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Antibacterial activity of the lipopetides produced by *Bacillus amyloliquefaciens* M1 against multidrug-resistant *Vibrio* spp. isolated from diseased marine animals

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Abstract In this work, the antibacterial activity of the lipopeptides produced by *Bacillus amyloliquefaciens* M1 was examined against multidrug-resistant *Vibrio* spp. and *Shewanella aquimarina* isolated from diseased marine animals. A new and cheap medium which contained 1.0 % soybean powder, 1.5 % wheat flour, pH 7.0 was developed. A crude surfactant concentration of 0.28 mg/ml was obtained after 18 h of 10-l fermentation and diameter of the clear zone on the plate seeded with *Vibrio anguillarum* was 34 mm. A preliminary characterization suggested that the lipopeptide N3 produced by *B. amyloliquefaciens* M1 was the main product and contained the surfactin isoforms with amino acids (GLLVDLL) and hydroxy fatty acids (of 12–15 carbons in length). The evaluation of the antibacterial activity of the lipopeptide N3 was carried out against *S. aquimarina* and nine species of *Vibrio* spp.. It was found that all the *Vibrio* spp. and *S. aquimarina* showed resistance to several different antibiotics, suggesting that they were the multidrug resistance. It was also indicated that all the *Vibrio* spp. strains and *S. aquimarina* were sensitive to the surfactin N3, in particular *V. anguillarum*. The results demonstrated that the lipopeptides produced by *B. amyloliquefaciens* M1 had a broad spectrum of action, including antibacterial activity against the pathogenic *Vibrio* spp. with multidrug-resistant profiles. After the treatment with the lipopeptide N3, the cell membrane of *V. anguillarum* was damaged, and the whole cells of the bacterium were disrupted.

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Introduction

It is well documented that most bacterial infections in marine animals are caused by vibrios (Li and Woo 2003). However, use of antimicrobial substances has limited success in the prevention or curing of the bacterial diseases in marine animals (Zhu et al. 2006). The massive use of antimicrobial substances, especially different kinds of antibiotics, increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance and the horizontal transfer of resistance genes in different organisms so that multidrug resistance of many bacteria in maricultural areas is developed. The best alternative strategies to the use of antimicrobial substances are to use the natural products that have a broad spectrum of killing activity, including antibacterial activity against the pathogenic *Vibrio* spp. with multidrug-resistant profiles.

In recent years, it has been found that the cyclic lipopeptides produced by *Bacillus amyloliquefaciens* have potent antimicrobial activity and can be generally classified into three families or groups: surfactin, iturin, and fengycin (Wong et al. 2008). Although the cyclic lipopeptides have been found to have many potential commercial, therapeutic, and environmental applications because of their high yields, easy production, and stability, it is still unknown if they have killing activity against the pathogenic *Vibrio* spp. isolated from diseased marine animals. For example, a protein from *B. amyloliquefaciens* has antilisterial activity (Halimi et al. 2010), antibacterial, and antifungal activity (Hu et al. 2010). An extracellular metabolite (a biosurfactant) produced by *B. amyloliquefaciens* from mangrove forests has a

mosquitocidal activity (Geetha et al. 2011), and difficidin and bacilysin produced by *B. amyloliquefaciens* possess biocontrol activity against fire blight caused by *Erwinia amylovora* (Chen et al. 2009). However, it is still unknown if the lipopeptides produced by *B. amyloliquefaciens* can be used for control of the pathogenic *Vibrio* spp. with multidrug-resistant profiles. In this study, the lipopeptides produced by the bacterial strain M1 isolated from the mangrove systems were found to have potent activity against the pathogenic *Vibrio* spp. isolated from diseased marine animals, especially the pathogenic *Vibrio* spp. with multidrug-resistant profiles. Then, a new and cheap medium was developed for a large scale of production of the lipopeptides and purification of bioactive substances and preliminary mechanisms of killing activity against *Vibrio anguillarum* were examined.

