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# Axenic Culture of *Gyrodinium impudicum* Strain KG03, a Marine Red-tide Microalga that Produces Exopolysaccharide

**Joung Han Yim and Hong Kum Lee***

*Korea Polar Research Institute, KORDI, Ansan P.O. Box 29, Seoul 425 600, Republic of Korea*

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**An exopolysaccharide-producing microalgal dinoflagellate was isolated from a red-tide bloom and designated strain KG03. A bacteria-free culture of strain KG03 was achieved using a modified wash with phototaxis and antibiotic treatment. Combined treatment with neomycin and cephalosporin was the most effective for eliminating the bacteria associated with the microalgae. Strain KG03 was identified as *Gyrodinium impudicum* by analyzing the ITS regions of the 5.8S rDNA, 18S rDNA, morphological phenotype and fatty acid composition. The exopolysaccharide production and cell growth in a 300-ml photobioreactor were increased 2.7- and 2.4-fold, respectively, compared with that in a flask culture at the first isolation step.**

***Key words*:** *Gyrodinium impudicum*, axenic culture, exopolysaccharide, cell growth

Microbial exopolysaccharides have significant commercial value, particularly for the production of gels and the modification of the rheological properties of aqueous systems. They may replace the plant and macroalgal exopolysaccharides traditionally used in the food, pharmaceutical, textile and oil industries (Linton, 1990; Sutherland, 1990). Marine microalgae are potential sources of new biopolymers in industry. Several species are characterized by the presence of thick capsules surrounding their cells and by their ability to release polysaccharide material into the culture medium (Vincenzini *et al*., 1990).

Since the mid 1980s, blooms caused by *Gyrodinium impudicum* have occurred annually during September and October in Korean coastal waters in conjunction with harmful red-tide organisms, such as *Cochlodinium polykrikoides* (Park and Park, 1999). *G. impudicum* produces a mucous exopolysaccharide (Park and Park, 1999), and the sulfated polysaccharide extracted from *G. impudicum* is a selective inhibitor of encephalomyocarditis virus (EMCV) replication *in vivo* (Yim *et al*., 2004).

To investigate the production of sulfated exopolysaccharide in *G. impudicum*, it is essential to obtain an axenic culture. Several methods are used to obtain axenic cultures, including washing with sterile diluents, serial dilution, ultrasonic treatment and differential centrifugation (Pringsheim, 1951; Singh *et al*., 1982; Sako *et al*., 1992; Imai and Yamaguchi, 1994). In recent years, these methods have been used either in conjunction with antibiotics or have been replaced by treatment with antibiotics (Droop, 1967; Guillard, 1975) or other anti-bacterial agents, such as lysozyme (Doucette and Powell, 1998). The advantage of bactericidal treatments over physical dissociation methods is their ease of application, coupled with their effectiveness in removing bacteria from mucilaginous algal species that cannot easily be physically separated from the accompanying microflora (Droop, 1967). In this study, an improved axenic culture method for the exopolysaccharide-producing *G. impudicum* is reported.

* To whom correspondence should be addressed.
(Tel) 82-31-500-4511; (Fax) 82-31-406-2495
(E-mail) hklee@kordi.re.kr

## Materials and Methods

### *Sampling and culture conditions*

The uni-algal separation and axenic culture procedures used for red-tide microalgae are described in Fig. 1. The microalgal strains were originally isolated from seawater samples collected along the Kang-Gu and Gam-Po coastal regions of Korea, where a red-tide bloom occurred. The culture was maintained in f/2 culture medium (Stein, 1973) at 22°C under a circadian light intensity of 100 μE/$m^2$/sec: dark cycle of 14 h : 10 h. Separation of the uni-algal culture was evaluated using the method of Lewin (1959), with f/2 medium, under stereomicroscopy (Axiovert 10, Carl-Zeiss, Germany).

### *Isolation of associated bacteria from uni-algal cultures*

One milliliter of unialgal culture was taken from the stationary phase (at 21 days), and then serially diluted (10-fold dilutions from near to $10^{-9}$) in sterile seawater. Each

