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OPTIMAL GROWTH CONDITIONS AND ANTIOXIDATIVE ACTIVITIES OF *CYLINDROTHECA CLOSTERIUM* (BACILLARIOPHYCEAE)[1]

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We isolated the unialgal strain of *Cylindotheca closterium* (Ehrenb.) Reimann et J. C. Lewin and produced an axenic strain using an antibiotic cocktail of enriched f/2 artificial seawater medium. The optimal growth conditions were estimated under 27 different combinations of temperature, salinity, and nutrients, and mass culture was performed based on the best specific growth conditions. Its antioxidant activities were determined from the extracts of methanol, water, and enzymes (proteases and carbohydrases). The maximum specific growth rate (μ_{max}) varied from 0.63 to 0.97 · d^{-1}. The maximum cell density was 7.20 × 10^4 cells · mL^{-1}, while the μ_{max} was 0.82 · d^{-1} in culture conditions of 20°C, 30 psu (practical salinity unit), and "F" nutrient concentrations on day 10 of the culture period. The scavenging rates for 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical were 72.5% and 69.4% from Viscozyme and methanol extracts, respectively. The enzymatic extracts of *C. closterium* prepared by the hydrolyses of Amyloglucosidase (AMG) and Viscozyme showed 45.8% and 45.5% nitric-oxide-scavenging rates, slightly lower than the activity of alpha-tocopherol (α-tocopherol) but similar to butylated hydroxytoluene (BHT). The extract from methanol and water showed 44.8% and 44.4% scavenging rates, statistically similar with BHT. The metal-chelating activities of the Kojizyme, Alcalase, methanol, Viscozyme, and Neutrase extracts were 67.1, 53.9, 53.2, 52.1, and 50.2 %, respectively, five to six times higher than the commercial antioxidants. The AMG, Viscozyme, and Neutrase extracts showed a remarkable linoleic acid peroxidation inhibition, which was higher than BHT and statistically similar with α-tocopherol.

Key index words: **antioxidant activities; benthic diatoms; *Cylindrotheca*; growth characteristics; mass culture**

Abbreviations: **AMG, amyloglucosidase; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ROS, reactive oxygen species**

The growing knowledge regarding the role of free radicals and reactive oxygen species (ROS) in chronic degenerative diseases has prompted many researchers to investigate free radical scavenging compounds. Exogenous chemicals and endogenous metabolic processes in the human body or in food systems produce extremely dangerous ROS. In respiration, ~5% of the inhaled O_2 is converted to ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) (Bandyopadhyay et al. 1999). Even if there is a nonenzymatic and enzymatic protection mechanism in the body against ROS, "oxidative stress" can result. ROS attack the biological molecules, including lipids, proteins, enzymes, DNA, and RNA, resulting in cell or tissue injuries associated with atherosclerosis and carcinogenesis. In addition, oxidation is a major cause of food quality deterioration, destroying fat-soluble vitamins and causing the development of toxicants and discoloration (Yan et al. 1999, Ukeda et al. 2002). Antioxidants can inhibit the oxidation process by scavenging free radicals, chelating catalytic metals, and acting as oxygen scavengers (Shahidi and Wanasundara 1992). Commercial antioxidant supplements, such as butylated hydroxyanisol (BHA), BHT, α-tocopherol, propyl gallate (PG), and tert-butylhydroquinone (TBHQ) are used to reduce oxidative damage to the human body (Sherwin 1990, Gülçin et al. 2002).

The interest in antioxidants from natural sources is increasing as some synthetic antioxidants may have negative side effects when taken in vivo (Chen et al. 1992). Among the natural antioxidants, polyphenols in the human diet have been reported to contribute to decreasing cardiovascular diseases

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and exerting a beneficial health effect (Rice-Evans et al. 1995, Hotta et al. 2002). Natural antioxidants have been isolated from various plants, but microalgae have received less attention as a source of natural antioxidants. The high antioxidant action of C-phycocyanin from the blue-green alga *Arthrospira maxima* has been detected, and this natural product is able to scavenge alkoxyl and hydroxyl radicals (Romay et al. 1998). Sulfated polysaccharide of *Porphyridium* sp. has shown good free-radical-scavenging activities (Spitz et al. 2005). Several microalgae, such as *Chlorella*, *Spirulina*, and *Dunaliella* spp., are grown commercially for their pigments, such as β-carotene and phycocyanin. In the search for new compounds with therapeutic potential, microalgae in all classes have demonstrated antibacterial, antifungal, and anticancer properties (Metting 1996, Tredici 2004). However, studies on antioxidant properties of diatoms are very rare. Recently, we screened the benthic diatoms *Grammatophora marina* (Affan et al. 2006) and *Navicula incerta* (Affan et al. 2007a), which showed very good antioxidant activities, especially for metal-chelating activity and DPPH-free-radical-scavenging, nitric-oxide-radical-inhibition and hydroxyl-radical-scavenging activities.

As our knowledge of the antioxidant effects of diatoms has increased, *C. closterium* has been selected as one of the best candidates because it dominates in the coastal waters of Jeju Island, Republic of Korea (Affan and Lee 2004). For mass culture of *C. closterium*, optimal growth conditions were determined using different combinations of water temperature, salinity, and nutrient concentrations in a fixed light intensity. Smayda (1969) reported that a combination of temperature, salinity, and light played an important role in the cell division of diatoms. Moreover, changes in salinity and temperature appear to influence oceanic phytoplankton abundance (Hoshiai et al. 2003), and estimation of the optimal growth rate in different environmental conditions is very important for mass culture of benthic diatoms. Thus, after isolating an axenic strain of *C. closterium*, we determined the optimal environmental conditions for growth and estimated its antioxidant activities.

MATERIALS AND METHODS

Isolation of C. closterium. The isolation procedure was performed as described in Affan et al. (2006). In summary, benthic diatoms attached to a wavy plastic plate ("papan") were collected from the abalone culture hatchery of the National Fisheries Research and Development Institute (NFRDI), Jeju, Republic of Korea. The diatoms were removed from the papan with a brush and diluted with seawater. Then, a 1 mL diluted sample was transferred to a custom-made Sedgwick Rafter (S–R) counting chamber, and a single benthic diatom cell was isolated with a mouth-sucking micropipette (MSM) technique. To make the MSM, the tip of a glass pasture pipette was heated over an alcohol lamp, and the tip was drawn out. Then, transparent PVC tubing (DH.TUP002; Daehan, Seoul, Korea) was pushed inside the holder part of the glass pasture pipette, and finally the joint between the glass pasture pipette and PVC tubing was made airtight with vacuum latex tubing (DH.KA2232; Daehan). The total length of the MSM apparatus was 75 cm. The needle of the MSM was placed close to the target cell observed with an inverted microscope (Olympus IX 71, Tokyo, Japan), and gentle suction was applied.

