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Research Article
Growth Characteristics of Three Benthic Dinoflagellates in Mass Culture and Their Antioxidant Properties

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Abstract

Background and Objective: Benthic dinoflagellates are potentially useful for many biomedical and toxicological applications. *Amphidinium carterae* (JHWAC), *Prorocentrum rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) isolated from Jeju island, Korea were cultured in a photobioreactor to evaluate the growth performances in two culture media (IMK and f/2) with three different concentrations (1X, ½X and ¼X) and to determine their proximate chemical compositions and potential antioxidant effect. **Methodology:** Growth was examined based on cell counts and biomass was determined as dry weight. The antioxidant effect was monitored using Electron Spin Resonance (ESR) spectroscopy in terms of radical scavenging activity. **Results:** *Amphidinium carterae* (JHWAC) showed significantly higher cell density (68.91×10^3 cells mL^{-1}), growth rate (0.473 division day^{-1}) and biomass (0.226 g L^{-1} dry weight) production in the 1X f/2 medium, whereas *P. rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) showed best performances in the 1X f/2 and 1X IMK media, respectively. This reveals that the 1X f/2 medium can maximize the cell density and biomass of *A. carterae* (JHWAC) and *P. rhathymum* (JHWPMX1), whereas the 1X IMK medium does the same for *Symbiodinium* sp. (JHLSD1) in mass culture in a photobioreactor. Among the proximate chemical compositions of cultured strains, *A. carterae* (JHWAC) showed significantly higher crude carbohydrates (25%), proteins (21.5%) and lipids (6.3%). **Conclusion:** Among the methanol extracts of cultured dinoflagellates, *A. carterae* (JHWAC) showed the highest alkyl, DPPH and hydroxyl radical scavenging activities at IC_{50} values of 0.68 ± 0.44, 1.40 ± 0.54 and 1.67 ± 0.38 mg mL^{-1}, respectively.

Key words: Benthic dinoflagellates, biomass, bioreactor, growth, Jeju island, radical scavenging activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Marine benthic dinoflagellates have received great attention because of their importance as primary producers in the marine ecosystem, as well as their implication as causative agents in fish/shellfish toxicity events such as Ciguatera Fish Poisoning (CFP) in subtropical to tropical coasts[1,2]. Some of the most common benthic dinoflagellate species belong to the genera *Amphidinium*, *Coolia*, *Gambierdiscus*, *Ostreopsis* and *Prorocentrum* among others. Benthic dinoflagellates can produce powerful bioactive secondary metabolites and bio-toxins[3,4]. They have been recognized as potential sources bearing novel compounds for appraisal as pharmaceuticals[5,6] and are considered as valuable laboratory tools in the case of drug discovery as well as having other potential applications[7,8]. Hence, bioactive components can be isolated from benthic dinoflagellates and rendered in a range of biological activities, including antioxidant, cytotoxic, antitumor, antibiotic, antifungal, immunosuppressant and neurotoxic[5,9]. However, toxins and bioactive compounds from dinoflagellates are inaccessible in large quantities and quite expensive even in small amounts[5,10]. In order to utilize the toxins and other bioactive molecules produced by mass scale cultivation of dinoflagellates, study on their biomedical, toxicological, chemical, pharmacological and therapeutic potential is essential. In fact, the study of their growth, gain cell density, biomass productivity and physiology in large-scale cultures are considered worthwhile.

The effectiveness of the quantity of extracted bioactive components depends on the culturing systems and productivity of dinoflagellate strains. Several studies have reported the growth conditions optimization and toxin production from benthic dinoflagellate small-scale cultures[11-21]. However, it is often difficult to mass culture dinoflagellates and little effort has been devoted to the mass culture of dinoflagellates in bioreactors[22-25]. The maximum biomass concentration attained in a typical dinoflagellate photosynthetic culture[24] was below 1 g L^{-1}. Owing to the slow growth rates of dinoflagellates, large-scale cultures are necessary to produce sufficient biomass for the characterization of novel compounds and toxin production[22,26].

In a previous study, it was reported that the potential of small-scale culture (20 L) of several strains of benthic dinoflagellate isolated from Jeju island, Korea and their bioactivities[27]. In fact, there is no previous study on the large-scale photobioreactor culturing of these marine benthic dinoflagellates and exploration of their bioactive components from Jeju island, Korea. Hence, special effort is required to develop stable and reliable large-scale culture systems in photobioreactors for these strains. The aim of this study was to evaluate the growth and biomass productivity of three potentially important benthic dinoflagellates: *Amphidinium carterae* (JHWAC), *Prorocentrum rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) using two culture media in a column photobioreactor. Further, it was attempted to determine the proximate chemical composition and antioxidant activity of the methanol extracts of the cultured dinoflagellates using Electron Spin Resonance (ESR) spectroscopy.

