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

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Abstract

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

Key words

Fucoxanthin, Marine microalgae, Outdoor cultivation, *Pavlova*

Introduction

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

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

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

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

Materials and Methods

Strains and Laboratory-Scale Cultivation

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

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

1× Daigo's IMK		f/2		Walne's	
$NaNO_3$	200	$NaNO_3$	75	$NaNO_3$	100
Na_2HPO_4	1.4	$NaH_2PO_4 \cdot 2H_2O$	6	$NaH_2PO_4 \cdot 2H_2O$	20
K_2HPO_4	5	-		-	
NH_4Cl	2.68	-		-	
Fe-EDTA	5.2	$FeCl_3 \cdot 6H_2O$	3.16	$FeCl_3 \cdot 6H_2O$	1.3
Mn-EDTA	0.332	$MnCl_2 \cdot 4H_2O$	0.18	$MnCl_2 \cdot 4H_2O$	0.36
Na_2-EDTA	37.2	Na_2-EDTA	4.4	Na_2-EDTA	45
$ZnSO_4 \cdot 7H_2O$	0.023	$ZnSO_4 \cdot 7H_2O$	0.021	$ZnCl_2$	0.021
$CoSO_4 \cdot 7H_2O$	0.014	$CoSO_4 \cdot 7H_2O$	0.012	$CoCl_2 \cdot 6H_2O$	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.0073	$Na_2MoO_4 \cdot 2H_2O$	0.007	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.009
$CuSO_4 \cdot 5H_2O$	0.0025	$CuSO_4 \cdot 5H_2O$	0.007	$CuSO_4 \cdot 5H_2O$	0.02
H_2SeO_3	0.0017	-		-	
-		$Na_2SiO_3 \cdot 9H_2O$	10	-	
-		-		H_3BO_3	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

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

	1× Daigo's IMK	mIMK ($NaNO_3$)	mIMK (KNO_3)	mIMK ($CO[NH_2]_2$)	mIMK (NH_4Cl)
$NaNO_3$	200	200	-	-	-
KNO_3	-	-	200	-	-
$CO(NH_2)_2$	-	-	-	200	-
NH_4Cl	2.68	-	-	-	200
Na_2HPO_4	1.4	-	-	-	-
K_2HPO_4	5	5	5	5	5
Fe-EDTA	5.2	-	-	-	-
Mn-EDTA	0.332	-	-	-	-
Na_2-EDTA	37.2	37.2	37.2	37.2	37.2
$ZnSO_4 \cdot 7H_2O$	0.023	0.023	0.023	0.023	0.023
$CoSO_4 \cdot 7H_2O$	0.014	-	-	-	-
$Na_2MoO_4 \cdot 2H_2O$	0.0073	-	-	-	-
$CuSO_4 \cdot 5H_2O$	0.0025	0.0025	0.0025	0.0025	0.0025
H_2SeO_3	0.0017	-	-	-	-
Thiamine-HCl	0.2	-	-	-	-
Biotin	0.0015	-	-	-	-
Vitamin B12	0.0015	-	-	-	-

Pigment Analysis

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

Large-Scale Cultivation

OPMS 30543 was cultivated outdoors under natural sunlight using the following common cultivation systems: 1) 60 mm outer diameter and 5 mm thickness acrylic pipe photobioreactor (PBR), 2) 114 mm outer diameter and 5 mm thickness acrylic pipe PBR, 3) 216 mm outer diameter and 5 mm thickness acrylic pipe PBR, 4) 267 mm outer diameter and 5 mm thick acrylic pipe PBR, 5) 450 mm outer diameter and 0.1 mm thickness plastic bag, 6) 200 L polycarbonate open tank, and 7) 500 L raceway pond, in 50% artificial seawater containing 2× Daigo's IMK elements described above (Table 1). Agitation was performed by aeration at 0.25 mL/min for 1) and 2), and 0.1 mL/min for 3), 4), 5), and 6) except for the raceway pond, in which the flow rate was adjusted to 0.5 m/s by stirring with a paddle. During cultivation, the pH was adjusted to 8 by supplying 100% CO_2.