Thereafter, each cell was transferred to a multiwell plate for subculture in autoclaved seawater, which was filtered through a 0.45 μm nitrocellulose membrane filter (Millipore MF, Billerica, MA, USA) and enriched with F/2 medium nutrients (Aquacenter Inc., Leland, MS, USA), trace metals (Aquacenter Inc.), and sodium metasilicate anhydrous crystals (Na_2SiO_3). The subculture medium and A and B solutions of F/2 medium were added at 1 mL per 7.75 L seawater, plus 25 μL · L^{-1} trace element solution and 50 μg · L^{-1} sodium metasilicate anhydrous crystal. The isolation process and subsequent culture was repeated until a unialgal strain was obtained. To reconfirm the identify of the subcultured benthic diatom, samples were observed under a phase-contrast microscope (Carl Zeiss Axioplan, Oberkochen, Germany) at ×400 magnification. The strain identified as *C. closterium* was again streaked onto an agar plate with 2% agar (w/v), 0.04% F/2 (v/v) nutrient medium, and autoclaved seawater. After 5 d, the colony was transferred to a 250 mL flask containing 100 mL F/2-enriched culture medium and antibiotics. Seven different dosages of antibiotic cocktail were used (100–250 units · mL^{-1} penicillin, 100–250 μg · mL^{-1} streptomycin, and 200–500 μg · mL^{-1} neomycin). Doses were increased by 25 units penicillin · mL^{-1}, 25 μg streptomycin · mL^{-1}, and 50 μg neomycin · mL^{-1} (P 4083; Sigma-Aldrich Corp., St. Louis, MO, USA) (Fig. 1). About 10 mL of each *C. closterium* sample was transferred from the antibiotic medium to a 250 mL flask with 100 mL of culture medium. The cultured sample was streaked again on Bacto Agar (Becton Dickinson Comp., Sparks, MD, USA) media to observe for bacterial presence. In this way, we obtained an axenic strain of *C. closterium* for further study.

Determination of optimal growth conditions. Optimal growth conditions of *C. closterium* were determined using the modified method of Affan et al. (2007a). Artificial seawater was made by adding synthetic sea salt (Energy Savers Unlimited Inc., Carson, CA, USA) with distilled water. *C. closterium* was cultured under 27 combinations of the three parameters: water temperature (15°C, 20°C, and 25°C), salinity (25, 30, and 35 psu), and nutrient concentration ($F/4$ = 50%, $F/2$ = 100%, and F = 200%) (Table 1). For preparation of nutrient enrichment culture medium, the same ingredients and constituents were added as for initial subculture medium used in the axenic production (see above). Each 1 L flask with 250 mL culture media was inoculated with ~20 cells · mL^{-1} *C. closterium* from the prepared axenic culture. The cultures were grown under fluorescent lights (180 μmol photon · m^{-2} · s^{-1}) on a 14:10 light:dark (L:D) cycle for 2 weeks. Each treatment was performed with two replications. To assess cell density, a 1 mL sample was collected from each culture flask every 2 d and fixed with Lugol's iodine solution. The fixed sample was diluted and mixed by a vortex mixer (Scientific Industries Inc., Bohemia, NY, USA), and the cells were counted using an S–R counting chamber with an Olympus IX 71 inverted microscope at ×400 magnification.

We calculated the specific growth rate (μ), defined as the increase in cell density per unit time (Pirt 1975) and formulated as follows:

$$\mu(d^{-1}) = \frac{\ln(X_1/X_0)}{t_1 - t_0} \quad (1)$$

where X_0 and X_1 are cell density at the beginning (t_0) and end (t_1) of a selected time interval between inoculation and

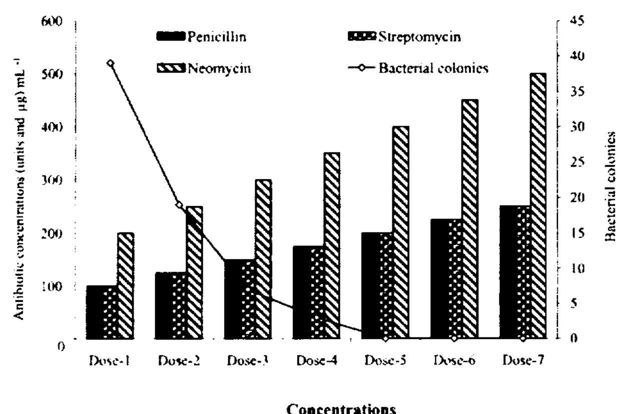

FIG. 1. Reduction in bacterial colonies with the increase in antibiotic concentrations over Dose 1 (penicillin 100 units · mL^{-1}, streptomycin 100 µg · mL^{-1}, and neomycin 200 µg · mL^{-1}). Each succeeding dose was increased by 25 units penicillin, 25 µg streptomycin, and 50 µg neomycin per mL.

TABLE 1. The maximum specific growth rate ($\mu_{max} \cdot d^{-1}$) at different temperatures (°C), salinity (psu), and nutrient concentrations (%) and peak growth period (d).

Culture conditions						
Treatment	(°C)	(psu)	(%)	$\mu_{max} \cdot d^{-1}$	Cell density ($\times 10^4 \cdot L^{-1}$)	d
A1	15	25	F/4	$0.63^f \pm 0.01$	3.83 ± 0.02	12
A2	15	25	F/2	$0.64^f \pm 0.01$	4.52 ± 0.02	12
A3	15	25	F	$0.66^{ef} \pm 0.02$	5.62 ± 0.03	12
A4	15	30	F/4	$0.63^f \pm 0.01$	3.93 ± 0.01	12
A5	15	30	F/2	$0.65^{ef} \pm 0.01$	4.70 ± 0.02	12
A6	15	30	F	$0.66^{ef} \pm 0.02$	5.59 ± 0.02	12
A7	15	35	F/4	$0.64^f \pm 0.02$	4.19 ± 0.01	12
A8	15	35	F/2	$0.65^{ef} \pm 0.2$	4.87 ± 0.01	12
A9	15	35	F	$0.81^b \pm 0.03$	6.59 ± 0.03	10
B1	20	25	F/4	$0.65^{ef} \pm 0.01$	4.92 ± 0.03	12
B2	20	25	F/2	$0.79^{bc} \pm 0.02$	5.32 ± 0.02	10
B3	20	25	F	$0.80^b \pm 0.01$	5.79 ± 0.02	10
B4	20	30	F/4	$0.65^{ef} \pm 0.02$	5.13 ± 0.02	12
B5	20	30	F/2	$0.80^b \pm 0.01$	5.80 ± 0.02	10
B6	20	30	F	$0.82^b \pm 0.02$	7.20 ± 0.02	10
B7	20	35	F/4	$0.66^{ef} \pm 0.03$	5.22 ± 0.01	12
B8	20	35	F/2	$0.79^{bc} \pm 0.01$	5.69 ± 0.03	10
B9	20	35	F	$0.80^b \pm 0.01$	5.55 ± 0.01	10
C1	25	25	F/4	$0.73^d \pm 0.01$	2.84 ± 0.01	10
C2	25	25	F/2	$0.97^a \pm 0.03$	4.54 ± 0.01	10
C3	25	25	F	$0.76^d \pm 0.03$	4.11 ± 0.02	8
C4	25	30	F/4	$0.93^a \pm 0.01$	3.33 ± 0.02	8
C5	25	30	F/2	$0.96^a \pm 0.02$	4.28 ± 0.02	10
C6	25	30	F	$0.75^d \pm 0.01$	3.74 ± 0.01	8
C7	25	35	F/4	$0.74^d \pm 0.01$	3.39 ± 0.03	10
C8	25	35	F/2	$0.95^a \pm 0.03$	4.04 ± 0.03	10
C9	25	35	F	$0.75^d \pm 0.02$	3.17 ± 0.02	8

The values represent the mean ± SD of specific growth rate cells · mL^{-1} determined from two replicate samples. Values followed by the superscript letters a, b, c, d, e, and f indicate a significant difference ($P < 0.05$) in different culture conditions.

maximum cell density, respectively. For the growth curve of each treatment, we counted two replicates and used the mean value.

