



# Development of a Method for Fucoxanthin Production Using the Haptophyte Marine Microalga *Pavlova* sp. OPMS 30543

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1 **Development of a Method for Fucoxanthin Production Using the Haptophyte Marine Microalga**  
2 ***Pavlova* sp. OPMS 30543**

3

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18 **Abstract**

19 The natural pigment fucoxanthin has attracted global attention because of its superior antioxidant  
20 properties. The haptophyte marine microalgae *Pavlova* spp. are assumed to be promising industrial  
21 fucoxanthin producers as their lack of a cell wall could facilitate the commercialization of cultured  
22 cells as a whole food. This study screened promising *Pavlova* strains with high fucoxanthin content to  
23 develop an outdoor cultivation method for fucoxanthin production. Initial laboratory investigations of  
24 *P. pinguis* NBRC 102807, *P. lutheri* NBRC 102808, and *Pavlova* sp. OPMS 30543 identified OPMS  
25 30543 as having the highest fucoxanthin content. The culture conditions were optimized for OPMS  
26 30543. Compared to f/2 and Walne's media, the use of Daigo's IMK medium led to the highest biomass  
27 production and highest fucoxanthin accumulation. The presence of seawater elements in Daigo's IMK  
28 medium was necessary for the growth of OPMS 30543. OPMS 30543 was then cultured outdoors  
29 using acrylic pipe photobioreactors, a plastic bag, an open tank, and a raceway pond. Acrylic pipe  
30 photobioreactors with small diameters enabled the highest biomass production. Using an acrylic pipe  
31 photobioreactor with 60 mm diameter, a fucoxanthin productivity of 4.88 mg/L/day was achieved in  
32 outdoor cultivation. Thus, this study demonstrated the usefulness of *Pavlova* sp. OPMS 30543 for  
33 fucoxanthin production in outdoor cultivation.

34

35 **Key words**

36 Fucoxanthin, Marine microalgae, Outdoor cultivation, *Pavlova*

37 **Introduction**

38 Fucoxanthin is synthesized by brown algae and diatoms as a major photosynthetic pigment;  
39 thus, it is the most abundant marine carotenoid and is widely distributed in nature (Dembitsky and  
40 Maoka 2007). Fucoxanthin has attracted considerable attention for use in the pharmaceutical,  
41 nutraceutical, and cosmetic industries because of its superior antioxidant properties (Peng et al. 2011).  
42 Fucoxanthin has also been studied for its anti-cancer activity in human cells (Hosokawa et al. 1999;  
43 Kotake-Nara et al. 2001), anti-type 2 diabetes and anti-obesity effects in mice and human cells  
44 (Gammone and d’Orazio 2015; Maeda et al. 2007), *in vitro* anti-cholesterol activity (Kawee-ai et al.  
45 2013), anti-inflammatory effects in rats (Shiratori et al. 2005), anti-angiogenic effects in human cells  
46 (Sugawara et al. 2006), anti-malarial effects against *Plasmodium falciparum* (Afolayan et al. 2008),  
47 and anti-hypertensive effects in rats (Ikeda et al. 2003; Sivagnanam et al. 2015), as well as for the  
48 treatment of Alzheimer’s disease (Kawee-ai et al. 2013). Currently, fucoxanthin is produced  
49 commercially from brown algae such as *Laminaria* spp. and *Undaria pinnatifida* and diatoms such as  
50 *Phaedactylum tricornutum* (Gayen et al. 2019). Algatechnologies Inc. supplies Fucovital™, which is  
51 manufactured from *P. tricornutum*, and this was the first fucoxanthin food ingredient product approved  
52 by the U.S. Food and Drug Administration (NDI 1048, 2017). Fucoxanthin obtained from diatoms  
53 such as *Chaetoceros gracilis* and *Odontella aurita* also have potential industrial applications  
54 (Tokushima et al. 2016; Xia et al. 2018). Culture conditions such as light and nutrients have been  
55 reported to affect microalgal fucoxanthin production (Xia et al. 2013; Gómez-Loredo et al. 2016; Lu  
56 et al. 2018; Yang and Wei 2020). In *O. aurita*, cultivation in a high nitrate medium led to high  
57 fucoxanthin content and volumetric fucoxanthin production (Xia et al. 2013). In *P. tricornutum*,  
58 tryptone and urea were examined as supplemental nitrogen sources, and tryptone was found to improve  
59 cell growth and fucoxanthin production (Yang and Wei 2020).

60 In addition to brown algae and diatoms, haptophyte microalgae of *Pavlova* spp., such as *P.*  
61 *lutheri* and *P. pinguis*, can produce fucoxanthin (Hiller et al. 1988; Lananan et al. 2013). The marine  
62 microalga *P. lutheri*, which can produce considerable amounts of polyunsaturated fatty acids (PUFAs),  
63 is commonly employed as a larval feed in aquaculture (Brown et al. 1997; Guihéneuf and Stengel  
64 2013), and its PUFA yield is increased via random mutagenesis (Meireles et al. 2003). *P. pinguis*  
65 contains abundant docosapentaenoic acid (Milke et al. 2008). As *Pavlova* spp. do not have a cell wall  
66 (Green 1980); they can be commoditized as whole foods without the need to extract intracellular  
67 fucoxanthin. Thus, *Pavlova* spp. are considered valuable fucoxanthin producers. However, there are  
68 no quantitative reports regarding fucoxanthin production by *Pavlova* spp.

69 In the present study, screening of several *Pavlova* spp. to identify a strain with high  
70 fucoxanthin content revealed that *Pavlova* sp. OPMS 30543 is a promising producer. Culture  
71 conditions for OPMS 30543 were examined and optimized, and factors affecting biomass and  
72 fucoxanthin production were investigated in laboratory experiments. Large-scale and outdoor

73 cultivation of OPMS 30543 was also conducted using various culture facilities.

