

VIABILITY, FATTY ACID COMPOSITION, AND STRUCTURE OF THE CORALLINE ALGA *CORALLINA PILULIFERA*

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Abstract: The decrease in the seaweed flora in some rocky areas, known as algal whitening or barren ground, is associated with some species of coralline algae. To determine the biological characteristics of a representative species of branched coralline alga, the number of medullary tiers was counted and ranged from 12 to 16. The 18S rDNA, *psbA*, and *rbcL* genes were used to confirm the identification of *Corallina pilulifera*. Measuring viability using triphenyl tetrazolium chloride showed highly viability from December to January. Cultural conditions of 16 °C, 16 h light:8 h dark cycle, and 40 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity were optimal for maintaining the viability of the coralline alga for up to three days. The fatty acids included 31.4% ω -3 eicosapentaenoic acid. Scanning electron microscopy of the surface structure revealed unique round wells about $7.9 \pm 1.3 \mu\text{m}$ in diameter. The coralline alga, preventing fleshy seaweeds, may be used as a potential template for the creation of new environmentally friendly biomimetic antifouling material against the attachment of soft foulants, especially micro- and macroalgae.

Key words: *Corallina pilulifera*, fatty acid, tissue structure, viability.

Resumen: La disminución de la flora de algas en algunas zonas rocosas, conocidas como el blanqueamiento de algas o de tierra árida, se asocia con algunas especies de algas coralinas. Para determinar las características biológicas de una especie representativa de alga coralina ramificada se contó el número de hileras medulares que varió de 12 a 16. Los genes 18S rDNA, *psbA* y *rbcL* se utilizaron para confirmar la identificación de *Corallina pilulifera*. La viabilidad se midió utilizando cloruro de trifeniltetrazolio y ésta fue alta de diciembre a enero. Las condiciones de cultivo: 16 °C, 16 h luz:8 h oscuridad ciclo y una intensidad lumínica de 40 $\mu\text{E m}^{-2} \text{s}^{-1}$ fueron óptimas para mantener la viabilidad de la alga coralina por un máximo de tres días. Los ácidos grasos incluyen 31.4% de ω -3 ácido eicosapentaenoico. La microscopía electrónica de barrido permitió observar la superficie tiene pozos redondos únicos de aproximadamente $7.9 \pm 1.3 \mu\text{m}$ de diámetro. El alga coralina, la prevención de algas carnosas, se puede utilizar como una plantilla para la creación de nuevo material anti-incrustante biomimético contra la unión de suciedades blandas, especialmente micro y macroalgas.

Palabras clave: ácidos grasos, *Corallina pilulifera*, estructura tisular, viabilidad.

Seaweed is an important source of food and shelter; its biomass takes up carbon in marine environments, and it is a source of chemicals for commercial applications (Thomas, 2002). Coralline red algae abound in nearshore areas and strongly influence the benthic community. When coralline algae are growing, the rock surfaces appear pink, while the fleshy seaweed flora disappears from the rocky areas. In marine environments, this phenomenon is generally called

algal whitening, barren ground (Tokuda *et al.*, 1994), coralline flats, or deforested areas. Now it is recognized as a natural hazard adversely affecting marine ecosystems and damaging commercial fishing areas. Although, biological (Kitamura *et al.*, 1993; Agatsuma *et al.*, 1997) and physical (Masaki *et al.*, 1984; Johnson and Mann, 1986) factors may be sufficient to prevent the recruitment of fleshy seaweeds, allelopathic bromoform (Ohsawa *et al.*, 2001) and fatty acid