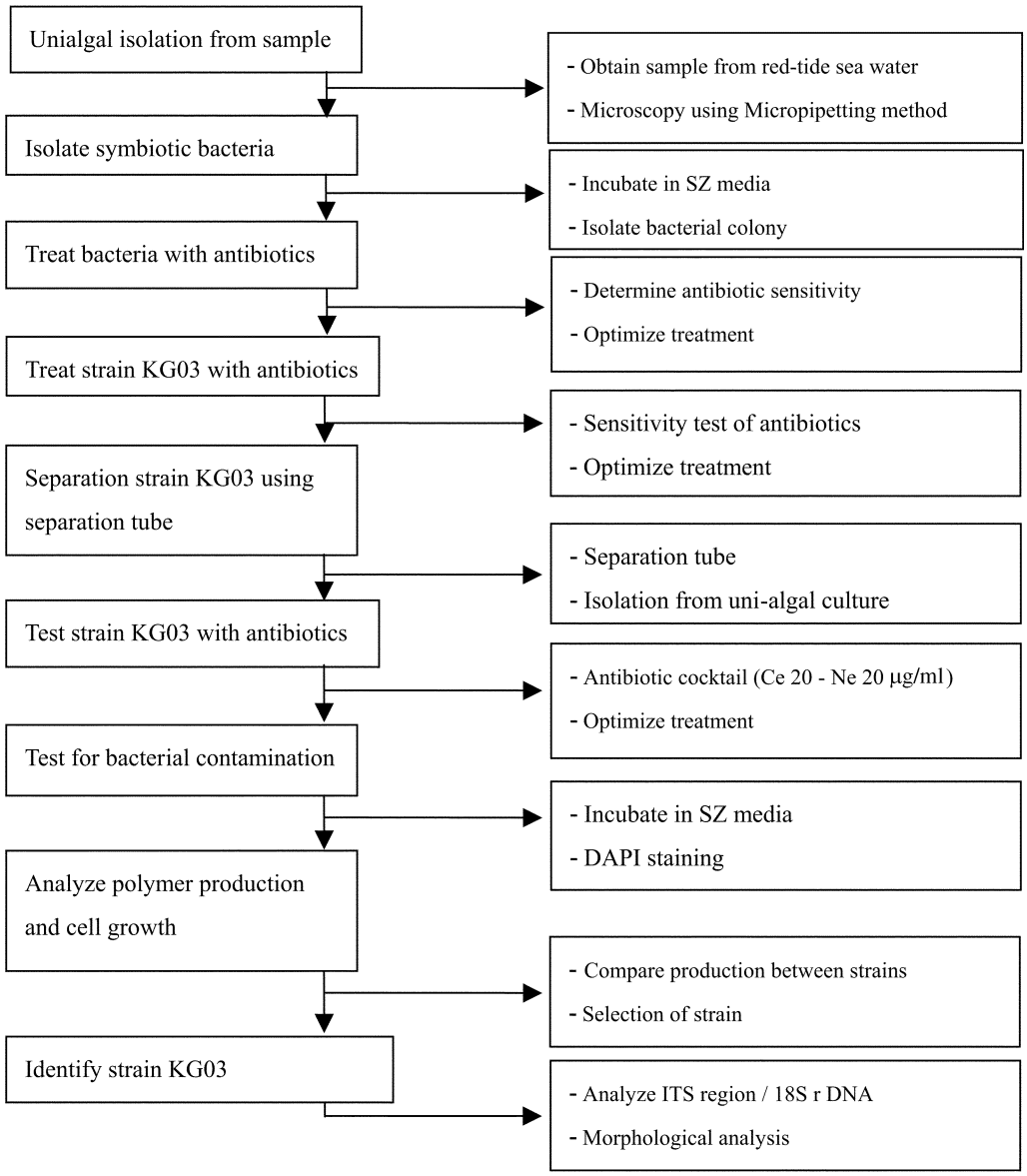



**Fig. 1.** The procedure used for axenic culture and analysis of strain KG03.

dilution (100 μl) was spread on ZoBell2216e agar plate medium and incubated for 5 days at 20°C. To obtain a pure culture, bacterial colonies from the agar plates generating between 20 and 100 colonies were selected and individually replated onto marine agar. The bacterial isolates were categorized using colony morphology, and then stored in marine broth plus 10% (v/v) glycerol at -70°C for antibiotic testing.

### *Antibiotic resistance profiling of the uni-algal culture and associated bacteria*

Isolated bacteria were inoculated into 2 ml of SZoBell media and incubated for 24 h in a shaking incubator (20°C, 120 rpm). Each bacterial suspension (200 μl) was incubated in SZoBell medium, containing chloramphenicol, polymyxin-B, ampicillin, streptomycin, neomycin, imipenem, penicillin-G and cephalosporin, at 20°C for 72 h, at concentrations of (10, 20, 50, 100, 150, 200 and 250 μg/ml. The antibiotic sensitivity was quantified using colony-forming units (CFU/ml) on SZoBell2216e agar plates. To determine the antibiotic sensitivity of *G. impudicum*, uni-algal cultures (about $1\times10^2$ cells/ml) were incubated under the same culture conditions and by examining the cell growth with fixation in 1% Lugol's solution. The cell count was performed by direct observation with a stereomicroscope in a 2 ml Sedgwick-Rafter Chamber.

### *Washing the uni-algal culture using a separation tube*

The wash method used a separation tube, with a modification to that used by Imai and Yamaguchi (1994). The procedure used to wash dinoflagellates is shown in Fig. 2.

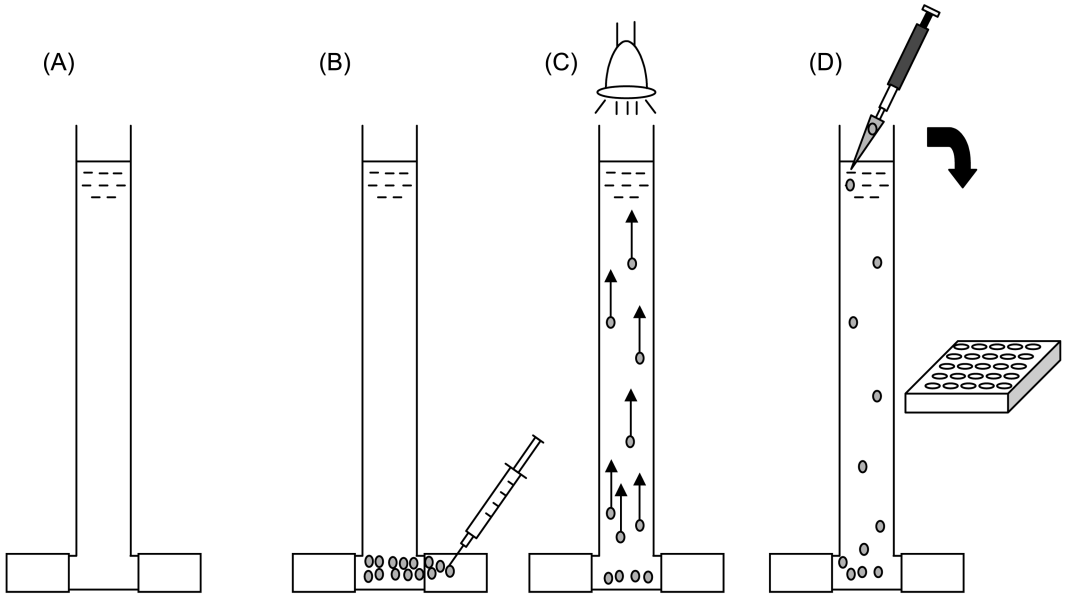



**Fig. 2.** The algal wash procedure using phototaxis in an algal separation tube. (A) Fill separation tube with sterile f/2 medium (10 mm, Φ×240 mm, L); (B) Inject unialgal culture of strain KG03; (C) Let the separation tube stand for 18 min under illumination to allow strain KG03 to swim up to the surface of the medium in the separation tube (light intensity: about 200 μE/$m^2$/sec); (D) Inoculate sterile f/2 medium containing antibiotics in a 24 well plate with separated cells from the surface of the medium in the separation tube.

A sterilized separation tube (240 mm long ×10 mm i.d.), with a silicon tube at the lower end, was filled with sterile f/2 culture medium (Fig. 2A). The contaminated uni-algal culture (about $1\times10^2$ cells/ml) was aspirated into a syringe and gently injected into the culture medium through the silicon tube (Fig. 2B). The separation tube was left standing on a clean bench for 18 min at room temperature, with a light intensity of 200 μE/$m^2$/sec at the top of tube (Fig. 2C). Strain KG03 swam to the surface of the culture medium in the separation tube, and was pipetted aseptically (ca. 0.5 ml) into 1.5 ml of antibiotic f/2 medium (cephalosporin at 20 μg/ml and neomycin at 20 μg/ml) in 2.0 ml, 24 well micro-plates (Corning, USA) (Fig. 2D). The cell growth was observed directly using stereomicroscopy.

