Watsuji et al. Annals of Microbiology (2021) 71:25
https://doi.org/10.1186/s13213-021-01636-6

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE Open Access

Effect of temperature and dissolved oxygen on gravity sedimentation of the unicellular alga *Dunaliella salina*

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Abstract

Purpose: The microalgae *Dunaliella salina* is mainly cultivated as a source of natural β-carotene, but it can also be an important source of other nutrients such as protein, carbohydrate, and lipids. Harvesting and dewatering are considered the most expensive processes in the biomass production, so we proposed gravity sedimentation as a cost-effective method. The effect of temperature and dissolved oxygen concentration on the gravity sedimentation of normal cells and carotenoid-accumulating cells of *Dunaliella salina* was investigated in this study.

Methods: *Dunaliella salina* was cultivated in an f/2 culture medium at two different salinities, 12.5% (2.2 M NaCl) and 17.5% (3.3 M NaCl). Carotenoid-accumulating cells were cultivated in a medium without a nitrogen source by removing $NaNO_3$ from the f/2 medium and at two different salinities, 12.5% and 17.5%. For gravity sedimentation tests, 10 mL of the suspended culture media were transferred to conical tubes and wrapped with aluminum foil for shading. The tubes were incubated at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C for 4 h and 18 h. For the gravity sedimentation under aerobic and anaerobic conditions, 10 mL of the suspended culture media were transferred to conical tubes and then purged with 20% oxygen and 80% nitrogen or 100% nitrogen for 10 min and wrapped with aluminum foil. The tubes were incubated at 25 °C for 0.5 h, 1 h, 2 h, and 3 h.

Result: Recovery rates differed with temperature, salinity, and time. The recovery rate of normal cells and carotenoid-accumulating cells reached 79 to 96% at 20 °C, 25 °C, and 30 °C at 12.5% and 17.5% of salinity and after 4 h. The recovery rate of both normal and carotenoid-accumulating cells in gravity sedimentation experiments was not significantly affected by changing initial dissolved oxygen in the cell-cultured media.

Conclusion: Considering that gravity sedimentation at the optimum growth temperature for *D. salina* presented high recovery rates, gravity sedimentation is expected to be a cost-effective method to harvest *D. salina* from open pond cultivation systems.

Keywords: Gravity sedimentation, *Dunaliella salina*, Temperature effect, Dissolved oxygen effect

Background

Microalgae are photosynthesizing microorganisms that use sunlight to produce carbohydrates, proteins, lipids, etc. (Ben-Amotz et al., 1985; Chew et al., 2017; Spolaore et al., 2006). Raw materials or products obtained from microalgae can be widely used in the production of food and supplements, feed, medicine, fertilizers, cosmetics, and raw material for different industries such as the fermentation industry (Ariede et al., 2017; Chisti, 2018; Milledge, 2011). The advantage of using microalgae is that they grow around 10–50 times faster than terrestrial plants (Yen et al., 2013). We have paid attention to the unicellular green alga *Dunaliella salina* for the creation of an ecological and carbon-neutral society that does not release extra carbon dioxide, while able to conduct industry. *D. salina* possesses outstanding halotolerance

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(up to 5 M NaCl) and accumulates high amounts of β-carotene in the cell during the growth under salt, light, and nutrient stress conditions (Coesel et al., 2008a; Fisher et al., 1994; Pourkarimi et al., 2020). Tolerance of *D. salina* to extreme salt conditions makes it grow in hypersaline environments, reducing predators, especially the ciliate *Fabrea salina* and the brine shrimp *Artemia salina* (Elloumi et al., 2009; Post et al., 1983). Thus, as a source of valuable β-carotene, *D. salina* has been commercially cultivated at salt ponds in Israel, the USA, and Australia (Ben-Amotz, 1995; Borowitzka, 1997; Spolaore et al., 2006). Open-air cultures are preferred in large-scale commercial algal cultures because of the higher cost for closed culture systems (Borowitzka, 1999). For instance, *D. salina* is cultured in large open-air ponds (up to 250 ha) without artificial mixing and bubbling in Australia. This represents an advantage because open-air cultures of the other main commercial microalgae such as *Chlorella* and *Spirulina* species require artificial mixing or additional energy input (Borowitzka, 1999). Therefore, it can be said that open-air culture systems of *D. salina* is economically viable.

