J Ind Microbiol Biotechnol (2014) 41:593–599
DOI 10.1007/s10295-013-1393-0

SHORT COMMUNICATION

SIMB
Society for Industrial Microbiology and Biotechnology

Algicidal metabolites produced by *Bacillus* sp. strain B1 against *Phaeocystis globosa*

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Received: 26 August 2013 / Accepted: 10 December 2013 / Published online: 27 December 2013

Abstract The bloom of *Phaeocystis globosa* has broken out frequently in the coastal areas of China in recent years, which has led to substantial economic losses. This study shows that *Bacillus* sp. strain B1, which was previously identified by our group, is effective in regulating *P. globosa* by excreting active metabolites. Heat stability, pH stability and molecular weight range of the algicidal compounds from strain B1 were measured and the results demonstrated that the algicidal activities of these compounds were not affected by pH or temperature variation. The algicidal compounds extracted with methanol were isolated and purified by ODS-A column chromatography and HPLC. The algicidal compounds corresponding to peaks 2–5 eluted from HPLC were further analysed by quadrupole time-of-flight mass spectrometry (Q-TOF–MS). PeakView™ Software determined the compounds corresponding to peaks 2–5 to be L-histidine, *o*-tyrosine, *N*-acetylhistamine and urocanic acid on the basis of the accurate mass information, the isotopic pattern and MS–MS spectra. Furthermore, these compounds were also able to eliminate *Skeletonema costatum*, *Prorocentrum donghaiense* and *Heterosigma akashiwo*. This is the first report of bacteria-derived algicidal compounds being identified only by Q-TOF–MS and PeakView™ Software, and these compounds may be used as the constituents of algicides in the future.

Keywords Algicidal compounds · Harmful algal blooms · *Phaeocystis globosa* · *Bacillus* sp. · Strain B1 · Q-TOF–MS

Introduction

Harmful algal blooms (HABs) have become global marine disasters owing to their tremendous damage to marine ecosystems, fisheries, tourism and human health [2, 18]. At present, physical, chemical and biological methods are the three major methods in controlling HABs. In particular, biological methods have drawn more attention on account of their lower cost and less secondary pollution than physical and chemical methods [3, 31]. Various strains of algicidal bacteria have been identified by the research of algal-bacterial interactions, such as *Pseudomonas* sp. [9], *Pseudoalteromonas* sp. [23], *Flavobacterium* sp. [19], *Bacillus* sp. [16], *Rhodococcus* sp. [12] and *Alteromonas* sp. [10]. In general, algicidal bacteria can inhibit or kill algal cells by the direct or indirect mode. The direct mode means that algicidal bacteria attack and dissolve algal cells via cell-to-cell contact, while the indirectly mode refers to algicidal bacteria eliminating algal cells through secreting active metabolites [8, 24]. Most identified algicidal bacteria are responsible for the algicidal effects by the indirect mode, suggesting that it is necessary to isolate and identify active metabolites.

Although a large number of algicidal bacteria have been determined, only a few algicidal compounds have been identified owing to the difficulty of their isolation, purification and enrichment. Several identified algicidal

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compounds include proteins [1, 11], polypeptides [4, 7], amino acids [25, 28] and antibiotics [17]. Moreover, most algicidal bacteria and active metabolites are specific to algal species and most attention has focused on *Alexandrium tamarense* [24, 31], *Heterosigma akashiwo* [26], *Microcystis aeruginosa* [25, 27, 28], etc. However, few published studies concern the isolation and identification of algicidal compounds from bacteria towards *Phaeocystis globosa* (*P. globosa*).