Mass culture and sample preparation for antioxidant assay. *C. closterium* was grown in a 20 L polycarbonate transparent bottle

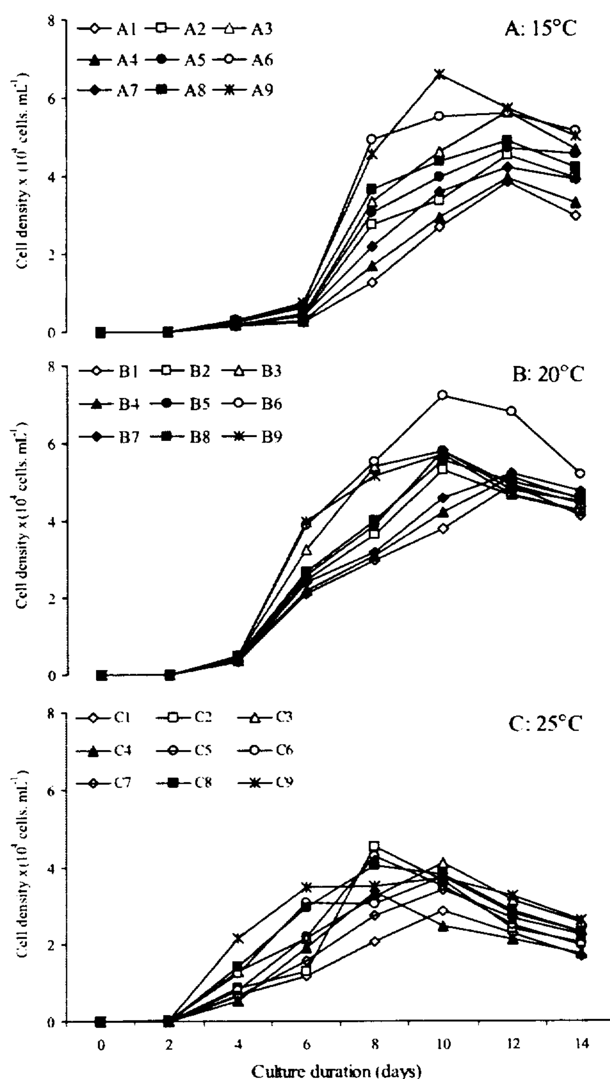

FIG. 2. Growth curves of *Cylindrotheca closterium* at 15°C, 20°C, and 25°C water temperatures, with F/4 (50%), F/2 (100%), and F (200%) nutrient concentrations and 25, 30, and 35 psu. Culture conditions: A1 (25 psu, F/4 nutrients), A2 (25 psu, F/2 nutrients), A3 (25 psu, F nutrients), A4 (30 psu, F/4 nutrients), A5 (30 psu, F/2 nutrients), A6 (30 psu, F nutrients), A7 (35 psu, F/4 nutrients), A8 (35 psu, F/2 nutrients), and A9 (35 psu, F nutrients) in 15°C water. Conditions B (20°C water) and C (25°C water) same as A with regard to salinity and nutrient concentrations.

(Nalgene, Rochester, NY, USA) under the optimal growth conditions identified in the previous experiment (i.e., B6 culture conditions in Fig. 2) using artificial seawater enriched with F/2 nutrients at the same irradiance as given above. After 2 weeks of culture, aeration was stopped to allow the *C. closterium* cells to settle overnight, and the supernatant removed by siphon. The residual algal biomass was centrifuged (HA 1000-3, Hanil Science Industrial Co., Incheon, Korea) at 226*g* for 3 min before being transferred to a plastic ziplock bag and stored at −70°C for 24 h. Afterward, the sample was freeze-dried at −50°C at 50 m Torr.

Antioxidant assay chemicals. Methanol (95%); the five carbohydrate degrading enzymes, including Viscozyme L, Celluclast 1.5 L FG, AMG 300 L, Termamyl 120 L, Ultraflo L; and

the five proteases, including Protamex, Kojizyme 500 MG, Neutrase 0.8 L, Flavourzyme 500 MG, Alcalase 2.4 L FG were donated from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). All the chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich Corp. or Fluka Co. (Buchs, Switzerland). Other chemicals used were 99% or greater purity.

Preparation of 80% methanolic and enzymatic extracts of C. closterium. Five grams of freeze-dried powder sample was mixed with 500 mL of 80% MeOH and kept in a shaking incubator at 25°C for 24 h. Afterward, the extract was filtered, and the filtrate was obtained for the activity test. For the enzyme extracts, 1 g of freeze-dried powder sample was mixed with 100 mL of distilled water. The pH of each reaction mixture was adjusted with 1 M HCl/NaOH. The optimum pH and temperature for the respective enzymes were similar to those reported by Heo et al. (2003). Each enzyme was added at a 1% ratio and incubated for 24 h. These mixtures were filtered, and the enzyme activity of the hydrolysates was inactivated by heat (100°C for 10 min). Finally, the pH of each hydrolysate was adjusted to 7.0 with HCl/NaOH. Both the methanolic and enzymatic extracts were adjusted to a final concentration of 2 mg $\cdot$ mL^{-1}. The activity of the extracts was compared with the commercial antioxidants, BHT and α-tocopherol, which were dissolved in ethanol (2 mg $\cdot$ mL^{-1}).

DPPH-free-radical-scavenging activity. This assay involved the scavenging of stable DPPH radicals by the radical-scavenging components of *C. closterium* extracts, using a modified method of Brand-Williams et al. (1995). A 2 mL fraction of *C. closterium* extract was mixed thoroughly with 2 mL freshly prepared 3×10^{-5} M DPPH solution in DMSO. The reaction mixture was incubated for 1 h, and the absorbance of the supernatant was measured at 517 nm using an Opron 3000 UV-VIS spectrophotometer (Hanson Tech. Co. Ltd., Seoul, Korea).

Superoxide-anion-scavenging assay. The superoxide-anion-scavenging effect was tested following Nagai et al. (2003). The reaction mixture consisted of 0.48 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 mL of 3 mM xanthine, 0.02 mL of 3 mM EDTA, 0.02 mL of 0.15% BSA, 0.02 mL of 0.75 mM NBT (nitro blue tetrazolium salt), and 0.02 mL of *C. closterium* extract. After incubation at 25°C for 10 min, the reaction was started by adding 6 mU xanthine oxidase and maintained at 25°C for 20 min. The reaction was stopped by adding 0.02 mL of 6 mM $CuCl_2$. The absorbance was measured in a Sunrise microplate reader (Tecan Co. Ltd., Salzburg, Austria) at 560 nm.

