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Optimum growth condition and microcystin utilization in bacteria isolated from a lake and a rice field in Japan

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Abstract

Three *Sphingomonas* strains, S5, S6, and 7CY, capable of degrading hepatotoxin microcystins produced by a blue-green alga, were grown to evaluate the optimum growth condition in LB medium under various conditions as follows: temperature: 25, 30, 35, 40, 45, 50°C; pH: 6.0, 7.0, 8.0, 9.0, 10.0; and the density of ingredients of the medium: 1/4-fold, 1/2-fold, 1-fold. Under these conditions, S5, S6, and 7CY strains showed at 35°C the highest *kg* in 1/2-fold LB at pH7.0, in 1/2-fold LB at pH8.0, in LB at pH7.0, respectively. Average generation times were 1.8 in S5, 1.7 in S6, and 2.4 hours in 7CY under each optimum condition. When microcystin-LR, -LY, -LW, and -LF were added to S6 cell suspension, S6 cells were able to degrade all these microcystins, and the rate of microcystin-LR degradation was $0.59\mu g \cdot ml^{-1} \cdot day^{-1}$. S6 strains, furthermore, grew much faster in minimal medium in the presence of microcystin-LR than in the absence of the toxin. These results indicate that environmental factors such as pH and temperature which regulate the growth of microcystin-decomposing bacteria are considerably close to those of toxic blue-green algae. It is, therefore, suggested that water bodies where toxic blue-green algae are dominant should be convenient for these bacteria as well, and that they should take up microcystins as nutrient after toxic blue-green algae disappear. It should be noted that the rate of decomposing microcystins by S6 strain is high enough to exhaust microcystin-LR guideline value ($1\mu g \cdot \ell^{-1}$) recommended by WHO and the usual concentration of microcystins produced by blue-green algae in eutrophic water bodies.

Introduction

The water blooms and surface scums of blue-green algae have broken out in eutrophic lakes, ponds and reservoirs all over the world. The massive accumulation of blue-green algae is hazardous and brings out problems such as the release of moldy smell and putrid odor, the spoiling of landscape, and the mortality of aquatic organisms. Among species of the blue-green algae, genera *Microcystis, Anabaena, Oscillatoria,* and so on produce neurotoxins and hepatotoxins (Carmichael., 1994).

Microcystins produced principally by genus *Microcystis* distributed as one of the most common blue-green algae all over the world are cyclic heptapeptide hepatotoxins consisting of D-alanine (Ala), D-glutamic acid (Glu), D-β-methylaspartic acid (β-Me-Asp), N-methyldehydroalanine (Mdha), Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid and two L-amino acids) (Fig.1, Rinehart *et al*., 1994). Microcystins are potent inhibitors of protein phosphatases 1 and 2A from animals and higher plants and exhibit acute toxicity to the mammals. Once microcystins are taken up by animals, they are transported into hepatocyte, cause an excessive phosphorylation of protein phosphatases, and act as carcinogenesis promoter.

Francis (1878) reported that the deaths of wild and domestic animals were attributed to the toxins produced by *Nodularia* which occurred in brackish water region in Australia. Ever since, investigations have been made on the causes of domestic animals death by ingesting cyanobacterial scums in UK, Europe and USA (Turner *et al*., 1990; Lawton and Codd, 1991; Frazier *et al*., 1998; Mez *et al*., 1997). In Japan, wild birds which ingested cyanobacterial blooms died in a local pond (Matsunaga *et al*., 1999). Microcystins produced by blue-

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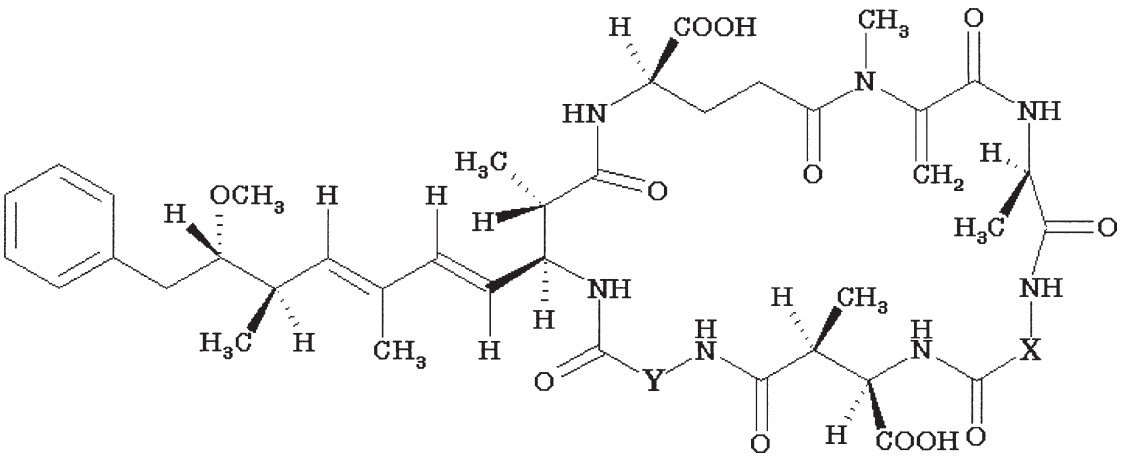

Fig. 1. Structure of microcystins where X and Y represent variable L-amino acid.

green algae are commonly found in drinking water in many countries. In 1996, in Brazil over fifty patients died by ingestion of dialyzed water heavily contaminated by microcystins (Dunn, 1996). Although various kinds of technique have been developed to remove and/or decompose these toxins in aquatic environments such as chlorination, ozonization, and activated carbon treatment and so on, they might be very costly or synthesize by-product toxins during the treatment process.

