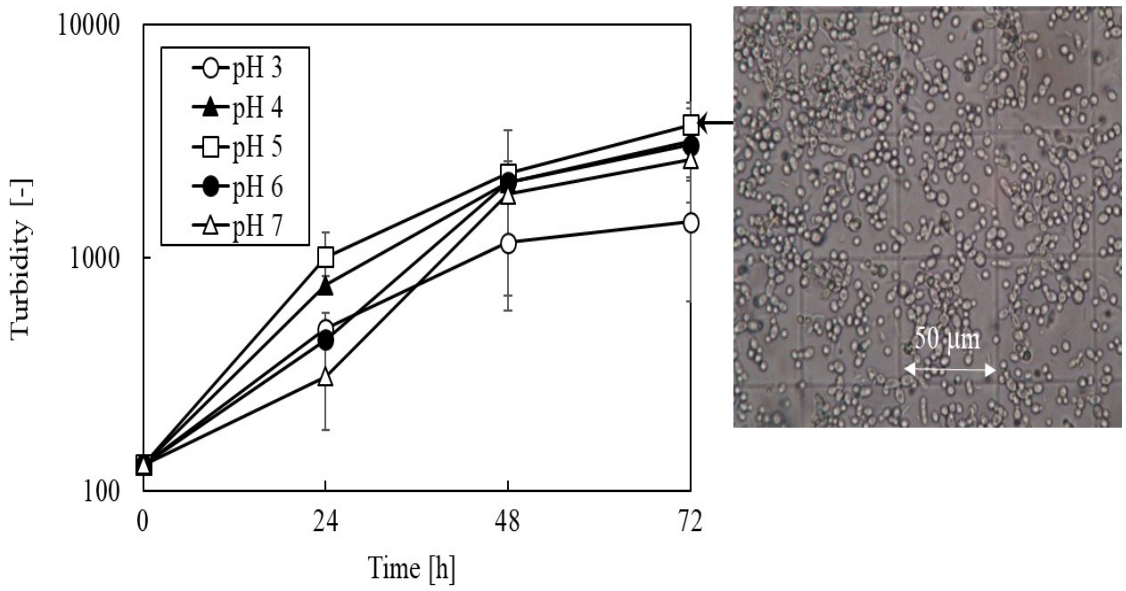

Figure 2.9. Growth of microorganisms originated in the miso-processing wastewater at 25°C as affected by pH adjustment using 0.1M HCl and 0.1M NaOH and the micrograph after 72 h. Bars indicate standard deviation (n = 3).

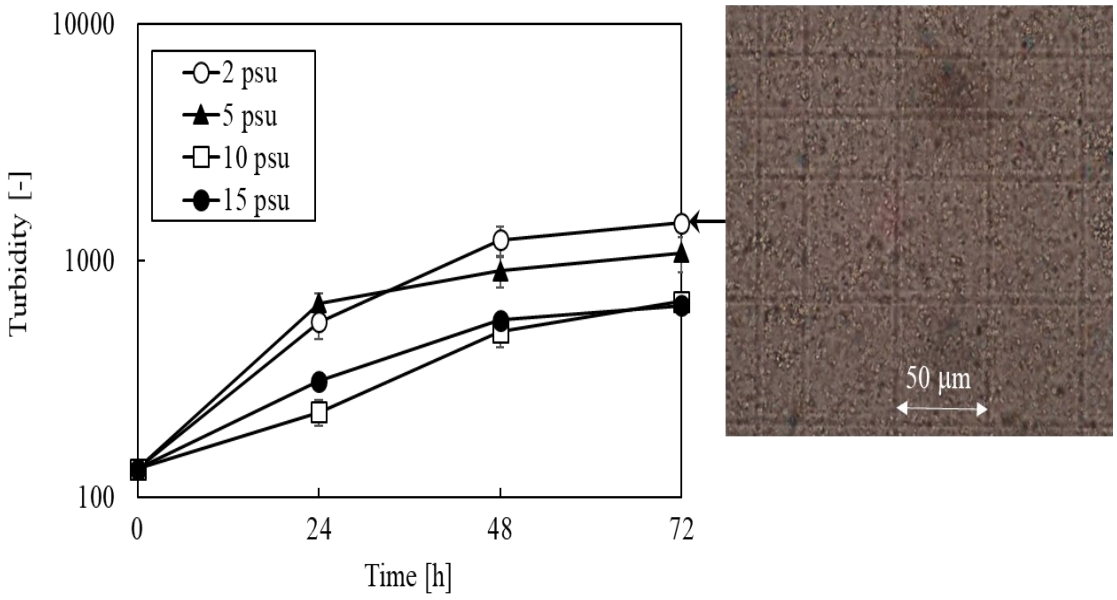

Figure 2.10. Growth of microorganisms originated in the bean boiling wastewater at 25°C as affected by salinity adjustment using NaCl and the micrograph after 72 h. Bars indicate standard deviation (n = 3)

2.5. Conclusions

The four strains of *Aurantiochytrium* sp. were isolated from mangrove leaf samples namely L2R, L2Y, L3W, and L4Y. The strain L3W showed the high specific growth rate of 0.27 h^{-1}, and the 18S rDNA sequence analysis showed that this was a novel strain *Aurantiochytrium* sp. *Aurantiochytrium* sp. L3W grew at the ranges of temperature from 15°C to 35°C, pH from 3 to 9, and salinity from 0.3 PSU to 70 PSU, and the optimal condition for its biomass and lipid productions existed at 25°C, pH6—8, and 15—30 PSU.

Culturing *Aurantiochytrium* sp. L3W at 25°C, 30 PSU, and pH7, the resultant biomass contained 270 mg/g of FAs including 135 mg/g of DHA, 11 mg/g of pentadecanoic acid and 9.3 mg/g of margaric acid. As pH decreased or increased from 7, the abundance of FAs in the biomass of *Aurantiochytrium* sp. L3W became lower. However, production of DHA and these two OCFAs was confirmed at a range of pH from 3 to 9. The observed growth characteristics

of *Aurantiochytrium* sp. L3W in terms of pH and salinity tolerance might be advantageous in application of this strain for removal of organic compounds in food processing wastewater and production of FAs.

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Chapter 3: Removal of Organic Carbon and Nitrogen from Miso-production Wastewater and Simultaneous Production of PUFAs

3.1. Introduction

Thraustochytrids, such as *Aurantiochytrium*, are heterotrophic microorganisms and known to produce valuable polyunsaturated fatty acids (PUFAs), in particular docosahexaenoic acid (DHA) (Gao et al., 2013). DHA has received worldwide attention because of its beneficial effects on human health (Zhang et al., 2018, Shang et al., 2017). DHA supplements have been manufactured from fish oil abundant in PUFAs (Ward and Singh, 2005). Furthermore, DHA is also an essential constituent for fish growth, and therefore fish oil has been spiked to fish feed in fish aquaculture (Torrecillas et al., 2015). Since PUFAs have a great deal of potential in industries, the biomass of thraustochytrids has drawn attention as a new PUFAs source.

PUFAs production by thraustochytrids has been extensively studied (Raghukumar et al., 2008). Because of the heterotrophic nature of *Aurantiochytrium*, food industry wastewater such as *shochu* (traditional Japanese hard liquor) distillery wastewater (Yamasaki et al., 2006), coconut water (Unagul et al., 2007), waste syrup (Iwasaka et al., 2013), and corn steep liquor (Wang et al., 2019) have been applied for cultivation of thraustochytrids with successful PUFAs production. From the economical viewpoint, utilization of food processing wastewater is a good option for thraustochytrids cultivation because such wastewater contains significant usable nutrients and lacks toxic and hazardous substances that inhibit the growth of such useful microorganism (Tan et al., 2014).

Soybeans are used as raw materials in numerous Japanese foods such as miso (fermented condiment) of which manufacturing process produces wastewater (Hokamura et al., 2017). In the production of one ton of miso, 740 liters of the steamed soybean wastewater are generated with an oxygen demand of 32,000 ppm (Kimura et al., 1997). In Japan, over 100 million liters of wastewater is generated annually from soybean processed foods such as miso and considered to be a serious environmental pollutant (Hokamura et al., 2017). As introduced in Chapter 3, *Aurantiochytrium* sp. L3W was cultivated in the miso-processing (MP) and bean-boiling (BB) wastewater samples and attained removal of dissolved organic carbon (DOC) and dissolved nitrogen (DN) and simultaneous production of PUFAs. However, high concentrations of DOC and DN remained after the cultivation.

From a viewpoint of wastewater treatment, remaining DOC and DN in the used culture solution should be minimized. In parallel, this implies that yields with respect to the consumed carbon and nitrogen need to increase. In order to address these matters, the BB and MP wastewater samples were diluted and applied for cultivation of *Aurantiochytrium* sp. L3W, and effects of dilution on DOC and DN removals from the BB and MP wastewater samples, biomass and lipid productions were investigated.

3.2. Material and Methods

3.2.1. *Thraustochytrid and wastewater samples*

The thraustochytrid used in this study was *Aurantiochytrium* sp. strain L3W. This strain was pre-incubated for three days in 790 By+ medium, which consists of 5.0 g D(+)-glucose, 1.0 g polypeptone, and 1.0 g yeast extract in 1.0 L of seawater (Smallwood, 2010). The culture used for the inoculum was grown at 25 °C in a rotary cell culture system at 70 rpm. The BB and MP wastewater samples were obtained from a miso soup factory in Kure, Hiroshima Prefecture, Japan. Prior to the usage, the wastewater samples were allowed to stand overnight to allow suspended solids to settle out, and the supernatant was filtered through a 0.5-μm glass fiber filter (GC 50, Advantec, Japan).

The DOC and DN concentrations were analyzed by injecting the filtered samples into a total organic carbon analyzer (TOC-VSCN, Shimadzu, Japan) attached with a total nitrogen chemiluminescence measurement unit (TNM-1, Shimadzu, Japan). Salinity and pH values were measured using a combined electrical conductivity–pH meter (LAQUAtwin, Horiba, Japan). **Table 3.1** summarizes the water quality characteristics of the BB and MP wastewater samples. Note that the DOC and DN concentrations were different from that in the BB and MP wastewater samples used in Chapter 2 because of the different sampling day.

Table 3.1. Water quality of the bean boiling (BB) and miso processing (MP) wastewater samples.

Sample	pH	Salinity (PSU)	DOC (mg/L)	DN (mg/L)	C/N*
BB	4.0	3.5	10200	600	20
MP	3.2	6.4	2800	400	8.2
Control (790 By+)	7.0	30	5000	380	15

*Ratio (DOC/DN) was calculated on the molar basis.

