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Effects of Environmental Factors on Cyanobacterial Production of Odorous Compounds: Geosmin and 2-Methylisoborneol

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Geosmin and 2-methylisoborneol (2-MIB), responsible for earthy or musty smell, are a major concern for safe drinking water supplies. This study investigated the effects of environmental factors on odorous compound production and cell growth in cyanobacterial strains. *Anabaena* sp. FACHB-1384, a 2-MIB producer, was sensitive to low temperature (<20°C). However, geosmin producers, *Anabaena* sp. Chusori and *Anabaena* sp. NIER, were sensitive to high light intensity (>100 μmol/m^2/sec), but not to low temperature. Geosmin concentrations increased under higher nitrate concentrations, being linearly proportional to cell density. A P-limited chemostat showed that P-stress decreased the geosmin productivity and extracellular geosmin amount per cell in *Anabaena* sp. NIER. However, only 2-MIB productivity was reduced in *Planktothrix* sp. FACHB-1374 under P-limitation. The extracellular 2-MIB amount per cell remained constant at all dilution rates. In conclusion, high light intensity and P-stress can contribute to the lower incidence of geosmin, whereas 2-MIB reduction could be attainable at a lower temperature.

Keywords: Geosmin, 2-methylisoborneol, chemostat, cyanobacteria, *Anabaena*, *Planktothrix*

Introduction

The naturally occurring compounds geosmin and 2-methylisoborneol (2-MIB) are responsible for musty or earthy tastes and odors in water (Fig. 1). Both geosmin and 2-MIB have extremely low odor thresholds to humans, below 10 ng/l [1]. Geosmin and 2-MIB do not pose a health risk; however, the presence of these compounds in drinking water can lead to consumer distrust. The odorous compounds (OCs) in drinking water supplies have been a major concern throughout the world. These OCs frequently appear in aquatic systems and result in strong odor even at extremely low concentrations. In drinking water treatment plants, common processes such as screening, flocculation, sedimentation, and chlorination did not prove useful for their complete removal [2, 3]. Geosmin and 2-MIB are produced by some cyanobacteria and actinomycetes [4]. Cyanobacteria that have been associated with OCs include *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Oscillatoria*, *Phormidium*, *Planktothrix*, and *Pseudanabaena* [5]. *Anabaena* is a common geosmin-producing genus in eutrophic lakes and rivers [6, 7]. In the case of 2-MIB, *Planktothrix* is a major OC-producing genus [8], but the incidence of 2-MIB problems has been much less frequent than that of geosmin. *Planktothrix*, accordingly, has not been scrutinized as much as *Anabaena*.

Phylogenetic and evolutionary studies of the geosmin synthesis gene (*geoA*) recently reached to suggest that this gene has been spread by horizontal gene transfer and has a common origin with the 2-MIB gene [9]. qPCR for *geoA* showed a better correlation with geosmin concentration than *Anabaena* cell counting data [10]. Compared with such achievement in genetic studies, physiological research is still bewildering. Universal, optimal conditions for cyanobacterial OC production have not been found. It is likely that different strains have their own specific optimal conditions.

Temperature and light intensity are the major factors that affect the growth of cyanobacteria. Most cyanobacteria are known to prefer high temperature (>20°C) for growth, but *Anabaena spiroides* can grow even at temperatures lower than 8°C [11]. Many filamentous cyanobacteria have the

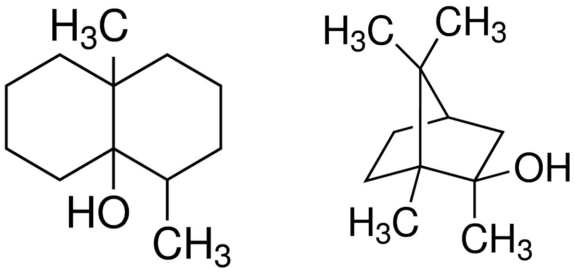

Fig. 1. Structures of geosmin and 2-methylisoborneol (2-MIB).

