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Potentiality of benthic dinoflagellate cultures and screening of their bioactivities in Jeju Island, Korea

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Eleven strains of benthic dinoflagellates (*Amphidinium carterae* (D1), *Prorocentrum rathymum* (D2), *Symbiodinium* sp. (D3), *Coolia malayensis* 1 (D4), *Ostreopsis ovata* 1 (D5), *Ostreopsis ovata* 2 (D6), *Coolia malayensis* 2 (D7), *Amphidinium operculatum* 1 (D8), *Heterocapsa psammophila* (D9), *Coolia malayensis* 3 (D10) and *Amphidinium operculatum* 2 (D11)) were collected in Jeju Island, Korea and cultured in 20 L carboys after establishing unialgal cultures. Their growth potential and biomass productivity were evaluated using two different culture media (IMK and f/2 medium); it was found that IMK medium has the potential to culture benthic dinoflagellates compared to commonly used f/2 medium. Among the benthic dinoflagellates, *A. carterae* (D1) had the maximum cell density (148.6×10^3 cells mL⁻¹), growth rate (0.317 ± 0.01 divisions day⁻¹) and biomass (0.260 ± 0.03 g L⁻¹ dry weight) in IMK medium at 20 days of culture. Also, screened bioactivities among the methanolic extracts of cultured dinoflagellates showed *A. carterae* (D1) to have the highest antioxidant and anti-inflammatory effect and *O. ovata* 1 (D5) had the highest anticancer activity compared to the other strains. Taken together, this is the first report on the growth potential and biomass production of benthic dinoflagellate strains isolated from Jeju Island in appropriate culture medium as well as their importance in potential pharmacological applications.

Key words: *Amphidinium carterae*, benthic dinoflagellates, biomass, bioactivities, culture conditions, Jeju Island.

INTRODUCTION

Dinoflagellates are the largest group of eukaryotic microalgae with approximately 2000 living species (Taylor et al., 2008). Marine benthic dinoflagellates are unicellular organisms that have received a great attraction due to their importance as primary producers in the marine ecosystem and also due to their encounter

with potentially harmful species, which causes economic losses (particularly in the aquaculture, recreation and tourism industries) as follows: death of fish/shellfish through toxicity, human health problems (for example, paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), azaspiracid poisoning (AZP), diarrhetic

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Abbreviations: PSP, paralytic shellfish poisoning; NSP, neurotoxic shellfish poisoning; AZP, azaspiracid poisoning; DSP, diarrhetic shellfish poisoning; CFP, ciguatera fish poisoning; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ESR, electro spin resonance; DMEM, Dulbecco's modified eagle medium; RPMI, Roswell park memorial institute; FBS, fetal bovine serum; NO, nitric oxide; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; DMSO, dimethylsulfoxide.

shellfish poisoning (DSP) and ciguatera fish poisoning (CFP) in subtropical to tropical coasts (Godhe et al., 2002; Gilbert et al., 2005; Hallegraef, 1993; Lehane and Lewis, 2000). Some of the most common benthic dinoflagellate species in tropical and subtropical marine environments belong to the genera, *Amphidinium*, *Coolia*, *Gambierdiscus*, *Ostreopsis*, *Prorocentrum*, among others. Benthic dinoflagellates possess unique structures with powerful bioactive secondary metabolites (Kita et al., 2005; Kobayashi et al., 2003; Onodera et al., 2005) and bio-toxins (Daranas et al., 2001). In particular, different types of toxins have been detected in different species of benthic dinoflagellates such as *Amphidinium carterae* (Haemolysins) (Nakajima et al., 1981; Yasumoto et al., 1987), *Prorocentrum lima* (okaidic acid and dinophysistoxins) (Murakami et al., 1982), *Prorocentrum rathymum* (water-soluble fast-acting toxins and hemolytic effects) (Nakajima et al., 1981; Tindall et al., 1989), *Coolia monotis* (cooliatxin) (Holmes et al., 1995) and *Ostreopsis* sp. (toxic butanol-soluble compound, palytoxin analogue) (Elbrächter and Faust, 2002; Nakajima et al., 1981; Usami et al., 1995).

Over the years, only a few studies have been done in evaluating the specific bioactivities of cultured dinoflagellates (Camacho et al., 2007; Nakagawa et al., 1998). They have been recognized as potential sources bearing novel compounds for appraisal as pharmaceuticals (Camacho et al., 2007; Dragunow et al., 2005). Moreover, bioactive components are gaining the interest of researchers because of their potential usefulness in many applications. Toxic metabolites have been reported from cultured dinoflagellates and are considered as valuable laboratory tools in the case of drug discovery (Elbrächter and Faust, 2002; Nakajima et al., 1981). Hence, bioactive components can be isolated from benthic dinoflagellates and rendered in a range of biological activities, including cytotoxic, antitumor, antibiotic, antifungal, immunosuppressant and neurotoxic (Camacho et al., 2007; Wright and Cembella, 1998).

Dinoflagellates' bioactive molecules are inaccessible in large quantities and this severely hampers research in potential applications of these compounds (Camacho et al., 2007). Some of the toxins which can be extracted from dinoflagellates are quite expensive, even in small amounts (Belarbi et al., 2003; Kobayashi and Tsuda, 2004). In order to utilize the toxins and other bioactive molecules produced by mass scale cultivated dinoflagellates, research on biomedical, toxicological, chemical, pharmacological and therapeutic potential is essential. In fact, the study of their growth, high cell density, high productivity and physiology in cultures is considered a worthwhile.

Biomass production and utilization of microalgae culture has been quite successful recently (Sevilla et al., 2010); however, little effort has been devoted to mass culture of dinoflagellates in bioreactors (Beuzenberg et al., 2012; Grünwald et al., 2013; Rodríguez et al., 2010b;

Walid et al., 2011). The effectiveness of the quantity of extracted bioactive components depends on the culturing systems and productivity of dinoflagellate strains. For the optimization of growth conditions and toxin production of benthic dinoflagellate, various kinds of research have been conducted in different parts of the world (Dixon and Syrett, 1988; Kitaya et al., 2008; Morton et al., 1992; Nascimento et al., 2012; Pistocchi et al., 2011; Rhodes and Thomas, 1997; Tanimoto et al., 2013; Tosteson et al., 1989; Vidyarthna and Granéli, 2012; Yamaguchi et al., 2012; Zimmermann, 2006). However, most studies were only done for small-scale cultures. The maximum biomass concentration was attained below 1 g L^{-1} in a typical photosynthetic culture of dinoflagellate (Rodríguez et al., 2010b). Due to slow growth rates of dinoflagellates, large volume of culture is necessary to produce sufficient material for characterization of novel compounds and toxin production (Beuzenberg et al., 2012; Rodríguez et al., 2010a). In fact, there is no previous research on culturing of marine dinoflagellates and exploration of their bioactive components from Jeju Island, Korea. Hence, a special effort must be done in developing stable and reliable culture systems for the desirable culture strains. Therefore, the aim of this study was to evaluate the potentiality of growth and biomass productivity of benthic dinoflagellates using two culture media, which were collected from different coastal sites of the Jeju Island in Korea. Then, the biomasses of the 11 selected strains of benthic dinoflagellates were screened to evaluate the anti-inflammatory, anticancer and antioxidants effects using chemical and *in vitro* assays.