In this study, the algicidal compounds produced by *Bacillus* sp. strain B1 against *P. globosa* were isolated by ODS-A column chromatography and HPLC on the basis of the polarity of these compounds. ODS-A column chromatography, a kind of C_{18} reversed-phase silica gel column chromatography, possesses high hydrophobicity and is applied to isolate polar to medium non-polar drugs, organic compounds, biological molecules and natural products. Subsequently, the algicidal compounds purified by HPLC were analysed by Q-TOF–MS and PeakView™ Software to determine their chemical structures on the basis of their MS spectra, isotope signals and MS–MS spectra. To our knowledge, this is the first article to identify algicidal compounds from bacteria by using Q-TOF–MS and PeakView™ Software. Most algicidal compounds from bacteria can not be determined by IR or NMR owing to their low content and the difficulty of enrichment. However, trace compounds can be also identified by highly sensitive Q-TOF–MS and PeakView™ Software, which provides an effective approach for identifying trace amounts of algicidal compounds.

Materials and methods

Bacterial strain and growth conditions

Bacillus sp. strain B1 (GenBank accession number JN 228893) was previously isolated from the bloom of *P. globosa* in Zhuhai, China by our group and its phylogenetic tree is shown in Fig. 1 [15]. The strain B1 was cultured in 2216E medium (peptone 5 g, yeast extraction 1 g, ferric phosphorous acid 0.01 g, agar 15 g, pH 7.6–7.8, mixed capacity to 1 l water) at 30 °C with shaking at 160 rpm.

Algal strains and growth conditions

The species of harmful algae, *P. globosa*, *Heterosigma akashiwo* (*H. akashiwo*), *Prorocentrum donghaiense* (*P. donghaiense*) and *Skeletonema costatum* (*S. costatum*), were kindly supplied by Professor Songhui Lv, Jinan University, China and cultivated in modified f/2 medium [6] at 20 ± 1 °C under an illumination of 4,000 lx in 12:12 (light/dark). Modified f/2 medium used manmade seawater with a salinity of 25 ‰ instead of natural seawater.

Algicidal activity assays

Algicidal activities of metabolites from strain B1 were determined by chlorophyll-a content variation of *P. globosa*. A 20-ml algal culture solution was centrifuged at 2,784×*g* for 10 min and the precipitate was collected. Subsequently, the precipitate was extracted with 5 ml 90 %

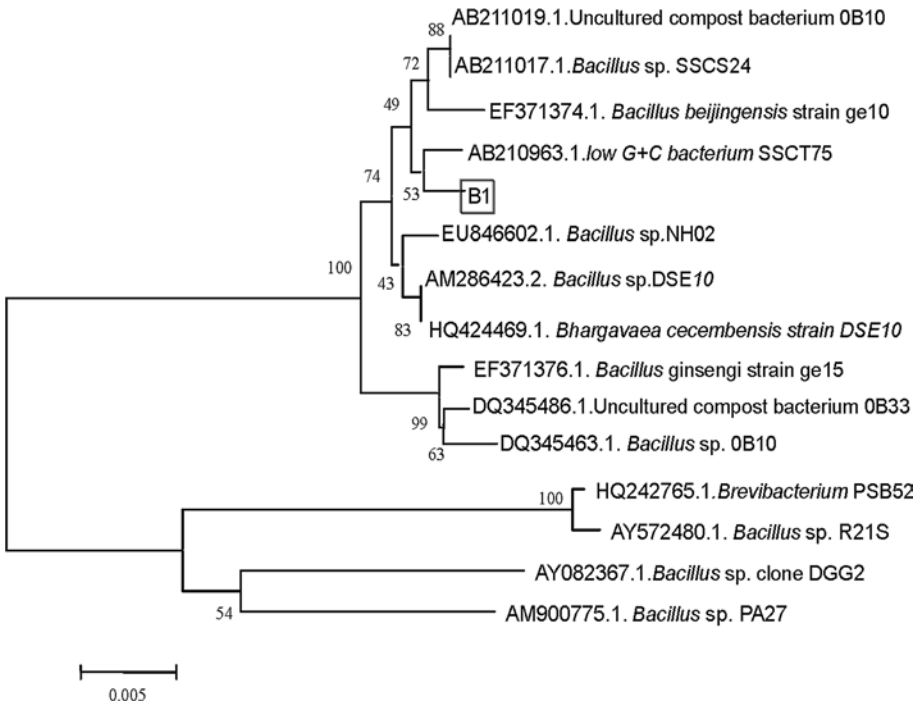

Fig. 1 Phylogenetic tree of strain B1 based on 16S rDNA [15]. It indicates that strain B1 is most related to *Bacillus* sp. SSCT75