This research aimed to establish a cost-effective technique for collecting *D. salina* as an inexpensive biomass. Methods available for harvesting or recovery of cultivated microalgae include centrifugation, flotation, filtration, flocculation, gravity sedimentation, electrophoresis techniques, and combined methods such as flocculation–sedimentation and flocculation–flotation (Chatsungnoen and Chisti, 2016; Junior et al., 2020; Milledge and Heaven, 2013; Okoro et al., 2019; Uduman et al., 2010). Since microalgal recovery by gravity sedimentation is an additive-free recovery method that utilizes gravitational force and enables reuse of culture medium, it has a clear advantage in terms of cost (Li et al., 2020; Najjar and Abu-Shamleh, 2020). However, this method is suitable for relatively large (ca. > 70 mm) microalgae such as *Spirulina* (Velan and Saravanane, 2013). Thus, smaller-sized microalgal cells, including *D. salina,* lead to high cost in recovery from the culture medium (Grima et al., 2003; Mata et al., 2010; Verma et al., 2010). Given that the production of microalgae-derived products is a growing and promising market worldwide, low-cost raw materials and large production quantities are necessary. Thus, the applicability of gravity sedimentation as a cost-effective method for microalgal recovery should be considered, especially for large-scale production of biomass. Even though the settling velocity of *Dunaliella* cells in gravity sedimentation has been measured (Eppley et al., 1967), very limited research has been done on this harvesting method. Scientific articles published in the last few years include sedimentation as a harvesting method, but they neither show experimental results, nor propose any design for harvesting microalgae from mass culture. Also, the effect of temperature and dissolved oxygen on gravity sedimentation of *D. salina* has not yet been identified as far as we know. Thus, in this study, recovery rates of *D. salina* cells in gravity sedimentation were investigated at a wide range of temperatures, under aerobic and anaerobic conditions. Additionally, normal cells and carotenoid-accumulating cells of *D. salina* were used in the gravity sedimentation experiments.

Results and discussion

D. salina cultivated in the absence of nitrogen source accumulate β-carotene in their cells (Coesel et al., 2008b). When exposed to such nutrient stress, the cells change from ovoid to spherical in shape and from green to orange in color due to β-carotene accumulation (Ramos et al., 2011). In the present study, *D. salina* cultivated in the absence of nitrogen source at a salinity of 12.5% and 17.5% indicated such morphological and coloristic features. The carotenoid-accumulating cells cultured at 12.5% salinity were shown in Fig. 1a as a representative of the different salinities. On the other hand, *D. salina* cultivated in the presence of a nitrogen source at a salinity of 12.5% and 17.5% indicated normal morphological and coloristic features. The normal cells cultured at 17.5% salinity were shown in Fig. 1b as a representative of the different salinities. The cell concentrations of *D. salina* cultured in the absence of nitrogen source at a salinity of 12.5% and 17.5% were 1.4×10^6 cells/mL and 1.8×10^6 cells/mL, respectively. The cell concentrations of *D. salina* cultured in the presence of nitrogen source at a salinity of 12.5% and 17.5% was 7.0×10^5 cells/mL and 2.4×10^6 cells/mL, respectively.

The *D. salina* cells in the media were collected by gravity sedimentation under dark conditions between 4 and 50 °C. The statistical significance of differences in cell recovery rates at different temperatures and settlement time was determined by unpaired t test analysis. Results showed statistically significant difference between temperatures ($P < 0.05$) in 13 to 70% of all tested cases. Normal cells cultured in 17.5% salinity and settled for 4 h showed the highest variability in recovery rate between temperatures. Collection by gravity sedimentation was more suitable for the carotenoid-accumulating cells than for normal cells (Fig. 2). The recovery rate of the carotenoid-accumulating cells in the medium with 12.5% salinity was 89.2 to 99.7% for 18 h at any given temperature (Fig. 2a). The recovery rate of the carotenoid-accumulating cells and the normal cells in medium with 12.5% salinity was higher than that with 17.5% salinity at each temperature and incubation time (Fig. 2). In particular, the recovery rate of both cells in a short period of time (4 h) was remarkably different between 12.5 and 17.5% salinity (Fig. 2). However, the

