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# Accelerating of Pink Pigment Excretion from Cyanobacterium *Oscillatoria* by Co-Cultivation with *Anabaena*

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**The freshwater cyanobacterium *Oscillatoria* BTCC/A 0004 excretes pink pigment containing lipoproteins with molecular weights of about 10 kDa. This pigment has surfactant properties with strong emulsification activity toward several hydrocarbons. This extracellular metabolite was suspected as toxin or allelochemical in their habitat. In this study, I investigated the effect of co-cultivation of *Oscillatoria* with *Anabaena variabilis* on the pigment excretion to explore the physiological roles of this pigment in its natural environment. The dead or viable cells and medium of *A. variabilis* were added into *Oscillatoria* cultures. Results showed that co-cultivation of free viable cells of *A. variabilis* enhanced the excretion of pigment without effect on the cell growth. Co-cultivation with viable cells in separated method and dead cells did not influenced the pigment production. The addition of *A. variabilis* medium was slightly increased the excretion of the pigment. Those results indicated that direct contact with *A. variabilis* caused *Oscillatoria* released a certain signaling compound.**

Key words: *Oscillatoria, Anabaena variabilis*, biosurfactant, cyanobacterium, pink pigment

## INTRODUCTION

Biosurfactants are produced by a wide variety of organisms ranging from microorganisms to plants and animals. The heterotrophic microorganisms such as bacteria, yeasts, and fungi produce surfactants with various chemical structures and molecular weights (Navon-Venezia *et al*. 1995; Desai & Banat 1997). On the other hand, biosurfactants produced by cyanobacteria (blue green algae) have not been reported so far, although they have received much attention as rich sources of bioactive compounds. However, it is expected that amphipathic extracellular metabolites frequently found in cyanobacterial cultures such as lipoproteins (Maeda & Omata 1997), lipopeptides (Burja *et al*. 2002), and lipopolysaccharides (Shah *et al*. 1999) will exhibit surfactant properties.

The investigation of the pink pigment containing lipoprotein from cyanobacterium *Oscillatoria* isolated from Jatiluhur dam (West Java, Indonesia) exhibited biosurfactants properties (Susilaningsih unpublished data). In particular, it has strong emulsified hydrocarbon and high fatty acids. They were higher than commercial synthetic surfactant such as a Triton X-100. A further evaluation of the lipoprotein activities showed that the compounds were effective for inhibiting protozoa growth and paddy weed germination. However, they were less effective for antimicrobial agents (Susilaningsih 2003). The yield of crude extracellular pigment was approximately 5 g dry weight/1 l culture and 1 g of crude extracellular pigment contained approximately 52 mg of proteins.

Based on those evidences, the excretion of the compounds by *Oscillatoria* may act as an anti protozoa or an allelochemical agents in their natural environments. The interaction between two organisms usually produce some compounds which sometimes could be active compounds to protect their life. Therefore, the aim of this study is to elucidate this phenomenon, by conducting co-cultivation experiments.

## MATERIALS AND METHODS

**Cultivation Conditions of *Oscillatoria*.** *Oscillatoria* BTCC/A 0004 was cultivated in 80 ml of modified C medium in a petri dish (150 mm in diameter; 15 mm in depth; Becton Dickinson, Falcon, USA). The medium consisted of (per liter); 5 g $KNO_3$, 0.1 g $KH_2PO_4$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeCl_2$, 2.86 mg $H_3BO_3$, 1.81 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.018 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.075 mg $CuSO_4 \cdot 5H_2O$ (pH 7.5). The initial cell concentration was adjusted to an optical density of 0.05 at 680 nm ($OD_{680nm}$). An $OD_{680\,nm}$ value of 1 is equal to 13 mg dry cell weight/ml. The dish was placed in a transparent cultivation box and incubated at 25 °C under aeration containing 1% $CO_2$ at 4 ml/min and illuminated with 30 W/m$^2$ white fluorescent light. Cell growth was monitored by measuring $OD_{680\,nm}$ of the culture sampled at indicated times.