MATERIALS AND METHODS

Dinoflagellates isolation and culture

Dinoflagellate strains were collected from sand and macroalgae from the Coast of Jeju Island, Korea in 2011. The species were identified by morphological feature using light microscope (Zeiss Axioplan 2, Germany) with digital camera (Axiocam ERc5s) and epifluorescence microscope (violate excitation ca 430 nm, blue emission ca 490 nm; Zeiss Axioplan 2, Germany; Axiocam ICm1 digital camera) with Calcofluor White M2R (Fritz and Triemer 1985). Cell size was measured for the preliminary identification of the dinoflagellates. Taxonomic identification was done based on our previous research (Shah et al., 2013). Identification was also confirmed by molecular analysis (genomic DNA extraction, LSU rDNA D1D3 region sequenced and phylogenetic analysis) (unpublished data). Summary of the benthic dinoflagellates used for this study is presented in Table 1.

Single cells were isolated by the capillary pipette washing method under an inverted microscope (Olympus 1X71, Olympus, Tokyo, Japan) and transferred to a 24-multiwell plate containing 2 mL of IMK culture medium (Nihon Pharmaceutical Co., Ltd., Japan) (Yamaguchi et al., 2012). IMK medium contained the following components: 2.35 mM NaNO₃, 50.1 μM NH₄Cl, 9.86 μM Na₂HPO₄, 28.7 μM K₂HPO₄, 12.3 μM Fe-ethylenediaminetetraacetic acid (Fe-EDTA), 0.871 μM Mn-EDTA, 111 μM Na₂EDTA, 0.01 μM CuSO₄·5H₂O, 0.03 μM Na₂MoO₄·2H₂O, 0.08 μM ZnSO₄·7H₂O, 49.8 nM CoSO₄·7H₂O, 0.91 μM MnCl₂·4H₂O, 0.0132 μM H₂SeO₃, 0.001

Table 1. List of benthic dinoflagellates from Jeju island, Korea used for 20 L culture.

Sample no.	Species/strains	Collected location		Isolation date	Cell size	
		Name	Latitude/ longitude		Length (μm)	Width (μm)
D1	<i>Amphidinium carterae</i> (JHWAC)	Hwasun	33° 14' 22.38" N 126° 19' 55.67" E	15.08.2011	13.4 \pm 1.6	9.4 \pm 1.1
D2	<i>Prorocentrum rhathymum</i> (JHWPMX1)	Hwasun	33° 14' 22.38" N 126° 19' 55.67" E	27.11.2011	34.5 \pm 1.3	22.7 \pm 0.8
D3	<i>Symbiodinium</i> sp. (JHLSD1)	Hyupjae	33° 23' 38.88" N 126° 14' 23.02" E	29.09.2011	10.5 \pm 1.3	8.2 \pm 0.6
D4	<i>Coolia malayensis</i> 1 (JHACO6)	Hamo	33° 12' 39.86" N 126° 15' 38.23" E	14.05.2011	32.2 \pm 1.4	34.8 \pm 0.8
D5	<i>Ostreopsis ovata</i> 1 (JHAOS5)	Hamo	33° 12' 39.86" N 126° 15' 38.23" E	11.06.2011	51.46 \pm 2.1	34.5 \pm 1.7
D6	<i>Ostreopsis ovata</i> 2 (JHWOS13)	Hwasun	33° 14' 22.38" N 126° 19' 55.67" E	15.08.2011	53.46 \pm 1.1	36.5 \pm 1.3
D7	<i>Coolia malayensis</i> 2 (JHWCO1)	Hwasun	33° 14' 22.38" N 126° 19' 55.67" E	26.10.2011	33.5 \pm 0.7	36.8 \pm 0.9
D8	<i>Amphidinium operculatum</i> 1 (HLAM2)	Hyupjae	33° 23' 38.88" N 126° 14' 23.02" E	13.07.2011	33.6 \pm 2.5	22.4 \pm 2.9
D9	<i>Heterocapsa psammophila</i> (JLHET1)	Hyupjae	33° 23' 38.88" N 126° 14' 23.02" E	29.12.2011	26.1 \pm 1.1	19.8 \pm 0.8
D10	<i>Coolia malayensis</i> 3 (JHLCO6)	Hyupjae	33° 23' 38.88" N 126° 14' 23.02" E	13.07.2011	33.2 \pm 1.1	37.8 \pm 0.5
D11	<i>Amphidinium operculatum</i> 2 (SIAM1)	Sinyang	33° 31' 29.86" N 126° 51' 40.50" E	17.06.2011	32.6 \pm 2.1	24.4 \pm 2.2

μM vitamin B₁₂, 0.006 μM biotin, and 0.593 μM thiamine-HCl. After two weeks, the isolated dinoflagellates were confirmed to be growing and then were sub-cultured in screw capped test tubes (15 mL) with a round bottom containing 10 mL $\frac{1}{4}$ IMK medium as stock culture. Natural seawater (35 psu of salinity) collected from Jeju Coast was filtered through GF/F (47 mm, Whatman) filter, diluted with distilled water to adjust salinity to 30 psu and used for preparation of culture media after autoclaving at 121°C for 40 min and filtering (0.21 μm , Millipore). The stock cultures were then incubated in duplicate at 20°C under approximately 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of cool-white fluorescent illumination on a 12:12 h L:D cycle.

Stock cultures of dinoflagellate strains were gradually scaled up to 30 mL, 300 mL, 1 L and 3 L flasks under the above laboratory conditions. For the 20 L carboys, the culture conditions had small changes, as culture water was treated with sodium hypo chloride solution containing 9% active chlorine (at the rate of 1.1 mL L⁻¹ seawater) for 30 min for chemical sterilization, and after that, sodium thiosulphate (at the rate of 0.12 g L⁻¹ seawater) was added to neutralize chlorine in the water; also medium level of aeration was provided. From all the scale up stages, inoculums of exponential growing phase were used to start the following cultures. Cultures grown in glass flasks (1 and 3 L) were initiated by inoculation of 10 to 20% volume of 300 mL culture stock (maintained by monthly transfer) and cultures grown in carboys (20 L) were initiated by inoculation of 10 to 20% volume of 3 L cultures.

Experimental design

To evaluate the growth potential and biomass production of the

total 11 benthic dinoflagellate strains (Table 1) cultured in IMK medium (Nihon Pharmaceutical Co., Ltd., Japan) (Yamaguchi et al., 2012) and f/2 medium (Guillard, 1979), the batch cultures were run in duplicate for 40 days. The experimental procedure started with the inoculation of late exponential phase cultures from 3 L with an initial cell density of approximately $1 - 3 \times 10^3$ cells mL⁻¹ into 20 L carboy.

Growth rates

Growth rate was measured every five days from the cultured strains. Cells in 50 mL aliquots were fixed with 1 % formalin solution and then direct counts were made with a light microscope using the Sedgwick-Rafter (S-R) cell. Specific growth rate (SGR; μ , day⁻¹) was defined as the increase in cell density per time (Pirt, 1975); it was formulated as follows

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

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 (Guillard, 1979).

$$\text{Divisions per day, Div. day}^{-1} = \mu / \ln 2 \quad (2)$$

Dinoflagellate cell harvest

For biochemical analysis, 11 strains of dinoflagellate were harvested

separately from the cultures (20 L of cultures at exponential growing phase) by centrifuging (5000 rpm for 10 min in 250 mL centrifuge bottle) with VS-24SMTi high speed refrigerated centrifuge (Vision Scientific Co. Ltd, Daejeon Si, Korea). Cells were prepared for dry weight biomass following Zhu and Lee (1997) and biomass was expressed as g/L. The cultures were kept at -80°C and subjected to dry freeze using dry freeze system (Samwon Freezing Engineering Co. Busan, Korea).

