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Effect of Antibiotic Treatment on Toxin Production by *Alexandrium tamarense*[1]

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Objective Impact of the presence of bacteria associated with a marine dinoflagellate, *Alexandrium tamarense* CI01, on the growth and toxin production of the algae in batch culture was investigated. **Methods** Pronounced changes in the activities of the algal culture were observed when the culture was treated with different doses of a mixture of penicillin and streptomycin. **Results** In the presence of antibiotics at the initial concentration of 100 u/mL in culture medium, both algal growth and toxin yield increased markedly. When the concentration of antibiotics was increased to 500 u/mL, the microalgal growth was inhibited, but resumed in a few days to eventually reach the same level of growth and toxin production as at the lower dose of the antibiotics. When the antibiotics were present at a concentration of 1 000 u/mL, the algal growth was inhibited permanently. **Conclusions** The results indicate that antibiotics can enhance algal growth and toxin production not only through their inhibition of the growth and hence competition for nutrients, but also through their effects on the physiology of the algae.

Key words: *Alexandrium tamarense*; Harmful algal bloom; Antibiotic; Toxin; Saxitoxin

INTRODUCTION

Harmful algal blooms (HAB), many of which produce toxins, are now a worldwide recognized a serious seafood safety, environmental and economic problems[1]. Hong Kong has suffered considerable economic losses from frequent occurrences of HAB, some of which are highly toxic[2,3]. The toxins associated with HAB are among the most potent non-proteinacious poisons. There are several classes of HAB toxins, such as diarrhetic, amnesic, and paralytic shellfish toxins (PST). Our previous studies have focused on PST, which consist of highly toxic saxitoxin (STX) and some 20 less-toxic saxitoxin derivatives[4]. These potent neurotoxins act by blocking sodium channels, leading to paralysis[5].

In natural environments, marine microalgae coexist with bacteria. Although Silva isolated an intracellular bacterium from the toxic dinoflagellate Gonyaulax (Alexandrium) tamarense, which induced PST production when inoculated into cultures of three non-toxic dinoflagellate algae[6], recent investigations have produced controversial results regarding the role of bacteria in the PST production by toxic algae[7,8].

In preliminary studies, we found that the associated bacteria might compete with algae

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for the limited phosphate in the medium and drive the algae into a stationary phase to produce high yields of toxins, as secondary metabolites. When the bacteria were inhibited by the use of antibiotics (penicillin-G and streptomycin), the algal growth and toxin production were enhanced by certain doses of antibiotics and suppressed by other doses, indicating that the algae are also susceptible to the antibiotics. Although the effects of antibiotics on bacteria are clear, whether there is an effect of antibiotics on algal cells remains unknown.

MATERIALS AND METHODS

Microorganisms

The strain of *Alexandrium tamarense* CI01 used was isolated in the South China Sea near Hong Kong waters[9] and was used to produce C2 toxin in our laboratory[10].

Culture Maintenance and Inoculums

The algal cells were grown in natural seawater supplemented with the nutrient enriched K-medium[11] at a salinity of 31‰, which was filtered through a 0.22 μm durapore (millipore) filter. Autoclaved medium was used for axenic cultures. The cultures were maintained stationary in 50 mL of medium in 250 mL flasks, under a 14 h/10 h light/dark cycle, light intensity of 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent tubes at 23.5℃. The stock cultures, 7-10 days old in the log phase of growth, were used as the inoculum. The size of the inoculum was 5% in all experiments unless specified otherwise.

Measurement of Algal Cell Number and Total Bacterial Cells

Each 100 μL of liquid culture was mixed with 900 μL of seawater and supplemented with 10 μL of Lugol's solution. After thoroughly mixed, 100 μL of aliquots was pipetted into wells of a 96-well microtitre plate, and the algal cells were visualized and counted under a light microscope.

Each 100 μL volume of an appropriate dilution of algal cultures was plated on agar plates prepared with marine broth (MBA; Difco). The inoculated MBA plates were incubated at 23.5℃ for 14 days, and the total viable colonies were read directly by a minicount colony counter (IPI). The number of colony-forming units (cfu) was used to calculate the total bacterial cells in the algal cultures.