Materials and methods

Bacterial strains and media

B. amyloliquefaciens M1 (collection number 2A00620 at the Marine Microorganisms Culture Collection of China) which could produce high level of lipopeptides was isolated from mangrove systems. *Vibrio harveyi* (collection number 2A00720 at MCCC) and *V. anguillarum* (collection number 2A00721 at MCCC) were isolated from diseased rainbow trout. *Vibrio parahaemolyticus* (collection number 2A00722 at MCCC) and *Vibrio vulnificus* (collection number 2A00723 at MCCC) were isolated from diseased silver sea bream. *Vibrio salmonicida* (collection number 2A00724 at MCCC) was isolated from Atlantic salmon. *Shewanella aquimarina* (collection number 2A00725 at MCCC), *Vibrio fischeri* (collection number 2A00726 at MCCC), *Vibrio splendidus* (collection number 2A00727 at MCCC), and *Vibrio septicus* (collection number 2A00728 at MCCC) were isolated from diseased sea cucumber. *Vibrio ichthyoenteri* (collection number 2A00729 at MCCC) was isolated from diseased Japanese Flounder. All the bacteria were grown in 2216E liquid medium (5.0 g of peptone, 1.0 g of yeast extract, and 0.1 g of Fe_3PO_4 per 1,000 ml seawater, pH 7.6). The medium for production of lipopeptides contained 1.0 % soybean cake powder, 1.5 % wheat flour, pH 7.0.

Measurement of drug resistance of the pathogenic *Vibrio* spp. and *S. aquimarina*

The nine strains of *Vibrio* spp. and *S. aquimarina* were aerobically cultivated in 2216E liquid medium at 28 °C for 24 h. The 6-mm diameter sterile Oxford cups (6×10 mm) were put on the 2216E plates seeded with the different bacterial cultures. Finally, 180.0 μl of the most commonly used antibiotics (100.0 μg/ml) was added to each cup, respectively and incubated at 28 °C for 48 h, and the clear zones around the Oxford cups were observed.

Molecular identification of the bacterial strain

The strain M1, one of the bacterial strains used in this study was identified using the molecular methods. The total genomic DNA of the bacterial strain was isolated and purified by using the methods as described by Sambrook et al (1989). Amplification and sequencing of 16S rDNA from the bacterial strain were performed according to the methods described by Chi et al. (2007). The common primers for amplification of 16S rDNA in the bacterial strain were used, the forward primer was M1-f (5′-CAAGTCGAGCGGACAGATG GGAGCT-3′), and the reverse primer was M1-r (5′-AGCT CCCATCTGTCCGCTC GACTTG-3′). The sequence (the accession number 16S rDNA was KF406338) obtained above was aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). The sequences which shared over 98 % similarity with currently available sequences were considered to be the same species, and multiple alignments were performed by using Clustal X1.83, and phylogenetic trees were constructed by MEGA 4.0 (Tamura et al. 2007).

Optimization of the medium and cultivation conditions for production of lipopeptides

Effects of different concentrations of soybean cake powder (from 0.5 to 4.0 %) and wheat flour (from 1.0 to 3.0 %), different pHs (from pH 5 to 9), and temperatures (24, 28, 32, and 37 °C) on production of lipopeptides by *B. amyloliquefaciens* M1 were optimized at flask level.

Preparation of the crude bioactive substance

B. amyloliquefaciens M1 was aerobically cultivated in a 250-ml flask containing 50 ml of the medium for production of lipopeptides by shaking at 180 rpm and 28 °C for 21 h. The culture was centrifuged at 4 °C and 12,000×*g* for 10 min, and the supernatant of the culture obtained was filtrated using the membrane with the pore of 0.22 μm, and the filtrate was kept at 4 °C for 2–4 h. The pH of the filtrate was adjusted to 2–3 using 6 M HCl solution, and the mixture was continuously kept at 4 °C for 8 h until the precipitation was completed. The precipitate was collected by centrifugation at 7,000×*g* and 4 °C for 5 min, and the pellets obtained were washed immediately with 0.1 M HCl solution two times and with distilled water one time. The treated pellets were dissolved in 100 ml of the distilled water, and the solution was adjusted to 7.0 using 1.0 N NaOH. The solution was vaccum frozen, and the solid was obtained. The bioactive substance in the solid was extracted with methanol three times. After methanol in the extract was evaporated by reduced pressure, the solid dissolved in methanol was used as the crude bioactive substance, and the solution was decolored using active carbon.

Antibacterial assay of the crude bioactive substance

V. anguillarum, *V. splendidus*, *V. harveyi*, *V. parahaemolyticus*, *V. septicus*, *S. aquimarina*, *V. fischeri*, *V. salmonicida*, *V. vulnificus*, and *V. ichthyoenteri* were cultivated in liquid 2216E marine medium at 28 °C for 24 h. The 6-mm diameter sterile Oxford cups (6×10 mm) were put on the 2216E marine medium seeded with the bacterial cultures. Finally, 200.0 μl of the crude and decolored bioactive substance obtained above was added to each cup, respectively, and incubated at 28 °C for 48 h, and the diameter of the clear zone was used as a measure of the antibacterial activity.

