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Characterization of Bioactive Extracellular Pink Pigments

Produced by Tropical Freshwater Cyanobacteria

Applied Environmental Biology Laboratory

Graduate School of Pharmaceutical Sciences

Osaka University

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Contents

General Introduction

Cyanobacteria are well known as promising source of valuable chemicals for human usage. They have been identified as one of the most attractive group of organisms for novel bioactive natural products [1]. Especially, cyanobacteria in tropical area are very wide in diversity and they are potent producers of unique metabolites which exhibit interesting bioactivities. However, most of them remain unexplored. Therefore, screening of cyanobacteria producing bioactive metabolites has been a critical part to discover novel useful chemicals applicable to various fields.

Most bioactive metabolites synthesized by cyanobacteria are accumulated intracellularly, but several of them are excreted, such as polypeptides [2] and polysaccharides [3, 4]. Some researchers reported that cyanobacteria may derive an advantage from the excretion of such compounds to inhibit the growth of competitors [5] or transmit the signals to other microorganisms [6, 7]. According to this knowledge, the extracellular metabolites have high possibilities to possess useful activities for many applications such as herbicides, pesticides, medicine and so on. With respect to bioprocess production, secretion of products from cells would have an advantage, because it can simplify or remove the extraction and purification steps.

Moreover, the production of extracellular pigment is very unique phenomenon, since most of extracellular metabolites produced by cyanobacteria have no color. In the previous study, Hirata *et al.* reported that tropical freshwater cyanobacteria *Nostoc spongiaforme* TISTR 8169 isolated in Thailand produced an extracellular violet pigment. The pigment, named nostocine A, exhibited growth inhibitory activity to various organisms. Nostocine A generates superoxide anion radical in the presence of oxygen and NAD(P)H in the manner similar to paraquat [8, 9].

In this study, I found that two tropical freshwater cyanobacterial strains, *Oscillatoria* sp. BTCC/A0004 and *Scytonema* sp. TISTR 8208, produce pink pigments extracellularly (Fig. 1). They were isolated in Indonesia and Thailand, respectively. The pigments are expected as novel cyanobacterial metabolites since there are no such pigments which have been reported so far. The objective of this study is to characterize the production of the novel extracellular pink pigments and evaluate their bioactivities.
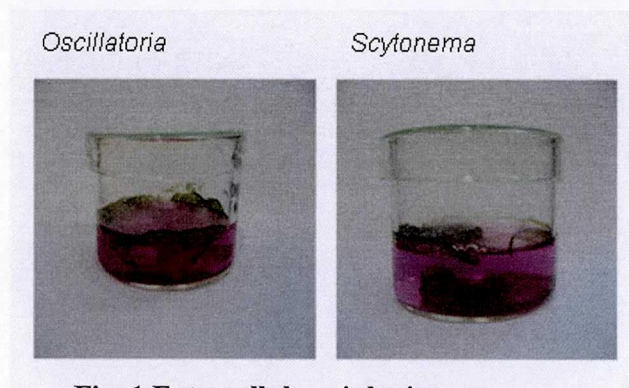
Fig. 1 Extracellular pink pigments produced by *Oscillatoria* and *Scytonema*.

Chapter 1

Characterization of extracellular pink pigments produced by cyanobacteria

Oscillatoria sp. and *Scytonema* sp.

1.1. Introduction

As mention in general introduction, I found that two tropical freshwater cyanobacteria *Oscillatoria* sp. BTCC/A0004 and *Scytonema* sp. TISTR 8208 released pink pigments into medium. Since there are no such pigments which have been reported so far, the characterization of the pigments is priority objective.

At first, I confirmed that the pigments were excreted from actively growing cells. Next I tried purification of the pigments. Finally, the physical properties of purified pigments were analyzed.

1.2. Materials and methods

1.2.1. Algal strains and cultivation conditions

Oscillatoria sp. BTCC/A0004 and *Scytonema* sp. TISTR 8208 were obtained from The Indonesian Institute of Sciences in Indonesia and the Thailand Institute of

Scientific and Technological Research (TISTR) in Thailand, respectively. All reagents were purchased from Nacalai Tesque, (Kyoto, Japan), except where noted.

Oscillatoria sp. and *Scytonema* sp. were sub cultured in test tubes (3 cm i.d. x 20 cm) containing 50 ml of modified C medium at pH 7.5 with the following composition: (per liter) 5 g KNO_3, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.005 g $FeCl_2$, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2.4H_2O$, 0.22 mg $ZnSO_4.7H_2O$, 0.018 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, and 0.075 mg $CuSO_4.5H_2O$. The cells were cultivated with aeration (10 ml min^{-1}, 1 % CO2) at $25^{\circ}C$ under 50 µmol photons m^{-2} s^{-1} of continuous illumination using white fluorescence lamps. For measurement of cell growth and pigment production, 4-day-old seed cultures of both *Oscillatoria* sp. and *Scytonema* sp. were transferred to fresh media and adjusted to 0.1 of OD_{680}. Incubation conditions were the same as for seed cultures except for light intensity was 100 µmol photons $m^{-2} s^{-1}$.

1.2.2. Determination of growth and pink pigment concentration

The time courses of cell growth were monitored by measuring OD_{680}. 1 of OD_{680} value corresponds to 0.99 mg cell dry weight ml^{-1}. The extracellular pink pigment concentration was determined by measuring Abs_{560} of culture media.

1.2.3. Purification of pink pigment

The concentrated media of 8-days-old *Oscillatoria* and *Scytonema* cultures including the extracellular pigments were applied to ultrafiltration using 10,000 Dalton molecular weight cut of filter (Amicon Ultra, Millipore, Billerica, MA, USA). The pigments were purified by gel filtration chromatography on a Superdex 75 column (30 mm i.d. x 1000 mm) associated with AKTAprime (Amersham Biosciences, Uppsala, Sweden). Equilibration of column and elution of pigments were performed with 50 mM sodium phosphate buffer, pH 7.0. Eluates were monitored by absorbance at 280 nm of wavelength. Fractions with pink color were collected and subsequently dialyzed against 1 liter of milli-Q water for 24 h at 4°C with three changes of milli-Q water. The dialysates were centrifuged at 10,000 x g for 20 min at 4°C and the supernatants collected were refrigerated for further investigation. Intracellular pigments were obtained by resuspension of cells in the modified C medium, then subjected to freeze-thaw cycles of -20°C and stored in dark for 24 h at 4°C. The crude extracts were centrifuged at 10,000 x g at 4°C for 10 min and the supernatants were subsequently lyophilized and refrigerated for further investigation.

1.2.4. Electrophoresis

The molecular weights of pink pigments were determined according to ref. [15]. Native-PAGE and SDS-PAGE were performed using a mini-protean II electrophoresis unit (Bio-Rad, USA) with a 4 % stacking gel and a separating gel of 10 % or 12 %, respectively. The separation was carried out at 30 mA for 1 h. NuPAGE (Invitrogen) was used as molecular mass marker. In addition, R-phycoerythrin (R-PE) derived from *Corallina officinalis* (Sigma, USA) was used as the pink pigment standard. Proteins were stained with Bio-safe Coomassie (Bio-Rad, USA).

1.2.5. Spectrophotometric analysis

The absorption spectra of the pink pigments were determined using a U-2000 Spectrophotometer (Hitachi, Japan). The scan wavelength range was 300-700 nm. The fluorescence emission spectra were determined using Fluorescence spectrophotometer F-2500 (Hitachi, Japan) with 498 nm of excitation wavelength. All spectra were recorded at room temperature.

1.2.6. Stability to light irradiation

Stability of the pigments against light irradiation was determined according to ref. (13). The solution examined in quartz cuvet was putted on the chamber and

immersed by running water in a thermostat at 25°C. The sample was exposed to strong

white light (1000 μmol photons $m^{-2} s^{-1}$) at different period. The maximum absorbance

(Abs_{560}nm) of the pigment solution was adjusted at approximately 1.0 before irradiation.

Change of absorption values were monitored spectrophotometrically (U-2000, Hitachi,

Japan).

1.3. Results

1.3.1. Cell growth and pink pigment production

In order to understand correlation of cell growth and extracellular pink pigment production of *Oscillatoria* and *Scytonema*, the time courses of them were monitored (Fig. 2).
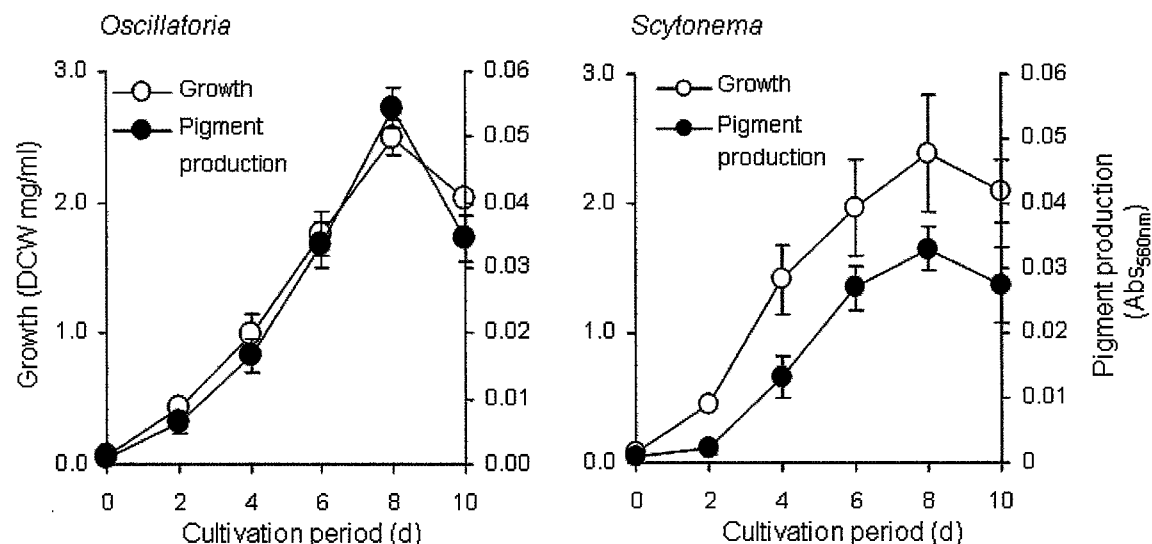
Fig. 2 Cell growth and extracellular pink pigment production of *Oscillatoria* and *Scytonema*. The strains were cultivated in the modified C medium with aeration (10 ml min^{-1}, 1 % CO_2 in air) at 25°C under 100 photons μmol m^{-2} s^{-1} of continuous illumination using white fluorescence light. DCW is dry cell weight. Values are the mean of ± SDs of three independent experiments.

As shown in the Figure 2, typical growth phases in the batch culture including lag (up to day 2), exponential growth (from day 2 to day 8) and declining growth (after day 8) phases were observed. The time courses of pink pigment productions by both

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strains were well correlated with cell growth. The amount of the pigments in the media

significantly increased on exponential growth phase. These results indicated that the

production of the pigments occurred in actively growing cells. The growth decreased at

day 10 might be due to limitation of the nutrient in the media and influence to cells

death. Under microscope investigation, several broken cells were found at day 10 (data

not shown). Consequently, the pigment productions were also decreased.

