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Effects of temperature, photosynthetic photon flux density, photoperiod and O_2 and CO_2 concentrations on growth rates of the symbiotic dinoflagellate, *Amphidinium* sp.

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Abstract Symbiotic dinoflagellates of the species *Amphidinium* are expected to be pharmaceutically useful microalgae because they produce antitumor macrolides. A microalgae production system with a large number of cells at a high density has been developed for the efficient production of macrolide compounds. In the present study, the effects of culture conditions on the cellular growth rate of dinoflagellates were investigated to determine the optimum culture conditions for obtaining high yields of microalgae. *Amphidinium* species was cultured under conditions with six temperature levels (21–35°C), six levels of photosynthetic photon flux density (15–70 μmol photons m^{-2} s^{-1}), three levels of CO_2 concentration (0.02–0.1%), and three levels of O_2 concentration (0.2–21%). The number of cells cultured in a certain volume of solution was monitored microscopically and the cellular growth rate was expressed as the specific growth rate. The maximum specific growth rate was 0.022 h^{-1} at a temperature of 26°C and O_2 concentration of 5%, and the specific growth rate was saturated at a CO_2 concentration of 0.05%, a photosynthetic photon flux density of 35 μmol photons m^{-2} s^{-1} and a photoperiod of 12 h day^{-1} upon increasing each environmental parameter. The results demonstrate that *Amphidinium* species can multiply efficiently under conditions of relatively low light intensity and low O_2 concentration.

Keywords Algae · *Amphidinium* sp. · Photosynthetic photon flux density · Specific growth rate · Symbiotic dinoflagellate · Temperature

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Introduction

The amphidinolides are a series of unique cytotoxic macrolides isolated from the dinoflagellate *Amphidinium* sp. that has been separated from marine acoel flatworms of the genus *Amphiscolops* (Kobayashi and Tsuda 2004; Kobayashi and Kubota 2007). Up to now, 39 amphidinolides have been reported (Oguchi et al. 2007). Amphidinolides H (Kobayashi et al. 1991, 2000) and B (Ishibashi et al. 1987; Kobayashi et al. 1989; Bauer et al. 1994), which were initially isolated from species of the marine dinoflagellate *Amphidinium* (strains Y-25 and Y-5, respectively), are 26-membered macrolides possessing unique structures such as an allyl epoxide and an *S*-cis-diene, and exhibit potent cytotoxicity (IC_{50} 0.0045–0.00014 mg mL^{-1}) against cultured tumor cells in vitro and antitumor activities in vivo. More recently, a new series of potently cytotoxic *Amphidinium* macrolides, designated iriomoteolides, have been isolated from the benthic *Amphidinium* strain HYA024 (Tsuda et al. 2007a, b). These macrolides are, therefore,

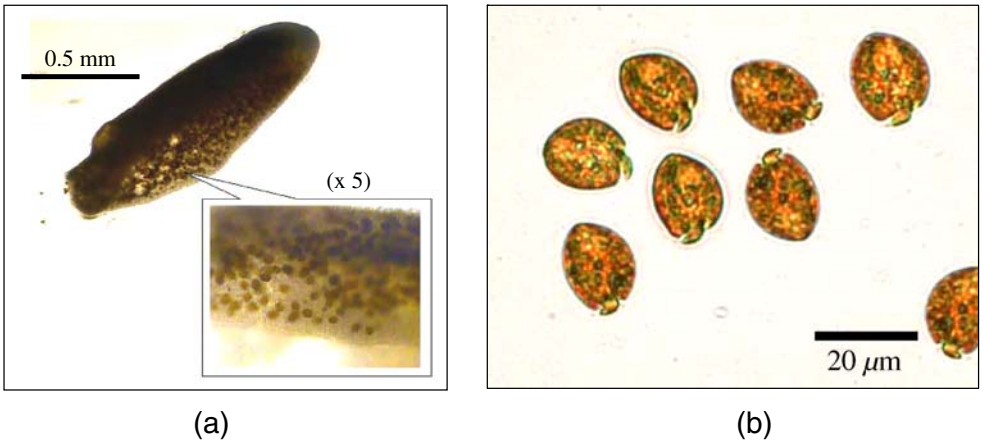

Fig. 1 **a** *Amphiscolops* species containing dinoflagellate cells. **b** Dinoflagellates of *Amphidinium* sp. separated from *Amphiscolops*