acetone in the fridge for 24 h. The extracted solution was centrifuged at 2,784×*g* for 10 min and the absorbance of the extracted supernatant was measured at the wavelengths of 630, 645 and 665 nm. The chlorophyll-a content was calculated by the following formula [22]:

$$C = 11.6 \times A_{665} - 1.31 \times A_{645} - 0.14 \times A_{630}\ (\mathrm{mg/L})$$

The chlorophyll-a content of the control group without algicidal compounds and the experimental group with algicidal compounds was measured after 5 days of cultivation. The algicidal rate was calculated according to the following formula:

$$\text{Algicidal rate}\ (\%) = \frac{C_c - C_E}{C_c} \times 100$$

C_C represents the chlorophyll-a content of the control group and C_E represents the chlorophyll-a content of the experimental group.

Preparation of algicidal compounds

Strain B1 was cultured in 4 l of 2216 medium at 30 °C with shaking at 160 rpm for 7 days. The bacterial cultures were centrifuged at 3,456×*g* for 10 min to collect the supernatant and the supernatant was filtered through 0.22-μm filterable membranes to completely remove bacteria. The bacteria-free supernatant was evaporated to dryness by using a rotary evaporator. The dehydrated compounds were dissolved in 200 ml ethyl acetate and then the supernatant of ethyl acetate (ingredient E) was collected. Similarly, the residual precipitate was soluble in 200 ml methanol and the supernatant of methanol (ingredient M) was collected. The insoluble matters were dissolved in water (ingredient W). Thus, the metabolites from strain B1 cultures were divided into three ingredients E, M and W on the basis of their polarity.

Purification of algicidal compounds

The algicidal ingredient (E, M or W) was applied to a 100-g ODS-A (GHODSB12S50, 50 μm, 120 Å) column and eluted with eight volume ratios of water/methanol (1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7) in sequence. Eight ingredients were obtained and the algicidal activity was measured. The ingredient that exhibited the best algicidal activity toward *P. globosa* was further isolated and purified by HPLC (Agilent 1100 series).

Identification of algicidal compounds

The algicidal compounds eluted from HPLC were analysed by Q-TOF–MS (AB SCIEX TripleTOF™ 5600+ System with Accelerator TOF™ Analyser and Electrospray Ionization source). Formula Finder of PeakView™ Software deduced the algicidal compounds according to the data of MS spectra, isotope signals and MS–MS spectra. The Fragments Pane of PeakView™ Software further verified the chemical structures of algicidal compounds via comparison of the experimental MS–MS fragments with the theoretical MS–MS fragments.

Results and discussion

Algicidal mechanisms and characteristics of algicidal compounds

Superoxide dismutase (SOD) and catalase (CAT) are effective in eliminating intracellular reactive oxygen species (ROS), thereby protecting cells from the potential damage of ROS [30]. Malondialdehyde (MDA) can indicate the extent of lipid peroxidation, which reflects the oxidative damage of the cellular membrane [21]. The tests demonstrated that the supernatant of strain B1 cultures could significantly inhibit SOD and CAT activities of *P. globosa* cells. The SOD and CAT activities separately declined by 73 and 48 % after treated with strain B1 supernatant for 4 days, which contributed to accumulated intracellular ROS (Fig. 2a, b). The high level of ROS increased the MDA content and the lipid peroxidation of *P. globosa* cells (Fig. 2c). Eventually, the cellular membrane of *P. globosa* was broken down and intracellular compounds were released.

In addition, our present studies showed that strain B1 supernatant retained its algicidal activity after being treated at different temperatures (20, 40, 60, 80, 100 and 120 °C), which illustrated that the supernatant has good heat stability (Table 1). The algicidal activity of the supernatant was also not affected by pH change (Table 1). The algicidal rate of the residue of strain B1 supernatant was less than 10 % after being dialysed in 500 Da interception dialysis bags, indicating that the molecular weights of the algicidal compounds were less than 500 Da.