(Kim *et al.*, 2004; Luyen *et al.*, 2009) substances may also inhibit the settlement or germination of seaweed spores. Consequently, coralline algae may also prevent fouling by fleshy seaweeds. Biomimetics or biomimicry is known as a multidisciplinary subject for the design of materials and machines using the structure and function of biological systems as models. To invent antifouling materials in an environmentally friendly use, biologically inspired design of biomimetics of coralline structure and process would offer a creation of new material. For the production of biomimetic coralline, it is required to select the healthy coralline tissue as a template. In nature, most coralline algae are pink and have almost indistinguishable shapes. Seasonal changes in articulated coralline algae can also cause confusion, even in their identification (Baba *et al.*, 1988; Akioka *et al.*, 1999). Some polyunsaturated fatty acids (PUFAs) from crustose coralline have shown potent lytic activity against fleshy seaweed spores (Luyen *et al.*, 2009). Therefore, in an attempt to make a biomimetic coralline alga material, we first had to identify the species that is used as a potential template, select the best conditioned tissues as standard material, analyze the fatty acid composition, and observe the fine surface structure.

Materials and methods

Plant material. Coralline algae were collected monthly from the rocky intertidal area at Cheongsapo (35° 09' 28" N, 129° 11' 47" E), on the east coast of Busan, Korea, during 2011 and 2012. The samples were transported in a container with seawater to the laboratory. After rinsing well with autoclaved seawater to remove epiphytes and debris, the tissues were sonicated three times with 30 s pulses of an ultrasonic water bath (low-intensity frequency of 90 kHz) to remove other microepiphytes.

Medullary tier. The coralline algae were fixed in SUSA solution (4.5 g mercuric chloride, 0.5 g sodium chloride, 20 ml 40% formalin, 4 ml trichloroacetic acid, and 80 ml distilled water) for 12 h and then transferred to formalin solution for 24 h. The fronds were fixed for another 24 h in 10% neutral buffered formalin solution (100 ml 40% formaldehyde, 800 ml distilled water, 4.0 g monosodium phosphate, and 6.5 g anhydrous disodium phosphate). The tissues were dehydrated through an alcohol series from 70 to 100% and cleaned with three changes of xylene. Specimens embedded in paraffin wax were cut at 5 μ m thickness with a rotary type microtome (Reichert-Jung 820; Leica, Wetzlar, Germany) and stained with hematoxylin and eosin (Clark, 1981).

Molecular identification. Approximately 0.2 g of the coralline alga was chopped into very tiny pieces and placed in a microtube. DNA was extracted using LiCl, following Hong *et al.* (1995). The 18S rDNA gene, encoding the ri-

bosomal RNA in the small subunit of eukaryotic cytoplasmic ribosome was amplified by polymerase chain reaction (PCR) with the universal primers 18sF (5'-CAACCTGGTTGATCCTGCCAGT-3') and 18sR (5'-GATCCTTCTGCAGGTTACCTACGGAA-3'; Bird *et al.*, 1992). The *psbA* gene encoding the D1 protein of the photosystem II reaction center complex in the photosynthetic organ was amplified by PCR with the primers *psbAF* (5'-ATTGCATT-CGTTGCTGCTCCTC-3') and *psbAR* (5'-GTGAACCA-GATTCCTACTACAGGC-3'; Kim *et al.*, 2006). The *rbcL* gene, known as RuBisCO or ribulose-1,5-bisphosphate carboxylase/oxygenase in photosynthesizing chloroplasts, was amplified by PCR with the primers *rbcLF* (5'-GCAGGT-GAATCATCTACAGCAAC-3') and *rbcLR* (5'-GCTTGA-ATACCGTCTGGATGACC-3'; Freshwater *et al.*, 1994). The PCR cycling parameters consisted of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1.5 min, and one final 72 °C for 10 min. The amplification products were sequenced using the same primers (SolGent, Daejeon, Korea). The sequences were edited and manipulated using MEGA3 (Kumar *et al.*, 2004). Phylogenetic trees were inferred using the neighbor-joining algorithm (Saitou and Nei, 1987) in MEGA3 with bootstrap analysis of 1,000 bootstrap replications.