### *Assessing bacteria-free strains*

To detect the presence of the culturable bacteria remaining in the wash and antibiotic-treated uni-algal cultures, SZo-Bell liquid media was inoculated with the microalgal culture, and incubated at 20°C for 5 days. The purity of the established cultures was confirmed by direct observation with DAPI (4'-6-diamidino-2-phenylindole) staining and fluorescence microscopy (Carl-Zeiss, Germany).

### *Morphological observation of the separated bacteria-free strain*

The morphological characteristics were observed using light, scanning electron (SEM) and transmission electron microscopies (TEM), as described by Hanson *et al*. (2000).

### *DNA extraction and amplification of the ITS regions*

An approximately 50 ml sample of bacteria in the exponential phase was harvested by centrifugation (3,000 rpm) for 10 min at room temperature, and transferred to a 1.5-ml Eppendorf tube. Before DNA extraction, the pellets were frozen at -20°C until required. As shown in Table 1,

**Table 1.** The sequences of the oligonucleotide primers used to amplify and determine the ITS region and 18S rDNA gene in strain KG03

| Primer name | Primer sequences (5' - 3') | Primer size | Reference |
|---|---|---|---|
| ITS-1 | 5'-CCA AGC TTC TAG ATC GTA ACA AGG TCC GTA GGT-3' | 30 | Cho *et al.*, 2001 |
| ITS-2 | 5'-CCT GCA GTC GAC AAT GCT TAA TTC AGC GG-3' | 29 | Cho *et al.*, 2001 |
| gy18F1[1] | 5'-CAC CTG GTT GAT CCT GC-3' | 17 | In this study |
| gy18R1[2] | 5'-GCT TGA TCC TTC TGC-3' | 15 | In this study |
| gy18F2[1] | 5'-AGT TGG ATT TCT GTT GG-3' | 17 | In this study |
| gy18R2[2] | 5'-TCA CTT GCG ACC ATA CT-3' | 17 | In this study |

[1]F; forward, [2]R; reverse

total genomic DNA was extracted, and the ITS-1, ITS-2 and 5.8 rDNA genes amplified using the polymerase chain reaction (PCR), as described by Cho *et al*. (2001), using the primers ITS-1 and ITS-2.

***Alignment and phylogenetic analyses***

For the phylogenetic analysis, nearly full-length 18S rDNA sequences were amplified by PCR, using the primers gy18F1, gy18R1, gy18F2 and gy18R2 (Table 1). The PCR mixture consisted of 5 μl of 10× PCR mix (final concentrations: 50 mM KCl, 0.01% gelatin and 10 mM Tris-HCl at pH 9), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl of each primer, 1 μl of template DNA and 2.5 units of *Taq* polymerase (TaKaRa, Japan) in a final volume of 50 μl. PCR was performed in a thermal cycler (Biometra, Germany) with an initial denaturation at 95ºC for 5 min and then 20 cycles at 94ºC for 1 min, 55ºC for 1 min and 72ºC for 7 min. A final extension was performed at 72ºC for 7 min. The PCR products were separated using agarose gel electrophoresis, and purified with a Highpure PCR Product Purification PCR kit (Roche, Germany). The 18S rDNA sequences were evaluated using the NCBI (National Center for Biotechnology Information) Advanced BLAST search program to identify the sequences of closely related organisms. The related sequences were aligned preliminarily using the default settings of ClustalW (Thompson *et al*., 1994), and complete sequence alignments performed using PHYDIT (Chun, 1995) and manual comparison. The phylogenetic analysis was performed with PHYLIP, and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei, 1987).

***Fatty acid analysis***

For the fatty acid analysis, cells were harvested by filtration (Whatman, GF/C, 0.47 mm, USA) and frozen (-20ºC) until analyzed. A modification to the cold solvent extraction method described by Cho *et al*. (2001) was used.

***Cell growth and exopolysaccharide production***

The uni-algal strain KG03 was cultured in 25 ml f/2 medium, as the basal medium, for 19 days at 22ºC, with 100 μE/$m^2$/sec. It was precultured in 250 ml Erlenmeyer flasks containing 50 ml of medium for 7 days on a reciprocal shaker at 80 rpm. The cell concentration in the initial phase was $1\times10^3$ cells/ml after inoculation.

To examine the cell growth, 100 μl aliquots of liquid culture were mixed with 900 μl of f/2 media, supplemented with 5 μl Lugol's solution. Then, 100 μl aliquots were pipetted into the wells of a 96 well micro-plate, and the cells counted directly under light microscopy in triplicate. The cell growth was calculated as the specific growth rate $\mu = ln\, X_2 - ln\, X_1 / t_2\, t_1$, where $X_2$ and $X_1$ are the cell densities (cells/ml) at times $t_2$ and $t_1$ (days), respectively (Fogg and Thake, 1987), and $k = [3.322 / (t_2 - t_1)] \times \log (X_2 / X_1)$ (Robert and Guillard., 1973).

To separate the exopolysaccharide, the cells were removed from the culture medium by centrifugation at 10,000 × *g* for 10 min at 4ºC. As the medium contained no carbohydrate, the exopolysaccharide production was determined as the total carbohydrate concentration, which was assayed using the phenol-sulfuric acid method, with glucose as a standard (Dubois *et al*., 1956), in 100 μl aliquots of cell-free culture broth. The biomass concentration was calculated as $x$ (mg/l)=0.0013×cell numbers (cells/ml)+4.103, ($r^2$=0.976). The exopolysaccharide production was calculated using the yield and specific production indices ($Y_{p/x}=dP/dX$) and ($Q_p$), respectively, where $X$ is the cell biomass (mg/l) and $P$ the exopolysaccharide concentration (mg/l).

## Results and Discussion

***Uni-algal culture and isolation of bacterium from cultures***

The isolation of a single algal unit for growth in suitable medium is required to establish a clonal, uni-algal culture. For the uni-algal culture of strain KG03, uni-algal separation was evaluated under stereomicroscopy, using the microcapillary pipette method, with f/2 medium. The stereomicroscopic observation showed the red-tide samples contained various marine dinoflagellates, including *Procentrum* sp., *Ceratium* sp. and *Cochlodinium polykrikoides*. Under stereomicroscopy, *G. impudicum* was similar to *C. polykrikoides*. The cells of *G. impudicum* (12-25 μm) were considerably smaller than those of *C. polykrikoides* (20-35 μm). The morphotype of a four-cell chain of *G. impudicum* was asymmetric, with the lowest cell being smaller than the upper; the chains of cells were maintained, and were light black in 1% Lugol's solution compared with *C. polykrikoides*. A sample of *G. impudicum* was washed mildly three times using distilled seawater, filtered through a cellulose membrane (10 μm, Millipore, USA) to eliminate bacterial contamination and then precultured in f/2 medium for two weeks. In the pre-culture of *G. impudicum*, the number of two- to four-cell chains increased, and moved faster with increasing culture time. For the axenic culture, the four-cell chains were separated from the culture, as the morphotype of the *G. impudicum* four-cell chains could be distinguished from that of the *C. polykrikoides* four-cell chains.

