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RESEARCH PAPER

Characterization of Cell Viability in *Phaeocystis globosa* Cultures Exposed to Marine Algicidal Bacteria

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Abstract The bloom of *Phaeocystis globosa* has occurred frequently in the southern coastal areas of China in recent years, which has led to substantial economic losses. This study investigated the effects of culture broth of strains Y1 and Y4 isolated from algal blooms in Zhuhai, China on physiological characteristics and cell viability of *P. globosa*. The increase in the levels of reactive oxygen species (ROS) in *P. globosa* cells exposed to strains Y1 and Y4 culture broth were detected, indicating that the algal cells suffered from oxidative damage. The surplus ROS induced the increase of malondialdehyde (MDA) contents and the activities of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT). The decrease in protein content indicated that strains Y1 and Y4 culture broth inhibited cell growth. The contents of pigments decreased after 96 h treatment, indicated that oxidative stress destroyed pigment synthesis. Furthermore, flow cytometry coupled with the propidium iodide stain and chlorophyll auto-fluorescence was used to investigate cell viability. Results showed that chlorophyll fluorescence intensities and cell integrity decreased with time of exposure, which demonstrated that strains Y1 and Y4 culture broth could change membrane permeability and resulted in the loss of these photosynthetic pigments. The isolated strains were identified as *Bacillus* sp. by culture morphology, biochemical reactions, and homology research based on 16S rDNA. Overall, these findings suggested that oxidative stress caused from *Bacillus* sp. potentially destroyed pigment synthesis and cell membrane integrity, and ultimately led to the lysis of the algal cells.

Keywords: harmful algal blooms, *Phaeocystis globosa*, algicidal bacteria, reactive oxygen species, flow cytometry

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1. Introduction

In recent decades, environmental pollution has become more and more serious. Nutrient enrichment in aquatic environments, especially nitrogen and phosphorus, has promoted the growth of harmful algal blooms (HABs) [1]. HABs in coastal waters have caused much negative impacts on the marine ecosystems, fisheries, tourism and human health [2,3].

Aimed at terminating HABs, several methods have been proposed for the control of HABs, including physical, chemical and biological methods. However, physical and chemical methods are expensive and impractical for application, and may bring about the secondary pollution to the marine environments [4]. Biological methods, especially algicidal bacteria, which are economical and feasible ways to solve HABs problems compared with other methods, have drawn more and more attention around the world [5,6]. In general, algicidal bacteria can inhibit algal growth or lyse algal cells by the indirect or direct mode. Studies

indicated that the indirect mode, which refers to algicidal bacteria through secreting extracellular substances to lyse algal cells (allelopathy), are most commonly observed [7,8].

The mechanisms of allelochemical inhibition on algal growth mainly focused on four pathways: reduction of pigment contents, destruction of cell structures, alteration of enzymatic activities and influence on algal photosynthesis [9-11]. The physiological and biochemical responses in aquatic organisms through allelochemical stresses have been extensively studied. As an adaptative response, aquatic organisms can increase antioxidant defenses to eliminate reactive oxygen species (ROS), which include superoxide dismutase (SOD), peroxidases (PODs), catalase (CAT) and low molecular weight compounds, such as carotenoids and glutathione, and thus avoid oxidative damage [11,12].

Algal growth measurement by means of traditional techniques needs to provide bulk measurements and does not allow us to observe possible intercellular differences within the cellular population analysed [13,14]. In this respect, flow cytometry (FCM) for studying the response of microalgal cells has been an alternative to these more traditional techniques. FCM enables single cells with different features or physiological states to be counted and examined based on quantification of scattered and fluorescent light signals [15], and allows the rapid detection of cell functions in large numbers by using a variety of biochemically specific and fluorescent molecules [16]. Recently, flow cytometry has been used to characterize the responses of microalgal cells exposed to paraquat and allelochemicals in terms of structural and physiological parameters, such as cell size and complexity, chlorophyll fluorescence, and cell viability [14,16,17].