ability to adapt to a low temperature by controlling the levels of pigments [12]. An inverse relation between the chlorophyll and geosmin content and photon influence rate was found, suggesting a similar biosynthetic pathway for these two compounds [13]. Although previous works examined the effects of light intensity and temperature, they investigated each factor separately, but not a combined effect of light and temperature [7, 14]. Since the interaction effect between different environmental factors could complicate the interpretation of the experimental results, PhotoBiobox [15] was used to get a whole combination of light intensity and temperature. PhotoBiobox, a high-throughput, tablet-sized, low-cost photobioreactor, is equipped with a controllable LED array and a combination of four water-cooling blocks offering different light intensities and temperatures. Because a wide range of light intensity and temperature could be realized in a small unit (96-well plate), it makes it possible to screen an optimal condition with the least effort.

The importance of nitrogen and phosphorus in bloom formation has been reported repeatedly [16, 17]. Phosphorus and nitrogen levels in aquatic habitats are the main factors promoting the odor problem caused by *Anabaena* spp. [14]. However, the growth of nitrogen-fixing cyanobacteria such as *Anabaena* spp. was shown to be dependent on phosphorus source [18]. This led us to look into the effect of phosphorus on OC-producing cyanobacteria by using a P-limited chemostat. Although a batch culture is commonly used in the comparison of environmental effects on growth, the physiological status is not constant but changing instantaneously. A chemostat has the benefit of culturing cyanobacteria in a steady state by limiting one environmental factor, while keeping the others sufficient [19]. Furthermore, a chemostat made it possible to maintain a stable culture of OC-producing cyanobacteria, which grew slowly and often became unstable in batch cultivation.

In this study, we optimized and investigated the effects of temperature and light intensity in a PhotoBiobox. The effects of nitrogen and phosphorus concentrations on OC production by cyanobacteria were also examined in batch and continuous culture systems.

Materials and Methods

Microalgae Collection and Preculture

Anabaena sp. Chusori was isolated in August 2014 from Daecheong Reservoir in Korea by the micropipetting method [20]. *Anabaena* sp. NIER was obtained from the National Institute of Environmental Research, Korea. It was isolated from North Han River in Korea. *Anabaena* sp. FACHB-1384 and *Planktothrix* sp. FACHB-1374 were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, China. These were isolated from Lushui Reservoir and Shang Lake in China, respectively. All OC-producing microalgae strains were precultivated in 250 ml culture flasks (Cell Culture Flask, SPL, Korea) with BG11 medium under a light intensity of 60 μmol/m^2/sec without shaking.

Detection of Odorous Compounds

GC/SPME analysis needs preprocessing for high sensitivity. The assay sample (1 ml) was prepared and transferred to a 15 ml vial (15 ml Clear Vial, Screw Top Hole Cap with PTFE/Silicone Septa; Supelco, USA) with a magnetic bar. The vials were incubated in a heating block at 40°C for 30 min with continuous stirring at 400 rpm on a hotplate stirrer (MSH-20D; Daihan, Korea), which promoted the accumulation of gaseous compound in the vial headspace. Polydimethylsiloxane (PDMS)-coated fiber (Stableflex 2 cm SPME Fiber PK3, 50/30 μm DVB/Carboxen; Supelco, USA) was inserted into the vial headspace until the absorption equilibrium of volatile organic compounds was reached. After extraction, the fiber with the absorbed compound was inserted in a GC injector at 250°C, 5 min for thermal desorption. An HP-5MS (Agilent Technologies, USA) column was equipped for the GC/SPME analysis by gas chromatography (GC-2010 Plus; Shimadzu, Japan). The GC/SPME analytical conditions are shown in detail in Table 1 [21].

Table 1. Analytical conditions of GC/SPME for detection of odorous compounds [21].

