Exploration in antioxidant and photosynthetic system of a marine algicidal Bacillus on four HABs species
Exploration in antioxidant and photosynthetic system of a marine algicidal *Bacillus* on four HABs species

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Abstract:

A novel marine bacterium strain B1, initially showed 96.4% of algicidal activity on *Phaeocystis globosa*. Under this, three other harmful algal species (*Skeletonema costatum*, *Heterosigma akashiwo* and *Prorocentrum donghaiense*) were chosen to study the algicidal effects of strain B1, and the algicidal activities were 91.4%, 90.7% and 90.6%, respectively. In order to explore the algicidal mechanism of strain B1 on these four harmful algal species, the characteristic of antioxidant and photosynthetic system were studied. Sensitivity to strain B1 supernatant, enzyme activity and gene expression varied with algal species, while the algicidal patterns were similar. Strain B1 supernatant increased malondialdehyde contents, decreased chlorophyll a contents as well as changed total antioxidant and superoxide dismutase activity, restrained *psbA*, *psbD* and *rbcL* genes expression which eventually results in the algal cells death. The algicidal procedure was observed using field emission scanning electron microscopy, which indicated that algal cells were lysed and cellular substances were released. These findings suggested that antioxidant and photosynthetic system of these four algal species was destroyed under strain B1 supernatant stress. This is the first report to explore and compare the mechanism of a marine *Bacillus* against harmful algal bloom species of covered four phyla.

**Keywords:** Harmful algal blooms, Algicidal *Bacillus*, Gene expression, Morphologic damage, Oxidative damage.
Introduction

Harmful algal blooms (HABs) breaks out more frequently worldwide, and have aggravated pollution, economic losses and disease hazard to human health in recent years (Hallegraeff 1993; Briand et al. 2008; Mohamed 2014). In particular, *Skeletonema costatum*, *Heterosigma akashiwo*, *Phaeocystis globosa* and *Prorocentrum donghaiense* are notorious HABs species, with the frequent outbreak in the Chinese coastal area (Mayali and Azam 2004; Zheng et al. 2013). Approaches have been carried out to prevent or remove HAB occurrences, involving the application of physical and chemical methods (Wang et al. 2005; Costas and Lopez-Rodas 2006). However, they are limited due to non-target toxicity and secondary pollution (Anderson 1997, 2009; Pierce et al. 2004). Biological methods, including bacteria, fungi, virus, macrophytes and protozoa (Nakashima et al. 2006; Su et al. 2007; Brussaard et al. 2007; Jeong et al. 2008; Graham and Strom 2010), have gained increasing attention. The algicidal bacteria are the main group of producers of extracellular active substances, which have been widely studied to the growth-inhibiting of harmful algae (Furusawa et al. 2003; Amaro et al. 2005; Kim et al. 2008; Kim et al. 2009; Yang et al. 2013). Most of these reported bacteria have a relatively species-specific algicidal effect on algae. For example, Li et al. isolated *Bacillus* sp. Lzh-5 and demonstrated that its compounds showed strong algicidal activity against *Microcystis aeruginosa* (Li et al. 2015). Only a few algicidal bacteria had a relatively wide host range and exerted activities on a variety of freshwater algae species (Liao and Liu 2014), which are seldom reported to indicate the parallel algicidal effect against marine algal species of covered four phyla.

Current studies about the algicidal mechanism have researched the change of malondialdehyde (MDA), antioxidant enzymes and photosynthetic genes in algal cells (Shi et al. 2009; Zhang et al. 2013a). MDA is an important product of the lipid peroxidation inside the cells, which reflects the
degree of oxidative damage in algal cells. Those antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) can scavenge free radicals, catalyze antioxidant defense reactions so as to inhibit oxidative damage caused by the radicals (Elbaz et al. 2010). Photosynthesis relies on the transcript of photosynthetic genes in photosystems. \textit{Psb}A and \textit{psb}D encode two core proteins, D1 and D2 of photosystems (Marder et al. 1987), and \textit{rbc}L encodes the large subunit of ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO) (Asada et al. 1998). Their expressions are used to explore whether there was damage in the photosynthetic processes. Investigations mainly focused on the algicidal bacteria associated with the growth of \textit{Microcystis aeruginosa} and \textit{Alexandrium tamarense} (Wang et al. 2012; Zhang et al. 2013b; Li et al. 2014). However, few reports demonstrated the change and characteristic of antioxidant and photosynthetic system for \textit{S. costatum}, \textit{H. akashiwo}, \textit{P. globosa} and \textit{P. donghaiense} species, especially on the gene expression.