3.2.2. Cultivation of the Aurantiochytrium sp.L3W using wastewater

The BB and MP wastewater samples were diluted 20 times, 10 times, and 5 times using MilliQ water. The diluted and undiluted BB and MP wastewater samples were adjusted to pH7 and 30 PSU using 1M NaOH and NaCl. After autoclave treatment at 120°C for 15 min, 100 ml of the sterilized wastewater samples were added in a 500 ml Erlenmeyer flask, and the precultured *Aurantiochytrium* sp.L3W was inoculated at 10^4 cells/ml. In the control experiment, the 790 By+ medium was used. Cultivation of *Aurantiochytrium* sp.L3W was carried out in triplicates at 25°C with a 70-rpm rotation. During the cultivation period, the cell numbers were periodically measured through microscopic observation using a Thoma hemocytometer (0.1 mm depth, Matsuyoshi, Japan). For the determination of the maximum growth, 10 ml of the culture solution was centrifuged at 8232 *g* for 15 min, and the cells pellet was washed twice with pure water. Finally, the sample was filtered through a glass fiber filter with 1.6 μm pore size (GF/A, Whatman, UK) and dried at 100°C for 3h followed by weighing.

3.2.3. Total lipid and fatty acid (FA) analyses

The salt-removed cell pellet after 72 h of cultivation was obtained from 50 ml of the culture solution by centrifugation as described above. After overnight lyophilization, 20 mg of the biomass was subjected to solvent extraction using a 2:1 mixture of chloroform: methanol to determine total lipid content (Folch et al., 1957).

To analyze the fatty acids in the lipid fraction, 20 mg of the lyophilized biomass was spiked with 100 µl of n-hexane solution containing undecanoic acid at 0.2 mg/L as an internal standard (Biller et al., 2014). The biomass was then subjected to extraction and methylation with a methylation kit (Nacalai Tesque, Japan). After purifying the methylated sample with a Simplified Liquid Extraction (SLE) tube (Strata, Phenomenex, USA), the samples were analyzed using a gas chromatograph equipped with a flame ionization detector (GC7820A, Agilent, USA) and DB-17 column (30 m × 0.250 mm × 0.5 µm, Agilent, USA). One microliter of the sample was injected in splitless mode at 300 °C with He as the carrier gas. The column temperature was set to 40 °C for 5 min followed by a gradual increase of the temperature at 4 °C/min to 250 °C and then at 30 °C/min to 280 °C. The temperature was finally held at 280 °C for 2 min[16]. A 37-component fatty acid methyl ester mixture (Sigma-Aldrich, USA) was used as the fatty acid methyl ester standard sample.

3.2.4. Statistical analysis

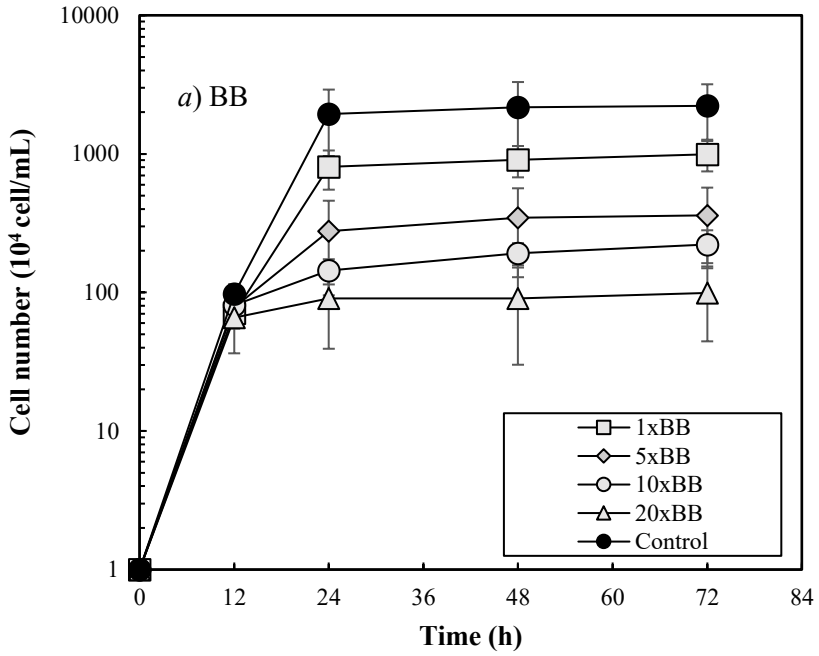
The statistical significance of cell growth and lipid/biomass production was determined by a one-way analysis of variance (ANOVA) followed by a bivariate Tukey comparison test. Statistical significance was accepted when $p < 0.05$.

3.3. Results and Discussion

3.3.1. Growth of Aurantiochytrium sp. L3W

Figure 3.1 shows the growth curve of *Aurantiochytrium* sp. L3W in the autoclaved BB and MP wastewater samples. *Aurantiochytrium* sp. L3W culture in the control media, diluted, and undiluted BB and MP samples quickly reached the logarithmic phase and achieved the stationary phase after 48 h. This cultivation experiment was stopped after 72 h following similar growth trends of most thraustochytrids and based on the expected maximum biomass production (Naganuma et al., 2006).

Comparing the growth of *Aurantiochytrium* sp. L3W in each BB and MP wastewater samples, significant differences were confirmed between non-diluted and 5 times dilutions ($p < 0.0002$) and between 5 times and 10 times dilutions ($p < 0.0348$) after 72 h. Although the differences were insignificant between 10 times and 20 times diluted BB ($p = 0.08171$) and MP ($p = 0.7183$) wastewater samples, the cell numbers under the 10 times dilution were apparently higher than the 20 times dilution. These results showed that less initial DOC and DN concentrations resulted in lowering the growth of *Aurantiochytrium* sp. L3W in the ranges of DOC and DN concentrations (**Table 3.1**).



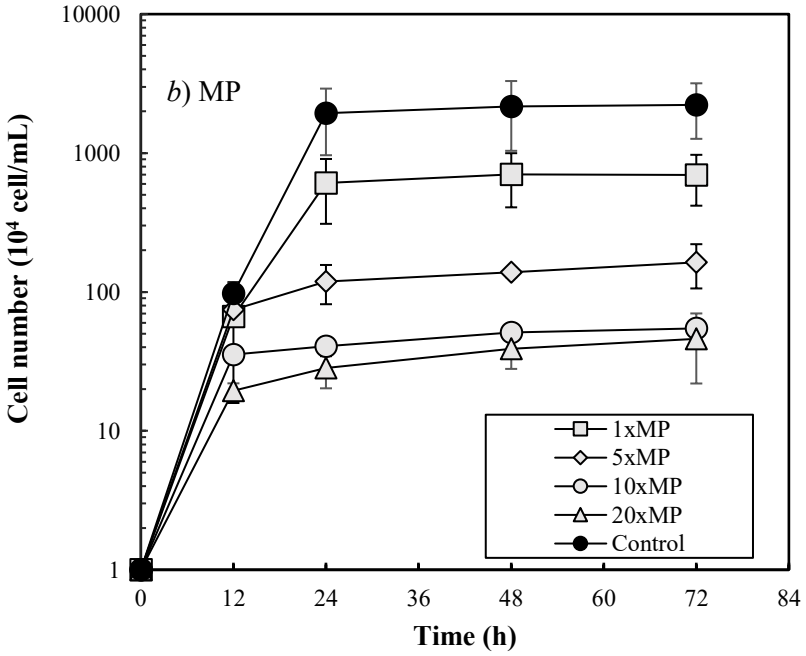


Figure 3.1. Growth curve of *Aurantiochytrium* sp. L3W in the pH- and salinity-adjusted BB (*a*) and MP (*b*). Bars indicate standard deviation (n=3).

3.3.2. Carbon and Nitrogen Consumption

Changes in DOC and DN concentrations during the cultivation of *Aurantiochytrium* sp. L3W in each wastewater sample are summarized in **Fig. 3.2**, where consumption by *Aurantiochytrium* sp. L3W was shown in the reduction of DOC and DN concentrations. The highest DOC and DN consumption were observed in the undiluted MP and BB wastewater, and the concentrations of removed DOC and DN were respectively 6876 mg/L and 354 mg/L in the undiluted BB wastewater sample and 1814 mg/L and 164 mg/L in the MP wastewater samples. Higher consumption of DOC and DN in the wastewater may be good in terms of utilization of resources; however, the remaining DOC and DN concentrations in the BB and MP wastewater samples were highest in the case of undiluted condition.

On the other hand, more dilution resulted in the less DOC and DN concentrations remaining after the cultivation with two exceptions for DOC in the 10 times and 20 times diluted MP wastewater samples. Comparing the C/N ratio in the BB and MP wastewater (**Table 3.1**), the latter was less abundant in DOC. In addition, the initial DOC concentration in the MP wastewater was lower. Collectively, a plausible explanation for no effect of 10 times and 20 times dilution on the remaining DOC concentration in the MP wastewater sample might be high contribution of the released metabolites from *Aurantiochytrium* sp. L3W.

