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Factors Affecting Antibiotic Production in Bioreactors with Immobilized Algal Cells

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ABSTRACT

A fresh-water, nitrogen-fixing blue-green alga (cyanobacterium), *Scytonema* sp. No. 11 (TISTR 8208), was isolated from a paddy field in northern Thailand. This alga produced bioactive substances and secreted them into the culture medium. These substances have antibiotic activity toward *B. subtilis*, and mitogen activity. The production of antibiotics was easily monitored with a spectrophotometer, because they are produced concomitantly with colored substances.

The conditions for antibiotic production were investigated and optimized with respect to pH, temperature, nitrogen source, and light intensity. Immobilization of cells was investigated in connection with its subsequent application to photobioreactors. The filamentous nature of this alga enabled cell immobilization in porous carriers of polyurethane foam. The porosity of the carrier was the most important factor for maximum holding of the filaments and, thus, for the highest productivity. Light intensity and CO_2 supply affected antibiotic production in bioreactors with the immobilized biocatalyst. These results are presented along with the design and characterization of a new photobioreactor.

Index Entries: Algae; bioreactor; antibiotic

INTRODUCTION

Many bioactive compounds are produced by algae. Most of them have been reported from red and brown marine algae (*1–3*). In the case of

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fresh-water algae, some bioactive compounds have been found in chlorophyta (4). With regard to the production of such compounds in cyanophyta or blue-green algae, the production of the secondary metabolite "cyanobacterin" from *Scytonema hofmanni*, which has a strong inhibitory effect on algal cells and higher plants, has been reported (5). Additionally, highly cytotoxic and antimycotic macrolides have been isolated from *Scytonema pseudohofmanni* (6). Also, two new macrolides with high cytotoxicity against KB and NIH/3T3 cells have been isolated from *Oscillatoria acutissima* (7). However, the production of bioactive compounds in a photobioreactor with immobilized algal cells has never been reported (8).

Several techniques have been utilized for algal cell immobilization; the most popular method is the entrapment of algal cells in polysaccharides (agar, agarose, alginate, and carrageenan). Most of the techniques have been applied to H_2 production (8,9). Nevertheless, one of the most successful studies has been the production of hydrogen and ammonia in a bioreactor with the immobilization of a symbiotic blue-green alga, *Anabaena azollae*, on polyurethane foam (10).

In our studies, the crude sample of violet solution that was secreted by *Scytonema* sp. No. 11 was tested for its bioactive properties. This compound showed antibiotic properties toward gram-positive *Bacillus subtilis* and also mitogen activity on mouse spleen cells. Because this alga has a filamentous form, it can secrete extracellular products as bioactive compounds. Owing to these properties, it was selected as a suitable model to study the development of a photobioreactor with immobilized algal cells for antibiotic production. In addition, the effects of light intensity and CO_2 concentration on algal growth and antibiotic production in the bioreactor were also investigated.

MATERIALS AND METHODS

Microorganism

The filamentous N_2-fixing blue-green alga (BGA) *Scytonema* sp. No. 11 (TISTR 8208) was obtained from the Culture Collection (Bangkok MIRCEN), Thailand Institute of Scientific and Technological research (TISTR). This algal strain was isolated from a paddy field in the northern part of Thailand (Pitsanulok province).

Culture Methods

This alga was grown in a defined medium modified from N-free medium for N_2-fixing blue-green algae (11). The medium composition is shown in Table 1. Sodium nitrate was added as a nitrogen source to stimulate growth and antibiotic production.

