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The Sterolic Properties of Heterotrophic *Tetraselmis suecica*

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The heterotrophic production method for *Tetraselmis suecica*, a suggested alternative to photoautotrophic one in the economic sense, was studied in terms of cell growth and sterolic property. The alga in the 10 mM organic carbon (glucose) manifested cell growth. However, the alga produced by the heterotrophic method showed a unique property of sterol determined with an aid of GC and GC-MS. The photoautotrophic control *T. suecica* contained 6 detectable sterol species: cholesta-5,22-dien-3β-ol, ergost-5-en-3β-ol, cholest-5-en-3β-ol, 24-methylcholesta-5,22-dien-3β-ol, 24-methylcholesta-5,24-dien-3β-ol, 24-ethylchlolesta-5,24-dien-3β-ol, 24-methylcholesta-5-en-3β-ol, and 24-ethylchlolesta-5en-3β-ol. We discuss the sterolic properties of the alga along the heterotrophic progress, particularly focusing on the availability of the method in the aquaculture of bivalves which normally need sterols as a dietary source.

Key words: Heterotrophic culture, *Tetraselmis suecica*, Sterols, Bivalve aquaculture

Introduction

Some microalgae have an ability to grow heterotrophically as well as autotrophically. These algae utilize organic carbons and other nutritious compounds under dark condition when appropriate growth elements are served in the culture medium. The heterotrophic behavior of alga has drawn attention because it can be a cost-effective alternative to photoautotrophic one by offering the possibility of volumetric cell growth under organic carbon-enriched culture media (Chen and Johns, 1995; Chen, 1996). While some species in the method has economic potential for hatchery-based shellfish seed production (Barclay et al., 1994; Shi et al., 1997), a problem still remains unsolved. One of the controversial key issues is that the method often results in less production of specific compounds probably from generation of senescent cells (Wright et al., 1980; Tsavalos and Day, 1994).

Phytosterol composition of an alga is extremely important when the item is considered as a food for bivalve aquaculture because sterols are essential in the nutrition of all animals, although many invertebrates possess only a limited capacity for the synthesis (Nes and McKean, 1977; Holden and Patterson, 1991; Teshima, 1991; Danton et al., 1999; Nes, 2000; Park et al, 2002). The importance of alga-contained sterols was also well documented by Wikfors et al. (1991) who reported that dietary sterols did influence the growth of juvenile eastern oyster more significantly than essential fatty acids by showing that the algal sterols (amount and composition) accounted 35% of the difference observed in the oyster growth between the algae, compared with fatty acids which were responsible for 28% of the difference.

Previously, we found that the heterotrophic behavior of two useful microalgae (*Isochrysis galbana* and *Tetraselmis suecica*) were manifest although they lost sterol composition along their apparent growing activities (Park et al., 2002; Jo et al., 2004). Here, we detail the heterotrophic behavior of *T. suecica* in different organic carbon concentrations and their ensuing change in phytosterols which are essential for normal bivalve growth.

Materials and Methods

Algal culture

Heterotrophic method for the algal culture was based on our previous way (Park et al, 2002; Jo et al., 2004). In brief, a *Tetraselmis suecica* strain was cultured in 3 L round flasks in the f/2 medium

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as inocula under illumination with photosynthetic active radiation (PAR) at about 140 mol $m^{-2}s^{-1}$ and light cycle 24L:0D. The inocula at mid-logarithmic growth phase were added in the f/2 medium to make the cell concentration, 5.21×10^5 cells mL^{-1} and contained in 5 liter round flask enriched with different glucose concentrations 0, 5, 10, 25, and 50 mM glucose in f/2 medium. The cultures were agitated and aerated with filtered air (Midisart 2000, 0.45 m TPFE) and maintained illumination-free at 20± 0.5℃ for three weeks. A special care was given to all the cultures to keep them from being contaminated. The cultures replicated in 3 times were sacrificed weekly for a sterol analysis after determination of cell growth with the aid of haematocytometer.

Sterol analysis

Sterols were analyzed by the method of Jo et al. (2004) which was slightly modified from Vron et al.'s (1996a). Samples were obtained by centrifugation (5,000×g for 5 min), and freeze-dried. Freeze-dried biomass (.0 g) was extracted in 2:1 (v:v) dichloromethane-methanol using a sonicator. The extracted samples were applied to Vron et al.'s (1996a) method to locate free sterol (FS), steryl ester (SE), steryl glycoside (SG), and acylated steryl glycoside (ASG) on TLC (Merck 60 F254) with the aid of standard *Rf* values. The samples were saponified for SE and hydrolyzed for ASG and SG as described in Vron et al. (1996a, b). After acetylation, free and conjugated sterols were applied to GC (HP 6890) equipped with a FID (flame-ionization detector) a column (HP-1). The carrier gas was operated at 1 mL min-1 of helium. The temperature was raised from 150 to 300℃ at 5℃ min^{-1} and held at 300℃ for 20 min. Injector and detector temperatures were 28℃ and 320℃, respectively. Sterols were further identified by gas chromatography-mass spectrometry (GC-MS) by comparing the mass spectra with literature and a database, while quantification was based on an internal standard, 5*a*-cholestane.