Viability assay. To measure the viability of the coralline tissue, the assay of Park *et al.* (2006) was used. Briefly, 1 ml of 0.8% 2,3,5-triphenyltetrazolium chloride (TTC) in seawater containing 50 mM Tris-HCl buffer (pH 8.0) was added to 0.05 g of tissue in a 1.5-ml microtube and incubated in darkness for 1.5 h at 20 °C under mineral oil. The triphenyl formazan that formed in the tissue was extracted with 0.6 ml of 0.2 N potassium hydroxide in 75% ethanol by heating for 15 min at 60 °C. The triphenyl formazan was quantified by measuring the absorbance at 475 nm. To determine the optimal conditions for maintaining viability, 0.25 g of the tissue was cultured in 50 ml of seawater under 40 μ E m⁻² s⁻¹ light intensity on a 16 h light:8 h dark cycle at 16 °C for 3 days as the standard conditions.

Scanning electron microscopy. Healthy tissue collected on January 11, 2012 was washed with Milli-Q water (Millipore, Billerica, MA) and dried under vacuum before scanning electron microscopy (SEM) analysis. For SEM images, tissues were mounted on conductive carbon tabs of a SEM post (Ted Pella, Inc., Redding, CA), sputter-coated using a Desk-II coater equipped with a gold target (Alfa Aesar, Ward Hill, MA), and imaged in a scanning-electron microscope (JSM-6700F; JEOL, Tokyo, Japan). To determine the elemental composition of parts of the tissues, the tissues were analyzed using energy-dispersive X-ray spectroscopy. The standards for carbon, oxygen, and calcium were calcium carbonate, silicon dioxide, and wollastonite, respectively.

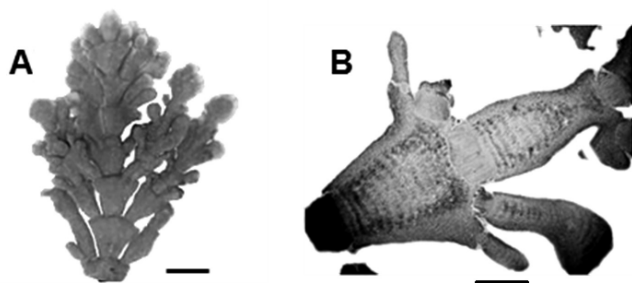


Figure 1. Tissue of the articulated coralline alga (A) and medullary tiers in longitudinal sections through the axial intergeniculum (B). The bars in A and B indicate 2 and 0.8 mm, respectively.

Fatty acid analysis. Fatty acids were determined by gas chromatographic quantification of their methyl esters (FAMES), which were prepared using slightly modified method from the AOAC (2000). Total lipid was extracted from the dried samples using a Soxhlet extractor. Then, FAMES were prepared with 5 ml of methylation solution (1 sulfuric acid:20 methanol:10 toluene) and heated at 100 °C for 1 h. Gas chromatography-mass spectroscopy (GC-MS) analysis was conducted using a 6890 network GC system with a 5973N Mass Selective Detector (MSD) (Agilent Technologies, Palo Alto, CA). The oven was started at 50 °C and held for 1 min, and then ramped up to 320 °C at 5 °C min⁻¹. The MSD was operated based on electron ionization.

Results

Eight samples of dominant coralline algae from different barren grounds and different seasons were collected. They were pinkish to reddish with a whitish apex and were gene-

rally characterized by their calcareous composition (Figure 1A). They typically colonized rocky substrates and formed turfs in the intertidal area exposed to waves. The number of medullary tiers per intergeniculum and other characters were observed after staining with hematoxylin and eosin (Figure 1B). The number of tiers of medullary cells per intergeniculum was between 12 and 16. The intergenicular length was approximately 1.0 - 1.4 mm. The alga had pinnate fusing branches that were somewhat fan-shaped, with broad intergenicula, and truncated at the ends. The main base of the branch was almost as wide as it was long and the branches were somewhat subspherical. The frond height ranged from 3 - 7 cm. To ascertain the species identification, we determined partial 18S rDNA, *psbA*, and *rbcL* gene sequences. They were then aligned and analyzed using the neighbor-joining method to construct a dendrogram. The 1,156-bp 18S rDNA sequence from base 412 to 1,567 was compared to the sequences of 20 species of coralline algae obtained from the NCBI database to infer the phylogenetic relationships (Figure 2A). The 18S rDNA sequence had 99% homology with the *Corallina* sp. sequence (GenBank accession # FM180100.1). Similarly, the 881-bp *psbA* plastid gene sequence from base 31 to 911 was compared with the sequences of 14 species of coralline algae obtained from the NCBI database (Figure 2B). The *psbA* gene sequence shared 100% homology with the *Corallina pilulifera* sequence (GenBank accession # DQ787634.1). Likewise, the 943-bp *rbcL* plastid gene sequence from base 226 to 1,168 (Figure 2C) shared 100% homology with the *C. pilulifera* sequence (GenBank accession # DQ787558.1). Therefore, the species was identified as the articulated coralline alga *C. pilulifera* using 18S rDNA, *psbA*, and *rbcL* gene identification. Using the TTC method, we measured the viability

