

論文 / 著書情報
Article / Book Information

Title	Extinction of cells of cyanobacterium <i>Anabaena circinalis</i> in the presence of humic acid under illumination
Authors	Bing-kun Sun, Yasunori Tanji, Hajime Unno
Citation	Appl. Microbiol. Biotechnol., Vol. 28, No. , pp. 823-828
Pub. date	2006,
Note	This is a post-peer-review, pre-copyedit version of an article published in Appl. Microbiol. Biotechnol.. The final authenticated version is available online at: http://dx.doi.org/http://dx.doi.org/10.1007/s00253-006-0327-4 .

Bing-kun Sun¹, Yasunori Tanji¹, Hajime Unno^{2*}

Extinction of cells of cyanobacterium *Anabaena circinalis* in the presence of humic acid under illumination

(1)Graduate school of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B-47
Nagatsuta-cho, Midoriku, Yokohama 226-8501, Japan

(2)Dept. of Environmental Chemical Engineering, Kogakuin University, 1-24-2 Nishi-shinjuku,
Shinjuku, Tokyo, 163-8677 Japan

* Hajime Unno

E-mail address: hajime-unno@bio.titech.ac.jp

Tel.: +81 3 3340 2687; fax: +81 3 3340 2687.

Abstract

Laboratory experiments targeting the effect of humic acid (HA) on the cell lysis of cyanobacterium *Anabaena circinalis* have been performed. Light irradiation was found to be an important factor for the cell lysis phenomenon, whereas intracellular H₂O₂ might be a chemical factor for the process. An exogenous H₂O₂ concentration of 1.0mg l⁻¹ was determined as the threshold for cell survival. Our results indicated that HA or its possible product(s) of photochemical reaction can induce damage to intracellular catalase under artificial illumination, which leads intracellular H₂O₂ to be accumulated to an abnormally high concentration, eventually resulting in cell death. Moreover, H₂O₂ released into the culture from dead cells can damage other cells, which in turn brings about the population extinction.

Introduction

Water blooms have become a nuisance for water resource management, especially algal blooms produced by cyanobacteria, in that toxic substances, foul odors and unpleasant tastes accompany them (Paerl et al. 2001, Mbeunkui et al. 2002). Both the appearance and the disappearance of such algal blooms are natural phenomena, taking place suddenly in a random fashion (Steven and Glombitza 1972). However, the mechanism that triggers the water blooms to disappear is important for taking countermeasures against them. Concerning this issue, two hypotheses have been proposed to explain the fate of cyanobacterial blooms. The first is that the cells are lysed by ecological reasons such as cyanophages, bacteria, fungus and amoebae; the second is that blooms are simply dispersed by destratification of the water body due to storms or tidal changes (Yamamoto 1988, Sakamoto et al. 1998). However, in addition to these, other biochemical aspects are considered to be involved in the phenomena.

Anabaena circinalis, a filamentous toxin-producing cyanobacterium, is widely distributed throughout the world. It commonly grows in lakes and can be responsible for water blooms (Beltran and Neilan 2000). In a previous study, the rapid cell lysis of *A.circinalis* caused by the presence of humic acid (at a concentration as low as <1.0mg-C l⁻¹) was reported (Sun et al. 2005). The effects of HA on algal growth has not completely been elucidated yet, although a hypothesis was proposed as follows: UV irradiation of humic substances in the culture medium might produce peroxides via the Fenton reaction, and thereby leads to damage algal cells (Gjessing and Kallqvist 1991). Unfortunately, no reliable study has been reported to verify the hypothesis.

In this study, we attempted to clarify the mechanism(s) of *A.circinalis* cell lysis occurring in the presence of HA. These analyses will contribute to the protection of water environments from cyanobacterial blooms.

Materials and methods

Chemicals

Humic acid (HA), horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂) were purchased from Wako Pure Chemical Co. (Osaka, Japan), catalase was purchased from Sigma Aldrich Japan (Tokyo, Japan).

Strain and culture conditions

The axenic strain, *Anabaena circinalis* NIES-41, from the NIES-Collection (the National Institute for Environmental Studies, Japan) was used. The strain was cultivated with CT medium (Table 1). The concentration of Fe was adjusted when necessary.