Our group has isolated strains Y1 and Y4 from the bloom of *P. globosa* in Zhuhai, China, and the strains showed strong algicidal activities against *P. globosa* through the indirect mode. The algicidal bacteria have the potential to be used to inhibit algal growth and thus to regulate harmful algal blooms. However, the specific algicidal mechanisms of bacteria on *P. globosa* have rarely been considered. In this study the effects of strains Y1 and Y4 culture broth on cell viability of *P. globosa* and the physiological characteristics of the cells, including pigment contents, the level of ROS, protein content, oxidative indicator of MDA, the enzyme activities of SOD and CAT, and fluorescence intensity and cell integrity, were investigated through exposure tests using HPLC, laser scanning confocal microscope, enzyme activity and flow cytometry technologies. The isolated strain Y4 was identified as *Bacillus* sp. by morphology and 16S rDNA gene sequence analysis. The objective of our study was to elucidate the mechanisms of strains Y1 and Y4 culture broth against *P. globosa*.

2. Materials and Methods

2.1. Bacteria cultures

Strains Y1 and Y4 were isolated from the bloom of *P. globosa* in Zhuhai, China. Strain Y1 was identified as *Bacillus* sp. by our group (GenBank accession number DQ531607). The strains Y1 and Y4 were cultured in 2216E broth (peptone 5 g/L, yeast extract 1 g/L, ferric phosphorous acid 0.1 g/L, dissolved in natural seawater, pH 7.6 ~ 7.8) at 30°C with shaking at 160 rpm.

2.2. Algal cultures

P. globosa was kindly provided by Professor Songhui Lv, Jinan University, China and maintained in modified f/2 medium under an illumination of 4,000 lx in 12:12 (light/dark) at 20 ± 1°C, which used manmade seawater with a salinity of 25‰ instead of natural seawater [18].

2.3. Pigment content

The pigments were extracted from the concentrated algal samples in an aqueous solution of acetone (90%, v/v) after 96 h of exposure to strains Y1 and Y4 culture broth, and analysed by using high-performance liquid chromatograph (HPLC, Agilent 1100 series) equipped with an autosampler, an Agilent pump, an ultraviolet diode array (UV-DAD) detector and a ZORBAX SBC18 column (5 μm, 4.6 mm × 250 mm, Agilent, USA). The mobile phase consisted of (A) ultrapure water (0.02% TFA) and (B) acetonitrile (0.02% TFA) programmed as follows: 90% A for 5 min, decreased to 10% A in 30 min, with 10 min to re-establish the initial conditions. The flow rate was 1.0 mL/min and the column temperature was maintained at 20ºC. The samples were filtered through 0.45 μm membrane filters and the injection volume was 20 μL. The absorbance was detected at 436 nm.

2.4. Assays for ROS

The intracellular ROS were detected by using the 2'7'-dichlorofluorescin diacetate (DCFH-DA) probe. The DCFH-DA was used at a final concentration of 20 μmol/mL and was incubated with suspended cells for 20 min at room temperature. Then the cells were immediately washed three times with 0.1 M Phosphate Buffer Solution (PBS), and suspended in 500 μL 0.1 M PBS. The fluorescent product DCF was observed by using a Meta Duo Scan Laser Scanning Confocal Microscope with excitation wavelength at 488 nm and emission wavelength at 525 nm.

2.5. Lipid peroxidation and antioxidative enzyme activity analysis

Twenty-five milliliters of culture samples were collected to

extract enzymes by following the method in Qian *et al*. [19]. The lipid peroxidation level was determined by malondialdehyde (MDA) content according to Dogru *et al*. [20]. The superoxide dismutase (SOD) activity was determined by following the method of Trenzado *et al*. [21]. The catalase (CAT) activity was measured according to Qian *et al* [22]. The activity of each enzyme was expressed on a protein basis.

2.6. Analysis of cell viability

Propidium iodide (PI) is a vital dye that intercalates with double-stranded nucleic acids to produce red fluorescence when excited with blue light at 488 nm, and a membrane-impermanent nucleic acid-staining dye which only penetrates cells with damaged membranes. The PI was used at a final concentration of 10 μmol/mL. The samples were stained with PI and incubated for 15 min at room temperature before being analysed with the flow cytometer [14].

Cell integrity in *P. globosa* cultures after exposure to strains Y1 and Y4 culture broth was studied by flow cytometry. A flow cytometer (BD FACSAria) which equipped with an argon-ion excitation laser (488 nm) and forward (FS) and side (SS) light scatter detectors was used. A standard fluorescence detector (FL2 detector, 610 nm) was used to detect cells stained with PI nucleic acid stain and a red fluorescence detector (FL4 detector, 650 nm) was used to detect the auto-fluorescence from chlorophyll in the cells. The flow rates of algal cells were controlled at 100 ~ 400 cells/sec for the analysis. For each analysed parameter, data were recorded in a logarithmic scale.