MATERIALS AND METHODS

Dinoflagellate species and culture conditions: Three benthic dinoflagellates, *Amphidinium carterae* (JHWAC), *Prorocentrum rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) were collected and identified in 2011 from the coast of Jeju island, Korea, according to Shah *et al.*[28]. Single cell isolation and unialgal pure stock culture preparation and maintenance of benthic dinoflagellate strains were carried out following the methodology described in a previous study[27]. Stock cultures of dinoflagellate strains were gradually scaled up into 30 mL, 300 mL, 1 L and 3 L flasks and 20 L carboys to use as inoculums for the photobioreactor. In 20 L carboy cultures, culture water was treated with sodium hypochlorite solution containing 9% active chlorine (at a rate of 1.1 mL L^{-1} seawater) for 30 min for chemical sterilization. After that, sodium thiosulfate (at a rate of 0.12 g L^{-1} seawater) was added to neutralize chlorine in the water. A medium level of aeration was provided. From all scale up stages, inoculums of exponentially growing phases were used to start the following cultures. Cultures grown in glass flasks (1 and 3 L) were initiated by inoculation of 10-20% by volume of 300 mL culture stock (maintained by monthly transfer) and cultures grown in carboys (20 L) were initiated by inoculation of 10-20% by volume of 3 L cultures.

Photobioreactor system: A vertical column photobioreactor system in the Fisheries Seed Research Center, Ocean and Fisheries Research Institute, Jeju, Korea was used in the present study, shown in Fig. 1. The photobioreactor consisted of 12 columns (diameter 35 cm, made with transparent flexible polyethylene sheet) placed in two rows, with six columns each. The first and sixth columns in the first row were comparatively larger in height (160 cm) than the other columns (145 cm). The columns in the second row all had equal height (145 cm). All columns were connected to each other at the bottom to facilitate culture water to circulate

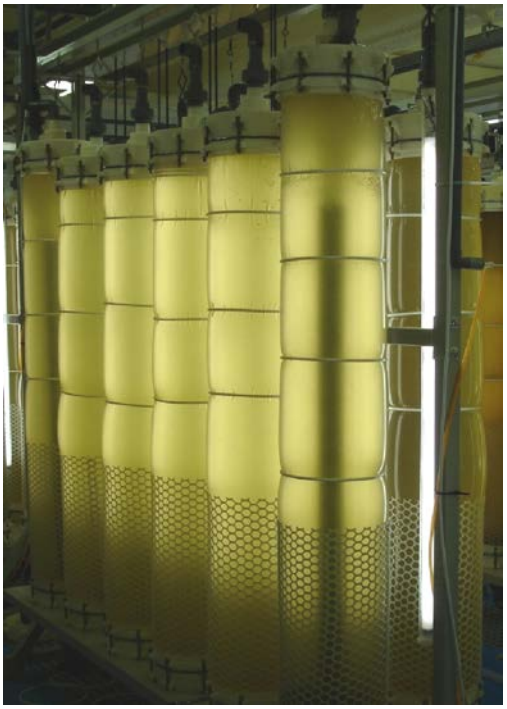


Fig. 1: Schematic of the column photobioreactor used for culturing benthic dinoflagellates in the present study

between them the other through aeration and each column was fitted with an inlet and outlet system. Culture seawater (30 psu) was UV treated, heat sterilized (110°C) and stored in an overhead steel tank overnight to cool down before being filtered (filter pore size 0.2 µm) and supplied through the inlet to the bioreactor for culturing dinoflagellates. Culture media was added through the inlet of the first column. Moderate aeration (filtered through 0.2 µm pore size filters) was provided through the bottom of the first and sixth columns of the first row. After the complete mixing of culture media with the seawater, exponentially grown dinoflagellates cultured in 20 L carboys were inoculated through the inlet of the first column of the photobioreactor. In two columns (one in the first row and one in the second), blank spaces (approximately 10 L each) were maintained to facilitate mixing and continuous circulation of culture water between columns. The total capacity of the photobioreactor system was approximately 700 L (each column contained 60 L). The photobioreactor was maintained in a temperature-controlled room at 20±1°C. Illumination was provided by a 1:1 combination of a cool-white (38W, Philips, Eindhoven, the Netherlands) light bulb and one column of the bioreactor was at an irradiance of approximately 40-50 µmol photons m^{-2} sec^{-1} with a 12:12 h light/dark (L/D) cycle.