expected to be good candidates for antitumor drugs with a novel mechanism of action because their chemical structure is unlike that of conventional anticancer materials. Nevertheless, development of these compounds as anticancer drugs has not been smooth due to limited sample amounts.

Development of a production system for *Amphidinium* cells separated from *Amphiscolops* is desirable in order to produce macrolide compounds efficiently. However, the construction of a mass culture system for *Amphidinium* production has not yet been established because environmental control of the mass culture of *Amphidinium* cells at a high density is difficult using conventional culture methods. Microalgal cell growth rates are affected by environmental parameters such as temperature, light intensity and period, and gas composition in the culture system, as well as the combination of all these factors. Few papers have focused on environmental control of cell cultures of symbiotic *Amphidinium*. In order to establish a symbiotic *Amphidinium* production system with a large number of cells and a rapid turnover rate, the appropriate combination of environmental parameters needs to be determined.

This research was initiated to optimize culture condition for symbiotic *Amphidinium* production. The present study is a starting point for the optimization of the culture. Effects of variable culture conditions on the cell growth rate of dinoflagellates were investigated to determine the potentially optimum culture conditions for obtaining high yields of this microalga. Effects of temperature, photosynthetic photon flux density, photoperiod and CO_2 and O_2 concentrations on growth rates of dinoflagellate cells were assessed using a hanging-drop culture system.

Materials and methods

The dinoflagellate *Amphidinium* sp. (strain HYA002) was separated from the internal cells of the marine acoel

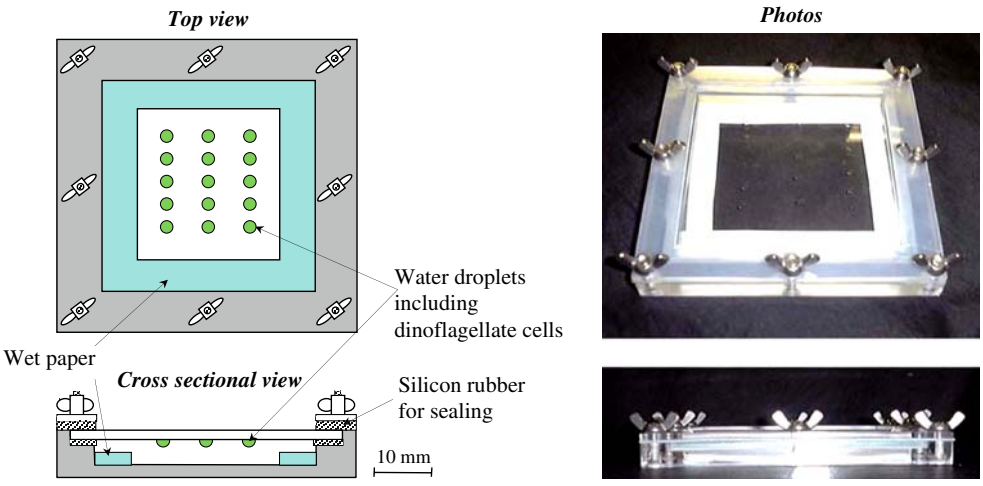

Fig. 2 Apparatus of the culture vessel with water droplets

flatworm *Amphiscolops* sp. (Fig. 1), which was collected off Sunabe beach, Okinawa, Japan (Oguchi et al. 2007). The voucher specimen and the SSU rDNA gene are deposited at the Center for Advanced Marine Core Research, Kochi University.