Blue-green algae are formed as water blooms and scums in lakes and reservoirs from late spring to early autumn in Japan when water bodies become rich in nutrient. Most of microcystins synthesized intracellularly are localized inside the cells when cells grow vitally, and they are released into surrounding waters after cell lyses. Since microcystins are chemically very stable, they are not susceptible to heat, UV irradiation, etc. in natural aquatic environments and to the attack of any proteases excreted by organisms co-existing with toxic blue-green algae, resulted in persisting in water bodies. Jones and Orr (1994) observed high concentration of microcystins (1300-1800$\mu g \cdot \ell^{-1}$) in lake water for 9 days, and then, they were degraded. These facts suggest that chemically stable microcystins might be decomposed by microorganisms in aquatic environment. Microcystins-degrading bacteria have been isolated from natural waters (Park *et al*., 2001; Maruyama *et al*., 2004; Tsuji *et al*., 2006; Imanishi *et al*., 2005; Ho *et al*., 2007; Edwards *et al*., 2008; Lemes *et al*., 2008) and Saito *et al*. (2003) resolved the gene involved in the degradation of microcystins.

Ishii (2002) and Ishii *et al*. (2004) isolated seven strains of microcystin-degrading bacteria from an irrigated rice field and Lake Suwa where heavy water blooms of *Microcystis* are frequently observed. In particular, three strains, S5, S6, and 7CY possessed potent microcytin-degrading activity (Ishii, 2002). All of these strains were able to degrade microcystin-RR and, in addition, S5 cells were capable of decomposing nodularin-Har while 7CY cells degraded nodularin-Har only in the presence of microcystin-RR (Ishii, 2002; Ishii *et al*., 2004). The results of 16S rDNA gene sequence analysis of S5, S6, and 7CY strain showed 98% homology of *Sphingomonas* sp. INF1597, 99% homology of *Sphingomonas koreaonsis*, and 94% homology of *Rhizomonas suberifaciens* and *Blastomonas hatatoria*, respectively (Ishii, 2002), suggesting that these three bacteria should be assigned to the genus *Sphingomonas*.

The present study describes optimum growth condition for microcystin-degrading S5, S6, and 7CY strains. The degradability and utilization of microcystins produced by highly toxic *Microcystis* PCC-7820 in S6 strain are also investigated.

Materials and Methods

Organisms

Toxic blue-green algae, *Microcystis* PCC-7820 was obtained from Pasteur Culture Collection, Paris, France. Microcystin-degrading *Sphingomonas* strains, S5 and S6, isolated from an irrigated rice field, Funakoshi, Shizuoka, Japan and 7CY, from Lake Suwa, Nagano, Japan (Ishii, 2002). The algal cells were grown in an Erlenmeyer flask containing BG11 medium (Allen, 1968) under 12L-12D cycle at 8$\mu E \cdot m^{-2} \cdot s^{-1}$ (fluorescent lamps, Toshiba) on the surface of the flask at 23°C. Microcystin-degrading bacterial cells were grown in an Erlenmeyer flask containing LB medium (Atlas, 1995) at 30°C in the dark.

Growth condition for S5, S6 and 7CY

S5, S6, and 7CY cells were grown in LB medium on orbital shaker at 100rpm in the dark under various conditions as follows: the density of the medium: LB, 1/2-fold LB, 1/4-fold LB; temperature:25, 30, 35, 40, 45, 50°C; pH: 6.0, 7.0, 8.0, 9.0, 10.0. An aliquot of cell suspension was withdrawn every two hours and the value of absorbance at 550nm (A_{550}) of cell suspension used as an indicator of growth was determined by spectrophotometer (UV-2100, Shimadzu). When the values of A_{550} are plotted against time, the growth rate constant (kg) is expressed in the following equation where t_1 and t_2 ($t_1 < t_2$) represent any two points during exponential growth phase, and n_1 and n_2, A_{550} values at time t_1 and t_2, respectively (Watanabe and Tamiya, 1965).

$$kg = \frac{1}{(t_2 - t_1)} \log(n_2/n_1)$$

The growth of these three strains in minimum M9 medium instead of LB medium was also measured to study on the availability of microcystins by them. S5, S6, and 7CY cells grown under the condition described previously were harvested by centrifugation at 6,100×g at 20°C for 5 min, washed three times with M9 medium or the one supplemented with microelements (A&A: Allen and Arnon, 1955; Arnon *et al*., 1955; BG11: Allen, 1968), resuspended in M9 medium, transferred to an Erlenmeyer flask containing 50ml of M9 medium, and grown on orbital shaker at 120rpm in the dark at 35°C. An aliquot of cell suspension was withdrawn every two hours and the A_{550} value was measured.