2.7. Identification of strain Y4 by phylogenetic analysis

Morphological observations were carried out by using Gram-staining. Conventional biochemical tests were examined by using the methods described by Dong and Cai [23], and bacterial strains were identified by polymerase chain reaction (PCR) amplification of the 16S rDNA gene. The total DNA was isolated by using a method described by Volossiouk *et al*. [24]. The amplification of the 16S rDNA was conducted with two primers: forward: 5'-CGAGCG CAACCCTTAATCTT-3', and reverse: 5'-AGTTCCCCA GTTTCCAATGA-3'. The thermal profile was 35 cycles of denaturation for 1 min at 94°C, annealing for 2.5 min at 55°C, extension for 2.5 min at 72°C, and within a final elongation step of 7 min at 72°C. The 16S rDNA amplified with PCR was purified and sequenced. A comparison of nucleotide sequences was performed with the BLASTN database (http://www.ncbi.nlm.noh.gov/BLAST) at the National Center for Biotechnology Information (NCBI). A neighbor-joining phylogenetic tree was made by using the Molecular Evolutionary Genetics Analysis (MEGA) 2.0 program.

2.8. Data analysis

Mean and standard deviation values were calculated for three independent replicate experiments. Data are presented as mean values ± standard error of the means.

3. Results

3.1. Effects of strains Y1 and Y4 culture broth on pigment contents

Pigment content has been used as biomarker of exposure to algicide in plants including algae [25]. In this study, fucoxanthin, diatoxanthin, xanthophyll and chlorophyll-a were detected in the cells of *P. globosa* (data not shown). As shown in Fig. 1, the pigment contents of *P. globosa* were obviously reduced with respect to control group after 96 h of exposure to strains Y1 and Y4 culture broth. The contents of fucoxanthin and diatoxanthin were reduced by about 90% compared to the control, and the reduction of xanthophyll and chlorophyll-a contents was about 80% with respect to the control. The reduction in pigment contents exposed to strain Y1 culture broth was slightly more affected than that exposed to strain Y4 culture broth.

3.2. Effects of strains Y1 and Y4 on ROS levels and protein contents

The ROS levels and the contents of total proteins were determined to assess the oxidative damage in the cells of *P. globosa* and the growth inhibition of *P. globosa* by strains Y1 and Y4 culture broth. The results are shown in Fig. 2. It can be seen that weak fluorescence of DCF was present in the control group (Fig. 2A), and the fluorescence intensity was obviously increased after 48 h of exposure to strains Y1 and Y4 culture broth (Figs. 2B and 2C). The

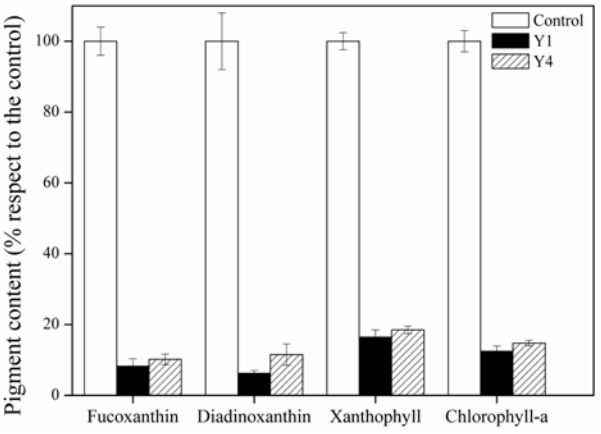

Fig. 1. Pigment contents, expressed as percentage with respect to control, of *P. globosa* cultures after exposure to strains Y1 and Y4 culture broth for 96 h with concentration of 1.5% (V/V). All error bars indicate standard error of the three replicates.

Fig. 2. Effects of strains Y1 and Y4 culture broth on the forms of intracellular ROS in *P. globosa* after 48 h exposure with concentration of 1.5% (V/V). (A) Control of *P. globosa*; (B) culture of *P. globosa* exposed to strain Y1; (C) culture of *P. globosa* exposed to strain Y4.