Table 1
Defined Medium

Compound	Concentration, g/L
NaCl	0.07
$MgSO_4 \cdot 7H_2O$	0.38
$CaCl_2$	0.08
K_2HPO_4	0.60
$NaNO_3$	1.50
$Fe_2(SO_4)_3 \cdot 6H_2O$	0.01
Titriplex III	0.027
H_3BO_3	0.003
$MnSO_4 \cdot 4H_2O$	0.002
$NaMoO_4 \cdot 2H_2O$	0.008
$ZnSO_4 \cdot 7H_2O$	0.0003
$CuSO_4 \cdot 5H_2O$	0.00008
$CoCl_2$	0.00002
pH 7.5	

Algal inoculum was prepared in a 2-L bottle incubated in a water bath at 30°C, and continuously illuminated by cool-white fluorescent lamps at a light intensity of 60 W/m². The culture was sparged with 5% (v/v) CO_2 in air at a flow rate of about 100 mL/min for algal cell agitation and CO_2 supply.

Photobioreactor Setup and Operation for the Selection of Suitable Algal Cell Culture Conditions

The characteristics of the glass-column bioreactor used are illustrated in Fig. 1 (a). Details of the bioreactor dimensions are summarized in Table 2.

Three kinds of algal cultivation conditions were studied in these bioreactors—suspension culture and two immobilization cultures. For algal cell immobilization, two systems employing different shapes of polyurethane foam (PU; pore size number HR-13) as immobilization support material were used. One consisted of free-floating 1×1×1 cm polyurethane foam blocks (PU-blocks) (Fig. 1 [b]). The other is composed of anchored 1×1×40 cm polyurethane foam strips (PU-strips) fixed on a stainless-steel ring to prevent flotation (Fig. 1 [c]). This immobilizing structure was placed vertically and acted as "artificial seaweed" in the bioreactor. The polyurethane foam HR-13 was used in this study, because it has given the best biomass holding with the highest antibiotic production when compared with the other number of HR-8, HR-20, and HR-30 (data not shown).

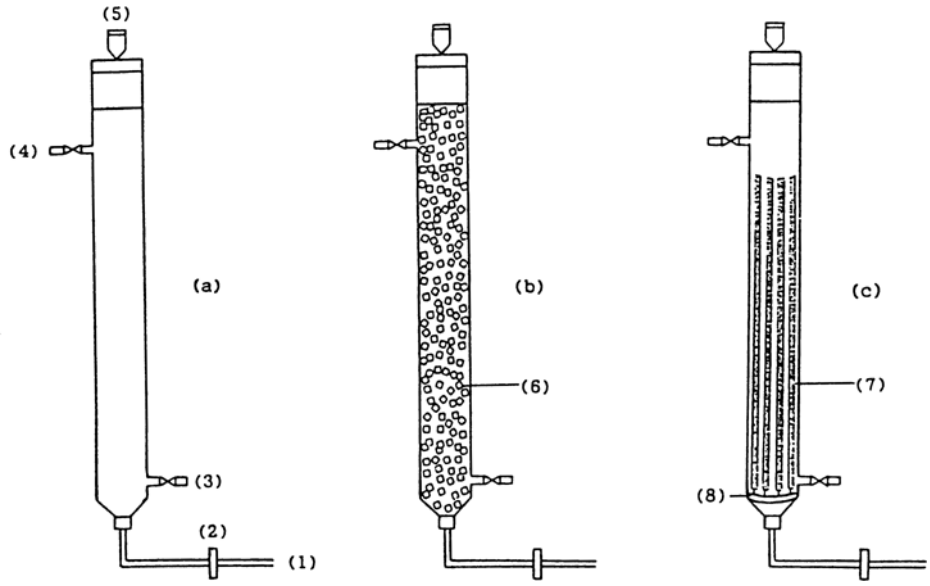

Fig. 1. Characteristics of photobioreactors. (a) Photobioreactor for suspension culture. (b) Photobioreactor with free-floating polyurethane foam blocks. (c) Photobioreactor with anchored polyurethane foam strips. ([1], air inlet; [2], air filter; [3] and [4], sampling port; [5], air outlet; [6], polyurethane foam block; [7], polyurethane foam strip; [8], stainless-steel ring).