All culture experiments were conducted at a constant temperature (27.0±0.5°C) and shaking rate (100 rpm). Lighting was set at an illumination of 33.3 μmol m⁻² s⁻¹ by fluorescent lamps. For inoculums, preliminary cultivations were carried out to logarithmic phase (5–12day) in 200 ml Erlenmeyer flasks. All test cultivations were carried out at 5ml scale in 10ml glass test tubes.

Preparation of humic acid solution

HA solution was prepared by equilibrating solid HA with pure water (Milli-Q plus, Millipore, USA) without pH adjustment (for details see Sun et al. 2005). The concentration of HA solution was examined by a total organic carbon analyzer (TOC-VCPH, SHIMADZU, Japan).

Chlorophyll-a analysis

Chlorophyll-a was measured before and after each cultivation experiment following the standard method (APHA, AWWA, WEF 1998) to evaluate the active biomass. An optical density (OD) meter BioSpec-1600 (SHIMADZU, Kyoto, Japan) was used.

Lighting

Illumination was effected by putting glass tubes onto a shaker where the light energy level was at 33.3 μmol m⁻²s⁻¹. Darkness was simulated by wrapping tubes tightly in aluminum foil. In order to create a series of illuminated periods of time after inoculation, first inoculated, then cultivated under illumination for a predetermined period (0min, 1min, 10min, 1h, 6h, 12h and 24h), and subsequently cultivated for a further 24h in darkness to stabilize the lighting effect (refers to Fig.2 “Illuminated after inoculation”, while “illumination” in Fig.1 simply means cultivated under illumination for 24h). For setting a series of illumination periods of time before inoculation, the HA-containing media was first exposed to light, then inoculated in darkness, and subsequently cultivated for 24h in darkness (refers to Fig.2 “Illuminated before inoculation”, while “darkness” in Fig.1

simply means cultivated in darkness for 24h).

Preparation of media for predetermined Fe concentration

Solutions of four different Fe concentrations were prepared, i.e., a) Fe: 0 and EDTA: 3mg l⁻¹, b) Fe: 0, c) Fe: 2.2μM, and d) Fe: 22.0μM. To avoid Fe contamination, the procedure for solution preparation was as follows. Pure water was used to prepare all solutions. Tubes and other glassware were soaked in 6 M HCl solution for at least 24h and rinsed extensively with pure water. Cells for inoculation were separated from batch cultures by centrifugation for 3 times, each time the supernatant of 90% of total volume was replaced with Fe-free fresh medium aiming to remove excess Fe.

Prevention of cell lysis by catalase and inhibition of the function of catalase by HA

A catalase solution (62,500 Units ml⁻¹; One unit is defined as the quantity necessary for decomposition of 1.0μmole of H₂O₂ per min at pH 7.0 at 25°C) was prepared by dissolving solid catalase into pure water and filtration (0.22μm pore, Millipore, USA). Completion of an enzymatic reaction requires a certain period of hours, before inoculation (in tests for prevention of cell lysis) or addition of H₂O₂ (in tests for inhibition of the function of catalase by HA), preliminary incubations of catalase and HA were done.

Procedure of H₂O₂ detection by Horseradish peroxidase (HRP)

H₂O₂ detection was achieved by detecting the increase in intermediate compound derived from HRP and the decrease in free HRP due to H₂O₂ decomposition using a spectrometer (for details, see Boveris et al.1972). Their absorption maximums at 417nm and 402nm respectively were used for the present study.

Results

Effect of lighting on cell lysis

The effect of lighting on the cell lysis of *A.circinalis* was examined by cultivation under different conditions of illumination. After 24h of cultivation, the illuminated culture in medium containing 1.0mg-C l⁻¹ of HA resulted in a small chlorophyll-a value of around 105±92 μg l⁻¹ (n=3) corresponding to 6.7% of its initial value of 1560 μg l⁻¹, which showed the extinguishment of cells, while its color changed from dark green to brown and then bleached altogether (Fig.1). In contrast, cells cultivated in darkness with the same HA concentration resulted in a chlorophyll-a value of 1320±15 μg l⁻¹ (n=3) corresponding to 84.6% of the same initial value, and its color remained green, which showed that the cells did not die out. In contrast, chlorophyll-a in cultures that were cultivated in media containing 0.1mg-C l⁻¹ of HA or none at all showed small changes (<±15%) despite being subjected to different illumination conditions, i.e. light or darkness. These results suggest

that when HA concentration is at 1.0mg-C l^{-1} , lighting is an important factor involved in the cell lysis phenomenon.