Fermentation

The seed cultures were prepared by inoculating the strain M1 grown on a LB agar slant into 250-ml Erlenmeyer flasks that contained 50 ml of LB liquid medium and cultivating it at 28 °C for 12 h with vigorous shaking. The fermentation was carried out in the 10-l fermentor [BIOQ-6005-6010B, Huihetang Bio-Engineering Equipment (Shanghai) CO-LTD] equipped with baffles, a stirrer, alkali pump, heating element, oxygen sensor, and temperature sensor. Seed cultures were prepared as described above. One hundred and forty milliliters of the seed culture (OD_{600nm}=3.0) was transferred into 7 l of the sterile medium for production of lipopeptides. The fermentation was performed under the conditions of the agitation speed of 200 rpm, aeration rate of 200 l/h, the temperature of 28 °C, and the fermentation period of 27 h. Only 10.0 ml of the culture was collected in the interval of 3 h and was centrifuged at 12,000×*g* and 4 °C for 5 min and antibacterial activity, amylase activity, and protease activity in the supernatant obtained were determined as described above and below. The cell number in the culture during the 10-l fermentation was also determined as described below.

Measurement of amylase activity

The mixture containing 200.0 μl of 1.0 % soluble starch dissolved in 40 mM citrate-Na_2HPO_4 buffer (pH 6.5) and 100.0 μl of the supernatant of the culture of the bacterial strain M1 was incubated at 40 °C for 30 min. After that, the mixture was heated at 100 °C for 10 min to stop the reaction immediately. The liberated reducing sugar was determined using Nelson-Somogyi method (Spiro 1966). The mixture containing 200.0 μl of 1.0 % soluble starch dissolved in 40 mM citrate-Na_2HPO_4 buffer (pH 6.5) and 100.0 μl of the inactivated supernatant at 100 °C for 10 min was used as the control. One unit of amylase activity was defined as the amount of enzyme causing release of reducing sugars equivalent to 1.0 μM of reducing sugar from soluble starch per minute under the assay conditions.

Assay of protease activity

The supernatant (0.2 ml) was mixed with 0.4 ml of 1.0 % casein solution in glycine–NaOH buffer (0.05 M, pH 7.0), pre-incubated at 45 °C for 30 min. The mixture was incubated at 45 °C for 30 min, and 0.7 ml of 10 % TCA (trichloroacetic acid) solution was added to the mixture immediately to stop the reaction. The reaction mixture was centrifuged at 10, 000×*g* and 4 °C for 10 min. Tyrosine content in the mixture was determined colorimetrically at 650 nm by using Folin–phenol reagent (Lowry et al. 1951). The mixture containing 0.2 ml of the inactivated supernatant at 100 °C for 5 min, and 0.4 ml of 1.0 % casein solution was used as the control. The protease activity was defined as the amount of the enzyme that liberated 1.0 μg of tyrosine per minute under the conditions used in this study.

Determination of the cell number in the culture

The cell number in the culture was determined using dilution plate method.

Isolation and purification of the bioactive substances

Two hundred microliters of the crude and decolored bioactive substance was applied to thin-layer chromatography plate precoated with silica gel GF254 (10–40 μm, Qingdao Marine Chemical Factory GF254 silica gel plate), and the plate was developed in the chamber containing the solvent system of chloroform–methanol–glacial acetic acid (10:1:0.055). After the TLC plate was taken out and dried, the separated substances were detected at 254 nm of UV light on a Beckman DU 640 spectrophotometer, and the bands with different Rf were marked. Each band was scraped off the plate, and antibacterial activity of each band was determined as described above. The antibacterial substance was separated on the semi-preparative HPLC using an ODS column [HPLC: YMC-Pack ODS-A (5.0 μm, 10×250 mm, flow rate of the mixture of methanol, water (60: 40) was 4.0 ml/min)]. HPLC-ECD spectra of the purified bioactive substance were run on a Chiralpak IC column (5 μm, 150×4.6 mm, hexane/isopropanol eluent, 1 ml/min of flow rate) and were recorded in stopped-flow mode on a JASCO J-810 electronic circular dichroism spectropolarimeter equipped with a 10-mm HPLC flow cell. The data of molecular mass of the separated substances were obtained using ESIMS on a Q-TOF Ultima Global GAA076 LC mass spectrometer.