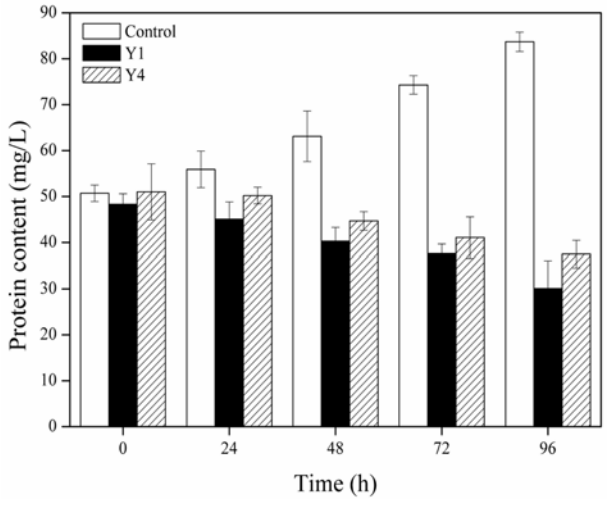

Fig. 3. Effects of strains Y1 and Y4 culture broth with concentration of 1.5% (V/V) on protein contents of *P. globosa*. All error bars indicate standard error of the three replicates.

ROS levels in *P. globosa* cells exposed to strain Y1 culture broth was higher than that exposed to strain Y4 culture broth. The cellular protein contents in treatment groups obviously decreased compared to the control (Fig. 3). After 96 h of exposure to strains Y1 and Y4 culture broth, the protein contents in treatment samples were reduced to 35.9 and 45.1% of the protein contents in the control sample, respectively.

3.3. Effects of strains Y1 and Y4 on lipid peroxidation and antioxidative enzyme activities

Fig. 4 showed the effects of strains Y1 and Y4 on lipid peroxidation and antioxidative enzyme activities. Exposure to strains Y1 and Y4 culture broth induced an increase in MDA contents in *P. globosa* (Fig. 4A), and MDA contents increased with exposure time. After 24 h and 96 h of exposure, the MDA contents increased from 1.13 and 1.19 times to 2.05 and 1.7 times, respectively. Cellular enzymatic activities including SOD and CAT were determined to investigate the cellular defense responses induced by exposure to strains Y1 and Y4 culture broth (Figs. 4B and

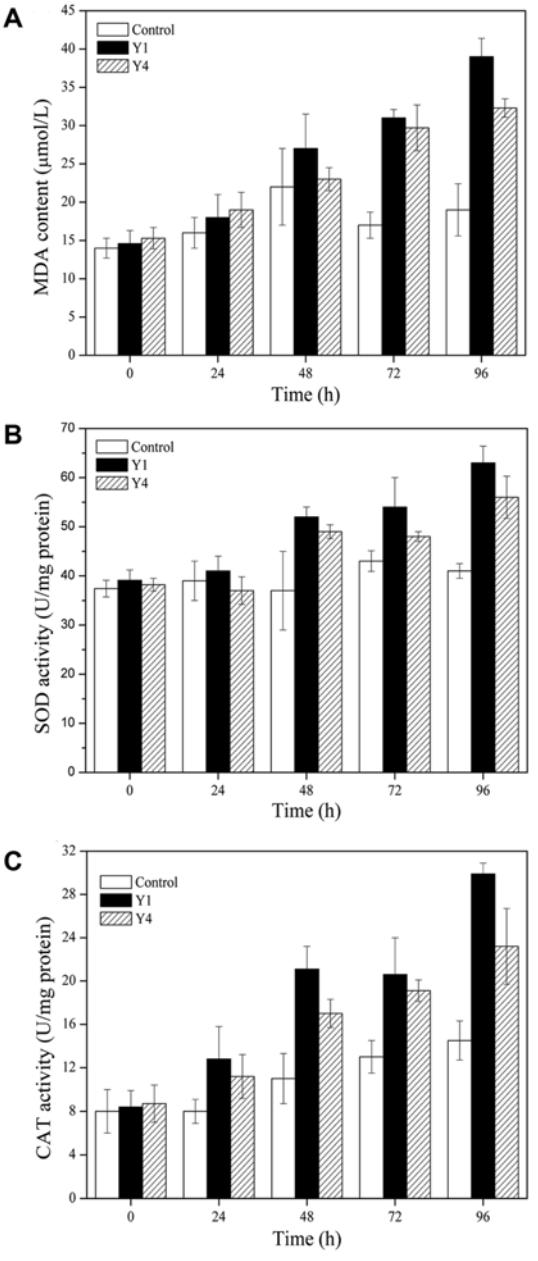

Fig. 4. Effects of strains Y1 and Y4 culture broth with concentration of 1.5% (V/V) on (A) MDA, (B) SOD, (C) CAT contents of *P. globosa*. All error bars indicate standard error of the three replicates.