Table 2
Characteristics of Bioreactor

	Dimensions
Vessel	
Height	60.0 cm
Inside diameter	7.2 cm
Conical profile bottom	
Internal height	3.5 cm
Smaller inside diameter	3.0 cm
Volume	
Maximim volume	2.3 L
Working volume	2.0 L
Immobilization support material	
Polyurethane blocks (320 pieces)	1×1×1 cm^3
Total available immobilizing volume	320.0 cm^3
Total available surface area	1920.0 cm^2
Polyurethane strips (eight pieces)	1×1×40 cm^3
Total available immobilizing volume	320.0 cm^3
Total available surface area	1296.0 cm^2

Algal cells of 10.0 g fresh wt (ca. 2.0 g dry wt) were inoculated to a 2.3-L bioreactor containing 2.0-L of medium. The experiment was conducted under the conditions mentioned above.

Effect of Light Intensity and CO_2 Concentration on Algal Growth and Antibiotic Production

Effects of light intensity and CO_2 concentration on growth and antibiotic production in the bioreactors with immobilized algal cells of *Scytonema* sp. No. 11 were studied. The effect of light intensity was compared among three different intensities—30, 60, and 90 W/m^2. The CO_2 concentration was studied at four levels—air only (0.04% CO_2), 5%, 10%, and 15% CO_2 in air.

Measurement of Antibiotic Production

The concentration of crude antibiotic expressed concomitantly with the secretion of violet pigment from algal cells into the culture medium can be measured by a spectrophotometer at a maximum wavelength of 537 nm. The inhibition activity of violet-colored medium on the growth of *Bacillus subtilis* (ATCC 6633) is shown in Fig. 2.

RESULTS AND DISCUSSION

Effect of Bioreactor Algal Cell Cultivation Conditions on the Growth and Antibiotic Production of *Scytonema* sp. No. 11

It was clearly shown that although this alga was immobilized on the same polyurethane foam material, the different conditions of the free-floating PU-blocks and anchored PU-strips resulted in considerable differences in growth and production. From Fig. 3, it can be seen that all the bioreactors started producing antibiotic at the same time, but continued at different production rates. The algal cell immobilized on PU-blocks showed much lower productivity than the others. The suspension culture gave a lower final concentration of antibiotic than the culture immobilized on anchored PU-strips, because it reached stationary (production) phase faster. The cells immobilized on PU-strips showed the best results in both growth and production when compared with suspension culture and immobilization culture on PU-blocks (Fig. 4). However, growth as biomass increment and production as the average production rate showed only slight differences between the suspension culture and immobilization culture on PU-strips, at 1.2 and 1.6 g/L and 0.03 and 0.04 $OD_{537\ nm}$/d, respectively.

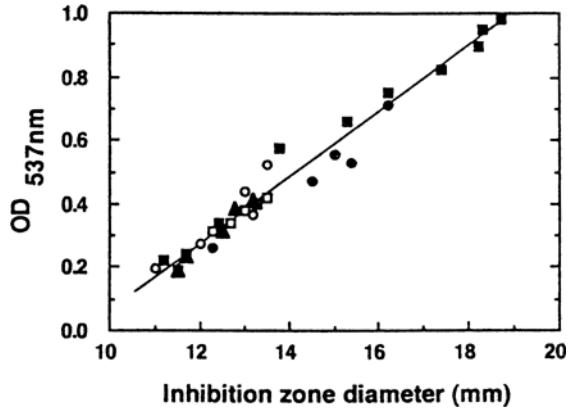

Fig. 2. Relationship between concentration of violet-colored culture broth ($OD_{537\,nm}$) and inhibition zone diameter. (Violet-colored culture broth was produced under various cultivation conditions of suspension culture, 60 W/m², 5% CO_2 [○]; immobilization culture, 60 W/m², 5% CO_2 [●]; immobilization culture, 90 W/m², 5% CO_2 [■], immobilization culture, 30 W/m², 5% CO_2 and [□] immobilization culture, 60 W/m², 15% CO_2 [▲]). (The antibiotic assay [3,12] was carried out in 9-cm Petri dishes containing 25 mL of nutrient agar seeded with 1% of an inoculum of 10^4 cells/mL of *Bacillus subtilis* [ATCC 6633]. Filtered sterilized crude samples were applied to the plate by placing 200-μL aliquots into wells [0.8 cm diameter] cut into the agar. All determinations were made at least in duplicate. A positive control with streptomycin [Meiji] was used [200 μL of a 25 μg/μL solution]. Inhibition zones were read 24 h after incubation at 37°C).