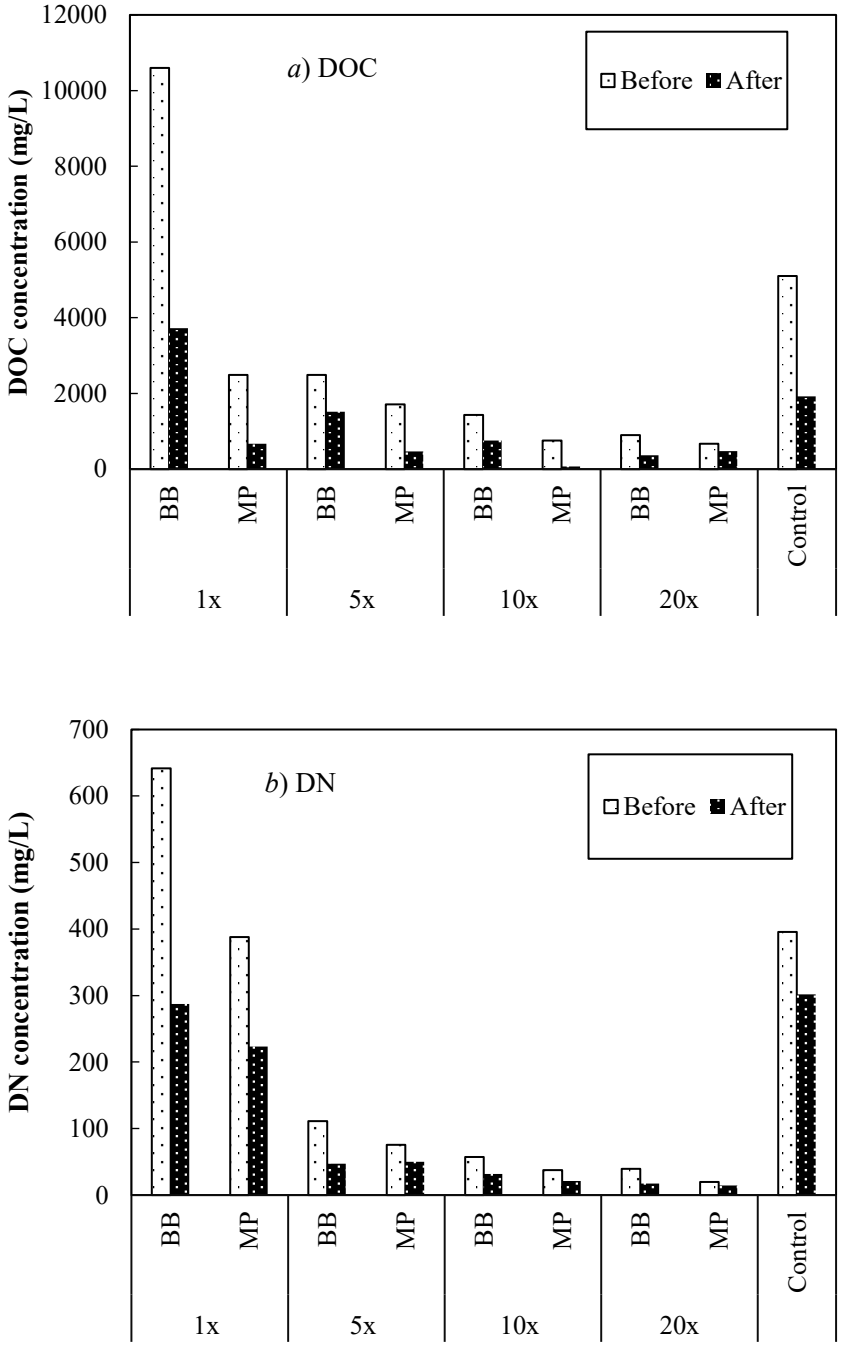


Figure 3.2. Dissolved organic carbon (DOC)(*a*) and dissolved nitrogen (DN)(*b*) concentrations in the BB and MP wastewater samples before and after cultivation of *Aurantiochytrium* sp. L3W.

According to the Sewerage Act in Japan, the sewage discharge standards for biological oxygen demand (BOD) and nitrogen in wastewater have been set by the national and local government, and these are dependent on industries and amounts of daily wastewater discharge. Taking the highest values set by the Higashihiroshima-shi municipal government, the standards for BOD and nitrogen are 600 mg/L and 120 mg/L, respectively (Higashihiroshima-shi, 2020). Because the initial DN concentration became less than 120 mg/L by dilution at 5 times, the diluted BB and MP wastewater samples naturally met the sewage discharge standard for nitrogen after cultivation of *Aurantiochytrium* sp. L3W. Applying approximately 1:2 ratio of organic carbon to BOD (Matsumoto et al., 1988), remaining DOC concentration should be less than 300 mg/L to meet the sewage discharge standard for BOD; however, both 20 times diluted BB and MP wastewater samples did not. In order to attain further reduction, longer cultivation duration and reuse of the used wastewater samples in a continuous multi-stage cultivation process could be feasible option.

3.3.3. Lipid and dry biomass

Figure 3.3 shows the production of biomass and lipid by *Aurantiochytrium* sp. L3W in the BB and MP wastewater samples. As for the MP wastewater, significant differences were confirmed in the biomass and lipid productions between non-diluted and 5 times diluted samples ($p = 0.0151$ and $p = 0.0005$, respectively) by one-way ANOVA analysis; however, the differences between the 5 times and 10 times dilutions and between 10 times and 20 times dilutions were insignificant. On the other hand, a difference of biomass production was significant between non- diluted and 5 times diluted BB wastewater samples ($p = 0.0135$), but the difference of lipid production was insignificant. In addition, we could not find significant differences by comparing the biomass and lipid productions in the MP wastewater samples at

the dilution levels more than 5 times; however, the biomass and lipid production apparently became lower, as the wastewater samples were diluted more.

A previous study reported that the abundance of glucose positively affected cell growth as well as lipid accumulation of several thraustochytrids up until the range of 30,000-50,000 mg/L (Guo et al., 2018). In addition, the DOC concentration in the wastewater samples used for thraustochytrids in the previous studies were 30,000mg/L in the *shochu* distillery wastewater (Yamasaki et al., 2006), 20,000 mg/L in the brewery spent yeast lysate (Ryu et al., 2013), and 50,000 mg/L in the cheese whey spiked medium (Humhal et al., 2019). Although the composition of organic compounds in the BB and MP wastewater might be different from that in the wastewater tested in the previous studies (Yamasaki et al., 2006); Ryu et al., 2013; Humhal et al., 2019), it was reasonable to conclude that dilution of the BB (10,200 mg/L DOC) and MP (2,800 mg/L DOC) wastewaters was unnecessary to obtain the highest biomass and lipid production from *Aurantiochytrium* sp. L3W.

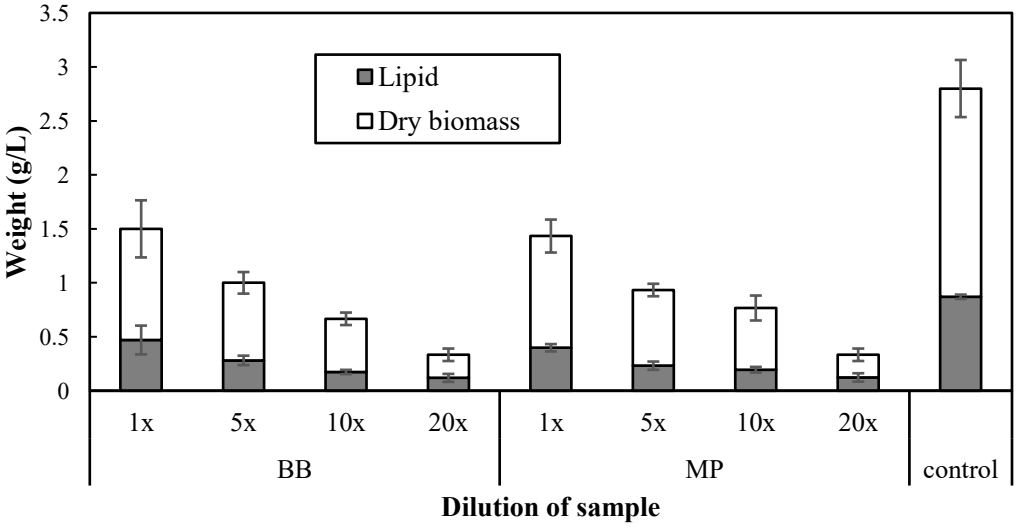


Figure 3.3. Dry biomass and lipid production by *Aurantiochytrium* sp. L3W in the BB and MP wastewater samples. Bars indicate standard deviation (n=3)

In contract, dilution was an option to improve the yields of biomass with respect to the consumed DOC and DN. Dividing the biomass production (**Fig. 3.3**) by the removed DOC and DN by *Aurantiochytrium* sp. L3W (**Fig. 3.2**), we calculated the yield. As shown in **Fig. 3.4**, the remarkable increase of biomass yield was observed by dilution in terms of the DOC and DN consumptions. Because of the inconsistency in the order of remaining DOC concentration in the MP wastewater samples (**Fig. 3.2**), we could not compare the yield between the BB and MP wastewater samples; however, the yield with respect to the consumed DN was higher in the MP wastewater samples. This result suggests that nitrogen sources in the MP wastewater sample might be more available for *Aurantiochytrium* sp. L3W. Future research should address compositions of organic compounds as well as their availability for thraustochytrids in BB and MP wastewater.

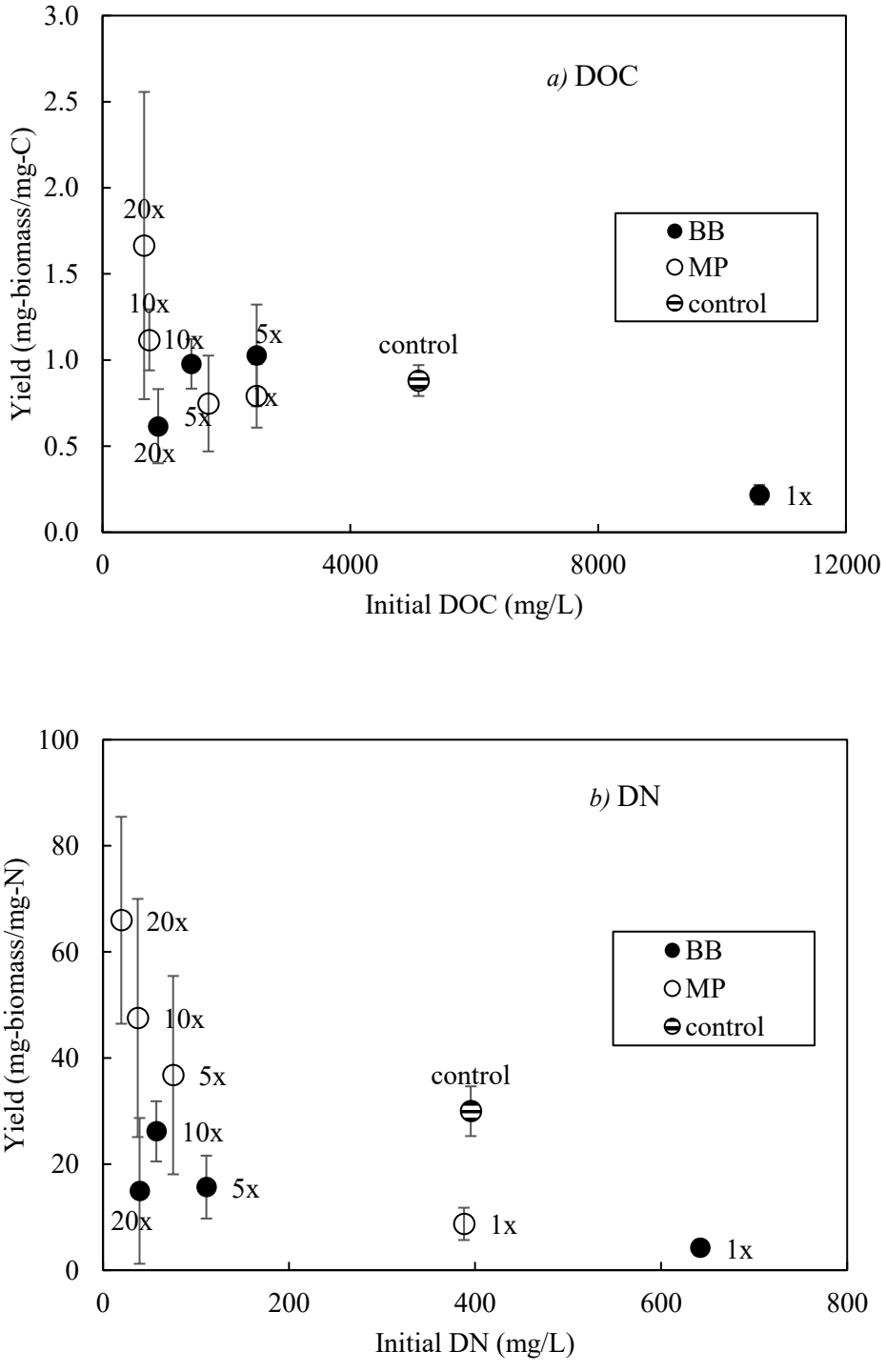


Figure 3.4. Yield of *Aurantiochytrium* sp. L3W biomass with respect to the dissolved organic carbon (DOC)(*a*) and dissolved nitrogen (DN)(*b*) consumptions