Extraction of Intracellular Toxins

Each 1 mL of liquid culture was pipetted into a micro- centrifuge tube. The cells were pelleted by centrifugation (12 000 rpm, 5 min, at room temperature), washed with milli-Q water and re-pelleted again. The cells were resuspended in 500 μL of 50 mmol/L acetic acid and then disrupted by freezing and thawing followed by sonication on ice. The toxin extracts were obtained as the supernatant was centrifuged (16 000 rpm, 18 min, at 4℃), and stored at -20℃ until analysis.

HPLC Analysis of Toxins

A method for PST modified from that of Oshima *et al.* established in our laboratory was used[12]. It is an isocratic HPLC with post-column derivatization and fluorescence detection.

Our hardware consists of a Hewlett-Packard model 1100 HPLC equipped with PCRS and fluorescence detector and an Intersil C8-5 column, 15 cm×4.6 cm i.d. Three mobile phases were used: S-, G- and C-buffer for the separation of saxitoxins, gonyautoxins and C toxins, respectively. Post-column oxidation was specified as follows. Water RXN 1000 reaction column was operated at 85℃, the oxidant solution contained 7 mmol/L periodic acid and 50 mmol/L potassium phosphate, pH 9.0, and at a flow rate of 0.4 mL/min. Acid solution contained 0.5 mol/L acetic acid at a flow rate of 0.4 mL/min. The fluorescence detection was performed with excitation at 330 nm and emission at 390 nm.

RESULTS

It was clear that the growth rate of bacteria was faster than that of algal cells in batch culture. In two days the bacterial cell number reached the maximum at 3.2×10^6 cells/mL, then decreased rapidly and remained at about 1.0×10^4 cells/mL. However, the microalgal cells needed more than four days to reach a maximum of 1.2×10^4 cells/mL, then decreased slowly to 0.8×10^4 cells/mL (Fig.1). When the mixture of antibiotics (Penicillin-G and Streptomycin) was added into the medium, bacterial cells were killed or inhibited from 7 h to 11 h according to the concentrations of antibiotics (Fig. 2). The results indicated that marine bacteria present were sensitive to the antibiotics.

It was interesting that the microalgal cells were also affected by antibiotics, and both the growth and toxin production responded differently to varying doses of antibiotics. When the mixed antibiotics (Penicillin-G and Streptomycin) were added at a final concentration of 100 u/mL in the medium, the number of microalgal cells and toxin production were enhanced markedly compared with the control groups. With the final concentration of 500 u/mL, the growth of the microalgal cells was inhibited, and after more than 5 days the cells recovered and grew slowly to their highest in another 7 days. However, when the final concentration was 1 000 u/mL, the microalgal cells were killed completely (Figs. 3 and 4). It was clear that optimal concentrations of the antibiotics would have a positive effect on the microalgae, and the growth and toxin production could be promoted markedly.