Hydrogen-peroxide-scavenging activity. Hydrogen-peroxide-scavenging activity was determined according to Rice-Evans et al. (1995). A sample was prepared in a 96-microwell plate by mixing 80 μL of *C. closterium* extract, 100 μL of PBS (0.1 M, pH 5.0), and 20 μL of 10 mM H_2O_2, and the sample was incubated at 37°C for 5 min. Then, 30 μL ABTS (2,2-azinobis (3-ethylbenthiazoline)-6-sulfonic acid) (1.25 mM) and 30 μL peroxidase (1 unit $\cdot$ mL^{-1}) were added, and the mixture was incubated at 37°C for 10 min. The absorbance was recorded at 405 nm.

Hydroxyl-radical-scavenging activity. The hydroxyl-radical-scavenging effect was determined according to Chung et al. (1997). A Fenton reaction mixture (200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 200 μL of 10 mM EDTA, and 200 μL of 10 mM 2-deoxyribose) was mixed with 1.2 mL of 0.1 M PBS (pH 7.4) and 200 μL of *C. closterium* extract. Then, 200 μL of 10 mM H_2O_2 was added, and the mixture was incubated at 37°C for 4 h, after which 1 mL of 2.8% trichloroacetic acid (TCA) and 1 mL of 1% 2-thiobarbituric acid (TBA) were added, and the mixture was kept in a bath of boiling water for 10 min. After cooling, the mixture was centrifuged for 5 min at 395*g*, and the absorbance was measured at 532 nm using an Opron 3000 UV-VIS spectrophotometer.

Nitric-oxide-radical inhibition assay. The nitric-oxide-radical inhibition effect was determined following Garrat (1964). A mixture of 2 mL of 10 mM sodium nitroprusside in 0.5 mL of PBS (pH 7.4) and 0.5 mL of *C. closterium* extract was incubated at 25°C for 150 min. From this, 0.5 mL was removed and added to 1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was added, and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm using an Opron 3000 UV-VIS spectrophotometer.

Metal-chelating assay. Metal-chelating ability was determined according to Decker and Welch (1990). The mixture sample was prepared by adding 5 mL of *C. closterium* extract to a solution of 0.1 mL of 2 mM $FeCl_2$. The reaction was started by adding 0.2 mL of 5 mM ferrozine solution. The reaction mixture was incubated, with shaking, at room temperature for 10 min. The absorbance was measured at 562 nm using an Opron 3000 UV-VIS spectrophotometer.

Determination of antioxidant activity using the ferric thiocyanate (FTC) method. This assay was performed following Kikuzaki and Nakatani (1993). The mixture, consisting of 2 mL of *C. closterium* extract (100 mg $\cdot$ L^{-1}), 2 mL of 2.51% linoleic acid in ethanol, 4 mL of 0.05 M PBS (pH 7.0), and 2 mL of distilled water, was kept in the dark at 40°C. From this mixture, 0.1 mL was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. After 5 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl was added. The samples were kept in dark at 40°C between absorbance measurements. The absorbance was measured at 500 nm wavelength on every 24 h interval for 7 d.

Determination of total phenolic content. The total phenolic content was determined using a protocol similar to that of Chandler and Dodds (1983). The mixture consisted of 1 mL of *C. closterium* extract, 1 mL of 95% ethanol, 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min, and then 1 mL of 5% Na_2CO_3 was added. The sample was thoroughly mixed and placed in the dark for 1 h. The absorbance was measured at 725 nm.

Determination of total flavonoids and polysaccharide content. The total flavonoid content was determined by the colorimetric method described by Woisky and Salatino (1998). To 0.5 mL of *C. closterium* extracts, 0.5 mL of 2% $AlCl_3$ ethanol solution was added and kept for 1 h at room temperature, and then the absorbance was measured at 420 nm. Total flavonoid content was calculated as kaempferol from the calibration curve. The polysaccharide content was determined by phenol-sulfuric method described by Dubois et al. (1956).

Scavenging-activity/chelating-ability calculation. The radical-scavenging activity/chelating ability was calculated using the following equation:

$$[1 - (A_i - A_j)/A_c] \times 100 \tag{2}$$

where, A_i is the absorbance of the *C. closterium* extract mixed with reactive oxygen or nitrogen compounds, A_j is the absorbance of the *C. closterium* extract without the reactive compounds, and A_c is the absorbance of the control.

Statistical analysis. All the experiments to determine growth characteristics were conducted in duplicate ($n = 2$). The experiments to estimate antioxidant activity were conducted in triplicate ($n = 3$). The mean values of each treatment were compared using one-way analysis of variance (ANOVA) followed by Tukey's tests. A *P*-value of <0.05 was considered

significant. All statistical analyses were performed using SPSS Statistical Software, version 11.5 (Edinburgh, Scotland).

RESULTS

Isolation of unialgal strain and axenic strain of C. closterium. MSM technique for the isolation of benthic diatom was more effective and less time consuming than serial dilution and agar plate culture isolation technique or direct pipetting. The unialgal strain of *C. closterium* was isolated using the MSM technique from the S–R counting chamber after observation with an inverted microscope. Finally, the unialgal strain cells were grown into the 12-multiwell cell culture plate. During the isolation and subculture, many bacteria were present around broken *C. closterium* cells. The antibiotics penicillin, streptomycin, and neomycin were used individually to eradicate the bacteria from the unialgal strain stock but failed to result in an axenic strain of *C. closterium*, even though the dose was lethal to *C. closterium*. After an antibiotic cocktail was applied to the subculture medium, a bacteria-free strain of *C. closterium* was obtained above dosages of 200 units penicillin · mL^{-1}, 200 μg streptomycin · mL^{-1}, and 400 μg neomycin · mL^{-1} (Fig. 1).

Growth characteristics. The *C. closterium* grew under all culture conditions with different cell densities showing good sigmoial growth curves (Fig. 2). The cell densities were the greatest in the 20°C water temperature condition (Fig. 2B), followed by 15°C (Fig. 2A) and 25°C (Fig. 2C). The maximum cell densities occurred either 10 or 12 d after inoculation in all treatments except the 25°C water temperature condition (Fig. 2). In 15°C water, with various salinities and nutrient concentrations, the μ_{max} varied from 0.63 to 0.81 · d^{-1}, averaging 0.66 · d^{-1} (Fig. 2A), and occurred on day 12 after being inoculated in the all "A" culture conditions except the A9 treatment where the μ_{max} occurred on day 10 (Table 1). The maximum cell density (6.59×10^4 cells · mL^{-1}) occurred at 0.81 · d^{-1} on day 10 after being inoculated with 35 psu salinity and F nutrient concentration (Table 1). The maximum cell density at each condition showed positive correlation with nutrient concentration ($r = 0.94$).