3.3.4. Fatty acid production

Figure 3.5 shows the fatty acid production in the BB and MP wastewater samples, where the total fatty acid content was higher in the undiluted BB wastewater sample than that in the undiluted MP wastewater sample. Although dilution was carried out up to 20 times, it was consistent that myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and docosahexaenoic acid (C22:6, DHA) were the major fatty acids accounting for more than 50% of the total fatty acids in the biomass of *Aurantiochytrium* sp.L3W. Among the four fatty acids, DHA was abundant in the biomass of *Aurantiochytrium* sp. L3W cultured in the undiluted BB and MP wastewaters, and the DHA content was 101 mg-DHA/g for the BB wastewater and 93.2 mg-DHA/g for the MP wastewater, respectively.

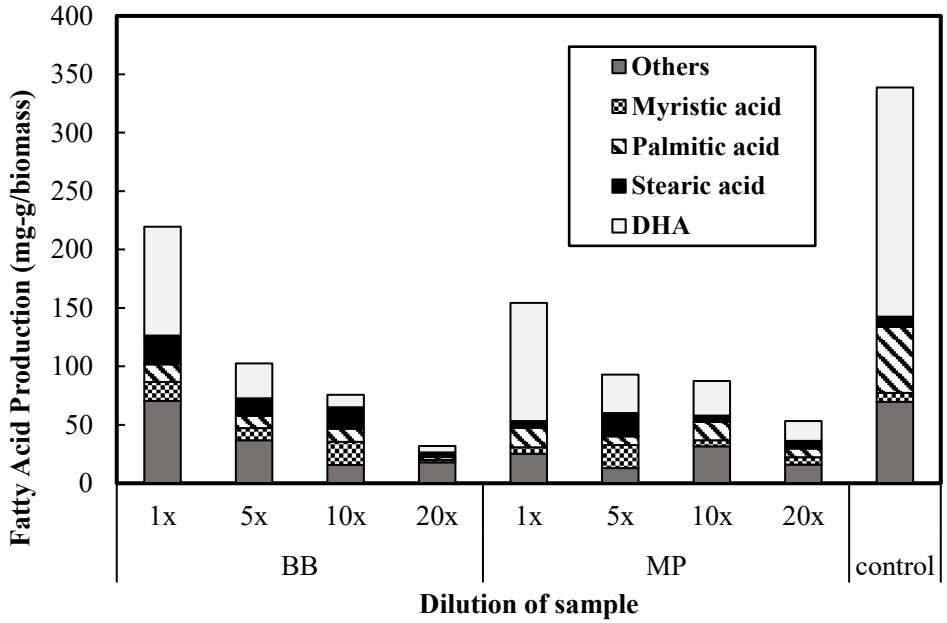


Figure 3.5. Fatty acid production of *Aurantiochytrium* sp. L3W in the BB and MP wastewater samples.

Although dilution resulted in less dominance of DHA, the DHA content in the biomass of *Aurantiochytrium* sp. L3W was 10.7 mg/g in the 10 times diluted BB wastewater sample and 16.8 mg/g in the 20 times diluted MP water sample. The DHA content from *Aurantiochytrium* sp. L3W was comparable to *Schizochytrium* sp. KH 105 (10 mg/g) cultured using the *shochu* distillery wastewater (Yamasaki et al., 2006) and *Aurantiochytrium* sp. KRS 101 (15.0 mg/g) cultured using the brewery spent yeast lysate (Ryu et al., 2013); however, *Aurantiochytrium* sp. L3W has a potential of containing DHA at higher levels (**Fig. 3.5**).

In this experiment, *Aurantiochytrium* sp. L3W was cultivated in a batch system. In order to manage better water quality and improvement of DHA production after culturing *Aurantiochytrium* sp. L3W, a continuous cultivation system may be applied under the optimized culture condition for better yield.

3.4. Conclusions

Dilution of the wastewater samples resulted in the less biomass and lipid production by *Aurantiochytrium* sp. L3W in the BB and MP wastewater; however, the remarkable increase of biomass yield was attained by dilution in terms of the DOC and DN consumptions. By diluting more than 5 times, the DN concentration in the BB and MP wastewater samples was maintained within the range in the sewage discharge standard for nitrogen set by the Higashihiroshima-shi municipal government. However, the remaining DOC was estimated to still exceed the sewage discharge standard for BOD, though the wastewater samples were diluted at 20 times. In general, dilution and sterilization of BB and MP was not required as a pre-treatment process prior to cultivation of *Aurantiochytrium* sp. L3W given that it could still readily consume the available dissolved organic carbon and nitrogen from the wastewater samples.

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Chapter 4: Application of *Aurantiochytrium* sp. L3W for food-processing wastewater treatment in combination with PUFAs production for fish aquaculture

4.1. Introduction

Thraustochytrids such as *Aurantiochytrium* are heterotrophic microorganisms that are known to produce valuable polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA). DHA and EPA have received worldwide attention because of their beneficial effects on human health (Zhang et al. 2016; Shang et al. 2017). DHA supplements have been manufactured from fish oil, which contains high concentrations of PUFAs (Ward and Singh 2005). Furthermore, satisfactory growth of fish requires that DHA and EPA be constituents of their food, and fish oil has therefore been added to the fish feed used for fish aquaculture (Torrecillas et al. 2017). Because PUFAs have a great deal of potential for use by the feed industry, the culture of thraustochytrids has been given serious consideration as a new source of PUFAs.

Production of PUFAs by thraustochytrids has been extensively studied (Trovão et al., 2020). Because of its high content of organic matter, food processing wastewater may be a cost-effective way to culture heterotrophic bacteria, although sterilization of large amount of wastewater may be required as a pretreatment (Yamasaki et al. 2006; Unagul et al. 2007; Ryu et al. 2013; Humhal et al. 2019; Rashid et al. 2020). However, the requirement for sterilization may reduce the cost-effectiveness, despite the use of the organic material available in the wastewater to grow the bacteria culture. Furthermore, in the context of wastewater engineering,

the need to remove pollutants, e.g. in the form of dissolved organic carbon and nitrogen, should also be addressed, but no study has addressed this particular issue.

Because thraustochytrids are halophilic, adjustment of salinity may result in improvement of biomass production (Chen et al. 2016). Addition of salt, which will be required to increase the salinity of the wastewater, may be advantageous because the increase of salinity may adversely impact contaminating microorganisms present in the wastewater. For example, a previous study demonstrated that raising the salinity helped to prevent a die-off of algae by inactivating the cilliates that appeared in a mixture of secondary-treated sewage and anaerobically digested effluent used for microalgal cultivation (Nakai et al., 2020).

Once successful cultivation of thraustochytrids in wastewater is achieved, then consideration of how to most effectively use the biomass will need to address the three feeding strategies that have been investigated to replace the fish oil in fish food: (i) enriching larval brine shrimp or rotifers with live thraustochytrid cells prior to feeding them to fish, (ii) directly feeding traustochytrids in the form of spray-dried or freeze-dried pellets, and (iii) formulating a fishmeal that includes thraustochytrid-derived oil as an ingredient (Fossier-Marchan et al., 2018). Although no pretreatment step may be preferable in terms of process simplification, a raw material composed of thraustochytrid biomass has not been used in fish aquaculture. Confirmation of the digestibility of thraustochytrid biomass by fish enzymes would be the first step to exploring this possibility.

In the present study, we tested the feasibility of using thraustochytrids to treat wastewater discharged from a miso factory and to simultaneously produce PUFAs. The thraustochytrid biomass produced in this step was incorporated into feed for aquacultured fish. To carry out this study, we cultivated thraustochytrids in sterilized and unsterilized bean-boiling (BB) wastewater and miso-processing (MP) wastewater. We measured the removal of dissolved organic carbon (DOC) and dissolved nitrogen (DN) as well as the production of PUFAs during

this step. Finally, we investigated the digestibility of the thraustochytrid biomass by enzymes extracted from fish stomachs.

4.2. Materials and Methods

4.2.1. Thraustochytrid and wastewater samples

The thraustochytrid used in this study was *Aurantiochytrium* sp. L3W, because of its high specific growth rate and its durability to the acidic condition. This strain was pre-incubated for three days in 790 By+ medium, which consists of 5.0 g D(+)-glucose, 1.0 g polypeptone, and 1.0 g yeast extract in 1.0 L of seawater (Smallwood 2010). The culture used for the inoculum was grown at 25 °C in a rotary cell culture system at 70 rpm.