Results

Screening of *Pavlova* Strains for Fucoxanthin Production

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

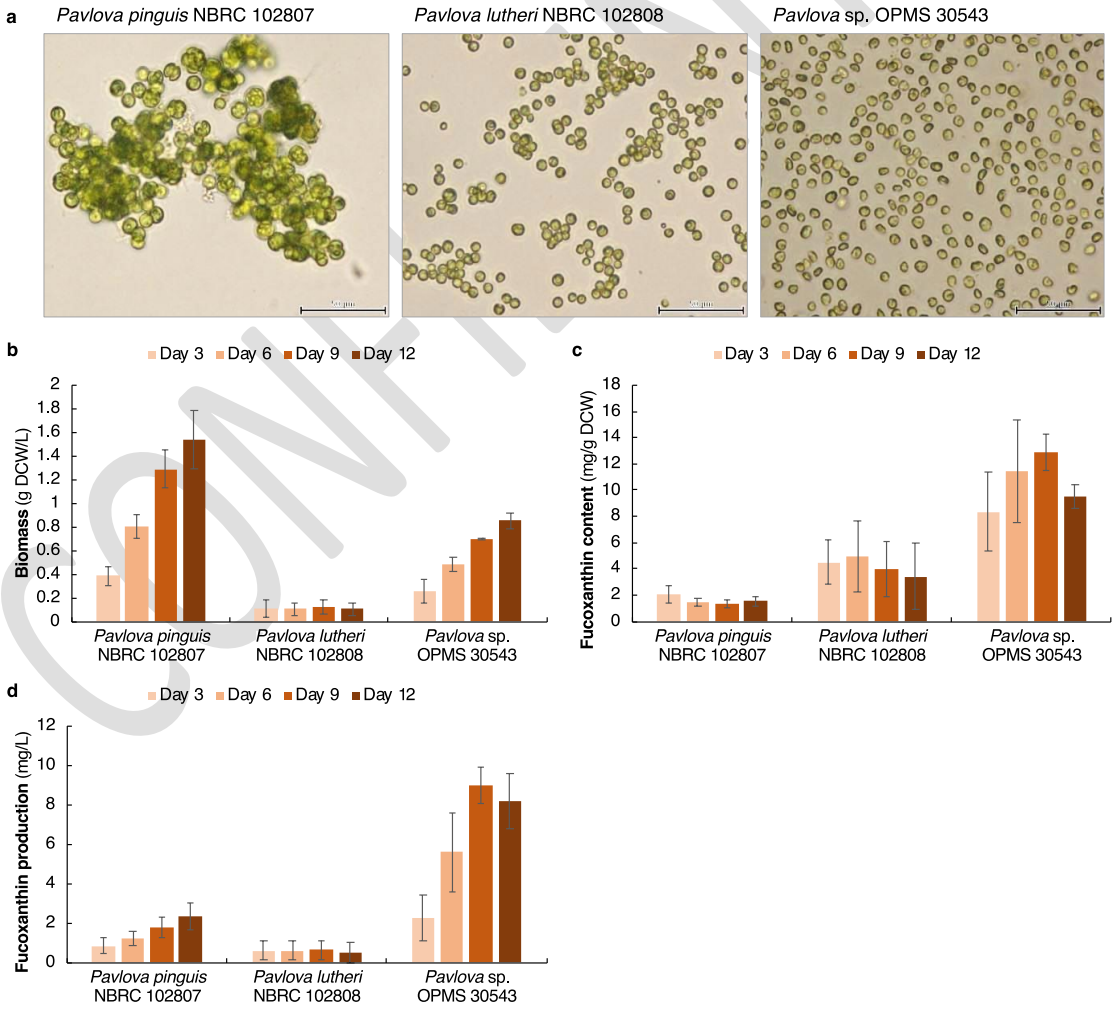

Fig. 1 Comparison of three *Pavlova* strains. **a** Microscopic images of *Pavlova* cells. Scale bars: 50 µm.