Solvent extraction and sample preparation

The lyophilized benthic dinoflagellate strains were grounded separately into fine powder and homogenized. Then the homogenized samples were sonicated (ultra sound-assisted extraction) at 25°C for 90 min for three times, using (80%) methanol. Crude methanol extracts were concentrated by evaporating the solvent under reduced pressure, using rotary evaporator (Fisher Scientific, Loughborough, UK) and each of the samples was prepared into 100 mg mL⁻¹ concentration. For the determination of antioxidant activity, dilution was done using deionized water and for the *in vitro* assays, dilution was done using Dulbecco's phosphate-buffered saline (DPBS).

DPPH radical scavenging assay

Spin trapping is the most direct method for the detection of highly reactive free radicals, which can overcome the sensitivity problem inherent for the detection of endogenous radicals in biological systems. 1,1 diphenyl-2-picrylhydrazyl (DPPH) is a free radical donor which can be detected via electro spin resonance (ESR) spectrometer. DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine, JOEL, Japan) by the technique described by Nanjo et al. (1996). A 60 µL of each sample was added into 60 µL of DPPH (60 µmol L⁻¹) in ethanol. After 10 s of vigorous mixing, the solutions were transferred into 100 µL teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adduct was determined on an ESR spectrometer exactly 2 min later. The measurement conditions were as follows: Central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10⁵; temperature, 298 K.

Cell culture

The murine macrophage cell line (RAW 264.7) and a human promyelocytic leukemia tumor cell line (HL-60) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). RAW 264.7 cell line was cultured in Dulbecco's modified eagle medium (DMEM) and HL-60 cell line was grown in Roswell Park Memorial Institute (RPMI-1640) medium. Both media were supplemented with 100 U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin and 10% fetal bovine serum (FBS). The cells were incubated and maintained in an atmosphere of 5% CO₂ at 37°C. These cells were sub-cultured every two days and cells in exponential phase were used throughout the experiments.

Determination of nitric oxide (NO) production

RAW 264.7 cells (1×10⁵ cell mL⁻¹) were placed in a 24-well plate and after 24 h the cells were pre-incubated with concentrations (25 and 50 µg mL⁻¹) of the sample at 37°C for 1 h. Then further incubation was done for another 24 h with LPS (1 µg mL⁻¹) at the same temperature. After the incubation, quantity of nitrite accumulated in the culture medium was measured as an indicator

of NO production (Lee et al., 2007). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min and the optical density at 540 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Sunrise, Tecan Co. Ltd., Australia). The fresh culture medium was used as a blank in every experiment.

Cytotoxicity assessment using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay

The cytotoxicity of methanol extracts of unialgal cultures against the RAW 264.7 cells and the cancer cells (HL-60) was determined using a colorimetric MTT assay. Cells were seeded in a 96-well plate at a concentration of 1 × 10⁵ cells mL⁻¹. 24 h after seeding, the cells were treated with the extracts. Cells were then incubated for an additional 24 h at 37°C. MTT stock solution (50 µL; 2 mg mL⁻¹ in PBS) was then added to each well to a total reaction volume of 250 µL. After 3 h of incubation, the plates were centrifuged (800 × g, 5 min) and the supernatants were aspirated using an aspirating pipette attached to the vacuum. The formazan crystals from each well were dissolved in 350 µL of dimethylsulfoxide (DMSO), and the absorbance was measured with an ELISA plate reader at 540 nm.

Statistical analysis

Statistical significance between the growth rates was determined by analysis of variance using the software program Graph Pad InStat ver.3, Microsoft Excel 2007 and Duncan's multiple range tests (DMRT).

RESULTS

Dinoflagellates' growth and biomass production

A total of eleven benthic dinoflagellate strains were cultured in two different media *viz.*, IMK and f/2 to estimate their growth characteristics and biomass production. In the present study, maximum cell density, maximum growth rate and biomass production of the strains varied as 12 to 148 × 10³ cells mL⁻¹, 0.15 to 0.31 divisions/day and 0.06 to 0.26 g/L, respectively. After 10-15 days of inoculation, dinoflagellate strains started to grow rapidly until they reached their maximum cell numbers (Figure 1). All strains reached their maximum cell density on the 20th day of culturing period except D11 (*A. operculatum* 2) and D4 (*C. malayensis* 1), which reached their maximum on the 25th day, in f/2 medium.

Amphidinium species including *A. carterae* (D1), *A. operculatum* 1 (D8) and *A. operculatum* 2 (D11) showed maximum cell density ($P < 0.05$), growth rates (divisions day⁻¹) ($P > 0.05$) and biomass production ($P > 0.05$) in IMK medium compared to f/2 medium. Among these three strains, the highest cell density (148 × 10³ cells mL⁻¹), growth rate (0.317 ± 0.01 div.day⁻¹) and biomass (0.26 ± 0.03 g/L) were recorded in *A. carterae* (D1) (Figures 1, 2 and 3). *Symbiodinium* sp. (D3) got maximum cell density ($P < 0.05$), growth rate ($P > 0.05$) and biomass production ($P > 0.05$) faster in IMK medium than in f/2