**Preliminary Test of *Oscillatoria* Co-Cultivation with Several Microalgae.** Freshwater cyanobacteria, *Anabaena cylindrica* NIES 19, *A. variabilis* NIES 23, *Microcystis aeruginosa* f. *aeruginosa* NIES 44, and *Synecocystis* sp. PCC 6803 were grown in modified BG 11 medium. Freshwater eukaryotic algae, *Euglena gracilis* (Osaka Prefecture University collection), *Chlamydomonas reinhardtii* IAM C 238, *Chlorella fusca* var. *vacuolata* IAM C 28, *C. pyrenoidosa* IAM C 212, and *Carteria inversa* NIES 422 were grown in

Bristol modified medium. *Euglena gracilis* was grown in Bold basal medium. Halotolerant cyanobacteria, *Spirulina maxima* BTCC/A 0008 and *S. platensis* BTCC/A 0011, and halotolerant green algae, *Dunaliella tertiolecta* ATCC 30929 and *D. salina* (Dunal) Teod UTEX LB 1644, were grown in Zarrouk medium and F-2 medium, respectively.

All algae were pre-cultivated in 200 ml of their respective media in a test tube (40 mm in diameter) under aeration containing 1% $CO_2$ at 30 ml/min and illuminated with 30 W/m$^2$ white fluorescent light. The cells were harvested in the exponential phase and resuspended in fresh medium at a concentration that gave an $OD_{680\,nm}$ of 0.1. Two milliliters of the cell suspension was transferred into a 24-well culture plate (Becton Dickinson, Falcon, USA) and then 0.1, 0.5, or 1of final $OD_{680nm}$ of fresh *Oscillatoria* cells was added and cultivated under the same conditions as those for *Oscillatoria.* The cells were monitored by cell counting number under microscope and the extracellular pigment was measured by spectrophotometer at ë 560 nm three times during cultivation.

**Co-Cultivation of *Oscillatoria* with *Anabaena*.** *Anabaena variabilis* and *A. cylindrica*, were used to stimulate pigment production by *Oscillatoria*. *Anabaena* was cultivated in 200 ml of culture vessel in BG 11 medium. In the logarithmic phase, the cells and medium were separated and both of the materials were collected. Both of the cells and medium were intact directly inoculated or packed in dialysis membrane (Seamless Cellulose Tubing, Viskase Sales Corp, Japan) to separate substances which having molecular weights higher than 7000 Dalton. The amounts of the cells were about 0, 0.3, 0.6, 0.9, and 1.3 mg cells/ml cultures. Concentration of the medium were 0, 20, 40, 80, and 160 ml/200 ml cultures. The experiments were conducted in triplicate and pigment production was monitored every two days by measuring absorbance at ë 560 nm.

In further treatment, *Oscillatoria* was cultivated in the presence of *A. cylindrica* culture. The concentrate was prepared by lyophilization of the culture medium in lag (3-day-old), logarithmic (8-day-old), and stationary (14-day-old) growth phases. The amounts of introduced media were about 0.25, 0.50, 100, and 200% volume of *Oscillatoria* cultures. The experiments were conducted in triplicate and pigment production was monitored every two days by measuring absorbance at ë 560 nm. Cells were harvested at the end of cultivation and cell growth was evaluated in terms of dry cell weight.

## RESULT

**Cultivation Conditions of *Oscillatoria* sp.** The pigment from *Oscillatoria* was released in the exponential phase, then suddenly decreased after 16 days (Figure 1). The maximum production of pink pigment was approximately 5 g dry weight 1 l culture. The pink pigment was consisting of 75% protein and 25% lipid (lipoprotein).

**Preliminary Test of Co-Cultivation of *Oscillatoria* with Several Microalgae.** The co-cultivation of *Oscillatoria* and several prokaryotic microalgae (*Microcystis*, *Synecocystis*, and *Spirulina*) showed that they did not or less accelerate the pink pigment production (Table 1). However, *Dunaliella* accelerated the pink pigment production and *Anabaena* highly accelerated the pink pigment production (Table 1). In addition, the results of co-cultivation the *Oscillatoria* with the eukaryotic microalgae showed the consortia accelerated the pink pigment production. To seek further information specific co-cultivation *Oscillatoria* with *Anabaena* was attempted.