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

Examination of Culture Medium for OPMS 30543

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

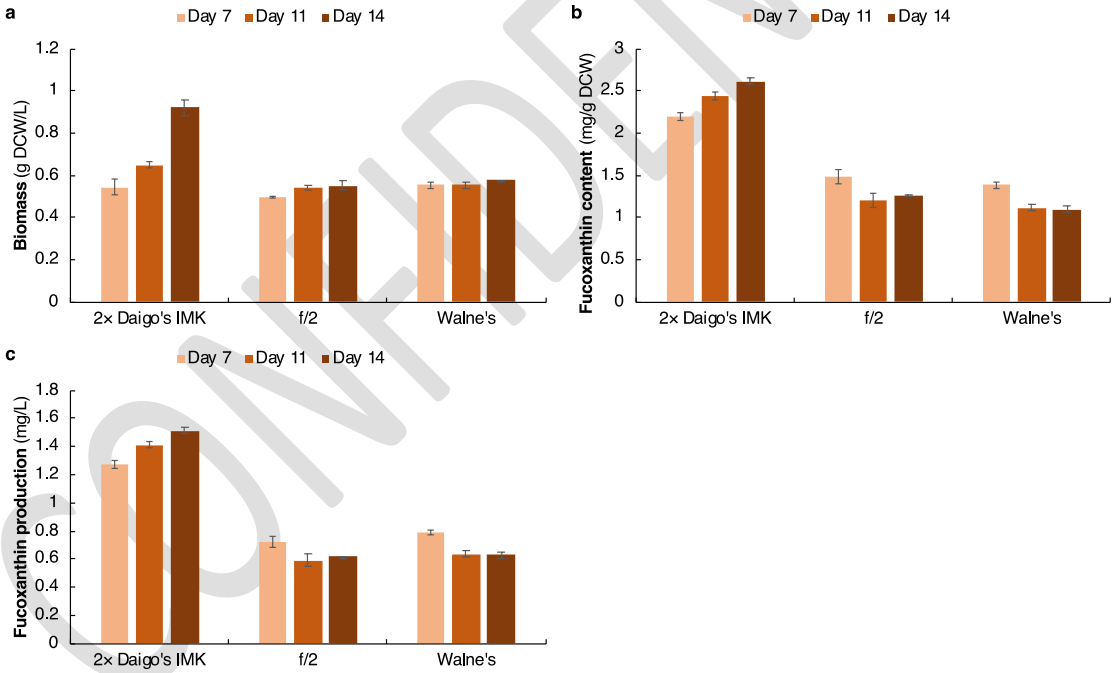

Fig. 2 Comparison of different media for OPMS 30543 cultivation. **a** Biomass. **b** Fucoxanthin content. **c** Fucoxanthin production. Cells were statically cultivated in 200 mL Erlenmeyer flasks with a 100 mL working volume of 50% seawater containing either 2× Daigo's IMK, f/2, or Walne's elements.

Examination of Culture Conditions for OPMS 30543

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

with different concentrations of seawater, biomass production was observed only in the presence of seawater; OPMS 30543 did not grow in 0% seawater medium (Fig. 3a). The highest biomass of 6.16 g DCW/L on day 14 was achieved in the medium with 50% seawater. The effect of varying the culture pH by supplying CO_2 gas to the medium was also examined (Fig. 3b). OPMS 30543 biomass production was reduced when the pH was adjusted to 6, whereas the highest biomass of 3.78 g DCW/L on day 6 was observed when pH was adjusted to 8. Culture temperature was investigated over the range of 15–35 °C (Fig. 3c). Within this temperature range, OPMS 30543 produced higher biomass at higher temperatures, and cultivation at 35 °C resulted in the highest biomass production of 3.32 g DCW/L on day 6. Thus, cultivation in 50% seawater medium at 35 °C and pH 8 was determined to be the optimal condition for OPMS 30543 biomass production.