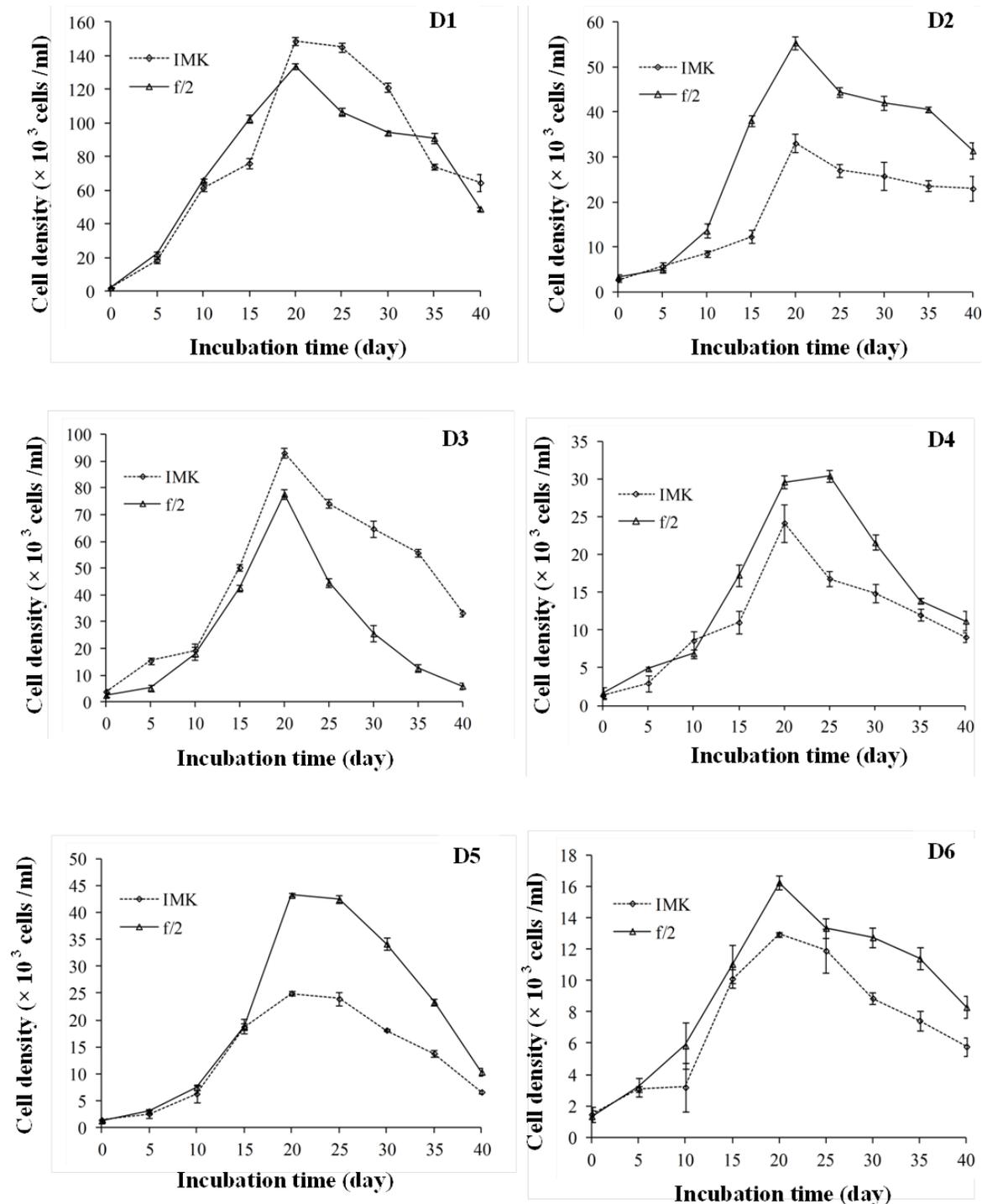


Figure 1. Growth curve (cell density) of benthic dinoflagellate strains cultured in 20 L culture. D1, *A. carterae*; D2, *P. rhathymum*; D3, *Symbiodinium* sp.; D4, *C. malayensis* 1; D5, *O. ovata* 1; D6, *O. ovata* 2; D7, *C. malayensis* 2; D8, *A. operculatum* 1; D9 *H. psammophila*; D10, *C. malayensis* 3; D11 *A. operculatum* 2.

medium (Figures 1, 2 and 3). On the contrary, *P. rhathymum* (D2), *H. psammophila* (D9) and two *Ostreopsis ovata* strains (D5 and D6) showed maximum cell density ($P < 0.05$), growth rate ($P > 0.05$) and

biomass production ($P > 0.05$) faster in f/2 medium than in IMK medium (Figures 1, 2 and 3).

Among the three *C. malayensis* strains, *C. malayensis* 1 (D4) and *C. malayensis* 3 (D10) had maximum cell

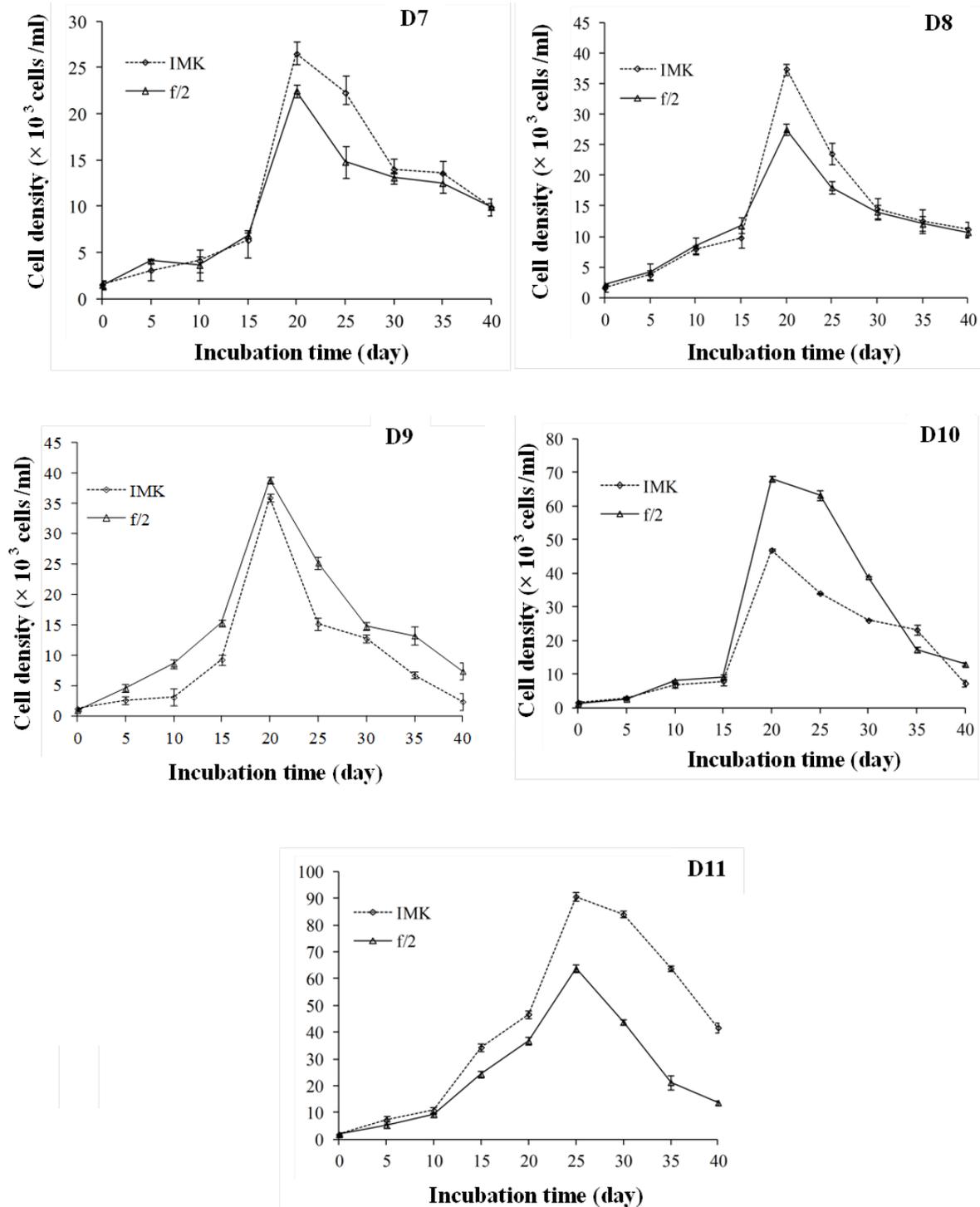


Figure 1. Contd.

density ($P < 0.05$), growth rates ($P > 0.05$) and biomass production ($P > 0.05$) in f/2 medium than in IMK medium, whereas *C. malayensis* 2 (D7) had better performances only in IMK medium (Figures 1, 2 and 3). When ANOVA was performed for IMK and f/2 medium separately,

maximum cell density, maximum growth rate and biomass production of *A. carterae* (D1) were statistically significant ($P < 0.05$) compared to all the eleven cultured strains. The other ten strains were not significant ($P > 0.05$).