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74 **Materials and Methods**

75 **Strains and Laboratory-Scale Cultivation**

76 *Pavlova pinguis* NBRC 102807 and *P. lutheri* NBRC 102808 were obtained from the  
 77 National Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation.  
 78 *Pavlova* sp. OPMS 30543 was isolated from brackish water from Okinawa Main Island, Japan.  
 79 Microalgae were photoautotrophically cultivated in artificial seawater (Marine Art SF-1, Tomita  
 80 Pharmaceutical, Tokushima, Japan) enriched with either Daigo's IMK (FUJIFILM Wako Pure  
 81 Chemical Corp., Osaka, Japan), f/2 (Guillard and Ryther 1962), or Walne's (Walne 1970) elements  
 82 (Table 1). Culture conditions were as follows, unless otherwise noted in the figure legends: 800 mL of  
 83 medium in 1 L sterilized bottles, illumination with white fluorescent lamps at an intensity of 150  $\mu\text{mol}$   
 84  $\text{photons}/\text{m}^2/\text{s}$  with a 12 h:12 h light/dark cycle, and continuous aeration of 0.25 mL/mL/min. Cells  
 85 were harvested using 0.7  $\mu\text{m}$  pore size glass fiber filter paper GF/F (Cytiva, Tokyo, Japan), washed  
 86 with distilled water, and dried at 120 °C for 2 h before measurement of dry cell weight (DCW). To  
 87 examine alternative nitrogen sources for Daigo's IMK, media were prepared as shown in Table 2.  
 88

89 **Table 1.** Nutrients in seawater media (mg/L)

1× Daigo's IMK		f/2		Walne's	
NaNO <sub>3</sub>	200	NaNO <sub>3</sub>	75	NaNO <sub>3</sub>	100
Na <sub>2</sub> HPO <sub>4</sub>	1.4	NaH <sub>2</sub> PO <sub>4</sub> · 2H <sub>2</sub> O	6	NaH <sub>2</sub> PO <sub>4</sub> · 2H <sub>2</sub> O	20
K <sub>2</sub> HPO <sub>4</sub>	5	-	-	-	-
NH <sub>4</sub> Cl	2.68	-	-	-	-
Fe-EDTA	5.2	FeCl <sub>3</sub> · 6H <sub>2</sub> O	3.16	FeCl <sub>3</sub> · 6H <sub>2</sub> O	1.3
Mn-EDTA	0.332	MnCl <sub>2</sub> · 4H <sub>2</sub> O	0.18	MnCl <sub>2</sub> · 4H <sub>2</sub> O	0.36
Na <sub>2</sub> -EDTA	37.2	Na <sub>2</sub> -EDTA	4.4	Na <sub>2</sub> -EDTA	45
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	0.023	ZnSO <sub>4</sub> · 7H <sub>2</sub> O	0.021	ZnCl <sub>2</sub>	0.021
CoSO <sub>4</sub> · 7H <sub>2</sub> O	0.014	CoSO <sub>4</sub> · 7H <sub>2</sub> O	0.012	CoCl <sub>2</sub> · 6H <sub>2</sub> O	0.02
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	0.0073	Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	0.007	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> · 4H <sub>2</sub> O	0.009
CuSO <sub>4</sub> · 5H <sub>2</sub> O	0.0025	CuSO <sub>4</sub> · 5H <sub>2</sub> O	0.007	CuSO <sub>4</sub> · 5H <sub>2</sub> O	0.02
H <sub>2</sub> SeO <sub>3</sub>	0.0017	-	-	-	-
-	-	Na <sub>2</sub> SiO <sub>3</sub> · 9H <sub>2</sub> O	10	-	-
-	-	-	-	H <sub>3</sub> BO <sub>3</sub>	33.6
Thiamine-HCl	0.2	Thiamine-HCl	0.1	Thiamine-HCl	0.01
Biotin	0.0015	Biotin	0.0005	Biotin	0.0002

Vitamin B12      0.0015    Vitamin B12      0.0005    Vitamin B12      0.01

90

91 **Table 2.** Nutrients in modified IMK (mIMK) media (mg/L)

	1× Daigo's IMK	mIMK (NaNO <sub>3</sub> )	mIMK (KNO <sub>3</sub> )	mIMK (CO[NH <sub>2</sub> ] <sub>2</sub> )	mIMK (NH <sub>4</sub> Cl)
NaNO <sub>3</sub>	200	200	-	-	-
KNO <sub>3</sub>	-	-	200	-	-
CO(NH <sub>2</sub> ) <sub>2</sub>	-	-	-	200	-
NH <sub>4</sub> Cl	2.68	-	-	-	200
Na <sub>2</sub> HPO <sub>4</sub>	1.4	-	-	-	-
K <sub>2</sub> HPO <sub>4</sub>	5	5	5	5	5
Fe-EDTA	5.2	-	-	-	-
Mn-EDTA	0.332	-	-	-	-
Na <sub>2</sub> -EDTA	37.2	37.2	37.2	37.2	37.2
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	0.023	0.023	0.023	0.023	0.023
CoSO <sub>4</sub> · 7H <sub>2</sub> O	0.014	-	-	-	-
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	0.0073	-	-	-	-
CuSO <sub>4</sub> · 5H <sub>2</sub> O	0.0025	0.0025	0.0025	0.0025	0.0025
H <sub>2</sub> SeO <sub>3</sub>	0.0017	-	-	-	-
Thiamine-HCl	0.2	-	-	-	-
Biotin	0.0015	-	-	-	-
Vitamin B12	0.0015	-	-	-	-

92

93 **Pigment Analysis**

94        Approximately 10 mg of dried cells was suspended in 1 mL of acetonitrile, mixed by  
95 vortexing for 1 min, and disrupted by sonication for 10 min. After centrifugation at 10,000 × g for 2  
96 min, the supernatant was analyzed by high-performance liquid chromatography (Shimadzu, Kyoto,  
97 Japan) under the following conditions: reverse-phase column, COSMOSIL 5C<sub>18</sub>-AR-II, 4.6 mm I.D.  
98 × 150 mm (Nacalai Tesque, Kyoto, Japan); column oven temperature, 40 °C; mobile phase, 80%  
99 acetonitrile aqueous containing 0.1% formic acid; flow rate, 1 mL/min; detection, 450 nm using a  
100 photodiode array detector. Fucoxanthin signals were identified and quantified using a standard curve  
101 generated using the fucoxanthin standard (FUJIFILM Wako Pure Chemical Corp.).