***Antibiotic resistance profiling of bacteria from uni-algal cultures and uni-algae in culture***

Bacteria isolated on ZoBell2216e agar medium from a uni-algal culture of *Gyrodinium impudicum* were separated into distinct morphotypes (Table 2). The antibiotic sensitivities of the bacterial isolates were assessed using a range of antibiotics; all strains were sensitive to chloramphenicol at 50 μg/ml, neomycin at 20 μg/ml, imipenem at 10 μg/ml and cephalosporin at 20 μg/ml (Table 3). To examine the antibiotic resistance of *G. impudicum*, uni-

**Table 2.** Morphological characterization of assorted symbiotic bacteria in the uni-algal culture of strain KG03

| Isolated bacteria | Form | Color | Texture |
|---|---|---|---|
| 01KG03-1 | IR | I | TL |
| 01KG03-2 | IR | I | TL |
| 01KG03-3 | C | O | TL |
| 01KG03-4 | C | O | TL |

IR; irregular, C; Circular, I; Ivory white, O; Cream, TL; Translucent

**Table 3.** Sensitivity of the bacteria to antibiotics and the parentage of the isolates removed following antibiotic treatment

| Antibiotics | Concentration (μg/ml) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | | | | 20 | | | | 50 | | | | 100 | | | | 200 | | | |
| | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| Chloramphenicol | +++ | +++ | ++ | +++ | + | ++ | + | + | – | + | – | + | – | + | – | + | – | – | – | – |
| Polymixicin-B | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | – | + |
| Ampicillin | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | +++ | +++ | ++ | ++ | ++ | ++ | + | + | + | + | ++ | – |
| Penicillin-G | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | + | + | ++ | + | + | + | + |
| Streptomycin | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | +++ | ++ | + | + | ++ | – | – | – | + | – | – | – |
| Neomycin | + | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Imipenem | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Cephalosporin | ++ | + | + | – | – | + | – | + | – | – | – | – | – | – | – | – | – | – | – | – |

*Cultured in SZoBell media, 20°C, 120 rpm shaking incubation for 3 days; A: 01KG03-1, B: 01KG03-2, C: 01KG03-3, D: 01KG03-4; Cell numbers: +++ (<$10^6$ colonies), ++ ($10^3$-$10^6$ colonies), + (<$10^3$ colonies), – (antibiotics resistance)

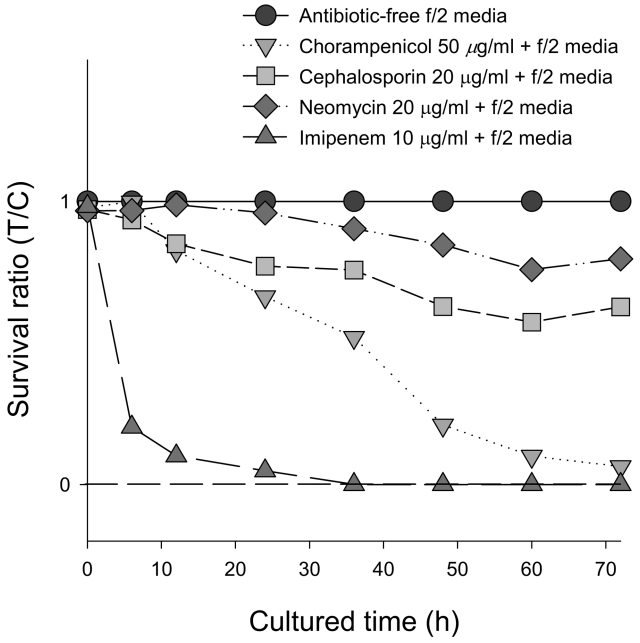



**Fig. 3.** Relative survival ratio of uni-algal strain KG03 after antibiotic treatments. The survival ratio (s/r) was corrected relative to the control value of 1. T: treated cells with antibiotics; C: cells without antibiotics.

algal cultures were treated with the four antibiotics at the same concentrations. The cell survival ratios (s/r) of *G. impudicum* in antibiotic-containing media were compared with that in antibiotic-free f/2 media; *G. impudicum* was sensitive to chloramphenicol (s/r = 0 in 36 h) and imipenem (s/r = 0 in 72 h), but resistant to neomycin (s/r = 0.79 in 72 h) and cephalosporin (s/r = 0.62 in 72 h). Imipenem was more effective at reducing bacterial numbers than the other antibiotics. *G. impudicum* was sensitive to chloramphenicol and imipenem (Fig. 3). Bacteria remained in uni-algal cultures when treated with neomycin and cephalosporin alone at 20 μg/ml, while combined treatment with neomycin and cephalosporin eliminated the associated bacteria for up to 19 days.

***Axenic culture of uni-algal culture using the wash method***

A uni-algal culture was separated and washed using a separation tube. Using an inoculum of about $1\times10^2$ cells/ml, the uni-algal culture was aspirated into a syringe, and gently injected into the culture medium through a silicon tube. The separation tube was left standing on a clean bench for 18 min at a light intensity of 200 μE/$m^2$/sec. Eighteen minutes was considered sufficient time for the algae to swim to the surface of the 240-mm-long separation tube, given a swimming velocity of 1.33 cm/min for the alga in the separation tube (10 mm, Φ×240 mm, L), as determined under stereomicroscopy. The washed cells were counted under stereomicroscopy; one to three cells were added to each well of a 24-well plate and incubated for 7 days under the maintenance conditions. Imai and Yamaguchi (1994) obtained axenic cultures of 13 species of marine phytoflagellates using the wash method, but failed to isolate an axenic culture of phytoflagellates with

mucilaginous material on the cell surface owing to the embedded bacteria. Therefore, antibiotic treatment is important, as *G. impudicum* produces a mucilaginous exopolysaccharide.

### *Assessing the bacteria-free strain*

After growing the antibiotic-treated, washed cells, an axenic test was performed using ZoBell2216 agar culture and the DAPI staining method. To verify the cells in the axenic culture, strains KG03, KG09 and GJ01 were tested, using a DAPI staining method, to confirm their purity. Moreover, no bacteria were visible in the cultures of the three strains, either attached to the cells or free-living in the culture media. No strain can acquire bacteria-free status if the associated bacteria are embedded in mucilaginous material (exopolysaccharide) on the cell surface. The axenic cultures were subsequently passaged twice over a 90-day period in antibiotic-free media, and their bacterial status was rechecked, as described above. These axenic cultures remained bacteria-free. The possibility that non-culturable bacteria remained in the dinoflagellate cultures was also assessed using epifluorescence microscopy (Doucette and Powell, 1998).