Extraction of microcystins

Microcystins in *Microcystis* PCC-7820 were extracted according to the method described by Lawton *et al*. (1994). The cells of *Microcystis* PCC-7820 were grown at *ca*. 21°C in a 19ℓ glass culture bottle containing 18ℓ of BG11 medium under continuous illumination from a bank of fluorescent lamps at 117μE•m^{-2}•s^{-1} on the surface of the bottle with aerating sterile air. The cells were harvested by vacuum filtration equipments through GF/C glassfiber filter (ϕ90mm, Whatman, England) and by centrifugation at 6,100×g for 5 min at 4°C, lyophilized, and stored at -20°C. The dried material of cells was suspended in 70% (v/v) methanol-0.1% (v/v) trifluoroacetic acid (TFA) and stirred for 1h at room temperature to extract microcystins. The extracted sample was centrifuged at 6,100×g for 5min at 4°C, and the supernatant was evaporated (R-114 and WJ-20, Shibata) at 40°C. The dried sample was suspended in 13.5 % methanol, and applied to Sep-Pak Vac C18 cartridges (1cc, Waters) which had been pre-conditioned with 20ml of methanol and 50 ml of H_2O. The cartridge was washed once with 100ml of H_2O, and eluted serially with 20ml of 30% methanol, 40ml of 70% methanol, and 20ml of methanol. 70% methanol fraction was applied again to Sep-Pak Vac C18 cartridge, and eluted serially with 20ml of 40% methanol, 40ml of 70% methanol, and 20ml of methanol. All fractions were analyzed by high performance liquid chromatography (HPLC).

Degradation of microcystins

Microcystin degradation was evaluated by the method reported by Ishii *et al*. (2004). S5, S6 and 7CY cells, grown on orbital shaker at 120rpm in LB medium in the dark at 35°C for two days were harvested by centrifugation at 6,100×g for 5 min at 20°C, washed three times with M9 medium, and resuspended in M9 medium. Microcystin-LR fraction prepared from PCC-7820 cells was added to an Erlenmeyer flask containing 50ml of M9 medium (final concentration of microcystin-LR: 20μg•ml^{-1}). They were grown on orbital shaker at 140rpm in the dark at 35°C and an aliquot of cell suspension was withdrawn at intervals, filtered by membrane filter (0.45μm, Millex-LH, Millipore), and analyzed on HPLC.

HPLC analysis

Determination of microcystin was carried out by the method established by Lawton *et al*. (1994). Microcystins were identified on analytical HPLC with photodiode array detector (Shimadzu). The equipment consists of degasser (DGU-12A), liquid chromatograph (LC-10AS), communications bus module (CBM-10A), spectrofluorometric detector (RF-10A), column oven (CTO-10AC), diode array detector (SPD-M10AV), manual injection (7725/7725i, 9725/9725i), and a Symmetry C_{18} column (5μm; 250×4.6mm, Waters). The eluent under linear gradient condition was a combination of Milli-Q water (Millipore) and acetonitrile, both containing 0.05% (v/v) TFA (Lawton *et al*., 1994). The flow rate was 1ml•min^{-1} at 25°C for analysis and the value of A_{238} was monitored. The HPLC data were analyzed by CLASS LC10 software (Shimadzu).

Results and Discussion

Growth condition for microcystin-degrading bacteria

Figs.2 and 3 show kg as a function of growth tem-

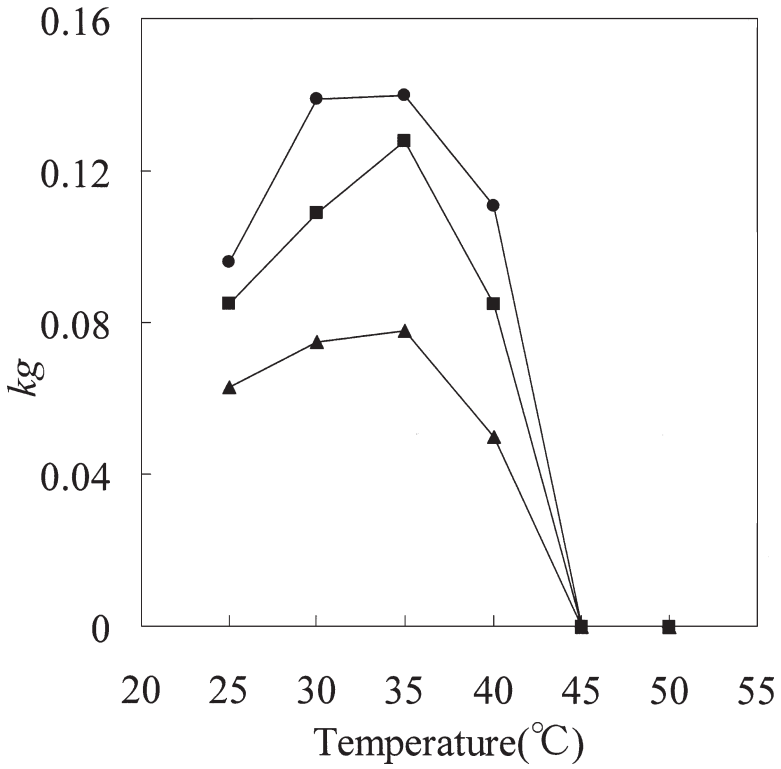

Fig. 2. *kg* as a function of growth temperature.
The cells of S5 (■) and S6 (●) were grown in 1/2-fold LB medium, and 7CY (▲), in LB.