102

103 **Large-Scale Cultivation**

104 OPMS 30543 was cultivated outdoors under natural sunlight using the following common  
105 cultivation systems: 1) 60 mm outer diameter and 5 mm thickness acrylic pipe photobioreactor (PBR),  
106 2) 114 mm outer diameter and 5 mm thickness acrylic pipe PBR, 3) 216 mm outer diameter and 5 mm  
107 thickness acrylic pipe PBR, 4) 267 mm outer diameter and 5 mm thick acrylic pipe PBR, 5) 450 mm  
108 outer diameter and 0.1 mm thickness plastic bag, 6) 200 L polycarbonate open tank, and 7) 500 L  
109 raceway pond, in 50% artificial seawater containing 2× Daigo's IMK elements described above (Table  
110 1). Agitation was performed by aeration at 0.25 mL/min for 1) and 2), and 0.1 mL/min for 3), 4), 5),  
111 and 6) except for the raceway pond, in which the flow rate was adjusted to 0.5 m/s by stirring with a  
112 paddle. During cultivation, the pH was adjusted to 8 by supplying 100% CO<sub>2</sub>.

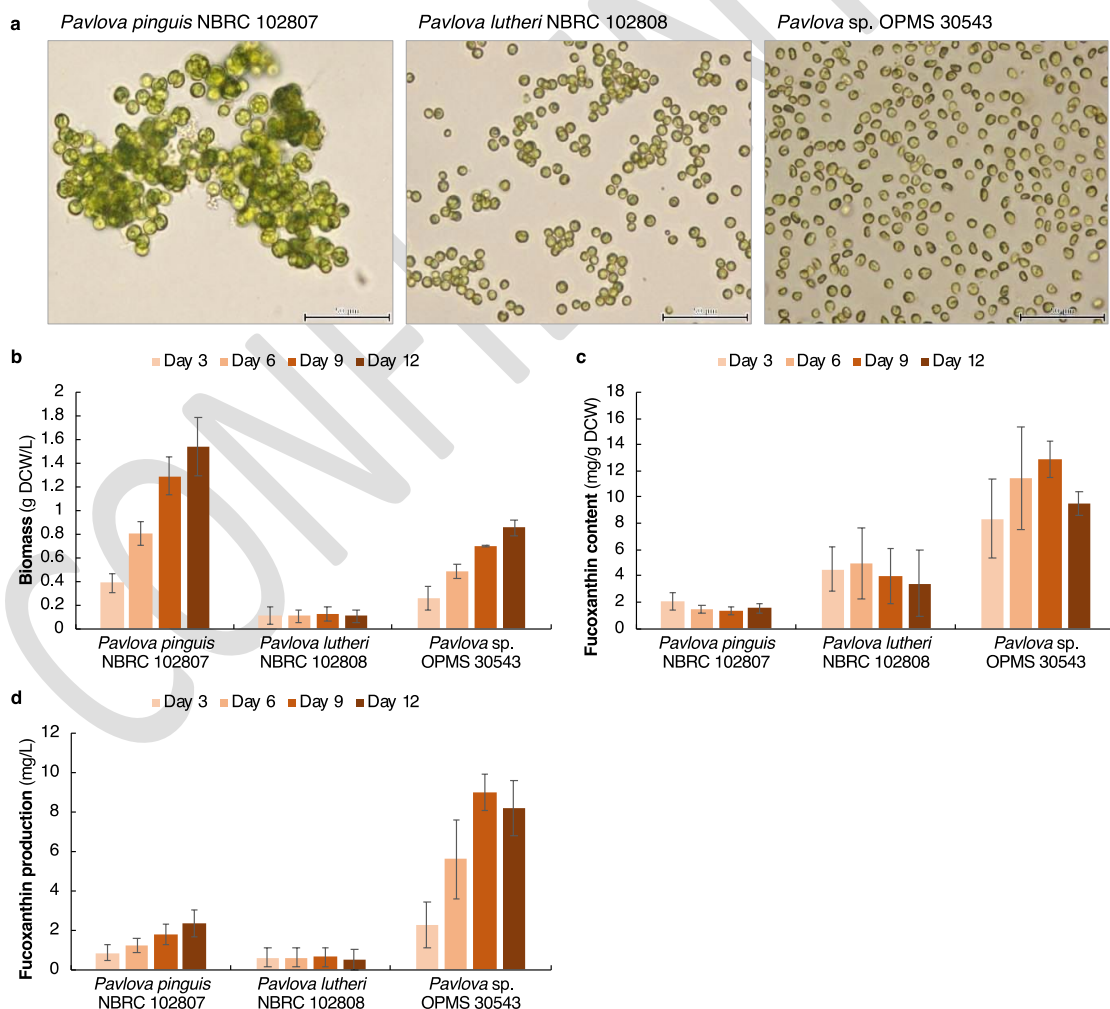
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113 **Results**

114 **Screening of *Pavlova* Strains for Fucoxanthin Production**

115 To develop a fucoxanthin production method using *Pavlova* spp., three strains (i.e., *P.*  
 116 *pinguis* NBRC 102807, *P. lutheri* NBRC 102808, and *P. sp.* OPMS 30543) were examined in this  
 117 study (Fig. 1a). The strains were cultured in 50% seawater containing 2× Daigo’s IMK at 25 °C to  
 118 identify a promising strain with high fucoxanthin production. Strain NBRC 102808 exhibited the  
 119 lowest biomass production, whereas NBRC 102807 exhibited the highest biomass production, 1.54 g  
 120 DCW/L at day 12 (Fig. 1b). In contrast, among these *Pavlova* strains, strain NBRC 102807 exhibited  
 121 the lowest fucoxanthin content (2.06 mg/g DCW, day 3) (Fig. 1c). OPMS 30543 exhibited measurable  
 122 biomass production of 0.85 g DCW/L over 12 days and achieved the highest fucoxanthin content,  
 123 12.88 mg/g DCW at day 9. Fucoxanthin production (calculated by multiplying the biomass and  
 124 fucoxanthin content) of 9.01 mg/L at day 9 was achieved by OPMS 30543, which was higher than that  
 125 of strains NBRC 102807 (2.32 mg/L, day 12) and NBRC 102808 (0.61 mg/L, day 9) (Fig. 1d). Thus,  
 126 OPMS 30543 was identified as a promising *Pavlova* strain for fucoxanthin production.