### *Identification of the bacteria-free strain KG03*

Fig. 4A and 4B show a transverse section of strain KG03 and the main cellular organelles. The cell was enclosed by single-layered plasmalemma and was not covered by a cell wall or theca. The nucleus was located almost in the center of the cell, and the chloroplast was separated from the cell surface layer. The mitochondria were surrounded by an electron-dense, double membrane and possessed many tubular cristae in the endoplasm, separated from the cytoplasm. Numerous chloroplasts lay along side many of the double thylakoid lamella, which abutted the entire length of the chloroplast. The chloroplasts were surrounded by inner and outer membranes, which were separated from each other. As shown in Figs. 3C and 4D, the average cell body length was 17.3±2.3 μm (range: 14.2 20.7 μm, n=32); the average width was 15.4±2.8 μm (range: 12.4 17.9 μm, n=32). The cells were flattened dorsoventrally, with an average thickness to body length ratio

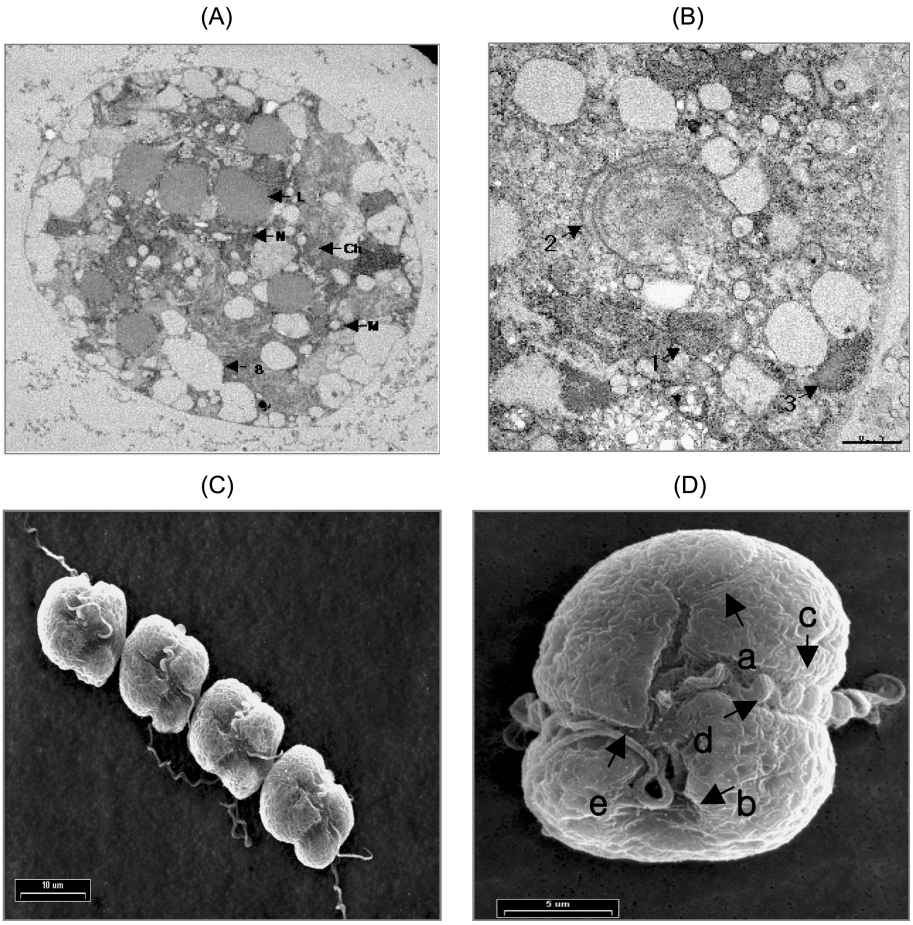
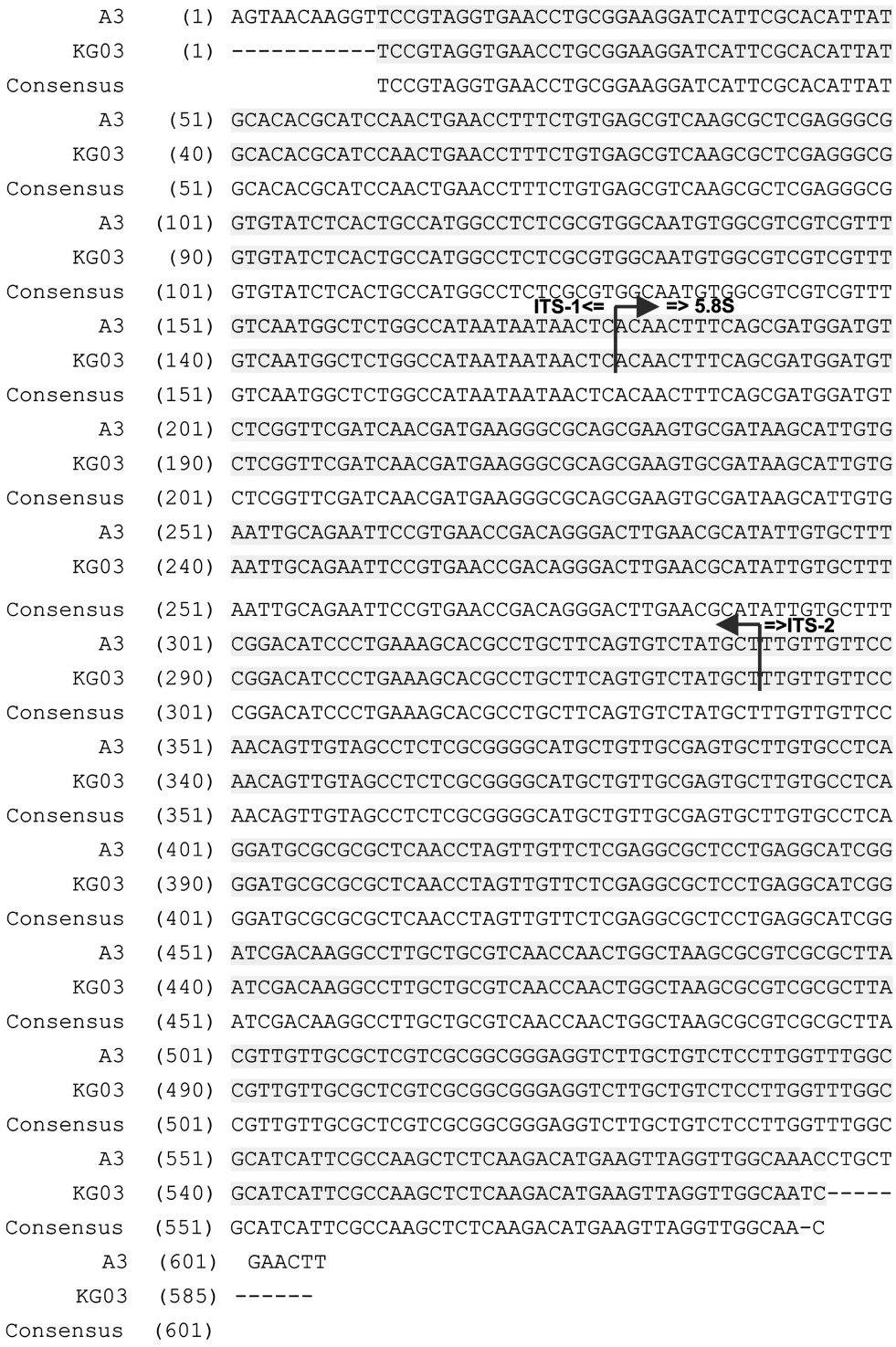