A novel marine bacterium, the algicidal \textit{Bacillus} sp. B1, with indirect algicidal activity against the \textit{P. globosa} was isolated from decayed algal blooms and identified by 16s rDNA sequence (Li Qiang, 2012). To better understand and compare the inhibitory mechanism of strain B1 supernatant on \textit{S. costatum}, \textit{H. akashiwo}, \textit{P. globosa} and \textit{P. donghaiense} species, further studies were carried out in this regard. Algicidal activity and basic characteristic in antioxidant and photosynthetic system for these four algal species were implemented during the algal lysis. The cell morphology was observed using field emission scanning electron microscopy (FESEM). In addition, the gene expressions of these algal species after exposure to strain B1 supernatant were also investigated. This is the first report to explore the algicidal effect of a marine algicidal bacterium on four harmful algae species deep into the genes expression.
Materials and methods

2.1 Four algal cultures and strain B1 supernatant

2.1.1 Algal cultures

Four algal species (*Skeletonema costatum*, *Heterosigma akashiwo*, *Phaeocystis globosa* and *Prorocentrum donghaiense*) cultures were obtained from the Algal Culture Collection, institute of Hydrobiology, Jinan University (Guangzhou, China). Cultures were prepared in f/2 medium under the light conditions of 12 h/12 h light-dark cycle at 20±1 °C (Light incubator GXZR0328). The test was started until the concentration of algae reached approximately 10^7 cells mL^-1.

2.1.2 Bacterial strain and supernatant preparation

Strain B1 was previously isolated by our group and its GenBank accession number is JN 228893 (Fig.1). Strain B1 were cultivated in 2216E medium at 30 °C (200 rpm) for five days, then centrifuged at 6000 rpm for 10 min. The supernatant was collected after passing through a 0.22 µm Millipore membrane and stored at 4 °C until use.

2.1.3 Determination of algicidal activity

Chlorophyll a was used to determine algicidal activity. When the algae grew to the logarithmic growth phase, strain B1 cell-free supernatant and 2216E liquid medium were added to the treatment groups at a proportion of 1.0% (v/v), and the culture conditions were the same as algal culture (2.1.1). Cultures without adding supernatant or bacterial media were prepared as controls. An aliquot of 20 mL algal culture was removed daily and centrifuged at 4000 rpm for 10 min to collect algal cells. The algal cells were stored in the dark at 4 °C for 24 h within 5 ml acetone (95%). The acetone extract was centrifuged at 4000 rpm for 10 min, and then measured the absorbance at wavelengths of 665 nm, 645 nm and 630 nm by 721 Vis spectrophotometer. The Chlorophyll a was calculated using the
Chlorophyll a (mg/L) = 11.6×$A_{665}$-1.31×$A_{645}$-0.14$A_{630}$

and the algicidal activity was calculated as the formulae (Hoshaw et al. 1973).

Algicidal activity (%) = (1- Nt/Nc) ×100 %

Where Nt presented the Chlorophyll a content of the treatment groups with supernatant and Nc presented the control groups without adding supernatant or bacterial media.

2.2 Oxidative stress analysis

Lipid peroxidation was measured by MDA and antioxidant ability was assayed by enzyme (SOD, CAT, and POD) activities. When the algae grew to the logarithmic growth phase, strain B1 supernatant was added to the treatment groups at a proportion of 1.0% (v/v) but not the control groups, and the culture conditions were the same as algal culture (2.1.1). Sample treatment method of algae for analysis was as follows: algal cells were collected by centrifuged at 6000 rpm for 15 min in the condition of 4 °C, PBS (0.05 mol L$^{-1}$, pH 7.8) was added to the cells and ground these into homogenate in ice bath, then collected supernatant of homogenate in the condition of 4 °C and stored in the -70 °C until use.