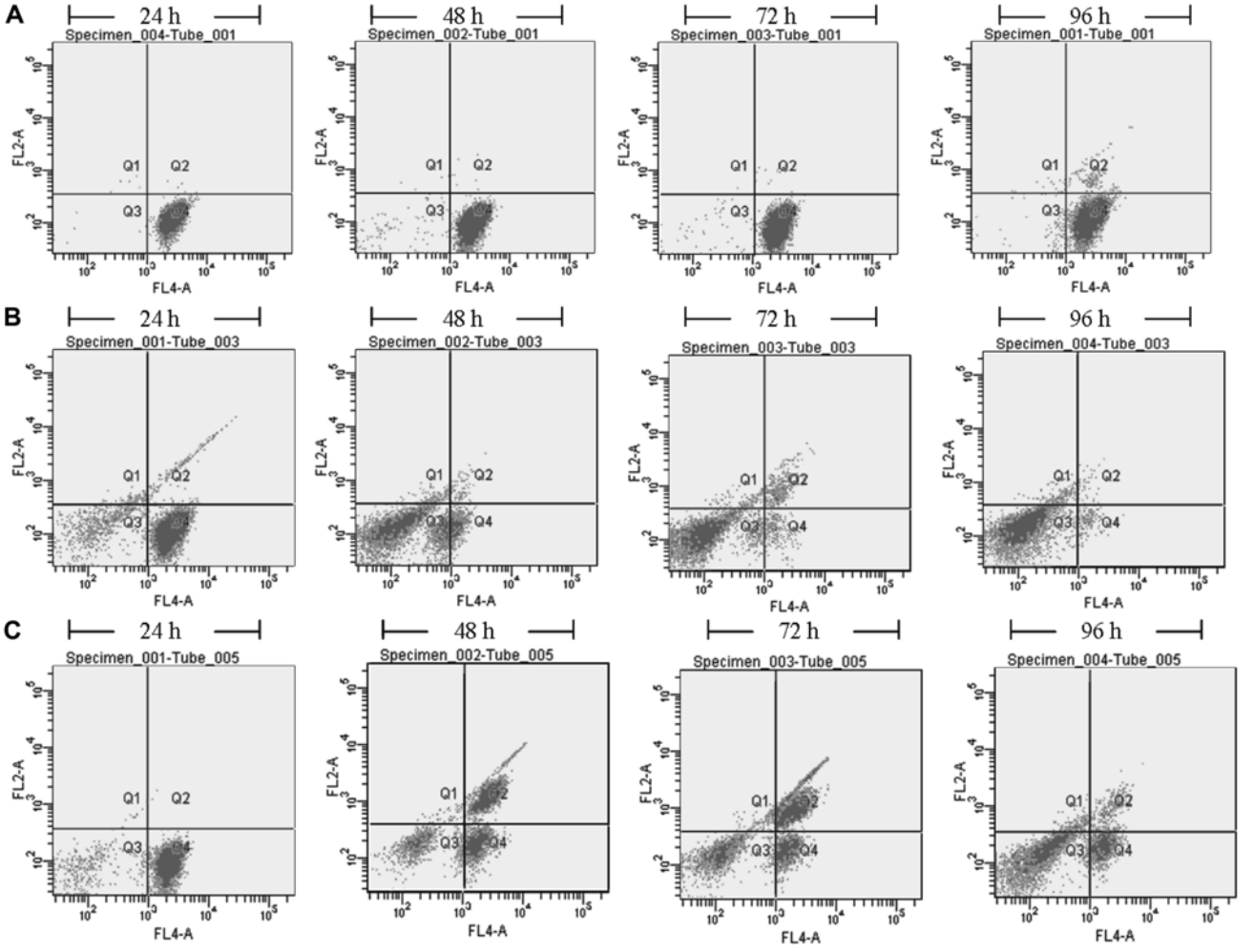

Fig. 5. Two-dimensional fluorescence results for *P. globosa* exposed to strains Y1 and Y4 culture broth with concentration of 1.5% (V/V). (A) Control of *P. globosa*; (B) culture of *P. globosa* exposed to strain Y1; (C) culture of *P. globosa* exposed to strain Y4. (FL4: chlorophyll-a fluorescence; FL2: PI fluorescence)

4C). Fig. 4B showed that SOD activities increased with the increase of exposure time. When algal cells were treated with strains Y1 and Y4 culture broth for 96 h, the activity values were 1.63 and 1.41 times of the control value, respectively. After 24 and 96 h of exposure, CAT activity values increased from 1.6 to 2.06 times and 1.4 to 1.64 times, respectively (Fig. 4C). It was also observed that the effects of lipid peroxidation and antioxidative enzyme activities exposed to strain Y1 culture broth were larger than the effects of those of exposure to strain Y4 culture broth.