In 20°C water, the μ_{max} was 0.65–0.82 · d^{-1}, averaging 0.75 · d^{-1} among several salinities and nutrient concentrations (Fig. 2B). The μ_{max} occurred later among those media where the nutrient concentration was F/4 (Table 1). The highest cell density (7.20×10^4 cells · mL^{-1}) occurred at the μ_{max} of 0.82 · d^{-1} on day 10 after inoculation in medium with 30 psu salinity and F nutrient concentration (Table 1). The maximum cell density occurrence showed positive correlation with nutrients ($r = 0.70$).

In 25°C water, the μ_{max} was 0.73–0.97 · d^{-1}, averaging 0.80 · d^{-1} among various salinities and nutrient concentrations. The μ_{max} occurred faster in "C" culture conditions than that of "A" and "B" culture conditions (Table 1). The maximum cell density (4.54×10^4 cells · mL^{-1}) occurred on day 8 after being inoculated, while the μ_{max} was 0.97 · d^{-1} in a culture medium with 25 psu salinity and F/2 nutrient concentration (Table 1).

Among the 27 culture conditions, the highest cell density for harvesting was 7.20×10^4 cells · mL^{-1} at the μ_{max} of 0.82 · d^{-1} on day 10 after being inoculated in a culture medium with 20°C, 30 psu salinity, and F nutrient concentration (Table 1). The mass culture of *C. closterium* was performed in Nalgene bottles under the greatest μ_{max} culture conditions ("B_6"). Average cell density in the bottles was 7.29×10^4 cells · mL^{-1}, and average biomass production was 1.6 g · L^{-1} (dry weight basis) after 2 weeks of culture.

Antioxidant activities. The DPPH-free-radical-scavenging rate of *C. closterium* varied from 31.1% to 72.5% among the methanolic, water, and enzymatic (five proteases and five carbohydrases) extracts. The Viscozyme extract of *C. closterium* had the highest activity (72.5%), followed by the methanol (69.4%), Ultraflo (63.8%), Neutrase (60.8%), water (59.3%), and Protamex (58.2%) extracts. The remaining extracts had less scavenging activity (Table 2). All the extracts had DPPH-scavenging activity significantly inferior to the commercial antioxidants (α-tocopherol and BHT), except Viscozyme extract, which showed statistically similar activity with α-tocopherol. The hydrogen-peroxide-scavenging rate was 4.6%–48.4% among the all extracts, with the highest achieved using the methanolic extract, but it was significantly lower than the commercial antioxidants (Table 2). The superoxide-scavenging activity was 29.1% to 53.0% among the extracts, with methanolic extract (53.0%) showing the highest, followed by water (50.0%), AMG (47.8%), and Kojizyme (42.8%). The scavenging activity of the above-mentioned extracts was significantly higher than other extracts, but lower than that of the commercial antioxidants (Table 2). The hydroxyl-scavenging rate was 10.5%–58.3% among all the extracts, with the methanolic extract exhibiting the highest (58.3%), followed by water extract (49.5%). All the extracts showed statistically lower scavenging activity compared to the commercial antioxidants (Table 2). The nitric-oxide-scavenging activity rate was 31.5%–45.8% among all extracts. The AGM (45.8%) and Viscozyme (45.5%) extracts showed higher nitric-oxide-scavenging activity among all the extracts, followed by methanolic (44.8%) and water (44.4%) extracts. The scavenging activities of AGM and Viscozyme were lower than α-tocopherol but similar to BHT. The activities of methanolic and water extracts from *C. closterium* were statistically similar to BHT (Table 2). All the extracts had strong metal-chelating effects that were significantly higher ($P < 0.05$) than that of α-tocopherol and BHT (10.3% and 11.5%, respectively). All the

TABLE 2. Antioxidative effects of different *Cylindrotheca closterium* enzymatic, methanolic, and water extracts.

Extracting solvents	Polyphenol ($mg \cdot 100\ g^{-1}$)	Flavonoid ($mg \cdot 100\ g^{-1}$)	Polysaccharide ($mg \cdot 100\ g^{-1}$)	DPPH-scavenging activity (%)	Hydrogen-peroxide-scavenging activity (%)	Hydroxyl radical activity (%)	Superoxide-anion-scavenging activity (%)	Nitric-oxide-scavenging activity (%)	Metal-chelating activity (%)
Viscozyme	557.7 ± 22	32.1 ± 1.1	7.11 ± 0.1	$72.5^{b} \pm 0.21$	$4.6^{m} \pm 0.11$	$20.7^{h} \pm 0.15$	$42.3^{d} \pm 0.09$	$45.5^{b} \pm 0.02$	$52.1^{d} \pm 0.11$
Celluclast	455.3 ± 24	31.7 ± 1.1	2.28 ± 0.1	$37.7^{g} \pm 0.19$	$14.6^{i} \pm 0.12$	$10.6^{m} \pm 0.09$	$34.5^{f} \pm \pm 0.5$	$42.8^{d} \pm 0.03$	$24.3^{k} \pm 0.11$
AMG	304.7 ± 18	24.7 ± 1.6	6.01 ± 0.1	$52.7^{ef} \pm 0.15$	$24.3^{e} \pm 0.11$	$14.1^{k} \pm 0.21$	$47.8^{c} \pm 0.6$	$45.8^{b} \pm 0.02$	$33.7^{i} \pm 0.10$
Termamyl	391.4 ± 21	29.2 ± 1.7	3.52 ± 0.3	$54.9^{ef} \pm 0.19$	$17.9^{h} \pm 0.11$	$10.5^{n} \pm 0.19$	$25.5^{j} \pm 0.05$	$42.4^{d} \pm 0.05$	$35.6^{h} \pm 0.09$
Ultraflo	623.9 ± 32	51.2 ± 1.5	2.99 ± 0.1	$63.8^{cd} \pm 0.13$	$23.5^{f} \pm 0.10$	$19.1^{i} \pm 0.13$	$31.8^{g} \pm 0.5$	$37.1^{e} \pm 0.05$	$23.50^{l} \pm 0.15$
Protamex	358.6 ± 18	36.6 ± 1.8	2.89 ± 0.1	$58.2^{def} \pm 0.28$	$7.0^{l} \pm 0.18$	$12.5^{l} \pm 0.11$	$29.1^{h} \pm 0.8$	$32.5^{j} \pm 0.03$	$32.4^{j} \pm 0.11$
Alcalase	534.2 ± 29	52.6 ± 2.1	1.88 ± 0.1	$38.2^{g} \pm 0.11$	$4.6^{m} \pm 0.15$	$24.3^{g} \pm 0.18$	$34.5^{f} \pm 0.9$	$31.8^{k} \pm 0.02$	$53.9^{b} \pm 0.12$
Flavourzyme	434.1 ± 21	41.2 ± 1.2	2.33 ± 0.1	$31.1^{g} \pm 0.18$	$12.5^{k} \pm 0.13$	$17.9^{j} \pm 0.15$	$39.3^{e} \pm 0.02$	$34.4^{f} \pm 0.02$	$47.4^{f} \pm 0.11$
Neutrase	642.8 ± 24	60.3 ± 2.6	5.81 ± 0.1	$60.8^{de} \pm 0.14$	$25.00^{d} \pm 0.11$	$30.8^{e} \pm 0.11$	$31.2^{g} \pm 0.13$	$31.5^{k} \pm 0.08$	$50.3^{e} \pm 0.09$
Kojizyme	553.1 ± 29	43.4 ± 2.1	5.77 ± 0.1	$49.9^{f} \pm 0.14$	$13.0^{j} \pm 0.10$	$27.9^{f} \pm 0.10$	$42.8^{d} \pm 0.7$	$32.1^{j} \pm 0.06$	$67.1^{a} \pm 0.10$
Methanol	532.15 ± 27	39.3 ± 2.2	5.58 ± 0.2	$69.4^{bc} \pm 0.19$	$48.1^{c} \pm 0.11$	$58.3^{c} \pm 0.21$	$53.0^{b} \pm 0.10$	$44.8^{c} \pm 0.03$	$53.2^{c} \pm 011$
Water	517.8 ± 21	34.8 ± 1.6	4.79 ± 0.1	$59.3^{de} \pm 0.17$	$20.2^{g} \pm 0.11$	$49.5^{d} \pm 0.12$	$50.0^{b} \pm 0.11$	$44.6^{c} \pm 0.05$	$40.9^{g} \pm 0.11$
α-Tocopherol				$73.6^{b} \pm 0.13$	$60.1^{b} \pm 0.13$	$76.6^{b} \pm 0.11$	$50.5^{b} \pm 0.09$	$56.9^{a} \pm 0.05$	$10.3^{n} \pm 0.12$
BHT				$77.6^{a} \pm 0.14$	$62.5^{a} \pm 0.18$	$79.7^{a} \pm 0.10$	$63.2^{a} \pm 0.11$	$44.4^{c} \pm 0.06$	$11.5^{m} \pm 0.09$