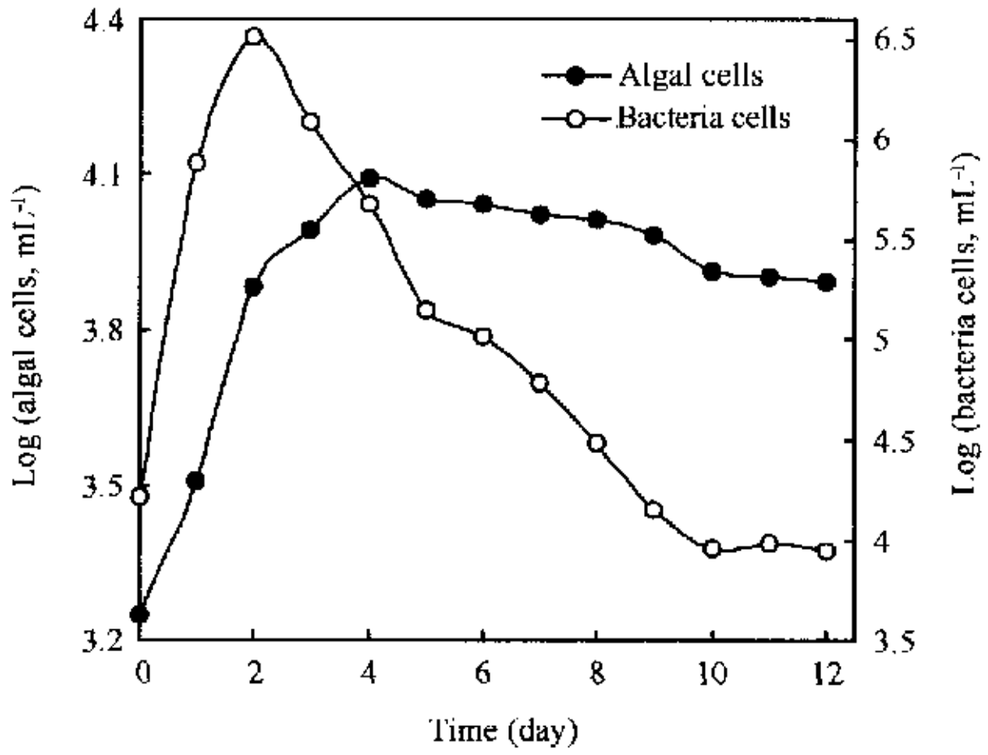

FIG. 1. Growing dynamics of total bacterial cells in the culture of ATCI01.

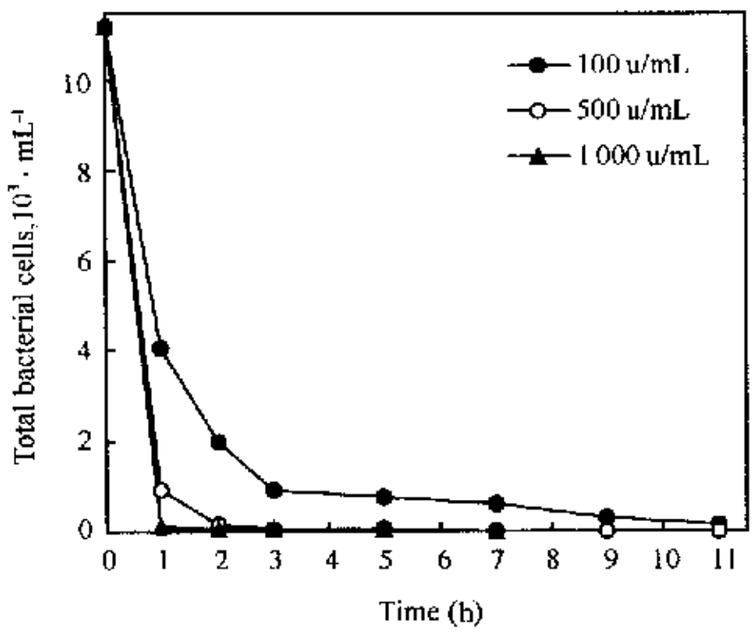

FIG. 2. Inhibition of antibiotics on the growth of total bacterial cells in the culture of ATCI01.

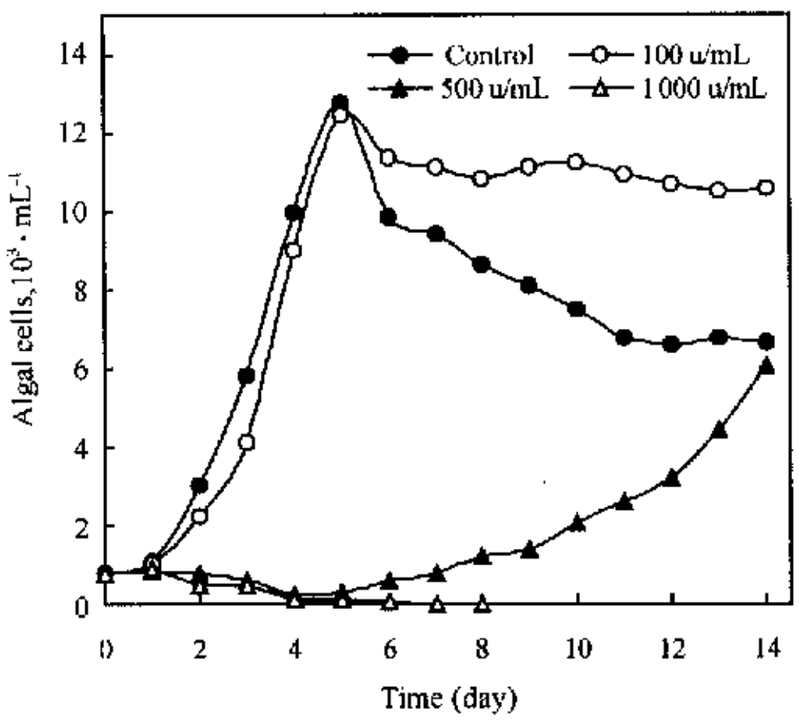

FIG. 3. Effect of concentrations of antibiotics on growth of ATCI01.