**Fig. 4.** Scanning and transmission electron microscopic images of strain KG03.
A. Cross section of whole cell of strain KG03, major cellular components as follueus: nucleus (N), chloroplast (Ch), mitochondria (M), Lipid (L), Starch (S), B. 1; double layer of mitochondria, 2; double layer of chloroplast, 3; pustule, C. 4-chained cell, D. a; epicon, b; hypocon, c; sulcus, d; transverse flagellum, e; longitudinal flagellum.

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        A3    (1) AGTAACAAGGTTCCGTAGGTGAACCTGCGGAAGGATCATTCGCACATTAT
      KG03    (1) -----------TCCGTAGGTGAACCTGCGGAAGGATCATTCGCACATTAT
Consensus                    TCCGTAGGTGAACCTGCGGAAGGATCATTCGCACATTAT
        A3   (51) GCACACGCATCCAACTGAACCTTTCTGTGAGCGTCAAGCGCTCGAGGGCG
      KG03   (40) GCACACGCATCCAACTGAACCTTTCTGTGAGCGTCAAGCGCTCGAGGGCG
Consensus   (51) GCACACGCATCCAACTGAACCTTTCTGTGAGCGTCAAGCGCTCGAGGGCG
        A3  (101) GTGTATCTCACTGCCATGGCCTCTCGCGTGGCAATGTGGCGTCGTCGTTT
      KG03   (90) GTGTATCTCACTGCCATGGCCTCTCGCGTGGCAATGTGGCGTCGTCGTTT
Consensus  (101) GTGTATCTCACTGCCATGGCCTCTCGCGTGGCAATGTGGCGTCGTCGTTT
        A3  (151) GTCAATGGCTCTGGCCATAATAATAACTCACAACTTTCAGCGATGGATGT
      KG03  (140) GTCAATGGCTCTGGCCATAATAATAACTCACAACTTTCAGCGATGGATGT
Consensus  (151) GTCAATGGCTCTGGCCATAATAATAACTCACAACTTTCAGCGATGGATGT
        A3  (201) CTCGGTTCGATCAACGATGAAGGGCGCAGCGAAGTGCGATAAGCATTGTG
      KG03  (190) CTCGGTTCGATCAACGATGAAGGGCGCAGCGAAGTGCGATAAGCATTGTG
Consensus  (201) CTCGGTTCGATCAACGATGAAGGGCGCAGCGAAGTGCGATAAGCATTGTG
        A3  (251) AATTGCAGAATTCCGTGAACCGACAGGGACTTGAACGCATATTGTGCTTT
      KG03  (240) AATTGCAGAATTCCGTGAACCGACAGGGACTTGAACGCATATTGTGCTTT
Consensus  (251) AATTGCAGAATTCCGTGAACCGACAGGGACTTGAACGCATATTGTGCTTT
        A3  (301) CGGACATCCCTGAAAGCACGCCTGCTTCAGTGTCTATGCTTTGTTGTTCC
      KG03  (290) CGGACATCCCTGAAAGCACGCCTGCTTCAGTGTCTATGCTTTGTTGTTCC
Consensus  (301) CGGACATCCCTGAAAGCACGCCTGCTTCAGTGTCTATGCTTTGTTGTTCC
        A3  (351) AACAGTTGTAGCCTCTCGCGGGGCATGCTGTTGCGAGTGCTTGTGCCTCA
      KG03  (340) AACAGTTGTAGCCTCTCGCGGGGCATGCTGTTGCGAGTGCTTGTGCCTCA
Consensus  (351) AACAGTTGTAGCCTCTCGCGGGGCATGCTGTTGCGAGTGCTTGTGCCTCA
        A3  (401) GGATGCGCGCGCTCAACCTAGTTGTTCTCGAGGCGCTCCTGAGGCATCGG
      KG03  (390) GGATGCGCGCGCTCAACCTAGTTGTTCTCGAGGCGCTCCTGAGGCATCGG
Consensus  (401) GGATGCGCGCGCTCAACCTAGTTGTTCTCGAGGCGCTCCTGAGGCATCGG
        A3  (451) ATCGACAAGGCCTTGCTGCGTCAACCAACTGGCTAAGCGCGTCGCGCTTA
      KG03  (440) ATCGACAAGGCCTTGCTGCGTCAACCAACTGGCTAAGCGCGTCGCGCTTA
Consensus  (451) ATCGACAAGGCCTTGCTGCGTCAACCAACTGGCTAAGCGCGTCGCGCTTA
        A3  (501) CGTTGTTGCGCTCGTCGCGGCGGGAGGTCTTGCTGTCTCCTTGGTTTGGC
      KG03  (490) CGTTGTTGCGCTCGTCGCGGCGGGAGGTCTTGCTGTCTCCTTGGTTTGGC
Consensus  (501) CGTTGTTGCGCTCGTCGCGGCGGGAGGTCTTGCTGTCTCCTTGGTTTGGC
        A3  (551) GCATCATTCGCCAAGCTCTCAAGACATGAAGTTAGGTTGGCAAACCTGCT
      KG03  (540) GCATCATTCGCCAAGCTCTCAAGACATGAAGTTAGGTTGGCAATC-----
Consensus  (551) GCATCATTCGCCAAGCTCTCAAGACATGAAGTTAGGTTGGCAA-C
        A3  (601)  GAACTT
      KG03  (585) ------
Consensus  (601)
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**Fig. 5.** Sequence alignment of the ITS-1, ITS-2 and 5.8S rDNA regions. A3, *Gyrodinium impudicum* A3; KG03, strain KG03.

of about 0.8. The morphotype appeared concave ventrally, the epicone was conical / hemispherical and the hypocone hemispherical, with an atypical notch caused by the sulcus. The ribbon-shaped transverse flagellate lay in the cingulum, curving around the cell in a counter-clockwise direction. The cingulum was slightly permitted, and the sulcus extended from the antapex. The left sulcal border was generally slightly convex, but sometimes had a small indentation.