**Co-Cultivation of *Oscillatoria* with *Anabaena*.** Co-cultivation *Oscillatoria* with *A. cylindrica* showed that the addition of intact (free) cells and packed cells in dialysis tube of *A. cylindrica*, both enhanced the pink pigment production (Figure 2a, b). The highest enhancement of the pink pigment production treated by free cells in *Oscillatoria* cultures was the 0.63 mg cells of *A. cylindrica.* That resulted

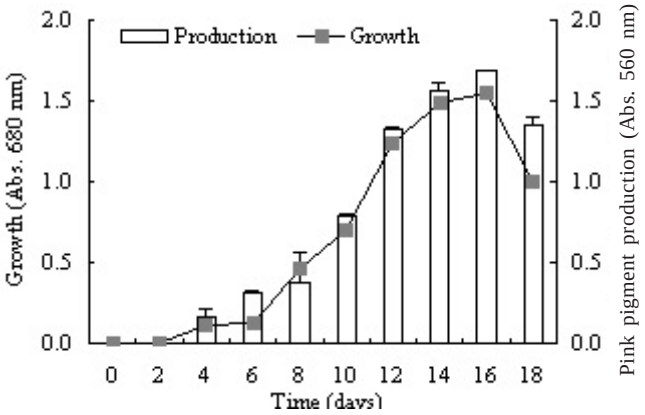


Figure 1. Time courses of growth and pink pigment production in *Oscillatoria*

Table 1. Acceleration of pink pigment production by *Oscillatoria* sp. by various microalgae

| Strain | Acceleration level |
|---|---|
| Fresh water microalgae | |
| Cyanobacteria | |
| *Oscillatoria* BTCC/A 0004 | control |
| *A. variabilis* NIES 23 | +++ |
| *A. cylindrica* NIES 19 | +++ |
| *M. aeruginosa* f. *aeruginosa* NIES 44 | + |
| *Synecocystis* sp. PCC 6803 | + |
| Eukaryotic microalgae | |
| *E. gracilis* | ++ |
| *C. reinhardtii* IAM C 238 | ++ |
| *C. fusca var. vacuolata* IAM C 28 | + |
| *C. pyrenoidosa* IAM C 212 | + |
| *C. inversa* NIES 422 | ++ |
| Haloterant microalgae | |
| Cyanobacteria | |
| *S. maxima* BTCC/A 0008 | - |
| *S. platensis* BTCC/A 0011 | - |
| Eukaryotic microalgae | |
| *D. tertiolecta* ATCC 30929 | ++ |
| *D. salina* (Dunal) Teod UTEX LB 1644 | ++ |

-: no acceleration, +: less acceleration, ++: acceleration, +++: high acceleration

in addition at production around 0.2 (Absorbance ë $560_{nm}$) unit (Figure 2a). In the second treatment, the addition of 1.30 mg packed cells of *A. cylindrica* in *Oscillatoria* culture gave the highest result, i.e. ± 0.22 (Figure 2b). In both treatment, almost all of the highest productions were achieved in the late exponential phase of *Oscillatoria* growth (Figure 2a, b).

The addition of *A. cylindrica* used media into *Oscillatoria* cultures showed that the media packed in the dialysis membrane enhanced the pink pigment production (Figure 2d). The highest yield was shown in the culture medium resulted 0.3-0.4 pink pigment production unit. However, the free/intact media addition treatments did not influence the pink pigment production (Figure 2c).

The further effect of the *A. variabilis* was investigated in the next experiments. The addition of *A. variabilis* in *Oscillatoria* cultures showed that only the free cells (intact) treatments enhanced the pink pigment production (Figure 3a-d). The highest result was achieved by concentration of 1.3 mg free cells addition, which is around 0.23 (Figure 3a). Therefore, for further investigation only co-cultivation with *A. cylindrica* was observed.

The addition of lyophilized *A. cylindrica* medium in *Oscillatoria* cultures showed that stationary phase of the *Anabaena* accelerated the pink pigment production, both of free and packed cells in dialysis tube treatments (Figure 4). The highest culture concentration gave the highest result, i.e. 0.2-0.31.

## DISCUSSION

To investigate the allelopathic function of the pigment, *Oscillatoria* was co-cultivated with several freshwater cyanobacteria and green algae that are commonly found in aquatic environments in Southeast Asia. Among them, only co-cultivation with *Anabaena* of both *A. cylindrica* and *A. variabilis* markedly enhanced the pigment production (Table 1). Growth of *Oscillatoria* in presence of other algae was slightly suppressed compare to cultivated alone. According to Demain and Fang (2000), secondary metabolites are useful as competitive weapons.