To estimate the effect of light energy on cell lysis, cultures of two types, i.e., illumination after and before inoculation, were maintained. Each culture was maintained under a different illumination period. Shown in Fig.2 are data from the two types of culture. No significant difference was observed among the “illuminated before inoculation” group. On the other hand, in the “illuminated after inoculation” group, an explicit decreasing trend of chlorophyll-a value with increasing illumination time was observed when the illumination time was 1h or longer, while no difference was observed in the culture where illumination time was shorter than 10 min. The relative amounts of chlorophyll-a preservation after cultivation in the “illuminated after inoculation” group were 81%, 58.8%, 35.6%, 14.1% and 2.2%, for illumination periods of 0-10min, 1h, 6h, 12h and 24h, respectively. This result indicated that lighting is a crucial factor for the cell lysis phenomenon under the presence of HA; particularly, that exposing the culture for 24h is enough to kill all the cells and makes them depigmented, even the light energy is essential for algal photosynthesis.

Since light energy was supplied constantly at $33.3\mu\text{mol m}^{-2}\text{ s}^{-1}$ and the initial cell number was $7.9\times 10^5\text{ cells ml}^{-1}$, the average number of photon captured by each cell was calculated as: 7.7×10^{11} and 4.6×10^{12} quanta cell^{-1} , after 10min and 1h, respectively. It follows that the threshold of light energy for producing substantial cell damage can be estimated to be 10^{11} - 10^{12} quanta cell^{-1} levels.

Factors affecting cell lysis in the presence of HA

A probable mechanism for the cell lysis under illumination is the Fenton reaction, which occurs in the presence of Fe ion. A series of experiments was carried out under different Fe concentrations. The data shown in Fig.3 indicated scarce dependence of Fe concentration on the chlorophyll-a value, suggesting that Fe was not noticeably involved in cell lysis.

Table 2 shows the detection of H_2O_2 by the HRP method. H_2O_2 was detected in cases where illumination and HA were both present or cells were disrupted with sonication, whereas it was not detected in the HA solution. These results indicate that the detected H_2O_2 originated intracellularly, not from HA or a product of possible photoreaction.

Following on from the above experimental results, an investigation of the effect of H_2O_2 , which was not produced by the Fenton reaction but rather produced intracellularly and excreted upon cell death/lysis, was deemed necessary.

The effect of extracellular H_2O_2 on *A.circinalis* cell behavior was examined. The results are summarized in Fig.4. H_2O_2 concentration when less than 0.5mg l^{-1} did not affect the chlorophyll-a value at all, while a H_2O_2 concentration of up to 1.0mg l^{-1} caused the chlorophyll-a value to decrease to half that of the H_2O_2 -free case. Cells in all the cultures incubated in media containing higher H_2O_2 concentrations (3.0 and 5.0mg l^{-1}) died out.

Therefore, the H_2O_2 concentration of 1.0mg l^{-1} can be considered as a threshold for the survival of *A.circinalis* cells.

Catalase is an enzyme commonly found in most living cells. Since it catalyzes the decomposition of H_2O_2 into water and oxygen, it detoxifies the activated oxygen species H_2O_2 and thereby can protect the living cells from dying. Assuming that H_2O_2 is involved in cell lysis, then addition of catalase to the medium should protect the cells. Figure 5 shows the effect of catalase on cell culture morphology after 24h incubation with media containing catalase. Compared with the lack of remarkable differences in those incubated in the HA-free media (Fig.5 a, b, c), there were obvious differences in cells incubated in media containing HA (Fig.5 d, e, f). In the culture incubated in medium containing HA but no catalase (Fig.5 d), the trichomes fragmented into single cells, and furthermore almost all cells changed into blackish debris. In contrast, in media containing both HA and catalase (Fig.5 e, f), a large number of trichomes and intact cells survived. In addition, there were more living cells and longer trichomes in the culture with a higher catalase concentration (Fig.5 f) than the other (Fig.5 e). These results implied that catalase can prevent the cells of *A.circinalis* from cell lysis induced by HA, and therefore that H_2O_2 is involved in the cell lysis.