3.4. Effects of strains Y1 and Y4 on cell viability

Fig. 5 showed the change of cell fluorescence in *P. globosa* before and after the addition of strains Y1 and Y4 culture broth at different times of the assay. In Fig. 5, the fluorescence intensities produced by chlorophyll-a (FL4 detector) and that produced by the PI dye (FL2 detector) stained on nucleic acids were shown in a two-dimensional plot. This type of two-dimensional fluorescence intensity plot was similar to that presented by Brussaard *et al*. [26]. Q4 quadrant represents integral cells, for which chlorophyll-a intensity was strong and PI intensity was low. In Q1 and Q3 quadrant, low chlorophyll-a fluorescence intensities were observed, indicating that the chlorophyll-a was degraded [26]. Q2 quadrant represents cells that were ruptured, for which chlorophyll-a and PI intensities were strong, indicating that the PI dye stained the nucleic acids within the cells. As shown in Figs. 5B and 5C, chlorophyll-a fluorescence intensity decreased gradually and the cells shifted to Q1 and Q3 quadrant with time of exposure. At the same time, PI fluorescence intensity increased gradually and the cells shifted to Q2 quadrant with treatment time prolonged.

The two-dimensional fluorescence data were further analyzed to quantify the effects of strains Y1 and Y4 culture broth on cell integrity of algal cells as shown in

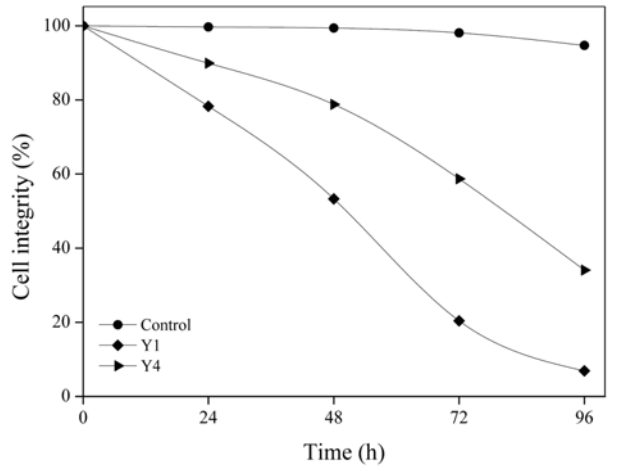

Fig. 6. Cell integrity of *P. globosa* with and without exposure to strains Y1 and Y4 culture broth with concentration of 1.5% (V/V).

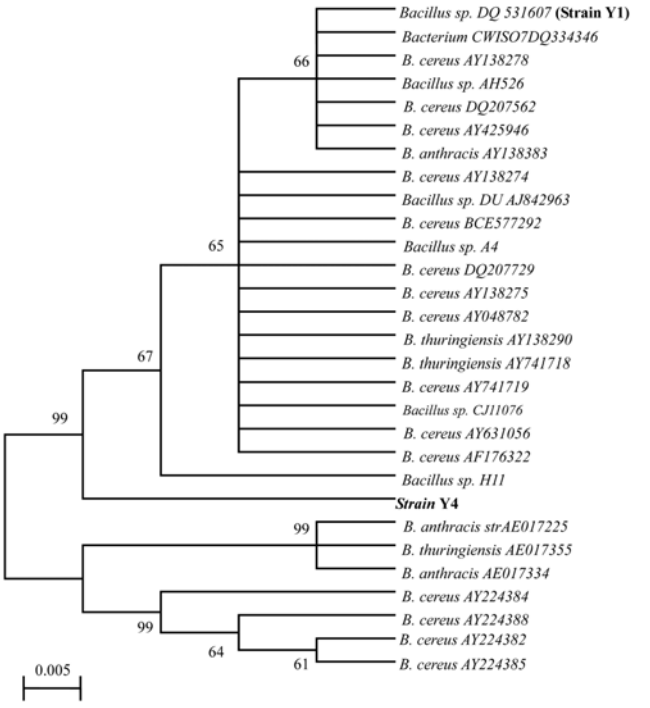

Fig. 7. Phylogenetic tree of strains Y1 and Y4 based on 16S rDNA sequences showing positions of strains Y1 and Y4, and representatives of *Bacillus* sp. The scale bar represents 0.005 substitutions per nucleotide position.