The experimental system for evaluating the effects of environmental variables on the growth rate of microalgal cells is shown in Fig. 2. The culture system was set up with a hanging-drop culture device according to the procedure reported by Kitaya et al. (1998, 2005). *Amphidinium* cells were cultured in water droplets (3 μL in volume each) in a vessel (100 mL in volume). Each water droplet is held on the surface of a translucent plastic plate and contains initially about ten *Amphidinium* cells. The culture vessel is completely sealed after placing water droplets with *Amphidinium* cells and controlling the internal CO_2 and O_2 concentrations at set levels. The vessel was sealed in a box in which the gas composition had been controlled beforehand. Changes in the gas composition in the vessel were almost negligible during the experiment. The number of water droplets in each treatment was at least five. The vessel was submerged in water at constant temperature. The water layer above the vessel cut out long-wave radiation from the light source, which would otherwise cause a heat load inside the vessel.

Wet paper inside the vessel kept the relative humidity at almost 100% and thus inhibited evaporation from the water droplets. The basal culture solution was sea water. Provasoli's Marine Water Enrichment solution (Provasoli 1963) as a supplemental nutrition, and $NaHCO_3$ as a carbon source for photosynthesis, were mixed with the basal culture solution. The initial pH in the culture solution was adjusted to 7.5. The salinity of the culture solution was adjusted to 3.3%.

The number of dinoflagellate cells in each water droplet was counted with a microscope at 70x magnification. The specific growth rate was used as a standardized index for the growth rate of cells. The specific growth rate (μ) was determined in the logarithmic multiplication stage by the following equation; $\mu = (\ln N_2 - \ln N_1) / (T_2 - T_1)$, where N_1 and N_2 are the cell numbers at times T_1 and T_2, respectively. In the present experiment, T_1 and T_2 were 48 and 144 h, respectively, after the start of the culture. The doubling time or the mean generation time (t_d), is given by the equation $t_d = \ln 2 / \mu$.

Cell growth rates determined in the hanging-drop culture method were compared with those in a conventional culture method to certify the hanging-drop culture method. In the conventional culture method, *Amphidinium* was cultured in aerated containers (210×140×70 mm depth and 800 mL medium volume) with the same culture condition as that in the water droplet method except culture vessel size and medium volume.

In a preliminary experiment, *Amphidinium* was cultured in solutions with four levels of Provasoli's Marine Water Enrichment concentrations (0–2%) and four levels of $NaHCO_3$ concentrations (0–10 mmol) to determine the chemical composition of the basal culture solution.

In the main experiment, *Amphidinium* was cultured at six different temperatures (21–35°C), four photosynthetic photon flux densities (PPFD, 15–70 μmol photons m^{-2} s^{-1}; measured on the inner surface of plastic cover of the hanging-drop culture device), three photoperiods (6–24 h), three CO_2 concentrations (0.02–0.1%), and three O_2 concentrations (0.2–21%). The light source was fluorescent lamps (FPL55EX-N, Matsushita Electric, Osaka, Japan). PPFD was controlled by covering each vessel with neutral shading films.

Results

Cells multiplied almost exponentially by cell division in both the conventional culture and the hanging-drop culture (Fig. 3). The cell density reached about 6.6×10^4 cells mL^{-1} in the conventional culture, while the cell density and total number of cells per hanging droplet were about 3.3×10^4 cells mL^{-1} and 98 cells per hanging droplet, respectively, 144 h after starting the culture. Specific growth rates determined with the hanging-drop culture and the conventional culture methods showed good correlation (r=0.97) as shown in Fig. 4. It was confirmed that the specific growth rate can be estimated accurately with the water droplet method. Specific growth rates increased significantly with increasing concentration of Provasoli's Marine Water Enrichment solution from 0 to 0.5% and then gradually

