International Journal of Applied Engineering Research ISSN 0973-4562 Volume 15, Number 11 (2020) pp. 1098-1102

Algicidal Activity of *Bacillus* sp. DC-64 and Its Effect of Thermal and pH Concentration

Seong-Yun Jeong

Department of Biomedical Science, Daegu Catholic University, Gyeongsan 38430, Korea.

Abstract

In order to exploit the algicidal bacterium as a useful biological agent against harmful algal blooms (HABs), marine bacterial strain was isolated. The inhibitory effect for the growth of HAB species was examined. The isolated strain DC-64 exhibited significant algicidal activity against *Cochlodinium polykrikoides* and *Alexandrium catenella*. The isolated algicidal bacterium was identified as *Bacillus* sp. The optimal conditions for growth of the strain DC-64 were 30℃, pH 7.0, and salinity 20‰. This strain did not grow at temperatures < 10℃, or at a pH < 4.0, while grow at various salinity. In addition, it was demonstrated that the supernatants of DC-64 were very effective at killing of HAB species. Aa a result of heat treatments of 100℃ for 30 min, algicidal activity of strain DC-64 was heat-tolerant. This result indicates that the algicidal activity of DC-64 is potentially useful in controlling HABs.

Keywords: Cochlodinium polykrikoides, Alexandrium catenella, Algicidal bacterium, Algicidal activity, Dinoflagellate

INTRODUCTION

The list of planktonic algal species that are potentially involved in HABs comprises about 90 toxic species and about 200 noxious species. The causative organisms of HABs usually belong to six algal groups: diatoms, dinoflagellates, haptophytes, raphidophytes, cyanophytes, and pelagophytes. The incidence of HABs has recently increased in frequency as a long-term and large-scale trend (Hallegraeff, 1993; Anderson, 1997). HABs can affect marine organisms by depleting oxygen, clogging their gills, altering their food supply, or producing toxin (Anderson and Garrison, 1997; Boesch *et al*., 1997).

Recent HABs have been frequently outbroken in Korean coastal waters since 1990s, and they have threatened public human health and fisheries industries. In an effort to solve these problems, several techniques have been used to manage blooms, including mechanical, physical, chemical, and biological control method. Biological methods are advantageous compared with other control methods. The dinoflagellate *Alexandrium* spp. (Dinophyceae) is one of the main PSP producers and shows harmful effects on shellfish and human health (GEOHAB, 2001). *Cochlodinium polykrikoides* (Margalef) is the causative organism led to outbreaks of HABs in Korea. The unarmored chain-forming dinoflagellate *C. polykrikoides* is one of the most noxious blooming species responsible for mass mortalities of fish aquaculture in Korea and Japan in the last three decades (Kim *et al*., 2007).

In order to isolate algicidal bacteria, algicidal effects of marine bacteria were tested against *Alexandrium catenella* and *C. polykrikoides*. Then, the isolated strain was identified and characterized. It was discovered possible algicidal bacterium that can use to control HABs as biological agent.

MATERIALS AND METHODS

Algal cultures

Alexandrium catenella and Cochlodinium polykrikoides were supplied by the National Fisheries Research & Development Institute (NFRDI), Republic of Korea. A. catenella and C. polykrikoides belong to dinophyceae. All algal cells were maintained in an f/2-Si medium (Guillard and Ryther, 1962) at 20°C, pH 8.0, with cycles consisting of 12 h of darkness and 12 h of cool white fluorescent light (120 μmol photons $m^{-2}s^{-1}$).

Isolation and screening of algicidal bacterium

Water samples were collected from coastal surface water in Masan Bay, Republic of Korea. Samples were serially diluted and 0.1 ㎖ aliquots of each dilution were spread onto PPES-II (Taga, 1968) agar plates, followed by incubation for seven days at 20℃. Individual colonies of distinct morphology were streaked onto PPES-II agar plates for purification and frozen at –70℃ in 20% glycerol.

In experiments testing bacterial effects on algal culture, it was used 24-well plates. Each well contained 1 ㎖ of algal culture, to which 0.5 ㎖ of a bacterial culture had been added. The plates were monitored at a magnification of ×200. A bacterial strains exhibiting algicidal activity against *A. catenella* and *C. polykrikoides* were selected for further study.