To confirm that HA inhibits the function of catalase, the following phenomena were observed: after 4h incubation with 1.0mg-C l^{-1} HA and under light, catalase (24Units ml^{-1}) no longer induced H_2O_2 (5 mg l^{-1}) decomposition, while in those lacking either HA or illumination, H_2O_2 (5 mg l^{-1}) was decomposed completely within 10min. This observation showed that HA or its possible product(s) of photoreaction can inhibit the function of catalase.

Discussion

In relation to the algicidal effect of UV-radiation of water containing humic substances, growth inhibition of the green alga *Selenastrum capricornutum* has been studied and a hypothesis proposed, specifically that UV irradiation of humic substances in the culture medium might produce peroxides and further transform them into the more powerful oxidizing free radical species $\text{OH}\cdot$ via Fenton reaction, and thereby leads to damaged algal cells (Gjessing and Kallqvist 1991). Although practical limitations prevented measurement of activated oxygen species such as O_2^- and $\text{OH}\cdot$, the data obtained for H_2O_2 made possible a suggestion on the mechanism of cell lysis of *A.circinalis* induced by HA. No H_2O_2 was produced from HA solution under illumination. Therefore, it can be concluded that the hypothesis proposed by Gjessing and Kallqvist is not the present case.

In fact, a large number of studies have reported that an oxidation reaction could be the reason for cell damage. For instance, growth on urea provoked peroxidation of lipid and then rapid death of the cyanobacterium *Synechococcus* sp. Strain PCC 7002 (Sakamoto et

al. 1998); H₂O₂ induced the release of Mn²⁺ from the oxygen-evolving complex and therefore brought about degradation of the photosynthetic pigments and cell lysis of the cyanobacterium *Anabaena variabilis* (Samuilov VD et al. 2001, 2004); also H₂O₂ was found to inhibit light-dependent O₂ evolution in studies of membrane preparations of spinach PSII with inactivated catalase (Sheptovitsky and Brudvig 1998).

The accumulation of H₂O₂ can cause critical damage to cyanobacterial cells. However, this condition is considered to be not only caused by adding H₂O₂ exogenously, but probably also by the inactivation of catalase intracellularly. Since H₂O₂ is produced in aerobes as a result of the partial reduction of O₂ and has toxicity to cells themselves, aerobic organisms have developed methods to induce its decomposition: heme-containing catalase and/or Mn-containing catalase (Wu et al. 2004). Because the present study has confirmed that HA or its product(s) can inhibit the function of catalase, the cell lysis mechanism of *A.circinalis* can probably be described as follows: HA or its photoreaction product(s) cause to inactivate catalase under illumination, which leads accumulation of H₂O₂ to an abnormally high concentration, eventually resulting in cell death. Moreover, the H₂O₂ released from dead cells can damage other cells in a culture, and therefore bring about the population's extinction. However, further questions about the details of this mechanism remain, such as: what product was formed by the photoreaction which inactivates catalase of *A.circinalis*, and what part of the chemical structure of catalase was attacked by it? These issues should be investigated using extracts of catalase from *A.circinalis* in the future.

Considering that humic substances are the most abundant organic matter on the Earth, and have gentle, regulative functions between the biosphere and inanimate objects, it is important to comprehend the relations between HA and living things (Frimmel 2001). The insight obtained here would also be helpful to the improvement of water environments; especially those which are troubled with cyanobacterial blooms.

Conclusions

To elucidate the mechanism of cell lysis of *A.circinalis* caused by the presence of HA, various experiments relating to several considerable factors, including light, Fe, H₂O₂ and catalase were conducted. The energy provided by light is an important factor in the cell lysis phenomenon caused by the presence of humic acid. Fe seems to have no significant influence on the cell lysis. H₂O₂ is a potential contributor to cell lysis. An exogenous H₂O₂ concentration of 1.0mg l⁻¹ is the threshold for the survival of *A.circinalis* cells. Catalase can protect the cells of *A.circinalis* from lysis. It was confirmed that HA or its possible reaction product(s) can inhibit the function of catalase.

Acknowledgments

This study was supported in part by the 21st Century COE Program, Ministry of

Education, Culture, Sports, Science and Technology, Japan. We would like to thank Mr. Aidan Synnott (Tokyo Institute of Technology, Japan), who provided critical opinions and helpful English checking for this paper. We are also grateful to Dr. Kazuhiko Miyanaga (Tokyo Institute of Technology, Japan) for his helpful advices.