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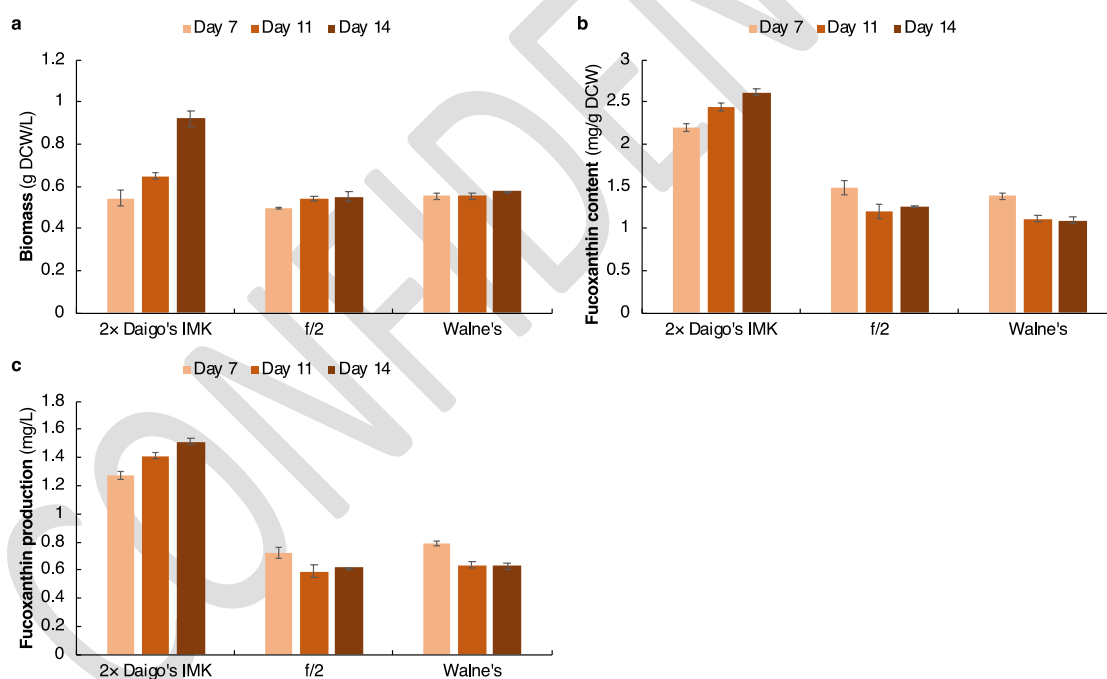
128 **Fig. 1** Comparison of three *Pavlova* strains. **a** Microscopic images of *Pavlova* cells. Scale bars: 50 µm.

129 **b** Biomass. **c** Fucoxanthin content. **d** Fucoxanthin production.

130

### 131 Examination of Culture Medium for OPMS 30543

132 To determine the optimal medium for fucoxanthin production, biomass, and fucoxanthin  
133 content were investigated using OPMS 30543 grown in 50% seawater enriched with either 2× Daigo's  
134 IMK, f/2 (Guillard and Ryther 1962), or Walne's (Walne 1970) elements (Table 1). Among these  
135 conditions, cultivation in 2× Daigo's IMK medium resulted in higher biomass (0.92 g DCW/L) relative  
136 to f/2 (0.55 g DCW/L) and Walne's (0.56 g DCW/L) media after 14 days of cultivation (Fig. 2a). In  
137 addition, the fucoxanthin content of OPMS 30543 grown in 2× Daigo's IMK medium was significantly  
138 higher (2.62 mg/g DCW, day 14) than that of cells grown in f/2 (1.48 mg/g DCW, day 7) or Walne's  
139 (1.39 mg/g DCW, day 7) media (Fig. 2b). Fucoxanthin production of 1.51 mg/L on day 14 was  
140 achieved by culturing cells in 2× Daigo's IMK medium, which was double the production of cells  
141 grown in medium containing f/2 (0.73 mg/L, day 7) or Walne's (0.79 mg/L) elements (Fig. 2c). Thus,  
142 these data suggest that the use of 2× Daigo's IMK was the most suitable for maximizing OPMS 30543  
143 biomass and fucoxanthin production.



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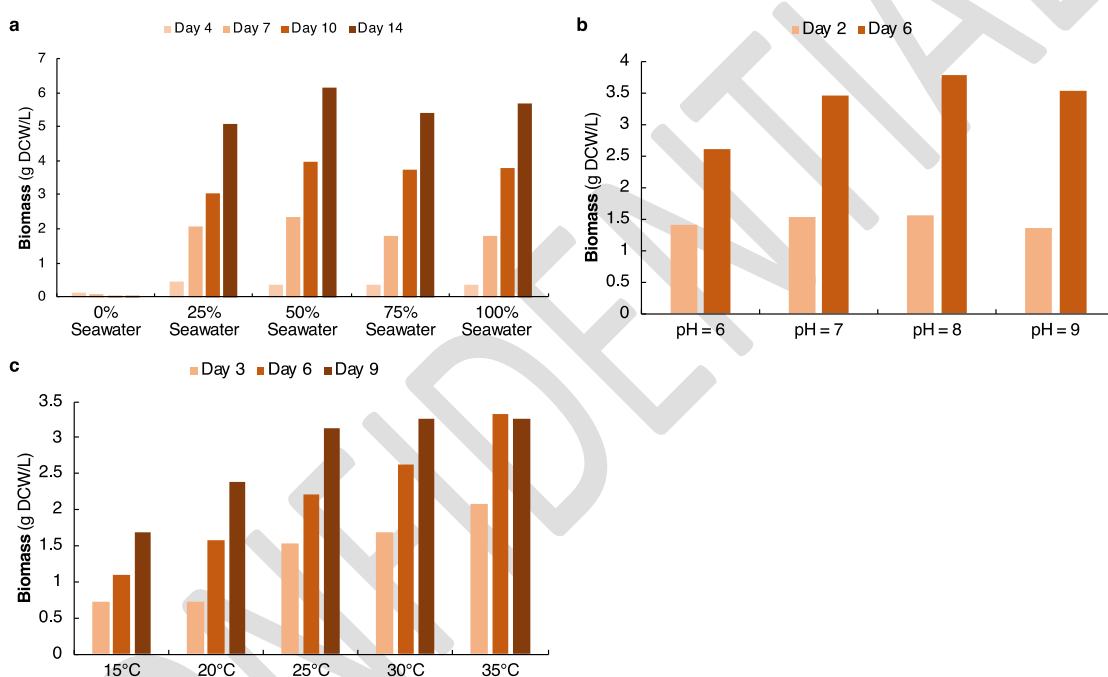
145 **Fig. 2** Comparison of different media for OPMS 30543 cultivation. **a** Biomass. **b** Fucoxanthin content.  
146 **c** Fucoxanthin production. Cells were statically cultivated in 200 mL Erlenmeyer flasks with a 100 mL  
147 working volume of 50% seawater containing either 2× Daigo's IMK, f/2, or Walne's elements.