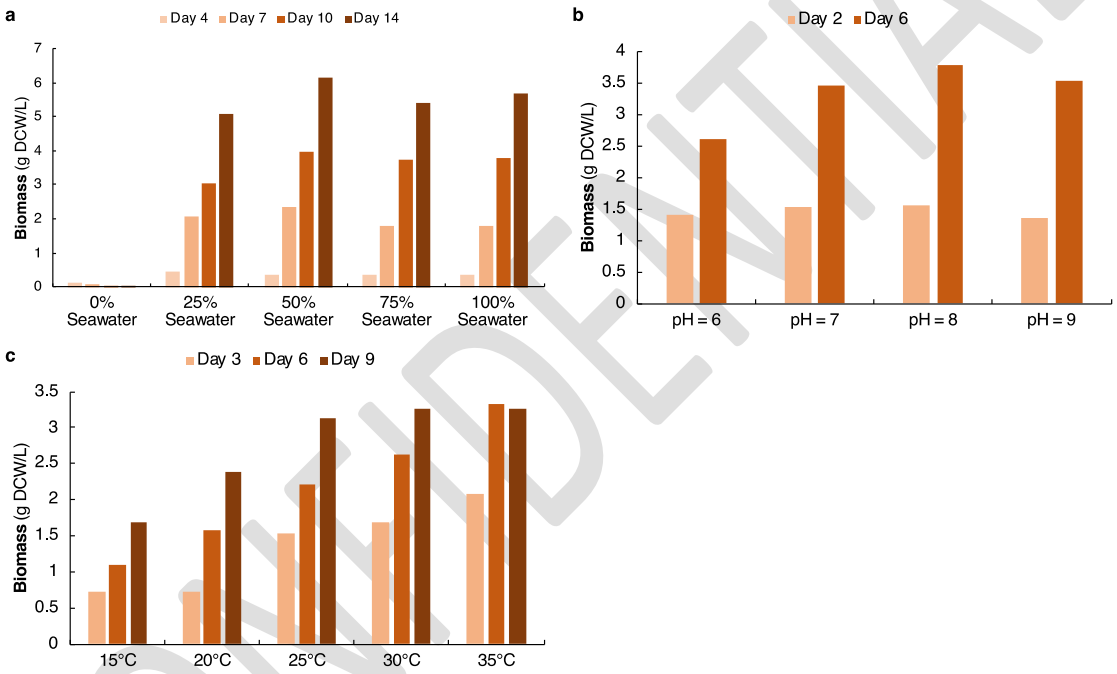

Fig. 3 Comparison of culture conditions for OPMS 30543. **a** Seawater concentration in medium. **b** pH, adjusted by supplying CO_2 gas to the culture. Cultures were illuminated with red, blue, and white LEDs at a total intensity of 300 μmol photons/m^2/s with a 12 h:12 h light/dark cycle. **c** Culture temperature.

Modification of IMK Medium by Replacing Nitrogen Sources and Adding Carbon Sources

To further improve OPMS 30543 biomass production and fucoxanthin content, the effect of varying the nitrogen source in the medium was examined. The modified IMK medium was prepared by replacing $NaNO_3$ in 1× Daigo's IMK with either $NaNO_3$, KNO_3, $CO(NH_2)_2$, or NH_4Cl (Table 2). After 9 days of cultivation, cells cultured in the modified IMK medium containing KNO_3 exhibited the highest biomass of 1.8 g DCW/L (Fig. 4a). Both urea $CO(NH_2)_2$ and NH_4Cl were found to be 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 $NaNO_3$-containing medium resulted in higher
176 fucoxanthin content (12.74 mg/g DCW) than in media with KNO_3 (5.57 mg/g DCW), $CO(NH_2)_2$ (8.38
177 mg/g DCW), or NH_4Cl (7.80 mg/g DCW) (Fig. 4b). Fucoxanthin production was the highest when
178 $NaNO_3$ was used as the nitrogen source (Fig. 4c). Fucoxanthin production of OPMS 30543 grown in
179 modified IMK medium containing $NaNO_3$, KNO_3, $CO(NH_2)_2$, or NH_4Cl was 17.84, 10.03, 13.24, and
180 6.40 mg/L, respectively. Thus, these data suggest that $NaNO_3$ 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× Daigo's IMK.
185 Each of the additional carbon sources increased biomass production compared to that with the normal
186 1× 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.