FIG. 4. Effect of concentrations of antibiotics on toxin production of ATCI01.

In order to better understand the effects of antibiotics on algal cells, the microalgae were incubated for 10, 15 and 24 hours respectively with 1 000 u/mL, 500 u/mL and 100 u/mL of antibiotic mixtures, so that all bacterial cells could be killed, and then two groups of experiments were carried out. 1) After filtration onto a sterile filter, the pretreated microalgal cells were inoculated into freshly autoclaved antibiotic free medium flasks. 2) The pretreated microalgal suspension was used as the inoculum, and 10% inoculum was directly inoculated into freshly autoclaved medium which still contained 100 u/mL, 50 u/mL and 10 u/mL of antibiotics, respectively.

In the first experimental group, all the antibiotic pretreatments had a positive effect on the growth and toxin production of algal cells. A higher cell density and toxin productivity of 1.6×10^3 cells/mL and 565.2 μg/L were obtained by cultures treated with 100 u/mL of antibiotics, which was 1.3 and 2.1 fold higher respectively than that in the controls (Figs. 5 and 6). When a lower dose of antibiotics was kept in the medium, although it also promoted the growth of algal cells, the benefit to toxin production was increased only by 1.3 fold. However, when the concentration of antibiotics was kept as high as 100 u/mL, the growth and toxin production of algal cells were inhibited (Figs. 7 and 8).

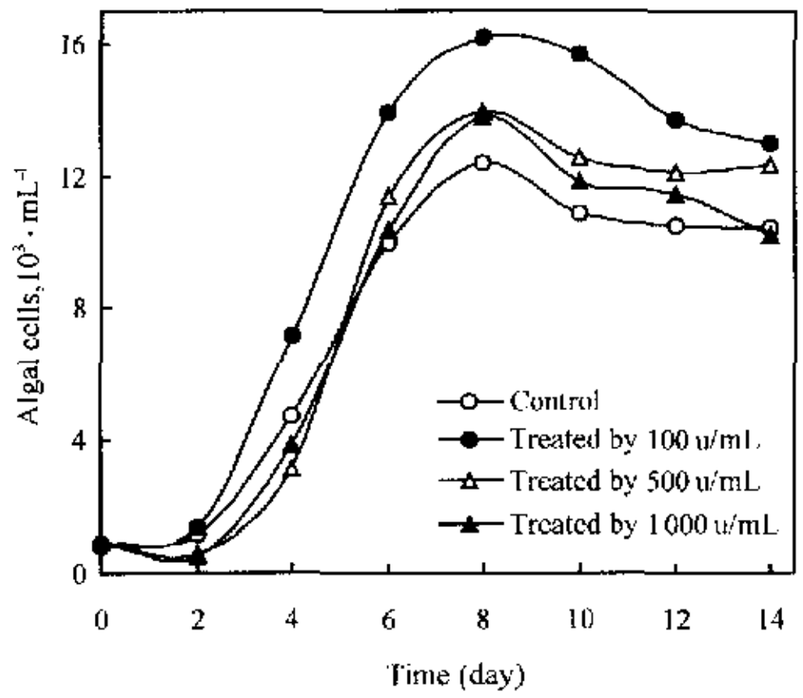

FIG. 5. Growth of algal cells treated by different dose of antibiotics and cultured in antibiotics free medium.