MDA content was determined following the thiobarbituric acid method by its extinction coefficient (155 mM$^{-1}$ cm$^{-1}$) at a wavelength of 532 nm in addition to the nonspecific absorption at 600 nm and expressed as nmol mg$^{-1}$ protein (Zhang et al. 2014). The lipid peroxidation levels were determined from the thiobarbituric acid (TBA) reacting to the substance contents through the determination of MDA (Shiu and Lee 2005). The total volume of the reaction mixture for the MDA assay was 4 mL, including 2.0 mL TBA (0.6%) and 2.0 mL supernatant of homogenate.

SOD activity was measured by using nitro blue tetrazolium photoreduction method (Beauchamp and Fridovich 1971; Cakmak and Marschner 1992). The reaction mixture (6 mL) contained PBS (3 mL,
0.05 mol L\(^{-1}\), pH 7.8), methionine (0.6 mL, 130 mmol L\(^{-1}\)), EDTA-Na\(_2\) solution (0.6 mL, 100 \(\mu\)mol L\(^{-1}\)), riboflavin (0.6 mL, 20 \(\mu\)mol L\(^{-1}\)), nitro-blue tetrazolium (NBT, 0.6 mL, 750 \(\mu\)mol L\(^{-1}\)), \(\text{H}_2\text{O}_2\) (0.5 mL, 30\%), enzyme extract (0.1 mL), and the absorbance was measured at wavelengths of 560 nm. CAT was assayed by ultraviolet spectrophotometry with mixed system (3 mL), including \(\text{H}_2\text{O}_2\) solution (0.3 mL, 0.1 mol L\(^{-1}\)), PBS (1.5 mL, 0.05 mol L\(^{-1}\), pH 7.8), distilled water (1 mL), an enzyme extract (0.2 mL), and the absorbance was measured at wavelengths of 240 nm (Zhao et al. 2012; Kumar et al. 2008). POD was assayed according to the Guaiacol method with the reaction mixture (4 mL) of \(\text{H}_2\text{O}_2\) solution (1.5 mL, 0.2\%), guaiacol solution (1.0 mL, 0.2\%), PBS (1.0 mL, 0.05 mol L\(^{-1}\), pH 7.0), enzyme extract (0.5 mL), and the decrease in absorbance was measured at wavelengths of 470 nm (Civello et al. 1995).

2.3 Electron microscopy analysis

Field emission scanning electron microscopy (FESEM) was used to observe the cellular morphology and compared for the differences among the four kinds of algae after adding strain B1 supernatant (1.0\%, v/v). Algal cells collected from algal cultures were first fixed with 2.5\% of glutaraldehyde overnight and then washed with PBS (0.1 mol L\(^{-1}\), pH 7.2), dehydrated with gradient ethanol, passed through 100\% acetone and replaced with isoamyl acetate. After fully dried, the samples were mounted on copper stubs and sputter-coated with gold palladium. The observed results and photographs were used a FESEM (ZEISS ULTRA\(^{TM}\) 55, Germany).

2.4 RNA Extraction and Quantitative Real-time PCR Analysis

In order to find whether the strain B1 supernatant influence the thylakoid membrane electron transfer and the photosynthetic system, the algal photosynthetic gene expressions were determined by the quantitative real-time PCR (qRT-PCR). The algal culture conditions were the same as algal culture (2.1.1). Algal cultures were centrifuged at 2500 rpm for 5 min to collect precipitate, and the total RNA
was extracted following the Trizol Reagent (Life Technologies, 15596-018). Using electrophoresis to check RNA integrity and measuring the absorbance at 260 nm and 280 nm to determine RNA concentration and purity. We designed the negative controls during the experiment viz. samples without reverse transcription to check a possibility of amplification from contaminated genomic DNA. Reverse transcription step was taken in strict accordance with the TaKaRa PrimeScript™ RT reagent Kit (Perfect Real Time) instructions. The reaction conditions were run 15 min at 37 °C and 5 s at 85 °C. Primer pairs were listed in Table 1. The qRT-PCR using a Bio-Rad CFX96 Real-Time PCR System and SYBR premix EX Taq™ (2x) were run at the following conditions: 95 °C for 30 s, 45 cycles with 15 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C. Final concentrations were in a total volume of 10 µL, including 5 µL of SYBR premix EX Taq™ (2x), 0.5 µL of each primer (10 µmol L⁻¹), 0.5 µL of cDNA template and 3.5 µL dH₂O. Analysis of the melting curves verified the accuracy of each amplicon, and ABI prism SDS 7300 software was used to analyze the density of SYBR green I and determine the threshold cycle (Ct). The mRNA levels of the target genes in the microalgae incubated without B1 supernatant addition were used as calibrators. The reference gene of 18S rRNA was used to standardize the results, and the relative gene expression was quantified using the 2⁻ΔΔCt.