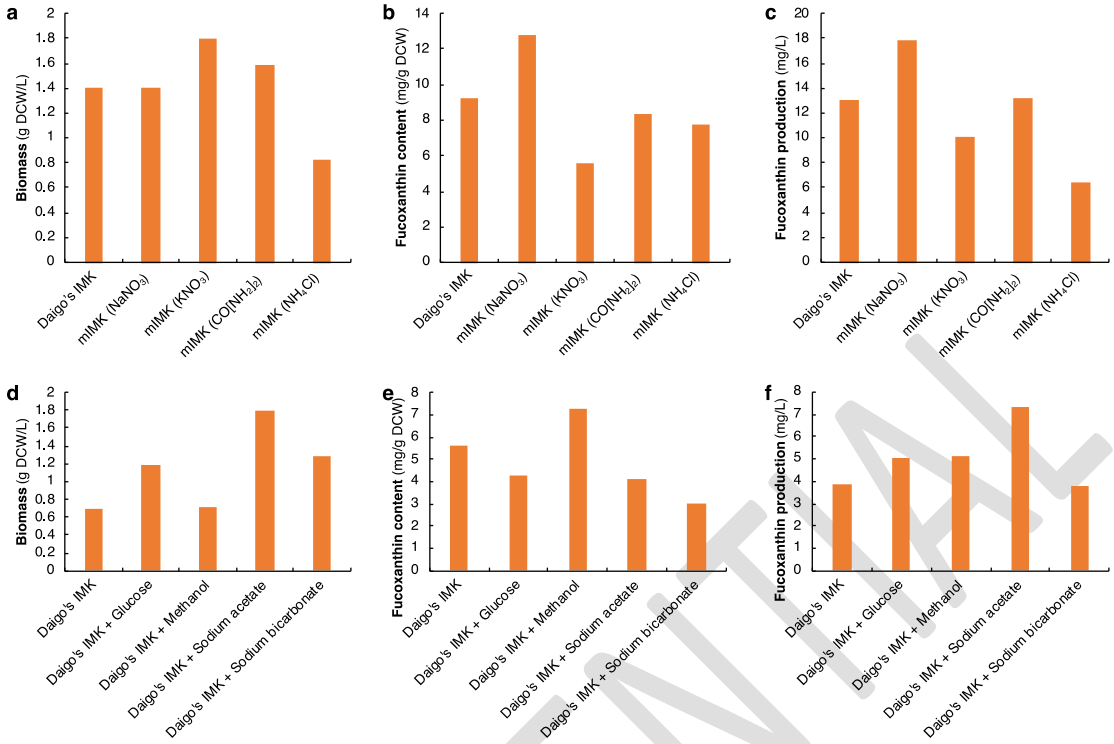

Fig. 4 Examination of alternative nitrogen sources and additional carbon sources. **a** Biomass, **b** fucoxanthin content, and **c** fucoxanthin production of cells grown in 50% seawater enriched with modified IMK and different nitrogen sources. **d** Biomass, **e** fucoxanthin content, and **f** fucoxanthin 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 μmol photons/m^2/s with a 12 h:12 h light/dark cycle.