GC	
Column	HP-5MS, 30 m × 0.25 mm I.D., 0.25 μm
Oven	50°C (5 min) to 250°C at 10°C/min
Carrier gas	Helium 34 cm/sec
Detector	FID, 300°C
SPME	
SPME fiber	30 μm PDMS (#57309)
Extraction	Headspace, 30 min with stirring, 40°C
Desorption process	5 min, 250°C

Solid-state geosmin (16423-19-1; Sigma-Aldrich, USA) and 2-MIB (2371-42-8; Sigma-Aldrich) were dissolved in methanol (67-56-1; Honeywell, Korea) to obtain a standard solution of 10 g/l. Standard curves were generated for OC quantitative analysis by serially diluting the standard with distilled water and then measuring by the GC/SPME method. Geosmin and 2-MIB standards were detected at 17.53 min and 13.89 min retention time (RT), respectively.

The geosmin peak at 17.53 min RT was detected for *Anabaena* sp. NIER and *Anabaena* sp. Chusori, but no 2-MIB peak was detected. On the contrary, the 2-MIB peak at 13.89 min RT was detected in *Anabaena* sp. FACHB-1384 and *Planktothrix* sp. FACHB-1374. Therefore, *Anabaena* sp. NIER and *Anabaena* sp. Chusori were confirmed as geosmin-producing cyanobacteria, and *Anabaena* sp. FACHB-1384 and *Planktothrix* sp. FACHB-1374 were 2-MIB-producing cyanobacteria.

Combined Effect of Light and Temperature on the Growth of OC-Producing Cyanobacteria: Application of the PhotoBiobox System

PhotoBiobox [15] was used to investigate the effect of light intensity and temperature in three OC-producing cyanobacteria. In a 96-well plate (08815014; Corning, USA) of the PhotoBiobox system, each well can have a different light intensity and temperature without interference from neighboring wells. Moreover, membrane impermeable to water but permeable to gas and light (BEM-1; Diversified Biotech, USA) kept moisture from evaporating. The vertical axis of the 96-well plate was set for the light intensity gradient and the horizontal axis for the temperature gradient. The light intensity and temperature conditions are shown in detail in Fig. 2.

Anabaena sp. NIER, *Anabaena* sp. Chusori, and *Anabaena* sp. FACHB-1384 were inoculated when these strains were at steady state (0.188 d^{-1} dilution rate) in a chemostat with a cell density of 2.4×10^6 cell/ml. Each culture (0.2 ml) was inoculated separately in each well, and cell growth was monitored by measuring the optical density (OD_{680}) using a 96-well plate reader (Sunrise, Tecan, Austria). The initial OD_{680} value was around 0.4 and cells were cultivated in PhotoBiobox for 60 h.

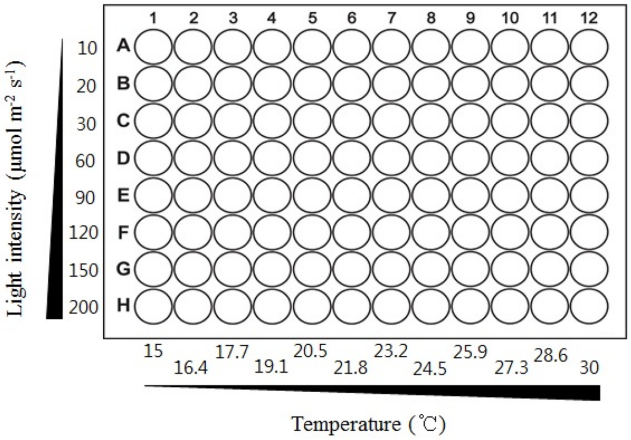

Fig. 2. Light intensity and temperature condition of PhotoBiobox.

Growth and OC Production under Different Nitrogen Concentrations

Geosmin production was measured in *Anabaena* sp. NIER and *Anabaena* sp. Chusori under different nitrogen concentrations with a light intensity of 50 μmol/m^2/sec and temperature of 25°C. Cultivation was performed in 250 ml flasks containing 160 ml of cell-mixed medium in batch culture. At each sampling point, 4 ml of culture was collected for odor analysis. Cells in the samples were disrupted by a bead beater (0.5 mm, 0.1 mm silica bead mix) and sonicated for 10 min to measure total geosmin. Extracellular geosmin was measured by using filtered culture media. Nitrate was used as a nitrogen source in the BG11 medium for cyanobacteria. Geosmin production was compared in three different nitrate conditions: N 250 (original BG11 nitrate condition, 250 mg/l), N 2.5 (BG11 100× diluted nitrate condition, 2.5 mg/l), and N- (nitrate-free condition). N 2.5 was similar to eutrophic water nitrogen concentration.