1.3.2. Comparison of intracellular and extracellular pigment profiles

To further confirm that the pigments were excreted by actively growing cells,

the intracellular and extracellular pigment profiles were compared. The concentrates of

media and cellular extracts from 6-day-old culture of both strains were subjected to

SDS-PAGE (Fig. 3).
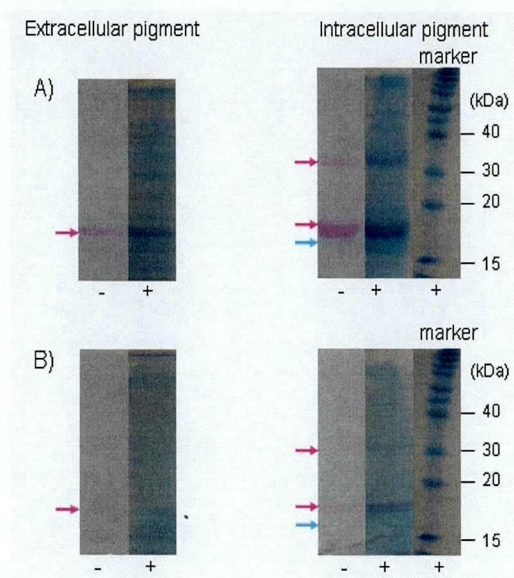

Fig. 3 SDS-PAGE profiles of extracellular and intracellular pigments produced by *Oscillatoria* (A) and *Scytonema* (B). Samples were concentrated media and cellular extracts of 6-day-old *Oscillatoria* sp. and *Scytonema* sp. cultures. The pink and blue bands in the gel were indicated by pink and blue arrows, respectively. Lane (-) and (+) show the gel before and after staining by CBB.

In both strains, the total profile patterns in the gel between extracellular and intracellular pigments were different. One pink band was observed in the medium concentrate. On the other hand, two pink bands and one blue band were observed in the concentrate of cellular extracts. After Coomassie brilliant blue (CBB) staining, the position and density of these bands and other bands were also different. These results suggested that the extracellular pigments were actively released into medium from growing cells rather than simple leakage from dead cells.

Similar results were obtained with *Oscillatoria* and *Scytonema* cultures in pigment production. The following experiments also showed no difference between pink pigments from *Oscillatoria* and *Scytonema* cultures. Consequently, only data on pink pigment from *Oscillatoria* are shown in the following manuscript.

1.3.3. Physical properties of the extracellular pink pigment

The pink pigment produced by *Oscillatoria* was purified by ultrafiltration and gel filtration with Superdex column (Fig. 4). Two fractions with strong absorbance at 280 nm of wavelength were obtained by gel filtration. One fraction possessed pink color and the other was colorless. This pink color protein was named OsPP.
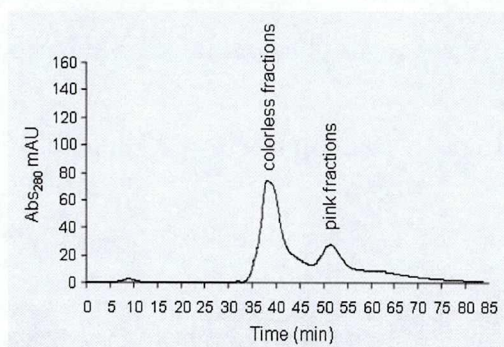
Fig. 4 Gel filtration chromatogram of pink pigment produced by *Oscillatoria* sp. The concentrated pigment was subjected to Superdex 75 column and elution with 50 mM of phosphate buffer. The elution profile was monitored by absorbance at 280 nm.

The pink fraction obtained from gel filtration was subjected to Native-PAGE and SDS-PAGE (Fig. 5). A Native-PAGE of the OsPP yielded one pink band with 250 kDa of molecular weight before staining with CBB. After staining, one blue band was observed at the same position of pink band. In addition, SDS-PAGE also showed one pink band in the gel with approximately 20 kDa of molecular weight. After staining with CBB, two bands were clearly observed with molecular mass of approximately 18 and 20 kDa, respectively. These results were confirmed that OsPP is an oligomeric protein with two types of subunits.
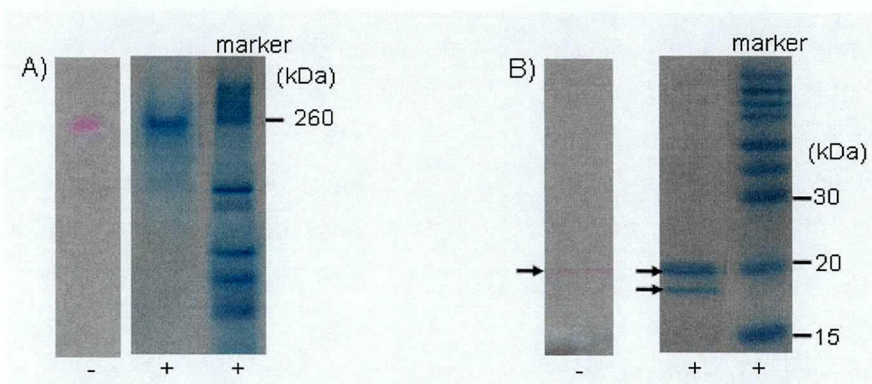
Fig. 5 Native-PAGE (A) and SDS-PAGE (B) of purified OsPP.
Lane (-) and (+) show the gel before and after staining by CBB

OsPP was water soluble proteins and presented as bright pink under white light. Furthermore, yellow fluorescence was observed under irradiation with UV-light at 365 nm (Fig. 6). The UV-VIS absorption spectrum of OsPP showed two peaks with absorption maxima at 560 and 620 nm. In addition, the fluorescence emission maximum wavelength of the OsPP was 580 nm with a 498 nm of excitation (Fig. 6).

These fluorescence properties seemed to be similar to one of phycoerythrin (PE). Furthermore, PE standard derived from *Corralina oficinalis* (CoPE) was analyzed with same method to OsPP (Fig. 7). In Native-PAGE, CoPE showed one pink band before staining with CBB and one blue band after staining with approximately 240 kDa of molecular weight. CoPE was water soluble and presented bright pink under white light. The fluorescence yellow was also observed under UV-light at 365 nm. In addition CoPE showed the fluorescence emission maxima at 580 nm with a 498 nm of excitation. These results strongly indicate that OsPP is PE-like protein.
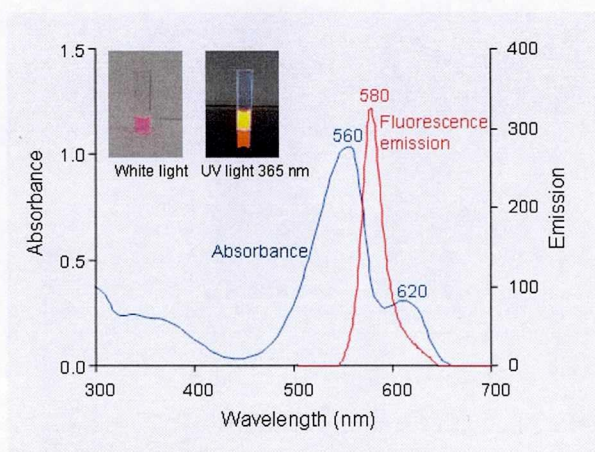
Fig. 6 Absorbance spectrum (blue line) and fluorescence emission spectrum with a 498 nm of excitation (red line) of purified OsPP.
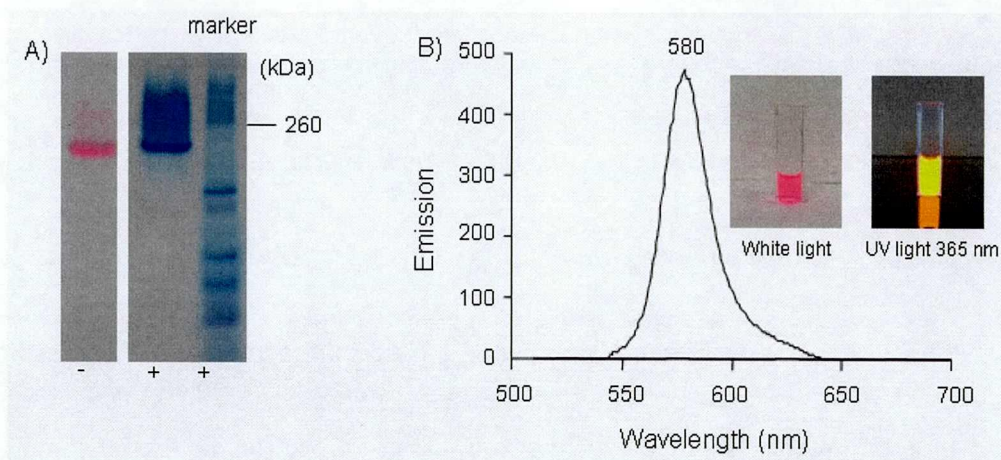
Fig. 7 Native-PAGE (A) and fluorescence emission spectrum (B) of phycoerythrin standard derived from *Corralina oficinalis*. The experimental conditions were the same to Fig. 5 and 6. Lane (-) and (+) show the gel before and after staining by CBB.

The OsPP showed more resistant than PE against light irradiation (Fig. 8). The absorption of OsPP at 560 nm was almost not changed after exposed to strong light (1000 μmol photons $m^{-2} s^{-1}$) up to 240 min. In contrast, at the same time the absorption of PE was decreased almost two fold.
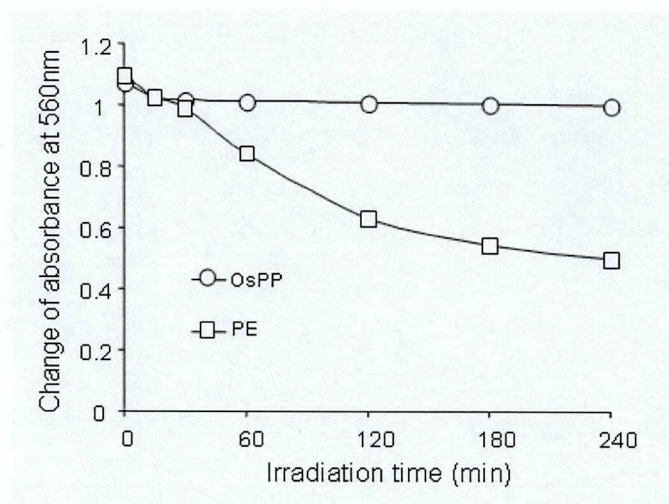
Fig. 8 Change in absorption of OsPP and PE under light irradiation. OsPP and PE solution were exposed to strong white light (1000 μmol photons $m^{-2} s^{-1}$) at different period. The initial absorbances at 560 nm of samples were adjusted at approximately 1.0. Change of absorption values were monitored spectrophotometrically (U-2000, Hitachi, Japan).

1.4. Discussion

In this study, I found novel extracellular pink pigments excreted by actively growing cells of cyanobacteria *Oscillatoria* sp. and *Scytonema* sp. The extracellular pink pigment production significantly increased during exponential growth phase and paralleled the cell growth (Fig. 2). Furthermore, different profiles of extracellular and intracellular pigments were clearly observed (Fig. 3). These results suggested that the extracellular pigments were actively released into medium from growing cells rather than simple leakage from dead cells.

The pigments were expected as novel cyanobacteria metabolites since there are no reports on such pigments. Native-PAGE analysis showed that the apparent molecular mass of OsPP was 250 kDa (Fig. 5). In addition, SDS-PAGE revealed that the OsPP is an oligomeric protein with two types of subunits. OsPP was water soluble and presented fluorescence yellow under UV-light at 365 nm. The absorption spectrum of purified OsPP showed two absorption maxima at 560 and 620 nm (Fig. 6). The emission spectrum of OsPP obtained with at 498 nm of excitation had a maximum fluorescence emission at 580 nm (Fig. 6). The physicals properties of OsPP as mentioned above were similar to PE standard (Fig. 7). PE is known as the most abundant type of phycobiliproteins in algae [10], which functions as light harvesting antenna pigment.

