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Growth Enhancement of the Microalga *Tetraselmis suecica* by an Extract of the Green Alga *Monostroma nitidum*

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Cell growth of the marine microalga *Tetraselmis suecica* was regulated by the addition of seaweed extracts in its culture medium. Of 26 species of seaweed tested, water-soluble extracts from *Monostroma nitidum* and *Pachymeniopsis elliptica* and methanol-soluble extract from *Sargassum confusum* enhanced the growth of *T. suecica* cells. The water extract of *M. nitidum* was the most effective, producing up to a 2-fold increase in cell density with the addition of 1 mg/mL of extract to the culture medium. Cell size, gross biochemical composition, fatty acids, and digestion efficiency all differed marginally between cultures of *T. suecica* grown with and without the *M. nitidum* extract.

Key words: Algal growth, Microalgae, Microalgal culture, *Monostroma nitidum*, Green alga, *Tetraselmis suecica*

Introduction

The mass culture of microalgae as feed for molluscs, crustaceans, and fish is an important component of the mariculture industry (Metting, 1996). Microalgal diets commonly provide essential nutrients and other growth-promoting factors. Insufficient amounts of highly unsaturated fatty acids, especially docosahexaenoic acid [DHA 22:6 (n-3)] and eicosapentaenoic acid [EPA 20:5 (n-3)], have caused high mortality and low growth rates, particularly during larval and early juvenile periods (Watanabe et al., 1989; Takeuchi et al., 1990). *T. suecica* possesses well-known nutritional qualities and is in great demand owing to its composition of protein, vitamins, pigments, and high quantities of EPA (Montaini et al., 1995; Robert et al., 2001). It is also a very mobile microalga and possesses four fragile flagella. However, most microalgae including *T. suecica* have difficulties in achieving high-density cultures, resulting in unreliable algal diet production. The development of new designs for biofermentors (Burgess et al., 1993) is one way to solve this problem. Another solution is the development of cell-growth activator substances to increase cell biomass and the economic feasibility of small-scale production (Cho et al., 1999). Growth activators for *T. suecica* were therefore screened in methanol- and water-soluble extracts of several seaweed tissues. Also examined in this study were changes in growth rate, biochemical composition, and digestion efficiency of *T. suecica* cultured with and without the most effective of these seaweed extracts, the water-soluble components extracted from *M. nitidum*.

Materials and Methods

Microalgal culture

The axenic Prasinophyte flagellate *Tetraselmis suecica* (CCAP-66; P-4) was selected through Percoll gradient centrifugation, antibiotic treatment, and growth on an agar medium (Cho et al., 2002) it was cultured in f/2 medium (Guillard and Ryther, 1962) with an initial cell density of 1.2×10^5 cells/mL. Each seaweed extract was added to the medium and cultured under 70 μmol/m^2/s light intensity at 18℃ for 8 d. Cells were counted under a microscope with a hemocytometer.

Seaweed extracts

Leafy thalli of 26 seaweed species were collected from the coast of Korea, dried, and then ground to

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powder using a coffee grinder for 5 min. To each 20 g powder, 1 L of methanol was added for 1 d to extract the methanol-soluble components. This was repeated three times, and the combined extracts were evaporated to dryness. One liter of distilled water was then added to the remaining powder to extract the water-soluble components. Stock solutions were prepared by the addition of 1 mL of methanol or distilled water to each 40 mg of dried extract. The stock solutions were filtered through a 0.22-μm filter before use.