Isolation and purification of algicidal compounds

The algicidal activity of different ingredients (E, M and W) extracted with different solvents from strain B1 cultures are summarized in Fig. 3. The result illustrated that ingredient M (compounds dissolved in methanol) was the most effective in killing *P. globosa* cells with an algicidal rate up to 92 %.

Considering the strong polarity of ingredient M, it was further isolated by ODS-A column chromatography and eight different ingredients were collected. The algicidal activity assays showed that ingredient 1, which was eluted with pure water, had the greatest algicidal effect on the bloom of *P. globosa* with an algicidal rate up to 88 %.

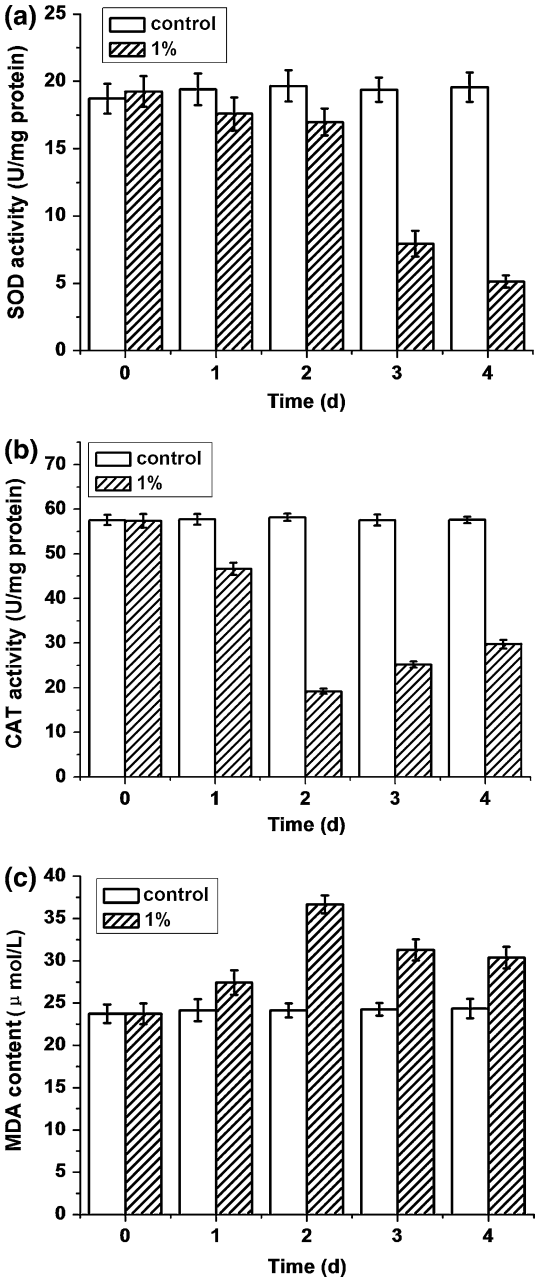

Fig. 2 Effects of strain B1 supernatant on **a** SOD activity, **b** CAT activity and **c** MDA content of *P. globosa*. 1 % means that 100 ml *P. globosa* culture was treated with 1 ml strain B1 supernatant. The assay methods of SOD activity, CAT activity and MDA content followed reported procedures [14, 30]

Ingredient 2 eluted with water/methanol (9:1, v/v) was able to eliminate *P. globosa* with an algicidal rate up to 63 %. In contrast, ingredients eluted with four volume ratios of water/methanol (6:4, 5:5, 4:6, 3:7) did not have obvious algicidal effects against *P. globosa* (Fig. 4).