The alignment of the DNA sequences of the internal transcribed spacers, ITS1 and ITS2 and the 5.8S rDNA, are shown in Fig. 5. The aligned *Gyrodinium impudicum*

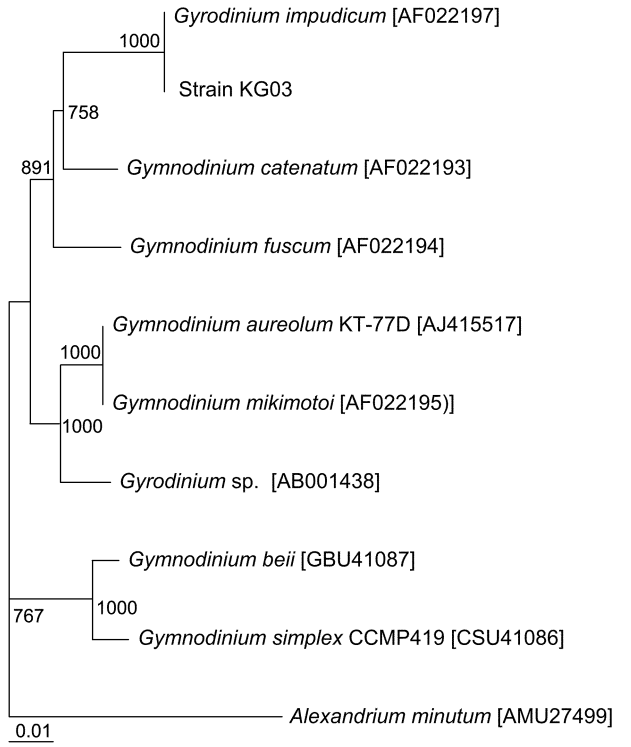


**Fig. 6.** Neighbor-joining tree showing the phylogenetic positions of strain KG03 and *Gymnodinium* species based on the 18S rDNA sequences.
The scale bar corresponds to approximately 0.1 changes per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch-points.

strain $A_3$ (Cho *et al*. 2001) and strain KG03 nucleotide sequences were 99.9% similar. The almost complete 18S rDNA sequence of strain KG03 was determined directly following PCR amplification. Phylogenetic analysis using 18S rDNA indicated that the nine strains included one *Alexandrium*, two *Gyrodinium* and six *Gymnodinium* strains. The 18S rDNA sequence of strain KG03 determined in this study consisted of 1,491 nucleotides, representing approximately 99.9% of the *Gyrodinium impudicum* AF022197 18S rDNA sequence. Phylogenetic trees based on the 18S rDNA sequences showed that strain KG03 falls within the cluster containing *Gymnodinium catenatum*, with 81.4% bootstrap support (Fig. 6). Strain KG03 exhibited levels of 18S rDNA similarity of 100, 96.0 and 96.6% to the strains *Gyrodinium impudicum*, *Gymnodinium catenatum* and *Gymnodinium fuscum*, respectively. The level of 18S rDNA similarity between strain KG03 and the other species included in the phylogenetic analysis was less then 90.8%. Adachi *et al*. (1996) suggested that the ITS region from *Alexandrium* was a useful genetic marker at the species and population levels; in particular, the ITS was thought to be a promising source for molecular markers.

As shown in Table 4, the contents of saturated ($C_{11}$-$C_{24}$), monoenoic ($C_{14}$-$C_{22}$), and polyenoic fatty acids ($C_{18}$-$C_{22}$) were 26.6, 0 and 73.2%, respectively, in strain KG03. Strain KG03 produced a wide range of unsaturated fatty acids, but produced no long-chain acids (above $C_{24}$); the major fatty acids were 16 : 0, 20 : 5 (n-5), and 22 : 6 (n-3) at 22.3, 16.7, and 33.9%, respectively. Strain KG03 contained especially high levels of eicosapentaenoic acid

**Table 4.** The fatty acid compositions of strain KG03

| Fatty acids | | *Gyrodinium impudicum* (%)[a] | Retention time (min) | Strain KG03 (%) |
|---|---|---|---|---|
| Saturated acids | 12 : 0 | 8.8 | – | |
| | 15 : 0 | 0.5 | – | |
| | 16 : 0 | – | 6.37 | 24.33 |
| | 18 : 0 | 5.1 | 11.32 | 1.77 |
| | 19 : 0 | | 13.23 | 0.16 |
| | 20 : 0 | 0.6 | – | – |
| | 22 : 0 | – | 21.39 | 0.48 |
| Sub-Total | | 15.0 | | 26.26 |
| Monoenic acids | 14 : 1 | 8.0 | – | – |
| | 16 : 1 | 28.4 | – | – |
| | | 36.4 | – | – |
| Sub-Total Polyenoic acids | 14 : 1 (n-11)[b] | – | 3.89 | 1.89 |
| | 16 : 1 (n-7) | – | 5.61 | 1.52 |
| | 16 : 4 (n-3) | – | 5.96 | 5.61 |
| | 18 : 1 (n-9) | – | 10.38 | 1.98 |
| | 18 : 1 (n-6) | 19.3 | – | – |
| | 18 : 1 (n-5) | – | 10.34 | 2.99 |
| | 18 : 1 (n-3) | 7.0 | – | – |
| | 18 : 2 (n-6) | 6.7 | 10.19 | 4.4 |
| | 18 : 3 (n-3) | 0.8 | 9.95 | 0.65 |
| | 18 : 3 (n-6) | – | 9.53 | 1.75 |
| | 18 : 4 (n-3) | – | 9.69 | 2.1 |
| | 20 : 2 (n-6) | 5.0 | – | – |
| | 20 : 5 (n-3) | 4.1 | 16.67 | 16.37 |
| | 22 : 2 (n-6) | 2.4 | – | – |
| | 22 : 6 (n-3) | 3.3 | 29.84 | 33.9 |
| | | 48.6 | | 73.16 |
| Sub-Total | $\sum$ n-3 | 15.2 | | 58.6 |
| | $\sum$ n-6 | 33.4 | | 6.15 |
| | $\sum$ n-7 | – | | 1.98 |
| | $\sum$ n-9 | – | | 1.52 |
| | $\sum$ n-11 | – | | 1.89 |
| Total | | 100 | | 99.84 |

a. Cho *et al*., (2001), Fatty acid composition calculated from the area of the peak (%). b. The fatty acids are described by *x* : *y* (n-*z*), where *x* is the number of carbon atoms, *y* the number of double bonds and *z* the position of the first double bond, counting from the methyl end of the molecule.