Further investigation was mainly using *Anabaena*. *Anabaena cylindrica* cells packed in the dialysis membrane enhanced the pink pigment production (Figure 2b), whereas packed *A. variabilis* cells exhibited no enhancement (Figure 3b). The growth of *Oscillatoria* co-cultivated with these two strains was slightly suppressed but not very different from that of *Oscillatoria* cultivated solely. These results indicate that *A. variabilis* requireds direct cell contact with *Oscillatoria* for the enhancement of pigment production, whereas *A. cylindrica* does not require the direct contact. Two hypotheses could explain the enhancement of pigment production by the packed *A. cylindrica* cells. One is the enhancement by a certain signaling substance released by this species. Another hypothesis is that the changes in medium composition as a result of consumption by co-existing algae trigger pigment

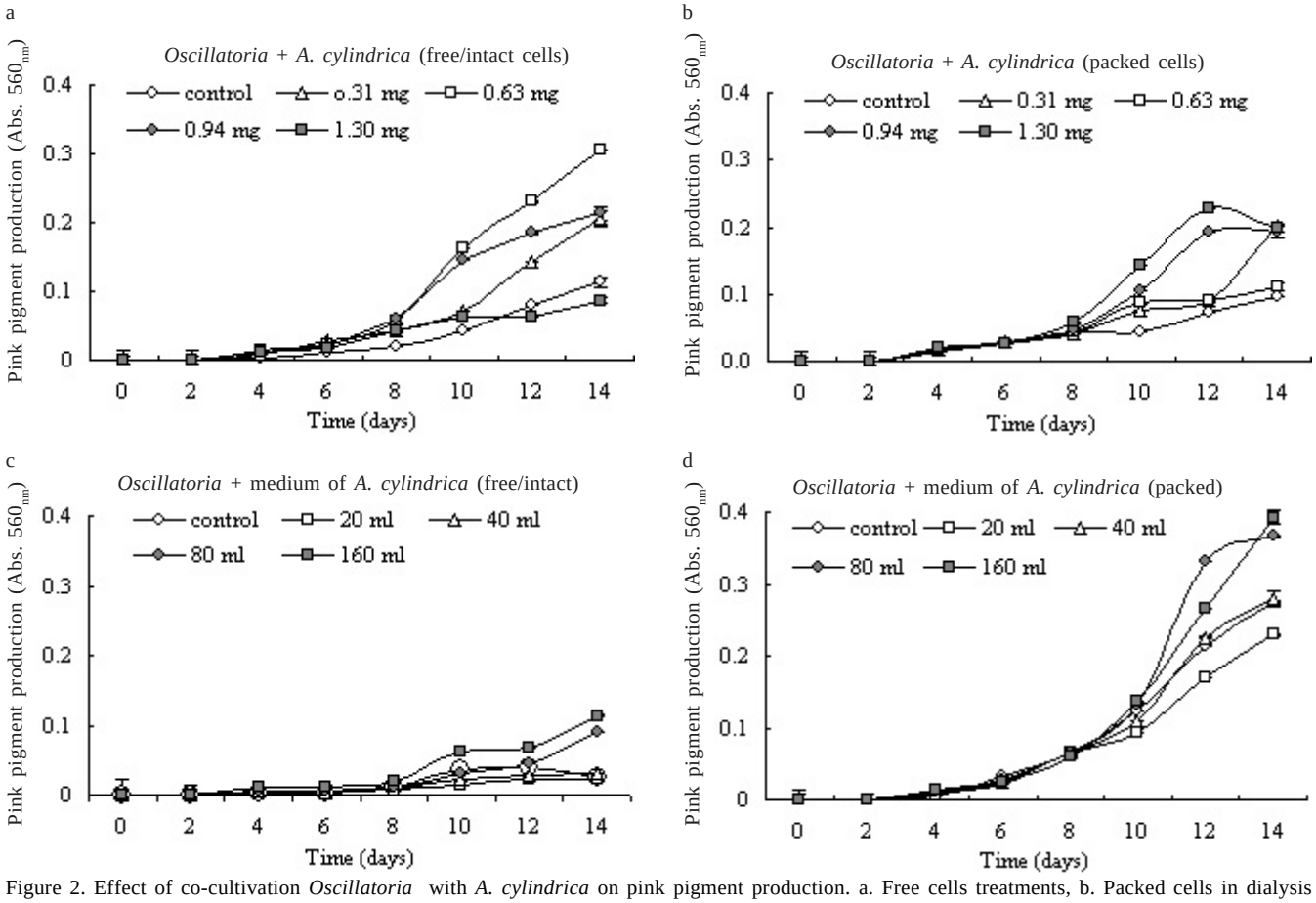


Figure 2. Effect of co-cultivation *Oscillatoria* with *A. cylindrica* on pink pigment production. a. Free cells treatments, b. Packed cells in dialysis membrane treatments, c. Free/intact media treatments, d. Packed media in dialysis membrane treatments.

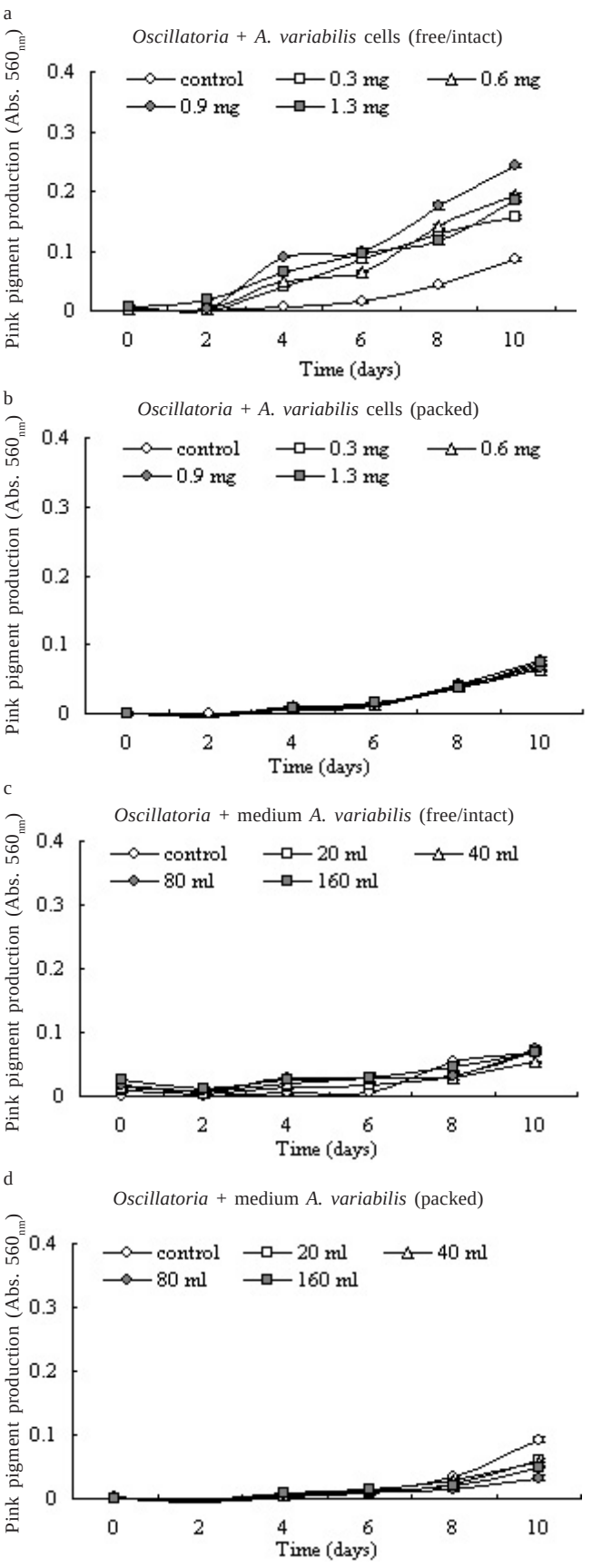


Figure 3. Effect of co-cultivation *Oscillatoria* sp. with *A. variabilis* on pink pigment production. a. Free cells (intact) treatments, b. Packed cells treatments, c. Free/intact media treatments, d. Packed media treatments.