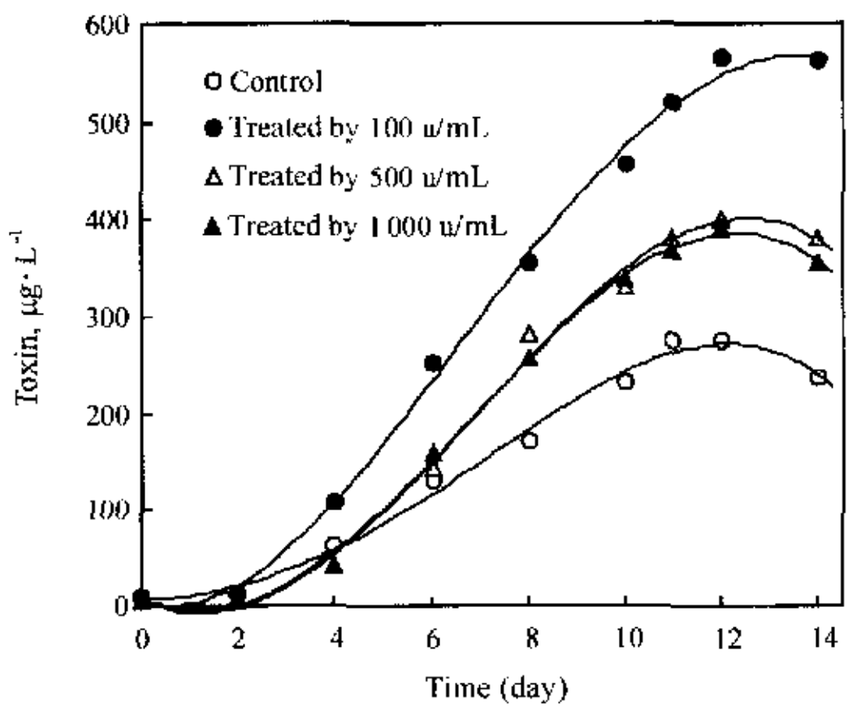

FIG. 6. Toxin production by algal cells treated by different dose of antibiotics and cultured in antibiotics free medium.

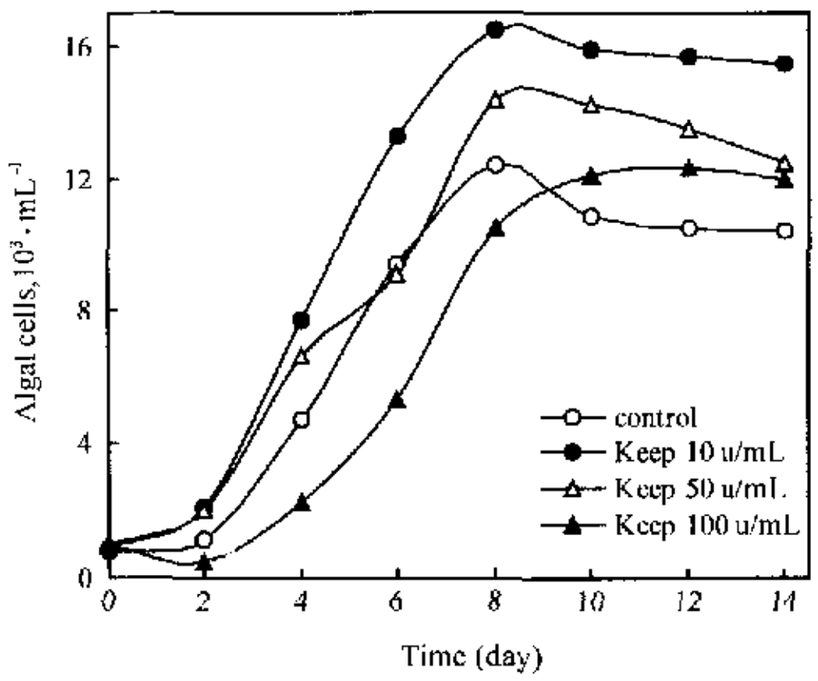

FIG. 7. Growth of algal cells treated with antibiotics and cultured in antibiotics containing medium.