2.5 Statistical analysis

Data were presented as means ± standard error and evaluated by the repeated measures ANOVA, with p<0.01 and p<0.05. All statistics was performed with Origin 8.0 for windows. All treatment groups were in triplicate.

3 Results

3.1 Algicidal effect
The algicidal activities of strain B1 supernatant inoculated into four algal species (*S. costatum*, *H. akashiwo*, *P. globosa* and *P. donghaiense*) were illustrated in Fig. 2. Strain B1 exhibited inhibitory effect to these four algal species and the maximum algal-lytic activity could be summarized in the following order of sensitivity: *P. globosa* (96.4%) > *S. costatum* (91.4%) > *H. akashiwo* (90.7%) > *P. donghaiense* (90.6%). Whereas the addition of 2216E medium showed no effect on the growth of four algal species compared to the controls. *S. costatum* (Fig. 2b) possessed higher algicidal levels of 34.2% after adding strain B1 supernatant for one day. After 3 and 4 days of the experiment, the algicidal activity was significantly increased in *P. globosa* cultures (Fig. 2a) in comparison to others (Fig. 2c and d), with the values raised from 40.4% to 87.7%. Moreover, high algicidal activity (>90%) could be seen in Fig. 2 when the treatment time was more than 5 days.

3.2 Effect of cellular MDA contents and antioxidative enzyme activity

MDA can reflect the degree of lipid peroxidation, and it is used for evaluating the degree of cell damage. Fig. 3 showed the MDA contents of strain B1 supernatant against the four algal species. During the first 3 days, the MDA contents increased significantly in all treatments. After 4 days, the MDA contents of *P. globosa* and *H. akashiwo* species have reached their highest level, which were 3.61 and 3.37 times (p<0.01) compared to the controls and then decreased, while that of *S. costatum* (p<0.05) and *P. donghaiense* treatments for 5 days were the highest, with 3.62 (p<0.05) and 2.92 times of the control values.

Cellular enzymatic activities including SOD, CAT and POD were determined to explore the cellular defense response of the four algal species induced by strain B1 supernatant (Fig. 4). Fig. 4a showed that SOD activity increased to maximum levels after 2 days of treatment to values of 1.46, 1.27 (p<0.05), 1.17 and 1.18 times compared to the controls in these four algal species, respectively. But it
significantly declined to lower level at all treatments as the treatment time was prolonged. It is worth noting that, *P. donghaiense* and *P. globosa* species responded more severely under the supernatant, they decreased significantly and the lowest levels were 27.6 and 24.8% of the controls after algal cells were treated for 5 days, while that of the *H. akashiwo* and *S. costatum* were 67.8 and 70.1% of the controls, respectively.

Fig. 4b illustrated that CAT activities showed a similar pattern between the four kinds of algae, where the activities were significantly increased within 3 days. The activity values after treatment for 3 days have reached its highest level, being 14.6, 9.55, 12.4 and 7.81 times (p<0.01) in algal cells of *P. donghaiense*, *S. costatum*, *P. globosa* and *H. akashiwo* compared with the controls. Subsequently, CAT activity decreased between 4 and 5 days exposure and eventually recovered to the initial level.

At the treatment for 1 d, POD activities rose to 1.54, 2.53, 1.54 and 1.77 times in algal cells of *P. donghaiense*, *S. costatum*, *P. globosa* and *H. akashiwo* compared to those of the controls (Fig. 4c). Then the POD activities at all treatments declined and were lower than that of the algal cells in controls. Remarkably, the activities of *S. costatum* and *P. globosa* species obtained the lowest values in 3 days and exhibited approximately 3.79% (p<0.01) and 65.1% decrease compared with the controls, while that of the lowest contents treatment of *P. donghaiense* and *H. akashiwo* were acquired in 4 days of incubation and manifested a 7.47% and 8.23% (p<0.01) decrease. After more than 4 days, POD showed that longer exposure times did not cause a significant increase in the activity of this enzyme between the four kinds of algae.