Ingredient 1 eluted from the ODS-A column needed to be further purified by HPLC because that it still contained various compounds. There were six pure peaks 1, 2, 3, 4, 5 and 6 in the HPLC chromatogram (Fig. 5) and the corresponding compounds were all collected. The biological activity assays demonstrated that the algicidal rates of compounds corresponding to peaks 1, 2, 3, 4, 5 and 6 were 30, 47, 50, 55, 56 and 28 %, respectively (Fig. 6). It followed that the compounds corresponding to peaks 2, 3, 4 and 5 had greater algicidal effect on *P. globosa* than those corresponding to peaks 1 and 6.

Table 1 Effects of pH or temperature variations on the algicidal activity of strain B1 supernatant

pH	Algicidal rate[a]	Temperature (°C)	Algicidal rate[a]
1	+	20	++
3	+	40	++
5	+	60	++
7	++	80	++
9	++	100	++
11	+	120	++

pH treatment: Each 1 ml strain B1 supernatant was inoculated into 100 ml *P. globosa* culture, and then the pH of *P. globosa* culture was adjusted to 1, 3, 5, 7, 9 or 11. Temperature treatment: The strain B1 supernatants treated at different temperatures for 2 h were added into *P. globosa* cultures with a final concentration of 1 % (v/v). After 5 days, the algicidal activities were determined as described in "Materials and methods"

[a] +, algicidal rate over 65 %; ++, algicidal rate over 85 %

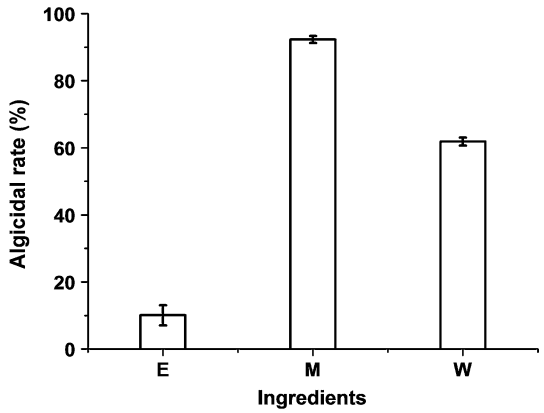

Fig. 3 Algicidal activities of the three ingredients from strain B1 against *P. globosa*. Ingredients E, M and W represented the compounds dissolved in ethyl acetate, methanol and water, respectively, and they were separately dissolved in 100 ml water. *P. globosa* was grown in 100 ml modified f/2 medium in the presence of 0.1 ml ingredient E solution, 0.1 ml ingredient M solution or 0.1 ml ingredient W solution for 5 days, and then the algicidal activity was determined as described in "Materials and methods"

Identification of algicidal compounds

In order to find out which kind of metabolites are essential in mitigating *P. globosa*, the algicidal compounds

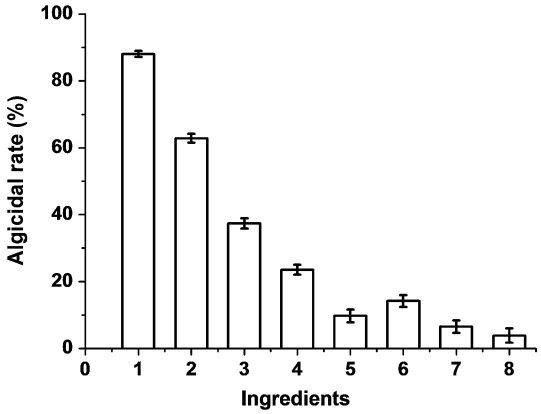

Fig. 4 Algicidal activities of the eight ingredients eluted from the ODS-A column towards *P. globosa*. Ingredients were separately dissolved in 20 ml water and 0.5 ml solution of each ingredient was separately added into 100 ml *P. globosa* culture for 5 days. The algicidal activity was determined as described in "Materials and methods"