The BB and MP wastewater samples were obtained from a miso soup factory in Hiroshima, Japan. Prior to use, the wastewater samples were left to stand overnight to allow suspended solids to settle out, and the supernatant was filtered through a 0.5-μm glass fiber filter (GC50, Advantec, Japan). The DOC and DN concentrations were analyzed by injecting the filtered samples into a total organic carbon analyzer (TOC-VSCN, Shimadzu, Japan) with attached total nitrogen chemiluminescence measurement unit (TNM-1, Shimadzu, Japan). Salinity and pH values were measured with a combination electrical conductivity–pH meter (LAQUAtwin, Horiba, Japan). **Table 3.1** summarizes the water quality characteristics of the BB and MP wastewater samples**.**

Table 4.1. Water quality characteristics of the wastewater samples

Wastewater	pH	Salinity (PSU)	DOC (mg/L)	DN (mg/L)
Miso Processing (MP)	4.2	6.4	7600	500
Bean Boiling (BB)	5.0	4.5	2900	400
790 By+ (control)	7.0	30	3900	320

4.2.2. Cultivation under sterile and unsterile conditions

We tested the potential of the BB and MP wastewater samples as culture media under sterile and unsterile conditions. Because the DOC and DN concentrations differed between the media derived from BB and MP wastewater and diluted wastewater would get better production, the two wastewater samples were diluted fivefold with ultrapure water. The diluted and undiluted BB and MP wastewater samples were adjusted to a salinity of 30 PSU using NaCl. However, the pH was not adjusted because the pH of the BB and MP wastewater samples were in the range within which *Aurantiochytrium* sp. L3W can grow well.

For both MP and BB, 600 ml non-diluted of each wastewater samples were divided into six 500 ml Erlenmeyer flask, each containing 100 ml of the wastewater samples. Three flasks were then autoclaved at 120 °C for 15 min for sterilization. For other dilution levels, 600 ml of each wastewater samples were prepared and then divided in similar manner. Both the sterilized and unsterilized media were inoculated with a log-phase strain of *Aurantiochytrium* sp. L3W at 10^4 cells/ml. We also inoculated *Aurantiochytrium* sp. L3W into 790 By+ medium as a control experiment. Cultivation of *Aurantiochytrium* sp. L3W was carried out in triplicate at 25 °C in a rotary cell culture system at 70 rpm for 72 h. During the cultivation period, cell numbers were periodically counted under a microscope at 400 × magnification with a Thoma hemocytometer (0.1-mm depth, Matsuyoshi & Co., Ltd., Japan). To measure dry weight biomass after 72 h of

cultivation, 10 ml of the culture solution was centrifuged at 8232 × *g* for 15 min, and the cell pellet was washed twice with pure water. Finally, the sample was filtered through a 0.7-μm pore size glass fiber filter (GF/F, Whatman, UK), and the residue was dried at 100 °C for 3 h followed by weighing.

4.2.3. Lipid analyses

A cell pellet after 72 h of cultivation was obtained from 50 ml of the culture solution by centrifugation as described above. After overnight lyophilization, 20 mg of the biomass was subjected to solvent extraction using a 2:1 mixture of chloroform: methanol to determine total lipid content (Folch et al. 1957). To analyze the fatty acids (FAs) in the lipid fraction, 20 mg of the lyophilized biomass was spiked with 100 μl of n-hexane solution containing at 0.2 mg/L undecanoic acid as an internal standard. The biomass was then subjected to extraction and methylation with a methylation kit (06482-04, Nakalai Tesque, Japan).

After purifying the methylated sample with a Simplified Liquid Extraction (SLE) tube (Strata, Phenomenex, USA), the samples were analyzed using a gas chromatograph equipped with a flame ionization detector (GC7820A, Agilent, USA) and DB-17 column (30 m × 0.250 mm × 0.5 μm, Agilent, USA). One microliter of the sample was injected in splitless mode at 300 °C with He as the carrier gas. The column temperature was set to 40 °C for 5 min followed by a gradual increase of the temperature at 4 °C/min to 250 °C and then at 30 °C/min to 280 °C. The temperature was then held at 280 °C for 2 min (Biller et al. 2014). A 37-component fatty acid methyl ester mixture (Sigma-Aldrich, USA) was used as the fatty acid methyl ester standard sample.

4.2.4. Digestion of Aurantiochytrium sp.L3W biomass using fish enzymes

Six individual rainbow trout (*Onchorhynchus mykiss*) with lengths of about 15 cm were starved in an aquaculture tank for three days and dissected in the ice bath at 0–4 °C. Each stomach was washed with distilled water, and samples of the stomach gut were then immediately frozen at –20 °C for later use. For extraction of digestion enzymes, 20 g-wet of the stomach-gut sample was homogenized at 4 °C in a mixture of 100 ml of 100 mM Tris-HCl buffer and 50 ml of 20 mM $CaCl_2$ (pH 8.0) (Cara et al. 2003). The homogenate was centrifuged at 600 × *g* for 10 min at 4 °C, and then the supernatant was further centrifuged for 10 min at 10,000 × *g* at 4 °C (Dinu et al. 2002). The pellets were suspended in the modified Tris-HCl buffer and centrifuged at 4000 × *g* for 10 min at 4 °C. The clear supernatant was used for subsequent studies.

Aurantiochytrium sp.L3W was cultured in 790 By+ medium for three days. The culture solution was then centrifuged at 8232 × *g* for 15 min at 25 °C. After the supernatant had been discarded, the pellet was washed with sterilized and sand-filtered seawater (salinity of 30). Finally, the pellet was suspended in about 6 ml of supernatant from the digestion enzyme extract, and it was incubated at 25 °C for 4 h. Before and after the incubation, cell numbers were counted with a microscopic and hemocytometer.

4.2.5. Statistical analysis

The statistical significance of cell growth and lipid/biomass production was determined by a one-way analysis of variance followed by a Tukey test. The data were assumed to be accepted $p < 0.05$.

4.3. Results and Discussion

4.3.1. Growth of Aurantiochytrium *sp.L3W and its consumption of DOC and DN*

Figure 4.1 shows the growth curve of strain L3W in the sterilized BB and MP wastewater samples at a salinity of 30. The experiment was suspended after 72 h because the increase of cell numbers in the control experiment was less than 10% between 48 h and 72 h. Similar growth of thraustochytrids has been reported in control cultures by Ma et al. (2015) and Ganuza et al. (2019).

Aurantiochytrium sp.L3W showed similar growth curves in the BB and MP wastewater samples, though the compositions of dissolved compounds might be different in addition to the initial DOC and DN concentrations (**Table 4.1**). In addition, the cell numbers in the BB and MP wastewater samples were lower than that in the 790 By+ medium. The plausible explanation might be that availability of substrates for *Aurantiochytrium* sp. L3W in both wastewater samples was similar but less than that in the 790 By+ medium used in the control experiment.

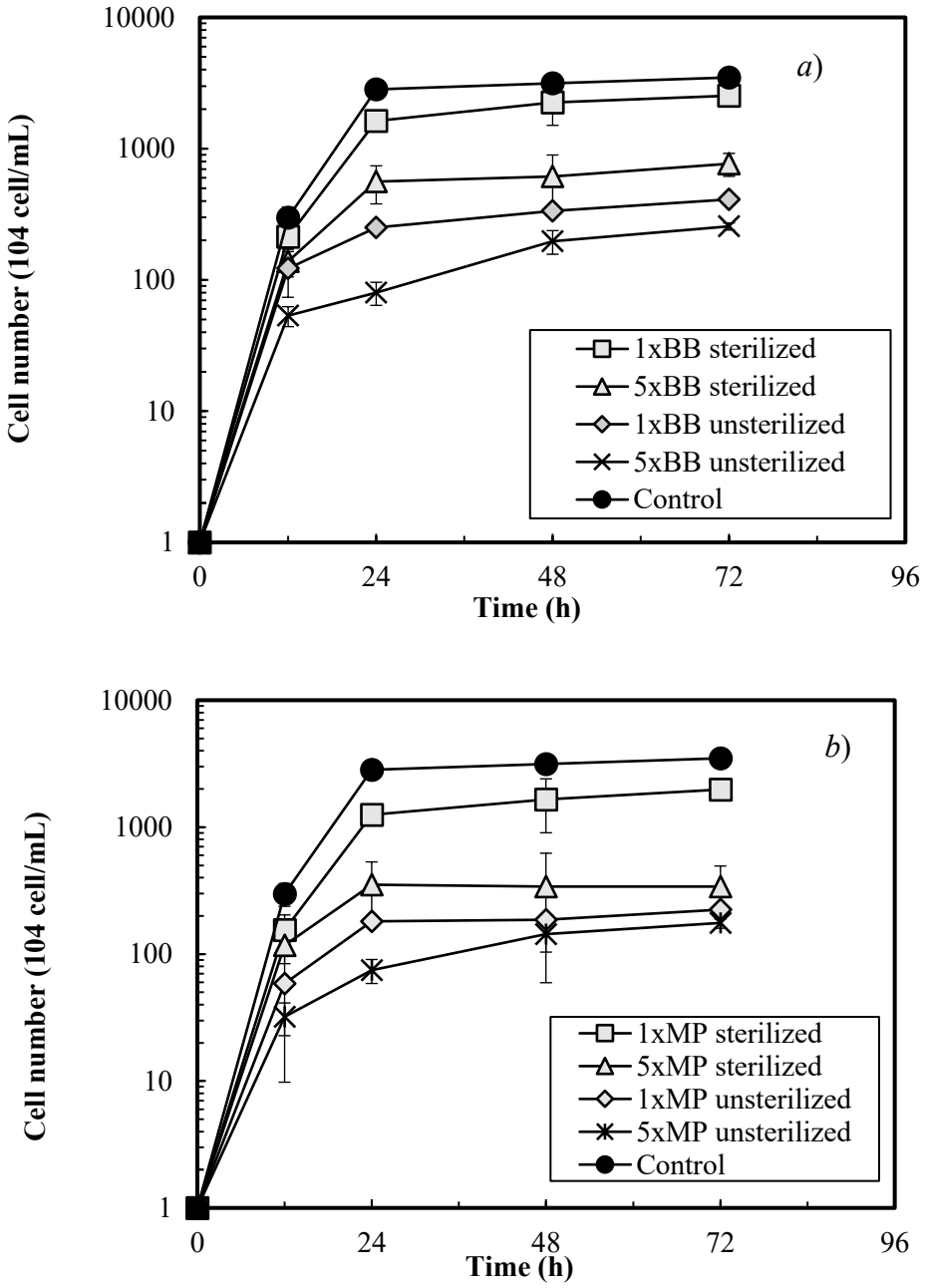

Figure 4.1. Growth curve of *Aurantiochytrium* sp. L3W in BB (*a*) and MP (*b*) wastewater with a salinity adjusted to 30 under sterile and unsterile conditions. Bars indicate standard deviation ($n = 3$).

Under sterile conditions, cell growth was higher in the undiluted BB and MP wastewater than in the fivefold-diluted wastewater ($p = 0.0022$ and 0.0099, respectively; **Fig. 4.1*a*** and **4.1*b***, respectively). When the unsterilized BB and MP wastewater samples were the growth media, the growth of *Aurantiochytrium* sp. L3W was lower in both BB ($p = 0.0006$) and MP ($p = 0.0421$) wastewater compared to the growth in sterilized wastewater. The cause of the lower growth might have been the presence of contaminating microorganisms such as bacteria and yeasts. Although sterilization may enhance the growth of *Aurantiochytrium* sp. L3W in terms of cell numbers, the results indicated that the BB and MP wastewater samples were suitable for cultivation of *Aurantiochytrium* sp.L3W without sterilization.