Chemostat Culture under P-Limitation

Four OC-producing cyanobacterial strains showed slow and unstable growth in batch cultures. Therefore, a continuous cultivation system (chemostat) was used for their stable growth. It helped to gather a large volume of samples and continuous data in a steady state. Chemostat reactors were installed with gas and culture medium in-out ports in 1-L glass wide-mouth bottles (GLS80; Duran, Germany). The working volume was 800 ml and the light intensity was 80 μmol/m^2/sec. We set the higher light intensity than previous batch culture because of the higher cell density (≥0.3 g/l). CO_2 was supplied into the chemostat reactor, after mixing with air at a final concentration of 1%, at a rate of 0.1 vvm. Fresh medium was continuously supplied by digital peristaltic pumps (EMP-600A; EMS Tech, Korea) into the chemostat reactor, at four different dilution rates (0.10 d^{-1}, 0.19 d^{-1}, 0.25 d^{-1}, 0.33 d^{-1}). The strains were cultivated at 25°C in well-mixed condition under phosphorus limitation, until they reached a steady state. When the cell density showed no increasing or decreasing trends for three consecutive days and changed no more than 5%, the chemostat was judged to be in a steady state. Ten-times diluted phosphorus concentration was used for P-limitation, which was measured by an ion chromatography system (ICS-1600; Thermo, USA). The dry cell weight (DCW) and OC concentration were measured at steady state for each dilution rate.

Results

Combined Effect of Light and Temperature in the PhotoBiobox System

PhotoBiobox was used to evaluate the effects of light intensity and temperature on the growth of three cyanobacteria