Relationship of algicidal activity and bacterial growth

The growth curve and algicidal activity of the algicidal bacteria were inspected every 3 h, over a 36-h period. The strains were inoculated with PPES-II (initial cell concentration; 1.0×10^3 cells/㎖) and incubated at optimal culture conditions, with shaking; optical density (O.D.) was then estimated every 3 h. The activity of the culture filtrate and each bacterial growth phase (every 3 h) on the growth of algal species were

investigated. Algal culture (1.0×10^4 cells/㎖) was placed in each well of a 24-well plate, together with each culture filtrate the plates were then cultured in algal culture conditions, as described above.

Impact and algicidal activity of algicidal bacterium culture supernatants on algal cultures

After cultivation on PPES-II, bacterial cultures were centrifuged at 6,000 × *g* for 20 min the supernatants were then filtered through 0.2-㎛ Millipore membranes. The algicidal activity of the bacterial culture supernatants on algal culture (1.0×10^4 cells/㎖) was tested using 24-well plates. Each well contained 1 ㎖ of algal culture, to which 10 ㎕, 50 ㎕, 100 ㎕, 500 ㎕, or 1,000 ㎕ (v/v) of culture supernatants were added. The plates were monitored at a magnification of ×200, depending on the size of the organism being tested. Several transects of each well were inspected, and the condition of the algae noted. The plates were inspected every 2 h for the first 7 h, and then less frequently over the next two days.

The algicidal activity of isolate strains was calculated, using the following equation (Kim *et al*., 2007): Algicidal activity (%) = (1–Tt/Ct)×100. Tt (treatment) and Ct (control) are the cell concentrations of algla culture with bacterial culture supernatants, and sterile PPES-II broth, respectively, after inoculation time (t).

To observe morphology change of algal cells, a suitable volume (10%) of the culture supernatants was inoculated to the cultures of algal species the treatment and was observed under light microscopy (Olympus CK40, Japan). In the case of control, equal volumes of sterile PPES-II broth were added to the algal cultures in the treatment instead of bacterial culture supernatants.

Properties of culture filtrate

To investigate the effect of heat treatment on algicidal activity, the filtrate from bacterial cultures grown in PPES-II broth was incubated in a water bath at 4, 10, 20, 30, 40, 50, 70, and 100℃ for 30 min. In addition, to investigate the effect of pH, the filtrate was suspended in 0.1 M citrate phosphate buffer with a range of pH 3 to 7 and 0.1 M Tris-HCl buffer with a pH of 8 to 10, and then kept in each buffer for 30 min. The treated filtrates were subsequently inoculated into algal cultures, to test the algicidal activity. PPES-II broth subjected to the same treatments was added to algal cultures as a control.

RESULTS

Identification and culture conditions of strain DC-64

Strain DC-64 was gram-positive rod (Fig. 1A), and non-pigmented in a PPES-II agar plate. Transmission electron microscope indicated that strain DC-64 was rod-shaped (Fig. 1B) and was approximately 1.2 μm long and 0.4 μm in diameter. The sequences of the DC-64 shared the greatest identity with *Bacillus* spp. (over 99% similarity). The strain was identified as *Bacillus* sp. via culture morphology, biochemical reactions, and homology research based on 16S rRNA gene, respectively. The optimal conditions for growth of the strain DC-64 were 30℃, pH 7.0, and salinity 20‰ (Fig. 2). This strain did not grow at temperatures < 10℃, or at a pH < 4.0, while grow at various salinity.

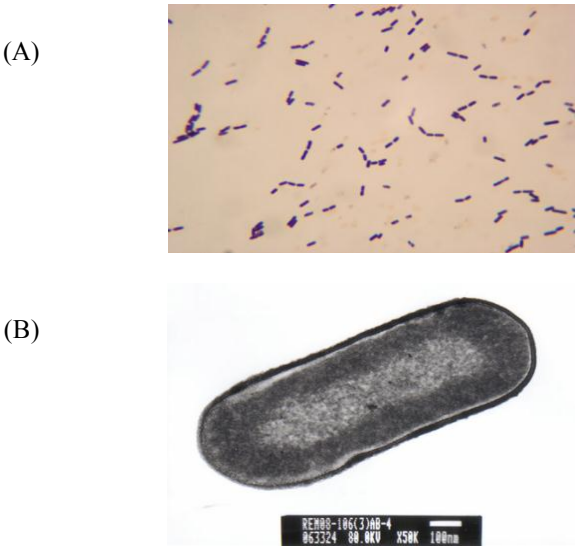

Fig. 1. Micrographs of strain DC-64. (A) Optical micrograph of gram staining and (B) transmission electron micrograph. Scale bar represents 100 nm in size.