Removal of DOC and DN by the growth of *Aurantiochytrium* sp. L3W is summarized in **Fig. 4.2** for the experiments where the reduction of DOC and DN concentrations in the BB and MP wastewater samples was confirmed. The results showed that *Aurantiochytrium* sp. L3W could remove DOC and DN from these food-processing wastewaters. The concentrations of DOC and DN that remained in all tested BB and MP wastewater samples exceeded 1025 mg/L and 840 mg/L, respectively. Although *Aurantiochytrium* sp. L3W used some of the DOC and DN in the BB and MP wastewater samples, parts of DOC and DN were remaining in the wastewater samples, most probably in the form of lactic and citric acids compounds (Matsuo, 2006**)**. Further treatment will therefore be necessary before the wastewater can be discharged.

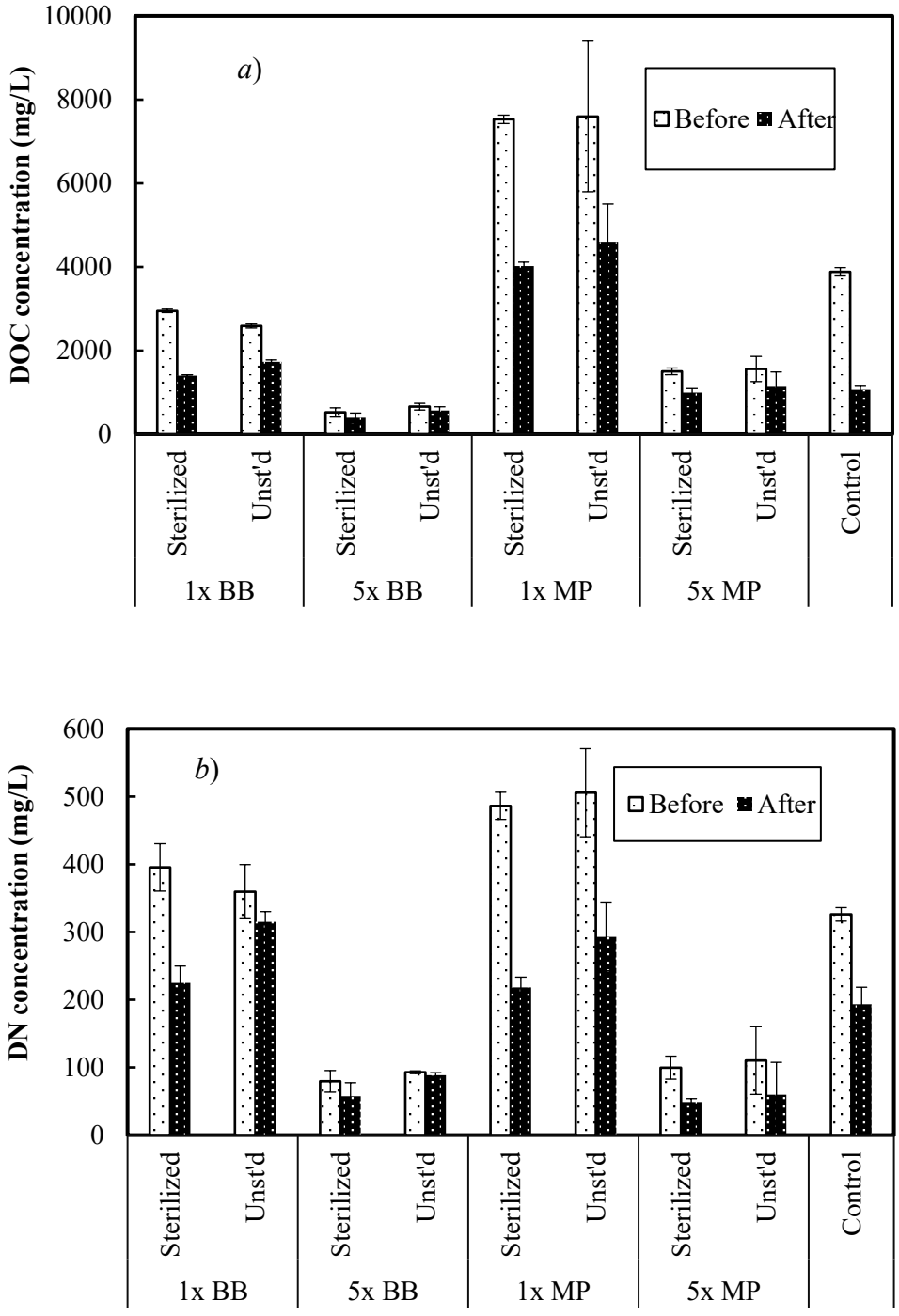

Figure 4.2. Dissolved organic carbon (DOC, *a*) and dissolved nitrogen (DN, *b*) concentrations in BB and MP wastewater with a salinity adjusted to 30 before and after cultivation of *Aurantiochytrium* sp. L3W under sterile and unsterile conditions. Bars indicate standard deviations ($n = 3$).

4.3.2. Biomass and lipid production

Figure 4.3 compares the biomass and lipid production of *Aurantiochytrium* sp. L3W in the BB and MP wastewater samples. Production was in the following order from highest to lowest: undiluted and sterilized, undiluted and unsterilized, fivefold-diluted and sterilized, and fivefold-diluted and unsterilized. Production on average was significantly higher in sterilized treatments. The one exception was lipid production in the undiluted and unsterilized BB wastewater sample, where there was a large standard deviation. This result might have been due to the fact that the measured biomass and lipid production in the unsterilized samples contained some contaminating microorganisms, because the order in terms of biomass was not the same as the order in terms of cell numbers (**Fig. 4.1)**.

Even though unsterilized conditions were less favourable for *Aurantiochytrium* sp. L3W, the results confirmed that biomass and lipid could be produced by this strain in BB and MP wastewater. In every comparison of diluted and undiluted treatments, the undiluted treatments resulted in significantly higher biomass production ($p = 0.0033$). Lipid production followed a similar pattern on average, but the differences were not statistically significant ($p = 0.4170$) because the results were more variable. Based on these results, it appears that dilution was unnecessary to achieve satisfactory production of biomass and lipid.

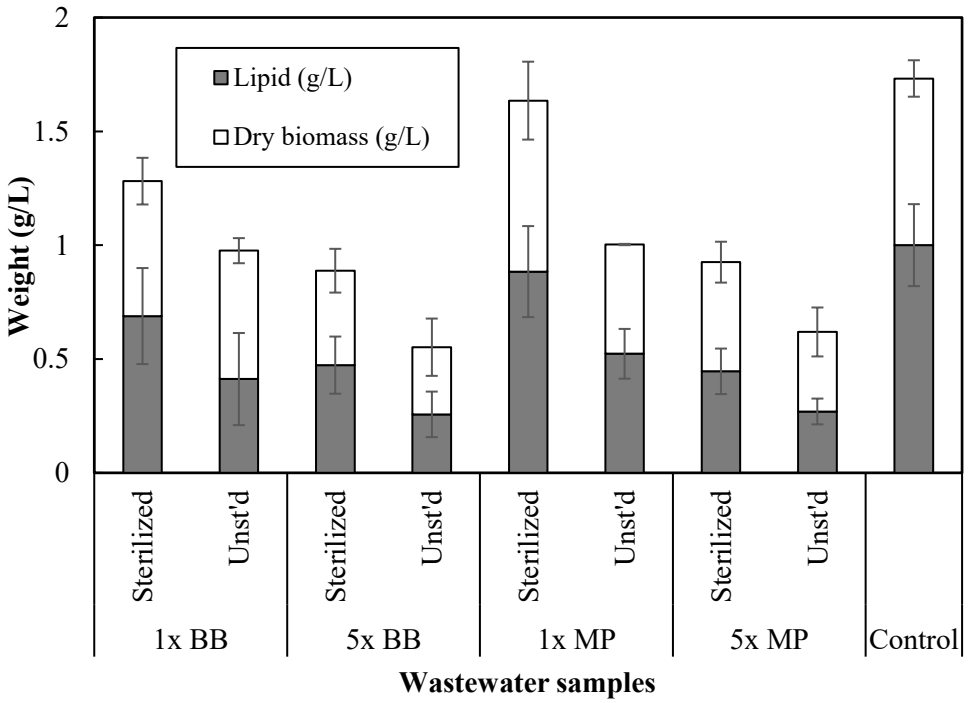

Figure 4.3. Dry biomass and lipid production by *Aurantiochytrium* sp. L3W in BB and MP wastewater with the salinity adjusted to 30 under sterile and unsterile conditions. Bars indicate standard deviations (n = 3).

Table 4.2 summarizes the amounts of FAs, including DHA and EPA, in the biomass of *Aurantiochytrium* sp. strain L3W produced in each treatment. Production of total FAs, DHA, and EPA by *Aurantiochytrium* sp.L3W in the sterilized BB and MP wastewater were almost the same; however, the production was lower in these treatments than production in By 790+ medium in the control experiment. In addition, the FA profiles differed among the three sterilized media **(Fig. 4.4, Table 4.2)**. Although the concentrations of DHA (C22:6) were the highest in the three sterilized media, the concentrations of docosadioneic acid (C22:2), EPA (C20:5), and heptanoic acid (C17:0) were the second, third, and fourth highest in the undiluted and sterilized BB and MP samples. In the By 790+ medium used in the control experiment, the concentration of palmitic acid (C16:0) was the second highest.

Synthesis of DHA via C16:0 in fatty acid synthase (FAS) pathway is known (Metz et al., 2001). In addition to FAS pathway, thraustochytrids have polyketide synthase (PKS) pathway to synthesize DHA (Ye et al., 2019). The different compositions of FAs between the control and the sterilized BB and MP wastewater (**Fig. 4.4**) might be due to differences of the dominant FAs synthesis pathway and/or its progress caused by different substrates, as a previous study confirmed the effect of different carbon and nitrogen sources in the substrate on FAs composition (Perez et al., 2019). The reason for the inconsistency between the undiluted and fivefold-diluted samples for each BB and MP treatment might be the variability of the analytical results due to the low detection levels.