148

### 149 Examination of Culture Conditions for OPMS 30543

150 To improve the biomass production of OPMS 30543, various culture conditions (i.e.,  
151 seawater concentration, pH, and temperature) were examined. When cultivated in 2× Daigo's IMK

152 with different concentrations of seawater, biomass production was observed only in the presence of  
 153 seawater; OPMS 30543 did not grow in 0% seawater medium (Fig. 3a). The highest biomass of 6.16  
 154 g DCW/L on day 14 was achieved in the medium with 50% seawater. The effect of varying the culture  
 155 pH by supplying CO<sub>2</sub> gas to the medium was also examined (Fig. 3b). OPMS 30543 biomass  
 156 production was reduced when the pH was adjusted to 6, whereas the highest biomass of 3.78 g DCW/L  
 157 on day 6 was observed when pH was adjusted to 8. Culture temperature was investigated over the  
 158 range of 15–35 °C (Fig. 3c). Within this temperature range, OPMS 30543 produced higher biomass at  
 159 higher temperatures, and cultivation at 35 °C resulted in the highest biomass production of 3.32 g  
 160 DCW/L on day 6. Thus, cultivation in 50% seawater medium at 35 °C and pH 8 was determined to be  
 161 the optimal condition for OPMS 30543 biomass production.



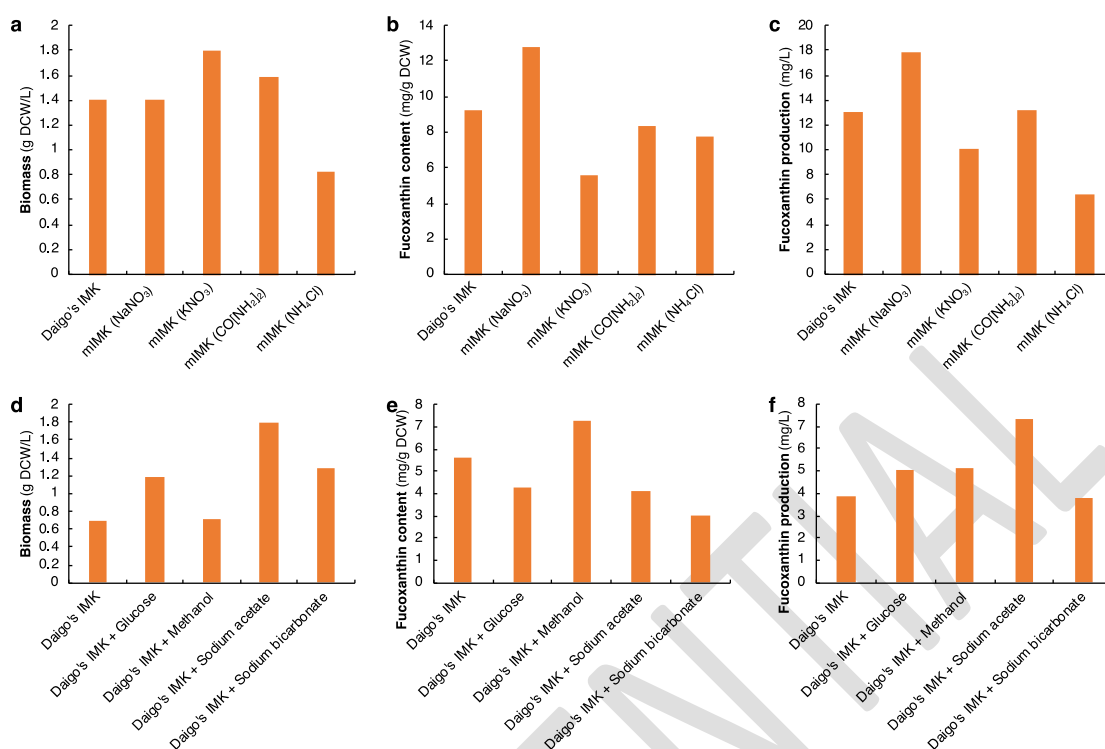
162  
 163 **Fig. 3** Comparison of culture conditions for OPMS 30543. **a** Seawater concentration in medium. **b** pH,  
 164 adjusted by supplying CO<sub>2</sub> gas to the culture. Cultures were illuminated with red, blue, and white  
 165 LEDs at a total intensity of 300 μmol photons/m<sup>2</sup>/s with a 12 h:12 h light/dark cycle. **c** Culture  
 166 temperature.

167  
 168 **Modification of IMK Medium by Replacing Nitrogen Sources and Adding Carbon Sources**

169 To further improve OPMS 30543 biomass production and fucoxanthin content, the effect of  
 170 varying the nitrogen source in the medium was examined. The modified IMK medium was prepared  
 171 by replacing NaNO<sub>3</sub> in 1× Daigo's IMK with either NaNO<sub>3</sub>, KNO<sub>3</sub>, CO(NH<sub>2</sub>)<sub>2</sub>, or NH<sub>4</sub>Cl (Table 2).  
 172 After 9 days of cultivation, cells cultured in the modified IMK medium containing KNO<sub>3</sub> exhibited  
 173 the highest biomass of 1.8 g DCW/L (Fig. 4a). Both urea CO(NH<sub>2</sub>)<sub>2</sub> and NH<sub>4</sub>Cl were found to be  
 174 available as nitrogen sources for OPMS 30543 cultivation, and biomass production of 1.58 and 0.82

175 g DCW/L at 10 days was observed, respectively. Use of  $\text{NaNO}_3$ -containing medium resulted in higher  
176 fucoxanthin content (12.74 mg/g DCW) than in media with  $\text{KNO}_3$  (5.57 mg/g DCW),  $\text{CO}(\text{NH}_2)_2$  (8.38  
177 mg/g DCW), or  $\text{NH}_4\text{Cl}$  (7.80 mg/g DCW) (Fig. 4b). Fucoxanthin production was the highest when  
178  $\text{NaNO}_3$  was used as the nitrogen source (Fig. 4c). Fucoxanthin production of OPMS 30543 grown in  
179 modified IMK medium containing  $\text{NaNO}_3$ ,  $\text{KNO}_3$ ,  $\text{CO}(\text{NH}_2)_2$ , or  $\text{NH}_4\text{Cl}$  was 17.84, 10.03, 13.24, and  
180 6.40 mg/L, respectively. Thus, these data suggest that  $\text{NaNO}_3$  is the best nitrogen source for  
181 maximizing OPMS 30543 fucoxanthin production.