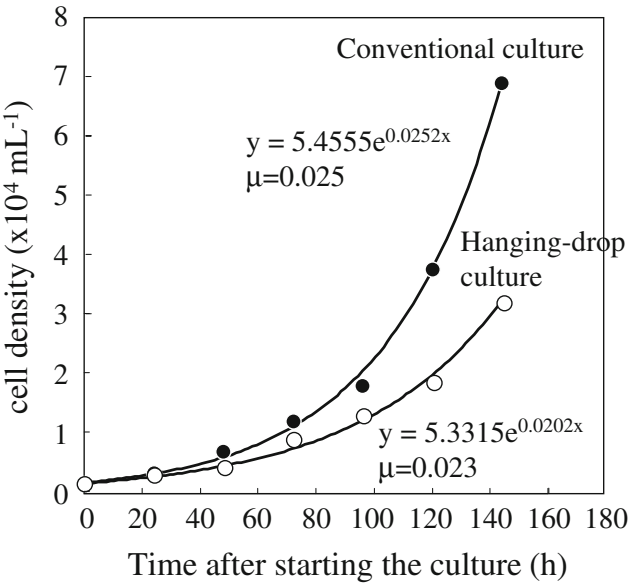

Fig. 3 Examples of cell densities over time with the hanging-drop culture and the conventional culture methods

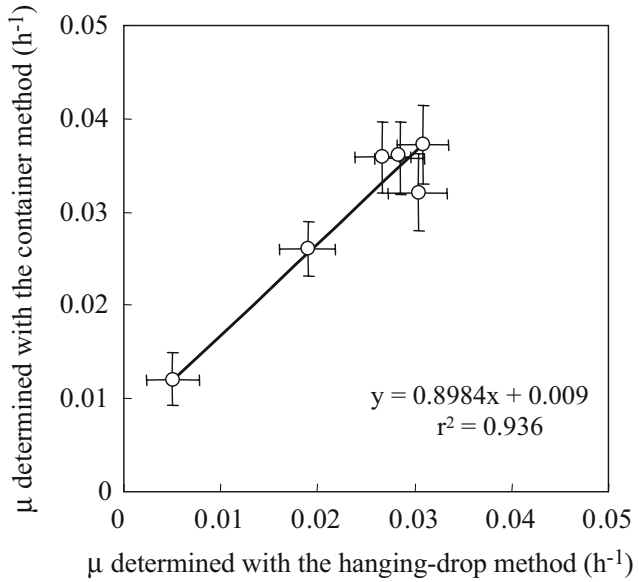

Fig. 4 Specific growth rates (μ) of *Amphidinium* cells determined with the hanging-drop culture and the conventional container culture methods. Volumes of culture media were 3 μL and 800 mL in the hanging-drop culture method and the conventional culture method, respectively. Means with standard deviations are shown (n=3–10)

increased at concentrations higher than 0.5%, although there were no significant differences among the treatments (Fig. 5). Specific growth rates slightly increased with increasing $NaHCO_3$ concentration from 0 to 2.5 mmol and then decreased significantly at $NaHCO_3$ concentrations higher than 5 mmol (Fig. 6). The optimum $NaHCO_3$ concentration for cell division was thus in the range of 2.5–5 mmol. Therefore, 1% Provasoli's Marine Water Enrichment solution and 5 mmol $NaHCO_3$ were used as basic supplements in the experiment for assessing appropriate culture conditions.