References

- APHA, AWWA, WEF (1998) Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, New York
- Beltran EC, Neilan BA (2000) Geographical segregation of the neurotoxin-producing cyanobacterium *Anabaena circinalis*, *Appl Environ Microbiol* 66:4468–4474
- Boveris A, Oshino N, Chance B (1972) The cellular production of hydrogen peroxide. *Biochem J* 128:617–630
- Frimmel FH (2001) Aquatic humic substances. In: Hofrichter M, Steinbuechel A (eds) *Biopolymers. Volume 1, Lignin, humic substances and coal*. Wiley-vch, Weinheim New York Chichester Brisbane Singapore Toronto, pp 301–324
- Gjessing ET, Kallqvist T (1991) Algicidal and chemical effect of u.v.-radiation of water containing humic substances. *Wat Res* 25:491–494
- Mbeunkui F, Richaud C, Etienne A.-L, Schmid R, Bachmann T (2002) Bioavailable nitrate detection in water by an immobilized luminescent cyanobacterial reporter strain. *Appl Microbiol Biotechnol* 60: 306 - 312
- Paerl HW, Fulton RS III, Moisander PH, Dyble J (2001) Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *ScientificWorldJournal* 1:76-113 DOI: 10.1100/tsw.2001.16
- Sakamoto T, Delgado VB, Bryant DA (1998) Growth on urea can trigger death and peroxidation of the cyanobacterium *Synechococcus* sp. Strain PCC7002. *Appl Environ Microbiol* 64:2361–2366
- Samuilov VD, Bezryadnov DB, Gusev MV, Kitashov AV, Fedorenko TA (2001) Hydrogen peroxide inhibits photosynthetic electron transport in cells of cyanobacteria. *Biochemistry (Moscow)* 66:640–645
- Samuilov VD, Timofeev KN, Sinitsyn SV, Bezryadnov DB (2004) H₂O₂-induced inhibition of photosynthetic O₂ evolution by *Anabaena variabilis* cells. *Biochemistry (Moscow)* 69:926-933
- Sheptovitsky YG, Brudvig GW (1998) Catalase-free photosystem II: The O₂-evolution complex does not dismutate hydrogen peroxide. *Biochemistry* 37:5052-5059
- Steven DM, Glombitza R (1972) Oscillatory Variation of a Phytoplankton Population in a Tropical Ocean. *Nature* 237:105 –107
- Sun BK, Tanji Y, UNNO H (2005) Influences of iron and humic acid on the growth of the cyanobacterium *Anabaena circinalis*. *Biochem Eng J* 24:195–201
- Yamamoto Y (1988) Cyanobacteria-lysing agents and their distribution patterns of lakes

and rivers in Japan. *Bulletin of Japanese Society of Microbial Ecology* 2:77–88 (in Japanese with English abstract)

Wu AJ, Penner-Hahn JE, Pecoraro VL (2004) Structural, spectroscopic, and reactivity models for the manganese catalases. *Chem Rev* 104:903–938

Table 1. Composition of CT medium

Substrate	Concentration (M)
TAPS buffer	2.06×10^{-3}
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	6.36×10^{-4}
KNO_3	9.89×10^{-4}
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.62×10^{-4}
β -glycerophosphate, disodium salt	2.31×10^{-4}
Vitamin B ₁	3.0×10^{-8}
Vitamin B ₁₂	7.4×10^{-11}
Biotin	4.1×10^{-10}
FeCl_3	2.2×10^{-6}
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.30×10^{-6}
ZnCl_2	2.20×10^{-7}
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	5.05×10^{-8}
Na_2MoO_4	9.92×10^{-9}
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	1.21×10^{-5}

Note: The pH of the medium was adjusted to 8.2 with HCl and NaOH.