Experimental design and growth rate measurements: The dinoflagellate strains were cultured in two media, IMK (Nihon Pharmaceutical Co., Ltd., Japan)[20] and f/2 (Aquacenter Ltd. USA)[29] with three different concentrations (1X, ½X and ¼X). Addition of all the chemical components, as described by Yamaguchi *et al.*[20] per liter of seawater was considered as 1X nutrient concentration. A general dose of f/2 media (addition of 1 mL of component A per 7.75 L of water and 1 mL of component B per 7.75 L of water) was considered as 1X nutrient concentration. Accordingly, ½X and ¼X concentrations were considered by reducing the amount of chemical components for both f/2 and IMK media. The batch cultures were run in triplicates for 27 days. The experiment started with the inoculation of a late exponential phase culture from 20 L with an initial cell density of approximately 500-1500 cells mL^{-1} into the photobioreactor.

Growth rate was measured every three days by direct cell counting using a Sedgwick-Rafter (S-R) cell according to Shah *et al.*[27]. Specific Growth Rate (SGR; µ day^{-1}) was defined as the increase in cell density per time[30] and calculated as follows:

$$\mu\ (day^{-1}) = \ln N_1 - \ln N_0 / t_1 - t_0 \quad (1)$$

where, N_0 and N_1 are the cell density at the beginning (t_0) and end (t_1) of the selected time interval between inoculation and maximum cell density, respectively. Growth rate as divisions per day was calculated using the following equation[29]:

$$\text{Division day}^{-1} = \mu \ln_2^{-1} \quad (2)$$

Dinoflagellate cell harvest, sample preparation: Dinoflagellate cells were harvested by centrifuging (5000 rpm for 10 min) in a VS-24SMTi high speed refrigerated centrifuge (Vision Scientific Co. Ltd, Daejeon, Korea). Cells were prepared for dry weight biomass according to Zhu and Lee[31] biomass was expressed as g L^{-1}. The cultures were kept at -80°C and subjected to freeze drying using a freeze dry system (Samwon Freezing Engineering Co. Busan, Korea).

Determination of proximate chemical composition: Proximate chemical composition of cultured dinoflagellates was determined according to the AOAC method[32]. Total nitrogen content was analyzed by the Kjeldahl method (Kejltec 8400, FOSS, USA). Crude protein content was determined by calculating a conversion factor of 6.25. Crude lipid content was determined by the Soxhlet extraction method with diethyl ether solvent (Soxtec 2050, FOSS, USA)

and crude ash content was determined by incineration of samples at 550°C in a muffle furnace (B180, Nabertherm GmbH, Germany). Moisture was determined by the oven-drying method at 105°C in a moisture analyzer (mb45, OHAUS, Switzerland). Crude fiber content was measured by a fibertec system (Fibertec 2010 Analyzer, FOSS, USA).

Solvent extraction and sample preparation: The lyophilized benthic dinoflagellate strains were grounded into fine powder and each of the materials were homogenized separately. Then the homogenized samples were sonicated (ultrasound-assisted extraction) at 25°C for 90 min 3 times using 80% methanol. Crude methanol extracts were concentrated by evaporating the solvent under reduced pressure using a rotary evaporator (Fisher scientific, Loughborough, UK) and each of the samples were prepared into 100 mg mL^{-1} concentration. For the determination of antioxidant activity, the dilution was carried out using deionized water.

Radical scavenging assay using Electrons Spin Resonance (ESR) spectrometry: The DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a free radical donor, which can be detected via ESR spectrometry (JES-FA machine, JOEL, Tokyo, Japan) following the technique described by Nanjo *et al.*[33]. The measurement conditions were as follows: Power 1 mW, modulation frequency 5×100 kHz, modulation width 0.8 mT, sweep width 10 mT, sweep time 30 sec, temperature 298 K. The hydroxyl radicals generated via the Fenton reaction reacted rapidly with the DMPO nitrone spin trap, the resultant DMPO-OH adduct was detectable with an ESR spectrometer[34]. According to the technique described by Hiramoto *et al.*[35], alkyl radicals were generated via AAPH.

Statistical analysis: Statistical significance between the growth rates was determined by analysis of variance using the software Graph Pad InStat ver.3, microsoft Excel 2007 and Duncan's Multiple Range Tests (DMRT).