Fig. 6. All the cells were expected to be integral before the addition of strain Y1 and Y4 culture broth, and the viability of *P. globosa* cells was effectively inhibited by the culture broth during the time of exposure. As can be seen in Fig. 6, the percentage of live cells decreased with the time of exposure to strains Y1 and Y4 culture broth, to 6.9 and 34.1% after 96 h exposure, respectively.

3.5. Identification of strain Y4

Strain Y4 was gram-positive, round and nonpigmented in 2216E medium. The 16S rDNA sequences of strain Y4 exhibited a high homology to the sequences of *Bacillus* sp., having 99.7% similarity through comparison with available sequences from the GenBank database (GenBank accession number: DQ531608). Phylogenetic tree based on 16S rDNA indicated that strain Y4 was most related to *Bacillus* sp. (Fig. 7).

4. Discussion

In coastal seawaters where red tides occur frequently, marine bacteria play an important role in reducing or developing algal blooms [27]. Algicidal bacteria can act as the key biological controllers in the rapid termination of phytoplankton blooms [28]. Lewitus *et al*. [29] reported that algicidal bacteria that are toxic to or prey upon raphidophytes (*e.g*., viruses and mixotrophic algae) act as important factors for terminating phytoplankton blooms. There are few reports in the literature on the effects of bacteria against *P. globosa*, and most of them only describe the relationship between alga and bacteria. In order to understand the mechanisms of strain Y1 and Y4 culture broth against *P. globosa*, the variations in pigment contents, levels of ROS, MDA, lipid peroxidation and antioxidative enzyme activity, fluorescence intensity and cell intergrity were determined.

ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) [30] and are produced during the mitochondrial electron transport chain, peroxisomes and chloroplast [31]. Superoxide radical ($O_2^{\cdot-}$) are produced as the byproducts of photosynthetic electron transport and are easily converted into hydrogen peroxide (H_2O_2) through chemical and enzymatic reactions. During photosynthesis, singlet oxygen ($^1O_2$) and hydroxyl radicals (·OH) are also generated [32]. Direct and indirect oxidative damages were caused by triggering an increased ROS level [31], and one of the responses of streptomycin and *C. vulgaris* to algicidal stress is the excessive production of ROS [19]. In the present study, it was observed that the levels of ROS in cells of *P. globosa* were obviously increased after 48 h of exposure to strains Y1 and Y4 culture broth (Figs. 2B and 2C). These results indicated that strains Y1 and Y4 culture broth induced algal cells to produce excessive ROS. These excessive ROS were not totally cleared by the algal cells and eventually caused cell damages. Cellular protein contents reflect the physiological states of cells. The increased protein contents in the control groups suggested that new proteins were synthesized. However, the contents of proteins

in the treatment groups decreased with extended treatment time (Fig. 3). The results implied that the external environmental stress could affect cellular protein synthesis in algal cells.

Yamauchi *et al*. [33] have pointed out that MDA is a major peroxidation product and is an indicator of lipid peroxidation, which reflects cellular oxidative damage under environmental stress conditions. Cell membranes are made of unsaturated phospholipids and are vulnerable to oxygen radical attack resulting in MDA accumulation [22,34,35]. In the present study, exposure to strains Y1 and Y4 culture broth increased the levels of MDA in *P. globosa* cells with exposure time (Fig. 4A), which indicated that strains Y1 and Y4 culture broth induced membrane lipid peroxidation and caused oxidative damage on cell membranes; This phenomenon was also observed by Kong *et al*. [36] and Qian *et al*. [34]. Under environmental stresses, various oxidases can be activated by intracellular ROS. ROS scavengers, such as SOD and CAT, protect against the potential damaging effects of ROS [37,38]. The production and scavenging by ROS are in dynamic homeostasis under normal environmental conditions such that excessive ROS cannot be accumulated in the algal cells. Our results indicated antioxidant enzymes activities were triggered to different degrees when exposed to strains Y1 and Y4 culture broth (Figs. 4B and 4C). A previous study also showed that *Bacillus* sp. B1 induced the increase of MDA, SOD and CAT activities in *P. globosa* [39]. When algal cells were exposed to allelochemicals, the same variation in SOD and CAT activities was also found [22,40].