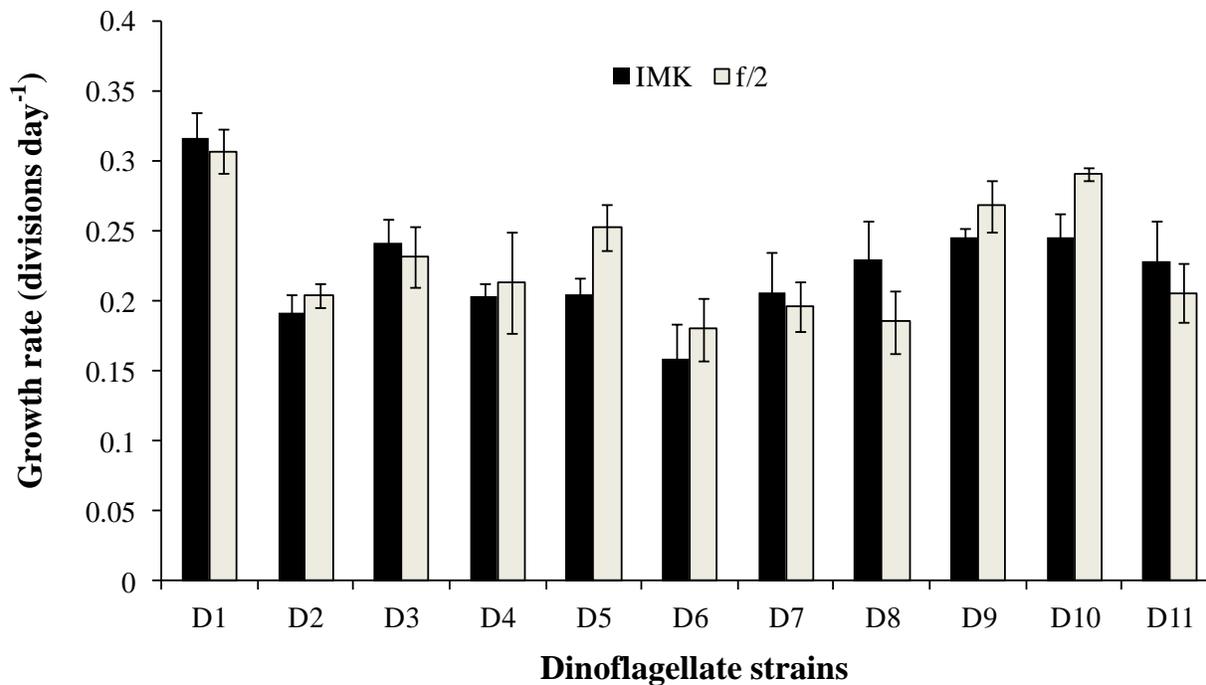


Figure 2. Growth rate (divisions day⁻¹) of benthic dinoflagellate strains cultured in 20 L culture. Strains are denoted as D1 (*A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values are expressed as means \pm SD in triplicate experiments.

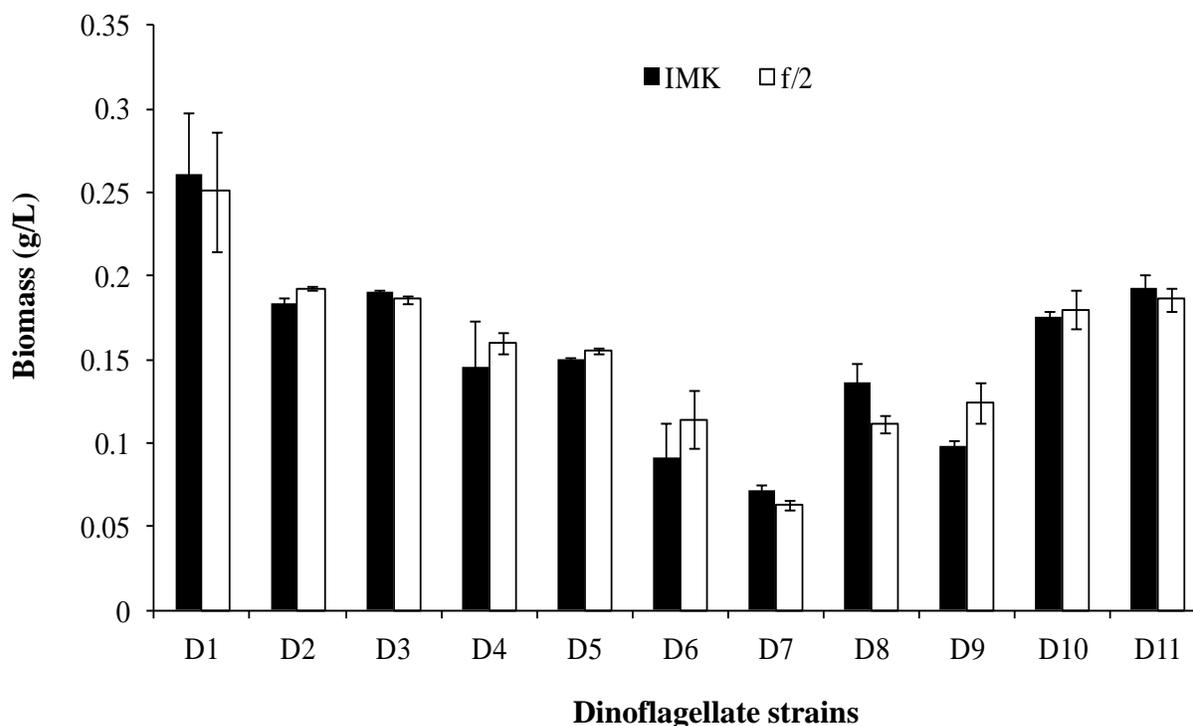


Figure 3. Biomass (g/L) production of benthic dinoflagellate strains cultured in 20 L culture. Strains are denoted as D1 (*A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values are expressed as means \pm SD in triplicate experiments.

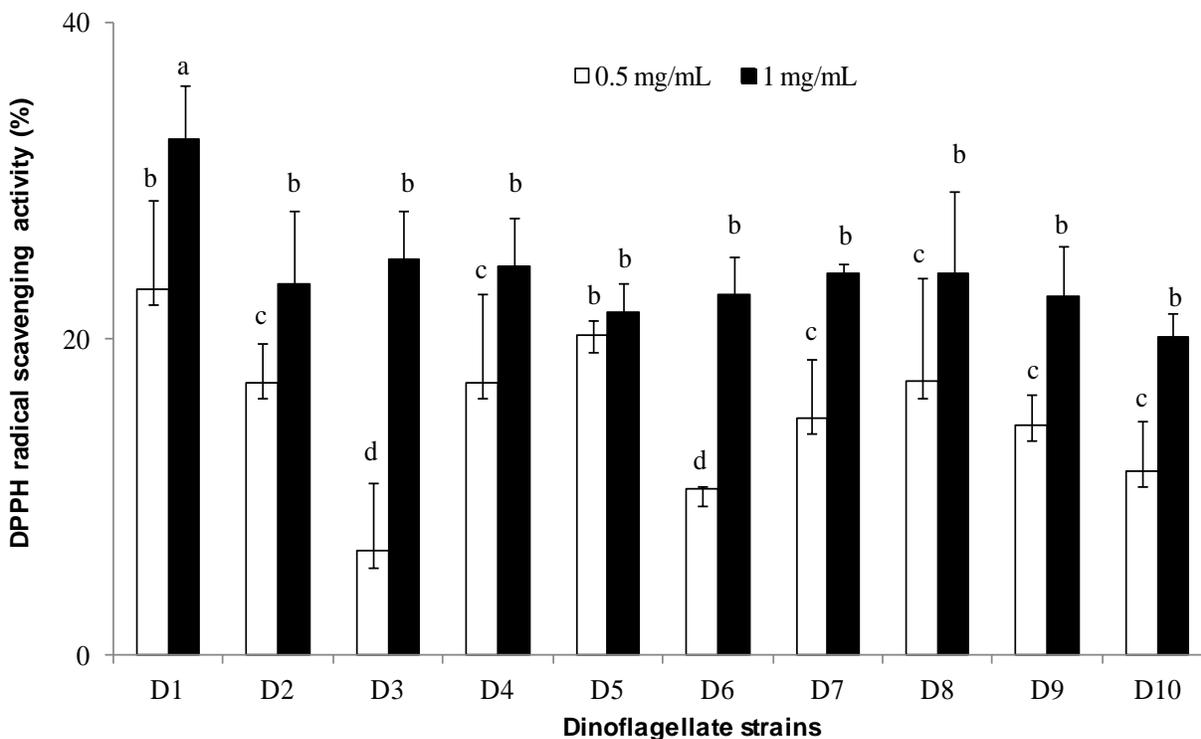


Figure 4. DPPH radical scavenging activity of 80% methanol extracts of dinoflagellate cultured strains by Electron Spin Resonance Spectroscopy. Strains are denoted as D1 (*A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values are expressed as means \pm SD in triplicate experiments. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