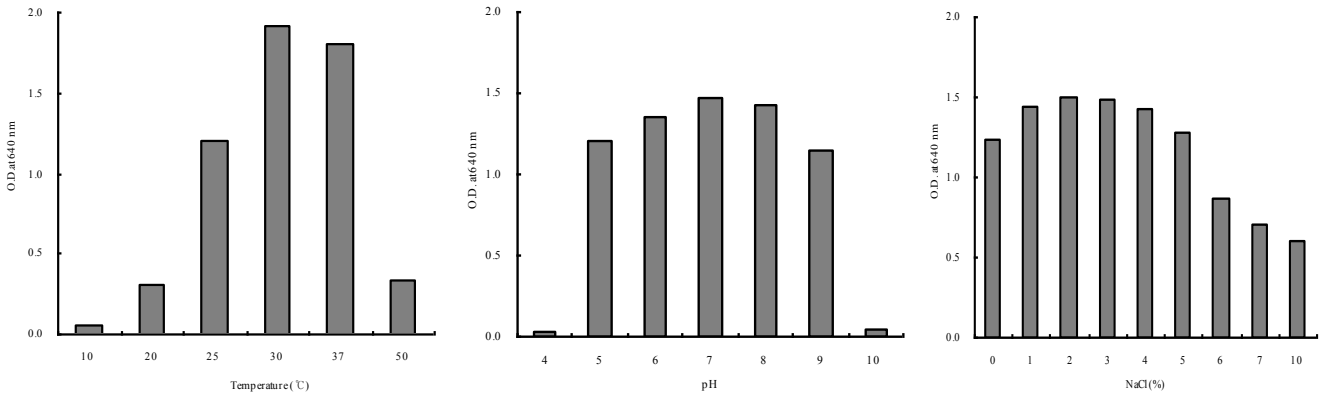

Fig. 2. Effects of temperature, initial pH and NaCl concentrations on the growth of *Bacillus* sp. DC-64 in PPES-II medium.