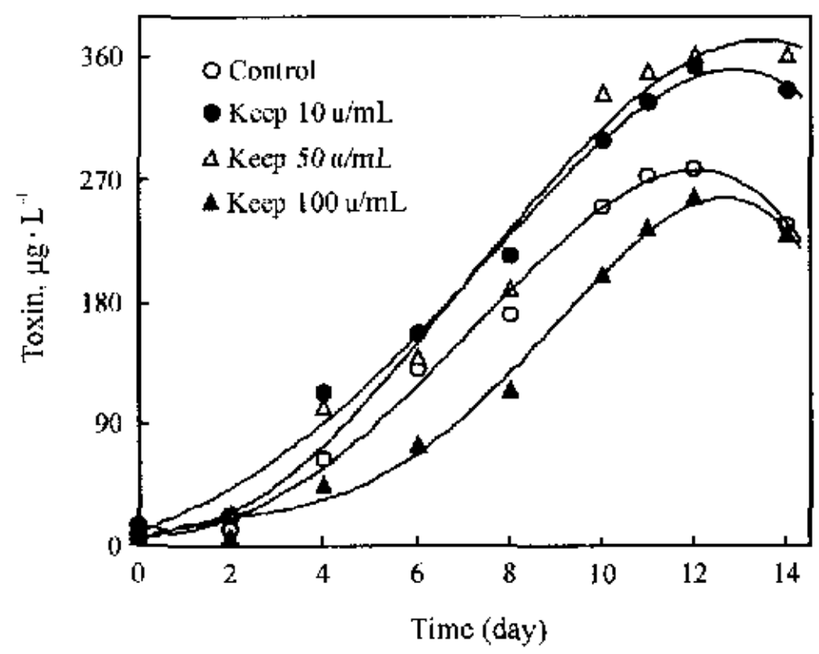

FIG. 8. Toxin production by algal cells treated with different dose of antibiotics and cultured in antibiotics containing medium.

On the basis of the above experiments, an axenic culture system was set up successfully. The results showed that the axenic cultural conditions of the growth and total toxin productivity of algal cells were higher than those in normal cultures. The antibiotics still kept the positive effect on algal cells. And a higher cell density and toxin productivity of 2.2 $\times 10^3$cells /mL and 644.5 μg/L was obtained by the axenic algal cells when 350 u/mL of antibiotics was added into the medium, which was 1.5 and 2.1 fold higher respectively than the control one (Figs. 9 and 10).

DISCUSSION

It is well known that penicillin inhibits the formation of peptide cross-linkages within the peptidoglycan backbone of the cell wall of Gram-positive bacteria and streptomycin is an inhibitor of protein synthesis in Gram-negative bacteria. Our research results showed that marine bacteria also could be inhibited or killed by the mixture of penicillin-G and streptomycin (Fig. 2). Therefore, an axenic culture of algae could be set up when some kinds of antibiotics were used to inhibit the growth of bacteria.

Although it has been known that dinoflagellate algae are the producers of paralytic shellfish toxins (PST), they are involved in a major class of harmful algal blooms (HAB). Recent investigations have produced controversial results regarding the role of bacteria in PST production by toxic algae. Some researchers believed that PST production originated in the intracellular bacteria associated with the algae, and others claimed that PST could be produced by the bacteria in the sea. We found that levels of toxin production were higher when certain doses of mixture of antibiotics were added into the culture medium than those still cultured with external bacteria. And under the axenic cultural conditions using antibiotic-treated cultures producing no bacterial colonies, the growth and toxin production increased markedly (Figs. 9 and 10), which indicated that intracellular bacteria were not responsible for the toxicity of cultures. Therefore, it is very clear that marine bacterial cells have no contribution to the total toxin production of algal cells, which agrees with the research results of Dantzer and Levin[13]. Furthermore, the results of Kim *et al.* supported our research, in which the axenic dinoflagellate strains of *A. tamarense* and *A. catenella* produced toxins at the levels similar to those of non-axenic cultures, indicating that bacterial activity is not involved in the toxin production[7].