The values represent the mean ± SD of $2\ mg \cdot mL^{-1}$ determined from triplicate samples. Values followed by the superscript letters a, b, c, d, e, f, g, h, and i indicate a significant difference ($P < 0.05$). AMG, Amyloglucosidase; BHT, butylated hydroxytoluene.

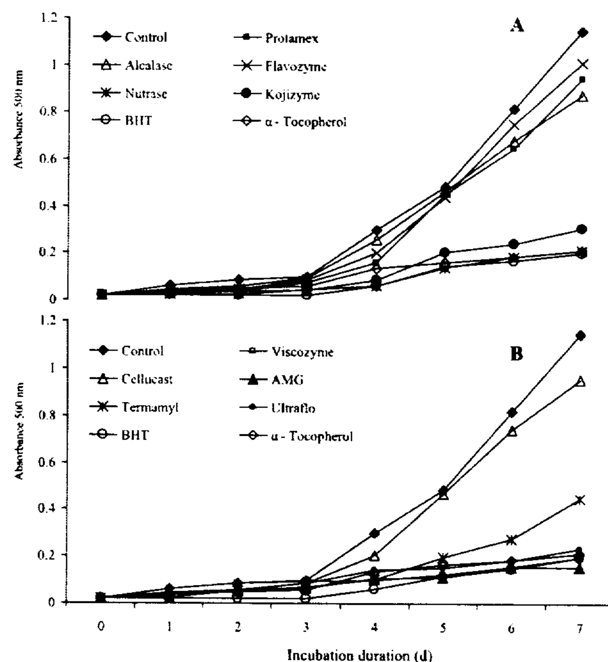

FIG. 3. Antioxidant activities of protease (A) and carbohydrase (B) extracts of *Cylindrotheca closterium*, compared to α-tocopherol and butylated hydroxytoluene (BHT) at $1\ mg \cdot mL^{-1}$ ethanol, as assessed by linoleic acid. AMG, Amyloglucosidase.

extracts showed two to six times higher scavenging activity than the commercial antioxidants. The activity rate was 23.5%–67.1% among all the enzymatic, methanolic, and water extracts with the highest from Kojizyme (67.1%), followed by Alcalase (53.9%), methanolic (53.2%), and Viscozyme (52.1%) (Table 2).

Antioxidant activity with ferric thiocyanate was determined at 40°C for 7 d. As shown in Figure 3, the linoleic acid emulsion without *C. closterium* extract (the control) resulted in an increase in lipid peroxidation, and the absorbance increased significantly ($P < 0.05$). Among all the extracts, the AMG, Viscozyme, and Neutrase extracts exhibited remarkable antioxidant activity, which exceeded that of α-tocopherol. No significant difference was observed among the activity of BHT and the AMG, Viscozyme, and Neutrase extracts ($P < 0.05$). None of the remaining extracts showed notable antioxidant activity compared to the commercial antioxidants.

Total phenolic content. The total phenolic content of the extracts varied with the solvents and enzymes used. The total phenolic content varied from 358 to 642 $mg \cdot 100\ g^{-1}$ among the various enzymatic, methanolic, and water extracts of *C. closterium*. The Neutrase extract exhibited the highest polyphenol content (642.8 $mg \cdot 100\ g^{-1}$), followed by Ultraflo (623.9 $mg \cdot 100\ g^{-1}$) and Viscozyme (557.7 $mg \cdot 100\ g^{-1}$; Table 2).

Total flavonoid and polysaccharide content. Different flavonoids access their target sites in different ways, and for that the total flavonoid was collected with

the extraction of different kinds of enzymes, methanol, and water from *C. closterium*. The total flavonoid content was 24.7 to 60.3 mg · 100 g^{-1}, and the highest flavonoid content was from the extract with Neutrase (60.3 mg · 100 g^{-1}), followed by Alcalase (52.6 mg · 100 g^{-1}) and Ultraflo (51 mg · 100 g^{-1}) (Table 2). The polysaccharide content was highest (7.11 mg · 100 g^{-1}) in the Viscozyme *C. closterium* extract, followed by AMG and methanol (Table 2).

DISCUSSION

Isolation for unialgal strain. Compared with serial dilution and agar plate culture isolation technique or direct pipetting, the MSM technique was the most efficient method of isolation of the diatom *C. closterium*. A single cell of the target species could be picked up using the MSM technique from the S–R counting chamber when a highly diluted natural sample was placed with the density of 3–5 cells · mL^{-1}. In comparison with the MSM technique, the serial dilution and agar plating take ~1 to 2 weeks to isolate the target benthic diatoms, as they need time to grow and to make colonies. Moreover, another species of benthic diatom or unwanted phytoplankton can also be attached to the target benthic diatom during agar plating since benthic diatoms produce EPS (extracellular polymeric substances, e.g., glycoconjugates) to which benthic diatoms can attach. Thus, MSM is the best technique for isolation of benthic diatoms.