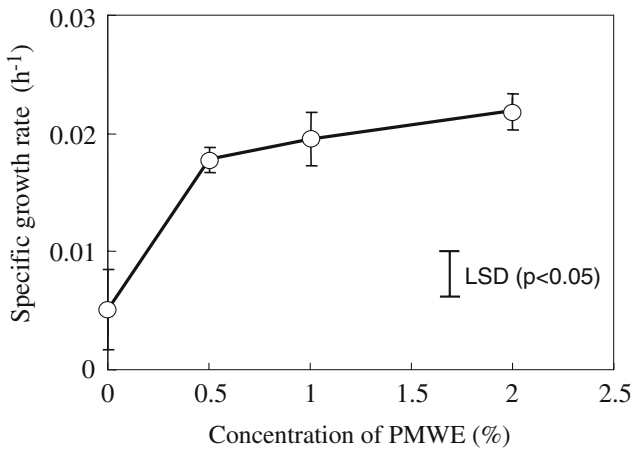

Fig. 5 Effects of Provasoli's Marine Water Enrichment concentration on specific growth rates of *Amphidinium* cells. Vertical bars on plots indicate standard deviations (n=10). Temperature: 28°C, O_2 concentration: 5%, CO_2 concentration: 0.05%, photosynthetic photon flux density (PPFD): 50 μmol photons m^{-2} s^{-1}, photoperiod: 24 h day^{-1}

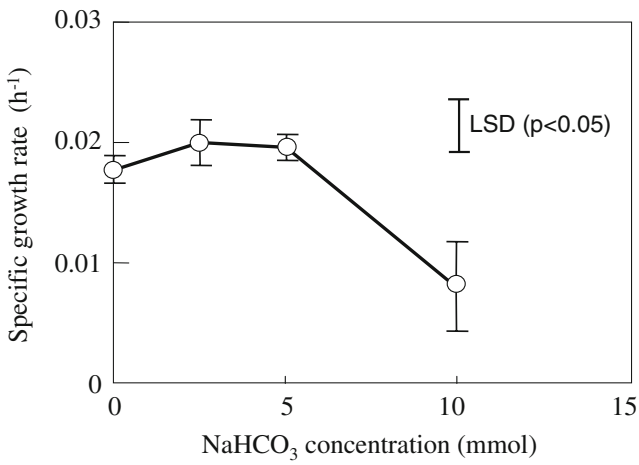

Fig. 6 Effects of $NaHCO_3$ concentrations on specific growth rates of *Amphidinium* cells. Vertical bars on plots indicate standard deviations (n=10). Temperature: 28°C, O_2 concentration: 5%, CO_2 concentration: 0.05%, PPFD: 50 μmol photons m^{-2} s^{-1}, photoperiod: 24 h day^{-1}

Specific growth rates doubled with increasing temperature from 21 to 26°C, and decreased with increasing temperature from 29 to 35°C (Fig. 7). The optimum temperature was 24–29°C .

Specific growth rates increased significantly by 50% with increasing PPFD from 15 to 35 μmol photons m^{-2} s^{-1} and reached a constant value at PPFD values greater than 35 μmol photons m^{-2} s^{-1} under continuous light (Fig. 8). Specific growth rates increased significantly by 80% with increasing photoperiod from 6 to 12 h day^{-1} and reached an almost constant value at photoperiods of 12 to 24 h day^{-1} (Fig. 9).

Specific growth rates tended to increase slightly with increasing CO_2 concentration from 0.002 to 0.05%, and

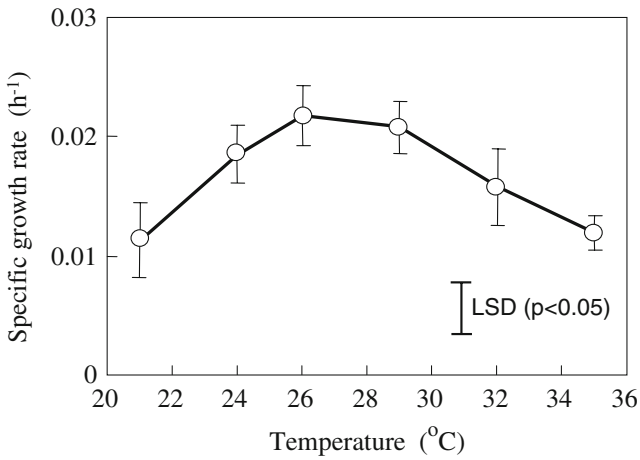

Fig. 7 Effects of medium solution temperatures on specific growth rates of *Amphidinium* cells. Vertical bars on plots indicate standard deviations (n=10). O_2 concentration: 5%, CO_2 concentration: 0.05%, PPFD: 50 μmol photons m^{-2} s^{-1}, photoperiod: 24 h day^{-1}