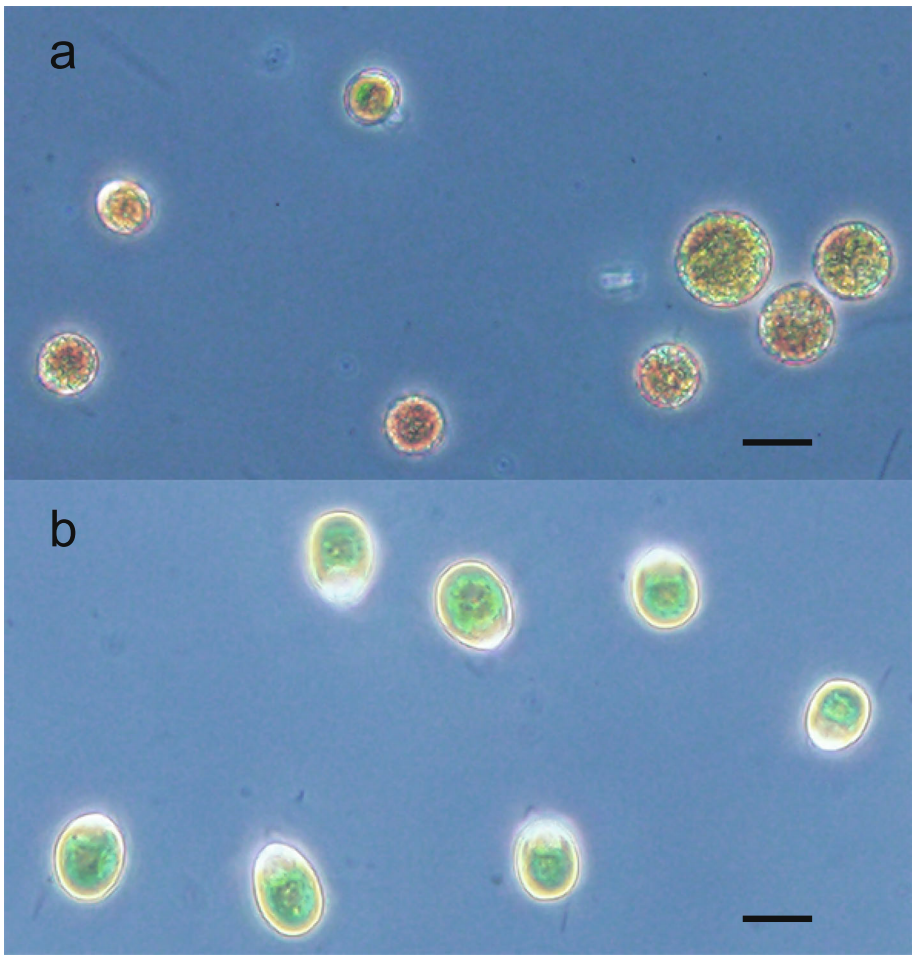

Fig. 1 *D. salina* cells cultivated under different nutrient conditions. **a** Cells cultivated in the absence of nitrogen source at 12.5% salinity. **b** Cells cultivated in the presence of nitrogen source at 17.5% salinity. All scale bars indicate 20 μm

recovery rate of both cells in the media with 12.5% and 17.5% salinity was one of the highest at the optimal growth temperature for *D. salina* (around 25 °C) and reached 79.4 to 95.6% at 20 °C, 25 °C, and 30 °C even after 4 h (Fig. 2). Additionally, the recovery rates of both cells in the media with 12.5% and 17.5% salinity tended to increase at over 40 °C (Fig. 2). The recovery rate of carotenoid-accumulating cells in both media increased at any given temperature by extending the incubation time; the recovery rate of normal cells in both media increased at temperatures below 20 °C and at temperatures over 40 °C by extending the incubation time (Fig. 2).