RESULTS

Dinoflagellates growth and biomass production: *Amphidinium carterae* (JHWAC) grew well in all media concentrations, except in the ¼X f/2 medium. The culture reached a significantly ($p<0.05$) higher cell density (68.91×10^3 cells mL^{-1}) in the 1X f/2 medium at day 15, followed by the 1X IMK medium were the maximum cell density (59.89×10^3 cells mL^{-1}) was reached at the same day (Fig. 2a). The maximum growth rate of *A. carterae* (JHWAC) varied from 0.161-0.473 division day^{-1}, which was significantly ($p<0.05$) higher (0.473±0.035 division day^{-1}) in the 1X f/2 medium and lower (0.161±0.014 division day^{-1}) in the ¼X f/2 medium (Table 1). The maximum biomass production (0.226±0.010 g L^{-1} dry weight) was significantly ($p<0.05$) higher in the 1X f/2 medium (Table 1). *Prorocentrum rhathymum* (JHWPMX1) reached a maximum cell density of 17.97×10^3 cells mL^{-1} in the 1X f/2 medium after 15 days of culture and the lowest cell density (5.80×10^3 cells mL^{-1}) was recorded in the ¼X f/2 medium at 21 days (Fig. 2b). There were significant ($p<0.05$) differences among the maximum cell density using different media concentrations. For *P. rhathymum* (JHWPMX1), the maximum growth rate varied between 0.176 -0.312 division day^{-1}. The significantly ($p<0.05$) highest (0.312±0.021 division day^{-1}) and lowest (0.176±0.055 division day^{-1}) growth rates were recorded in the 1X f/2 and ¼X f/2 media, respectively. The highest biomass (0.183±0.005 g L^{-1} dry weight) was also attained in the 1X f/2 medium (Table 1). The growth curve of

Table 1: Maximum cell density, growth rate and biomass production of three benthic dinoflagellates in photobioreactor mass culture

		Culture media					
Growth parameters	Species	1X f/2	½X f/2	¼X f/2	1X IMK	½X IMK	¼X IMK
Maximum cell density	*Amphidinium carterae*	68.91±1.28^{a}	45.54±0.601^{c}	8.380±0.576^{d}	59.890±0.862^{b}	56.810±0.869^{b}	54.970±0.3252^{b}
(cells×10^3 mL^{-1})	*Prorocentrum rhathymum*	17.97±0.176^{a}	8.90±0.141^{c}	5.800±0.261^{d}	11.800±0.141^{b}	8.710±0.229^{c}	6.760±0.180^{d}
	Symbiodinium sp.	13.82±0.53^{b}	8.99±0.173^{c}	2.850±0.148^{d}	15.860±0.197^{a}	8.320±0.180^{c}	3.560±0.162^{d}
Maximum growth rate	*Amphidinium carterae*	0.473±0.035^{a}	0.339±0.032^{a}	0.161±0.014^{c}	0.401±0.017^{a}	0.352±0.014^{ab}	0.308±0.012^{b}
(division day^{1})	*Prorocentrum rhathymum*	0.312±0.021^{a}	0.218±0.012^{bc}	0.176±0.055^{c}	0.275±0.014^{b}	0.216±0.012^{c}	0.197±0.016^{c}
	Symbiodinium sp.	0.304±0.016^{a}	0.195±0.024^{b}	0.103±0.018^{b}	0.320±0.018^{a}	0.244±0.017^{a}	0.157±0.019^{b}
Maximum biomass	*Amphidinium carterae*	0.226±0.010^{a}	0.145±0.020^{b}	0.080±0.013^{d}	0.186±0.005^{b}	0.156±0.012^{c}	0.123±0.058^{c}
yield (g L^{-1})	*Prorocentrum rhathymum*	0.183±0.005^{a}	0.125±0.021^{bc}	0.076±0.024^{cd}	0.157±0.005^{b}	0.125±0.007^{c}	0.100±0.0115^{d}
	Symbiodinium sp.	0.159±0.006^{a}	0.097±0.017^{b}	0.074±0.008^{b}	0.173±0.008^{a}	0.092±0.026^{b}	0.079±0.0202^{b}

Values represent the Mean±Standard Deviation from triplicate determination, $^{a-f}$Significant different ($p<0.05$) within the same row