When unsterilized wastewater samples were used, the fatty acid compositions differed. In the BB wastewater samples, the concentrations of DHA were consistently the highest; however, the concentrations of palmitic acid (C16:0) and oleic acid (C18:1) were the second and third highest, respectively. This difference might be due to contamination of bacteria and yeast (Bellou et al., 2016); however, the dominance of DHA suggests that *Aurantiochytrium* sp. L3W was still the major fatty-acid producer in the unsterilized BB wastewater samples.

Production of DHA was confirmed in the unsterilized MP wastewater samples (**Table 4.2**); however, the concentration of oleic acid (C18:1) was the highest, and the high concentration of linolelaidic acid (C18:2) was notable (**Fig. 4.5**). In addition, EPA production was not confirmed (**Table 4.2**). These results indicate the much greater impact that contaminating microorganisms had on the production of PUFAs by *Aurantiochytrium* sp. L3W in the MP wastewater samples than in the BB wastewater samples.

4.3.3. PUFAs production

Sterilized condition consistently produced larger amount of PUFAs compared to unsterilized condition for both wastewater samples at both dilution factors (**Fig. 4.4**), which could be attributed due to absence of competition from contaminating organisms. DHA had always been the largest proportion in the produced PUFAs condition, e.g. even unsterilized BB wastewater samples yielded 51.2% DHA by mass (**Table 4.2**). The relatively high concentration of salt in the salinity-adjusted wastewater samples may suppress the growth of contaminating microorganism(s).

When the growth media were the undiluted and sterilized BB and MP wastewater, the concentrations of DHA and EPA in the biomass of *Aurantiochytrium* sp. L3W were 96.2 mg-DHA/g and 1.79 mg-EPA/g for the BB wastewater and 97.8 mg-DHA/g and 1.40 mg-EPA/g for the MP wastewater (**Table 4.2**). These concentrations are higher than those previously reported in microorganisms: 10 mg-DHA/g and no EPA (Yamasaki et al. 2006), 15.0 mg-DHA/g and 0.14 mg-EPA/g (Ryu et al. 2013), and 43.6 mg-DHA/g and no EPA (Humhal et al. 2019).

When undiluted and unsterilized BB wastewater was the growth medium, the DHA and EPA contents (61.2 mg-DHA/g and 1.19 mg-EPA/g) were comparable to those achieved with fivefold-diluted and sterilized BB as the growth medium and still higher than the values in previous studies. These results confirmed the potential for use of BB and MP wastewater as growth media for PUFA production as well as DOC and DN removal using *Aurantiochytrium* sp. L3W. In addition, BB wastewater may be used after salinity adjustment without sterilization; however, use of MP as a growth medium may require suppression of contaminating microorganisms for better PUFA production.

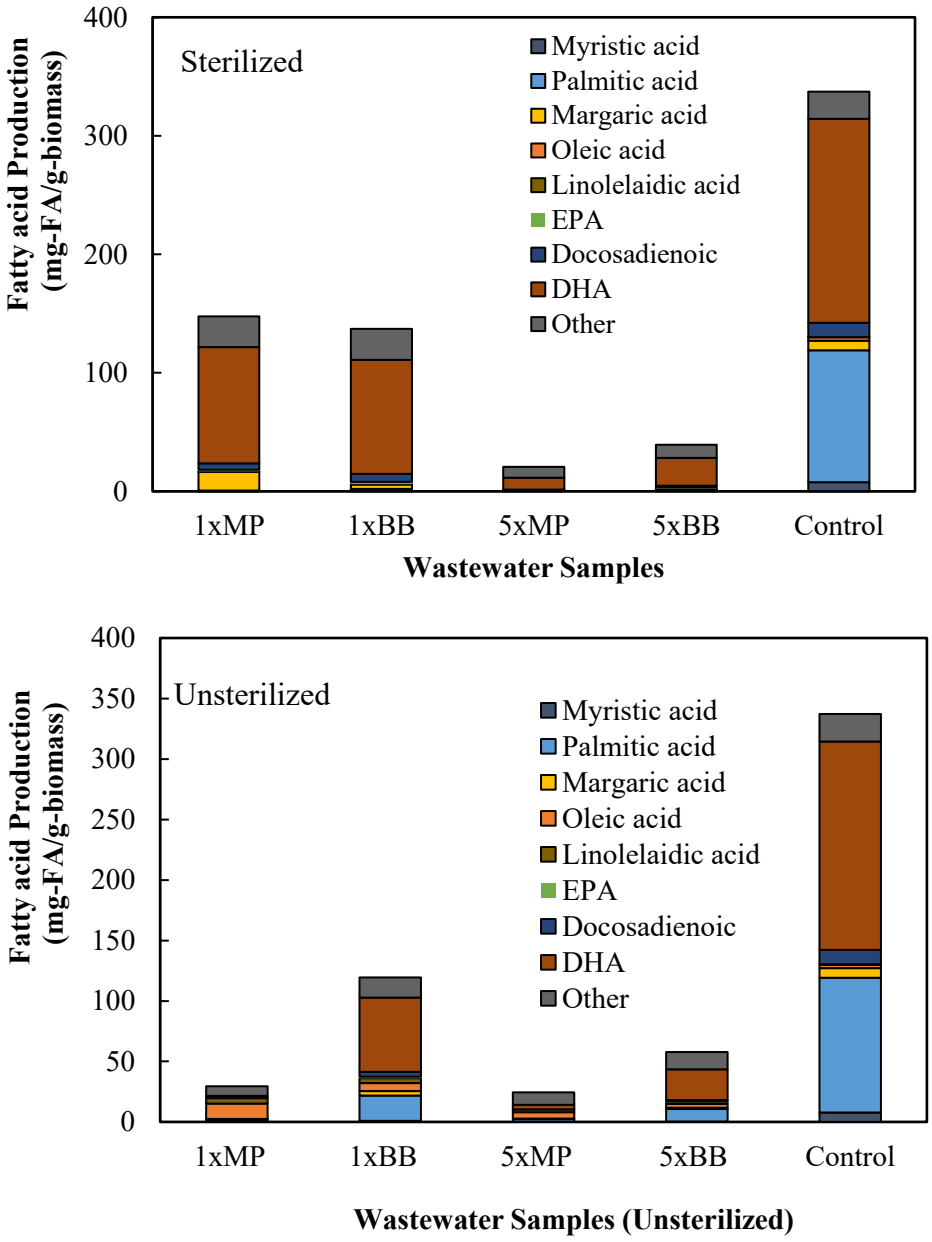

Figure 4.4. PUFA produced from cultivation on salinity-adjusted wastewater samples (30 psu) under sterilized (a) and unsterilized (b) condition.

Table 4.2. Fatty acid content of *Aurantiochytrium* sp. L3W cultivated in BB and MP wastewater samples.

FA contents	BB wastewater				MP wastewater				Control
	Sterilized		Unsterilized		Sterilized		Unsterile		
	1x	5x	1x	5x	1x	5x	1x	5x	
Total FAs	137	39.3	119.5	57.7	148	21	29	24	337
DHA	96.2	23.4	61.2	25.1	97.8	9.83	7.83	3.70	172
EPA	1.79	0.93	1.19	1.07	1.40	0.131	N.D.*	N.D.*	0.142
Oleic	0.34	0.29	6.64	3.25	0.21	N.D.*	12.8	5.26	2.89
Palmitic	0.63	0.62	20.9	10.2	0.43	0.39	1.44	1.92	111

*Not detected (<0.01 mg/g)

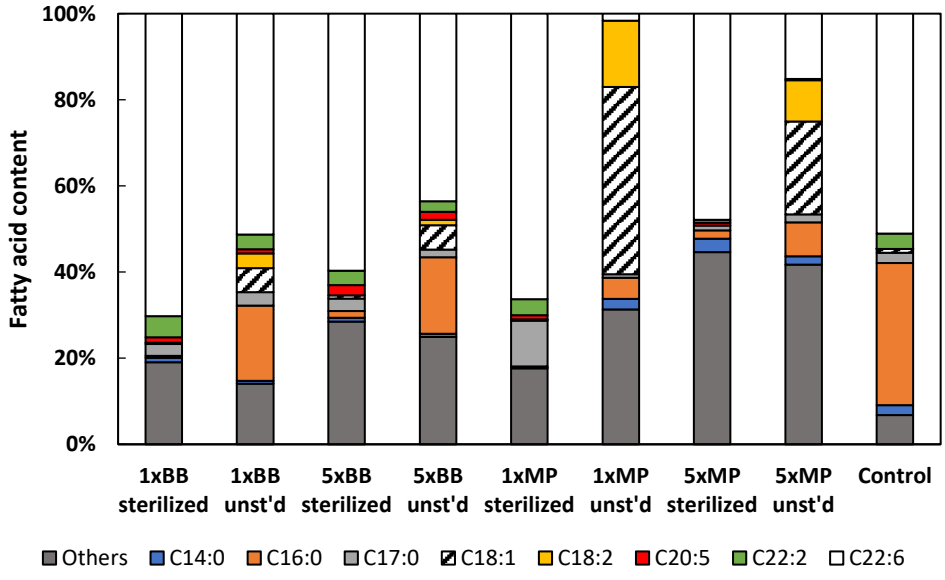

Figure 4.5. Percentages of PUFAs in *Aurantiochytrium* sp. L3W grown in BB and MP wastewater adjusted to a salinity of 30 under sterile and unsterile conditions.

4.3.4. Investigation of Aurantiochytrium sp. L3W biomass for fish feedstock

Figure 4.6 shows the changes of the cell shape of *Aurantiochytrium* sp.L3W by the enzymes extracted from the stomachs of rainbow trout. *Aurantiochytrium* sp.L3W cells were at first densely packed with a large, globular shape (**Fig. 4.6*a***). It has been reported that the cell walls of thraustochytrids consist of thin, flexible, and nearly circular scales of proteinaceous or mucopolysaccharide components (Darley et al. 1973). The disruption of the cell wall after 4 h (**Fig. 4.6*b***) confirmed the digestion of *Aurantiochytrium* sp.L3W biomass by the enzymes extracted from the fish stomachs. As mentioned before, the biomass of thraustochytrids has been incorporated into fish feed; however, dried biomass was used in those studies (Nobrega et al. 2019, Sevgili et al. 2019).

Because the drying process may cause protein denaturation and rupture the cell walls prior to digestion, we used the raw biomass of *Aurantiochytrium* sp.L3W in for the digestion experiment. The confirmation by the results of the enzymic digestion experiment that the biomass of *Aurantiochytrium* sp. L3W is digestible by fish provides the first evidence that the raw biomass of thraustochytrids can be used for fish aquaculture as a source of PUFAs in feed.