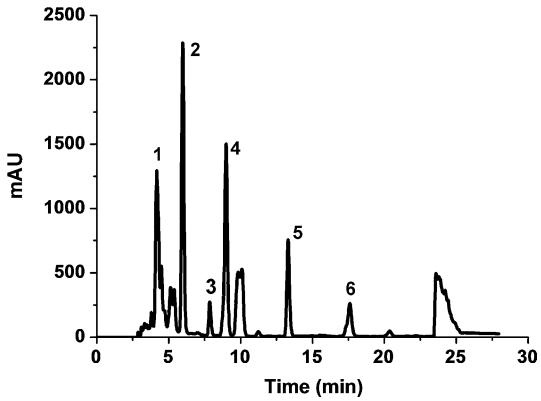

Fig. 5 HPLC chromatogram of ingredient 1. Chromatographic conditions: column type, $5C_{18}$-MS-II; column size, 10 mm ID × 250 mm; mobile phase, 0–20–21–28 min, water/methanol (99:1, v/v)–water/methanol (95:5, v/v)–water/methanol (0:1, v/v)–water/methanol (0:1, v/v); flow rate, 4 ml/min; detection wavelength, 254 nm; injection volume, 100 μl

corresponding to peaks 2–5 were analysed by Q-TOF–MS and PeakView™ Software, which are able to determine the trace compounds. Formula Finder of PeakView™ Software indicated that peak 2 was probably due to L-histidine on the basis of the accurate mass information, the isotopic pattern and MS–MS spectra (Tables 2, 3a). The Fragments Pane of PeakView™ Software further confirmed that peak 2 corresponded to L-histidine by matching the experimental MS–MS fragments with L-histidine's theoretical MS–MS fragments (Table 3a). Similarly, PeakView™ Software identified the compounds corresponding to peaks 3–5 as *o*-tyrosine, *N*-acetylhistamine and urocanic acid, respectively, by the same analytic methods (Tables 2, 3).

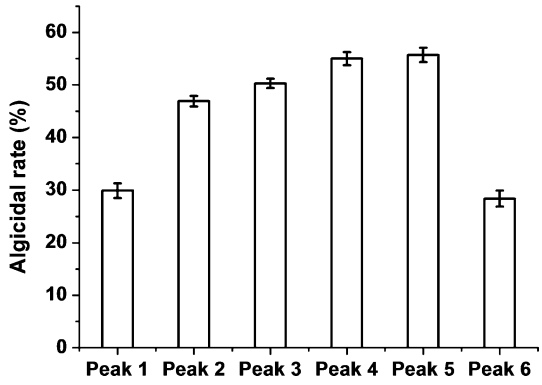

Fig. 6 Algicidal activities of the compounds corresponding to peaks 1–6 eluted from HPLC against *P. globosa*. The compounds were separately dissolved in 10 ml water and 1 ml solution of each ingredient was separately added into 100 ml *P. globosa* culture. After 5 days of cultivation, their algicidal activities were measured as described in "Materials and methods"

Table 2 Data of accurate mass spectra and isotope signals

Sample	Formula	Polarity type	Theoretical *m/z*	Experimental *m/z*	Error (ppm)
Peak 2	$C_6H_9N_3O_2$	+H	156.0768	156.0762	−3.6
Peak 3	$C_9H_{11}NO_3$	+H	182.0812	182.0811	−0.4
Peak 4	$C_7H_{11}N_3O$	+H	154.0975	154.0976	0.9
Peak 5	$C_6H_6N_2O_2$	+H	139.0502	139.0503	0.6

TOF–MS mode, *m/z* 50–1,000

Although several similar active metabolites from bacteria have been identified, these compounds are specific to other algal species rather than *P. globosa*, such as L-lysine and L-pyroglutamic acid to *Microcystis aeruginosa* [25, 27] and β-cyanoalanine to *Oscillatoria amphibia* [28]. Thus, this is the first article to report that several amino acids and their metabolites from strain B1 have pronounced effects on *P. globosa* and it provides a new systematic method for isolation and identification of algicidal compounds.