The efficiency of the two algal cell immobilizing systems was determined. Measuring the algal cell immobilization efficiency as the percentage of immobilized cells out of the total number of cells on a dry-weight basis, the free-floating PU-blocks could immobilize only 70% of the total cells, whereas the anchored PU-strips could immobilize as much as 97%. The biomass holding capability as dry cell weight per volume or per surface area in the anchored PU-strips was two and three times higher, respectively, than in the free-floating PU-blocks.

Although the surface area of the free-floating PU-blocks was 1.5 times greater than that of the anchored PU-strips, the nature of the PU-blocks can easily cause them to float when air bubbles are trapped in the pores. Thus, after a short time circulating in the medium, they floated and formed a dense column in the upper part of the medium. Because of this undesirable characteristic, algal cells in these culture conditions that could not immobilize remained in the medium as a suspension culture. Furthermore, the dense column of PU-blocks had a deleterious effect on immobilized algal cells by decreasing the light intensity, which in turn influenced cell growth and antibiotic production. In contrast to the free-floating PU-blocks, the PU-strips anchored vertically in the bioreactor demonstrated high efficiency in the immobilization of algal cells. Almost all the cells circulated by sparging air could be immobilized within 2 h.

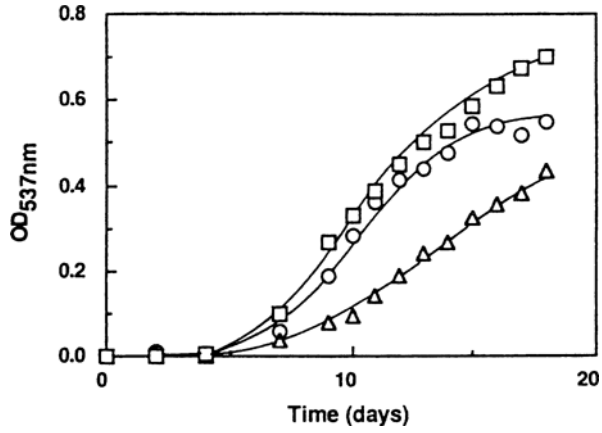

Fig. 3. Time-course of antibiotic production in photobioreactors with suspension and immobilization cultures of *Scytonema* sp. No. 11. ○ Suspension; △ PU-block; □ PU-strip.