Standard algal growth bioassays for assessment of phytotoxic effects provide important ecological information [41-43], but these tests cannot be used to determine the causes for growth inhibitions. On the other hand, flow cytometry has been introduced as a useful tool in studying different morphological and physiological features of microalgal cells [44]. This technique allows us to perform quantitative measurements of individual cells in large numbers and in appreciably short time [45]. In the present work, flow cytometry was used to characterize the response of *P. globosa* cells to algicidal bacteria.

A natural autofluorescence is provided by the pigments of phototrophic organisms like microalgae, mainly by chlorophyll-a. The physiological states of microalgal cells exposed to different stress factors have been studied by detecting chlorophyll-a fluorescence alterations [46,47]. A previous study showed that an oxidative breakdown of chlorophyll as a consequence of the generation of reactive oxygen species by paraquat action probably resulted in the progressive reduction of cell autofluorescence [48]. Results in the present study showed that chlorophyll-a flurescence of *P. globosa* cells gradually reduced with time of exposure to strains Y1 and Y4 culture broth (Figs. 5B and 5C), which indicated an almost total loss of these photosynthetic pigments. Pigment contents analysed by HPLC indicated a significant reduction of pigment contents in *P. globosa* cells exposed to strains Y1 and Y4 culture broth (Fig. 1), which was in accordance with the results obtained by flow cytometry. Overall, results showed that chlorophyll-a fluorescence and cellular pigment contents were significantly decreased by the bacterial culture broth and therefore the photosynthetic processes were inhibited. This was probably also related to the oxidative damages that can disrupt normal photosynthesis [12].

Determination of cell viability is one of the basic tasks in physiological analysis. Cell death is accompanied by a series of processes, among which are the loss or reduction of enzymatic activity or membrane integrity [49]. The detection of cell integrity by using nucleic acid dyes is a common and widely used technique. PI is one of the commonly used nucleic acid stain, which cannot penetrate into cells with intact membranes owing to its positive charge, and has been used to monitor cell viability in several studies [50,51]. In the present study, flow cytometry was used to analyze cell viability in *P. globosa* cultures exposed to strains Y1 and Y4 culture broth and it was based on changes occurring in membrane integrity. Results showed that PI fluorescence intensity increased with the time of exposure (Figs. 5B and 5C), which indicated that the cells had lost membrane integrity. Low chlorophyll-a fluorescence intensity indicated that the cells had lost most of the nucleic acids due to partial cell lysis or DNA breakdown. Therefore, the dead and low chlorophyll-a portions are considered to be ruptured cells for the analysis [16]. Fig. 6 showed that most of cells were ruptured after 96 h exposure of strains Y1 and Y4 culture brothand strain Y1 culture broth was more effective in lysing *P. globosa* cells than strain Y4 culture broth, which was in accordance with Figs. 1 and 2. The increase in the percentage of cells which have lost their membrane integrity observed in cultures exposed to strains Y1 and Y4 culture broth was probably related to the generation of oxygen free radicals, as reactive oxygen species have been implicated in the initiation of membrane damage by lipid peroxidation [52].

Studies have shown that about 50% of algicidal strains belong to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, about 45% of strains are members of *γ-Proteobacteria*, and the remaining strains are the Gram-positive genera *Micrococcus*, *Bacillus*, and *Planomicrobium* [53]. A strain closely related to *Bacillus* sp. is uncommon. Recently, Liu *et al*. [54] hypothesizes that algicidal bacteria may act as an important top-down control mechanism, which are gram-

positive organisms. Therefore, the algicidal bacteria, *Bacillus* sp. Y1 and Y4, can be expected to play an important role in controlling algal blooms.

5. Conclusion

The effects of *Bacillus* sp. Y1 and Y4 culture broth on physiological characteristics and cell viability in *P. globosa* were investigated. Results from the present study showed that strains Y1 and Y4 culture broth decreased cellular pigment contents, induced ROS production, and altered physiological states, enzymic antioxidant systems, chlorophyll fluorescence intensities and cell integrity in *P. globosa*. The excessive ROS in the algal cells caused oxidative damage to algal cells, and ultimately inhibited cell growth or lysed algal cells. Despite the algicidal substance is unclear, our results are great helpful to understand the algicidal mechanism and control HABs. Moreover, further studies could focus on the isolation and identification of the algicidal compounds from bacteria towards *P. globosa.*

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