Algicidal activity of the pure algicidal compounds against selected algae

The algicidal activity of L-histidine, *o*-tyrosine, *N*-acetylhistamine and urocanic acid against four selected algal strains is shown in Table 4. 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*. *P. globosa* was very sensitive to L-histidine at a concentration of 20 μg/ml, *o*-tyrosine at a concentration of 6 μg/ml, *N*-acetylhistamine at a concentration of 4 μg/ml and urocanic acid at a concentration of

Table 3 Comparison of the experimental MS–MS fragments with the theoretical MS–MS fragments by the Fragments Pane of PeakView™ Software

Sample	Experimental mass/charge (Da)	Intensity (%)	Error (Da)
3-a, peak 2 (L-histidine)	68.0499	20.42	0.000
	81.0462	4.92	0.002
	110.0703	100.00	0.001
	156.0774	8.22	0.001
3-b, peak 3 (*o*-tyrosine)	65.0423	11.44	0.004
	77.0415	26.32	0.003
	91.0562	100.00	0.002
	95.0511	48.27	0.002
	107.0508	17.56	0.002
	118.0660	7.42	0.001
	119.0501	93.04	0.001
	123.0449	63.55	0.001
	136.0762	74.02	0.001
	147.0444	16.42	0.000
	165.0550	13.52	0.000
3-c, peak 4 (*N*-acetylhistamine)	68.0531	15.61	0.004
	83.0628	7.32	0.002
	95.0624	100.00	0.002
	112.0885	11.72	0.002
	154.0981	6.05	0.001
3-d, peak 5 (urocanic acid)	66.0340	24.80	0.000
	93.0443	74.36	0.000
	121.0398	100.00	0.000

TOF–MS mode, *m/z* 50–1,000

Table 4 Algicidal activities of L-histidine, *o*-tyrosine, *N*-acetylhistamine and urocanic acid against selected algae

Compound	Target species[a]	Concentration of algicidal compounds (μg/ml)[b]							
		0.5	2	4	6	10	15	20	30
L-Histidine	Pg	–	–	–	–	–	+s	+	+
	Pd	–	–	–	–	–	–	–	–
	Ha	–	–	–	–	–	–	–	–
	Sc	–	–	–	–	–	–	–	–
o-Tyrosine	Pg	–	–	+s	+	+	+	+	+
	Pd	–	–	–	+s	+	+	+	+
	Ha	–	–	–	+s	+	+	+	+
	Sc	–	–	+s	+	+	+	+	+
N-Acetylhistamine	Pg	–	–	+	+	+	+	+	+
	Pd	–	–	+	+	+	+	+	+
	Ha	–	–	+	+	+	+	+	+
	Sc	–	–	+	+	+	+	+	+
Urocanic acid	Pg	+s	+	+	+	+	+	+	+
	Pd	–	+	+	+	+	+	+	+
	Ha	–	+	+	+	+	+	+	+
	Sc	–	+	+	+	+	+	+	+

[a] Pg, *P. globosa*; Pd, *P. donghaiense*; Ha, *H. akashiwo*; Sc, *S. costatum*. These four algae have formed blooms frequently in coastal areas of China in recent years

[b] +, inhibited; +s, slightly inhibited; –, not inhibited

2 μg/ml. Hence, in appropriate concentration ranges, L-histidine, *o*-tyrosine, *N*-acetylhistamine and urocanic acid all have notable algicidal effects against *P. globosa.*

Although marine algicidal bacteria and their active metabolites are considered to be vital bioagents in controlling HABs, their biological safety should be fully considered before their practical application [5, 20]. It has been reported that high levels of histamine and urocanic acid can lead to pathological effects on fish and other organisms [13, 29]. Therefore, attention should be paid to the

dosages of *N*-acetylhistamine and urocanic acid if they are used as the components of bioalgicides. As for L-histidine and *o*-tyrosine, their safe dosages with respect to marine organisms remain in need of further study. According to the discussion above, L-histidine, *o*-tyrosine, *N*-acetylhistamine and urocanic acid produced by strain B1 have the potential to become the components of algicides in the future.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Project No. 41076068) and the Joint Fund of National Natural Science Foundation of China-Guangdong (Project No. U1133003). All authors of this study have agreed to submit this manuscript to the *Journal of Industrial Microbiology & Biotechnology*.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments comply with the current laws of China.

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