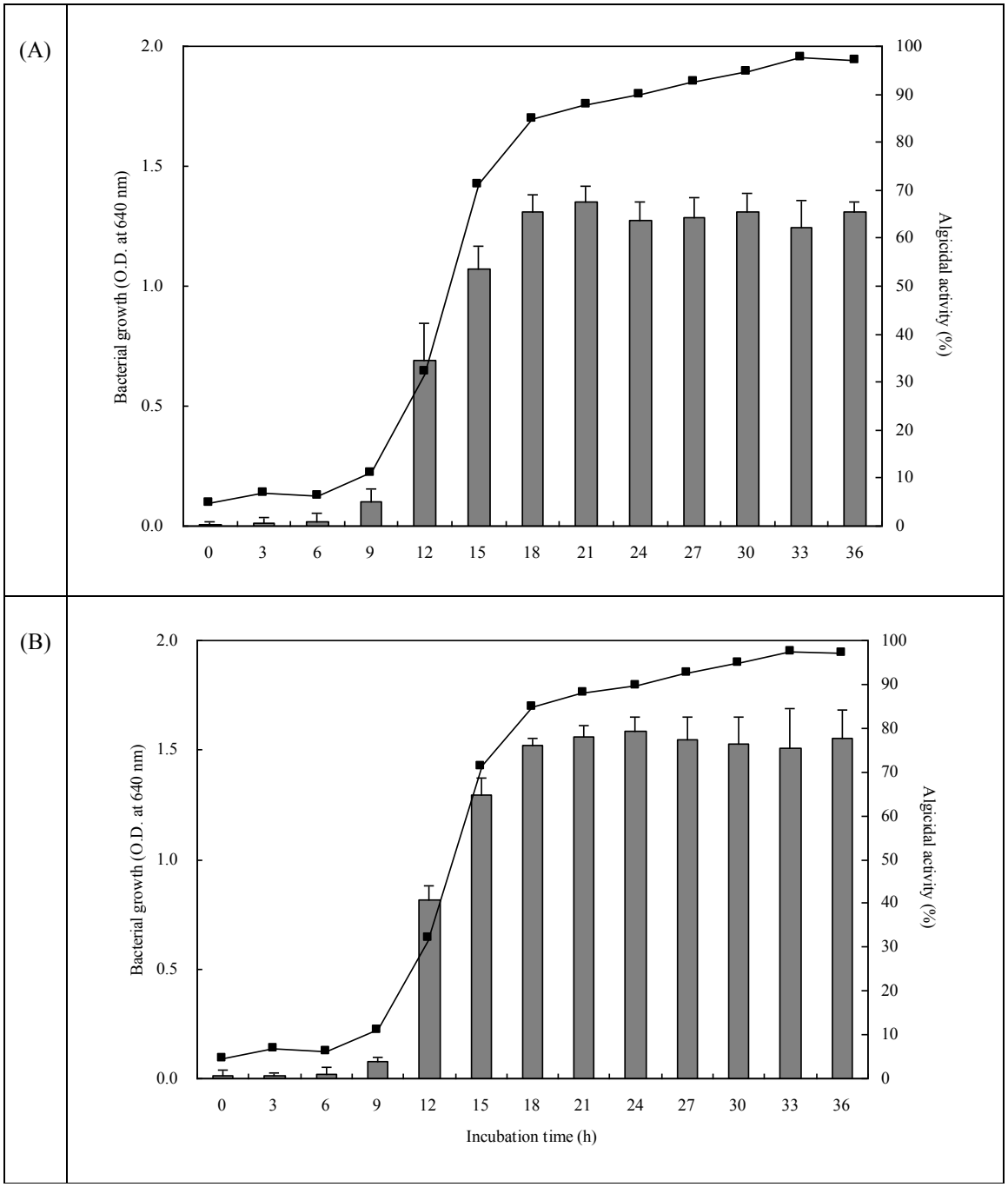

Fig. 3. Growth curve of *Bacillus* sp. DC-64 at optimal culture conditions (30℃, pH 7.0, and NaCl 2.0%) and algicidal activity by the culture supernatants of each growth phase of *Bacillus* sp. DC-64 against *A. catenella* (A) and *C. polykrikoides* (B). -■- = bacterial growth curve (O.D. at 640 nm). Bar graph= algicidal activity (control = algal cultures with PPES-II broth added).

Relationship of algicidal activity and bacterial growth

The growth curve and algicidal activity of the strain DC-64 were inspected every 3 h, over a 36-h period (Fig. 3A and B). It seemed that the algicidal activity of the isolated strain was bacterial growth-dependent, since the strongest algicidal activity occurred in early stationary-phase cultures, in treatments involving the addition of a bacterial culture filtrate.

Impact and algicidal activity of bacterial culture supernatants on *C. polykrikoides* cultures

The experiment testing for different concentrations of culture supernatant (*Bacillus* sp. DC-64) additions showed various levels of algicidal activity on the *C. polykrikoides* culture (Fig. 4). *C. polykrikoides* was killed by high-concentration rather than low-concentration additions. A concentration of < 5% showed weak algicidal activity against *C. polykrikoides*. Although all of the cells of *C. polykrikoides* remained motile,

their speed of motility had decreased markedly within 3 h, with a low concentration of culture supernatant (5% added).