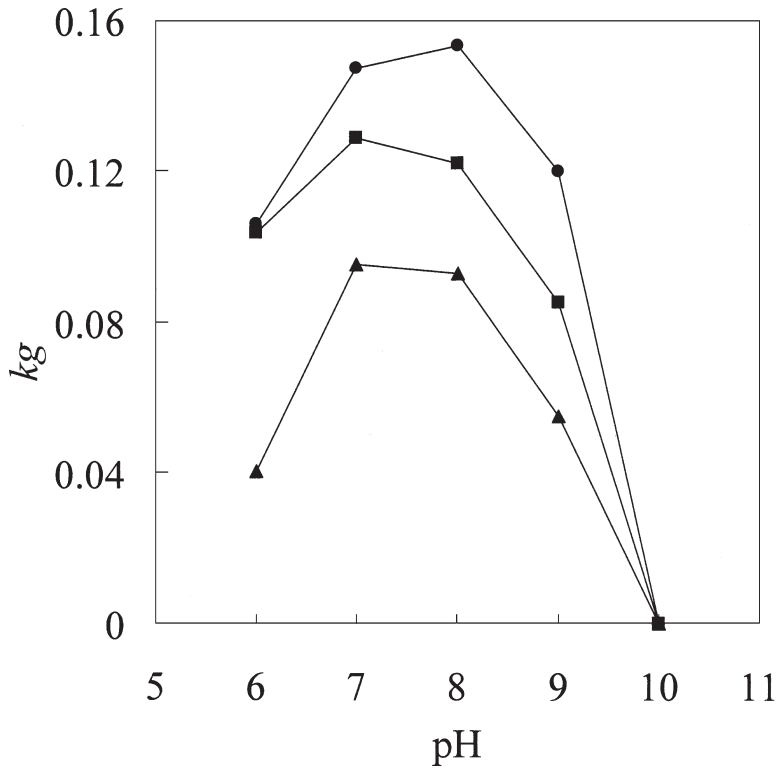

Fig. 3. *kg* as a function of medium pH.
The cells of S5 (■) and S6 (●) were grown in 1/2-fold LB medium, and 7CY (▲), in LB at 35°C.

perature and medium pH, respectively. The medium used in this study was 1/2-fold LB for S5 and S6, and 1-fold LB for 7CY since these strains had showed the most favorable growth at the concentration of LB described above among 1/4-fold, 1/2-fold, and 1-fold LB (data not shown). All three strains grew well between 25 and 40°C, but at 45 and 50°C, they could not (Fig.2). The highest growth rate was obtained at 35°C in all strains and *kg* values were 0.13 in S5, 0.14 in S6, and 0.078 in 7CY (Fig.2). It should be emphasized that the dependency of growth on temperature was more pronounced in S5 and S6 than in 7CY (Fig.2). At 35°C, in 1/2-fold LB in both S5 and S6 and in 1-fold LB in 7CY, pH-dependent curves were very similar among these strains and they failed to grow at pH10.0. Their highest growth rates were shown to be 0.13 at pH7.0 in S5, 0.15 at pH8.0 in S6, and 0.10 at pH7.0 in 7CY (Fig.3). As opposed to Fig.2, pH dependency of growth was most prominent in 7CY (Fig. 3). Bloom-forming *Microcystis* is frequently dominant in eutrophic water bodies including lakes and reservoirs where pH and temperature ranges are *ca.*7-11 and 25-35°C, respectively, suggesting that toxic *Microcystis* and *Sphingomonas* strains should be co-existent especially from late spring to early autumn in the habitat. In fact, 7CY strain is an isolate from Lake Suwa, Japan in which heavy water bloom of *Microcystis* is often observed (Ishii *et al*., 2004). Time courses of growth under the optimum conditions were shown in Fig.4. At exponential growth phase, S5 and S6 cells grew more rapidly than 7CY cells and the respective doubling time was 1.8, 1.7, and 2.4 hours (Fig.4).

Growth in minimal medium

Three *Sphingomonas* strains used in the present study grew well in LB medium as shown in Figs.2, 3, and 4, but it contains various kinds of organic carbon and nitrogen. Minimal medium should be employed to study whether these strains can utilize microcystins as a unique carbon and/or nitrogen source or not.

In Fig.5(a), M9 medium, well-established minimal medium for heterotrophic bacteria, was taken in S5, S6, and 7CY. The cells of S6 started to grow vigorously at 8 hours after inoculation whereas S5 and 7CY cells did not throughout the experiment (Fig.5(a)). In the latter strains, it is possible that their inability to grow in M9 medium would be due to lack of microelements such as Fe and so on in the medium. In Fig.5(b), modified M9 medium supplemented with microelements which is universally applied in cyanobacterial growth was used as growth medium for *Sphingomonas* strains. S5 and