Analysis of gross biochemical composition

Biomass dry weight was measured after washing with 0.5 M ammonium bicarbonate (pH 7.5) and drying at 90℃ for 1 d. Total carbohydrate expressed as glucose was determined by the phenol-sulfuric acid method (Kochert, 1978), using glucose as a standard. A common pigment, chlorophyll *a* (chl *a*), was extracted in 100% acetone, and the amount was calculated according to the chl *a* equation of Sterman (1988). Total lipid was extracted by the use of hexane and isopropanol (3:2) as a solvent (Radin, 1981) and was quantified gravimetrically. The amount of soluble protein in the cell was estimated according to the method of Lowry et al. (1951) after heating the cell suspension at 100℃ in 1 N NaOH for 2 h to obtain the complete solubilization of protein. Bovine serum albumin was used as the standard for protein determination. For fatty acid analysis, the alga *T. suecica* was cultured in f/2 medium with and without the water extract (1 mg/mL) of *M. nitidum* at 18℃ for 8 d. The cells were collected by centrifugation at 1,000 rpm for 10 min at 4℃. The algal pellet was frozen and lyophilized for fatty acid analysis. Methyl esters of fatty acids were prepared from 10 mg alga by *in situ* saponification and methylation with methanolic boron trifluoride (Whyte, 1988). Solutions were analyzed on a Perkin-Elmer Autosystem gas-chromatograph equipped with a flame ionization detector. Separation was accomplished on a db-wax column (30 m×0.3 mm ID, 0.25 μm film), programmed from 60℃ to 350℃ at 9℃/min. Nitrogen carrier gas was controlled at 21 cm/sec with a split ratio of 100:1. Identification was made by comparison with the equivalent chain length (ECL) of authentic fatty acid standards. Peaks of less than 0.2% of the total area were omitted from the profile. The weight percentage of fatty acids was estimated from the peak area on the chromatograph using heneicosanoic acid (21:0) as an internal standard.

Digestion efficiency

The feeding index of the microalga was determined using five each of *Crassostrea gigas* (Pacific oyster, 8±0.5 cm), *Mytilus edulis* (blue mussel, 4±0.5 cm), and *Venerupes philippinarum* (Japanese littleneck clam, 3±0.5 cm). The amounts of chl *a* (supplied amount amount remaining in the container) and phaeopigment (in digestive diverticula) were determined every hour during a 12-hr period, using spectrophotometric methods (Strickland and Parsons, 1963).

Statistics

The experiments were repeated at least three times with each independent assay. Mean values of the index were compared with the control using Student's *t*-test.

Results and Discussion

Effect of seaweed extracts

To confirm the existence of microalgal growth regulators in seaweed tissues, a methanol-soluble fraction and a water-soluble fraction were isolated from each seaweed species. When each methanol extract from 26 seaweeds was added to *T. suecica* culture medium at a concentration of 200 μg/mL, the extract from *Sargassum confusum* demonstrated a growth activation of 1.3-fold, while those from *Ishige foliacea*, *Endrachne binghaniae*, *Monostroma nitidium*, and *Ulva pertusa* showed growth inhibition of 0.2-0.3-fold (Table 1). When 200 μg/mL of the water extract was added to the medium, two seaweeds, *Monostroma nitidum* and *Pachymeniopsis ellioptica*, showed growth activations of 1.8-fold and 1.5-fold, respectively. None of the water extracts from the 26 seaweeds showed growth inhibition. The water extract of *M. nitidum* was shown to produce the greatest enhancement of the cell growth of *T. suecica* and was used in all further experiments.

Concentration of *M. nitidum* extract

T. suecica attained the stationary phase of growth within 8 d in f/2 medium under 70 μmol/m^2/s light intensity at 18℃. The half-time of the exponential phase was approximately 4 d; thus, cell numbers were measured at 4 d to determine growth enhancement or inhibition. The solvent methanol alone provided minimum inhibition of *T. suecica* growth at 0.5%. Methanol-soluble components were therefore always added to the medium at less than 0.5% methanol in the assay cultures. The water extract of *M. nitidum*, the most effective at enhancing cell growth of *T.*

Table 1. Effect of seaweed extracts on cell growth of the microalga *Tetraselmis suecica*[1]