The pigment has large absorption coefficient, high quantum yield in fluorescence, and high Stokes shift. Therefore, PE had potentially useful for a number of applications. It has been extensively investigated for usage as fluorescent dyes in bio-assay [11, 12]. Furthermore, it has also been studied for application to photodynamic therapy by making use of its photosensitising activity [13, 14].

Since the OsPP exhibited physical properties similar to PE, it might be substituted application instead of PE. Moreover, the OsPP showed more resistant to strong light irradiation than PE (Fig. 8). This better point will imply more useful application of OsPP.

In conclusion, results of this chapter demonstrated that pink pigments produced by *Oscillatoria* and *Scytonema* were excreted from actively growing cells and exhibited physical properties similar to PE.

Chapter 2

Bioactivities of extracellular pink pigment produced by *Oscillatoria* sp.

2.1. Introduction

In the chapter 1, I found that extracellular pink pigments produced by *Oscillatoria* and *Scytonema* were excreted from actively growing cells. Cyanobacteria may derive and advantage from the production of extracellular metabolites to combat of other organisms of the same habitat with function as allelochemicals [15]. In this regards, I assume that the pink pigments secreted into medium might possesses similar function as allelochemical exhibiting growth inhibitory activity against competing organisms in natural environment.

In addition, several metabolites produced by cyanobacteria and other phytoplankton possess functions as photosensitisers (PS) were also documented. Photosensitiser is the chemicals are able to generate reactive oxygen species (ROS) by light energy. PS is not toxic until exposed to light with specific wavelength, which can lead to energy transfer to oxygen and other substances, producing toxic reactive species such as singlet oxygen and free radicals which act on various constituent of the cells resulting in cell death [16].

Metabolites showing photosensitizing activity have been attractive attention, in particular, rise in microbial resistant to antibiotic requires the development of new antibacterial strategy. Photodynamic antimicrobial chemotherapy (PACT) utilizes light in combination with a PS to induce a phototoxic reaction present a promising alternative to the use of PS to combat resistant microbial [17]. On the other hand, application of photosensitiser for photodynamic therapy (PDT) on cancer treatment is already in clinical use. However, the most widely use of PS such as hematoporphyrin derivatives appear several limitations, such as low absorption coefficient in the phototherapeutic region and dermal photosensitivity resulted from prolonged retention of these agent in the skin [13]. These limitations have led to search for improved PS for PDT.

In this study, therefore bioactivities of OsPP to various microorganisms were investigated. To confirm the OsPP as photosensitiser, generation of reactive oxygen species from light-irradiated OsPP was analyzed. In addition, to consider useful application of OsPP as photosensitiser, growth inhibitory activity against cancer cells was also evaluated.

2.2. Materials and methods

2.2.1 Growth inhibition assay

To evaluate that extracellular pink pigment released to culture medium has potent growth inhibitory activity, 2.5 mg ml^{-1} of medium concentrates obtained from different cultivation period of *Oscillatoria* culture were added to culture of green algae *Chlorella fusca* and *Chlamydomonas reinhardtii*. Cells were incubated in the same condition with light 30 μmol photons m^{-2} s^{-1} and growth inhibitory activity was determined after incubation period 4 days.

To further evaluate the inhibitory concentration 50 % (IC_{50}) of the pigment, different concentrations (0.1, 0.5, 1, 5 and 10 mg ml^{-1}) of concentrated 8-day-old *Oscillatoria* culture media were added to culture of various microorganisms (Table 1). Green algae and cyanobacteria cultures were incubated in a cultivation box and supplied with air containing 1 % CO_2 and continuous illumination with white fluorescence light at 30 μmol photons m^{-2} s^{-1}. Growth inhibitory activities were determined after incubation period 4 days.

Two bacteria *Escherichia coli* and *Bacillus subtilis* were precultivated in the test tube containing 5 ml of Nutrient Broth at 37°C for 24 h in the dark. The cells then transferred to Erlenmeyer flask containing 100 ml of same medium and incubated until

the OD_{600} was obtained to 0.5. Two millilitre of the cell suspension was inoculated into a 24-well tissue culture plate containing 0.1, 0.5, 1, 5 and 10 mg ml^{-1} of concentrated 8-day-old *Oscillatoria* culture media. Growth inhibitory activities were observed after incubation of cells at 37°C for 24 h in the dark.

2.2.2. Effect of light irradiation on growth inhibitory activity of OsPP

To evaluate the effect of light irradiation on growth inhibitory activity of OsPP, green algae *C. fusca* cell was treated with 2 mg ml^{-1} of OsPP. *C. fusca* cell without OsPP was used as a control. The cells then exposed to light (200 µmol photons m^{-2} s^{-1}) with white fluorescence light or in the dark for 15, 30 and 60 min. After incubated of *C. fusca* cell with light-irradiated OsPP or non-irradiated one, the treated cells were picked up and transferred to fresh medium then incubated in same condition with light. Growth inhibitory activity was observed by measuring of growth *C. fusca* cell every two days.

2.2.3. *In vitro* analysis of reactive oxygen species (ROS)

The purified OsPP solution (250 µg ml^{-1}) in sodium phosphate buffer pH 7.0 was added by 12 µl of 100 mM 5,5,-dimethyl-1-pyroline-*N*-oxide (DMPO) (Labotex, Dojindo Chemical, Japan). The solution was mixed well by vortex mixing to introduce

air, incubated for 1 min at 25°C then irradiated by 200 μmol photons $m^{-2} s^{-1}$ white light or non-irradiated. The photogeneration of hydroxyl radicals or superoxide radical anions from the OsPP solution were analyzed by an electron spin resonance (ESR) spectrophotometer (JES-TE200, JEOL, Japan) under the following conditions: Field modulation frequency, 9.42338 GHz; power, 8.0 mW; magnetic field, 335±5 mT; field modulation width, 0.1 mT; time constant, 0.1 s; receiver gain, 200; sweep time, 2 min; temperature, 21°C. Typical signals of superoxide anion and hydroxyl radicals standard were obtained from reaction mixture of xanthine and xanthine oxidase and H_2O_2 and $FeSO_4$, respectively.

2.2.4. Growth inhibitory activity of OsPP against cancer cells

To evaluate the effect of light-irradiated purified OsPP against cancer cells, A549 (human lung cancer cell) was used as a target cell. The A549 cells were seeded in a 96-well flat-bottom culture plate at a density of 5×10^3 cells 100 μl^{-1} well^{-1} and cultured for 12-24 h at 37°C with 5 % CO_2. 20 μl of OsPP or PE solution were added to each cell at final concentration of 1, 0.3, 0.1, 0.03 mg ml^{-1} per well, and were then incubated for 1-3 h at room temperature with or without light exposure. Subsequently, cell viability was evaluated by WST-8 assay kit (Nacalai Tesque, Kyoto, Japan)

according to manufacture's protocol. Briefly, 10 µl of WST-8 solution was added to each well and the cells were incubated for 1 hour. Growth inhibitory activity was determined by measure OD460/600. OD value from untreated cells was designated 100 % as a control. Each point represents the mean ± SD from three independent cultures.

2.3. Results

2.3.1. Growth inhibition assay

Growth inhibitory activity of medium concentrates obtained from different cultivation period of *Oscillatoria* cultures are shown in the Figure 9. Growth of *C. fusca* and *C. reinhardtii* were inhibited by 2.5 mg ml^{-1} of medium concentrates of 6, 8 and 10-day-old cultures of *Oscillatoria* sp. when pink pigment was clearly detected in the medium. On the other hand, growth was not affected by the addition of the 4-day-old medium concentrates, in which pink pigment was not detected. Furthermore, increasing of concentration of medium concentrates tend to increase growth inhibitory activity (data not shown). These results indicated that extracellular pink pigment has potent growth inhibitory activity.
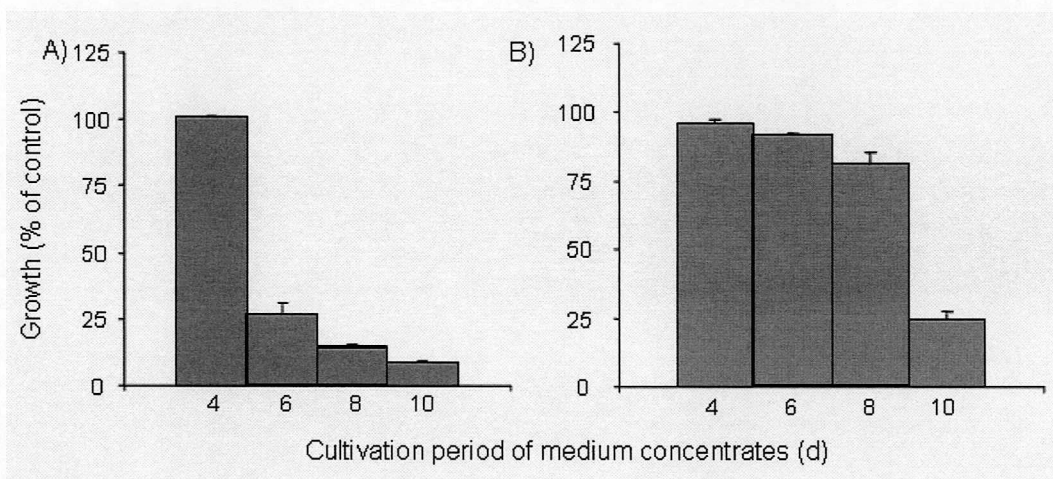

Fig. 9 Growth inhibitory activity of medium concentrate from different cultivation period of *Oscillatoria* cell against *C. fusca* (A) and to *C. reinhardtii* (B). Cells were cultivated in 2.5 mg ml^{-1} of 4, 6, 8 and 10-days-old cultures of *Oscillatoria* sp. and incubated in the light for 4 days. Values are the mean ± SDs of three independent experiments.

To further evaluation of growth inhibitory activity of the pigment, IC_{50} was investigated. Different concentrations (0, 0.1, 0.5, 1, 5 and 10 mg ml^{-1}) of concentrated 8-day-old *Oscillatoria* culture media were added to the culture of various microorganisms.

The growth inhibition against *C. fusca* and *C. reinhardtii* were concentration-dependently increased and IC_{50}s were 0.5 mg ml^{-1} and 6 mg ml^{-1}, respectively (Fig. 10). In contrast, IC_{50} was not observed at more than 10 mg ml^{-1} of medium concentrates against cyanobacteria and other bacteria tested (Table 1). These results indicated that OsPP has potent growth inhibitory activity against green algae specifically. In particular, the growth of *C. fusca* was intensively inhibited by the OsPP. Therefore, *C. fusca* cell was used for further investigation.
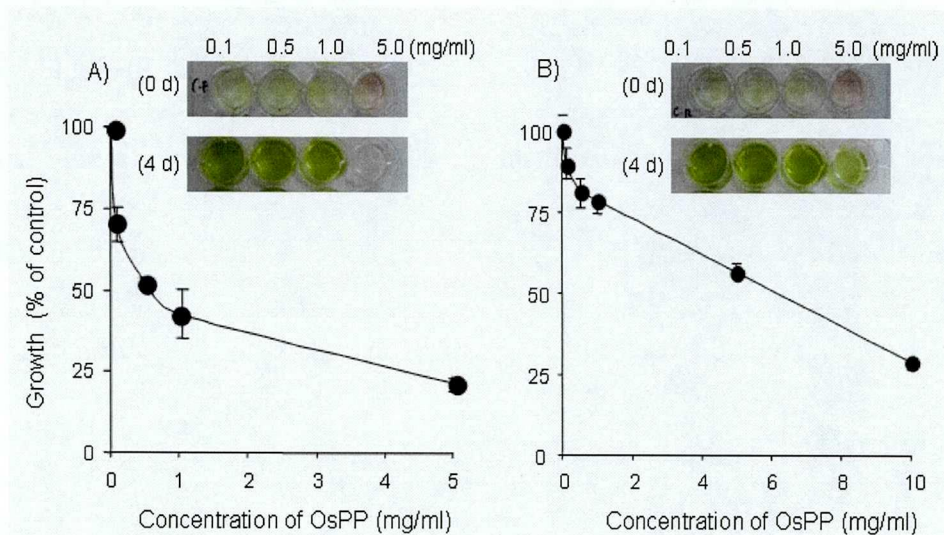

Fig. 10 Dose response of OsPP against *C. fusca* (A) and *C. reinhardtii* (B).
Cells were cultivated in various concentrations of OsPP and incubated in the light for 4 days. Value from untreated cell was designated 100 % as control. Values are the mean ± SDs of two independent experiments.