Screening of antioxidant, anticancer and anti-inflammatory activities

For the screening of bioactivity, the cultured benthic dinoflagellates were extracted in 80% methanol. Figure 4 shows the DPPH radical scavenging activities of the methanolic extracts from the eleven strains determined using ESR spectrometer. Among the extracts, only *A. carterae* (D1) had a significant ($P < 0.05$) DPPH radical scavenging activity (32.57 ± 3.44 %) at 1 mg mL^{-1} , compared to the other extracts. However, all the other methanolic extracts were not significant ($P > 0.05$) in terms of DPPH scavenging activity.

The inhibitory growth activity of the HL-60 cell was determined against methanolic extracts of the cultured benthic dinoflagellates as anticancer activity. Among the incubated methanolic extracts of the dinoflagellate strains at 25 and $50 \mu\text{g mL}^{-1}$ concentrations, the growth of HL-60 cells was suppressed significantly ($P < 0.05$) by D5 (*O. ovata* 1) and D8 (*A. operculatum* 1). Moreover, the determined cytotoxicity on HL-60 cells at $50 \mu\text{g mL}^{-1}$ concentration was reported as 52 and 42% against D5 (*O. ovata* 1) and D8 (*A. operculatum* 1), respectively (Figure 5).

Anti-inflammatory activity was performed as the inhibi-

tory effect of NO production (%) on LPS-induced RAW 264.7 macrophages. According to the results, the strongest inhibitory effect of NO production (app. 97%) was reported by D5 (*O. ovata* 1) at $50 \mu\text{g mL}^{-1}$ concentration. However, MTT assay showed that D5 (*O. ovata* 1) methanol extract increased the cytotoxicity of the RAW 264.7 macrophages by 73.5% at $50 \mu\text{g mL}^{-1}$ concentration compared to the control. Therefore, the examined activity did not comply with the anti-inflammatory activity. In addition, a significant inhibitory effect of NO production (%) was inferred from *A. carterae* (D1), *P. rhathymum* (D2) and *A. operculatum* 1 (D8) in comparison to the other strains. The MTT assay confirmed that there was no significant cytotoxicity at all in the treated concentrations and more than 100% cell viability was observed. Hence, these three dinoflagellate strains are described as having the potential to isolate anti-inflammatory active compounds (Figure 6).

DISCUSSION

This is the first attempt to evaluate and characterize the growth potential of benthic dinoflagellate species collected from Jeju Island, Korea. The maximum cell

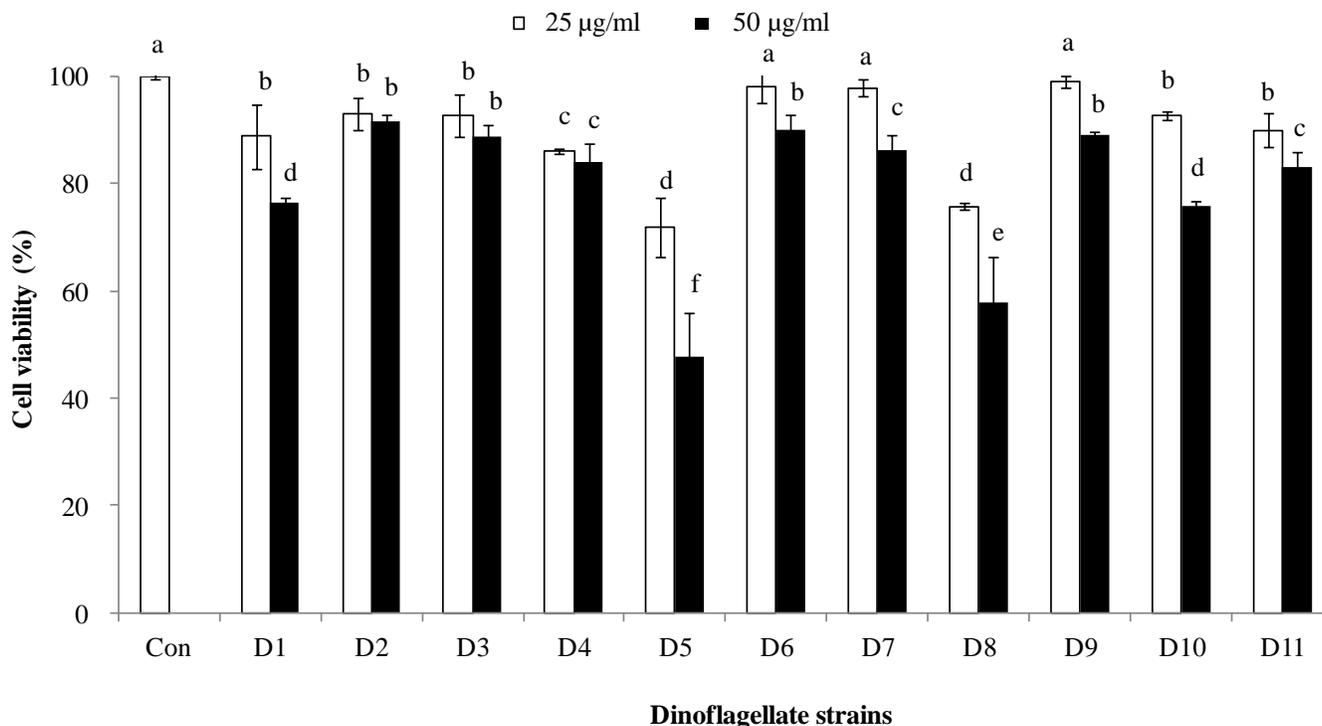


Figure 5. Inhibitory effect of the growth of cancer cells against 80% methanol extracts of dinoflagellate cultured strains on HL-60 cell lines. Cells were treated with the extracts at the indicated concentrations (25 and 50 $\mu\text{g mL}^{-1}$). After 24 h to treat the extracts cell viability was assessed by MTT assay. Values are expressed as means \pm SD in triplicate experiments. Strains are denoted as D1 (*A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

number of *A. carterae* (D1) obtained in the present study (148.60×10^3 cells mL^{-1} in IMK medium) was closer to the cell density ($1 - 6 \times 10^5$ cells mL^{-1}) reported by Thomas and Carr (1985). However, it was lower than other observations (23.3×10^5 cells mL^{-1}) for *A. carterae* cultured in f/2 medium with the modification of nitrogen and phosphorus concentrations at 33 psu, 25°C and 300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light intensity in 250 mL lab scale culture by Espinoza et al. (2011).