In this study, the carotenoid-accumulating cells had higher cell recovery rates through gravity sedimentation than the normal cells. The carotenoid-accumulating cells exhibited a marked decrease in motility compared with the normal cells under microscopic observation (data not shown). According to laboratory observations reported by Smayda (1970), phytoplankton sank to the bottom of the flask when they lost their flagella, were stressed to affect their motility, or were senescent (non-dividing cells, between maturity and death). Thus, high recovery rates of carotenoid-accumulating cells in gravity sedimentation can be attributed to their low motility. In any case, it is desirable that the carotenoid-accumulating cells with high commercial value are suitable for cell collection by gravity sedimentation. In the gravity sedimentation method, medium volume could be reduced to 10%. This method using gravity is compatible and easy to adapt to scaling-up cell recovery. Thus, recovery of *D. salina* cells by combining gravity sedimentation and centrifugation methods will significantly reduce power consumption when compared with centrifugation alone.

The recovery rates of both cells after 18 h of incubation increased as the temperature rose above 40 °C (Fig. 2). Moreover, it was observed under the microscope that

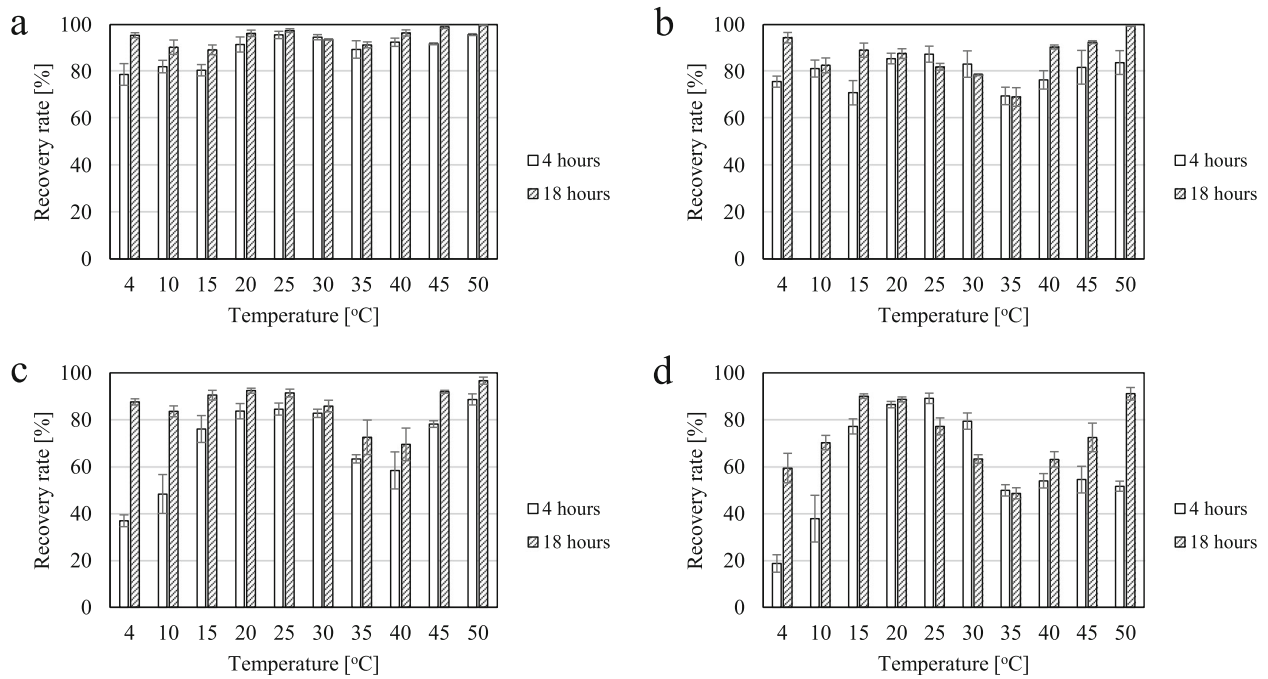

Fig. 2 Effect of temperature on gravity sedimentation of *D. salina* cells. **a** Recovery rate of carotenoid-accumulating cells cultured at 12.5% salinity. **b** Recovery rate of normal cells cultured at 12.5% salinity. **c** Recovery rate of carotenoid-accumulating cells cultured at 17.5% salinity. **d** Recovery rate of normal cells cultured at 17.5% salinity. The recovery rate of both cells in the gravity sedimentation was obtained after incubation for 4 h (white bar) and 18 h (hatched bars) at a given temperature. The recovery rate for each sample was determined in triplicate. All of the error bars represent the standard deviation of the mean