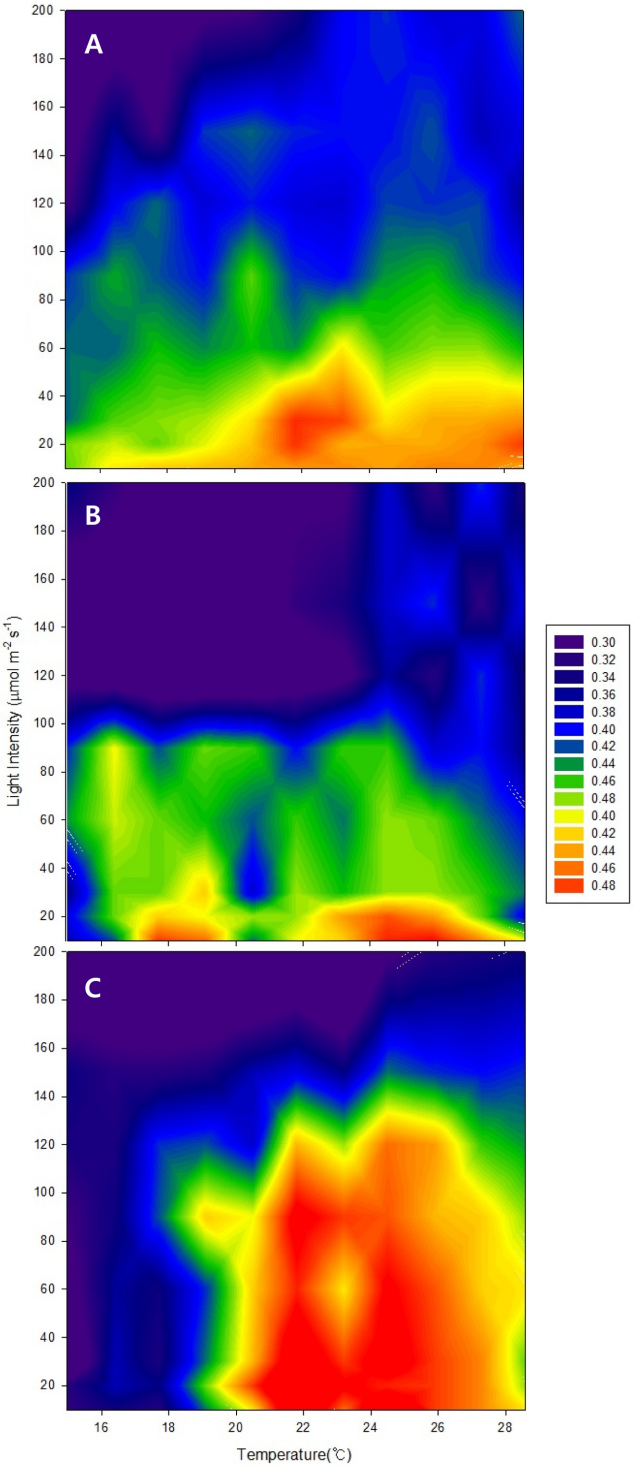

Fig. 3. Optical density (OD_{680}) of *Anabaena* sp. NIER (**A**), *Anabaena* sp. Chusori (**B**), and *Anabaena* sp. FACHB-1384 (**C**) under a light and temperature gradient in PhotoBiobox after 60 h.

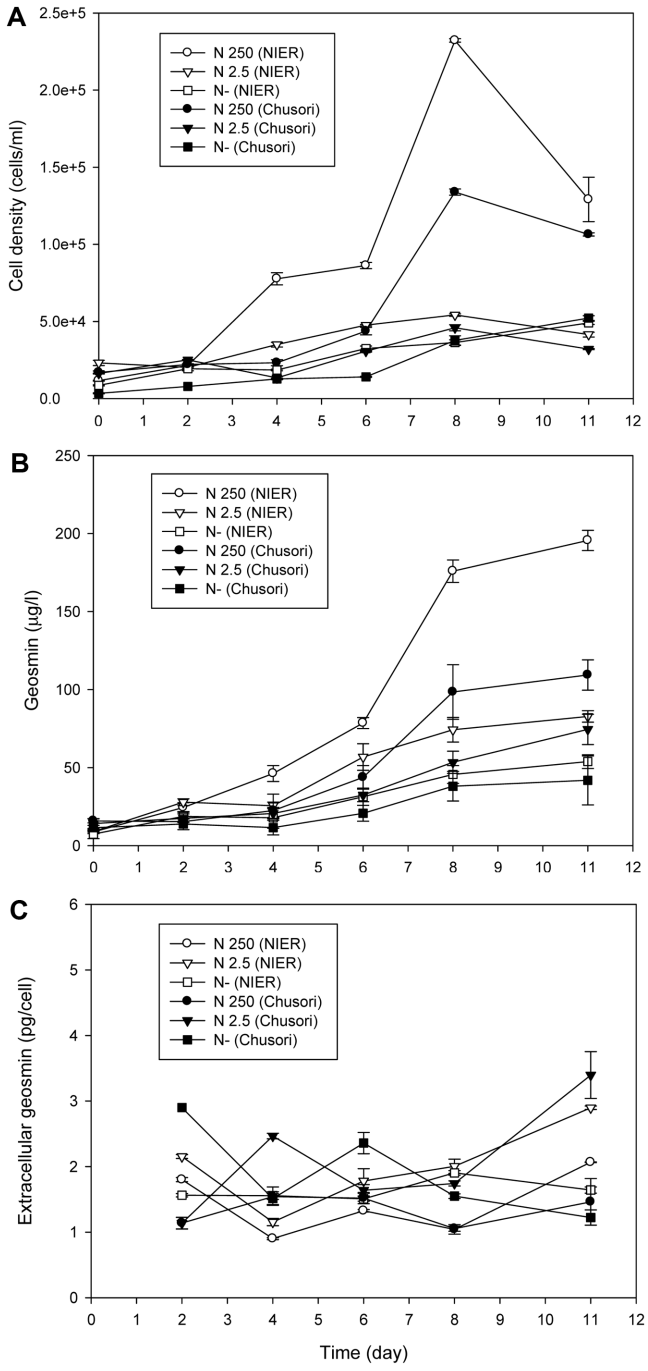

Fig. 4. Cell density (**A**), dissolved geosmin concentration (**B**), and extracellular geosmin per cell (**C**) of *Anabaena* sp. NIER and *Anabaena* sp. Chusori in nitrogen concentrations of 250, 2.5, and 0 (-) mg/l.