Table 1. Growth inhibitory activity 50 % (IC$_{50}$)
of OsPP to various microorganisms

Strain	IC$_{50}$ (mg ml^{-1})
C. fusca	0.5
C. reinhardtii	6
Oscillatoria sp.	> 10
Scytonema sp.	> 10
E. coli	> 10
B. subtilis	> 10

2.3.2. Effect of light irradiation on growth inhibitory activity of OsPP

To evaluate the effect of light irradiation on growth inhibitory activity of OsPP, green algae *C. fusca* cell was treated with 2 mg ml^{-1} of OsPP. *C. fusca* cell without OsPP was used as a control. After incubated of *C. fusca* cell with light-irradiated OsPP for 15, 30 and 60 min, the treated cells were picked up and transferred to fresh medium then incubated in same condition with light. Results from this experiment are shown in the Figure 11. Open circle symbols mean growth of *C. fusca* cell with light-irradiated OsPP and closed circle symbols mean growth of *C. fusca* cell in non-irradiated OsPP. In addition, open square and closed square symbols mean growth of *C. fusca* cell without OsPP, respectively.
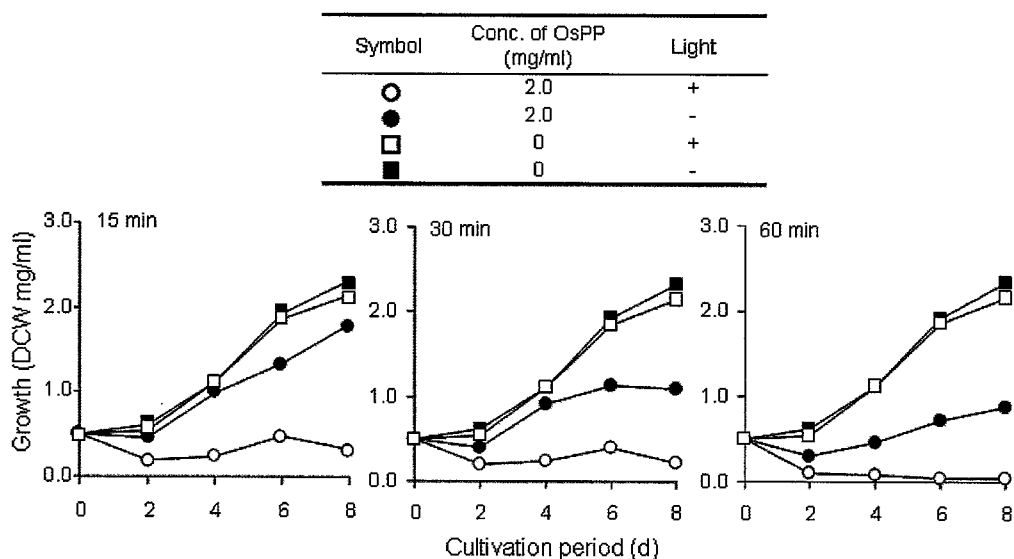
Fig. 11 Effect of light irradiation on growth inhibitory activity of OsPP against *C. fusca*.
C. fusca cell was treated by 2 mg ml^{-1} of light-irradiated OsPP or non-irradiated OsPP for 15, 30 and 60 min, respectively. *C. fusca* cell without OsPP was used as control. Conditions of treatment to *C. fusca* cell are indicated with symbols as shown in the figure. DCW is dry cell weight. Values are the mean ± SDs of two independent experiments.

It was clearly observed, growth of *C. fusca* cells was more strongly inhibited by light-irradiated OsPP than non-irradiated OsPP. Furthermore, the activity increased with increasing the period of light irradiation. In addition, when concentration of the OsPP was doubled (4 mg ml^{-1}), growth inhibitory activity of the OsPP to *C. fusca* cell was also increase nearly two fold in the irradiation times dependence manner (data not shown). These results indicate OsPP functions as photosensitiser agent.

2.3.3. *In vitro* analysis of ROS

To verify that the OsPP possesses photosensitising activity, ESR analysis of purified OsPP solution was performed (Fig. 12).
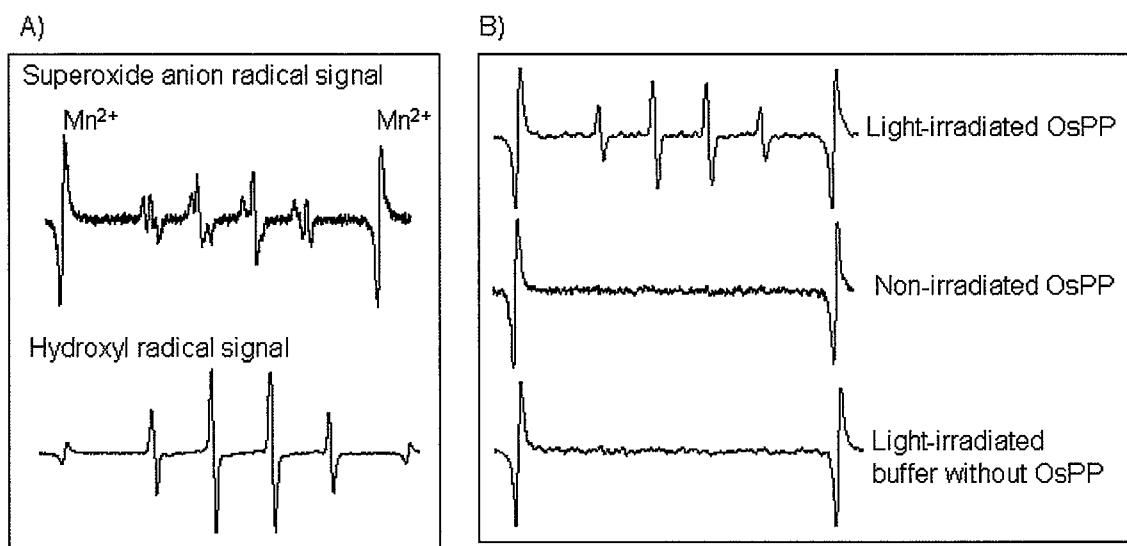
Fig. 12 ESR spectra of standards (A) and OsPP solution (B). Purified OsPP solution (250 $\mu g\, ml^{-1}$) containing DMPO (100 mM) were irradiated by 200 μmol photons $m^{-2}\, s^{-1}$ for 15 min. The ESR analysis conditions are presented in the materials and methods.

When signals in the right side graph were compared to typical spectra of standards, the typical spectrum of hydroxyl radicals were clearly observed from light-irradiated OsPP. In contrast, no signals of hydroxyl radicals were detected from non-irradiated OsPP or from light-irradiated buffer solution without OsPP. These results indicated that hydroxyl radicals were generated from light-irradiated OsPP.

To further confirm that hydroxyl radicals were generated by light-irradiated OsPP, effects of catalase (CAT) and dietylene triamine penta-acetic acid (DTPA) were

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investigated (Fig. 13). CAT and DTPA are well known as specific scavengers for

hydrogen peroxide and hydroxyl radicals, respectively.
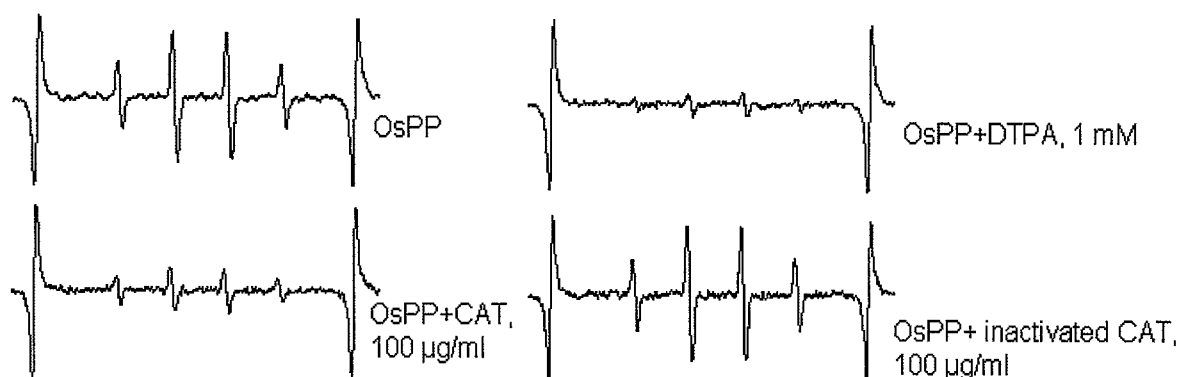
Fig. 13 ESR spectra of light-irradiated OsPP in the presence of catalase (CAT) and dietylene triamine penta-acetic acid (DTPA). The concentration of CAT and DTPA are shown in the Figure. Purified OsPP solution (250 μg ml^{-1}) containing DMPO (100 mM) were added by CAT and DTPA and irradiated by 200 μmol photons $m^{-2} s^{-1}$ for 15 min. The ESR analysis conditions are presented in the materials and methods.

As shown in the Figure 13, more than 50 % of hydroxyl radical signals were

inhibited by CAT addition, but addition of heat-inactivated CAT showed no effect.

These phenomena indicated that OsPP would generate hydrogen peroxide followed by

conversion to hydroxyl radical. Furthermore, addition of DTPA significantly reduced

the signal intensity of hydroxyl radicals. These evidences further confirm that hydroxyl

radicals were generated from light-irradiated OsPP.