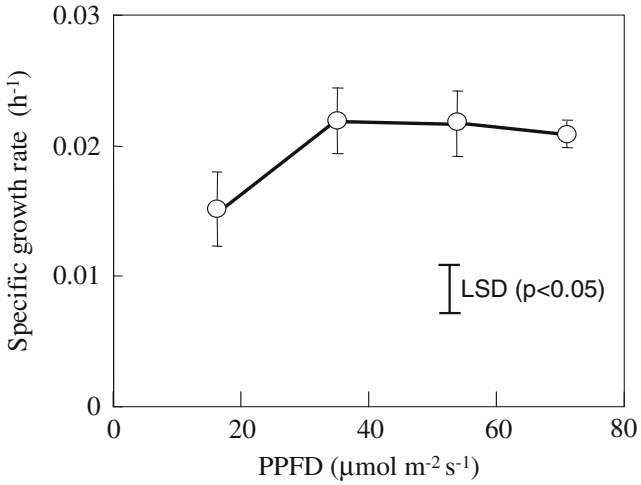

Fig. 8 Effects of PPFD on specific growth rates of *Amphidinium* cells. Vertical bars on plots indicate standard deviations (n=10). Temperature: 26°C, O_2 concentration: 5%, CO_2 concentration: 0.05%, photoperiod: 24 h day^{-1}

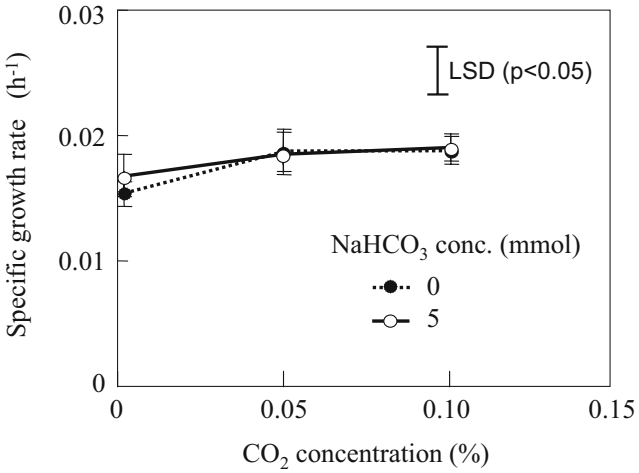

Fig. 10 Specific growth rates of *Amphidinium* cells as affected by CO_2 concentrations with or without $NaHCO_3$ in the medium solution. Vertical bars on plots indicate standard deviations (n=10). Temperature: 26°C, O_2 concentration: 5%, PPFD: 40 μmol photons m^{-2} s^{-1}, photoperiod: 24 h day^{-1}

reached a constant value at CO_2 concentrations of 0.05 to 0.1% regardless of $NaHCO_3$ concentration, although there were no significant differences among the 0.002 to 0.1% treatments (Fig. 10). Specific growth rates tended to increase slightly with increasing O_2 concentration from 0.3 to 5%, and then decreased with increasing O_2 concentration from 5 to 21% regardless of CO_2 concentration, although there were no significant differences among the treatments of 0.3–21% (Fig. 11).

The maximum specific growth rate was 0.022 h^{-1} at a temperature of 26°C and O_2 concentration of 5%, and the specific growth rate was saturated at a CO_2 concentration of 0.05%, a photosynthetic photon flux density of 35 μmol photons m^{-2} s^{-1} and a photoperiod of 12 h day^{-1}.