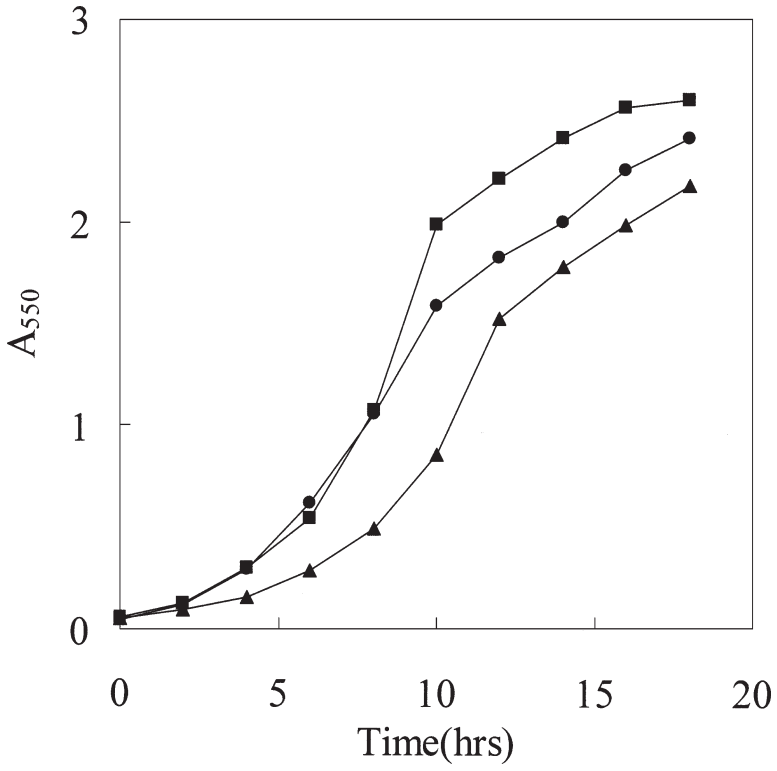

Fig. 4. Growth of S5, S6 and 7CY under optimal condition.
S5 cells (■) was cultured in 1/2-fold LB (pH 7.0), S6 (●), in 1/2-fold LB (pH 8.0), and 7CY (▲), in LB (pH 7.0) at 35°C.

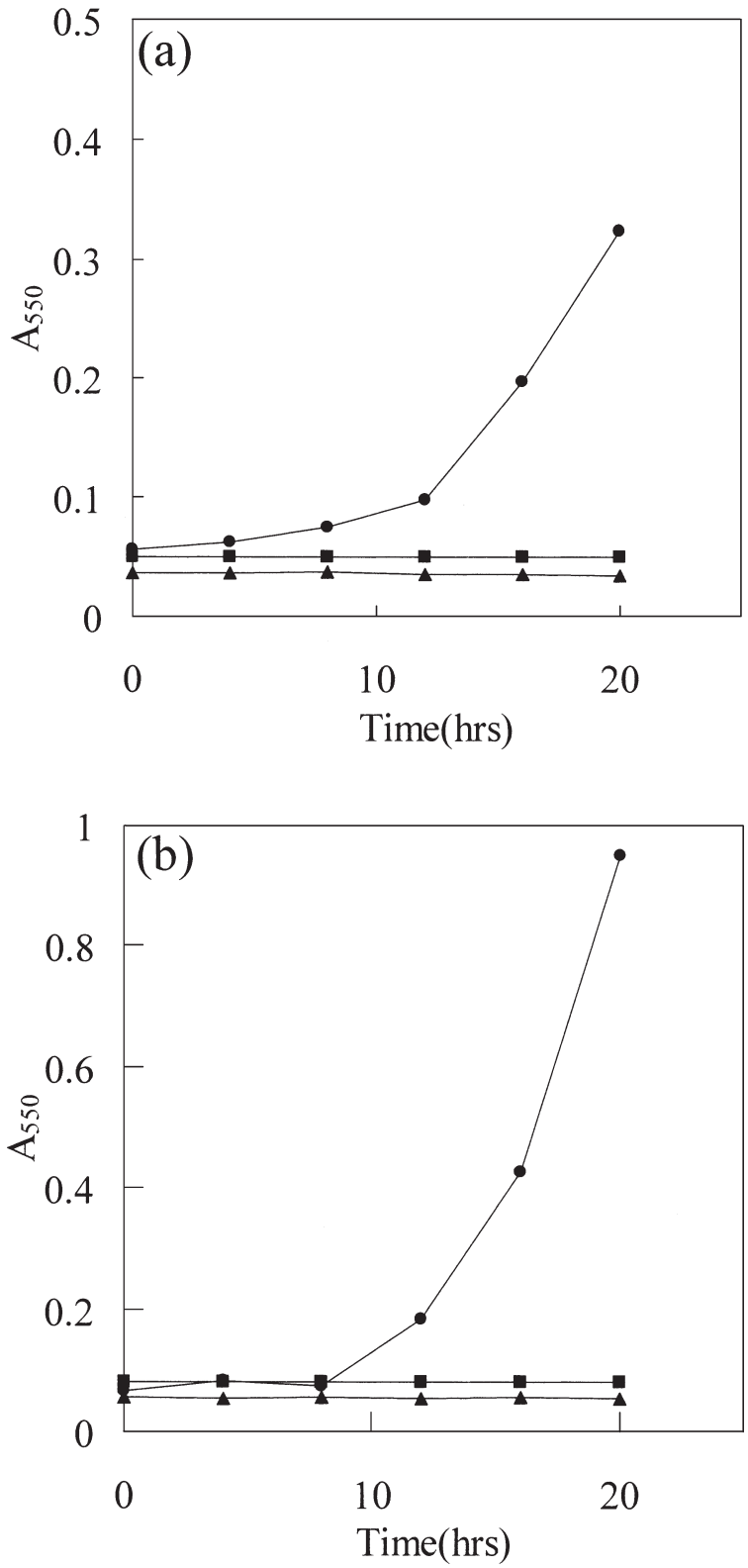

Fig. 5. Growth of S5, S6, and 7CY in M9 medium and in modified M9 medium. The cells of S5, S6, and 7CY were grown in M9 (a), or M9 supplemented with A&A and BG11 microelements (b). pH, temperature, and symbols were the same as in Fig.4.

7CY cells failed to grow, but S6 cells grew better than in M9 medium (Fig.5(b)). These results indicate that glucose and ammonium chloride play a role as unique carbon and nitrogen sources in S6 and that microelements are growth-limiting factors in M9 medium for S6 in contrast with S5 and 7CY.