(Fig. 3). Two geosmin-producing strains (*Anabaena* sp. NIER and *Anabaena* sp. Chusori) could grow under low light intensity (20–80 μmol/m^2/sec) but were inhibited at ≥100 μmol/m^2/sec. *Anabaena* sp. FACHB-1384, a 2-MIB producer, showed good growth up to 140 μmol/m^2/sec. All three strains exhibited relatively higher growth at

≥20°C. *Anabaena* sp. NIER and *Anabaena* sp. Chusori were well adapted to the low-temperature range. *Anabaena* sp. FACHB-1384 growth was inhibited remarkably at ≤18°C, but well adapted to a wide light intensity range.

Geosmin Production under Different Nitrogen Concentrations

The growth of *Anabaena* sp. NIER and *Anabaena* sp. Chusori was monitored under three different nitrogen concentrations (Fig. 4A). Both strains showed the highest growth in the highest nitrogen concentration (N 250), where the cell density was over 2 × 10^5 cells/ml after 8 days. Little difference in the cell densities was observed in low nitrogen concentrations (N 2.5 and N-), but the cell densities were 10 times lower than that of N 250. Dissolved geosmin concentration was highest (≥100 μg/l) at the N 250 condition in both the *Anabaena* strains (Fig. 4B). At lower N concentrations (N 2.5 and N-), dissolved geosmin concentration was lower than that of the N 250 condition. Although no clear difference was observed in growth curves between the N 2.5 and N- conditions, dissolved geosmin concentrations were different in the stationary phase. Extracellular geosmin amounts per cell were generally similar (1–2 pg/cell) even under different nitrogen concentrations (Fig. 4C).

OC Production in a P-Limited Chemostat

A continuous cultivation system (chemostat) was used for the growth of geosmin-producer *Anabaena* sp. NIER and 2-MIB-producer *Planktothrix* sp. FACHB-1374. The dry cell weight of *Anabaena* sp. NIER decreased slightly with increase in the dilution rate, whereas not much difference was observed in *Planktothrix* sp. FACHB-1374 (Figs. 5A and 5C). A slight increase in OC concentrations was observed in the two strains at higher dilution rates. The extracellular geosmin per cell in *Anabaena* sp. NIER showed increasing trend with the increase in dilution rate (Fig. 5B), but the extracellular 2-MIB per cell in *Planktothrix* sp. FACHB-1374 was not affected by the increase in dilution rate (Fig. 5D). However, the two OC productivities increased with dilution rates, indicating higher productivity of OCs at a faster growth rate. Higher geosmin productivity (ca. 6 times) was observed in *Anabaena* sp. NIER than 2-MIB productivity (ca. 3 times) in *Planktothrix* sp. FACHB-1374, as the dilution rate increased from 0.10 d^{-1} to 0.33 d^{-1}.

Discussion

Under different light and temperature ranges, the growth of *Anabaena* sp. NIER and *Anabaena* sp. Chusori was inhibited