Discussion

The symbiotic dinoflagellates used in this study exhibited a maximum specific growth rate equivalent to that of asymbiotic dinoflagellates, e.g., *Gyrodinium aureolum* (μ=0.015) (Dixon and Syrett 1988), *Ceratium furca* (μ=0.03) and *Ceratium fusus* (μ=0.023) (Baek et al. 2008). The conditions that provided the highest growth rate of the symbiotic dinoflagellates in this study would be similar to those inside the body of the host animal, *Amphiscolops*. The doubling time (t_d) was 31 h at a specific growth rate of 0.022 h^{-1}. The results demonstrate that symbiotic *Amphidinium* species can multiply efficiently under conditions of relatively low light intensity and low O_2 concentration.

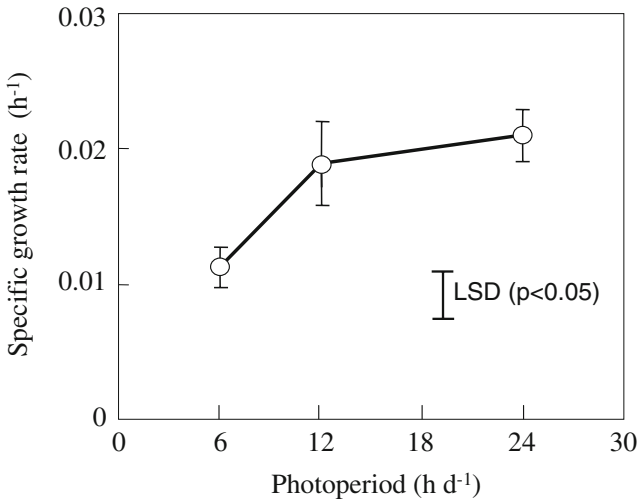

Fig. 9 Effects of photoperiods on specific growth rates of *Amphidinium* cells. Vertical bars on plots indicate standard deviations (n=10). Temperature: 26°C, O_2 concentration: 5%, CO_2 concentration: 0.05%, PPFD: 50 μmol photons m^{-2} s^{-1}

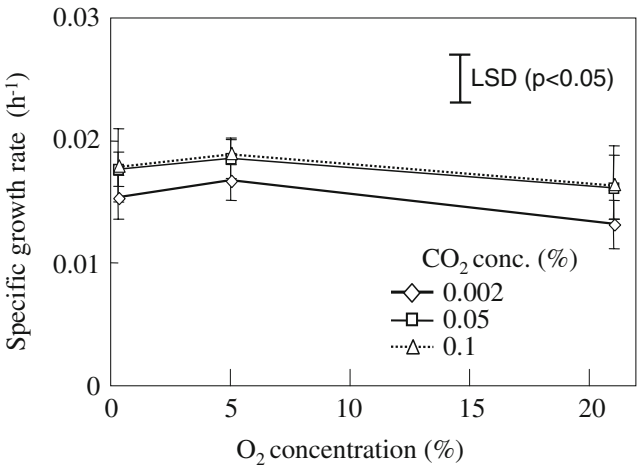

Fig. 11 Specific growth rates of *Amphidinium* cells as affected by O_2 concentrations at different CO_2 concentrations. Vertical bars on plots indicate standard deviations (n=10). Temperature: 28°C, PPFD: 50 μmol photons m^{-2} s^{-1}, photoperiod: 24 h day^{-1}

This experiment was conducted with algal cultures at low cell densities. Cell productivity is dependent not only on the growth rate but also on the population density. Moreover, the fragility of cells represents one of the biggest obstacles to the cultivation of dinoflagellates (e.g., Dixon and Syrett 1988). In order to establish a system to produce secondary metabolites such as amphidinolides, it is necessary to determine appropriate conditions for cellular multiplication at high cell densities and also for the efficient production of secondary metabolites in cells.

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