both cells lost motility at over 45 °C and after 4 h or more (data not shown). Therefore, it can be said that both cells that lost motility would precipitate—the higher the incubation temperature, the faster they lose motility leading to higher recovery rates of both cells after 18 h. In gravity sedimentation experiments of both cells at 17.5% salinity medium and 50 °C, it was observed that cells lost motility. Also, the difference in recovery rate of the normal cells after 4 h and 18 h was higher than that of the carotenoid-accumulating cells with low motility (Fig. 2c, d). These results also suggested that the motility decrease in *D. salina* cells was effective for precipitation. Except for high incubation temperature cases (above 40 °C), the recovery rates with both cells at 12.5% and 17.5% salinity media were the highest at 20 °C, 25 °C, and 30 °C (Fig. 2). The optimum growth temperature for *D. salina* is known to be 22 °C or 25 °C (Wu et al., 2016). Since the optimum temperature for growth is aligned with the temperature for gravity sedimentation, it can be said that the same temperature is required for both, the cultivation of *D. salina* in open ponds and the gravity sedimentation.

The normal and carotenoid-accumulating cells in the medium with 12.5% salinity had higher recovery rates than those with 17.5% salinity at a wide range of temperatures (Fig. 2). These results suggested that *D. salina* cells in the medium with lower specific gravity precipitate faster. Thus, culture medium with lower salinity is likely suitable for the gravity sedimentation. However, *D. salina* shows optimum growth at about 18–21% NaCl (Borowitzka and Borowitzka, 1990). Additionally, it is known that higher salinity (> 27% NaCl) in medium is required to result in the maximum β-carotene accumulation in *D. salina* and the effective prevention of microbial contamination in outdoor mass cultivation (Borowitzka and Borowitzka, 1990). Therefore, it seems better to perform the gravity sedimentation after lowering the medium salinity by adding seawater or water to the cultured medium, as done in this study. Furthermore, diluting the salt concentration of the medium increases the specific gravity of cultured cells compared with the medium. Thus, we can say that, in this study, motility decrease and relatively high specific gravity of *D. salina* cells might have led to gravity sedimentation.

The effect of dissolved oxygen on the gravity sedimentation of the carotenoid-accumulating and normal cells cultivated in the media with 12.5% salinity was investigated. The cell densities of the carotenoid-accumulating and normal cells in the media were 8.2×10^5 cells/mL and 7.2×10^5 cells/mL, respectively. Preparing cell-cultured media with different initial oxygen concentrations was achieved by 20% oxygen or nitrogen purge.

The incubation temperature in the gravity sedimentation was set to 25 °C to take economic costs into account.

The recovery rates of both cells under aerobic and anaerobic conditions increased with the sedimentation time (Fig. 3). The significance of differences in cell recovery rates under aerobic and anaerobic conditions at individual time points were determined by an unpaired t test. The differences in the recovery rates of the carotenoid-accumulating cells after 1 h (Fig. 3a) and the normal cells after 0.5 h (Fig. 3b) were of borderline significance (P = 0.07 and 0.09, respectively), but there were no statistically significant differences at other time points ($P > 0.1$). Therefore, it was indicated that recovery rate of both cells was not significantly affected by the difference of initial dissolved oxygen concentration in the cell-cultured media.

The effect of light and pH in culture medium on gravity sedimentation of *D. salina* cells was not investigated in this study. However, *D. salina* has exhibited phototaxis (Wayne et al., 1991) and *Dunaliella tertiolecta* cells in culture medium has aggregated at pH between 8.6 and 10.5 (Horiuchi et al., 2003), influenced by some