In our study, the maximum growth shown by *A. carterae*, (0.317 divisions day^{-1} in IMK medium) was similar with the growth rate (0.32 to 0.71 divisions day^{-1}) reported by Tomas et al. (1989). In addition, higher growth rate of *A. carterae* (D1) compared to other two *Amhidinium* strains, *A. operculatum* 1 (D8) and *A. operculatum* 2 (D11) in IMK medium indicates that *A. carterae* (D1) could be grown at its highest potential with the described culturing system. The maximum growth rate of *P. rhathymum* (D2) (0.204 divisions day^{-1} in IMK medium) was lower than the growth rate (app. 0.3 to <0.6 divisions day^{-1}) of closely related species, *P. mexicanum* collected from Knight key, Florida, USA and cultured in K medium (Keller and Guillard, 1985) under small scale stock cultures by Morton et al. (1992). *Symbiodinium* sp. (D3) cultured in this study (0.242 divisions day^{-1} in f/2

medium) showed almost similar growth rate of 0.30 d^{-1} in f/2 medium observed during the study of mixotrophic growth rate from *Symbiodinium* sp. (Jeong et al., 2012). Strains of *Coolia* spp. (D4, D7 and D10) showed the growth rate ranges from 0.19 to 0.29 divisions day^{-1} . This complies with the observations of Morton et al. (1992) who reported maximum growth rate of app. 0.2 to 0.6 divisions day^{-1} of *Coolia monotis* at 29°C, 33 psu in K medium (Keller and Guillard, 1985). Moreover, Rhodes and Thomas (1997) found the growth rate of 0.25 divisions per day when *C. monotis* was grown in GP medium (Loeblich and Smith, 1968). In our study, *O. ovata* strains (D 5 and D 6) showed maximum cell density of 12 to 43 $\times 10^3$ cells mL^{-1} , which is higher than that of other studies: for example, 10.1×10^3 cells mL^{-1} by Nascimento et al. (2012) and 4×10^3 cells mL^{-1} by Vidyarthna and Graneli (2012). In addition, growth rate of 0.15 to 0.25 divisions day^{-1} is similar to growth rate of 0.22 divisions day^{-1} mentioned by Nascimento et al. (2012). However, it was lower than 0.53 divisions day^{-1} (Guerrini et al., 2010), 1.07 divisions day^{-1} (Granéli et al., 2011) and 1.03 divisions day^{-1} (Yamaguchi et al., 2012). Dason and Colman (2004) cultured *Heterocapsa oceanica* in f/2 medium and found a growth rate of 0.344 divisions day^{-1} (calculated from doubling time) which was

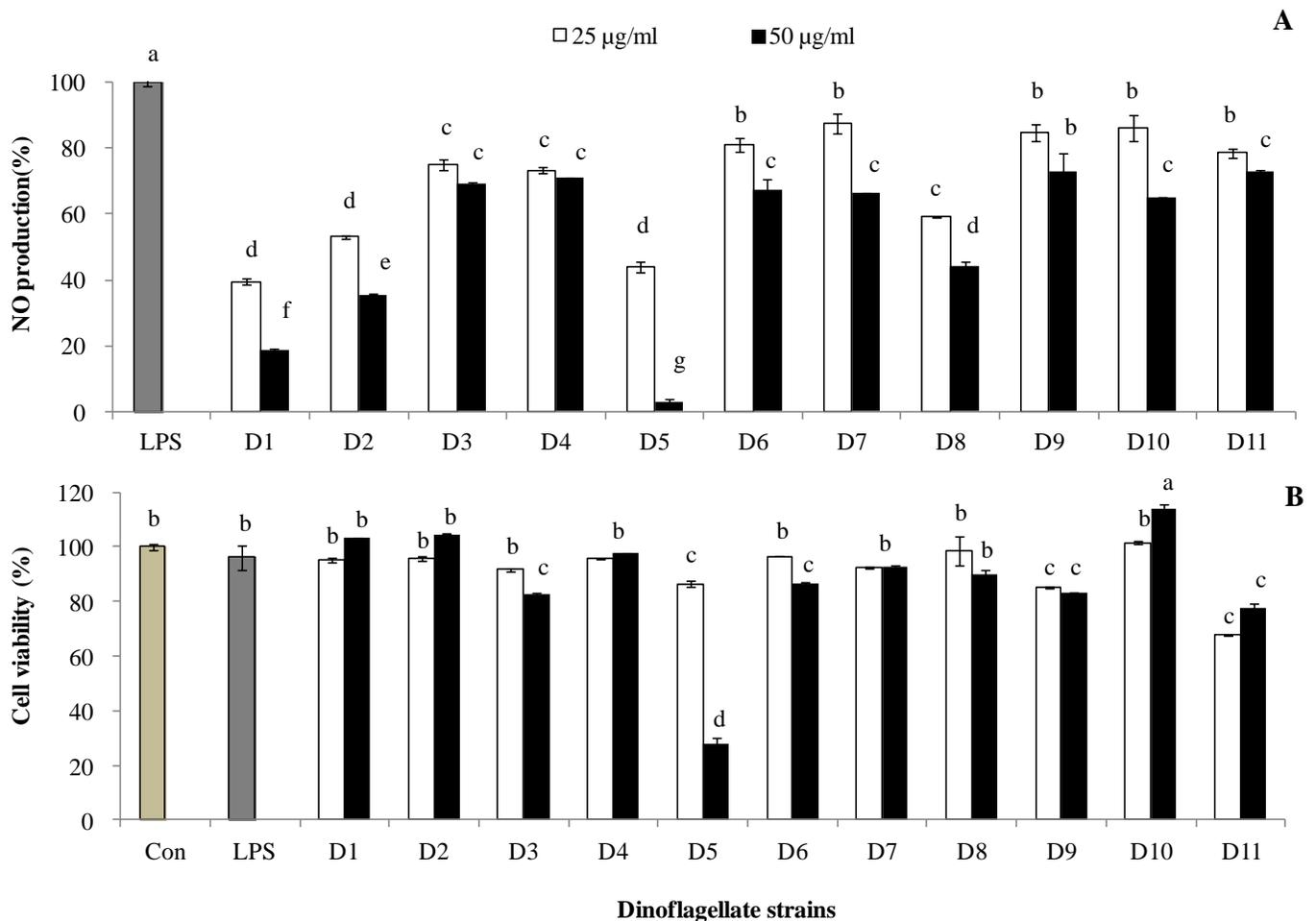


Figure 6. Inhibitory effect of 80% methanol extracts of dinoflagellate cultured strains. **(A)** on LPS-induced NO production in RAW 264.7 macrophages and **(B)** cell viability (%) in RAW 264.7 macrophages, respectively. Incubation of the extract concentrations (25 and 50 $\mu\text{g mL}^{-1}$) with cells in response to LPS ($1 \mu\text{g mL}^{-1}$) for 24 h, the NO levels in the medium was measured. Values are mean \pm SD of three determinations. Strains are denoted as D1 (*A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

slightly higher than the growth rate obtained by our strain of *Heterocapsa psammophila* (D9).

Dinoflagellates have commonly low growth rates, a complicated metabolism and low toxin productivity. This lower growth rate reflects in the lower chlorophyll *a* to carbon ratio (Chl *a* : C) as speculated by Tang (1996). The growth rates of dinoflagellates rarely double one per day (Thomas and Carr, 1985). Most of other economically important microalgae species have shown growth rates much higher than 1.0 d^{-1} ; for example, *Dunaliella tertiolecta* (1.4 d^{-1}), *Thalassiosira pseudonana* (1.8 d^{-1}) and *Chaetoceros calcitrans* (2.0 d^{-1}) (Thompson et al., 1990). Different chemicals have been experimented for maximizing the biomass production and CO_2 bio-fixation of microalgae (Nayak et al., 2013).