Axenic species. Bacteria decompose the microalgae and can also release or contain the bioactive compounds that can influence the antioxidant properties of microalgal extract. Thus, axenic culture of target microalgae is very important for long-term storage and for the determination of the exact chemical produced from the respective strain of microalgae. To make the axenic strain of *C. closterium*, we used antibiotic-enriched F/2 medium. The successful antibiotic dosages were lower than those (gentamycin, 0.05 mg · L^{-1}; penicillin-G, 16 mg · mL^{-1}; and streptomycin, 0.8 mg · mL^{-1}) used by Kotaki et al. (2000) to obtain the axenic culture of a domoic-acid-producing *Nitzschia* species. Cho et al. (2004) used a mixture of five antibiotics (ampicillin, 250 μg · mL^{-1}; gentamycin, 50 μg · mL^{-1}; kanamycin, 100 μg · mL^{-1}; neomycin, 500 μg · mL^{-1}; and streptomycin, 50 μg · mL^{-1}) to make a bacterial-free strain of *Isochrysis galbana*. However, we made the *C. closterium* axenic strain by using a mixture of three antibiotics, namely, penicillin, streptomycin, and neomycin. The optimal antibiotic dosages may be dependent on the bacterial load or the presence of specific bacteria, such as gram-negative or gram-positive species. Neomycin is an amylglycoside antibiotic, which is principally active against gram-positive bacteria, whereas streptomycin is mostly active against gram-negative bacteria, and the penicillin family is active against both (Guillard 2005). Thus, an antibiotic (penicillin, streptomycin, and neomycin) cocktail is more effective in suppressing the survival and growth or eradication of bacteria under benthic diatom culture conditions.

Growth characteristics. Estimation of growth rate is important data about microbial population activity, which can increase at exponential rates. Determination of growth characteristics under controlled and measurable conditions can be used to create a high cell density in mass-culture system of microalgae. Limitations of *C. closterium* growth were determined with different temperature, salinity, and nutrient concentrations. F/2 medium was used as a nutrient source due to its commercial availability, ease of use, and suitability for our goal of producing this alga commercially for industrial use. The cell density of *C. closterium* was higher at the low (15°C) and medium (20°C) water temperatures with high salinity than at the high (25°C) temperature, with cell density being the greatest at 20°C. The growth pattern of this species suggests that the moderate temperature may create the most favorable conditions for blooming.

In terms of salinity, *C. closterium* appears to be euryhaline, because it grew well, with different maximum cell densities, in a remarkable range of salinities (25–35 psu). A combination of moderate temperature with high salinity and nutrient concentrations may create favorable circumstances for blooms of this species, indicating that *C. closterium* is a eutrophic benthic diatom. Affan and Lee (2004) monitored the seasonal dynamics of phytoplankton and environmental factors in the coastal water of Jeju Island, Republic of Korea, and determined that *C. closterium* comprised >10% of the total phytoplankton in the late winter when the water temperature and salinity were 16°C and 34 psu, with highly available nutrient concentrations, especially when the total concentration of NH_4-N, NO_2-N, and PO_4-P was 6 μg-at · L^{-1}. Thus, we suggest that the maximum production of *C. closterium* commercially can be obtained at medium temperature (20°C) with medium salinity and higher nutrient concentrations.

Evaluation of antioxidant effects of C. closterium. Interest in antioxidants from natural sources, like plants, has been increasing because of their ability to reduce ROS by the production of polyphenolics and nitrogen-containing compounds, such as phytosterols and carotenoids. The use of natural antioxidants is safe and readily acceptable for consumers as those antioxidants are not chemically contaminated, and no more safety tests are required by legislation if the food components are "Generally Recognized As Safe" (Pokorny 1991). Seaweeds are a good source of natural antioxidants (Fujimoto and Keneda 1984, Cahyana et al. 1992, Ruperez et al. 2002, Athukorala et al. 2003), due to the variety of potential medicinal bioactive compounds

that they contain (Moore 1978, Konig et al. 1994). In contrast to terrestrial plants and seaweeds, microalgae have many advantages as an important source of antioxidants and other bioactive compounds due to their faster growth in much smaller areas under controlled culture conditions.

Aerobic organisms rely on antioxidants to resist environmental stresses. Numerous antioxidant defense systems protect them from damage by ROS and reactive nitrogen species (RNS). Among the microalgae, *Spirulina* sp., *Dunaliella* sp., *Botryococcus* sp., and *Haematococcus* sp. are being using as the important sources of antioxidants for human consumption (Herrero et al. 2005, 2006, Rao et al. 2006). Research into the antioxidant properties of benthic diatoms is still in its infancy. In our study, the antioxidant properties of *C. closterium* were determined using aqueous, methanolic, and enzymatic extractions.

The DPPH-free-radical-scavenging assay is widely used to evaluate the antioxidant efficacy of plant extracts. Free radicals are continuously produced in cells, either as metabolic by-products or as protectors against phagocytic pathogens (Cheeseman and Slater 1993). The methanolic extract of *C. closterium* and some enzymatic extracts, especially Viscozyme and Neutrase, exhibited higher free-radical-scavenging activities. The DPPH-free-radical-scavenging activity of the Viscozyme extract of *C. closterium* was statistically similar to α-tocopherol. Viscozyme could be recommended as a good solvent to extract the DPPH-free-radical-scavenging bioactive compound from *C. closterium*. DPPH-free-radical-scavenging activity may increase with phenolic content (Oki et al. 2002), but here free-radical scavenging activity increased with increasing polysaccharide content. DPPH-radical-scavenging activity increased due to the increase in polysaccharide content, which was extracted by the Viscozyme digest, and the polysaccharide content was significantly higher ($P < 0.05$) than those of other digests. Therefore, the digest might contain low-molecular-weight polysaccharides, which may be responsible for the results obtained in this study. The DPPH-free-radical-scavenging activity and polysaccharide content showed a positive correlation ($r = 0.68$). Spitz et al. (2005) reported good antioxidant activity from the polysaccharide of the red microalga *Porphyridium* sp. Thus, it can be suggested that the polysaccharide of the brown microalga *C. closterium* could be a good source for DPPH-free-radical-scavenging bioactive compounds.

H_2O_2 is an oxidant belonging to ROS with a weak reactive molecule due to the absence of radicals, but it may convert into more reactive species, such as singlet oxygen and hydroxyl radicals (Halliweill 1991), which can penetrate biological membranes. Therefore, the estimation of H_2O_2-scavenging effect can be a useful method to determine the antioxidant properties of any chemical or determining the ability of antioxidants to decrease the level of prooxidants, such as H_2O_2 (Pazdzioch-Czochra and Widenska 2002). Methanol extract of *C. closterium* had a good H_2O_2-scavenging ability, although it was statistically inferior to the commercial antioxidants.

Hydroxyl radical is the most reactive free radical and can be formed from a superoxide anion and hydrogen peroxide in the presence of copper or iron ions. In general, aromatic compounds or compounds with carbon–carbon multiple bonds undergo addition reactions with hydroxyl radicals and produce free radicals. In saturated compounds, the hydroxyl radical removes a hydrogen atom from the weakest C–H bond to yield a free radical (Korycka-Dahl and Richardson 1978). The resulting radicals can also react with oxygen and generate other free radicals. Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine (Ashok and Ali 1999). It can extract hydrogen atoms from phospholipid membranes and perform peroxidic reactions with lipids (Kitada et al. 1979). Among all the extracts of *C. closterium*, the methanolic extract showed the highest activity, but that activity was inferior to standard antioxidants.