Extracted microcystins from *Microcystis* PCC-7820

Microcystins from lyophilized PCC-7820 material were extracted in aqueous 70% (v/v) methanol-0.1% (v/v) TFA (Lawton *et al*., 1994) and analyzed on HPLC. Fig.6 shows the chromatogram of crude extract from *Microcystis* PCC-7820 cells (upper) and absorption spectra for peaks a, b, c, and d (lower). Since Lawton *et al*. (1994) reported that *Microcystis* PCC-7820 produced microcystin-LR, -LY, -LW, and -LF, peaks a, b, c, and d in Fig.6 were identified to be microcystin-LR, -LY, -LW, and -LF, respectively. Microcystins show two typical spectra, one with an absorption maximum at 238nm, which is exhibited by all except a few microcystins containing tryptophan and that gives an absorption maximum at 222nm (a peak c in Fig.6). More than those, there were several peaks of which some were unidentified microcystins and others were not, judging from their absorption spectra (Fig.6). To eliminate these contaminants, the crude extract was passed through Sep-Pak Vac C18 cartridge and eluted with various concentrations of methanol. All fractions obtained were analyzed by HPLC. Microcystin-LR, -LY, -LW, and -LF were detected in 70% methanol fraction while any microcystins were not present in 30% and 40% methanol fractions (data not shown).

Degradation of microcystins

It is reported that S6 cells are able to decompose microcystin-RR (Ishii, 2002), but degradability of other

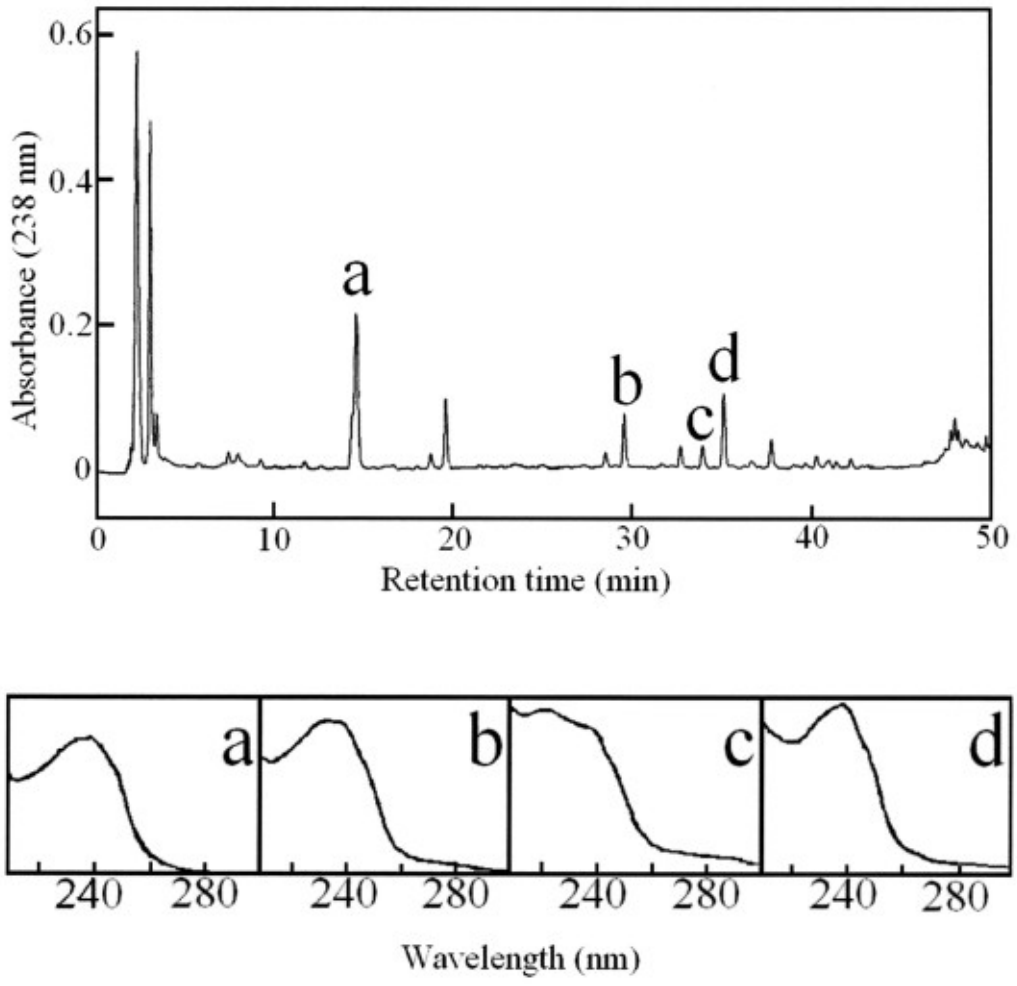

Fig. 6. HPLC Chromatogram and absorption spectra of microcystins extracted from *Microcysits* PCC-7820.
The eluent was a combination of water-0.05% (v/v) TFA and acetonitrile-0.05% (v/v) TFA. Linear gradient condition was described in Materials and Methods. The peaks a, b, c, and d represent microcystin-LR, -LY, -LW, and -LF, respectively.

microcystins by the strain which can only survive in minimal medium among three strains of *Sphingomonas* described here (Fig.5) have not yet been elucidated. Microcystins prepared from *Microcystis* PCC-7820 and treated twice through Sep-Pak C18 cartridge with 70% methanol were added to S6 cells in M9 medium (final microcystin-LR: 20μg•ml^{-1}). At the outset of experiment, the peak at 17.5min was microcystin-LR, at 30min, -LY, at 34min, -LW, and at 35min, -LF (upper in Fig.7). After 336 hours' incubation, the peak area of microcystin-LR decreased by 40% and the peaks of other microcystins apparently disappeared (lower in Fig.7).