2.3.5. Growth inhibitory activity of OsPP against cancer cells

To consider further useful application of OsPP as photosensitiser, evaluation of growth inhibitory activity against cancer cells was investigated (Fig. 14).
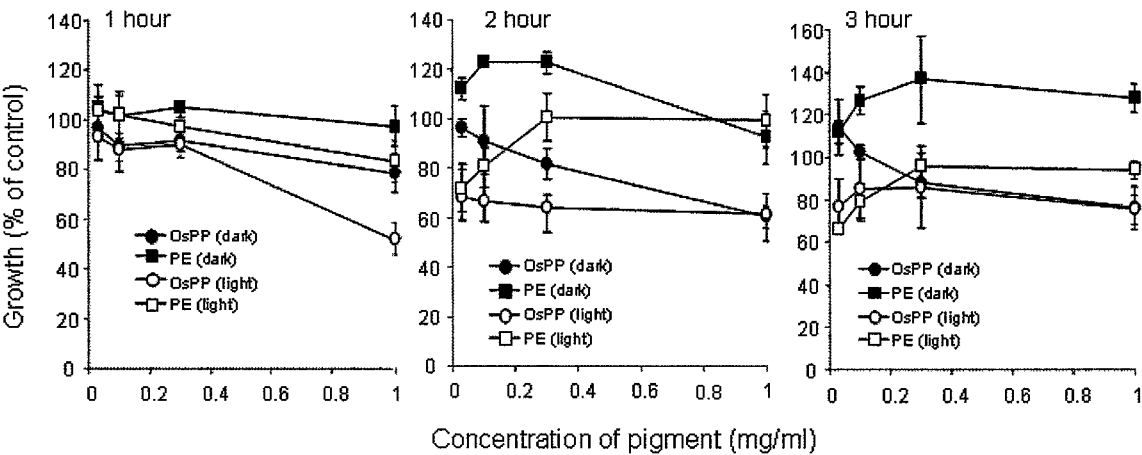
Fig. 14 Growth inhibitory activity of OsPP and PE against A549 cancer cell.

5×10^3 100 μl^{-1} well^{-1} cancer cells were treated by various concentration of light-irradiated OsPP and PE (open circle and square symbols) or non-irradiated (closed circle and square symbols) for 1-3 hour. Value from untreated cell was designated 100 % as control. Each point represents the mean ± SDs from three independent cultures.

Growth of cancer cells was strongly inhibited after cells were exposed to light-irradiated OsPP for 1 h than non-irradiated one. Similar phenomenon was observed under light irradiation period for 2 h at the concentration of OsPP less than 1 mg ml^{-1}. However, increasing light irradiation period for 3 h did not increase growth inhibitory activity of the pigment. Overall, growth inhibitory activity of OsPP was stronger than PE.

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2.4. Discussion

Several planktonic and terrestrial cyanobacteria are well known to produce a wide range of secondary metabolites [18]. In particular, extracellular metabolites might involve in allelopathic interactions to photoautotrophic organisms (algaecide). In the case of freshwater cyanobacteria, this algaecide produced in order to eliminate other photoautotrophic organisms in the same habitat particularly algae, which might compete for nutrients and light that would limit growth of the cyanobacteria [6].

OsPP was thought to function as allelochemicals in the natural environment. To confirm its function, growth inhibitory activities of OsPP to various organisms were investigated. OsPP inhibited the growth of green algae *C. fusca* and *C. reinhardtii* (Fig. 10), but did not inhibit the growth of cyanobacteria and bacteria (Table 1). These results indicated that growth inhibitory activity of OsPP was effective against green algae. To our knowledge, this is the first report to demonstrate that an extracellular pink pigment-like PE shows growth inhibitory activity against green algae.

Interestingly, the growth inhibition of OsPP against *C. fusca* cell occurred much greater in the light than in the dark (Fig. 11), indicating that OsPP functioned as photosensitizing agent. The proposed pathway for photochemical process of photosensitiser in photokilling organisms is presented in Figure 15.
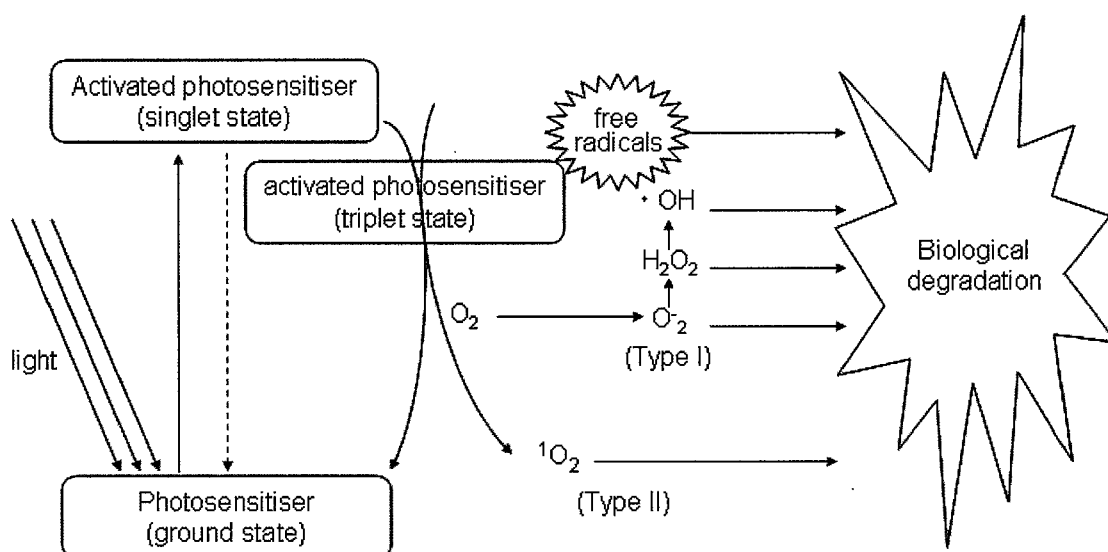
Fig. 15 Proposed pathways for generation of ROS from photosensitisers

When exposed to light of specific wavelength, the sensitizer is transformed from

its ground state into excited state (triplet state) via a short-lived excited singlet state.

The excited triplet can undergo two kinds of reactions; first, it can react with substrates,

such as the cell membrane directly and transfer an electron to form highly free radicals.

These radicals interact with oxygen to produce oxygenated products such as superoxide

anion (O_2^-) and hydroxyl radicals ($\cdot$OH) (type I reaction). Second, the triplet can

transfer its energy directly to oxygen, generating singlet oxygen $(^1O_2)$, a potent

oxidizing agent (type II reaction) [13, 16]. $^1O_2$ and $\cdot$OH are well known as the major

mediator of photochemicals to damage cells in many type of PS. They induce

deleterious effects including lipid peroxidation and membrane damage. It was also

reported that photokilling of photosensitisers act on multiple targets biomolecules such as genomic DNA, proteins and enzymes [19].

Growth inhibitory activity of OsPP was more effective against green algae (eukaryote) cells than against bacteria (prokaryote) cells. It was reported that eukaryotic cells were less sensitive to algaecidal than prokaryotic cells [20]. Moreover, hydroxyl radical was known specifically inhibiting photosystem II, which lack in prokaryotic cells. A number of herbicides including cyanobacterin, which is a toxin produced by cyanobacteria, induce damage to thylakoid membranes, resulting in loss of chlorophyll and inhibiting a site in the vicinity of photosystem II [21]. Increases in intracellular concentrations of hydroxyl radicals, caused by exposure of cells to photosensitizers, stimulated the apparent photodamage to PS II was also documented.

In addition, hydroxyl radical is also generated by green algae itself during photosynthesis which might further contribute to inhibition of their growth. Hirayama *et al.* demonstrated that hydroxyl radical was generated from the living cells and involved on photoinhibition of *Chlorella vulgaris* [22]. Inhibition of photosynthetic efficiency and the specific growth rate same to be coupled in *Spirulina maxima* [23].

C. reinhardtii showed more resistance to the growth inhibitory activity of OsPP than *C. fusca* (Fig. 10). Among green algae, *C. reinhardtii* is well known as tolerance

strain to oxidative stress. This alga has been used as model organism for studying adaptive responses in photosynthetic organisms [24]. *C. reinhardtii* possesses several enzymatic and non enzymatic defense mechanisms to the toxicity of ROS. The enzymatic defense mechanisms in *C. reinhardtii* are mainly based on the activity of some enzymes like CAT, gluthatione peroxidase (GSH), ascorbate peroxidase (APX), and superoxide dismutase (SOD). These enzymes eliminate different type of ROS. SOD is superoxide radical scavenger, while CAT and APX are hydrogen peroxide scavengers [25]. In the non enzymatic responses, glutathione, ascorbate, vitamin E and carotenoids work as scavengers of ROS.

OsPP does not inhibit the growth of *Oscillatoria* and *Scytonema*, presumably they have mechanisms for self-protection against their own bioactive metabolites and these mechanisms might be unique to photosynthetic organisms where protection to herbicidal compounds is required [21]. This phenomena further confirm that the growth of *Oscillatoria* decreased at day 10 (Fig. 2) was not by toxicity of the pigment but due to limitation of nutrient in the media.

Prokaryotic cells, especially Gram negative bacteria such as *E. coli* appeared to be more resistant to photodynamic action of PS, because the specific of cellular envelopes, in particular to the existence of the outer membrane. The outer membrane is

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essential barrier against the penetration of hydrophobic or high-molecular weight compounds into the cells, and it seems to prevent the binding of these compounds to the inner, cytoplasmic membranes. There is evidence that the binding of the PS to the cytoplasmic membrane is essential for the killing effect of the photodynamic action to occur. In the photodynamic action of PS, the production of ROS mediated by the exited triplet state of PS molecules should not be dependent on the binding of the PS to the cells. However, in some PS, the efficiency of photodynamic action was proportional to the incorporation of these PS in to the cells, suggesting that incorporation of the PS into the cells is important. Dependent on a particular chemical structure, some PS can bind or attach to the cells consequently causing much higher effectiveness of these compounds [26].

Other possibility that OsPP does not inhibit growth of cyanobacteria or bacteria is because the concentration of OsPP is low. 10 mg ml^{-1} of 8-days-old medium concentrates containing OsPP used in this study was corresponding to 122 µg ml^{-1} of purified pink pigment. This concentration might not quite enough to produce hydroxyl radicals in the level which the toxicity occurred against both cyanobacteria and bacteria strains (data not shown). It was reported that photosensitization of PS was dependent on the concentrations of the dyes. For example, light-irradiated phycocyanin less than 125

μg ml^{-1} did not affect to *E. coli* cell, at least when exposed for a maximum of 20-min illumination (2.4 X 10^5 J m^{-2}). Light-irradiated phycocyanin showed photosensitizing activities to the cell when its concentration reached at 250 μg ml^{-1} [27]. In addition, although a range of toxicity of hydrogen peroxide (acts as hydroxyl radical generation) against *E. coli* is as low as 0.7 mg l^{-1} (20.6 μM), the most common concentration of hydrogen peroxide for bioremediation has been 100 mg l^{-1} which corresponds to 2.94 μM [28].

Surprisingly, light-irradiated OsPP showed growth inhibitory activity against human lung carcinoma cell A549 (Fig. 14). Almost 50 % of cancer cells were inhibited after treated by light-irradiated OsPP (1 mg ml^{-1}) for 1 hour. In contrast, only 10 % of cancer cells were inhibited by non-irradiated one. However, prolong on light irradiation period (3 h) did not increase growth inhibitory activity of the pigment. In case of cancer cells, apoptosis has been shown to be a rapid and dominant form of cell death following PDT in multiple experimental setting. It was proposed that apoptosis was induced by hydroxyl radicals resulting in the suppression of cancer cells proliferation. However, molecular mechanism of cell death due to hydroxyl radicals on PDT is not fully recognized.

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Interestingly, growth inhibitory activity of light-irradiated OsPP was stronger than PE. Up to 1 mg ml^{-1} PE, increasing light irradiation period did not increased growth inhibitory activity of PE. These results indicated that purified OsPP possesses photosensitizing activity more effective than PE against cancer cell.

Recently, PE is considered to exhibit several advantageous over the presently used hematoporphyrin derivatives (HPD) in PDT, i.e., readily preparation and purification compared to HPD, high molar extinction coefficient, wide UV-visible absorption, no side effects and significantly reduction of normal tissue photosensitivity because of its fast metabolism *in vivo* [13]. Based on these points, it is greatly expected that OsPP will be new type of photosensitiser applicable on PDT.