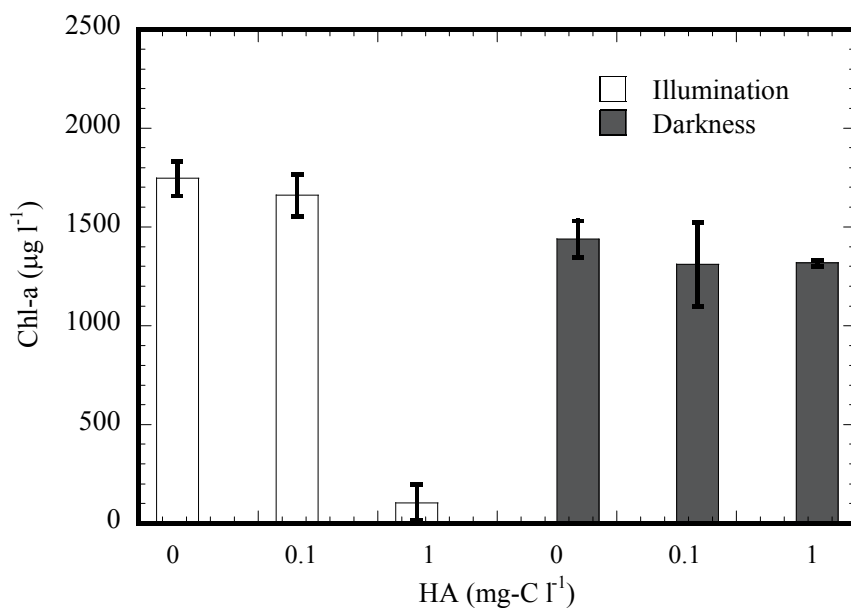


Fig.1 Effect of HA and lighting on cell growth

(All cultures were cultivated for 24h, from the same initial chlorophyll-a value of 1560 $\mu\text{g l}^{-1}$, under conditions of illumination (*open columns*) or darkness (*closed columns*). Values are means \pm SD of triplicate samples)

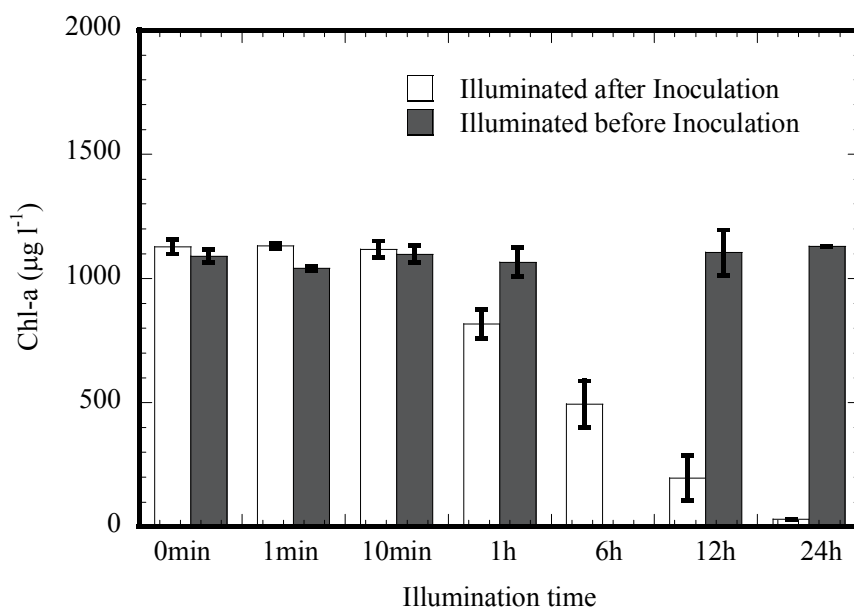


Fig.2 Illumination effect on cell growth

(“Illuminated after inoculation” (*open columns*) means that, after inoculation, the cells were cultivated under illumination for the periods of time indicated on the horizontal axis; the initial chlorophyll-a concentration was 1389 $\mu\text{g l}^{-1}$. “Illuminated before inoculation” (*closed columns*) means that the HA-containing media was first exposed to light for the periods of time indicated on the horizontal axis (no 6h setting), and then inoculated; the initial

chlorophyll-a concentration was 1128 $\mu\text{g l}^{-1}$. Values are means \pm SD of triplicate samples.)