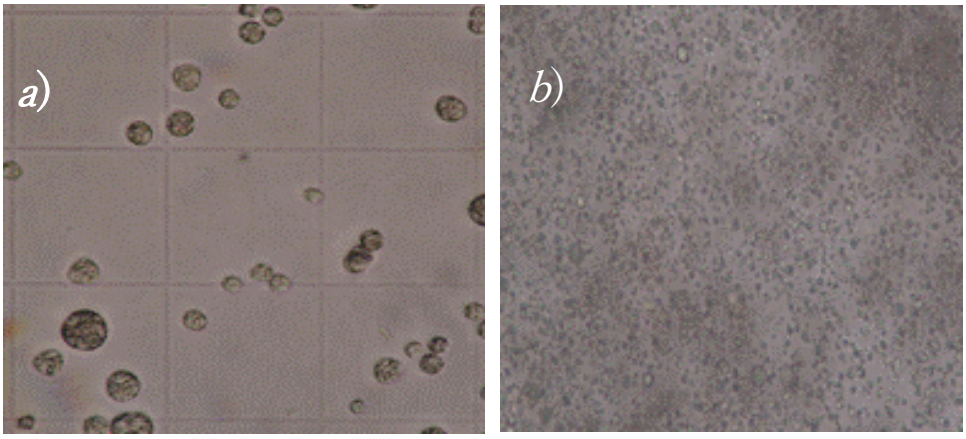

Figure 4.6. Changes in cell shape of *Aurantiochytrium* sp. L3W by enzymes extracted from rainbow trout stomachs. Control (*a*) and enzyme extract (*b*), 4 hours after L3W biomass addition.

4.4. Conclusions

Aurantiochytrium sp. strain L3W was cultivated in bean-boiling (BB) and miso-processing (MP) wastewater discharged from a miso factory. *Aurantiochytrium* sp. L3W could grow in salinity adjusted and unsterilized BB and MP wastewater and removed DOC and DN; however, additional treatment with different technologies might be necessary to reduce DOC and DN to acceptable concentrations. The amounts of DHA and EPA produced in the undiluted, sterilized wastewater were 96.2 mg/g and 1.79 mg/g, respectively, for BB wastewater and 97.8 mg/g and 1.40 mg/g, respectively, for MP wastewater. These results confirmed the potential to grow *Aurantiochytrium* sp. L3W in BB and MP wastewater to produce PUFAs and remove DOC and DN. We also confirmed that undiluted BB wastewater may be used after salinity adjustment to 30 PSU without sterilization. Finally, the enzymic digestion experiment showed that *Aurantiochytrium* sp. L3W biomass was digestible by enzymes extracted from rainbow trout. The result provides the first evidence that the raw biomass of thraustochytrids can be used for fish aquaculture as a source of PUFAs in feed.

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Chapter 5: Summary and Conclusions

Chapter 1 introduced usefulness of PUFAs for human consumption from a viewpoint of health benefits and aquaculture to maintain growth of fish and mentioned the fact that fish oil has been used as a conventional PUFAs production source. In addition, necessity of its alternation was pointed out due to issue of sustainability of fish supply, competition with direct consumption as food for human and the high price of the commodity, and thraustochytrids were introduced as a promising PUFAs source. After reviewing the previous studies related to production of PUFAs using thraustochytrids, challenges for practical and sustainable production of PUFAs using thraustochytrids were addressed by utilization of food processing wastewater as the culture media. Cultivation process under unsterile condition is desired to avoid energy-consuming pretreatment processes, such as sterilization. Such cultivation will require durable strain that is able to grow in unsterile condition against contaminating microorganism. Furthermore, examples for utilization of thraustochytrid biomass for fish aquaculture were introduced, and finally the objectives and flow of this study were explained.

Chapter 2 described isolation of strains of thraustochytrids, identification of the isolated strains and their growth and PUFAs production characteristics. These were done to address the requirement of thraustochytrids cultivation high salinity and low pH condition to reduce the growth of contaminating microorganism, instead of sterilization process. The four strains of *Aurantiochytrium* sp. were isolated from the mangrove leaf samples, and *Aurantiochytrium* sp. L3W showed the high specific growth rate of 0.27 h^{-1} at 25°C. In addition, the 18S rRNA gene sequence analysis showed that this strain is novel. *Aurantiochytrium* sp. L3W grew at the ranges of temperature from 15°C to 35°C, pH from 3 to 9, and salinity from 0.3 to 70 PSU. Under the optimal condition of 25°C, 30 PSU, and pH7, *Aurantiochytrium* sp. L3W produced 270 mg/g of FAs including 135 mg/g of DHA, 11 mg/g of pentadecanoic acid and 9.3 mg/g of margaric

acid. Furthermore, *Aurantiochytrium* sp. L3W produced DHA and these FAs at pH3 and pH9 of which has no prior study reported a strain of thraustochytrids capable to grow at pH3. The pH and salinity tolerance of *Aurantiochytrium* sp. L3W might be advantageous in its application for production of valuable FAs under competition with other microorganisms in unsterile wastewater.

Chapter 3 investigated effects of dilution of the MP and BB wastewater discharged from a miso factory on the removal efficiency of dissolved organic carbon and dissolved nitrogen from the wastewaters and biomass and PUFAs production by *Aurantiochytrium* sp. L3W for the compatibility. Dilution up to 20 times resulted in the less biomass and lipid productions, however the remarkable increase of biomass yield was attained in terms of the DOC and DN consumptions. By diluting more than 5 times, the DN concentration in the BB and MP wastewater samples was maintained within the range in the sewage discharge standard for nitrogen set by the Higashihiroshima-shi municipal government. However, the remaining DOC was estimated to exceed the sewage discharge standard for BOD despite 20 times dilution. In addition, DHA production by *Aurantiochytrium* sp. L3W was reduced by dilution; however, the DHA content in the biomass of *Aurantiochytrium* sp. L3W was 10.7 mg/g in the 10 times diluted BB wastewater sample and 16.8 mg/g in the 20 times diluted MP water sample. It was concluded that both BB and MP are applicable for cultivation both to remove DOC and DN as wastewater treatment effort and simultaneous PUFAs production, with the highest DHA was 101 mg/g-biomass from BB and 93.2 mg/g-biomass from MP. Dilution was found to increase the biomass yield per consumed DOC/DN despite the lower concentration of the produced biomass from the cultivaton.

Chapter 4 investigated usability of miso-processing (MP) and bean boiling (BB) wastewater discharged from a miso factory for cultivation of *Aurantiochytrium* sp. strain L3W and established a culture condition without sterilization. *Aurantiochytrium* sp. L3W removed 52% of the DOC and 37% of the DN from the sterilized BB wastewater and produced biomass that contained 137 mg/g of fatty acids (FAs), including 96.2 mg/g of DHA. Growth of *Aurantiochytrium* sp. L3W in the sterilized MP wastewater resulted in the production of biomass containing 147.6 mg/g of FAs, including 97.8 mg/g of DHA, and removal of 47% of the DOC and 55% of the DN from the wastewater. These results confirmed the usability of MP and BB wastewater for cultivation of strain L3W. *Aurantiochytrium* sp. L3W could grow in the salinity adjusted MP and BB wastewater samples to reduce possibility of contaminative microorganism without sterilization, although DHA and EPA productivity became less as compared to the sterilized wastewater. Finally, digestibility of its raw biomass by the enzymes extracted from the stomach of rainbow trout was investigated. The raw biomass of *Aurantiochytrium* sp. L3W was found to be digestible by the enzymes extracted from the stomachs of rainbow trout after 4h. These results confirmed the potential use of *Aurantiochytrium* sp. L3W to remove DOC and DN from food processing wastewater and to produce PUFAs and suggested that the produced biomass can be used as a fish feed additive.

Recommendations

Sterilization is a preferred pretreatment to maximize biomass and PUFAs production from thraustochytrids cultivation. Cultivation without sterilization is more suitable as wastewater treatment effort by DOC/DN removal, given the additional substrate consumption by contaminating microorganism.

Attaining good biomass production and balanced DOC/DN removal conforming to sewage discharge standard can be achieved by combination of cultivation and wastewater treatment plant using fed-batch fermentation can be applied.

List of Achievements

Journal Articles

1) Satoshi Nakai, Asmit Das, Yuya Maeda, **Nurlaili Humaidah**, Masaki Ohno, Wataru Nishijima, Takehiko Gotoh, Tetsuji Okuda "A Novel Strain of Aurantiochytrium sp. strain L3W and Its Characteristics of Biomass and Lipid Production Including Valuable Fatty Acids" ***Journal of Water and Environment Technology***, 19(1):24-34. (related to Chapter 2)
2) **Nurlaili Humaidah,** Satoshi Nakai, Wataru Nishijima, Takehiko Gotoh, "Effects of dilution on removal of organic carbon and nitrogen from miso-production wastewater by *Aurantiochytrium* sp. L3W and resultant fatty acid production" ***Environmental Science,*** 34(1):18-26 (related to Chapter 3)
3) **Nurlaili Humaidah,** Satoshi Nakai, Wataru Nishijima, Takehiko Gotoh, Megumi Furuta "Application of *Aurantiochytrium* sp. L3W for food-processing wastewater treatment in combination with polyunsaturated fatty acids production for fish aquaculture" ***Science of The Total Environment*** 743, 140735 (related to Chapter 4)

International Conference

1) **Nurlaili Humaidah,** Satoshi Nakai, Takehiko Gotoh, Megumi Furuta, Wataru Nishijima. "Cultivation of *Aurantiochytrium* sp. L3W for Food-processing Wastewater and Possible Utilization of Biomass for Fish Feed Additive", in: Water and Environment Technology Conference 2019 (WET2019), **Osaka University, Japan on the 13th–14th July 2019. (**related to Chapter 3)
2) **Nurlaili Humaidah**, Das Asmit, Satoshi Nakai, Takehiko Gotoh, Wataru Nishijima. "A Novel Strain, L3W of *Aurantiochytrium* sp. Applicable for Cultivation Food-processing Wastewater", in: 8th IWA Microbial Ecology and Water Engineering Specialist Conference (MEWE2019), **Hiroshima, Japan on 17-20 November 2019. (**related to Chapter 2)

Domestic Conference

3) **Nurlaili Humaidah**, Satoshi Nakai, Takehiko Gotoh, Wataru Nishijima, Yuya Maeda, Megumi Furuta. "Application of *Aurantiochytrium* sp. L3W for Food-processing Wastewater Treatment in Combination with DHA Production", in: 53rd Annual Conference of JSWE, **Yamanashi University, Japan. 3-5 March 2019 (**related to Chapter 4)

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