In conclusion, this study demonstrated that OsPP behave photosensitizing agent. It was proposed that hydroxyl radicals generated from the light-irradiated OsPP might totally or partially contribute to the photokilling and photoinhibition of OsPP against green algae and cancer cell. To consider further useful application of OsPP as photosensitiser, clarification of detail mechanism of its functions and evaluation of photosensitizing activity to cancer cells, pathogenic bacteria as well as eukaryotic microorganisms (fungi and yeast) are necessary.

Chapter 3

Effect of environmental factors on pink pigment production

3.1. Introduction

Results from the Chapter 1 demonstrated that OsPP exhibited physical properties similar to PE. PE has gained tremendous interest due to excellent spectroscopic properties. It has been extensively investigated and exploited commercially for usage such as for fluorescent dyes in bio-assay [11, 12]. In this regards, OsPP might be substituted chemicals of PE. In addition, OsPP has potent growth inhibitory activity against green algae and cancer cells with function as photosensitizer (Chapter 2). These results indicated that OsPP is promising substance for application in various fields. Therefore, investigation of its production is critical part to obtain high yield of the pigment.

In many papers, the effects of nutrient, light, pH, temperature and co-cultivation with competitive organisms on the synthesis of cyanobacterial metabolites were well documented [29-32]. It was expected that those factors might also stimulate the production of OsPP. Therefore, in this chapter the factors which stimulate cell growth and OsPP production were investigated.

3.2. Materials and methods

3.2.1. Algal strains and cultivation conditions

Oscillatoria sp. BTCC/A0004 was cultivated in the same conditions as described in the Chapter 1. Other cyanobacteria *Anabaena cylindrica* NIES 19, *Anabaena variabilis* NIES 23 and green algae *Chlorella fusca* IAM C-28 and *Chlamydomonas reinhardtii* IAM C-238 were obtained from the National Institute for Environmental Studies, in Japan. The cyanobacteria and green algae were cultivated in the same conditions to cultivation of *Oscillatoria* sp.

3.2.2. Effect of different media

Three different culture media named C, modified C and No 18 media, in which nutrient compositions are different were tested (Table 2). The cultivation methods were same to basal cultivation condition.

3.2.3. Effect of various temperature and pH

To evaluate the effect of various temperature and pH on the pigment production, *Oscillatoria* cell was cultivated under condition where temperature (25, 30, 40°C) and

pH (6.5, 7.5 and 8.5), respectively. Other parameters are same to basal cultivation condition.

Table 2. Chemical composition of three culture media (L^{-1})

Components	No 18	C	Modified C
NaCl (mg)	70	-	-
$MgSO_4\,7H_2O$ (mg)	380	40	50
$CaCl_2\,H_2O$ (mg)	106	-	-
$Fe_2(SO_4)_3\,nH_2O$ (mg)	10	-	-
$Na_2EDTA\,2H_2O$ (mg)	27	1000	-
K_2HPO_4 (mg)	600	-	100
H_3BO_3 (mg)	3	-	2.86
$MnSO_4\,4H_2O$ (mg)	2	-	-
$Na_2MoO_4\,2H_2O$ (mg)	8	2.5	-
$ZnSO_4\,7H_2O$ (mg)	0.3	22	0.22
$CuSO_4\,5H_2O$ (mg)	0.08	-	0.075
$CoCl_2\,6H_2O$ (mg)	0.03	4	-
Titriplex III (g)	0.07	-	-
$Ca(NO_3)\,4H_2O$ (mg)	-	150	-
KNO_3 (g)	-	0.1	5
$FeCl_3$ (mg)	-	196	-
$MnCl_2\,4H_2O$ (mg)	-	36	1.81
Tris (hydroxymethyl) aminomethane (mg)	-	500	-
$NaH_2PO_4\,2H_2O$ (mg)	-	50	-
Vitamin B_{12} (µg)	-	0.1	-
Biotin (µg)	-	0.1	-
Thiamin HCl (µg)	-	10	-
$FeCl_2$ (mg)	-	-	5
$(NH_4)_6Mo_7O_{24}\,4H_2O$ (mg)	-	-	0.018

3.2.4. Effect of light quality

To investigate the effects of light quality on cell growth and pink pigment production, the cells were cultivated in 100 ml of modified C medium in Petri dishes (6 and 9 cm in depth and diameter, respectively). The dish was placed in a transparent cultivation box and illuminated continuously with 30 µmol photons m^{-2} s^{-1} fluorescence light quality and aeration with 1 % CO_2 in air, 25°C. White, blue and green light were supplied with three 10 W fluorescence lamps (National, Japan). The pink light was supplied with three 10 W fluorescence lamps (NEC, Japan).

3.2.5. Effect of co-cultivation with competitors

To evaluate the effects of co-cultivation with competitors, cyanobacteria (*A. cylindrica* and *A. variabilis*) or green algae (*C. fusca* and *C. reinhardtii*) were cultivated together with *Oscillatoria* under the same conditions to cultivation of *Oscillatoria* alone.

3.3. Results

3.3.1. Effect of different culture media

In order to find the factors which stimulate cell growth and pink pigment production, the effects of various environmental factors were investigated. The effects of different media on cell growth and OsPP production were presented in Figure 16.

The highest cell growth and OsPP production were obtained in modified C medium. Modified C medium contains more nitrogen (KNO_3) than two other media evaluated (Table 2). It is well known that nitrogen is an essential element required for the growth and synthesis of various metabolites [29]. This might be one of the reasons why cell growth and OsPP production in modified C medium were higher than those in the other media.
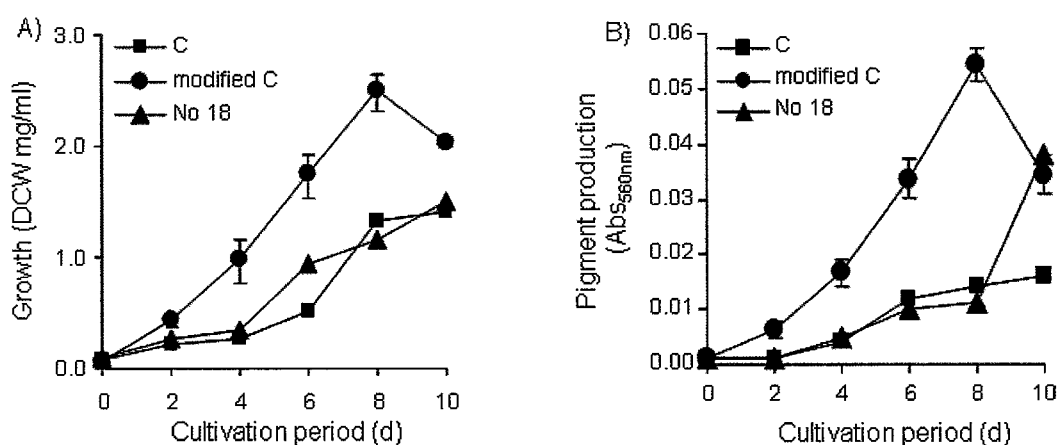

Fig. 16 Effect of different media on growth (A) and pigment production (B) of *Oscillatoria* sp. The cell was cultivated in various media in the light (100 μmol photons $m^{-2} s^{-1}$) and aeration of 1 % CO_2 in air at 25°C. DCW is dry cell weight. Values are the mean ± SDs of three independent experiments.

3.3.2. Effects of light quality

Light is the primary energy source and play an important role in photosynthetic organisms including cyanobacteria. There are several reports that light quality as well as light intensity showed significant effects on growth and metabolism in cyanobacteria [33-36]. The effects of light quality on cell growth and OsPP production are shown in Figure 17. The cell growth and OsPP production were stimulated significantly by pink light irradiation.
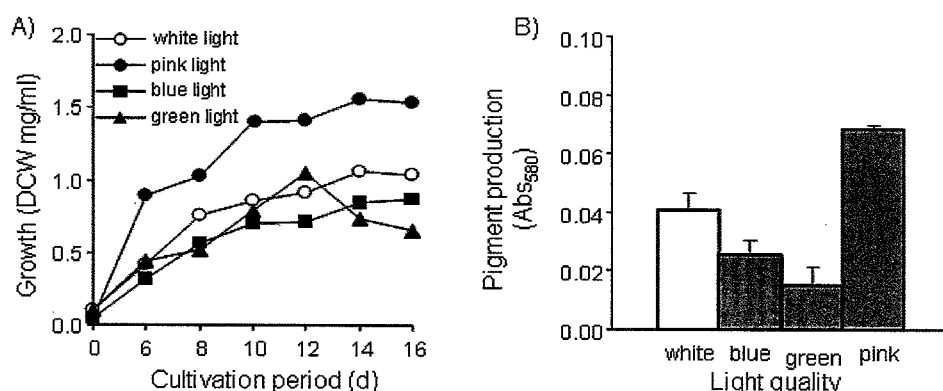

Fig. 17 Effects of light quality on growth (A) and pigment production (B) of *Oscillatoria* sp. Cells were cultivated under different wavelengths of light at 30 μmol photons $m^{-2} s^{-1}$. The production of OsPP in the medium was measured for 16-day-old *Osillatoria* sp. DCW is dry cell weight. Values are the mean ± SDs of three independent experiments.

3.3.3. Effect of co-cultivation with competitors

To evaluate the effect of competitors on pigment production, *Oscillatoria* was co-cultivated with cyanobacteria *A. cylindrica*, *A. variabilis* and green algae *C. fusca*

and *C. reinhardtii*, respectively. These strains are commonly found as co-existing

organisms in aquatic environment in tropical area. Among them, only weak stimulatory

effect was observed by co-cultivation of *Oscillatoria* with *A. cylindrica* (data not

shown).

The effect of other factors, such as light intensity, pH and temperature were also

investigated. However, no factors exhibiting significant stimulation on cell growth and

OsPP production (data not shown).

3.4. Discussion

Among the environmental factors were evaluated, the effects of different media and light quality show significant effect on cell growth and OsPP production. Three different media C, modified C and No 18 are known as culture media for cultivation of freshwater cyanobacteria. These media were also used for cultivation of several freshwater cyanobacteria available in our laboratory. Under modified C medium, the pink pigment in culture media was clearly observed on 6-day-old *Oscillatoria*, which is correspond to the absorbance value (Abs_{560}) of 0.02. On the other hand, the pigments from grown cells in No 18 and C media were observed on 10-day-old *Oscillatoria*, respectively.

The highest cell growth and OsPP production were obtained from grown cell in modified C medium (Fig. 16). The nitrogen concentration in modified C is higher (5 g L^{-1}) than C medium (0.1 g L^{-1}) or in No 18 (0 g L^{-1}). The correlations between nitrogen concentration and cells growth as well as on the pigment production were well documented. Nitrogen is an essential major element required for the synthesis of primary and secondary amino acids, proteins, nucleic acids, coenzymes, chlorophyll and other accessory photosynthetic pigment such as phycobiliproteins in cyanobacteria [29]. It was reported that the concentration of KNO_3 as nitrogen source was found to be an

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essential factor influencing the growth of *Synechocystis* sp. PCC 6701. In contrast, the growth and phycobiliproteins content in several cyanobacteria decreased during nitrogen starvation/limitation [37].