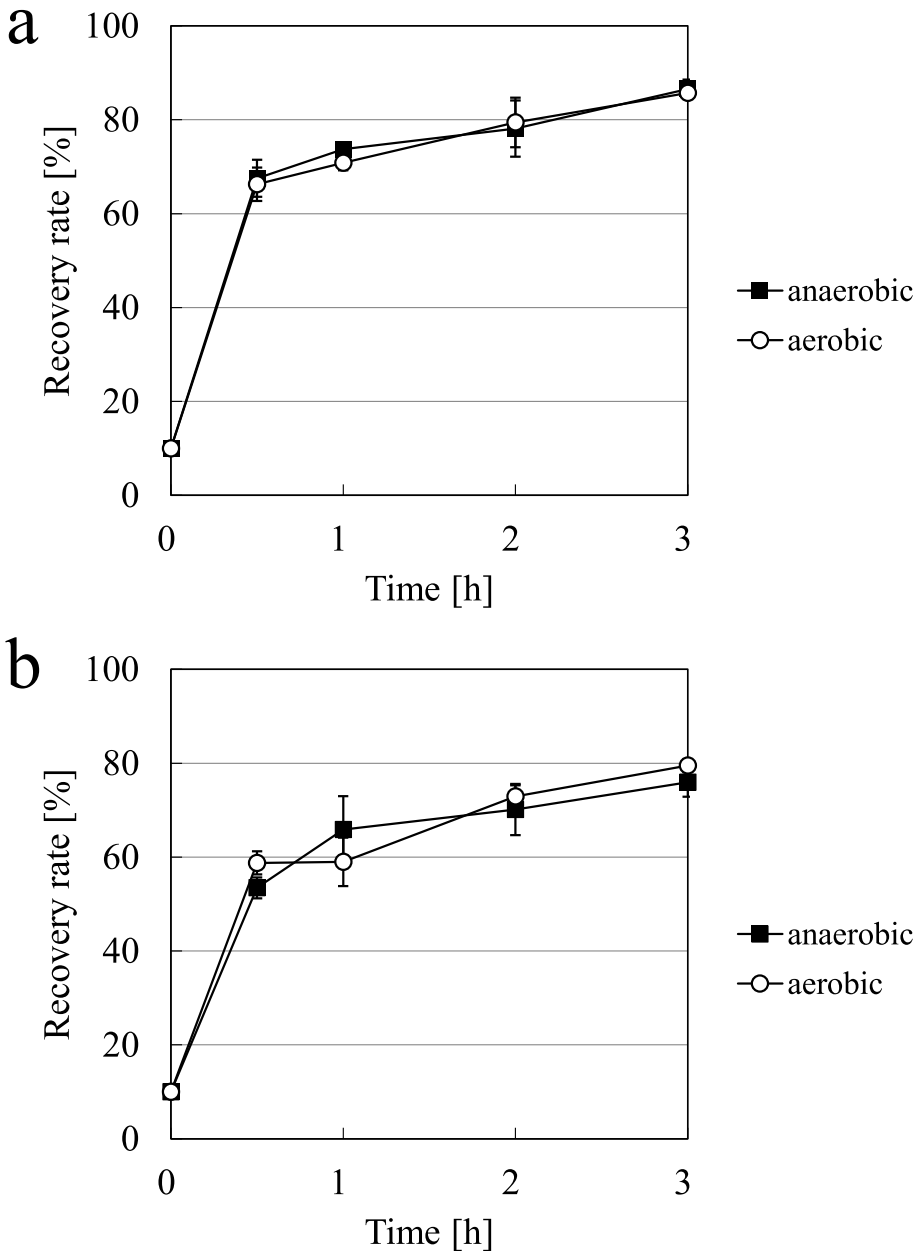

Fig. 3 Recovery rate of *D. salina* cells in gravity sedimentation under aerobic and anaerobic conditions. The recovery rates were examined at the indicated intervals for carotenoid-accumulating cells (**a**) and normal cells (**b**) under aerobic conditions (○) and anaerobic conditions (■)

precipitates such as calcium carbonate and magnesium hydroxide induced in culture medium at high pH (Şirin et al., 2012). Therefore, we must be careful to avoid light and high pH (> pH 8.6) in collection of *D. salina* cells by gravity sedimentation.

Conclusions

D. salina cells were collected by gravity sedimentation in this study. Even though high recovery rates were observed at low and high temperatures in some cases, the gravity sedimentation method ensured high recovery rates of the algal cells at 20 °C, 25 °C and 30 °C even after 4 h. This temperature range is in good alignment with the optimum temperature range for the microalgal growth. We can say that gravity sedimentation can be readily applied to *D. salina* cultivated in open ponds without cooling or heating systems, probably by transferring it to harvest storage tanks. This technology will be particularly useful to collect *Dunaliella salina* cells at low cost for subsequent raw material extraction in different industries.