Large-Scale Outdoor Cultivation of OPMS 30543

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

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

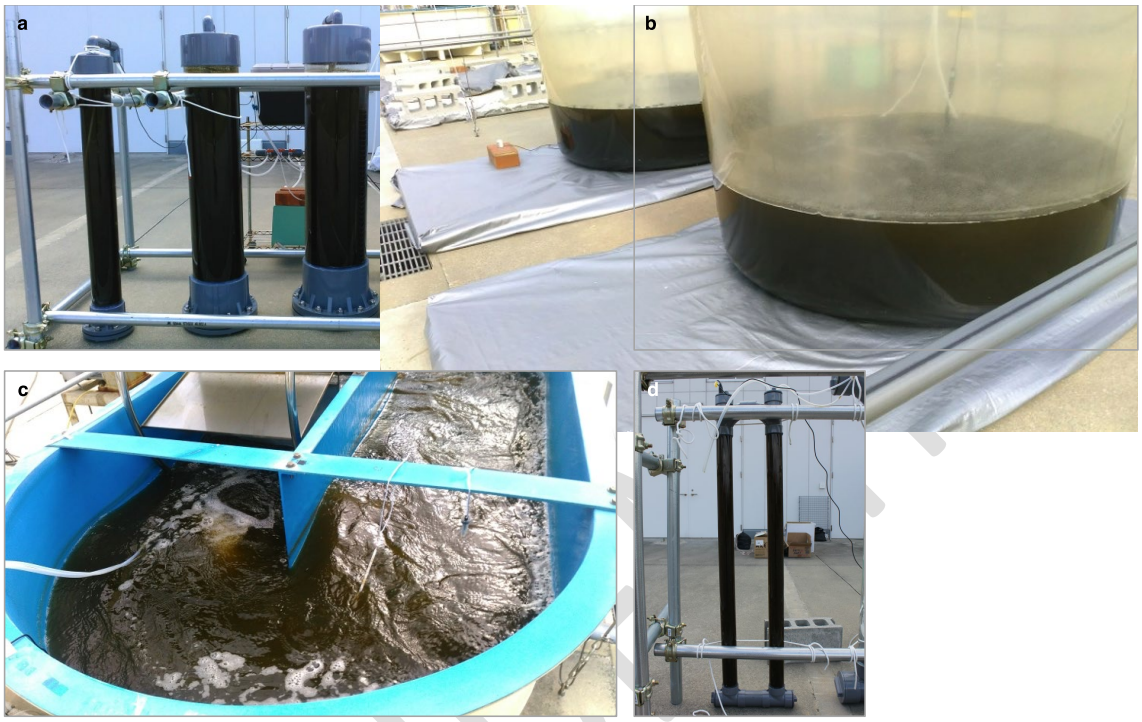

Fig. 5 Facilities used for outdoor cultivation. **a** Acrylic pipe photobioreactors (5 mm thickness with outer diameters of 114, 216, and 267 mm) and a plastic bag (0.1 mm thickness with 450 mm outer diameter). **b** 200 L polycarbonate open tank. **c** 500 L raceway pond. **d** Acrylic pipe photobioreactor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

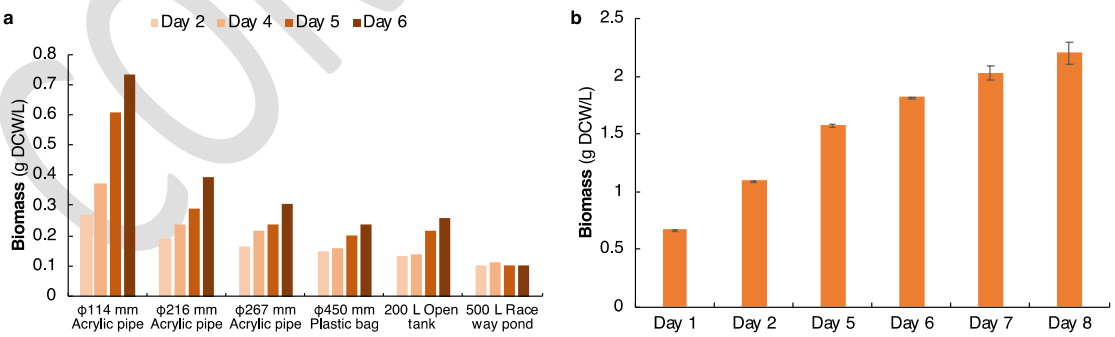

Fig. 6 Large-scale outdoor cultivation of OPMS 30543. **a** Biomass of OPMS 30543 cultivated using natural light in acrylic pipe PBRs (5 mm thickness with different outer diameters of 114, 216, and 267 mm), a plastic bag (0.1 mm thickness with 450 mm outer diameter), 200 L polycarbonate open tank, and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

Daigo's IMK was used as the medium. Aeration was provided except for the raceway pond. In the raceway pond, cells were stirred using a paddle. During cultivation, the pH was adjusted to 8 by blowing CO_2.

236 **Discussion**

237 In previous studies, *P. lutheri* and *P. pinguis* were examined as aquatic feed producers that
238 accumulate high levels of ω-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 $NaNO_3$) than f/2 (75 mg/L $NaNO_3$) or Walne's (100 mg/L $NaNO_3$) (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 $NaNO_3$ 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_3 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_3 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 Pelagophycea *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 $NaNO_3$, NH_4Cl, 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^{2+} and Ca^{2+} 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

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

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

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Declarations

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Competing Interests

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

Availability of data and material

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

Code availability

Not applicable.

Authors' Contributions

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