Species	MeOH extract (μg/mL)		Water extract (μg/mL)	
	200	20	200	20
Chlorophyta				
Codium fragile	26.0 ± 0.5	27.0 ± 0.1	25.0 ± 0.5	24.5 ± 0.5
Enteromorpha linza	27.5 ± 0.4	25.0 ± 0.6	25.5 ± 0.6	26.5 ± 0.5
Monostroma nitidum	8.0 ± 0.3	9.0 ± 0.4	44.0 ± 0.5**	31.5 ± 0.2
Ulva pertusa	8.5 ± 0.7	21.0 ± 0.5	27.0 ± 0.5	27.5 ± 0.3
Phaeophyta				
Colpomenia bullosa	27.0 ± 0.9	27.0 ± 0.4	28.0 ± 0.5	28.0 ± 0.1
Colpomenia sinuosa	25.0 ± 0.8	26.0 ± 0.5	27.0 ± 0.5	25.5 ± 0.2
Ecklonia cava	24.5 ± 0.7	26.5 ± 0.3	26.5 ± 0.7	26.5 ± 0.6
Endrachne binghamiae	7.0 ± 0.6**	16.0 ± 0.2**	25.0 ± 0.6	27.0 ± 0.5
Hizikia fusiformis	26.0 ± 0.4	26.0 ± 0.7	25.5 ± 0.5	27.5 ± 0.5
Ishige sinicola	5.0 ± 0.3**	4.0 ± 0.9**	26.0 ± 0.8	26.0 ± 0.2
Kjellmaniella crassifolia	26.0 ± 0.8	27.5 ± 0.8	28.0 ± 0.8	25.5 ± 0.3
Sargassum confusum	32.0 ± 0.1	38.5 ± 0.4*	26.5 ± 0.7	25.0 ± 0.4
Sargassum hornei	27.0 ± 0.9	28.0 ± 0.5	27.0 ± 0.6	26.0 ± 0.7
Sargassum sagamianum	27.5 ± 0.4	27.5 ± 0.5	26.0 ± 0.2	26.5 ± 0.5
Sargassum thunbergii	28.0 ± 0.5	27.0 ± 0.6	25.5 ± 0.2	28.0 ± 0.6
Scytosiphon lomentaria	28.5 ± 0.1	25.5 ± 0.7	25.0 ± 0.3	26.0 ± 0.5
Undaria pinnatifida	29.0 ± 0.6	25.0 ± 0.8	29.5 ± 0.5	25.0 ± 0.4
Rhodophyta				
Carpopeltis affinis	27.0 ± 0.5	27.5 ± 0.9	25.5 ± 0.4	26.5 ± 0.5
Chondrus ocellatus	28.0 ± 0.6	28.0 ± 0.5	26.5 ± 0.7	26.0 ± 0.5
Corallina pilulifera	28.5 ± 0.4	28.0 ± 0.6	24.0 ± 0.2	27.5 ± 0.4
Gigartina intermedia	27.5 ± 0.5	29.0 ± 0.7	24.5 ± 0.5	28.5 ± 0.6
Grateloupia prolongata	26.5 ± 0.7	27.0 ± 0.8	29.5 ± 0.3	29.0 ± 0.5
Grateloupia turuturu	27.0 ± 0.6	25.5 ± 0.9	29.0 ± 0.2	26.5 ± 0.4
Hypnea charoides	27.5 ± 0.5	26.0 ± 0.8	28.0 ± 0.5	28.0 ± 0.1
Pachymeniopsis elliptica	26.0 ± 0.6	26.5 ± 0.4	37.0 ± 0.4	33.0 ± 0.5
Porphyra yezoensis	25.5 ± 0.7	28.0 ± 0.5	26.5 ± 0.3	27.0 ± 0.3

[1]Reference culture reached $24.0 \pm 0.7 \times 10^5$ cells/mL. [1]Cell numbers presented are $\times 10^5$. [1]Data are the means ±SD from three independent assays. *$P<0.01$ and **$P<0.001$ as compared with control.

suecica, was tested at different concentrations in the culture medium (Fig. 1). At 1.0 mg/mL, the water extract demonstrated a strong growth enhancement, up to two-fold relative to the reference culture without the extract. By contrast, the methanol extract at 1.0 mg/mL inhibited growth to 0.3-fold of the reference culture.

Growth rate

The addition of the water extract at day 0, day 2, and day 4 of the culture of *T. suecica* activated cell growth (data not shown). At the stationary phase, on day 8, the cell density was 4.2×10^6 cells/mL with the addition of the extract at day 0, compared with 2.3×10^6 cells/mL in the reference culture. Not only the final cell mass of the culturebut also the specific growth rate was increased. The specific growth rate of the microalga was increased from 1.39/d in the reference culture to 1.76/d with the addition of the extract. Cell size (20 μm in diameter) was not different between cultures with and without *M. nitidum* extracts.