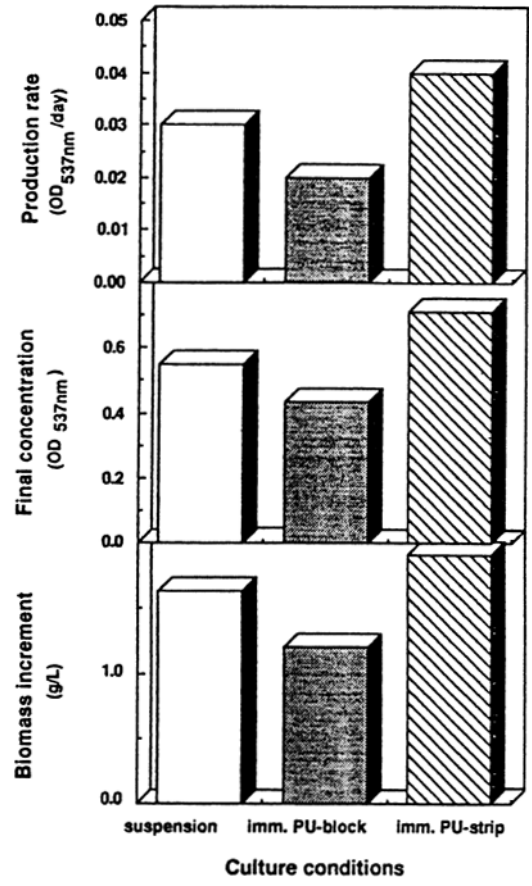

Fig. 4. Biomass increment, final concentration, and production rate of antibiotic in photobioreactors with suspension culture and immobilization cultures.

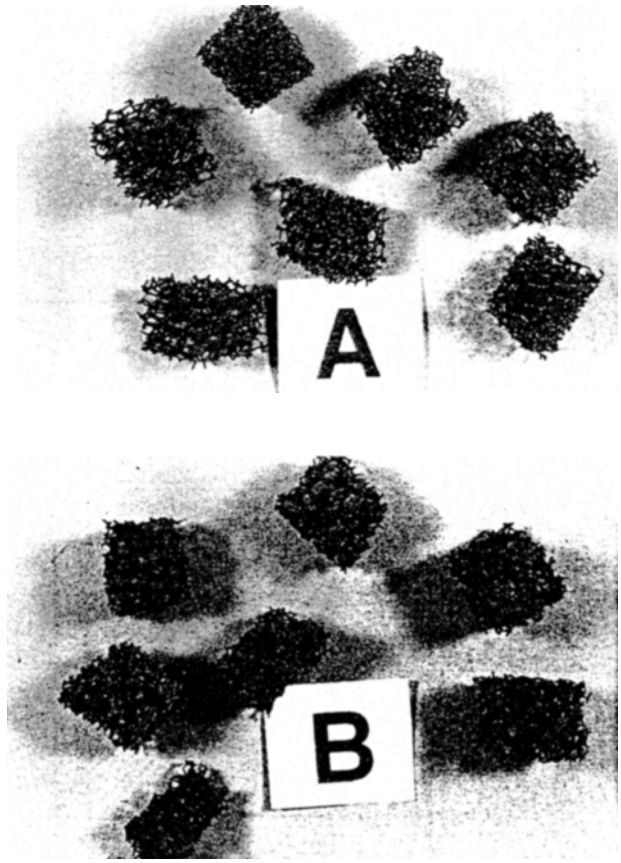

Fig. 5. Immobilization of *Scytonema* sp. No. 11 on PU-blocks. (A) PU-blocks without immobilized algal cells. (B) PU-blocks with immobilized algal cells.

Fixing the PU-strips on a stainless-steel ring not only prevents flotation, but can also lessen the problem of reduced light intensity caused by shading. Figures 5 and 6 show the immobilization of algal cells on PU-blocks and PU-strips, respectively. The density of algal filaments on PU-strips indicates suitable cultivation conditions.

Figure 4 shows that the immobilization of this alga on anchored PU-strips gave higher growth and antibiotic production than the suspension culture, indicating that the employment of a photobioreactor with algal cells immobilized on anchored PU-strips is more beneficial than using a suspension culture. Although in this study the final antibiotic productions in the suspension and immobilization cultures were not very different ($OD_{537\ nm}$ of 0.55 and 0.71, respectively), the immobilized cell bioreactor has a further advantage over the suspension cells bioreactor in terms of the ease of cell-product separation. Thus, based on the result obtained here, the photobioreactor with algal cells immobilized on anchored PU-strips appears to be the most suitable system to study extracellular product formation by algae.