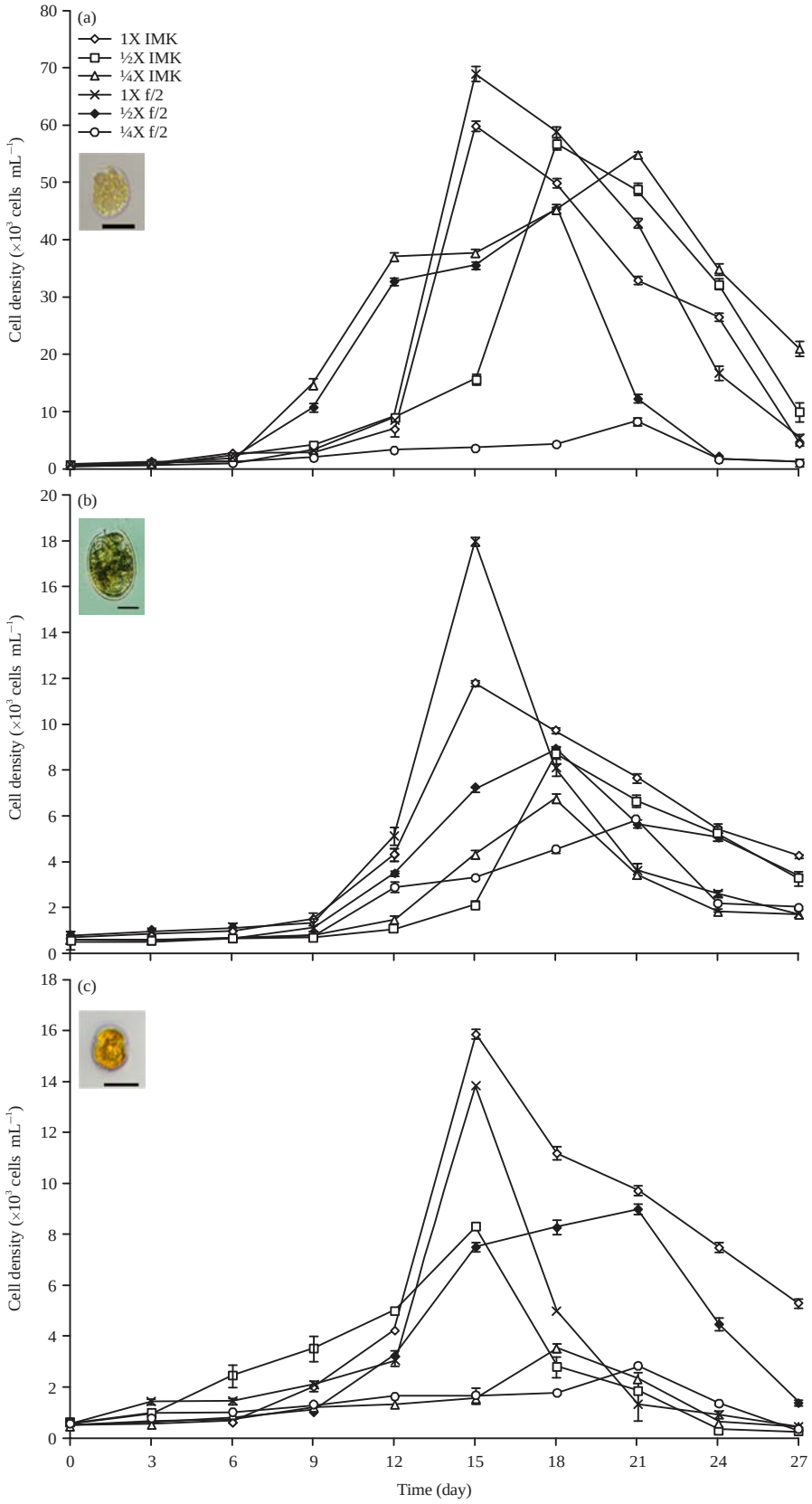


Fig. 2(a-c): Growth of three benthic dinoflagellate species in each medium, (a) *Amphidinium carterae* (JHWAC), (b) *Prorocentrum rhathymum* (JHWPMX1) and (c) *Symbiodinium* sp. (JHLSD1)

Table 2: Proximate chemical composition of three benthic dinoflagellate species (crude dry weight basis)

	Proximate chemical composition (%) dry weight basis*					
Species	Protein	Lipid	Carbohydrate	Fiber	Ash	Moisture
Amphidinium carterae	21.5±0.04^{e}	6.31±0.08^{c}	25.03±0.01^{d}	2.45±0.03^{b}	41.15±0.2^{a}	3.57±0.05^{a}
Prorocentrum rhathymum	20.5±0.05^{e}	5.21±0.01^{c}	20.90±0.04^{cd}	2.45±0.06 ^{b}	46.15±0.08^{ab}	4.79±0.07^{ab}
Symbiodinium sp.	6.03±0.05^{b}	0.44±0.04^{a}	12.70±0.02^{ab}	7.54±0.07 ^{c}	67.06±0.05^{bc}	6.23±0.01^{b}