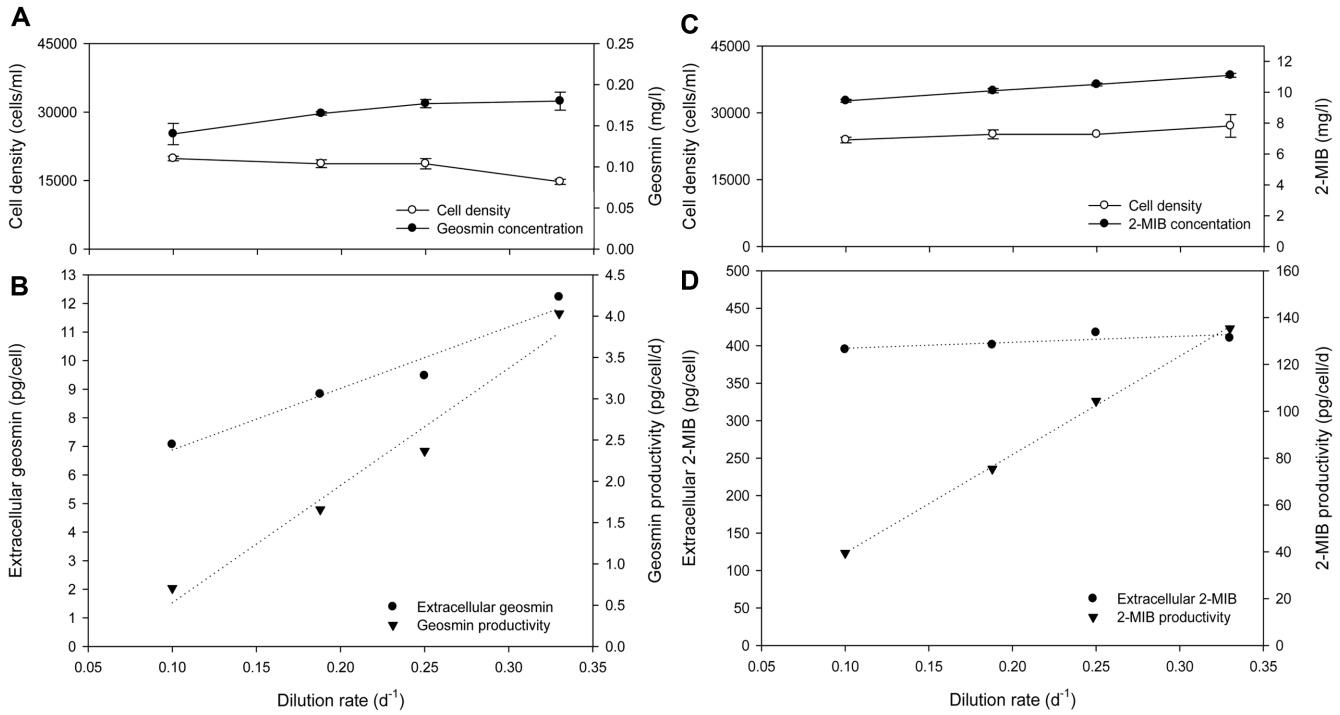

Fig. 5. *Anabaena* sp. NIER cell density, geosmin concentration (**A**), extracellular geosmin per cell, and geosmin productivity (**B**) and *Planktothrix* sp. FACHB-1374 cell density, 2-MIB concentration (**C**), extracellular 2-MIB per cell, and 2-MIB productivity (**D**) at different dilution rates in the chemostat.

more significantly at high light intensity (>100 μmol/m^2/sec) than at low temperature (<20°C) (Fig. 3). On the other hand, *Anabaena* sp. FACHB-1384 was adapted to a relatively wide range of light intensity (20–140 μmol/m^2/sec) but sensitive to low temperature. Saadoun *et al*. [14] reported that the growth and geosmin production of *Anabaena* sp. was not significantly dependent on temperature from 15°C to 30°C but was sensitive to high light intensity. Low temperature (<20°C) favored the growth of the two geosmin-producing *Anabaena* strains, whereas high light intensity (100–140 μmol/m^2/sec) favored the growth of the 2-MIB-producing *Anabaena* strain (Fig. 3). This indicates the possibility of sustained growth of geosmin-producing *Anabaena* strains even during the winter season. Historically, *Anabaena spiroides* blooms occurred in early winter in the North-Han River, Korea with geosmin concentration up to 1,640 ng/l [22].