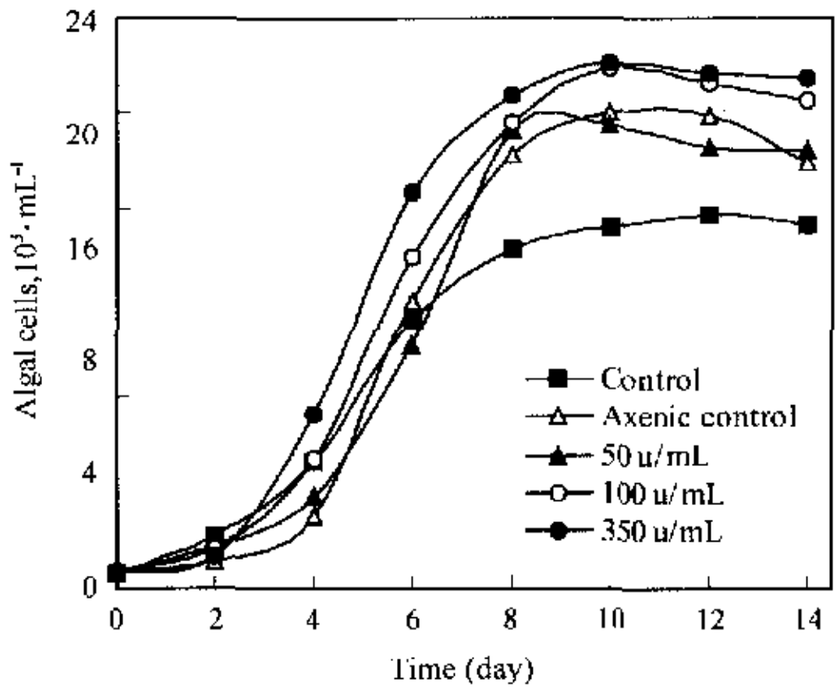

FIG 9. Effect of antibiotics on growth of ATCI01 under axenic conditions.

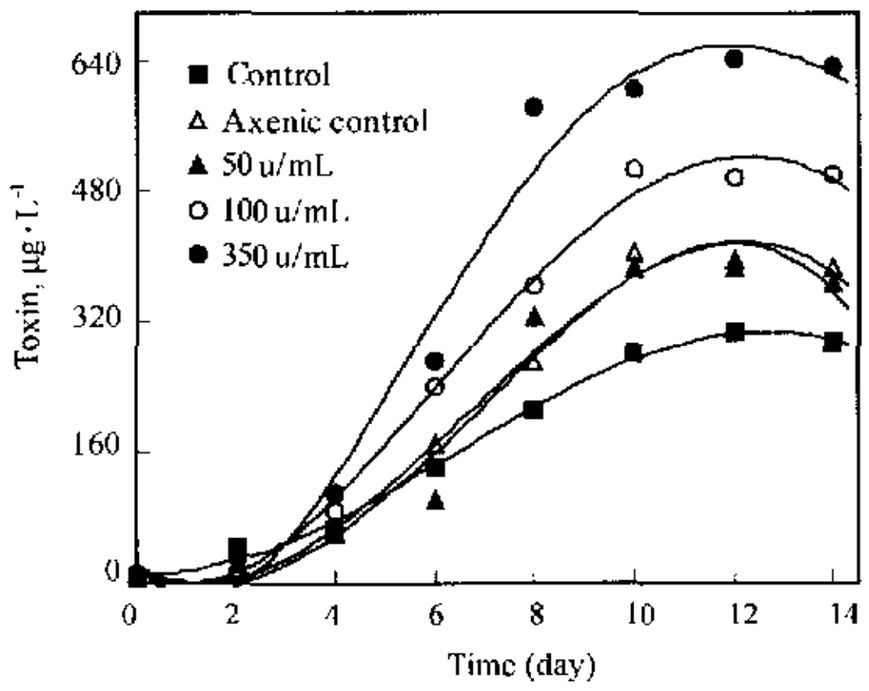

FIG. 10. Effect of antibiotics on toxin productin of ATCI01 under axenic cultures.

It was very interesting that algal cells were also sensitive to the mixture of antibiotics.

When the concentration of antibiotics was higher than 500 u/mL, the algal cells could be hurt, the growth and toxin production were inhibited. However, when a lower concentration of antibiotics was used, the growth and toxin production increased. The key reason seemed to be that inhibition of bacteria by antibiotics could make more nutrients available for algal cells, which have a positive effect on the growth and total toxin production of algal cells. Because algal cells and toxin production were induced to increase by antibiotics under axenic culture conditions (Figs. 7 and 8), therefore, the results indicated that physiological or genetic change of the algal cells would be another very important reason. However, this result was not in agreement with the research of Gonzalez *et al.*, in which cultures of benthic dinoflagellates treated with mixture of antibiotics (chloramphenicol, tetracycline and rifampicin) that inhibited prokaryote protein synthesis showed no significant increase in toxicity during static phase of culture growth[14].

Investigation of the effects of two antibiotics on ATCI01 has shown that the mixture of penicillin-G and streptomycin is not only effective in removing bacteria from the cultures of ATCI01, but also in enhancing the algal growth and toxin production when treated at a right dose. It means that a judicious use of the antibiotics will enhance the toxin productivity of cultured ATCI01. In addition, an axenic strain of ATCI01 could be obtained by this experimental system, which will be very useful in biosynthetic studies and production of 14C-labeled C2T. However, the antibiotics would be toxic to the cells of ATCI01 if the concentration of mixture of antibiotics is 500 u/mL or above.

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