Original Article

A Novel Strain of *Aurantiochytrium* sp. Strain L3W and Its Characteristics of Biomass and Lipid Production Including Valuable Fatty Acids

Satoshi Nakai a, Asmit Das a, Yuya Maeda a, Nuraili Humaidah a,b, Masaki Ohno c, Wataru Nishijima d, Takehiko Gotoh a, Tetsuji Okuda e

a Graduate School of Engineering, Hiroshima University, Higashi-Hiroshima, Japan
b Faculty of Vocational Studies, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia
c Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Science, Niigata, Japan
d Environmental Research and Management Center, Hiroshima University, Higashi-Hiroshima, Japan
e Faculty of Advanced Science and Technology, Ryukoku University, Otsu, Japan

ABSTRACT
Thraustochytrids such as *Aurantiochytrium* are heterotrophic and produce valuable fatty acids (FAs) and therefore expected as a tool for removal of organic compounds in wastewater and simultaneous production of FAs. The four strains of *Aurantiochytrium* sp. were isolated from the mangrove leaf samples, and among these the strain L3W showed the high specific growth rate of 0.27 1/h at 25°C. In addition, this strain 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, the strain L3W produced 270 mg/g of FAs including 135 mg/g of docosahexaenoic acid, 11 mg/g of pentadecanoic acid and 9.3 mg/g of margaric acid. Furthermore, the strain L3W produced these FAs at pH3 and pH9. The pH and salinity tolerance of the strain L3W might be advantageous in its application for production of valuable FAs under competition with other microorganisms in unsterile wastewater.

Keywords: *Aurantiochytrium* sp., ω-3 fatty acid, odd-chain fatty acid, physiological characteristic

INTRODUCTION

Thraustochytrids such as *Aurantiochytrium* and *Schizochytrium* are heterotrophic stramenopilan protists belonging to kingdom Chromista [1] and known to produce lipid consisting of fatty acids (FAs) including ω-3 FAs, such as docosahexaenoic acid (DHA), and odd-chain FAs (OCFAs) [1–3] and pentadecanoic acid [2–4], respectively. ω-3 FAs have received worldwide attention because of the beneficial effects on human health [1,4], and nowadays DHA supplements are manufactured. In addition, OCFAs have been received attention too because of the unique pharmacological properties [5]. Because of a great deal of potential of ω-3 FAs and OCFAs in industries, thraustochytrids have been focused as a new source of valuable FAs.

As thraustochytrids are heterotrophic in nature, organic compounds such as glycerol, glucose, fructose and yeast extract, peptone, urea have been used as the substrates [1,3,4,6,7], while sodium nitrate and ammonium sulfate may be used as nitrogen sources [3,4,8]. Due to expensive nature of commercial substrates, use of food processing wastewater [7–9] and liquid waste [10] 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.

Corresponding author: Satoshi Nakai, E-mail: sn4247621@hiroshima-u.ac.jp

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To conquer this dilemma, use of thraustochytrids exhibiting durable growth may be one of the options. In our preliminary experiments, adjustment to pH3 and to 10 PSU of salinity worked in suppressing microorganisms such as yeasts and bacteria originated in the unsterile miso processing and bean boiling wastewater samples (Table S1, Figs. S1 and S2). Thraustochytrids were once reported to have a broad pH tolerance of 5 to 8 for growth and DHA production [4], and a later study reported Aurantiochytrium sp. SD116 capable of growing at pH4 [3]. 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‰ [1,11]; however a paper reported Schizochytrium limacisporum strain SR21 showing a salinity tolerance of 0–200% of seawater [4,12]. As for temperature, 25–30°C is generally favor optimal growth [1,4].

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 [1], environmental tolerance might be genetically modified too.

The present study aimed at finding strains of thraustochytrids tolerant to 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.

**MATERIALS AND METHODS**

**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.

**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 [13], the polymerase chain reaction (PCR) was performed using Euk A/Euk B primers [14], 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 blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A neighbor joining phylogenetic tree was produced by using the MEGA X software [15]. 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 [16].

**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 × 10³–10⁴ cells/ml. The culture flasks were incubated in triplicate at a rotation rate of 70 rpm and 25°C in dark.

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 (H3PO4, NaH2PO4, Na2HPO4) to attain pH3 and pH6 or 50 mM of tris (hydroxymethyl) aminomethane to attain pH7, pH8, and pH9 together with 1 M HCl.

**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. For a statistical analysis of biomass and lipid productions, one-way analysis of variance (ANOVA) and Turkey test were conducted.