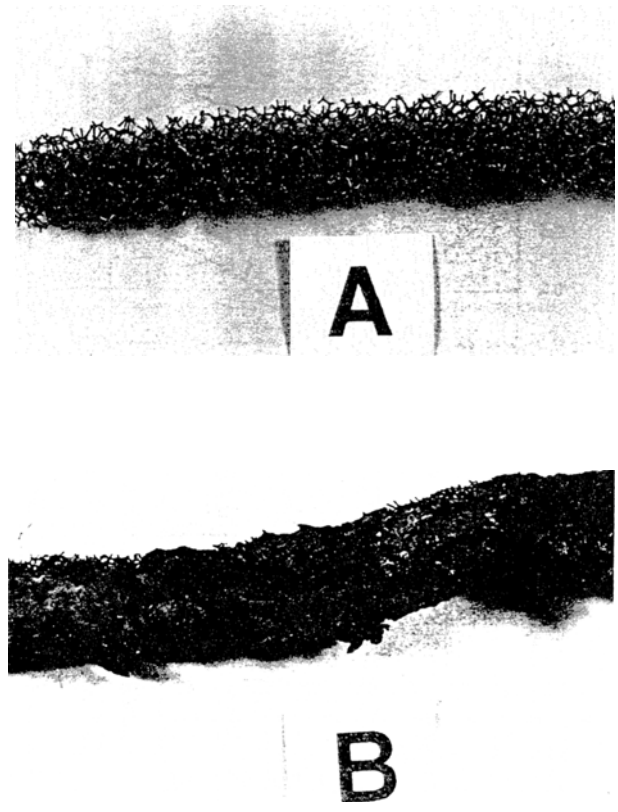

Fig. 6. Immobilization of *Scytonema* sp. No. 11 on PU-strip. (A) PU-strip without immobilized algal cells. (B) PU-strip with immobilized algal cells.

Effect of Light Intensity on Growth and Antibiotic Production in a Photobioreactor with Immobilized *Scytonema* sp. No. 11 on Anchored PU-Strips

Higher light intensity can enhance algal growth and the final concentration of antibiotic production. Figure 7 shows the decreasing production lag time by illuminating under higher light intensity (2, 4, and 6 d under illumination of 90, 60, and 30 W/m^2). Moreover, the biomass increment and final concentration of antibiotic under an illumination of 90 W/m^2 was two times higher than that under 30 w/m^2 (Fig. 8).

Although the result mentioned above showed that the immobilization of algal cells on PU-strips did not greatly affect growth and antibiotic production when compared with suspension culture, a higher light intensity resulted in better growth and antibiotic production in the photobioreactor with immobilized algal cells. It was also observed that the antimicrobial

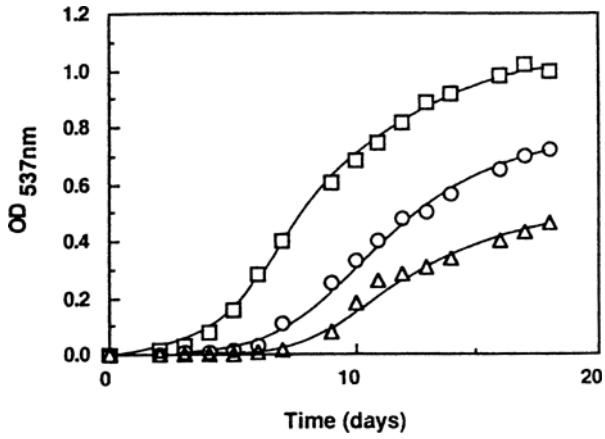

Fig. 7. Effect of light intensity on time-course of antibiotic production. □ 90 W/m²; ○ 60 W/m²; △ 30 W/m².