*Analysis was according to the AOAC methods. Values are Mean±SD of three determinations. Significant differences at p<0.05 were indicated with different letters in columns

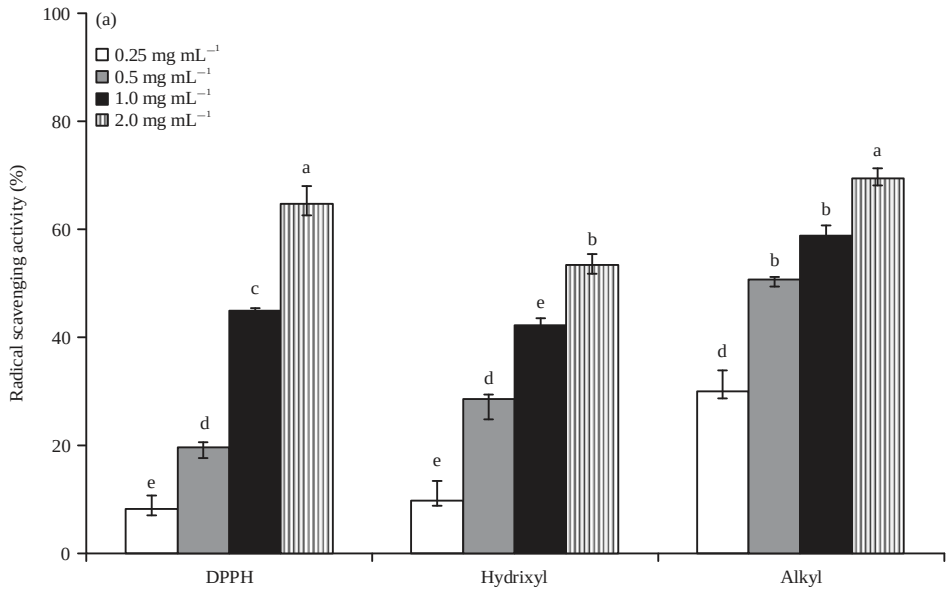
Symbiodinium sp. (JHLSD1) showed that this strain did not grow well until day 12 but later it reached the highest cell density in the 1X f/2 and 1X IMK media. The cell density was lower for the other culture media concentrations when compared to the 1X f/2 and 1X IMK media. One way ANOVA results showed that the significantly (p<0.05) highest cell density (15.86×10^3 cells mL^{-1}) was achieved in the case of the 1X IMK medium followed by 1X f/2 medium (13.82×10^3 Ml^{-1}) (Fig. 2c). The maximum growth rate varied from 0.103-0.320 division day^{-1} with an average of 0.220 division day^{-1}. Significantly higher (p<0.05) growth rates of 0.320±0.018 division day^{-1} and biomass production (0.173±0.008 g L^{-1} dry weight) were recorded in the 1X IMK medium but the difference from the highest biomass obtained in the 1X f/2 medium was not statistically significant (Table 1).

Proximate chemical composition and antioxidant activity: The proximate chemical composition (%) of the three cultured dinoflagellate strains was measured based on crude dry weight. All the samples had a high content of ash (%) compared to the other nutrients. Among the strains, *Symbiodinium* sp. (JHLSD1) showed the highest ash content at 67% and *A. carterae* (JHWAC) the least at 41% (Table 2). However, among the key nutrients that were determined in this assay, crude carbohydrates (25%), crude proteins (21.5%) and crude lipids (6.3%) were highest in *A. carterae* (JHWAC) compared to the other strains. Moreover, similar content of crude proteins and carbohydrates, (approximately 20%) were determined from *P. rhathymum* (JHWPMX1) and least values (6 and 12.7%) from *Symbiodinium* sp. (JHLSD1). Among the 80% methanol extracts of the cultured benthic dinoflagellates, *A. carterae* (JHWAC) showed the highest alkyl radical scavenging activity (IC_{50} value 0.68±0.44 mg mL^{-1}) compared to the other two strains, whereas *P. rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) showed IC_{50} values of 0.81±0.14 and 1.08±0.11 mg mL^{-1}, respectively (Fig. 3a). In addition, *A. carterae* (JHWAC) methanol extracts had the highest DPPH and hydroxyl radical scavenging activities at IC_{50} values of 1.40±0.54 and 1.67±0.38 mg mL^{-1}, respectively. Moreover, *P. rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) extracts showed comparatively lower DPPH (1.64±0.21 and 2.32±0.27 mg mL^{-1}) and hydroxyl (1.76±0.05 and 1.93±0.07 mg mL^{-1}) radical scavenging effects, respectively (Fig. 3b).