182 The effect of adding various carbon sources to the medium was also examined to enhance  
183 biomass and fucoxanthin production. Modified IMK medium was prepared by adding either glucose,  
184 methanol, sodium acetate, or sodium bicarbonate to 50% seawater enriched with  $1\times$  Daigo's IMK.  
185 Each of the additional carbon sources increased biomass production compared to that with the normal  
186  $1\times$  Daigo's IMK (Fig. 4d). After 4 days of cultivation, OPMS 30543 grown in medium with sodium  
187 acetate exhibited the highest biomass of 1.79 g DCW/L, whereas OPMS 30543 biomass in medium  
188 containing glucose, methanol, and sodium bicarbonate was 1.19, 0.71, and 1.28 g DCW/L, respectively.  
189 Use of medium containing methanol resulted in the highest fucoxanthin content (7.26 mg/g DCW)  
190 relative to medium containing glucose (4.25 mg/g DCW), sodium acetate (4.11 mg/g DCW), or  
191 sodium bicarbonate (2.99 mg/g DCW) (Fig. 4e). Fucoxanthin production was the highest when sodium  
192 acetate was added to the medium (Fig. 4f). Fucoxanthin production by OPMS 30543 grown with  
193 glucose, methanol, sodium acetate, and sodium bicarbonate was 5.06, 5.15, 7.36, and 3.83 mg/L,  
194 respectively. Thus, sodium acetate was suggested as the optimal carbon source for enhancing  
195 fucoxanthin production.



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**Fig. 4** Examination of alternative nitrogen sources and additional carbon sources. **a** Biomass, **b** fucoxanthin content, and **c** fucoxanthin production of cells grown in 50% seawater enriched with modified IMK and different nitrogen sources. **d** Biomass, **e** fucoxanthin content, and **f** fucoxanthin production of cells grown in 50% seawater enriched with 2× Daigo's IMK with additional carbon sources, illuminated with red, blue, and white LEDs at a total intensity of 300  $\mu\text{mol photons/m}^2/\text{s}$  with a 12 h:12 h light/dark cycle.

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#### Large-Scale Outdoor Cultivation of OPMS 30543

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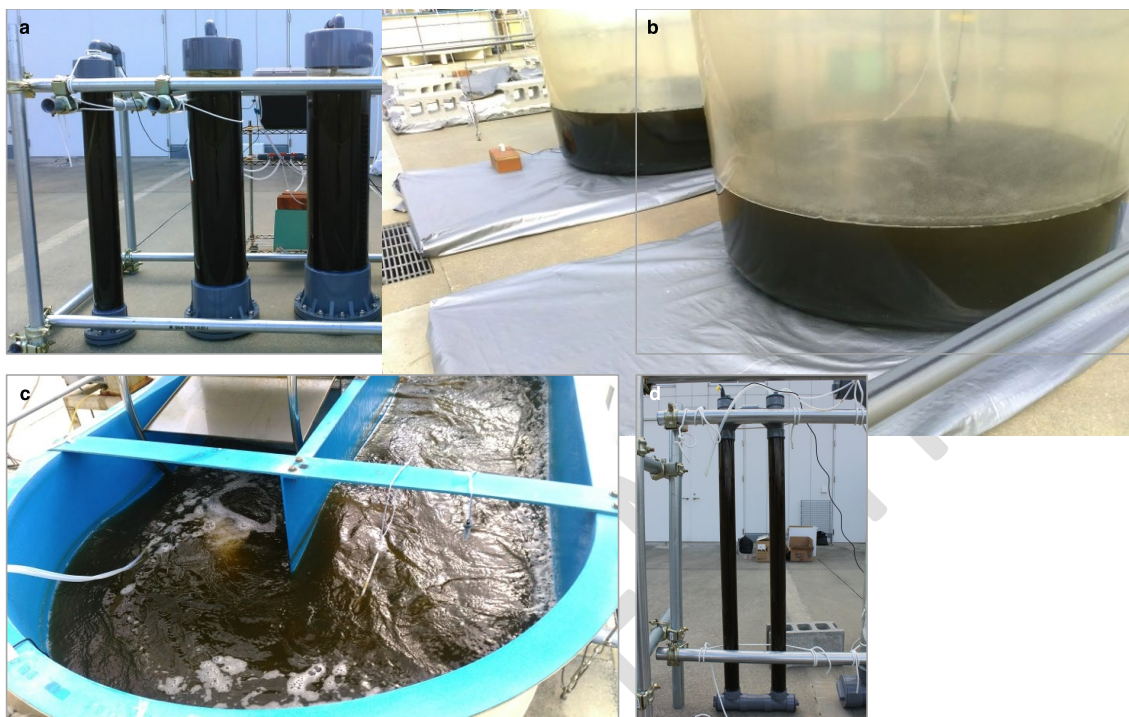
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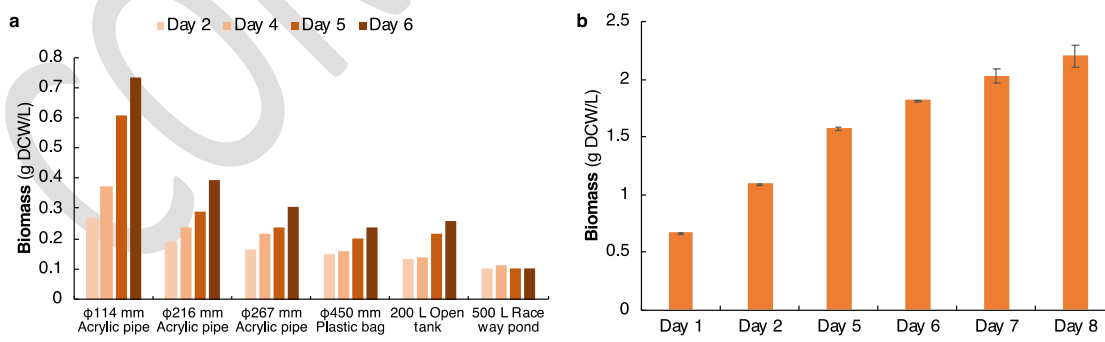
A large-scale outdoor OPMS 30543 cultivation test was performed to evaluate the potential of fucoxanthin production outdoors. Acrylic pipe PBRs (5 mm thickness with different outer diameters of 114, 216, and 267 mm), a plastic bag (0.1 mm thickness with 450 mm outer diameter), a 200 L polycarbonate open tank, and a 500 L raceway pond were used for cultivation (Fig. 5). Six days of cultivation outdoors in acrylic pipe PBRs with 114, 216, and 267 mm outer diameter produced biomass of 0.73, 0.39, and 0.31 g DCW/L, respectively (Fig. 6a). Cultivation using a plastic bag, a 200 L polycarbonate open tank, and a 500 L raceway pond produced 0.24, 0.26, and 0.10 g DCW/L, respectively, on day 6. Thus, the acrylic pipe PBRs with smaller outer diameters achieved higher biomass production than the plastic bag, open tank, or raceway pond. To further examine these results, OPMS 30543 was cultivated using an acrylic pipe PBR with a 60 mm outer diameter. Biomass of 1.82 g DCW/L and 2.20 g DCW/L were observed on days 6 and 8, respectively (Fig. 6b), both of which were higher than the biomass production achieved using the acrylic pipe PBR with a 114 mm outer