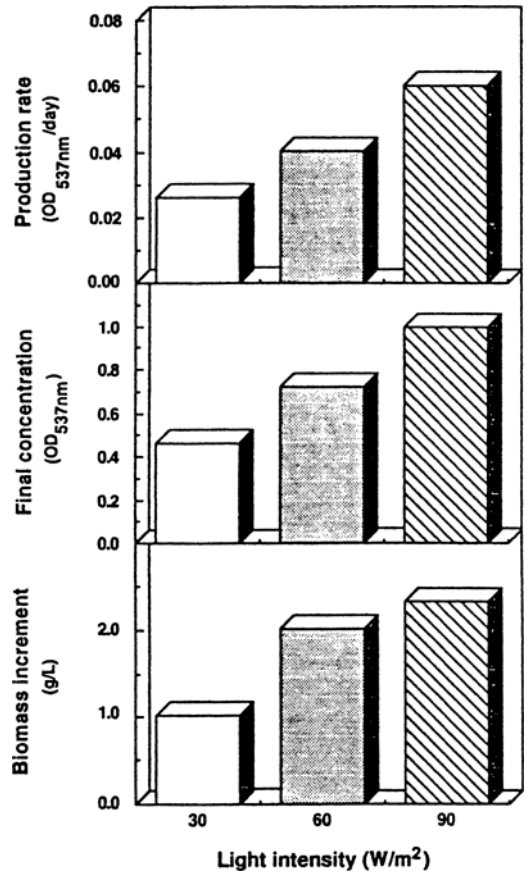

Fig. 8. Effect of light intensity on biomass increment, final concentration, and production rate of antibiotic.

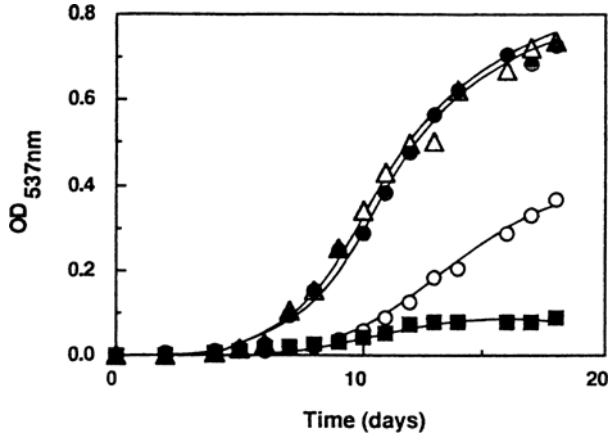

Fig. 9. Effect of CO_2 concentration on time-course of antibiotic production. ■ Air (0.04% CO_2); △ 5% CO_2; ● 10% CO_2; ○ 15% CO_2.

activity of *Chlorella*, for which the term "chlorellin" was used, increased with the age of the culture and was light dependent (*13*).

Effect of CO_2 Concentration on Growth and Antibiotic Production in a Photobioreactor with Immobilized *Scytonema* sp. No. 11 on Anchored PU-Strips

Sparging of air with various concentrations of CO_2 produced interesting results in term of growth and antibiotic production. Under the lowest and highest concentrations of CO_2 (air only [0.04% CO_2] and 15% CO_2 in air), there was a longer production lag time with lower antibiotic production, whereas almost the same production rate and final concentration of antibiotic production were found in the cultures cultivated under sparging 5 and 10% CO_2 in air (Fig. 9). In addition, the CO_2 concentration influenced algal growth: On sparging with air alone, the biomass increment was only 0.11 g/L, whereas an increment of up to 3.5 g/L was found in 15% CO_2 in air (Fig. 10).

These results showed that under air sparging without addition of CO_2, algal cells can grow only very slowly, and hence, there is very low antibiotic production. Not only did they grow slowly, but the cells also turned yellow-green in color.

Figure 11 shows N-utilization by algal cells under various concentrations of CO_2. About two-thirds of the nitrate were utilized by cells cultivated under 5, 10, and 15% CO_2 concentration. However, only slight utilization of nitrate was found in the case of the algal cells cultivated under sparging with air only. The pH range along the cultivation period was between 7.5 and 8.5 in all culture conditions.