DISCUSSION

This is the first report characterizing the growth potential of three potentially important benthic dinoflagellate species (*Amphidinium carterae* (JHWAC), *Prorocentrum rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1)) collected from Jeju island, Korea, in a large-scale photobioreactor culture system. The maximum cell number of *A. carterae* (JHWAC) obtained in the present study (68.91×10^3 cells mL^{-1} in 1X f/2 medium) was lower than previous observations ($1\text{-}6\times10^5$ cells mL^{-1}) reported by Thomas and Carr[36]. However, the maximum growth rate of this strain (0.473 division day^{-1} in 1X f/2 medium) was similar to the growth rates observed by Tomas *et al.*[37] (0.32-0.71 division day^{-1}) and Valenzuela-Espinoza *et al.*[38] (0.49-0.66 day^{-1}), for *A. carterae* cultured in f/2 medium modified with nitrogen and phosphorus concentrations (at 33 psu, 25°C and 300 µmol quanta m^{-2} sec^{-1} light intensity in 250 mL flask culture in lab scale). The maximum growth rate of *P. rhathymum* (JHWPMX1) in this study (0.312±0.021 division day^{-1} in 1X f/2 medium) was closer to the growth rate (approximately 0.3 to <0.6 division day^{-1}) of the closely related species *Prorocentrum mexicanum*, collected from Knight key, Florida, USA and cultured in K medium under small-scale stock cultures by Morton *et al.*[13]. The maximum growth rate of *Symbiodinium* sp. (JHLSD1) (0.320 division day^{-1} in 1X IMK medium) in this study had a similar growth rate of 0.30 division day^{-1} in the f/2 medium, observed during the study of mixotrophic growth rate of *Symbiodinium* sp. in small-scale culture in the lab[39].

Dinoflagellates commonly have low growth rates, with a complicated metabolism and low toxin productivity. This low growth rate is reflected in the lower chlorophyll a to carbon ratio (Chl a:C), as speculated by Tang[40]. The growth rates of dinoflagellates rarely double per day[36]. Most of the other economically important microalgal species have shown growth rates much higher than 1.0 day^{-1} for example, *Dunaliella tertiolecta* (1.4 day^{-1}), *Thalassiosira pseudonana*



(b)

	Radical scavenging activity IC_{50} (mg mL^{-1})		
Dinoflagellate strains	DPPH	Hydroxyl	Alkyl
Amphidinium carterae	1.40±0.54	1.67±0.38	0.68±0.44
Prorocentrum rhathymum	1.64±0.21	1.76±0.05	0.81±0.14
Symbiodinium sp.	2.32±0.27	1.93±0.07	1.08±0.11

Fig. 3(a-c): Antioxidant activity of cultured dinoflagellates determined using spin resonance electron spectroscopy, (a) Scavenging activity of the methanol extract of the cultured *Amphidinium carterae* (JHWAC) against DPPH, hydroxyl and alkyl radicals, (b) Determined IC_{50} (mg mL^{-1}) values of the methanol extracts of cultured dinoflagellates against DPPH, hydroxyl and alkyl radicals scavenging activity. Values are Mean±SD of three determinations. Values with different letters are significantly different at $p<0.05$ as analyzed by DMRT

(1.8 day^{-1}) and *Chaetoceros calcitrans* (2.0 day^{-1})[41]. In the present study, maximum cell density, growth rate and biomass yield of *A. carterae* (JHWAC) and *P. rhathymum* (JHWPMX1) were achieved in the 1X f/2 medium, while *Symbiodinium* sp. (JHLSD1) showed the best performance in the 1X IMK medium. These results indicate that the use of 1X f/2 medium could be considered suitable for exploring the growth potential of *A. carterae* (JHWAC) and *P. rhathymum* (JHWPMX1), whereas the 1X IMK medium could be considered suitable for culturing *Symbiodinium* sp. (JHLSD1) in the present photobioreactor mass culture system. Growth rates and cell yields of benthic dinoflagellate strains presented in this study are presumably attributed to the different types of media, size of the culture and culture conditions used in other studies. The determination of antioxidant activity is the key parameter in deducing other promising bioassays as well as exploring natural products. Notably, among the cultured benthic dinoflagellates, *A. carterae* (JHWAC) performed profound radical scavenging activities in ESR spectroscopy. The ESR spectroscopy is the most sensitive, direct and accurate method to detect free radical scavenging activity. It has been used to monitor reactive species, including DPPH, hydroxyl and alkyl radicals scavenging activity in terms of antioxidant effects at room temperature[42]. Reactive Oxygen Species (ROS) are the major causative agents for oxidative stress owing to imbalances between the natural antioxidant enzymes and ROS activities in the human body. Therefore, antioxidant compounds from marine sources play an important role in eliminating these reactive molecules. The ESR spectroscopic measurements can monitor the free radical scavenging activity because it does not interfere with the color of the extracts that derive from different organic solvents. Moreover, *A. carterae* (JHWAC) also showed the best culture conditions in the 1X f/2 media and obtained the highest biomass yield compared to the IMK media. This is attributed to the proximate chemical composition of *A. carterae* (JHWAC). Hence, it is shown that the crude nutrients are significantly correlated with the potent antioxidant activities. This can