Superoxide anion, which is a reduced form of molecular oxygen, is an initial free radical formed from mitochondrial electron transport systems. The effects of the superoxide anion can be exaggerated, as it produces other kinds of cell-damaging free radicals and oxidizing agents, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Liu and Ng 2000, Stief 2003). The superoxide anion can react with nitric oxide and form peroxynitrite, which can generate toxic compounds, such as hydroxyl radical and nitric dioxide (Halliweill 1991). The superoxide-anion-scavenging potential of *C. closterium* from methanolic and water extracts was greater than that of the enzymatic extract, and the activity was statistically similar to α-tocopherol, but inferior to BHT. Not only are superoxide radicals formed in the body but also the early products of protein glycation, such as the Schiff base and Amadori compound, which may be the key structural components involved in the generation of superoxide radicals (Ukeda et al. 2002). Therefore, *C. closterium* can be an important source for superoxide-anion-scavenging in the medicinal and food fields.

Nitric oxide (NO·) is a free radical with a single unpaired electron. Nitric oxide is formed from L-arginine by NO synthase (Fang et al. 2002). It can also be formed from the reaction of peroxyl radical and NO·, polluted air, and smoking (Noguchi and Niki 1999). Nitric oxide itself is not a very reactive free radical, but the overproduction of NO· is involved in ischemia reperfusion and neurodegenerative and chronic inflammatory diseases, such as rheumatoid arthritis. Nitric dioxide adds to double bonds and extracts labile hydrogen atoms initiating lipid peroxidation and production of free radicals.

Marcocci et al. (1994) reported that nitric oxide scavengers compete with oxygen, resulting in a lower production of nitric oxide. The carbohydrase (especially Viscozyme and AMG), methanol, and water extracts of *C. closterium* exhibit high nitric-oxide-scavenging activity and were significantly higher than BHT and statistically similar to α-tocopherol. The metabolite ONOO-(peroxynitrite) is extremely reactive, directly inducing toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation, and DNA modifications (Moncada et al. 1991, Radi et al. 1991). Thus, *C. closterium* is a good source of bioactive compounds that can scavenge nitric oxides.

Transition metals such as iron and copper play important roles in initiation and propagation steps of lipid oxidation. The initiation step of oxygen oxidation requires removal of a hydrogen atom. The presence of metal can accelerate the initiation step of lipid oxidation by the mechanism of $RH + M^{n+} \rightarrow R\cdot + H^{+} + M^{(n-1)+}$. Ferrozine complexes with ferrous ions and can initiate lipid peroxidation by the Fenton reaction and accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliweill 1991, Fridovich 1995). Metal chelator can reduce the concentration of catalyzing transition metals in lipid peroxidation (Duh et al. 1999). Furthermore, chelating agents that form σ-bonds with a metal are effective as secondary antioxidants because they reduce the redox potential and thereby stabilize the oxidized form of the metal ion (Gordon 1990). Metal chelators can convert metal ions into insoluble metal complexes or generate steric hindrance, which can prevent the interactions between metals and lipid intermediates. The Kojizyme enzymatic *C. closterium* extracts had a 6-fold, and other extracts had 2- to 5-fold, higher metal-chelating activity than commercial antioxidants. Thus, this species has a high ability to bind iron and copper.

C. closterium extracts were also compared with commercial antioxidants to evaluate the antioxidant effects, using the ferric thiocyanate (FTC) method to determine the amount of peroxide-formed emulsion during the incubation period. AMG, Viscozyme, Neutrase, and Ultraflo extracts of *C. closterium* showed higher antioxidant activities than those of BHT and α-tocopherol, which are commercial antioxidants. Those enzymatic digests reduced the formation of peroxide. Therefore, these results suggest that digestion by different enzymes releases different components from microalgal cells, which show potential antioxidant abilities. Digestion by different enzymes may cause the breakdown of higher-molecular-weight polysaccharides or proteins into smaller compounds. Additionally, polyphenol compounds can be released from inside the microalgal cells. These low-molecular-weight polysaccharides and peptides, and polyphenols may act as antioxidant compounds.

The Ultrafo and Neutrase *C. closterium* extracts showed high content of phenolic compounds. Although many studies have reported a highly positive correlation between total phenol content and antioxidant activity in various plant species (Vinson et al. 1998, Oktay et al. 2003), we did not find this correlation. The cells of *C. closterium* contain a large amount of soluble polysaccharides and insoluble fibers, which together with other cell wall materials, act as a physical barrier to the extraction of bioactive materials. Bioactive gel compounds in gel matrices also interfere with the extraction of bioactive compounds and lead to poor solubility in aqueous media. Surmounting such barriers with enzymatic hydrolysis of tissues or cells used as raw materials could allow desirable compounds to be extracted in high yields (Jeon et al. 2000, Nagai and Suzuki 2000). Viscozyme, AMG, and Neutrase extracts of *C. closterium* had higher polysaccharide content than other digests. The breakdown and release of high-molecular-weight polysaccharides may contribute to enhancing antioxidative activity (Ramos and Xiong 2002, Ruperez et al. 2002). Spitz et al. (2005) found good free-radical-scavenging effects from a sulfated polysaccharide of *Porphyridium* sp. However, the extracted polysaccharide in the current study had positive correlation on DPPH-scavenging ($r = 0.68$) and superoxide-scavenging ($r = 0.60$) effects. Moreover, enzymatic extraction possesses innovative advantages over conventional extraction procedures.

Diatom culture for biotechnological applications is still at an early stage of development, in contrast to the production for shellfish aquaculture. The applications of diatoms will depend on needs, uses, and potential bioactive compounds, along with advances in cultivation, including the lower cost of media and culture techniques. We used methanol, water, and different enzymes for extracting bioactive compounds from *C. closterium* extractions and then compared their antioxidant activities with that of commercial antioxidants. Although the methanolic and water extracts showed more activity in some assays (e.g., hydroxyl and superoxide), enzymatic hydrolysis significantly increased antioxidant activity in numerous assays. *C. closterium* showed more antioxidant activity in DPPH, superoxide, nitric oxide, and the metal-chelating assays than commercial antioxidants.

In our previous study of benthic diatoms (Affan et al. 2006, 2007a,b), *Grammatophora marina* showed very good antioxidant activities, especially for metal-chelating and DPPH-free-radical-scavenging activity (Affan et al. 2006). Neutrase and methanolic extract of *Navicula incerta* exhibited higher scavenging activity of DPPH and metal chelating than commercial antioxidants, such as α-tocopherol and BHT (Affan et al. 2007a). *Amphora coffeaeformis* showed higher scavenging activity for DPPH, hydroxyl, and metal chelating than BHT (Affan et al. 2007b). *Achnanthes longipes* hexane extract had approximately two times

higher nitric-oxide and metal-chelating scavenging activity than α-tocopherol and BHT (Affan et al. 2007b). These results indicate that benthic diatoms may be a good candidate for a natural antioxidant source and could be applied in the functional food field. Further studies are necessary to isolate and purify the biochemical compounds responsible for antioxidant effects and to characterize their in vivo antioxidant activity and related antioxidant mechanisms.

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