Because the strain L3W was novel and showed the excellent growth characteristic, the strain L3W was cultured at 25°C, pH7, and 30 PSU, and the resultant biomass was analyzed for FAs. In addition, we also investigated an effect of pH on the FAs production by analyzing the biomass of the strain L3W cultivated at pH3, 6, 7, 8, and 9. Briefly, the culture solution of the strain L3W was centrifuged at 3000 rpm for 20 min, and the biomass pellet was washed with MillQ 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.

**RESULTS AND DISCUSSION**

**Identification of the isolated strains**

By the isolation campaign, the four strains L2R, L2Y, L3W, and L4Y were isolated, and the sequence data of these 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 S2**). In addition, the neighbor joining phylogenetic tree showed the long branch length of strain L3W (**Fig. 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** spp. (**Table S2**).

**Growth and lipid production of the four strains**

**Figure 2** shows the growth curve of the strain L3W that demonstrated the fast growth, where the stationary phase appeared after 30 h. Analyzing the growth during the first 30 h of cultivation, the strain L3W showed a high specific growth rate of 0.27 1/h (**Fig. S3**). 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. In the previous studies, specific growth rates of other strains were reported to be 0.15 1/h for **Aurantiochytrium mangrovei** SK-02 [17], 0.12 1/h for **A. limacinum** [18], 0.077 1/h for **A. mangrovei** FB3 [19], and 0.60 1/d (0.025 h −1) for **Aurantiochytrium** sp. AF0043 [20]. In addition, specific growth rates of **Aurantiochytrium** sp. mh0186 (later identified as **A. limacinum** mh0186) (0.2 1/h) [2], **Aurantiochytrium** sp. KRS101 (0.08 1/h) [6], **Aurantiochytrium** sp. JMV1.1 (0.2 1/h) [21], and **Aurantiochytrium** sp. SW1 (0.05 1/h) [22] were estimated on the basis of the presented growth curves. Although the culture condition of the strain L3W was different from that in the previous studies [2,6,17–22] in terms of the temperature and composition of culture media, a comparison of the specific growth rates suggests that the strain L3W might demonstrate relatively fast growth as compared to the strains reported before.

**Figure 3** 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 *A. limacinum* mh0186 (33%) [2] and (45%) [23], *Aurantiochytrium* sp. KRS101 (43.4%) [6], *Aurantiochytrium* sp. AF0043 (31%) [20], and *Aurantiochytrium* sp. SW1 (48%) [22]. Since the strain L3W was a novel strain showing the high specific growth rate (Fig. 1 and Fig. S3) and biomass and lipid productions (Fig. 3), we further tested its growth characteristics.

**Growth characteristics of the strain L3W**

*Effect of temperature*

Figure 4 shows the effect of temperature on the growth of the strain 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...
Fig. 2 Growth curve of the strain L3W in the 790 By+ medium at 25°C. Bars indicate standard deviation (n = 3).

Fig. 3 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 values (n = 2). A difference was significant between the strains L3W and L2Y (p = 0.0088)
showed that 25°C was optimal for the strain 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 [24], from which we collected the isolation source for the strain L3W.

Temperatures of 25°C–30°C is generally favor for growth of thraustochytrids [4]. In the previous studies, similar optimal temperatures were reported for Aurantiochytrium sp. SD116 (25°C) [3] and Aurantiochytrium sp. KRS101 (28°C) [6], while no significant change was observed in growth of A. limacinum mh0186 at a range from 15°C to 35°C [2]. In addition, A. limacinum mh0186 could grow at 10°C, however, the growth was significantly lower than that at 15°C and 28°C [23]. The growth characteristics of the strain L3W in terms of temperature (Fig. 4) is similar to these strains of Aurantiochytrium sp., though the growth test was not performed at 10°C. Since 25°C was optimal for the strain L3W, further testing was performed at this temperature.

**Effect of salinity**

Figure 5 shows effects of salinity on the biomass and lipid productions by the strain L3W. The strain 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 the strain 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.

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‰ [4,11]. Later, 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 [1]. 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 [6]. As for Aurantiochytrium sp. SD116, it could grow...
Studies have shown that the strain L3W can grow at a range of salinity from 0 to 60 g-sea salt/L, and the optimal salinity was 15 g-sea salt/L [3]. In this study, we used the sand filtered seawater with salinity of about 30 PSU, and salinity optimal for growth of the strain L3W was 15 and 30 PSU (Fig. 5). This agreed with the previous studies [1,3,4,6]. In addition, the strain L3W could grow at 70 PSU which was about 200% of the sand filtered seawater, and its tolerance to high salinity was similar to that of *Schizochytrium limacinum* SR21 [12]. This growth characteristic might be suitable for utilization of saline food processing wastewater and liquid waste such as fish canning wastewater [25].