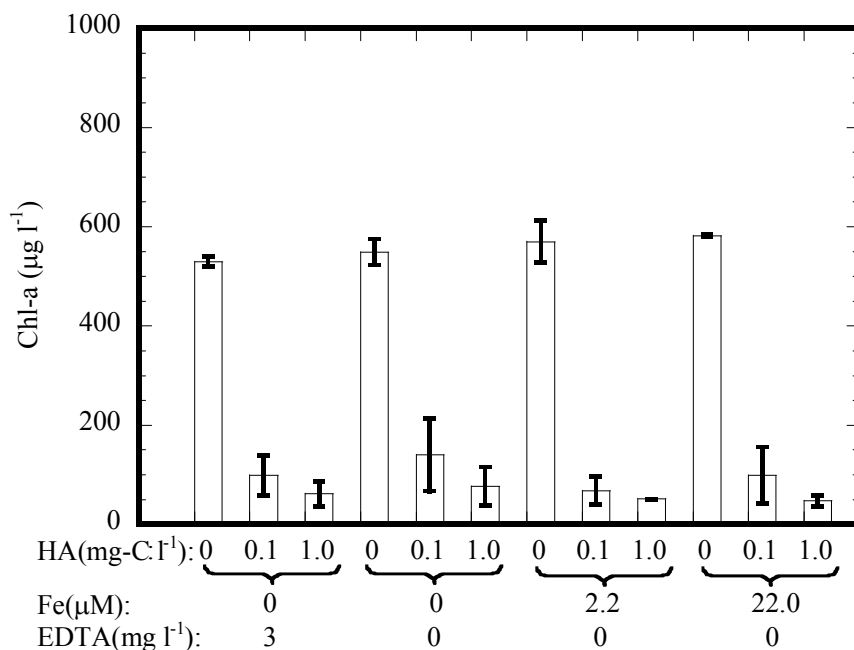


Fig.3 Comparisons of cell lysis under different Fe concentrations

(The chlorophyll-a values were measured after incubation for 24h under each of these conditions: Fe: 0 and EDTA: 3 mg l^{-1} , Fe: 0, Fe: 2.2 μM , and Fe: 22.0 μM . Values are means \pm SD of triplicate samples.)

Table 2 Detection of H_2O_2 from several media

	HA (1.0 mg-C l^{-1})		HA-free
	Illumination	Darkness	Illumination
Media with cells	+	-	-
Sonication treatment of above	+	ND	+
Media without cells	-‡	ND	-
Sonication treatment of above	ND	ND	-

* +: H_2O_2 was detected; -: H_2O_2 was not detected; ND: no detection.

‡ H_2O_2 was not detected even with addition of Fe^{2+} 100 μM

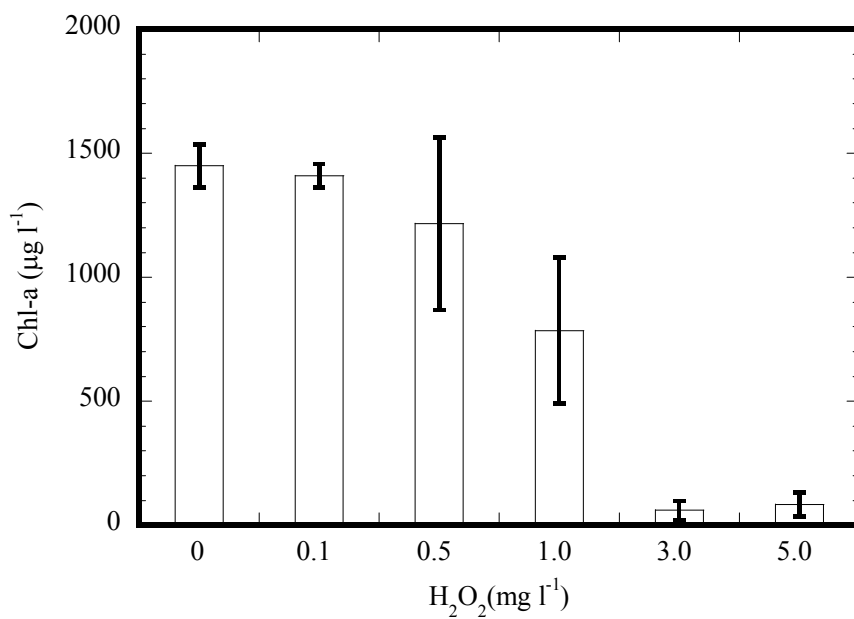


Fig.4 Susceptibility of cells to exogenous H₂O₂ in terms of chlorophyll-a
(The chlorophyll-a values were measured after incubation for 24h at different H₂O₂ concentrations. Values are means±SD of triplicate samples.)