Gross biochemical composition

Biomass dry weight was measured from approximately 3×10^4 cells after adjusting the cell numbers from the stationary phase culture with and without the water extract of *M. nitidum* (1 mg/mL). The values were calculated on a dry weight per cell basis. The dry weights of cells cultured with and without the water extract were similar (Table 2). The amounts of the gross biochemical composition of carbohydrate, chlorophyll *a*, lipid, and protein were also similar between the cells cultured with and without the water extract. Cellular fatty acid profiles were determined to ascertain if the lipid quality of the microalga as a feed organism had been altered by the addition of the water extract. The proportion of EPA (6.5-6.7%)

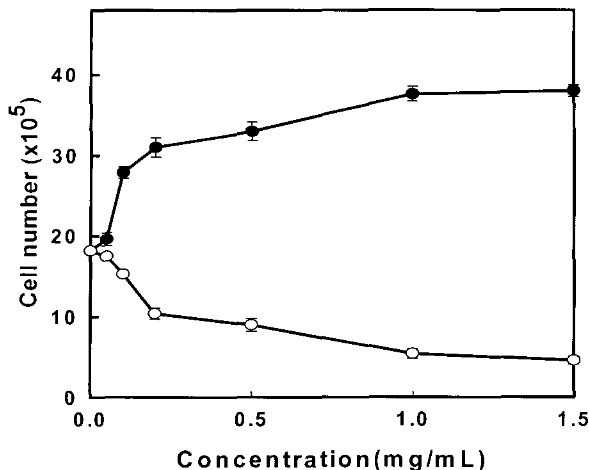

Fig. 1. Effect of extracts of the seaweed *Monostroma nitidum* on cell growth of the microalga *Tetraselmis suecica*. Each methanol extract (open circles) and water extract (solid circles) was added to f/2 medium with 1.2×10^5 cells/mL and cultured at 18℃ for 4 d. Values are means ± SD (n=3).

Table 2. Gross biochemical composition of *Tetraselmis suecica* cultured for 8 d in f/2 medium with and without the water extract of *Monostroma nitidum* (1 mg/mL)[1]

Parameter	*T. suecica* cultured without the extract	*T. suecica* cultured with the extract
Dry weight	2.0×10^{-8}	2.2×10^{-8}
Carbohydrate	4.2×10^{-10}	4.3×10^{-10}
Chlorophyll *a*	1.5×10^{-12}	1.8×10^{-12}
Lipid	3.1×10^{-9}	4.0×10^{-9}
Protein	4.6×10^{-9}	3.9×10^{-9}

[1]Values are expressed as g dry weight/cell.

was similar between lipids from *T. suecica* cultures with and without the addition of the water extract (data not shown). The sums of saturated, monoenoic, and polyunsaturated fatty acids were also similar.

Digestion efficiency

Digestion efficiencies of Pacific oysters, blue mussels, and Japanese littleneck clams were measured when fed *T. suecica* cultured with and without the water extract. The amount of phaeopigment in the bivalves relative to the uptake amount of chlorophyll *a* from the alga was stable after 5 hr, 5 hr, and 6 hr for the oyster, mussel, and clam, respectively. At these times, the digestion efficiency values were very similar for the cultures of the microalga with and without the addition (Table 3). Thus, no difference in bivalve feeding efficiency was evident when fed *T. suecica* cultured with and without the extract.

The gross biochemical composition and fatty acid profile of the cells were shown to vary with growth phase (Zhu et al., 1997) or nutrient concentration (Otero and Fabregas, 1997). However, in this experiment, no cellular changes were evident between cultures with and without the water extract of the seaweed *M. nitidum*, even though the stationary cells were twice as dense in the culture with added extract. This seaweed is edible and can now be aquacultured. The water extract has been shown to inhibit growth of the green alga *Enteromorpha prolifera* thallus (Cho et al., 2001) and to decrease the amounts of total cholesterol and low density lipoprotein (Jung et al., 1997). Further research is needed to elucidate the mechanism of growth enhancement in *T. suecica* cultures.

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Table 3. Digestion efficiency of bivalves fed *Tetraselmis suecica* cultured with and without the water extract of *Monostroma nitidium* (1 mg/mL)[1]

Bivalve species	*T. suecica* cultured without the extract	*T. suecica* cultured with the extract
Crassostrea gigas (Pacific oyster)	711/1248 (56%)	794/1346 (58%)
Mytilus edulis (Blue mussel)	1023/1624 (63%)	921/1530 (60%)
Venerupis hilippinarum (Japanese litteneck clam)	338/584 (58%)	341/610 (56%)

[1]Efficiency is expressed as the digested amount of phaeopigment (μg/L) per ingestion of chlorophyll *a* (μg/L).

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