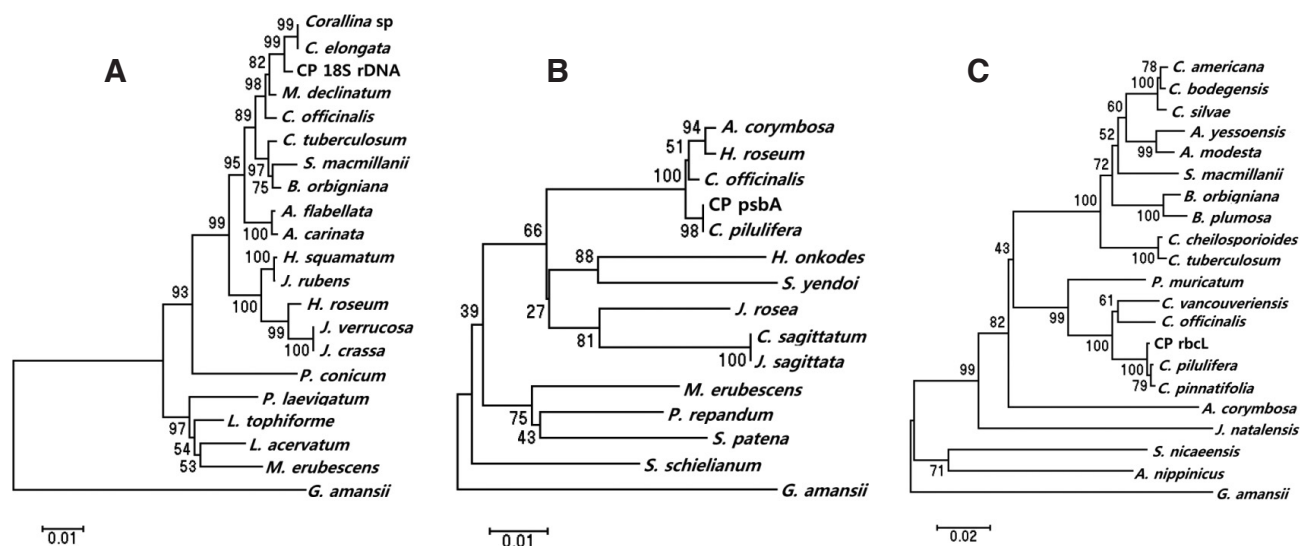


Figure 2. Phylogenetic dendrograms of the coralline alga (CP) based on 18S rDNA (A), *psbA* (B), and *rbcL* (C) sequences, and constructed using the neighbor-joining method. Numbers at nodes indicate the level of bootstrap support (1,000 replicates).