Materials and methods

Species and culture

Microalgae *Dunaliella salina* strain CS-744/01 was used in experiments. The alga was cultivated in liquid f/2 medium with a salinity of 12.5% (2.2 M NaCl) or 17.5% (3.3 M NaCl) (Guillard and Ryther, 1962). The desired salinity concentrations were reached by adding sea salt (Red Sea Salt used for aquaria), and the pH of the medium was adjusted to pH 8.2 with HCl. A preculture of 200 mL was inoculated into 800 mL of fresh medium in an open tank and incubated at 25 °C under 10,000 lux of continuous illumination. On the third day, the concentration of $NaNO_3$, $NaH_2PO_4{\cdot}2H_2O$, trace elements and vitamin mix were adjusted by adding 1 mL of stock solution, to reach concentrations specified in the f/2 culture medium. Also, water equivalent to evaporated water were added. The algal cells on the seventh day of cultivation were used for experiments as normal cells. Culture conditions of preculture were the same as those of main culture.

To obtain the algal cells accumulating carotenoids, the alga was cultivated in a medium without a nitrogen source. This was prepared by removing $NaNO_3$ from f/2 medium with a salinity of 12.5% (2.2 M NaCl) or 17.5% (3.3 M NaCl). The medium's pH was adjusted to pH 8.2 with HCl. Preculture was prepared as indicated above. Two hundred milliliters of preculture were inoculated into 800 mL of fresh medium without nitrogen source in an open tank and incubated at 25 °C under continuous illumination of 10,000 lux. One milliliter of each stock solution removing $NaNO_3$ from f/2 medium and water equivalent to evaporated water was added to the culture medium on the third day, and the algal cells on the seventh day of cultivation were used for experiments as carotenoid-accumulating cells.

Gravity sedimentation

The culture media after cultivation were adjusted to 12.5% (2.2 M NaCl) or 17.5% (3.3 M NaCl) of salinity with water and to pH 8.2 with NaOH solution. The grown cells in the media were collected by gravity sedimentation. Ten milliliters of the suspended culture media were transferred to 15 mL conical screw cap tubes (No. 352096; Falcon, Corning), and the tubes were wrapped with aluminum foil for shading. The tubes were stirred on a vortex mixer and incubated at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C for 4 h and 18 h. After the incubation, 9 mL of the supernatant in each tube was transferred to a new tube. Experiments were conducted in triplicate for each temperature and settlement time.

Gravity sedimentation under aerobic and anaerobic conditions

The salinity of culture media after cultivation was adjusted to 12.5% (2.2 M NaCl) or 17.5% (3.3 M NaCl) by adding water and to pH 8.2 using NaOH solution. Ten milliliters of the suspended culture media were transferred to 15 mL conical screw cap tubes. The culture medium in the tubes was purged with 20% oxygen and 80% nitrogen or 100% nitrogen for 10 min prior to the gravity sedimentation experiment, with the tubes wrapped with aluminum foil. The tubes were stirred on a vortex mixer and incubated at 25 °C for 0.5 h, 1 h, 2 h, and 3 h. After the incubation, 9 mL of the supernatant in each tube was transferred to a new tube. Experiments were conducted in triplicate for each tested case.

Observation and cell count

The cultured cells were observed under a phase-contrast Olympus BX53 microscope equipped to a CCD camera system. The number of the algal cells in the culture media and supernatants were counted using an improved Neubauer chamber and the microscope.

Acknowledgements

This work was supported in part by the Grant-in-Aid for Scientific Research granted by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT KAKENHI Grant Number 15K12262). We would like to express our deep appreciation to Dr. Ken Takai and Dr. Hiroyuki Yamamoto from the Japan Agency for Marine-Earth Science and Technology for providing the necessary resources, equipment, and facilities to conduct this research. The authors would also like to thank Mitsubishi Gas Chemical Company, Inc. for their support in the research fund.

Authors' contributions

TW conceived the original idea, carried out experiments, analyzed the data, and wrote the manuscript. AN contributed to the analysis of the results and to the writing of the manuscript. YM performed experiments. MK obtained

research fund and supervised the overall project. All authors discussed the results, read, commented, and approved the final manuscript.

Funding
Grant-in-Aid for Scientific Research granted by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT KAKENHI Grant Number 15K12262).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 11 March 2021 Accepted: 15 June 2021
Published online: 09 July 2021

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