### 3.3 Effects of cellular microstructure

FESEM analysis revealed morphological alterations of the cell surface in four kinds of algae treated with 1.0% (v/v) strain B1 supernatant for 0, 3, 5 days (Fig. 5). Those treated cells based on the FESEM
observations showed different degrees of damage in morphological characteristics compared with the normal and plump cells in control groups (A, D, G, and J). After 3 days, different degrees of deformation appeared, with the cells of *S. costatum* taking on several broken junctions between cells and the chains of cells while the cell surface of *H. akashiwo*, *P. globose* and *P. donghaiense* appeared as a melting and tortile phenomenon (B, E, H, and K). Cell membrane was severely deformed and split, and the cells were even broken with their contents leaked out after 5 days exposure. Ultimately, majority of the cells was completely cracking (C, F, I, and L), especially the cells of *P. globosa* were completely broken into pieces under visual fields of microscope.

3.4 Effects of photosynthesis-related genes

Fig. 6 showed the effects of the different supernatant concentrations of strain B1 on the three photosynthesis-related gene expressions in *H. akashiwo*, *S. costatum* and *P. globosa*. The abundances of all genes have been significantly inhibited by the B1 supernatant. After 96 h exposure of the 1.0% (v/v) supernatant, *psbA*, *psbD*, *rbcL* genes expression decreased to 43.0% (p<0.05), 31.7% (p<0.01), 44.1% (p<0.05) of *H. akashiwo*, 2.02% (p<0.01), 4.10% (p<0.05) and 12.6% (p<0.01) of *S. costatum* and 28.5% (p<0.01), 74.2% (p<0.05), 32.0% (p<0.05) of *P. globosa* compared with the controls. As to the 2.0% (v/v) treatment groups, gene expression of three algal species have significantly decreased compared to the controls. Obviously, *psbA*, *psbD*, *rbcL* genes expression of *H. akashiwo* decreased to 14.3%, 3.08% (p<0.01), 11.6% of the controls, *S. costatum* were 1.04% (p<0.01), 2.21% (p<0.01) and 10.7% (p<0.01) and *P. globosa* were 8.39% (p<0.01), 24.6% (p<0.01), 30.2% (p<0.05) of the controls. Three kinds of photosynthetic genes expression of *S. costatum* have fallen to very low levels regardless of the treatment concentration and all treatment groups genes expression decreased significantly compared with control groups at different concentration.
4 Discussion

Many marine algicidal bacteria have been obtained, most of which only lysed one kind of algae (Kim et al. 2008). Furthermore, studies seldom reported the algicidal mechanism of a *Bacillus* on different kinds of harmful algae (Tilney et al. 2014). Strain B1 showed the algicidal activity on *P. globosa, S. costatum, H. akashiwo* and *P. donghaiense* (Fig. 2), which belongs to *Pelagophyta, Bacillariophyta* *Raphidophyta, Dinophyta* respectively, suggesting that strain B1 had a relatively wide host range. It is reported that algicidal bacteria and active metabolites are specific to algal species (Zhao et al. 2014), but strain B1 has algicidal effect on the four kinds of algae is that the strain excrete specific and complex effective metabolites, such as L-Histidine, o-tyrosine, N-acetylhistamine and urocanic acid. L-Histidine was only toxic to *P. globosa*, whereas o-tyrosine, N-acetylhistamine and urocanic acid could also inhibit the growth of *P. donghaiense, H. akashiwo* and *S. costatum* (Zhao et al. 2014).

The physiological response, including MDA, SOD, CAT and POD, was implemented to reveal the oxidative damage. Especially, the MDA is an immediate index of the lipid peroxidation inside the cells. The significant elevation in MDA contents (Fig. 3) manifested the existence of serious oxidation on the four algal species. Zhang H. J also found that *Brevibacterium* sp. BS01 culture supernatant could make a significant increase in MDA contents when treated on *A.tamarense* (Zhang et al. 2013b). When the MDA increased on the first four days, the MDA could stimulate the algal cell proliferation (Zhao et al. 2012), and the increasing number of algal cells could cause more MDA than before. However, the MDA level of *H. akashiwo* and *P. globosa* treatment groups decreased after reaching the maximum might be their irreversible oxidative damage when treated with supernatant. Conclusions also have
been reported that the MDA contents increased acutely to the toxic level when exposed to L7-LPEALP, in which algal growth was inhibited and the cellular membrane system was damaged (Thomas and Wofford 1993; Zhao et al. 2012).