217 diameter. The fucoxanthin content on day 8 was 20.86 mg/g DCW, which was higher than that  
 218 achieved with any of the laboratory-scale cultivations in this study. Using a PBR with a 60 mm outer  
 219 diameter, biomass productivity of 0.23 g DCW/L/day and fucoxanthin productivity of 4.88 mg/L/day  
 220 were demonstrated in large-scale outdoor cultivation.



221  
 222 **Fig. 5** Facilities used for outdoor cultivation. **a** Acrylic pipe photobioreactors (5 mm thickness with  
 223 outer diameters of 114, 216, and 267 mm) and a plastic bag (0.1 mm thickness with 450 mm outer  
 224 diameter). **b** 200 L polycarbonate open tank. **c** 500 L raceway pond. **d** Acrylic pipe photobioreactor  
 225 (60 mm outer diameter).  
 226



227  
 228 **Fig. 6** Large-scale outdoor cultivation of OPMS 30543. **a** Biomass of OPMS 30543 cultivated using  
 229 natural light in acrylic pipe PBRs (5 mm thickness with different outer diameters of 114, 216, and 267  
 230 mm), a plastic bag (0.1 mm thickness with 450 mm outer diameter), 200 L polycarbonate open tank,  
 231 and 500 L raceway pond. **b** Biomass of OPMS 30543 cultivated outdoors under natural light in an  
 232 acrylic pipe PBR with a 60 mm outer diameter. In these experiments, 50% seawater enriched with 2×

233 Daigo's IMK was used as the medium. Aeration was provided except for the raceway pond. In the  
234 raceway pond, cells were stirred using a paddle. During cultivation, the pH was adjusted to 8 by  
235 blowing CO<sub>2</sub>.

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## 236 Discussion

237 In previous studies, *P. lutheri* and *P. pinguis* were examined as aquatic feed producers that  
238 accumulate high levels of  $\omega$ -3 fatty acids, including docosahexaenoic acid and eicosapentaenoic acid  
239 (Guihéneuf and Stengel 2013; Guihéneuf et al. 2015; Fernandes et al. 2020). However, these  
240 organisms have not been studied extensively for their use as fucoxanthin producers, despite several  
241 reports describing fucoxanthin production by *P. lutheri* (Hiller et al. 1988; Lananan et al. 2013) and  
242 the advantages of the lack of a cell wall in *Pavlova* spp. (Green 1980). To develop a useful fucoxanthin  
243 production method, this study first compared fucoxanthin production in three *Pavlova* strains and  
244 identified *Pavlova* sp. OPMS 30543 as a promising strain owing to its significantly higher fucoxanthin  
245 production than that of *P. pinguis* NBRC 102807 and *P. lutheri* NBRC 102808 (Fig. 1d).

246 To determine the optimal conditions for OPMS 30543 cultivation, three types of media were  
247 examined. The use of 2× Daigo's IMK medium resulted in higher fucoxanthin production than with  
248 either f/2 or Walne's medium (Fig. 2c). A likely reason is that 2× Daigo's IMK contains a much higher  
249 level of nitrate (400 mg/L NaNO<sub>3</sub>) than f/2 (75 mg/L NaNO<sub>3</sub>) or Walne's (100 mg/L NaNO<sub>3</sub>) (Table  
250 1). Nitrate supplementation has been reported to increase fucoxanthin production in the diatoms  
251 *Phaeodactylum tricornutum* and *O. aurita* (Xia et al. 2013; McClure et al. 2018). Nitrogen  
252 supplementation with tryptone improved fucoxanthin production in *P. tricornutum* (Yang and Wei  
253 2020). This study also investigated different nitrogen sources with which to modify 2× Daigo's IMK  
254 and found that the use of NaNO<sub>3</sub> resulted in the highest fucoxanthin accumulation (Fig. 4c).  
255 Microalgae growth and fucoxanthin generally show a positive relationship, except under some  
256 conditions such as nitrogen depletion, under which fucoxanthin content decreases (Xia et al. 2018). In  
257 this study, the modified IMK medium containing KNO<sub>3</sub> led to the highest biomass (Fig. 4a), although  
258 the fucoxanthin content was the lowest (Fig. 4b). This might be because the nitrogen source was  
259 depleted in the KNO<sub>3</sub> medium owing to the highest cell growth. The effect of the nitrogen source on  
260 fucoxanthin production has not been examined in detail in previous studies. Absorption and  
261 assimilation of different nitrogen sources were investigated in Pelagophyceae *Aureococcus*  
262 *anophagefferens*, which also accumulates fucoxanthin (Ou et al. 2018). Different from the results of  
263 this study, cultivation using urea resulted in the highest fucoxanthin content in this microalga  
264 compared to cultivation with NaNO<sub>3</sub>, NH<sub>4</sub>Cl, or glutamic acid. Although the effects differ among algae  
265 species, these results suggest that supplementation and type of nitrogen source are important factors  
266 affecting fucoxanthin accumulation.