**Table 5.** Comparison of cell growth and exopolysaccharide production of strains KG03, KG09 and GJ01

| Strains | Culture time (days) | Cell growth | | Carbohydrate production | |
|---|---|---|---|---|---|
| | | FCN[a] | $\mu_{max}$ (T)[b] | FCC[c] | $Y_{p/x}$[d] |
| KG03 | 19 | 27,432±1,231 | 0.44 (6) | 20.7±2.23 | 0.76 |
| KG09 | 19 | 26,901±2,312 | 0.41 (6) | 18.8±1.24 | 0.70 |
| GJ01 | 19 | 25,208±987 | 0.36 (6) | 16.7±0.31 | 0.67 |

Culture condition : f/2 media 20 ml, 20°C, light cycle 14 L/10 D, light intensity 100 μE/$m^2$/sec, inoculation size : $1\times10^3$ cells/ml.
a; FCN : final cell number (cells/ml), b; $\mu_{max}$ (T) : maximum specific cell growth (/d), c; FCC : final polysaccharide concentration (mg/L), d; $Y_{p/x}$ : yield index.

**Table 6.** Characterizations of cell growth and carbohydrate production of strain KG03 with different culture types

| Characterizations | Flask culture | Photoreactor | Changes to photoreactor |
|---|---|---|---|
| Cultured condition | 20 ml f/2 medium | 200 ml f/2 medium | – |
| Cultured time (days) | 19 | 19 | – |
| Final cell number[a] | 27,432 ± 1,231 | 65,500 ± 3,287 | + 3,8068 |
| $\mu_{max}$ (T)[b] | 0.389 (3) | 0.457 (3) | + 0.068 |
| $\mu k_{max}$ (T)[c] | 0.56 (3) | 0.66 (3) | + 0.1 |
| FCC[d] | 20.70 ± 1.23 | 46.81 ± 4.04 | + 26.1 |
| Final $Y_{p/x}$[e] | 0.76 ± 0.01 | 0.71 ± 0.13 | – 0.05 |
| Max $Y_{p/x}$ (T)[f] | 4.29 ± 0.65 (15) | 2.32 ± 0.22 (15) | – 1.97 |
| Avr. $Y_{p/x}$[g] | 0.51 ± 0.002 | 0.21 ± 0.04 | – 0.3 |
| Max. Rp (T)[h] | 8.41 ± 1.32 (15) | 10.83 ± 1.23 (15) | + 2.24 |
| Qp[i] | 15.62 ± 1.22 | 24.61 ± 2.33 | + 8.99 |
| Final pH | 7.82 | 7.99 | + 0.17 |

Culture condition : f/2 media, 20°C, light cycle; 14 L/10 D, 100 μE/$m^2$/s, inoculation volume; $1\times10^3$ cells/ml.
a; cells/ml, n=3, ±: standard deviation, b; maximum specific growth rate (days), c: maximum k value (days), d; final carbohydrate, e; final yield index, f; maximum yield index (/d), g; average of yield index, h; ratio of maximum yield to average yield indices, i; specific production index, j; 50 ml flask culture k: 300 ml photoreactor culture.

(EPA, 20 : 5, n-3) and docosahexanoic acid (DHA, 22 : 6, n-3). Cho *et al.* (2001) reported that *Gyrodinium impudicum* contained abundant palmitic acid and serial n-3 and n-6 polyunsaturated fatty acids, which gave a similar fatty acid profile.

***Cell growth and exopolysaccharide production of the bacteria-free strain***

As shown in Table 5, the production of exopolysaccharide and the cell growths of the three strains were analyzed to select the best exopolysaccharide-producing strain. The exo-polysaccharide productions of strains KG03, KG09 and GJ01 were 20.7 ± 2.2, 18.8 ± 1.24, and 16.7 ± 0.31 mg/L ($Y_{p/x}$; 0.76, 0.7, and 0.67), respectively. Consequently, strain KG03 was selected as the strain for exopolysaccharide production. The $\mu_{max}$ and $k_{max}$ of strain KG03 were 0.39 and 0.56/day, respectively. To analyze the exopolysaccharide (p-KG03) production, the cell growth and p-KG03 production of strain KG03 were investigated using flask and bioreactor cultures (Table 6). The production of p-KG03 and the cell growth in the 300-ml photoreactor were 46.8 ± 4.0 mg/l and 65,500 ± 3,287 cells/ml, respectively, representing 2.7- and 2.4-fold increases, respectively, compared with the values for 50 ml flask cultures. In the 300-ml photoreactor culture, the $\mu_{max}$ and $k_{max}$ were 0.46 and 0.66/day after 3 days of cell growth, and the final $Y_{p/x}$ was 0.71 for the production of p-KG03.

To investigate the production of exopolysaccharide in such organisms, it is essential to obtain an axenic culture. There are several optimized procedures for obtaining axenic cultures, but red-tide microalgae, such as *Cochlodinium polykrikoides* and *Gyrodinium impudicum*, produce exopolysaccharide mucus, which reduces the effectiveness of the procedures for these algae. As *G. impudicum* lacks a cell wall and theca, it was easily ruptured during older, physical separation methods, and the mucilaginous exopolysaccharide cannot easily be physically separated from the accompanying microflora. Therefore, an axenic culture was obtained using a combination of phototactic behavior and an antibiotic method. Subsequently, the pattern of exopolysaccharide production was analyzed in the axenic culture. In future studies, the axenic culture method should be simplified for use with various microalgae species.

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## Abbreviations

DAPI, 4'-6-diamidino-2-phenylindole; s/r, survival ratios; $Y_{p/x}$, yield index; *X*, cell biomass (mg/l); *P*, exopolysaccharide concentration (mg/l); FCN, final cell number (cells/ml); $\mu_{max}$ (T): maximum specific cell growth (time); FCC, final polysaccharide concentration (mg/l); $k_{max}$, maximum k value; Max $Y_{p/x}$, maximum yield index; Avr. $Y_{p/x}$, average of yield index; Max. Rp, ratio of maximum yield to average yield indices; $Q_p$, specific production index

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