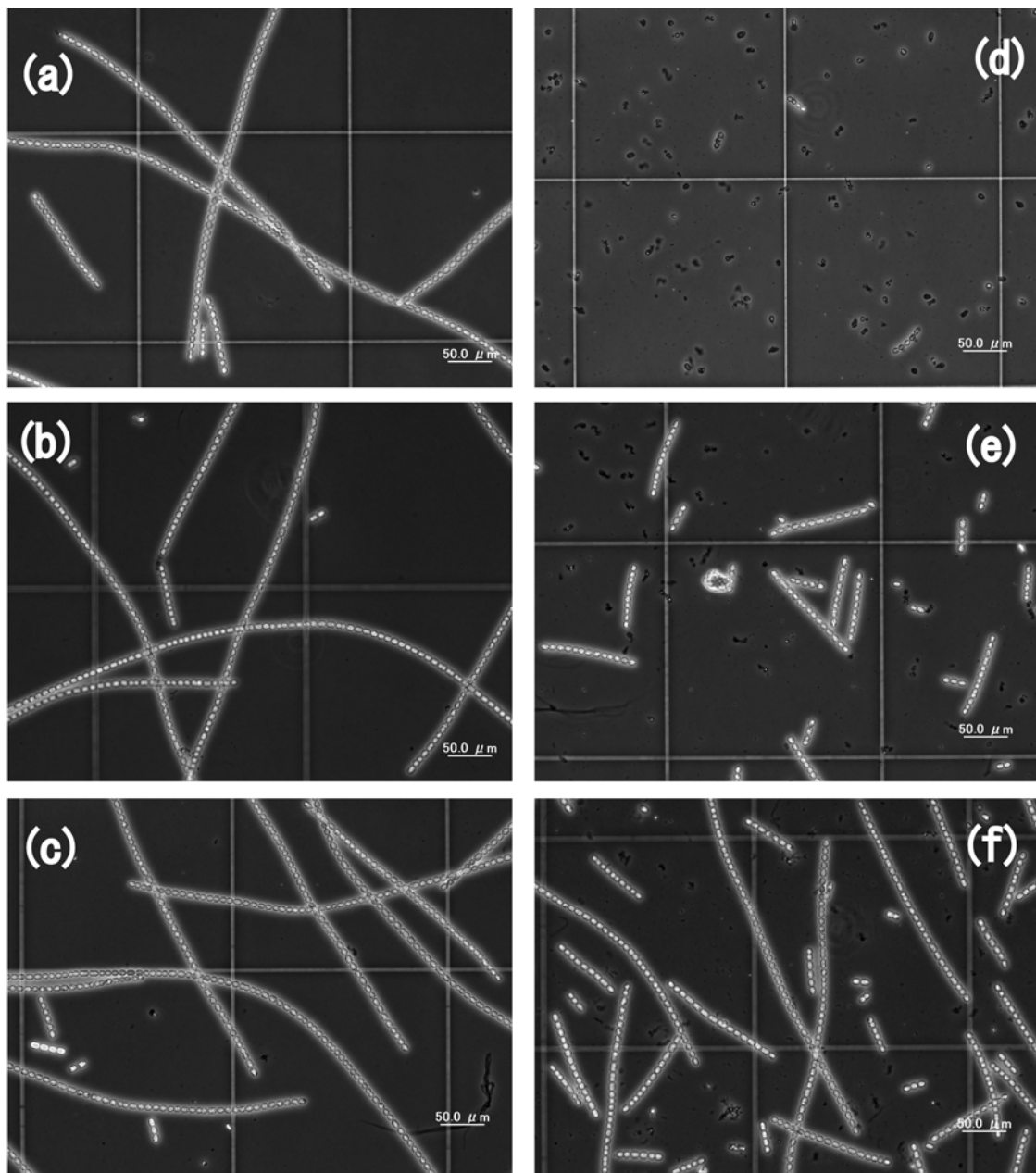


Fig.5 Observations of the effect of catalase on cell lysis

(The conditions were as follows: (a) HA-free, catalase-free, (b) HA-free, catalase: $1000 \text{Units ml}^{-1}$, (c) HA-free, catalase: $10000 \text{Units ml}^{-1}$, (d) HA: 0.1mg-C l^{-1} , catalase-free, (e) HA: 0.1mg-C l^{-1} , catalase: $1000 \text{Units ml}^{-1}$, (f) HA: 0.1mg-C l^{-1} , catalase: $10000 \text{Units ml}^{-1}$. The scale bar under pictures represents $100 \mu\text{m}$)