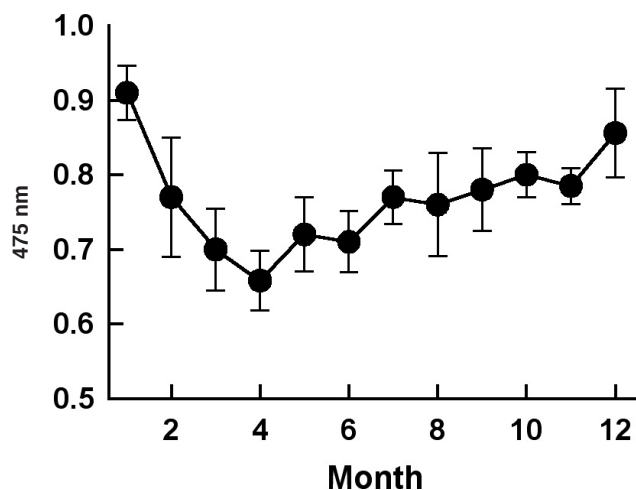


Figure 3. Seasonal variation in the viability of *Corallina pilulifera* tissue. The viability was quantified using the absorbance at 474 nm, and the values are the mean \pm s.d. of at least five independent assays.

of *C. pilulifera* tissues collected at the same site throughout the year (Figure 3) and quantified viability as the absorbance at 545 nm. The tissues collected in January had the greatest via-

bility, which then decreased gradually in the spring. Some tissues remained short or as unbranched fronds, and some disappeared from the rock surface. In late spring and summer, new buds appeared on epilithic basal crusts. In autumn, they started to grow, and most tissues recovered their viability. In December and January, the tissues again had the healthiest pink structure and greatest viability. Therefore, this period would be the best season to use as a model structure of a biomimetic antifouling material. To keep healthy tissues, we optimized the maintenance conditions using the TTC viability assay (Figure 4). The optimal temperature for incubation was 16 °C, the optimal light intensity was 40 $\mu\text{E m}^{-2} \text{s}^{-1}$ with white fluorescent light, and the optimal light period was a 16 h light:8 h dark cycle. Under these conditions, the tissues maintained the best viability for up to three days in a standing flask containing natural seawater. Therefore, the tissues were kept at the optimized conditions and used within three days. We determined the major fatty acid composition of healthy *C. pilulifera* tissue (Table 1). Of the fatty acids, 45.4% were PUFAs, with ω -3 eicosapentaenoic acid (EPA; C20:5) comprising 31.4%; the ω -6: ω -3 ratio was 0.45. The surface structure of the coralline tissue was examined using SEM. The tissue surface was covered with