In particular, *Amphidinium*, *Prorocentrum* and two *Coolia* strains grew better and showed a higher biomass

production in IMK culture medium; this may indicate the potentiality of this medium for benthic dinoflagellate culture. Growth rates and cell yields of benthic dinoflagellate strains presented in this study are presumably attributed to the difference of the kind of medium, size of the culture and culture conditions used in other studies.

As a consequence, IMK culture medium can be suggested for the growth performances of benthic dinoflagellates and for their culturing compared to the commonly used *f/2* culture medium. Moreover, maximum cell yield, growth rate and biomass yield of D1 (*A. carterae*) were comparatively better than that of the other dinoflagellate strains, which suggests that this species might be the most efficiently cultured in the present culture system.

Benthic dinoflagellates cultured in our study are commonly found associating with sea grasses, macro algae, dead corals, rocks, soft sediments and inverte-

brates in tropical, subtropical and temperate marine environment (Totti et al., 2010). Among the cultured strains, only *Ostreopsis* sp. is known to produce bloom mainly in tropical waters or in temperate areas during summer (Pistocchi et al., 2011; Parsons et al., 2012). High water temperatures, high irradiance and high remineralisation are factors that create an environment favouring the blossom growth of benthic harmful algal (Fraga et al., 2012). In the benthic environment, as cells are linked to the substrate, their relative movement to 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 (Atkinson, 2001). A variety of factors other than wave action and temperature may also be important in controlling and promoting the occurrence and intensity of the blossom of benthic dinoflagellates, for example, availability of macroalgal substrates, light intensity, precipitation and nutrients (Tindall and Morton, 1998).

Nutrient availability is to be considered as an important environmental factor for controlling and promoting the occurrence and intensity of the blossomy of benthic dinoflagellates (Pistocchi et al., 2011), whereas the relationship between benthic/epiphytic dinoflagellates and nutrient conditions is less clearer (Pistocchi et al., 2011). Vila et al. (2001) found no significant correlations between epiphytic dinoflagellate and nutrients, and the authors stated that mechanisms that trigger species abundance remain unclear (Vila et al., 2001). In the Veracruz Reef Zone (Gulf of Mexico), a lack of correlation was recorded between nutrient concentrations and the abundant benthic/epiphytic dinoflagellate assemblage (Okolodkov et al., 2007).

Therefore, the role of nutrients in supporting elevated dinoflagellate biomass is still uncertain. In fact, as reported by Tindall and Morton (1998), epiphytic/benthic dinoflagellates do not appear to be unique in their requirements for the two major limiting macronutrients, nitrogen and phosphorus.

In our culture system, temperature, salinity, light intensity and aeration were not in variable conditions and growth performance of eleven strains differed with the variation of culture medium. Growth performance of these strains could be different by the variation of other factors (for example, temperature) in the present culture system. Further research 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 dynamics of benthic dinoflagellates in coastal environments.

DPPH chemicals generate stable free radicals and are widely used to test the ability of antioxidant activity of compounds or extracts of marine sources as free radical

scavenging properties or hydrogen donor capacity. In this study, we attempted to assess the antioxidant effects of the methanol extracts from dinoflagellate strains by ESR method. In addition, inflammation is initiated due to the pathogenic invasion or injury to cells and tissues as a physiological process (Newton and Dixit, 2012). In fact, inflammatory mediators, such as NO, play an important role as the signaling molecule that is induced in macrophages. LPS acts as endotoxins for mammals and stimulate the RAW cells in terms of enhancing the NO concentration in the medium (Wadleigh et al., 2000). Hence, as a screening technique, we used to measure the NO accumulation inhibitory percentage in the RAW macrophages *in vitro* with the treated methanol extracts of dinoflagellates. Furthermore, anticancer activity was determined as the inhibitory effects of cancer cell growth *in vitro* followed by pre-treated cultured dinoflagellates methanol extracts. MTT assay was performed to determine the cell viability of cancer cells.

As a consequence, methanol extracts from the cultured dinoflagellates were screened against DPPH radical scavenging, inhibitory effect of NO production (%) on LPS-induced RAW macrophages and inhibitory effect of the growth of human leucemia (HL-60) cells; it showed the possible potentials needed to isolate secondary metabolites. Importantly, among the cultured benthic dinoflagellates, *A. carterae* (D1) and *O. ovata* 1 (D5) performed profound bioactivities against the determined activity assays. It also showed the best culture conditions in IMK media and obtained the highest biomass yield compared to the f/2 media. This is further furnished by separating its active components through the bioassay guided fractionations. In fact, Echigoya et al. (2005) have shown the potentiality of isolation of novel bioactive compounds from the cultured *A. carterae*. Moreover, a potent hemolytic and antifungal active compound (amphidinol 2) was isolated from the cultured *A. klebsii* (Paul et al., 1995). However, biochemical analysis proved that D5 (*O. ovata* 1) strain showed the highest anticancer activity among the cultured dinoflagellates strains and was successfully cultured in f/2 media. Previous studies reported that cultured dinoflagellates are prolific sources for lipid or fatty acid isolation (Rodriguez et al., 2010a). The screening results of our studies emphasized that available lipids including stanols, steroids and polyunsaturated fatty acids are potential metabolites for anti-inflammatory effects. On the other hand, available polyhydrox metabolites can be responsible for the antioxidant and anticancer activity of the cultured dinoflagellates.

More than 21,000 bioactive metabolites have been isolated from marine species over the years (Blunt et al., 2009). In fact, dinoflagellates have rendered many natural compounds useful for the field of drug discovery. Therefore, benthic dinoflagellates are described as alternative candidates for the isolation of bioactive metabolites with the pharmacological value for future

therapeutic applications (Estrada et al., 2007; Kobayashi and Tsuda, 2004; Mydlarz et al., 2003). However, many have failed to produce and culture dinoflagellates in laboratory scale to gain the desired bioactivities under the artificial culture media (Rodríguez et al., 2012). Despite that, in this study, 11 strains of the cultured benthic dinoflagellates were screened for antioxidant, anticancer and anti-inflammatory assays to figure out the available potentiality for isolation of bioactive metabolites. A few studies have been done on the isolation of new secondary metabolites from dinoflagellates; for example, Wu et al. (2005) have isolated a new unsaturated glycolipids from a cultured marine, *A. carterae*. In addition, a novel polyhydroxy metabolite, zooxanthellamide A was isolated from *Symbiodinium* sp. (Onodera et al., 2002), and another polyhydroxy compound with potent hemolytic activity, Amphidinol 2 was identified from *Amphidinium klebsii* (Paul et al., 1995). Therefore, in this study, cultured dinoflagellates would be the key sources for novel finding with respect to chemical, pharmacological and toxicological research.

Conclusions

This study reveals a useful culture system for scaling up benthic dinoflagellate using IMK and f/2 media successfully by demonstrating the growth potential of the strains collected from Jeju Island, Korea. Besides the common f/2 medium, IMK medium was proven to have the potential to culture some of benthic dinoflagellates in 20 L significantly. Among the cultured strains, *A. carterae* showed the highest antioxidant and anti-inflammatory effect *in vitro* assays. In addition, deemed bioactive potentials from the cultured strains in the respective media can be considered to extract novel secondary metabolites for future pharmacological and commercial applications. Furthermore, research can be carried out to explore the effects of salinity, light and temperature on the growth characteristics of benthic dinoflagellates by batch and continuous culture systems.

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