be further established by separating its active components through bioassay guided fractionations. In fact, Echigoya *et al*.[43] showed the potential of isolating novel bioactive compounds from cultured *A. carterae* (JHWAC). In many studies, metabolites from dinoflagellates have proven to be valuable laboratory tools and promising material for the lead compounds in drug discovery. In addition, some of the compounds produced from dinoflagellates are rendering for toxins, such as okadaic acid, a diarrhetic shellfish toxin and tumor promoter found in many dinoflagellates of the genera *Dinophysis* and *Prorocentrum*, which is used for studies of cellular regulation[44]. Moreover, a potent hemolytic and antifungal active compound (amphidinol 2) was isolated from the cultured *Amphidinium klebsii*[45]. Until now, only a few groups have attempted to develop conditions that led to improvements in product yields from dinoflagellates (e.g., Institute of Marine Biosciences, NRC, Halifax, Canada and IFREMER, Nantes, France). However, the slow growth rates of dinoflagellates would require large culture volumes in order to produce sufficient material for the characterization of secondary metabolites[46]. Hence, screening of the antioxidant effects of cultured marine dinoflagellates would access their potency in future therapeutic applications.

Benthic dinoflagellates cultured in the present study are commonly associated with seagrasses, macroalgae, dead corals, rocks, soft sediments and invertebrates in tropical, subtropical and temperate marine environments[47]. In the benthic environment, as cells are linked to the substrate, their relative movement in the water surrounding them depends more on water motion than on swimming. The efficiency of benthic species in nutrient uptake depends not only on their own physiological characteristics but also on water velocity according to the mass-transfer theory[48]. A variety of factors other than wave action and temperature may also be important in controlling and promoting the occurrence and intensity of the bloom of benthic dinoflagellates, for example, availability of macroalgal substrates, light intensity, precipitation and nutrients[49]. Nutrient availability is to be considered an important environmental factor for controlling the growth[15], whereas the relationship between benthic/epiphytic dinoflagellates and nutrient conditions is less clear[15]. The role of nutrients in supporting elevated dinoflagellate biomass is still uncertain. In fact, as reported by Tindall and Morton[49], epiphytic/benthic dinoflagellates do not appear to be unique in their requirements for the two major limiting macronutrients, nitrogen and phosphorus. However, high water temperature, high irradiance and high remineralization are factors that can create an environment favoring the bloom of benthic harmful microalgae[50]. In this culture system, temperature, salinity, light intensity and aeration were constant and the growth performance of the strains differed with variations of the culture medium. The growth performance of these strains could be different when other factors varied (for example temperature) in the present culture system. Further study with varying temperature, salinity, light intensity, aeration and nutrient concentration is necessary to clarify the growth physiology, such as temperature salinity tolerance and nutrition of these cultured strains with the present system, as well as to understand the mechanisms of the dynamics of benthic dinoflagellates in coastal environments.

CONCLUSION

This study has demonstrated the use of a scaling-up mass culturing system for the growth of dinoflagellates using a column photobioreactor. Among the three dinoflagellates, *A. carterae* (JHWAC) and *P. rhathymum* (JHWPMX1) can be grown successfully in 1X f/2 medium and *Symbiodinium* sp. can be cultured in 1X IMK medium. Proximate chemical composition evidenced on this cultured biomass for screening antioxidant activities of their methanol extracts using radical scavenging activity on electron spin resonance spectroscopy. The proposed method might be useful for the mass culture of important benthic dinoflagellates and the produced biomass can be utilized for extracting bioactive natural products for commercial applications.

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