Fig.8 shows the time-dependent degradation of microcystin-LR calculated from the data shown in Fig. 7. Microcystin-LR was degraded by S6 at relatively constant rate which was estimated to be 0.59μg•ml^{-1}•day^{-1} while microcystin-LR in the medium in the absence of S6 cells remained intact (Fig.8). Since Ishii (2002) reported that S6 degraded microcystin-RR at the rate of 0.56μg•ml^{-1}•day^{-1}, microcystin-RR and -LR are decomposed at nearly the same rate, suggesting that other microcystins should break down at the same rate. The biodegradation rates shown in S6 strain are consid-erably higher than in other microcystin-degrad ing bacteria such as Y2 strain (13μg•ml^{-1}•day^{-1} for -RR and 5.4μg•ml^{-1}•day^{-1} for -LR, Park *et al*., 2001) and *Burkholderia* (0.05μg•ml^{-1}•day^{-1} for -LR, Lemes *et al*., 2008).

The attempt was made on the utilization of cyanobacterial toxin, microcystin as nutrient required for microcystin-degrading S6 cells (Fig.9) since any microcystins prepared from *Microcystis* PCC-7820 were decomposed by them. Microcystin-LR was added to S6 cells in M9 medium supplemented with glucose (5 mM) and ammonium chloride (5 mM) at time zero and the value of A_{550} was monitored (Fig.9). The concentration of microcystin-LR in the medium was 0.61g•ℓ^{-1}, i.e. 0.36g C (carbon)•ℓ^{-1} and 0.045g N (nitrogen)•ℓ^{-1} and those of glucose (5 mM) and ammonium chloride (5 mM) were 0.36g C•ℓ^{-1} and 0.07g N•ℓ^{-1}, respectively. As shown in Fig.9, the cells with microcystin-LR grew much faster than those without microcystin-LR and *kg* value in the former ($kg = 1.8$) was twice as high compared with that

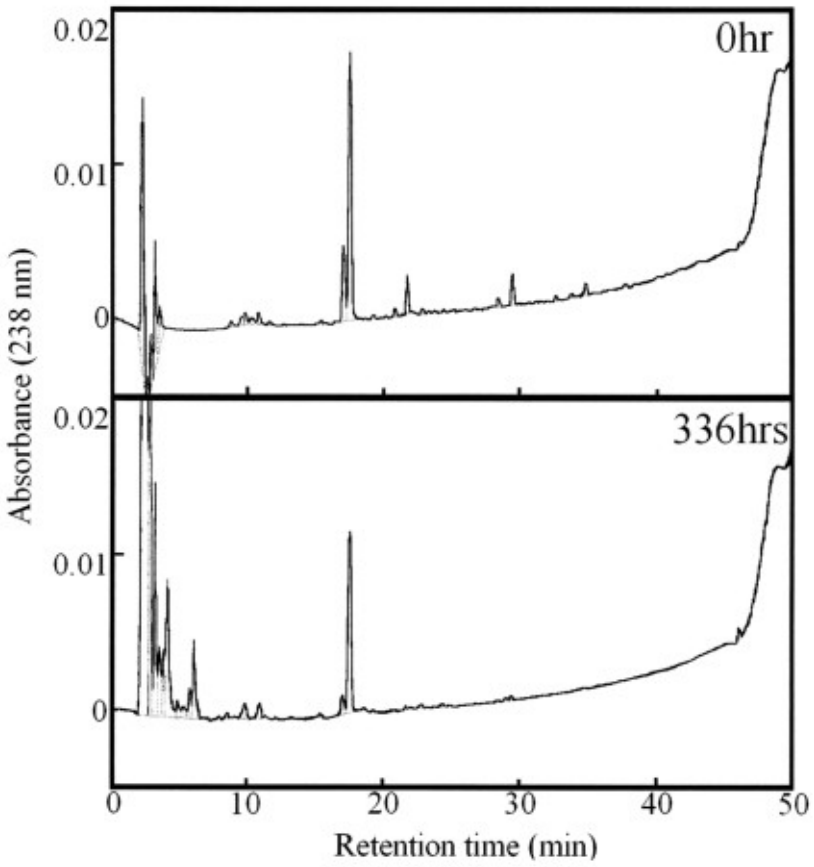

Fig. 7. HPLC chromatograms of microcystins in the presence of S6 cells.
The cells of S6 were grown at 35°C in M9 medium with a fraction containing microcystin-LR (20μg•ml^{-1}).
The cell suspension was withdrawn at time zero (upper) and 336hours (lower) and the filtrate was analyzed on HPLC.