SOD, CAT and POD are important antioxidases in cells, which protect organisms against damages caused by oxygen-free radicals and harmful substances (Luo et al. 2013). The mechanism on how algal cells could be killed was indirectly reflected through the variation of these biochemical indexes under strain B1 supernatant stress. Elevation of SOD and CAT activities meant that the algal cells started their antioxidant activity system to dispose the oxidation of external stresses (Fig. 4), and analogous result had been confirmed (Wang et al. 2012). The increased SOD activity in the first three days and decreased afterwards suggested that algae was controlled effectively, and even killed through the long-term exposure to supernatant. The SOD activities of *P. donghaiense* and *P. globosa* responded more severely than *H. akashiwo* and *S. costatum* by indicating the differences in sensitivity to supernatant. However, the enzyme activities of the four species had a similar trend. CAT, one of the key enzymes of the defense system of organism, can make hydrogen peroxide into oxygen and water, thereby preventing cell damaging by hydrogen peroxide. The fiercest defense reaction of CAT appeared at the very start and subsequently collapsed when exposure to the supernatant of strain B1 (Fig. 4b), which was consistent with previous studies (Sun et al. 2004). It could be concluded from the almost synchronous CAT activities of the four algae species that the activity of CAT was mainly affected by active substances, however, there were not obvious differences among these algae species. The decline in SOD and CAT activities both contributed to accumulated intracellular ROS, and high level of ROS increased the MDA content and the lipid peroxidation of algal cells (Zhao et al. 2014). The increase in POD activities of treatment groups suggested that the potential of resisting oxidative damage against
strain B1 supernatant has already begun (Fig. 4c), because normal algal cells will not accumulate the POD. Contrary to the SOD and CAT activities, the POD activities decreased down to the lowest and then increased (Fig. 4c). The POD activity of \textit{S. costatum} firstly showed a maximum ratio of the treatment group to the control group implying that \textit{S. costatum} had timely induction ability, and this was also reflected by the algicidal activity (Fig. 2) and electron microscopy images (Fig. 5-E). What is noticed here in the present research was that the effects of oxidation stress induced by strain B1 supernatant on \textit{P. globosa} were always obvious. It indicated that \textit{P. globosa} has a more sensitive response to the external stresses compared with the others. According to the results of MDA, SOD, CAT and POD activities, enzymes were seen to be directly involved in resisting supernatant stress in algal cells, and this conclusion was in agreement with previous studies (Zhang et al. 2014). By comparison, though there were significant differences on enzyme activities between the four kinds of algae, the change mechanism of the cell antioxidant system was similar under the oxidative stress.

In order to explore the effect of strain B1 supernatant on the photosynthetic system, causing the oxidative damage, the photosynthesis-related genes (\textit{psbA}, \textit{psbD} and \textit{rbcL}) expression were measured (Asada et al. 1998). Active substances might affect normal electron transport to produce surplus electrons in the photosynthetic process, and electronic transmission chain related genes (\textit{psbA}, \textit{psbD}) transcription could indicate the plant photosynthetic oxidation damage through the consumption of oxygen and formation of ROS (Zhang et al. 2013b). The effects of strain B1 supernatant on photosynthetic gene expression were strong and greatly influence the electronic transmission chain to cause oxidative injury (Fig. 6). No matter algal cells exposed to 1.0\% (v/v) or 2.0\% (v/v) strain B1 supernatant, all genes expressions were significantly decreased, especially in the \textit{psbA} gene expression of \textit{S. costatum}. Results had been reported that the \textit{psbA} and \textit{psbD} genes had different responses to
BS01 supernatant, but the mechanism is still not clear (Zhang et al. 2013b). *P. globosa* responded slowly to B1 supernatant, such as the not obvious algicidal activity on the first day (Fig. 2a) and not significant genes expression neither in the 1% (v/v) nor 2% (v/v) treatment groups (Fig. 6), which can be interpreted as its higher tolerance and antioxidant system’s ability. The decrease in photosynthesis related gene revealed the affected electron transport and generation of ROS (Zhang et al. 2013b).