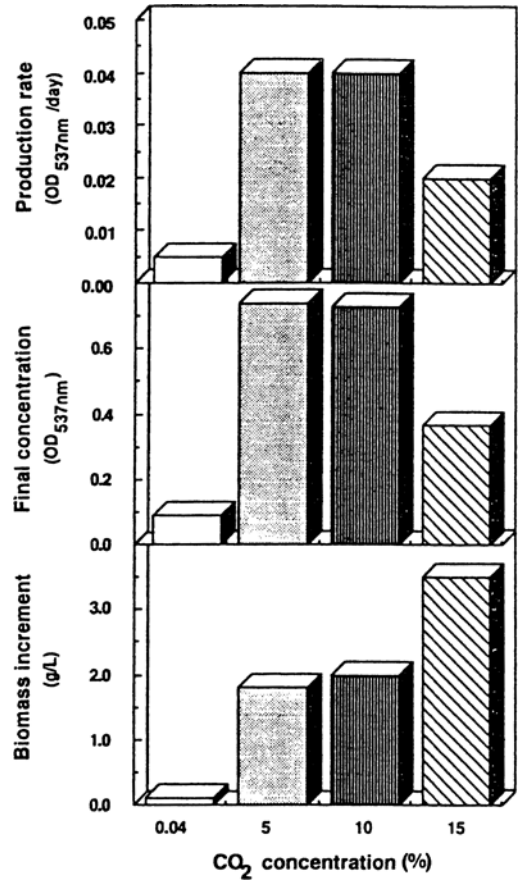

Fig. 10. Effect of CO_2 concentration on biomass increment, final concentration, and production rate of antibiotic.

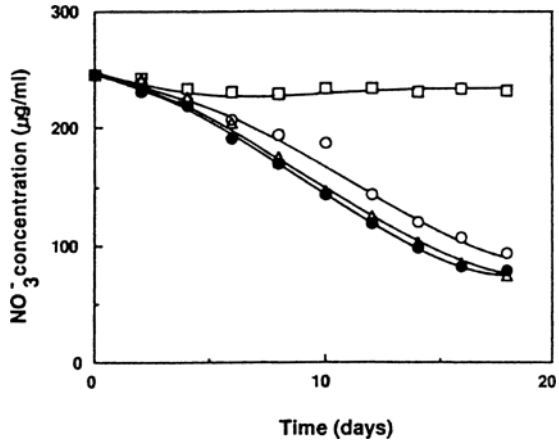

Fig. 11. Nitrate consumption of *Scytonema* sp. No. 11 cultivated at various CO_2 concentration. □ Air (0.04% CO_2); ● 5% CO_2; △ 10% CO_2; ○ 15% CO_2.

Usually, this alga can grow and produce antibiotic when cultured in an N-free medium with the sparging of air, because *Scytonema* strain is one of the N_2-fixing blue-green algae. Nevertheless, when it was cultivated in a medium with an added N-source, the formation of heterocysts, the sites of N_2-fixation, was depressed, and they disappeared. Thus, under the condition of sparging with air in an N-addition medium, some enzymes involved in nitrogen utilization may be repressed in this algal strain.

In contrast, under 15% CO_2 in air, with only the algal cell growth stimulated by the carbon source, the production of antibiotic may be affected by an imbalance of nutrient utilization. Under 5 and 10% CO_2 sparging, the utilization of nutrients for both growth and production can be in balance, and thus the biomass increment, average production rate, and final concentration of antibiotic under these two conditions were almost the same. This implied that there are no differences on algal growth and antibiotic production by sparging within the range of 5–10% CO_2 in air.

CONCLUSION

The photobioreactor with immobilized algal cells of *Scytonema* sp. No. 11 on anchored polyurethane foam strips was the best model for the production of antibiotic in comparison with photobioreactors employing a suspension culture and an immobilization culture on free-floating polyurethane foam blocks. Higher light intensity can decrease production lag time and, at the same time, can increase the final concentration of antibiotic. A suitable level of sparging with CO_2 (5–10%) is also necessary for algal growth and antibiotic production. The purification and identification of this antibiotic will be the subject of further studies.

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