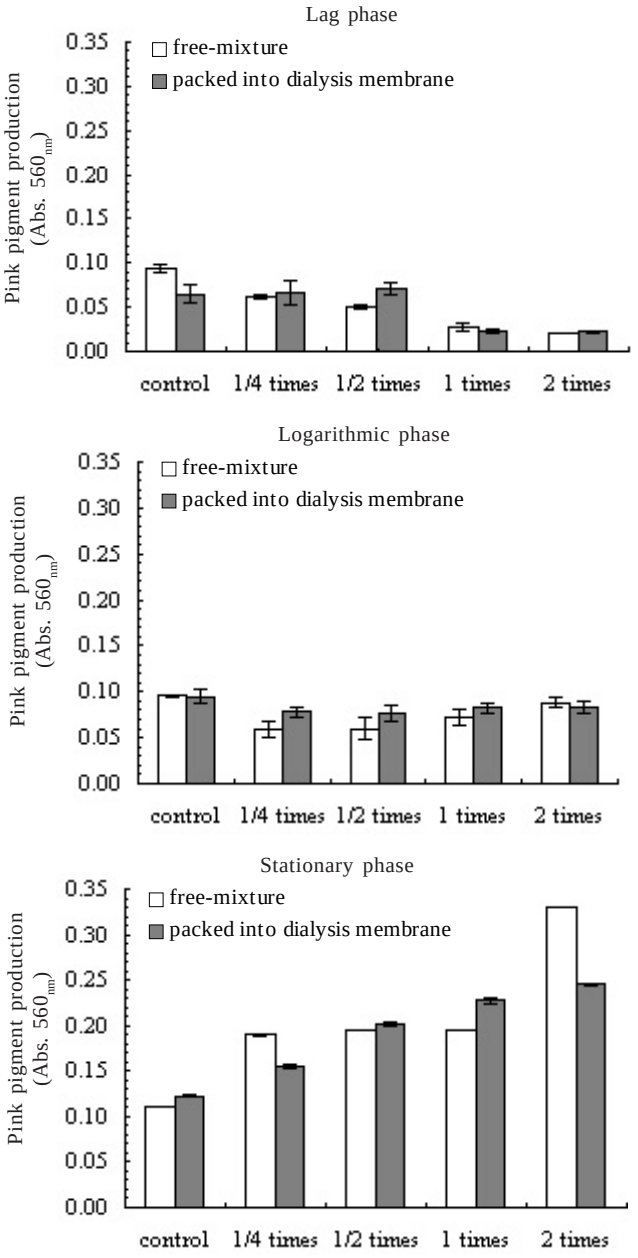


Figure 4. Effect of addition of medium concentrate of *A. cylindrica* culture in three different growth phases on pink pigment production.

production at an earlier growth period than that by *Oscillatoria* solely cultivated. Among the medium concentrates obtained from the *A. cylindrica* culture in three different growth phases, the pigment production was enhanced only by the concentrate in the stationary phase (Figure 4). Furthermore, the concentrate packed into dialysis membrane was also effective to enhance the production. Meanwhile, the growth of *Oscillatoria* was not affected by the addition of these concentrates. These results indicate that a certain signal compound with molecular weight lower than 7000 was released by *A. cylindrica* and may cause *Oscillatoria* release more pigment in response to that signal. This result supports the first hypothesis, i.e., the pigment may be synthesized and released as an allelochemical-like

product in response to a signaling substance released by the co-existing *A. cylindrica*.

Previous experiment showed that the pink pigment exhibited allelopathy function towards specific microalgae and bacteria with minimum inhibitory concentration ranging from 1-10 μM (Susilaningsih 2003). Along with the determination of the chemical structure of the pigment which has specific surfactant function, this information would be useful for its potential application as a pink pigment or an antibiotic compound. The pigment is suggested as an allelochemical-like function towards other organisms such as cyanobacteria breeding with *Oscillatoria* in natural aquatic environment. More detailed analysis of this unique phenomenon would give useful information to clarify allelopathic function in aquatic photosynthetic microorganisms. Furthermore, the enhancement of pigment production using allelopathic effect would be effective technique for the cultivation of *Oscillatoria* to supply the pigment for its practical application. Further investigation to determine the signal compound and its enhancing mechanism is in progress.

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