Statistical analysis

Statistical analysis of the data was carried out, using the Student's *T*-test.

Results and Discussion

Fig. 1 shows weekly growth curves of photoautotrophic and heterotrophic *Tetraselmis suecica.* The photoautotrophic algal batch maximized itself by week 1 and then decreased to total breakdown

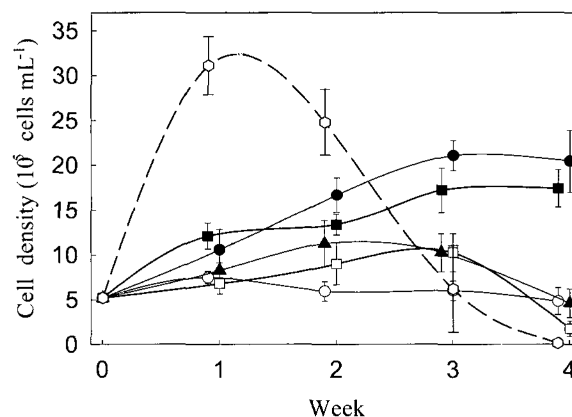

Fig. 1. Effect of glucose concentrations added on the heterotrophic behavior of *Tetraselmis suecica*. The alga shipped from CCMP (USA) was cultured under the compartmentalized illumination-free culture chambers of NFRDI Food Organism Culture Room. The cultures were aerated with filtered air and maintained under 20±0.5℃ for 4 weeks. The hexagon with dashed line represents photoautotrophic control. The other symbols with solid lines represent heterotrophic growth; white circle for glucose-free control, solid rectangle for 5 mM glucose, solid circle for 10 mM glucose, solid triangle for 25 mM glucose, and white rectangle for 50 mM glucose. Vertical bars represent standard error.

by week 4. The heterotrophic growth of the alga was clearly different from photoautotrophic one. In our culture system the cell growth of heterotrophic *T. suecica* was significantly affected by concentration of the organic carbon added. The cell growth difference was particularly significant from week 1 ($p< 0.05$) when comparison was made between concentration 10 mM and concentrations higher than 25 mM. The concentration-dependent difference more significant with time. For example, the differences after week 3 were of significance of $p<0.01$. Unlike in the fermentor (Chen, 1996), most apparent growth was in the 10 mM glucose experiment in our system. In the optimum growth condition, the cell growth was slow and steady for the first 3 weeks, reaching the maximum concentration, about 20×10^5 cells mL^{-1} by week 3. However, the cell growth failed to reach the the photoautotrophic control growth gained by the first week, about 30×10^5 cells mL^{-1}.

The glucose-free heterotrophic control remained constant for 4 weeks. Because of our weekly based measurement we could not determine the day, but it appeared that the alga needed at least days of time

to have it phase-changed from photoautotrophic to heterotrophic behavior. Similar results were obtained in heterotrophic *Isochrysis galbana* (Park et al., 2002). Unlike the phase change from photoautotroph to heterotroph, phase change vice versa was more rapid in *T. suecica* (Jo et al., 2004).

Table 1 summarizes the sterol composition along the progress of heterotrophic behavior of the alga. The alga at logarithmic growth (week 0) contained 6 sterols showing more than 1% of cholesta-5,22-dien-3β-ol (the most abundant sterol species at week 0); namely, cholesta-5,22-dien-3β-ol, ergost-5-en-3β-ol, cholest-5-en-3β-ol, 24-methylcholesta-5,22-dien-3β-ol, 24-methylcholesta-5,24-dien-3β-ol, and 24-ethylchlolesta-5,24-dien-3β-ol. The alga also showed two infinitesimal species, 24-methylcholesta-5-en-3β-ol and 24-ethylchlolesta-5-en-3β-ol at week 0. The relative amount of the total sterol to cholesta-5,22-dien-3β-ol, 295.5, increased to 321.0 by week 1. Thereafter, the relative amount decreased markedly, 222.5 by week 2, and 61.5 by week 3.