The effects of temperature and light intensity on OC-producing cyanobacteria have already been examined, but their combined effect was not confirmed [7, 14]. When they investigated temperature effect, light intensity was fixed and vice versa. However, the pattern of temperature effect could change at different light intensities, as shown in Fig. 3C. This is due to the interaction effects between environmental factors. Although a full factorial design of the experiment is the best way to grasp the whole picture of the combined effect of several factors, it necessarily requires higher cost, longer time, and larger space. PhotoBiobox could be the solution to such high-throughput screening experiments, especially for the study of temperature and light effects [15]. However, its small scale provides different culture conditions from flask cultivation. For example, the growth of filamentous microalgae was sometimes unstable, and a stationary phase was often reached earlier at a lower cell density in PhotoBiobox than in conventional culture conditions.

In batch-culture experiments, a high growth rate was observed under high nitrogen concentration, which corresponded to the result of Saadoun *et al*. [14]. However, a high concentration of nitrogen could not guarantee a higher geosmin production per cell (Fig. 4C). Thus, the increase in geosmin concentration was mainly due to the increase in cell numbers rather than any changes in the geosmin production per cell. Despite the decrease in cell number, a high geosmin concentration was observed even after 8 days of cultivation in high nitrogen concentration (Figs. 4A and 4B). This may be attributed to the leakage of geosmin during cell death, because cell death might release cell-associated geosmin into the surrounding water [23]. Moreover, the release of geosmin from damaged cells could be the main source of mal-odor in the drinking water.

An increase in OC production was observed with the higher growth rate in the chemostat. A higher dilution rate in a P-limited chemostat relieves P-stress by faster supply of medium nutrients. Therefore, it could be assumed that the phosphorus concentration could affect the cell growth rate and OC productivity. The geosmin amount per cell decreased at lower dilution rates (higher P-limitation), but 2-MIB per cell was not affected by P-limitation (Fig. 5). The geosmin amount per cell, however, was not affected by nitrate concentrations in batch cultivation (Fig. 4). The OC concentration seems to reflect simply cell density, but not to be regulated finely by environmental conditions. Another secondary metabolite of cyanobacteria, microcystin, showed a similar pattern. A major determinant of microcystin concentration was cell density, implying that the microcystin content per cell is fairly constant [24]. These secondary metabolites in cyanobacteria are thought to be produced constitutively, rather than being influenced by an external stimulus.

The 2-MIB productivity was higher than that of geosmin (Fig. 5). However, about 10 times higher geosmin concentration than 2-MIB has been measured in Korea [25, 26]. This indicated the dominance of geosmin-producing cyanobacteria in Korea. Moreover, 2-MIB is lost more easily from water compared with geosmin owing to a higher volatility and biodegradation [1, 27]. Therefore, geosmin-producing cyanobacterial strains would be more often problematic in causing reduced water quality in Korea, as compared with 2-MIB-producing strains.

Terpenoids are modified terpenes where methyl groups are moved or removed, or oxygen atoms added. The two terpene alcohols, namely geosmin and 2-MIB, are the most frequently found secondary metabolites in bacteria, filamentous cyanobacteria, myxobacteria, and in a small number of fungi [28]. Some terpenoids are reported to have allelopathic activities on aquatic consumer organisms such as rotifers and crustaceans [29]. Geosmin or 2-MIB production by filamentous cyanobacteria may have an allelopathic effect, which might help them for better adaptation to respective environmental conditions. For example, Ozaki *et al*. [30] confirmed a lytic activity on *Microcystis* sp., at concentrations of 3.0 mM geosmin and 2.5 mM 2-MIB.

In this study, geosmin-producing *Anabaena* strains were better adapted to lower temperature (<20°C) but were sensitive to high light intensity. However, 2-MIB-producing *Anabaena* grew well at a higher light intensity (100–140 μmol/m^2/sec), but was sensitive to low temperature.

The extracellular geosmin amount per cell was not affected by nitrogen concentrations but decreased with P-limitation, implying that geosmin production is more dependent on P than N. These results suggested that the incidence of 2-MIB problem would be more profound in the summer season, but geosmin has the potential of causing drinking water problems in all seasons. On the whole, the OC concentration was highly dependent on OC-producing cell density. Therefore, prevention of OC problems could be best attainable only by controlling the growth of OC producers.

Acknowledgments

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