**Effect of pH**

Effects of pH on the biomass and lipid productions by the strain L3W are shown in Fig. 6, where the biomass production was highest at pH 6, 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 results indicate that the optimal pH for the strain L3W exists in a range from pH 6 to 8. In addition, the averaged lipid content at pH 3 (67%) and pH 9 (49%) was higher than pH 7 (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 [3].

Thraustochytrids are known to have a broad pH tolerance at a range from 5 to 8 for growth [4]. For example, the optimal-initial pH values were reported pH 7 for *Aurantiochytrium* sp. KRS101 [6] and pH 6 for *Aurantiochytrium* sp. SD116 [3]. The optimal pH value for the strain L3W was similar to that reported before [3,4,6]; however, the strain L3W demonstrated the interesting growth characteristics in terms of pH tolerance. That is, it could grow at pH 3 and pH 9. The previous study showed that *Aurantiochytrium* sp. SD116 could grow at pH 4 and pH 9 [3]; however, to the best of our knowledge, no paper has reported a strain of thraustochytrid capable of growing at pH 3. The result suggests good potential of strain L3W for utilization of acidic food processing wastewater and liquid waste such as pineapple cannery waste [26].

**Potential of the strain L3W for FAs production**

Based on the effects of temperature, salinity and pH on the biomass and lipid productions by the strain L3W (Fig. 4–6), the strain 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. 7, the biomass of strain 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 1). Although the DHA content of strain 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 [2,12,27]. In addition, a fed-batch or continuous fermentation strategy may be used to improve the DHA productivity [1]. As for OCFAs, the concentrations of pentadecanoic acid (C15:0) and margaric acid (C17:0) were 11 mg/g and 9.3 mg/g, respectively.

The strain 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 L3W was suppressed out of the optimal condition (Figs. 3–5). Since the pH tolerance of the strain L3W is a unique property, we investigated an effect of pH on production of FAs by the strain 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 [4]; however, we confirmed production of DHA and pentadecanoic and margaric acids by the strain L3W at pH3 and 9.

As the pH value decreased or increased from 7, the abundance of FAs in the biomass of strain L3W became lower. On the other hand, the lipid content was not affected by pH at the tested range (Fig. 5). The previous studies confirmed production of squalene in addition to DHA by *Aurantiochytrium mangrovei* [18] and *Aurantiochytrium* sp. T66 [28] and xanthophylls by *Aurantiochytrium* sp. KH105 [9,10]. 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 [9,10,28], our future study will address effects of pH on lipid composition.

The growth characteristics of strain 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. S1 and S2, 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. In fact, we demonstrated that the strain L3W could grow in the unsterilized bean boiling wastewater (pH 5.0) of which salinity was adjusted to 30 PSU to produce FAs at 120 mg/g including DHA at 61.2 mg/g [29]. This suggests combinational use of pH and salinity tolerance may result in successful growth and DHA production, though the strain L3W could grow at pH 3. 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, pH 4) (unpublished data). Because of acidity and

Table 1 Comparison of the DHA content introduced in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DHA [mg/g-dry cell]</th>
<th>Medium</th>
<th>Ref.</th>
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</thead>
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<td>2</td>
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<tr>
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<td>Artificial</td>
<td>3</td>
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<tr>
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<tr>
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<td>214</td>
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<td>7</td>
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<tr>
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<td>115</td>
<td>Shochu wastewater</td>
<td>9</td>
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<tr>
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<td>32</td>
<td>Waste syrup</td>
<td>10</td>
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<td><em>Aurantiochytrium</em> sp. L3W</td>
<td>135</td>
<td>Artificial</td>
<td>This study</td>
</tr>
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</table>

* The strain was once reported as *Aurantiochytrium* sp. mh0186.
** The strain was once reported as *Schizochytrium*.
*** Optimization of the culture condition was carried out.

Fig. 7 Effect of pH on the FAs production by the strain L3W at 25°C and 30 PSU.
salinity, treatment and utilization of pickle production liquid waste remain challenging. Applicability of the strain L3W for various kinds of unsterilized food processing wastewater will be further tested in near future.

**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. The strain 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 the strain 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 strain 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 strain 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.

**ACKNOWLEDGEMENTS**

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**SUPPLEMENTARY MATERIALS**

Supplementary Materials file for this article is available at the link below.
https://www.jstage.jst.go.jp/article/jwet/19/1/19_20-087_/supplement/_download/19_20-087_1.pdf

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