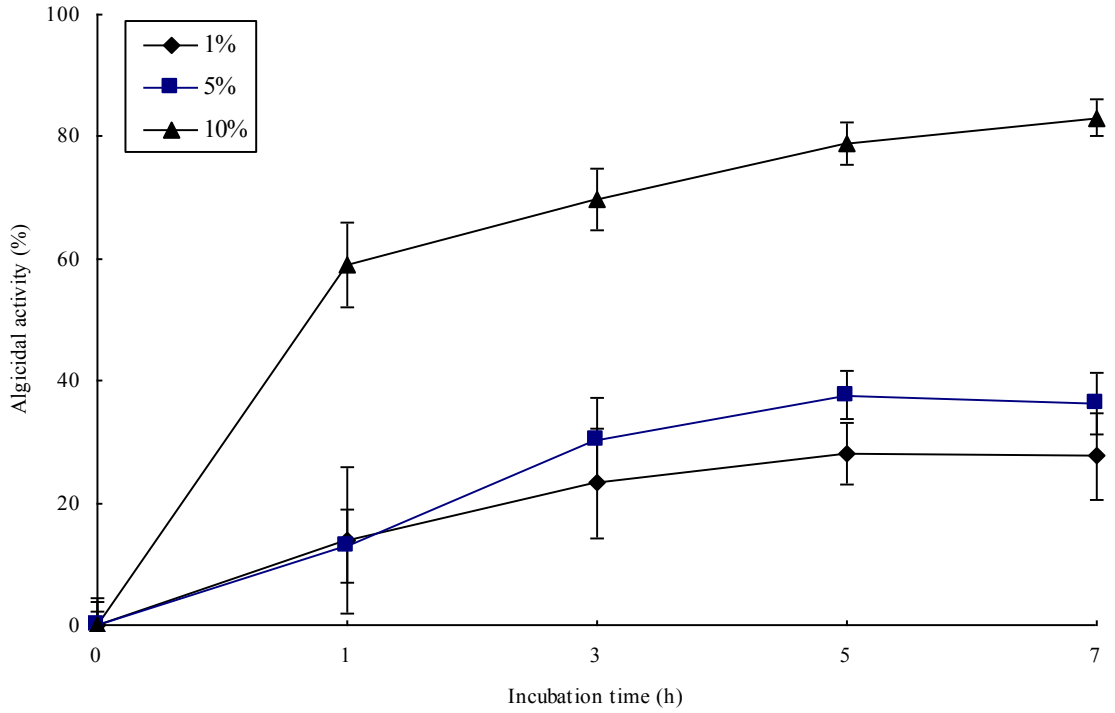

Fig. 4. Algicidal activity of the culture supernatants of *Bacillus* sp. DC-64 against *C. polykrikoides* at various concentrations(◆, 1% (10 ㎕); ■, 5% (50 ㎕); ▲, 10% (100 ㎕)). Control = algal cultures with PPES-II broth added. Data are expressed as mean ± standard deviation, from ten-time assays.

Properties of culture filtrate

The thermal stability after heat treatments was tested (Table 1). All filtrates after incubation at 4, 10, 20, 30, 40, 50, 70, and 100℃ for 30 min exhibited algicidal activity, indicating that the released algicidal compounds were heat-tolerant. A loss of algicidal activity occurred when the pH of the filtrates were adjusted to 3, 4 or 5, while algicidal activity continued in filtrates with a pH adjusted to 10.

Table 1. Thermal and pH stability of *Bacillus* sp. DC-64. For thermal stability, the culture supernatants were incubated at an indicated temperature for 30 min. For pH stability, the culture supernatants were suspended in 0.1 M citrate phosphate buffer and 0.1 M Tris-HCl buffer. And it was then kept in each buffer for 30 min. The relative algicidal activity was estimated for relative data, when the algicidal activity at 4°C and at pH 7.0 or 8.0 was defined as 100%. (data are expressed as mean ± standard deviation, from triplicate assays)

Treatments	Relative algicidal activity (%)
4 ℃	100.0 ± 8.5
20 ℃	93.3 ± 7.4
40 ℃	95.9 ± 7.2
50 ℃	95.9 ± 8.5
70 ℃	85.8 ± 5.5
100 ℃	84.6 ± 10.2
pH stability	
3	39.6 ± 32.7
4	63.5 ± 23.5
5	66.3 ± 23.1
6	74.4 ± 12.6
7	93.3 ± 7.0
8	100.0 ± 5.9
9	97.2 ± 11.6
10	93.3 ± 11.7

DISCUSSION

Algicidal strain closely related to *Bacillus* sp. was also unusual, because most algicidal bacteria belong to either the CFB group or the genus *Pseudoalteromonas* (Imai *et al*., 2001). Recently, Ahn *et al*. (2003) reported that a culture broth of *Bacillus subtilis* completely inhibited the growth of *Microcystis aeruginosa*, a bloom-forming cyanobacterium found in highly eutrophic lakes additionally, Mu *et al*. (2007) reported that the secreted metabolites of *Bacillus fusiformis* showed algicidal activity against *M. aeruginosa*, *Chlorella*, and *Scenedesmus*.