On the other hand, K_2HPO_4 as phosphorus source is also known as essential to algal growth because it was important roles in many cellular processes and it is maintaining pH of culture media by it buffering capacity [38]. Modified C medium containing phosphorus (100 mg L^{-1} as K_2HPO_4) might be appropriate concentration for growth of *Oscillatoria* strain. BG-11 medium supplemented with phosphorus (10 mg L^{-1} as K_2HPO_4) was significantly stimulated the cell growth and phycoerythrin production of *Nostochopsis lobatus* [38]. In contrast, high levels of phosphorus in medium was inhibited the growth of *Anabaena variabilis* [39]. It was reported that nitrate (KNO_3) and phosphate (K_2HPO_4) were identified as major factors of cell growth *Synechocystis* sp. PCC 6701 [37]. In this study, high cell growth and OsPP production obtained from modified C medium were agreed with these phenomena.

Light qualities as well light intensity play an important role on the cell growth and metabolite production in photosynthetic organisms, including cyanobacteria. For example, in *Rhodella reticulate,* the production of algal biomass is enhanced by about 5-6 times in response to increased light intensities from 18 to 215 $\mu E\ m^{-2}\ s^{-1}$ under white,

green or red light. At low light intensity, B-phycoerythrin content is preferentially

enhanced to 27 % under the influence of green light compared with red light [34]. The

phycocyanin content in the cyanobacteria *Synechococcus* sp. NKBG 042902 grown

under green or blue light is markedly low, while that of the cells grown under red light

is high [35]. The production of marennine, a blue pigment produced by the diatom

Halsela ostrearia, is controlled by blue-light radiation [32]. In addition, production of

micosporine-like amino acids (MAAs) and scytonemin in cyanobacteria, phytoplankton

and macroalgae has been observed as a response to counteract the damaging effect of

UV-radiation [36]. Therefore, the individual light regimen becomes a predominant

factor in affecting the productivity of algae.

It was reported that longer wavelength (540 nm) may not suitable for

chlorophyll and carotene synthesis but phycocyanin and PE significantly accumulated

under such wavelength. The chlorophyll synthesis was suited under white light and blue

light irradiation. Longer wavelengths such as green, yellow and red lights are the main

light adsorbed by photosystem II to produced highest phycocyanin and phycoerythrin

content in *Spirulina fussiformis*. This is because at higher light wavelength

phycobilisome is a highly efficient system for transferring energy to PS II reaction

center [40]. In addition, under red or pink light radiation, most of the photosynthetic

energy can be diverted into new biomass due to high carbon investment efficiency [33]. In conjunction to these phenomena, cell growth and extracellular pink pigment production of *Oscillatoria* strain were also stimulated proportionally under pink light radiation.

Microbial interactions in the co-cultivation system can be mutual and competitive. Studies concerning the competition between different microbes have been aimed at elucidating the synthesis of secondary metabolites [41]. The predominant hypothesis is that these secondary metabolites are synthesized and released to give the producing organism a competitive advantage by killing or inhibiting growth of other microbes [42].

In this study, co-cultivation of *Oscillatoria* with other cyanobacteria *A. cylindrica* stimulated the pigment production (data not shown). There are two hypotheses to explain the enhancement of OsPP production by co-cultivation system. One is the enhancement by the certain signal compound released by this strain. As have been reported, cell signalling during co-cultivation prevalent biochemical interactions among them, both stimulatory and inhibitory interaction [43, 44]. Another hypothesis is that changes in medium composition as a result of consumption by co-existing algae trigger pigment production at an earlier growth period than that by *Oscillatoria* sp.

cultivated alone. Increase of OsPP production during co-culture with other cyanobacteria was considered as one strategy to inhibit competitors for nutrients resources.

In conclusion, this chapter provides information that high cell growth and OsPP production were obtained from cultivation of *Oscillatoria* strains in modified C medium and under pink light irradiation. Modified C medium is simple in both chemical composition and preparation than the other media. Therefore, this medium has advantage for practical application on pink pigment production of *Oscillatoria* strain.

General Discussion

The work presented in this thesis focused on the characterization of extracellular pink pigments produced by tropical freshwater cyanobacteria *Oscillatoria* and *Scytonema* and evaluate their bioactivities. Firstly, it was demonstrated that the pink pigments were excreted extracellularly by actively growing cell. Different profiles between extracellular and intracellular pigments on the gel of SDS-PAGE showed the evidence of this phenomenon clearly. The pigments had approximately 250 kDa of molecular weight and two types of subunits, indicating that the pigments are oligomeric proteins. In addition, the pigments had the absorption maxima at 560 nm and 620 nm and the fluorescence maxima at 580 nm (Chapter 1). These results suggested that pink pigments exhibited physical properties similar to PE. PE is well known as one of phycobiliproteins in which function as light harvesting antenna pigment found in cyanobacteria and other prokaryotic algae. Although the pigments had physical properties similar to PE, the bioactivities of the pigments seem to be totally different in terms of cyanobacteria *Oscillatoria* and *Scytonema,* since the pigments are excreted extracellularly rather than accumulated intracellularly.

One high possibility about biological role of the pigment is that its functions as an allelochemical exhibiting growth inhibitory activity against other competing organisms in natural environment. These allelochemicals may also play a role in defense against potential predators and grazers, particularly aquatic invertebrates and their larvae. It was demonstrated that the OsPP has potent inhibitory activity specifically against eukaryotic green algae. Green algae such as *Chlorella fusca* and *Chlamydomonas reinhardtii* are known as competitors of cyanobacteria in the aquatic ecosystem. Therefore, it assumes that OsPP secreted into medium is one strategy to inhibit against competitors.

Growth inhibitory activity against green algae *C. fusca* occurred much greater in the light-irradiated OsPP than non-irradiated one, suggesting that OsPP is photosensitizing agent (Chapter 2). Several cyanobacteria and other phytoplankton metabolites are known to possess functions as photosensitisers. A photosensitiser is chemicals that are able to generate reactive oxygen species (ROS) by light energy. ROS including hydroxyl radical, hydrogen peroxide, superoxide anion and singlet oxygen are known as major mediator on various biological degradations resulting in the cell death.

In this study, signals of hydroxyl radical were clearly observed from light-irradiated OsPP. In contrast, no signal of hydroxyl radicals were detected from

non-irradiated OsPP or from irradiated buffer solution without OsPP. These results

indicated that hydroxyl radicals are major reactive oxygen species generated by light-

irradiated OsPP. ESR analysis of light-irradiated OsPP in the presence of DTPA,

confirmed that phenomenon. On the other hand, hydrogen peroxide was thought to be

generated by light-irradiated OsPP and act as a source of generation of hydroxyl radical.

More than 50 % of hydroxyl radical signals were inhibited by the addition of CAT, but

addition of heat-inactivated CAT showed no effect. Base on these results, the following

hypothesis is proposed for the hydroxyl radical generation from light-irradiated OsPP
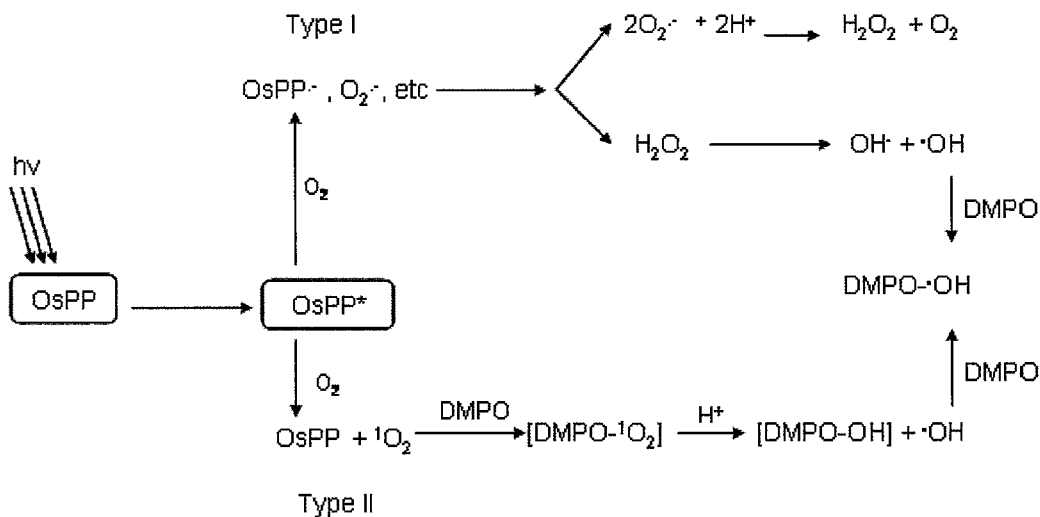
(Fig. 18).

Type I

$OsPP^-$, O_2^-, etc $\longrightarrow$

$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

$H_2O_2 \longrightarrow OH^- + \cdot OH$

$\downarrow$ DMPO

DMPO-$\cdot$OH

$\uparrow$ DMPO

hv

$\uparrow O_2$

OsPP $\longrightarrow$ OsPP*

$\downarrow O_2$

OsPP $+ {}^1O_2 \xrightarrow{\text{DMPO}} [DMPO-{}^1O_2] \xrightarrow{H^+} [DMPO-OH] + \cdot OH$

Type II

Fig. 18 Possible mechanisms of hydroxyl radical generation from OsPP under irradiation in the presence of DMPO.

Hydroxyl radicals are known specifically inhibiting photosystem II in photosynthetic organisms. They are inducing damage to thylakoid membranes, resulting in loss of chlorophyll, and inhibiting a site in the vicinity of photosystem II [23]. Therefore, growth of C. fusca was more strongly inhibited by light-irradiated OsPP as mentioned above might be caused by hydroxyl radical. From these results, it was strongly indicated that OsPP is photosensitizing agent.

Another interesting result during this work is the purified OsPP has potent growth inhibitory activity against cancer cells. Surprisingly, growth inhibitory activity of OsPP against A549 cancer cell was stronger than PE used in this study. Therefore, it is greatly expected that new type of photosensitiser applicable on PDT will be found in OsPP.

Base on these results, OsPP is promising substance for application in various fields. Therefore, strategy to obtain high OsPP production is important. Among the environmental factors were evaluated, the cell growth and OsPP production were stimulated significantly by high nitrogen concentration in the media and pink light irradiation (Chapter 3). Nitrogen is well known as an essential major element required for the synthesis of primary and secondary amino acids, proteins, nucleic acids, coenzymes, chlorophyll and other accessory photosynthetic pigments such as

phycobiliproteins in cyanobacteria. The availability of nitrogen is a key factor in regulating the cell growth and production of metabolites, including pink pigments. Nitrogen limitation in *Oscillatoria willei* BDU 130511 for example, resulted in the reduction of photosynthetic pigments such as chlorophyll *a*, carotenoids and phycocyanin. In addition, in photosynthetic organisms, N limitation triggers ordered degradation of phycobilisomes and thylakoid membranes [45].

Light, on the other hand, is a very important environmental factor for all photosynthetic organisms. Not only it is essential for photosynthesis but it is also involved in the regulation of many aspects of cell growth and development. It is a basic substrate for photosynthetic prokaryotes such as cyanobacteria. In particular, light quality is known to affects metabolic and physiological characteristic of algae. It is therefore cyanobacteria exhibit numerous responses to changes in the spectral of light quality. In this study, the highest cell growth and OsPP production were obtained under pink light radiation. Therefore, nitrogen concentration in the media and pink light radiation are key factors regulation cell growth and pink pigment production of *Oscillatoria* strain found in this study.

Overall, the work described in this thesis yielded very interesting results at both fundamental and application levels.

Conclusions

In this study, characterization of pink pigments produced by freshwater cyanobacteria, *Oscillatoria* sp. and *Scytonema* sp. and evaluation their bioactivities were concluded as follow:

1. Pink pigment produced by *Oscillatoria* and *Scytonema* were excreted from actively growing cells. The pigment produced by *Oscillatoria* sp. (OsPP) exhibits physical properties similar to phycoerythrin one of cyanobacterial photosynthetic pigments, phycobiliproteins.