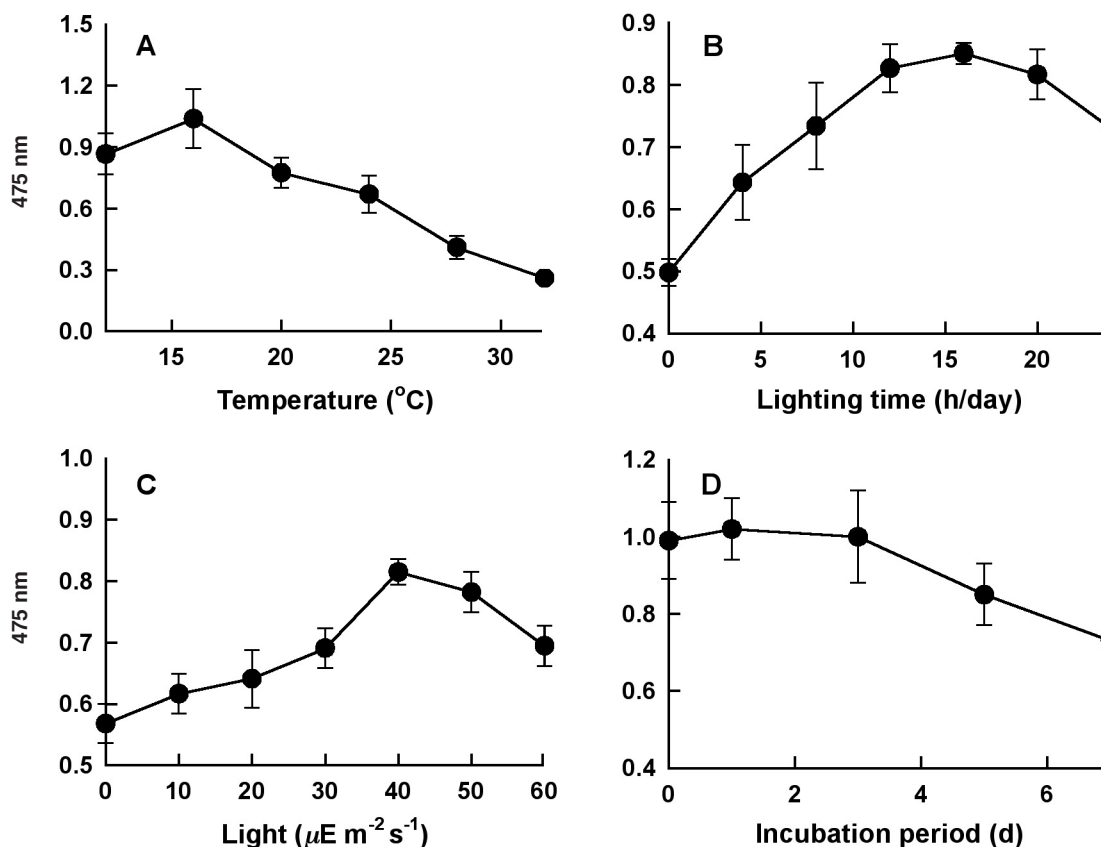


Figure 4. Effect of various parameters on the optimal maintenance of *Corallina pilulifera* tissue: A. incubation temperature, B. amount of light per day, C. light intensity, and D. incubation period under the optimized conditions. The viability was measured using the absorbance at 474 nm, and the values are expressed as the mean \pm s.d. of at least five independent assays.

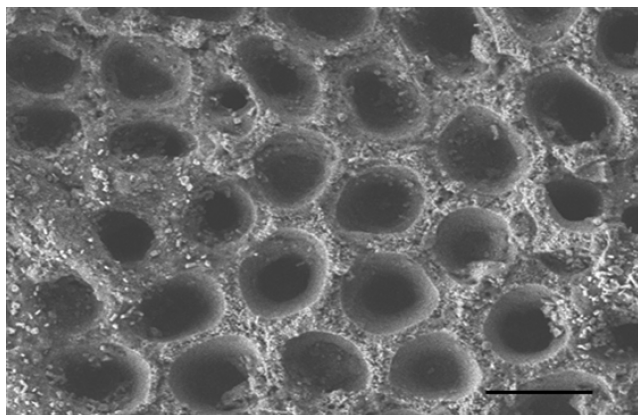
Table 1. Profile of the major fatty acids (% of total fatty acids) in *Corallina pilulifera* tissue collected on December 22, 2012.

Fatty acids	Relative amount (%)
C14:0	1.9
C16:0	28.8
C18:0	6.3
C18:1 ω -9	1.7
C18:2 ω -6	3.8
C18:3 ω -6	1.5
C20:4 ω -6	5.3
C20:5 ω -3	31.4
C22:0	4.5
C22:2 ω -6	2.3
C24:0	1.5
Saturated fatty acids	50.7
PUFAs	45.4

unique round dimples about $7.9 \pm 1.3 \mu\text{m}$ in diameter at the surface (Figure 5). Most of these dimple-shaped structures were irregular circles, forming cones vertically. SEM-based energy-dispersive X-ray spectroscopy showed that the relative elemental composition was 33% carbon, 56% oxygen, and 11% calcium by atomic percentage, or 23% carbon, 53% oxygen, and 24% calcium by weight.