Electron transfer started from the photosystem II and then O$_2$ accepts electrons to become ·O$_2$ (superoxide anion). Only the presence of SOD in the chloroplast can eliminate the ·O$_2$ to generate H$_2$O$_2$, far more important is both the ·O$_2$ and H$_2$O$_2$ in the process belonging to reactive oxygen molecules (ROS, Mehler’s reaction) (Jia et al. 2000), however, the activity of SOD was gradually decreased during the experiment. Gene expression levels of different algal species have different responses, which help to further understand the mechanism of active substances and promote the realistic feasibility.

The cell membrane acts as a barrier to prevent extracellular active substances of strain B1 passing freely through algal cells, and thus ensure a relatively stable environment within the cells, so that the orderly operation of the various biochemical reactions can be implemented (Veldhuis et al. 2001; Li et al. 2014). From the damage degree of the cells (Fig. 5), different sensitivity of the algal species to strain B1 supernatant can be seen. At the initial stage of the reaction, cell morphological damage of *S. costatum* was obvious, and the significant change also showed in the algicidal activity, CAT, POD and genes expression. It may be because the *S. costatum* filtrates had an autoinhibitory allelopathic effect on the growth phase of the species (Wang et al. 2013), and the effects of B1 supernatant and autoinhibitory allelopathic could combine to strengthen the algicidal effect. The morphological changes of *P. globosa* cell reacted slowly at beginning probably because its existing forms of capsule body that prevent the cells from damage (Huang et al. 2007). When the cells began to have morphological
damage, the intracellular MDA content, enzyme activity and gene expression almost synchronously changed. The change was also in accordance with the decrease in genes expression. Similar results were previously reported with *Microcystis aeruginosa* exposure to neo-przewaquinone A (Zhang et al. 2013a). Therefore, it can be inferred that various changes in algal cells influenced each other after adding strain B1 supernatant since enzymes changed significantly with the increase of MDA content, leading to the suppression of genes expression.

In summary, strain B1 supernatant could inhibit the growth of the four kinds of algae. Different algae have different sensitivity, including the change of algicidal activity and MDA contents, decrease in enzymes activities, damage of cell morphology and expression of genes. *P. globosa* algae had the highest tolerance to the algicidal substances, while *S. costatum* algae were more sensitive to strain B1 supernatant in the first day. Comparing the differences among the four kinds of algae may provide the potential inhibition mechanism. After understanding the algicidal procedure, it will help to control HABs with marine algicidal bacteria. However, further investigations are needed before practical application, such as tracking the active metabolites in the algicidal process.

Acknowledgement:

This work was supported by the Joint Fund of National Natural Science Foundation of China-Guangdong (Project No.U11330003) and t National Natural Science Foundation of China (Project No. 41076068).

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Table 1 Sequences of primer pairs used in real-time PCR

<table>
<thead>
<tr>
<th>Algae</th>
<th>Primer name</th>
<th>Forward Sequence (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phaeocystis</em></td>
<td><em>rbc</em>L</td>
<td>5′-GCTTGCTAACCTAAGTCCGA-3′</td>
<td>5′-GGACGACAAAGCTTACATCC-3′</td>
<td>NC021637</td>
</tr>
<tr>
<td><em>globosa</em></td>
<td><em>psb</em>A</td>
<td>5′-GCTTTTATCGCTGACCTCC-3′</td>
<td>5′-CACCATTATACACCCTATCC-3′</td>
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</table>
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Fig. 5 FESEM micrographs of algal cells after strain B1 supernatant treatment. Phaeocystis globosa (A-C), Skeletonema costatum (D-F), Heterosigma akashiwo (G-I), and Prorocentrum donghaiense (J-L) at 0, 3, 5 days, respectively.

Fig. 6 Relative transcriptional levels of strain B1 supernatant on Heterosigma akashiwo (1-3), Skeletonema costatum (4-6) and Phaeocystis globosa (7-9) that was exposed to different concentrations (0.0 %, 1.0 % and 2.0 %, orderly and respectively) for 96 h. All error bars correspond to the standard deviation. *p<0.05 and **p<0.01 indicate a significant level.
Table 1 Sequences of primer pairs used in real-time PCR

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<tr>
<th>Algae</th>
<th>Primer name</th>
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<th>Accession numbers</th>
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136x126mm (300 x 300 DPI)
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