Sterols are known to play an essential structural role in the lipid core of biological membranes. They also do metabolic function, such as regulators of membrane fluidity, substrates for the synthesis of numerous secondary metabolites, and biogenetic precursors of growth substances in the normal plant cells (Wojciechowski, 1991). However, in our research, the sterol level of *T. suecica* started to decrease as soon as the alga was set in the heterotrophic condition. The sterol level remained 59-68% of the control level by week 1. The decrease was more marked the rates of 14-45% by week 2, 8-18% by week 3, and 5-16% by week 4, depending on the growing activities coming from different organic carbon concentrations in the culture medium. Regarding the sterolic species, they differed from that studied previously (Vron et al., 1998) who found 24-methylcholesta-5,24 (24^1)-dien-3β-ol and 24-methylcholest-5-en-3β-ol most abundant in a strain of *T. suecica.* Previous works dealing with *Tetraselmis* sterol contents showed the genus seemed to be dividable into three sub-groups on the basis of major sterol species. In fact, Patterson et al. (1993) analyzed 11 *Tetraselmis* clones and compiled their results with others (Lin et al., 1982; Ballantine et al., 1979; Volkman, 1988). Therefore it is suggested that compositional difference of the algal sterol might be reflected from the differences in strain and growing history.

When the algal cell growth was compared with the sterol level, it was closely related with the sterol level in the photoautotrophic *T. suecica* (Fig. 1 and Table 1). One of the interesting points is that unlike in the photoautotrophic alga, the growth of the heterotrophic alga was not clearly related sterol level. The sterolic composition decreased with the commencement of the heterotrophic behavior, instead. Interestingly, the period the sterol decreased most significantly was while heterotrophic behavior was active in the 10 mM experiment (see Fig. 1 and Table 1). Similar results were also found in the heterotrophic growing *I. galbana* (Park et al., 2002), suggesting that algal cells growing heterotrophically do not require sterol level as highly as photoautotrophically

Table 1. The change of relative sterol composition in the heterotrophic *Tetraselmis suecica*

Culture method	Total sterol[1]				
	Week				
	0	1	2	3	4
Photoautotrophic control	295.5	321.0	222.5	61.5	-[2]
Heterotrophic culture					
at 0 mM glucose	295.5	201.6	47.5	25.6	29.9
at 5 mM glucose	295.5	221.9	132.1	52.5	43.0
at 10 mM glucose	295.5	188.0	55.0	49.0	48.5
at 25 mM glucose	295.5	194.8	43.2	23.6	21.2
at 50 mM glucose	295.5	174.7	47.4	27.4	15.3

[1]Total sterol is a sum of Cholesta-5,22-dien-3β-ol, Cholesta-5,22-dien-3β-ol, Ergost-5-en-3β-ol, Cholest-5-en-3β-ol, 24-Methylcholesta-5,22-dien-3β-ol, 24-Methylcholesta-5,24-dien-3β-ol, 24-Ethylcholesta-5,24-dien-3β-ol, 24-Methylcholesta-5-en-3β-ol, and 24-Ethylcholesta-5-en-3β-ol in terms of relative composition expressed as a percent chromatogram peak area to a peak area of cholest-5,22-dien-3β-ol at day 0 multiplied by 100.
[2]Failed to detect due to immeasurable cell counts.

growing cells for their growing activity.

Besides the nutritional importance, algal sterol composition is species specific, thus being used as a biomarker for culture condition (Wright et al., 1980) and species (Véron et al., 1996a; Véron et al., 1998; Leblond and Chapman, 2000, 2002). It has a unique metabolic pathway. For the biosynthesis of sterols, plants have a unique primary metabolism from animals for sterol biosynthesis. Unlike animals that synthesize the C27 cholestane-based members of the steroid family, plants synthesize C28 and C29 compounds in which an extra ethyl or methyl group is added to carbon-24 of the sterol side chain (Nes, 2000). In spite of its intrinsic metabolism, it is reasonable to expect that the sterol composition can be influenced by sterol methyltransferases (SMTs) which are principally influenced by physiological status of organisms (Nes et al., 1990, Nes, 2000).

This finding that the heterotrophic behavior of the alga is evident without cellular requirement of phytosterols can further explain the objectionable aspect of this method. This is particularly bringing some significant implication in the bivalve hatcherists who are interested in the introduction of heterotrophic method for bivalve aquaculture, arguing with the suggested heterotrophic potential as an alternative to photoautotrophic culture.

References

Ballantine, J.A., A. Lavis and R.J. Morris. 1979. Sterols of phytoplankton: effects of illumination and growth stage. Phytochemistry, 18, 1459-1466.

Barclay, W.R., K.M. Meager and J.R. Abril. 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J. Appl. Phycol., 6, 123-129.

Chen, F. and M.R. Johns. 1995. A strategy for high cell density culture of heterotrophic microalgae with inhibitory substrates. J. Appl. Phycol., 46, 43-47.

Chen, F. 1996. High cell density culture of microalgae in heterotrophic growth. TIBTECH, 14, 421-426.