Discussion

The species *Corallina pilulifera* was identified using anatomical characters, such as the tier number per intergeniculum (12-16) and intergenicular length (0.9-1.2 mm; Baba *et al.*, 1988; Akioka *et al.*, 1999). All eight samples of the coralline algae collected had the same range of tier numbers, which is a key character of *C. pilulifera*. Slight differences in the

**Figure 5.** Scanning electron micrograph of *Corallina pilulifera* tissue. Bar = 10 μm .

intergenicular length, width, frond height, branch, conceptacle, and intergenicular shapes were seen. Guiry and Guiry (2012) included four varieties of *C. pilulifera* and 29 entries of *C. officinalis* in AlgaeBase that were classified as uncertain. Morphological studies alone no longer suffice to identify species, as demonstrated by the apparent duplication of descriptions due to geographical, seasonal, and environmental differences. Differences in microhabitat conditions, such as desiccation, epiphyte loading, and the abundance of herbivores, can also alter the morphology (Akioka *et al.*, 1999). Therefore, we used molecular information to complement the physical variation. Molecular analysis based on 18S rDNA has been used to elucidate the division of Corallinoideae (Walker *et al.*, 2009), the classification of the order Corallinales with 35 species of coralline algae (Bailey and Chapman, 1998), and the relationships within the tribe Janieae (Kim *et al.*, 2007). The evolutionary history of the Corallinales has also been inferred from nuclear, plastidial, and mitochondrial genomes (Bittner *et al.*, 2011). In this work, the species was identified as the *C. pilulifera* using tier numbers, even without reproductive structures and characteristics, but with complementary support of 18S rDNA, *psbA*, and *rbcL* gene sequences.

Among the factors preventing the settlement or germination of fleshy seaweed spores, we found that PUFAs have potent lytic activity against algal spores (Luyen *et al.*, 2009). When preparing biomimetic coralline alga material, the antifouling activity could be enhanced by adding bioactive PUFAs. The fatty acids from healthy *Corallina pilulifera* tissue, 31.4% were ω -3 eicosapentaenoic acid (EPA; C20:5) and 14% were the other PUFAs. In a previous study, EPA showed strong lytic activity against algal spore with $\text{LC}_{50} = 2.1 \mu\text{g ml}^{-1}$ (Luyen *et al.*, 2009). Therefore, the preparation of biomimetic calcium carbonate nanoparticles with EPA is suggested to have potent antifouling activity as an environmentally friendly biocontrol. The mineral content of *C. pilulifera* was 28.5% carbonate, 18.4% calcium, 4.37% magnesium, 1.75% chloride, 0.31% iron, and 0.13% sodium (Yan, 1999). These high levels of essential minerals, especially calcium, magnesium, and iron, coupled with the low chloride, sodium, and potassium contents, make this species a potential mineral source for livestock, and further support this mineral composition as an environmentally friendly material.

Corallina pilulifera is an articulated coralline alga with a hard, abrasive calcareous skeleton. It dominates the intertidal zone on rocky substrate where strong waves occur. It produces a bromoperoxidase that acts on bromide and iodide, and the enzyme is potentially useful as a catalyst for biotransformation (Itoh *et al.*, 1985). The allelopathic bromomethane released by the alga can eliminate epiphytic organisms, and might induce the continuation of coralline flats in marine environments (Ohsawa *et al.*, 2001). Similarly, halogenated furanone compounds prevent biofilm forma-

tion of bacteria on the algal body (Gram *et al.*, 1996). Thus, addition of bromoperoxidase or halogenated compounds is recommended in the biomimetic material. The growth of the red tide microalga *Prorocentrum donghaiense* was inhibited by aqueous and methanolic extracts of *C. pilulifera* (Wang *et al.*, 2007). The methanolic extract of *C. pilulifera* also had an antioxidant protective effect on UVA-induced oxidative stress in human fibroblasts (Ryu *et al.*, 2009). Its ethanol extract inhibited the growth of the HeLa cancer cell line and induced apoptosis in a dose-dependent manner (Kwon *et al.*, 2007). The tissue surface of the coralline alga was covered with dimples. A rough-surfaced sphere with small dimples experiences less drag than a sphere with smooth surface, same like dimpled golf balls fly farther than non-dimpled balls. Fouling organisms or spores may be easily swept off and harder attached on the algal surface with dimples. Therefore, the information obtained from the *C. pilulifera* structure and antifouling substances can be used to make biomimetic materials and used as an environmentally friendly novel material to protect against the attachment of soft foulants, especially micro- and macroalgae.

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