The gram-positive genera comprised unusual algicidal bacteria (Skerratt *et al*., 2002); moreover, the gram-positive organisms do not comprise a major group of the water column, but are rather found in deep-sea sediment (Li *et al*., 1999). Liu *et al*. (2008) hypothesize that algicidal bacteria—which are gram-positive organisms—may act as an important top-down control mechanism.

In conclusion, this strain DC-64 exhibited the strongest algicidal activity against *A. catenella* and *C. polykrikoides*. The strongest algicidal activity occurred in stationary-phase cultures in the treatments involving the addition of bacterial cultures. We used culture filtrate in experiments testing algicidal activity, and the supernatant showed algicidal activity. It was expected that the algicidal bacterium, *Bacillus* sp. DC-64, can also function as an important controller.

REFERENCES

[1] Ahn, C.Y., S.H. Joung, J.W. Jeon, H.S. Kim, B.D. Yoon, and H.M. Oh. 2003. Selective control of cyanobacteria by surfactin-containing culture broth of *Bacillus subtilis* C1. *Biotechnol. Lett*. 25, 1137–1142.

[2] Anderson, D.M. 1997. Bloom dynamics of toxic *Alexandrium* species in the northwestern US. *Limnology and Oceanography* 42, 1009–22.

[3] Anderson, D.M. and D.J. Garrison. 1997. The ecology and oceanography of harmful algal blooms. *Special Issue of Limnology and Oceanography* 42, 1305.

[4] Boesch, D.F., D.M. Anderson, R.A. Horner, S.E. Shumway, P.A. Tester, and T.E. Whitledge. 1997. Harmful algal blooms in coastal waters: Options for prevention, control and mitigation. NOAA Coastal Ocean Program Decision Analysis Series No. 10. NOAA Coastal Ocean Office, Silver Spring, MD, p. 46.

[5] GEOHAB, 2001. Global Ecology and Oceanography of Harmful Algal Blooms, Science Plan. p. 87. *In*: Glibert, P., Pitcher, G. (eds.), SCOR and IOC, Baltimore and Paris.

[6] Guillard, R.R.L. and J.H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* (Hustedt) and *Detonula confervacea* (Cleve). *Gran. Can. J. Microbiol*. 8, 229–235.

[7] Hallegraeff, G.M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* 32, 79–99.

[8] Imai, I., T. Sunahara, T. Nishikawa, Y. Hori, R. Kondo, and S. Hiroishi. 2001. Fluctuation of the red tide flagellates *Chattonella* sp., (Raphidophyceae) and the algicidal bacterium *Cytophaga* sp., in the Seto Inland Sea, Japan. *Mar. Biol*. 138, 1043–1049.

[9] Kim C., H. Kim, C. Kim, and H. Oh. 2007. Life cycle of the ichthyotoxic dinoflagellate *Cochlodinium polykrikoides* in Korean coastal waters. *Harmful Algae,* 6, 104–111.

[10] Kim, J.D., B. Kim, and C.G. Lee. 2007. Alga-lytic of *Pseudomonas fluorescens* against the red tide causing marine alga *Heterosigma akshiwo* (Raphidophyceae). *Biological Control,* 41, 296–303.

[11] Li, L.N., C. Kato, and K. Horikoshi. 1999. Bacterial diversity in deep-sea sediments from different depths. *Biodivers. Conserv*. 8, 659–677.

[12] Liu, J., A.J. Lewitus, J.W. Kempton, and S.B. Wilde. 2008. The association of algicidal bacteria and raphidophyte blooms in South Carolina brackish detention ponds. *Harmful Algae,* 7, 184–193.

[13] Mu, R.M., Z.Q. Fan, H.Y. Pei, X.L. Yuan, S.X. Liu, and X.R. Wang. 2007. Isolation and algae-lysing characteristics of the algicidal bacterium B5. *J. Environ. Sci*. 19, 1336–1340.

[14] Skerratt, J.H., J.P. Bowman, G.M. Hallegraeff, S. James, and P.D. Nichols. 2002. Algicidal bacteria associated with blooms of a toxic dinoflagellate in a temperate Australian estuary. *Mar. Ecol. Prog. Ser*. 244, 1–15.

[15] Taga, N. 1968. Some ecological aspects of marine bacteria in the Kuroshio Current. *Bull. Misaki Mar. Biol*. 12, 65–76.