Danton, E. B. Vron and M. Mathieu. 1999. Influence of diet level on sterols of diploid and triploid oysters *Crassostrea gigas* (Thunberg). J. Exp. Mar. Biol. Ecol., 233, 259-267.

Holden, M.J. and G.W. Patterson. 1991. Absence of sterol biosynthesis in oyster tissue culture. Lipids, 26, 81-82

Jo, Q., E.J. Choy, D.W. Park and B.Vron. 2004. Sterol dynamics of heterotrophic *Tetraselmis suecica* and its nutritional implication in the bivalve aquaculture. Aquacult. Res., 35, 371-377.

Leblond, J.D. and P.K. Chapman. 2000. Sterols as biomarkers in *Gymnodinium breve*: Distribution in dinoflagellates. J. Phycol., 36, Suppl. 42.

Leblond, J.D. and P.J. Chapman. 2002. A survey of the sterol composition of the marine dinoflagellates *Karenia brevis*, *Kerena mikimotoi*, and *Karlodinium micrum*: distribution of sterols within other members of the class Dinophyceae. J. Phycol., 38, 670-682.

Lin, D.S., A.M. Ilias, W.E. Connor, R.S. Caldwell, H.T. Cory and G.D. Daves. 1982. Composition and biosynthesis of sterol in selected marine phytoplankton. Lipids, 17, 818-824.

Nes, W.R. and M.L. McKean. 1977. Biochemistry of steroids and other isopentenoids. University Park Press, Baltimore, pp. 629.

Nes, W.D., R.A. Norton, F.G. Crumley, S.J. Madigan and E.R. Katz. 1990. Sterol phylogenesis and algal evolution. Proc. Natl. Acad. Sci. USA., 87, 7565-7569.

Nes, W.D. 2000. Sterol methyl transferase: enzymology and inhibition. Biochim. Biophys. Acta, 1529, 63-88.

Park, D.W., Q. Jo, H.J. Lim and B. Vron. 2002. Sterol composition of dark-grown *Isochrysis galbana* and its implication in the seed production of Pacific oyster, *Crassostrea gigas*. J. Appl. Phycol., 14, 351-355.

Patterson, G.W., E. Tsitsa-Tzardis, G.H. Wikfors, P.K. Gladu, D.J. Chitwood and D. Harrison. 1993. Sterols of *Tetraselmis* (Prasinophyceae). Comp. Biochem. Physiol., 105, 253-256.

Shi, X.M., F. Chen, J.P. Yuan and H. Chen. 1997. Heterotrophic production of lutein by selected *Chlorella* strains. J. Appl. Phycol., 9, 445-450.

Teshima, S. 1991. Sterols of Crustaceans, Molluscs and Fish. In: Physiology and Biochemistry of Sterols, Patterson G.W. and W.D. Nes, eds. American Oil Chemists' Society, Champaign, Illinois, pp. 229-256.

Tsavalos, A.J and J.G. Day. 1994. Development of media for the mixotrophic/heterotrophic culture of *Brachiomonas submarina*. J. Appl. Phycol., 6, 431-433.

Véron, B., J.C. Dauguet and C. Billard.. 1996a. Sterolic biomarkers in marine phytoplankton. I. Free and conjugated sterols of Pavlova lutheri (Haptophyta). Eur. J. Phycol., 31, 211-215.

Véron, B., C. Billard, J.C. Dauguet and M.A. Hartmann. 1996b. Sterol composition of *Phaeodactylum tricornutum* as influenced by growth temperature and light spectral quality. Lipids, 31, 989-994.

Véron, B., J.C. Dauguet and C. Billard. 1998. Sterolic biomarkers in marine phytoplankton. II. Free and conjugated sterols of seven species used in mariculture. J. Phycol., 34, 273-279.

Volkman, J.K., H.R. Burton, D.A. Everitt and D.I. Allen. 1988. Pigment and lipid compositions of algal and bacterial communities in Ace Lake, Vestfold Hills, Antartica. Hydrobiol., 165, 41-57.

Wikfors, G.H., P.K. Gladu and G.W. Patterson. 1991. In search of the ideal algal diet for oysters: Recent progress, with emphasis on sterols (Abstract). J. Shellfish Res., 10, 292.

Wojciechowski, Z.A. 1991. Biochemistry of phytosterol conjugates. In: Physiology and Biochemistry of

Sterols, Patterson G.W. and W.D. Nes, eds. American Chemists' Society, Champaign, Illinois, pp. 361-395.
Wright, D.C., L.R. Berg and G.W. Patterson. 1980. Effect of cultural conditions on the sterols and fatty acids of green algae. Phytochemistry, 19, 783-785.

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