267 Among the *Pavlova* strains tested in this study, *P. pinguis* NBRC 102807 exhibited the  
268 highest biomass production (Fig. 1b). In contrast, *Pavlova* sp. OPMS 30543 could grow under a wide  
269 range of seawater concentrations, ranging from 25% to 100%, with similar biomass productivity (Fig.  
270 3a). This robustness toward salinity is a valuable characteristic for seawater cultivation. OPMS 30543  
271 did not produce biomass when cultured in medium with 0% seawater, possibly because Daigo's IMK



272 medium depends upon supplementation of Mg<sup>2+</sup> and Ca<sup>2+</sup> in seawater (Table 1). Of the three media  
 273 examined, 2× Daigo’s IMK provided the highest OPMS 30543 biomass production (Fig. 2a), probably  
 274 because it contained more nitrate than either f/2 or Walne’s media (Table 1). The effects of an  
 275 additional carbon source were also examined. This analysis revealed that the addition of glucose,  
 276 sodium acetate, or sodium bicarbonate to 2× Daigo’s IMK medium enhanced OPMS 30543 biomass  
 277 production (Fig. 4d). In haptophyte *Isochrysis galbana*, glycerol was found to be the best additional  
 278 carbon source to enhance biomass production, whereas acetate had no effect and glucose only slightly  
 279 enhanced the growth rate (Alkhamis and Qin 2013). Overall, these data suggest that the addition of a  
 280 suitable carbon is a promising approach for enhancing the biomass production of microalgae,  
 281 including OPMS 30543.

282 In the large-scale outdoor cultivation experiment, the acrylic pipe PBRs demonstrated higher  
 283 biomass production than the open tank or raceway pond (Fig. 6a). A possible reason for this result is  
 284 that the open tank and raceway pond were highly contaminated with bacteria, fungi, and protozoa  
 285 (data not shown). Among the acrylic pipe PBRs examined, those with a smaller diameter produced  
 286 higher biomass, most likely because the higher surface area-to-volume ratio contributes to more  
 287 efficient illumination. Using the 60 mm diameter acrylic pipe PBR, a fucoxanthin content of 20.86  
 288 mg/g DCW and fucoxanthin productivity of 4.88 mg/L/day was obtained after 8 days of cultivation  
 289 (Fig. 6b). Fucoxanthin content in various microalgae and macroalgae has been reported in previous  
 290 studies (Table 3). Microalgae such as haptophytes, diatoms, and chrysophytes generally show higher  
 291 fucoxanthin content than macroalgae. In diatoms, *P. tricornutum* and *Cylindrotheca closterium* were  
 292 reported to achieve 59.2 mg/g DCW and 25.5 mg/g DCW fucoxanthin content, respectively (McClure  
 293 et al. 2018; Wang et al. 2018). Chrysophytes *Mallomonas* sp. also showed a high fucoxanthin content  
 294 of 26.6 g/g DCW (Petrushkina et al. 2017). For commercialization of cultured cells as a whole food,  
 295 however, these microalgae would not be favorable because they have a cell wall. In this study, as a  
 296 cell wall-lacking microalga, *Pavlova* sp. OPMS 30543 achieved a fucoxanthin content of 20.86 mg/g  
 297 DCW, which is higher than that achieved with *Isochrysis* aff. *galbana* (Kim et al. 2012). Thus, *Pavlova*  
 298 sp. OPMS 30543 is a promising feedstock for fucoxanthin, characterized by both a high fucoxanthin  
 299 content and the absence of cell wall. With the development of a large-scale outdoor cultivation method  
 300 for OPMS 30543 fucoxanthin production as demonstrated in this study, the utilization of *Pavlova* cells  
 301 as whole foods has taken a step toward successful commercialization.

302  
 303

**Table 3.** Summary of fucoxanthin content in microalgae and macroalgae

			Fucoxanthin	
	Species	Cell wall	content (mg/g DCW)	References
Haptophytes	<i>Pavlova</i> sp.	Negative	20.86	This study

	<i>Isochrysis aff. galbana</i>	Negative	18.23	Kim et al. 2012
	<i>Isochrysis galbana</i>	Negative	15.8	Sun et al. 2019
	<i>Tisochrysis lutea</i>	Negative	16.39	Gao et al. 2020
Diatoms	<i>Chaetoceros gracilis</i>	Positive	2.24	Kim et al. 2012
	<i>Cylindrotheca closterium</i>	Positive	25.5	Wang et al. 2018
	<i>Nitzschia laevis</i>	Positive	12.0	Lu et al. 2018
	<i>Nitzschia</i> sp.	Positive	4.92	Kim et al. 2012
	<i>Odontella aurita</i>	Positive	18.47	Xia et al. 2013
	<i>Phaeodactylum tricornutum</i>	Positive	59.2	McClure et al. 2018
	<i>Thalassiosira weissflogii</i>	Positive	9.5	Marella and Tiwari 2020
Chrysophytes	<i>Mallomonas</i> sp.	Positive	26.6	Petrushkina et al. 2017
Brown algae	<i>Cystoseira hakodatensis</i>	Positive	2.01	Susanto et al. 2016
	<i>Cystoseira indica</i>	Positive	3.56	Fariman et al. 2016
	<i>Nizamuddinina zanardinii</i>	Positive	1.65	Fariman et al. 2016
	<i>Padina</i> sp.	Positive	1.97	Dang et al. 2017
	<i>Sargassum horneri</i>	Positive	2.12	Susanto et al. 2016
	<i>Sargassum linearifolium</i>	Positive	1.76	Dang et al. 2017
	<i>Sargassum siliquastrum</i>	Positive	1.99	Susanto et al. 2016
	<i>Sphaerotrichia divaricata</i>	Positive	1.15	Maeda et al. 2018
	<i>Undaria pinnatifida</i>	Positive	0.73	Xiao et al. 2012

304

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464 **Declarations**

465

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468

469 **Competing Interests**

470 A. Kanamoto was a CEO of OP Bio Factory at the time this study was conducted. A. Kanamoto  
471 participated in the experiments **as a representative** of OP Bio Factory. The corresponding author has  
472 full access to all the data in the study and is completely responsible for the data and its accuracy. All  
473 authors declare that they have no competing interests.

474

475 **Availability of data and material**

476 The data supporting the findings of this study are available within this article or from the corresponding  
477 author upon reasonable request. *Pavlova pinguis* NBRC 102807 and *Pavlova lutheri* NBRC 102808  
478 can be obtained from the National Biological Resource Center (NBRC).

479

480 **Code availability**

481 Not applicable.

482

483 **Authors' Contributions**

484 A. Kanamoto designed the study, conducted the experiments, and drafted the manuscript. Y. K., E. Y.,  
485 T. H., and A. Kondo commented on the study, helped interpret results, and revised the manuscript. All  
486 authors approved the final version of the manuscript.