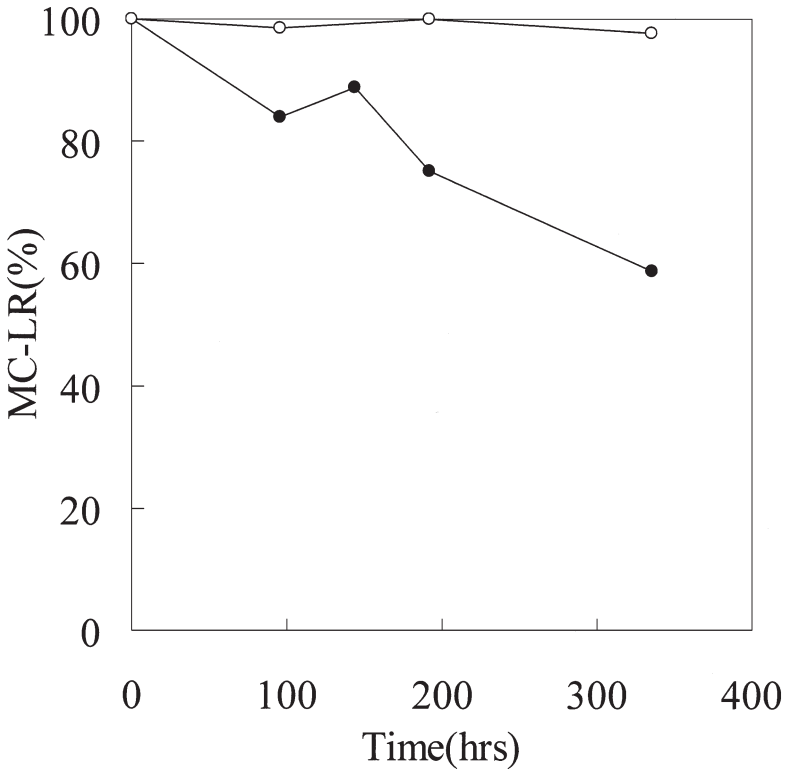

Fig. 8. Time course of microcystin-LR degradation in the presence of S6 cells.
The amount of microcystin-LR in M9 medium with a fraction containing microcystin-LR (20μg•ml^{-1}) in the absence (○) or presence (●) of S6 cells was estimated on HPLC.

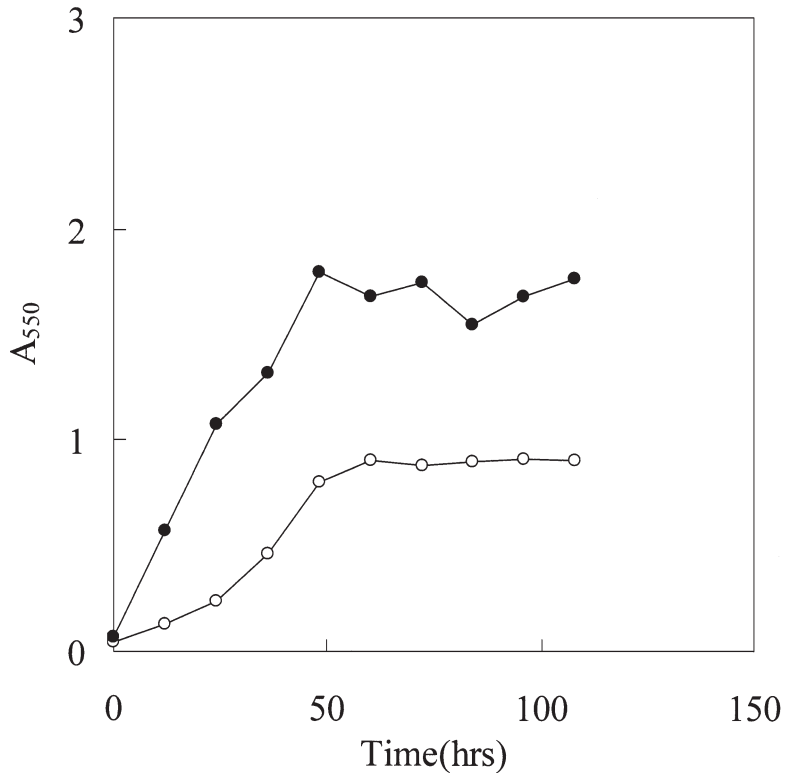

Fig. 9. Growth of S6 cells in M9 medium with microcystin-LR.
S6 cells were grown in M9 medium (5mM glucose + 5mM NH_4Cl) in the absence (○) or presence (●) of microcystin-LR (0.61mM) at 35°C.

in the latter ($kg = 0.91$), indicating that S6 cells decompose microcystin-LR to utilize for their nutrition.

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要　旨

日本の湖と水田から単離したバクテリアの最適生育条件と細菌によるミクロシスチンの利用

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アオコ毒素ミクロシスチンを分解するS5，S6，7CY株について，培養温度を25～50℃，pHを6.0～10.0，LB培地濃度を1倍，1/2倍，1/4倍として培養し，生育速度定数（*kg*）を比較した．S5株は35℃，pH7.0，×1/2LB，S6株は35℃，pH8.0，×1/2LB，7CY株は35℃，pH7.0,×1LBで最も高い*kg*を示し，これらの条件におけるそれぞれの株の平均世代時間は1.8，1.7，2.4時間であった．また，最少培地M9でこれら3株を培養したところ，S6株のみが生育した．さらにこのS6株にミクロシスチン-LR，-LY，-LW，-LFを添加したところ，本株はこれらすべてのミクロシスチンを分解し，-LRの分解速度は0.59$\mu g \cdot ml^{-1} \cdot day^{-1}$であった．さらに，この株にミクロシスチン-LRを添加した場合と添加しなかった場合で生育を比較したところ，-LRを添加した場合の生育速度は添加しなかった場合の2倍であった．以上の結果より，アオコとこれらの分解菌の生育条件はほぼ一致しており，アオコが発生する湖沼にはこのような分解菌も生息し，アオコが消滅したのちに湖水に残存するミクロシスチンを分解しているものと推定される．また，S6株はWHOが定めた飲料水の基準値（1$\mu g \cdot L^{-1}$）や自然環境においてアオコが発生している湖沼に存在するミクロシスチンを分解するには十分な能力を有すると考えられる．