2. The production of OsPP was growth-dependently occurred.

3. OsPP exhibited potent growth inhibitory activity specifically against green algae *Chlorella fusca* and *Chlamydomonas reinhardtii*.

4. Light-dependent growth inhibitory activity of OsPP might be caused by ROS generation, in which OsPP functions as a photosensitizer.

5. Cell growth and OsPP production were significantly stimulated by high concentration of nitrogen in media and pink light radiation.

From these results, extracellular pink pigments produced by *Oscillatoria* and *Scytonema* found in this study are novelties. OsPP exhibited physical properties similar

to PE and shows more resistant to light imply the high potential application of the pigment instead of PE. In addition, OsPP has potent growth inhibitory activity with functions as photosensitizer, inform the advantageous commercial development of the pigment for application in various fields. These are several progresses are obtained in this study. However, further investigation of the mechanisms in detail will surely be crucial to the possible application of the pigment for such purposes. In addition, the extracellular pink pigments have seemingly other biological roles, in which still much remains to be elucidated with respect to these roles.

References

[1] Singh, S., Kate, B.N., and Banerjee, U.C.: Bioactive compounds from cyanobacteria and microalgae: An Overview Critical Rev. Biotechnol., 25, 73-95 (2005).

[2] Sergeyenko, T.V., and Los, D.M.: Identification of secreted proteins of the cyanobacterium *Synechocystis* sp. strain PCC 6803. FEMS Microbiol. Lett., 193, 213-216 (2000).

[3] De Philippis, R., and Vincenzini, M.: Exocellular polysaccharides from cyanobacteria and their possible application. FEMS Microbiol. Rev., 22, 151-175 (1998).

[4] Otero, A., and Vincenzini, M.:   Extracellular polysaccharide synthesis by *Nostoc* strains as affected by N source and light intensity. J. Biotechnol., 102, 143-152 (2003).

[5] Volk, R.B.: Antialgal activity of several cyanobacterial exometabolites. J. Appl. Phycol., 18, 145-151 (2006).

[6] Berry, J.P., Gantar, M., Perez, M.H., Berry, G., and Noriega, F.G.: Cyanobacterial toxins as allelochemicals with potential applications as algaecides, herbicides and insecticides. Mar. Drugs, 6, 117-146 (2008).

[7] Weir, T.L., Park, S.W., and Vivanco, J.M.: Biochemical and physiological mechanism mediated by allelochemicals. Curr. Opin. Plant Biol., 7, 472-479 (2004).

[8] Hirata, K., Nakagami, H., Takashina, J., Mahmud, T., Kobayashi, M., In, Y., Ishida, T., and Miyamoto, K.: Novel violet pigment, nostocine A, an extracellular metabolite from cyanobacterium *Nostoc spongiaforme*. Heterocycl., 43, 1513-1519 (1996).

[9] Hirata, K., Yoshitomi, S., Dwi, S., Iwabe, O., Mahakhant, A., Polchai, J., and Miyamoto, K.: Generation of reactive oxygen species undergoing redox cycle of nostocine A: a cytotoxic violet pigment produced by freshwater cyanobacterium *Nostoc spongiaforme*. J. Biotechnol., 110, 29-35 (2004).

[10] Irmouli, A.V.G., Pons, L., Lucon, M., Villaume, C., Mrabet, N.T., Gueant, J.L., and Fleurence, J.: One-step purification of R-phycoerythrin from the red macroalga *Palmaria palmate* using preparative polyacrylamide gel electrophoresis. J. Chromatogr. B, 739, 117-123 (2000).

[11] Hildith, C.M., Balding, P., Jenkins, R., Smith, A.J., and Rogers, L.J.: R-phycoerythrin from the macroalga *Corallina officinalis* (Rhodophyceae) and application of derived phycofluor probe for detecting sugar-binding cites on cell membranes. J. Appl. Phycol., 3, 345-354 (1991).

[12] Glazer, A.N.: Phycobiliproteins a family of valuable, widely used fluorophores. J. Appl. Phycol., 6:105-112 (1994).

[13] He, J.A., Hu, Y.J., and Jiang, L.J.: Photodynamic action of phycobiliproteins: in situ generation of reactive oxygen species. Biochim. Bhiophys. Acta, 1320, 167-174 (1997).

[14] Zhang, S. P., Zhao, J.Q., and Jiang, L.J.: Photosensitized formation of singlet oxygen by phycobiliproteins in neutral aqueous solutions. Free Radic. Res., 33, 489-49 (2000).

[15] Volk, R.B.: Antialgal activity of several cyanobacterial exometabolites. J. Appl. Phycol., 18, 145-151 (2006).

[16] Dolman, D.E.J.G.J., Fukura. D., and Jain, R.K.: Photodynamic therapy for cancer. Nat. Rev. Cancer., 3, 380-387 (2003).

[17] Maisch, T., Baier, J., Frans, B., Maier, M., Landthaler, M., Szeimies, R.M., and Baumler, W.: The role of singlet oxygen and oxygen concentration on photodynamic inactivation of bacteria. PNAS, 104, 7223-7228 (2007).

[18] Ray, S., and Bagchi, S.N.: Nutrient and pH regulate algicide accumulation in cultures of the cyanobacterium *Oscillatoria laetevirens*. New Phytol., 149, 455-460 (2001).

[19] Wainwright, M.: Photodynamic antimicrobial chemotheraphy (PACT). J. Antimicrob. Chemother., 42, 13-28 (1998).

[20] Issa, A.A.: Antiobiotic production by the cyanobacteria *Oscillatoria angustisima* and *Calothrix parietina*. Environ. Toxicol. Pharmacol., 8, 33-37 (1999).

[21] Smith, G.D., Doan, N.T.: Cyanobacterial metabolites with bioactivity against photosynthesis in cyanobacteria, algae and higher plant. J. App. Phycol., 11, 337-344 (1999).

[22] Hirayama, S., Ueda R., and Sugata, K.: Detection of hydroxyl radical in intact cells of *Chlorella vulgaris*. Free Radic. Res., 23, 51-59 (1995).

[23] Ganesh, A.B., Manoharan, P.T., and Suraishkumar, G.K.: Response of the photosynthetic machinery of *Spirulina maxima* to induced reactive oxygen species. Biotech. Bioeng., 96, 1191-1198 (2007).

[24] Alvarez, A.M., Leisinger, U., and Eggen, R.I.L.: Adaptive responses in *Chlamydomonas reinhardtii*. Internatl. Microbiol., 2, 15-22 (1999).

[25] Yoshida, K., Igarashi, I., Mukai, M., Hirata, K., and Miyamoto, K.: Induction of tolerance to oxidative stress in the green algae, *Chlamydomonas reinhardtii*, by absisic acid. Plant Cell Environ., 26, 451-457 (2003).

[26] Drabkova, M., Marsalek, B., and Admiral, W.: Photodynamic therapy against cyanobacteria. Environ. Toxicol., DOI 10.1002/tox (2006).

[27] Padula, M., Boiteux, S., Felzenszwald, I., and Menezes, S.: Photodynamic action of phycocyanin: damage and repair. J. Photochem. Photobiol. B, 32, 19-26 (1996).

[28] Watts, R.J., Washington, D., Howsawkeng, J., Loge, F,J., and Teel, A.L.: Comparative toxicity of hydrogen peroxide, hydroxyl radicals and superoxide anion to *Escherichia coli*. Advance in Environmental Research, 7, 961-968 (2003).

[29] Liotenberg, S., Campbell, D., Rippka, R., Houmard, J., and de Marsac, N.T.: Effect of the nitrogen source on phycobiliproteins synthesis and cell reserves in a chromatically adapting filamentous cyanobacteria. Microbiol., 142, 611-622 (1996).

[30] Repka, S. J., Mehtonen, J., Vaitomaa, L., Saari, and Sivonen, K.: Effect of nutrient on growth and nodularin production of *Nodularia* strain GR8b. Microbiol. Ecol., 42, 606-613 (2001).

[31] Kearns, K.D., and Hunter, M.D.: Green algal extracellular products regulate antialgal toxin production in cyanobacterium. Environ. Microbiol., 2, 291-297 (2000).

[32] Mouget, J.L., Rosa, P., Vachoux, C., and Tremblin, G.: Enhancement of mareninne production by blue light in the diatom *Haslea ostrearia*. J. Appl. Phycol., 17, 437-445 (2005).

[33] Korbee, N., Figueroa, F.L., and Aguilera, J.: Effect of light quality on the accumulation of photosynthetic pigments, protein and micosporine-like amino acids in the red alga *Porphyra leucosticta* (Bangiales, Rhodophyta). J. Photochem. Photobiol. B, 80, 71-78 (2005).

[34] Mihova, S.G., Georgiev, D.I., Minkova, K.M., and Tchernov, A.A.: Phycobiliproteins in *Rhodella reticulate* and photoregulatory effects on their content. J. Biotechnol. 48, 251-257 (1996).

[35] Takano, H., Arai, T., Hirano, M., and Matsunaga, T.: Effect of intensity and quality of light on phycocyanin production by a marine cyanobacterium *Synechococcus* sp. NKBG 042902. Appl. Microbiol. Biotechnol., 43, 1014-1018 (1995).

[36] Sinha, R.P., Klisch, M., Groniger, A., and Hader, D.P.: Ultraviolet-absorbing/screening substances in cyanobacteria, phytoplankton and macroalgae. J. Photochem. Photobiol., B, 47, 83-94 (1998).

[37] Hong Seong-Joo and Lee Choul-Gyun.: Statistical optimization of culture media for production of phycobiliproteins by *Synechosytsis* sp. PCC 6701. Biotechnol. Bioprocess Eng., 13, 491-498 (2008).

[38] Pandey, U and Pandey, J.: Enhanced production of biomass, pigments and antioxidant capacity of nutritionally important cyanobacteria *Nostochopsis lobatus*. Bioresource Technol., 99, 4520-4523 (2008).

[39] Battah, M.G., Shabana, E.F., Kobbia, I.A., and Eladel, H.M.: Differential effect of thiobencarb toxicity on growth and photosynthesis of *Anabaena variabilis* with changes in phosphate level. Ecotoxicol. Environ. Saf., 49, 235-239 (2001).

[40] Madhyastha, H.K., Vatsala, T.M.: Pigment production in *Spirulina fussiformis* in different photophysical conditions. Biomol. Eng., 24, 301-305 (2007).

[41] Jin, Q., and Dong, S.: Comparative studies on the allelopathic effects of two different strains of *Ulfa pertusa* on *Heterosigma akashiwo* and *Alexandrium tamarense*. J. Exp. Mar. Biol. Ecol., 293, 41-55 (2003).

[42] Volk, R.B., and Furkert, F.H.: Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. Microbiol. Res., 161, 180-186 (2006).

[43] Petter, M.: Proteomic as a tool to study microbial interactions. Curr. Proteom., 1, 27-34 (2004).

[44] Mulderij, G., Mooij, W.M., Smolders, A.J.P., and Donk, E.V.: Allelopathic inhibition of phytoplankton by exudates from *Seratiotes aloides*. Aquat. Bot., 82, 284-296 (2005).

[45] Saha, S.H., Uma, L., and Subramanian, G.: Nitrogen stress induced change in the marine cyanobacterium *Oscillatoria willei* BDU 130511. FEMS Microbiol. Ecol., 45, 263-272 (2003).

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