Fluorescence microscopy

In order to check the lethal effect of the purified lipopeptide N3 on the plasma membrane, the sensitive bacterial *V. anguillarum* (10^7 cells/ml) were mixed with 1.0 ml of the purified lipopeptide

N3 (pH 6.5) and 1.0 M sorbitol, and the mixture was incubated under gentle shaking at 28 °C for 1 h. The cells were then harvested by centrifugation at 5,000×*g* and 4 °C for 5 min, washed three times with 1.0 M sorbitol, and resuspended again in 100 μl of 1.0 M sorbitol. After that, 30.0 μl PI (propidium iodide) solution (100.0 μg/ml) was added to the cell suspension, and the mixture was incubated for 30 min in the dark and at low temperature. Finally, the treated cells were observed under blue light with Olympus U-LH100HG fluorescent microscope with ×100 oil immersion objective. Images were recorded using the cellSens Standard software. The cells treated with the same amount of sterile water were used as the negative controls.

Scanning electron microscopy

The washed cells of the bacterial *V. anguillarum* were seeded on the assay medium. After incubation at room temperature for 20–30 min, the sterile Oxford cup was put on the medium, and 200.0 μl of the purified lipopeptide N3 was added to the cup and incubated at 28 °C for 2 days. The cells at the border of the clear zone were collected and used as the sample for the scanning electron microscopy. The untreated bacterial cells were used as the controls. The scanning electron microscope (JSM-840, JEOL, Japan) was used.

Preparation of bacterial protoplasts for use in assays of antibacterial activity of the lipopeptide N3

Bacterial protoplasts were prepared using the methods described by Da et al. (2005). *V. anguillarum* was grown in a 250-ml flask containing 50.0 ml of 2216E medium at 28 °C and 180 rpm for 24 h. At this time, 1.0 ml of the culture was transferred to a 250-ml flask containing 50.0 ml of fresh 2216E medium, and the culture was grown at 28 °C and 180 rpm for 24 h. The cells were harvested by centrifugation at 6,000×*g* and 4 °C for 10 min, washed three times with 0.01 M Tris–HCl (pH 7.0), and resuspended in 30.0 ml of 0.01 M Tris–HCl (pH 7.0) containing 0.5 M sucrose. Potassium ethylenediaminetetraacetate (EDTA, 0.1 M, pH 8.0) was added slowly over a period of 20 min to a final concentration of 0.01 M. The cells were shaken at 100 rpm and 37 °C for an additional 20 min to begin removal of the outer membrane. The cells were harvested by centrifugation at 3,000×*g* and 4 °C for 10 min and washed twice with SMM buffer (0.5 M sucrose, 20.0 mM sodium maleate, and 20.0 mM $MgCl_2$, pH 6.5). The cells were resuspended in 30.0 ml of the SMM buffer containing lysozyme (1.0 mg/ml of water). The cells were shaken at 37 °C and 100 rpm for 30 min to allow digestion of the peptidoglycan layer. EDTA solution (final concentration was 0.01 M) was added to the treated cells, and the mixture was continued to be incubated at 37 °C for 15 min. The complete formation of protoplasts was observed and confirmed under the microscope (Olympus CX21FS1, Olympus Corporation, Tokyo, Japan). The protoplasts were collected and washed with the SMM buffer by centrifugation at 3,000×*g* and 4 °C for 5 min. Then, the washed protoplasts were resuspended in the fresh SMM buffer, and the suspension was divided into three parts each of which contained 0.5 ml of the cell suspension. Finally, 100 μl of sterile water, 100 μl of the lipopeptide N3 solution (1.0 mg/ml), and 100 μl of SDS (1.0 mg/ml) suspension were added to each part, respectively, and the mixtures were incubated at 37 °C for 1 h, and results were recorded.

Results

Measurement of drug resistance of the pathogenic *Vibrio* spp. and *S. aquimarina*

In order to know if the pathogenic *Vibrio* spp. and *S. aquimarina* used in this study are resistant to the most commonly used antibacterial antibiotics, effects of the different antibacterial antibiotics on growth of *Vibrio* spp. and *S. aquimarina* were examined. The results in Table 1 indicated that all the pathogenic *Vibrio* spp. and *S. aquimarina* used in this study were resistant to several different antibiotics, suggesting that all of them were multidrug-resistant strains.

Screening of the bacterial strains against the pathogenic *Vibrio* spp.

Considering the problems that the commonly used antibiotics shown in Table 1 can cause to the environment and human health, alternative and more sustainable strategies are required. As the fast spread of multidrug-resistant pathogenic *Vibrio* spp. has increased the demand for new antibacterial drugs (Table 1), the bacterial strains that were isolated from the mangrove systems were screened in order to fight against the multidrug-resistant *Vibrio* spp. and *S. aquimarina* isolated from the diseased marine animals. It can be seen from the results in Table 2 that the bacterial strain M1 obtained in this study had high and wide spectrum of antibacterial activity against growth of all the pathogenic *Vibrio* spp. and *S. aquimarina* tested in this study. Therefore, the bacterial strain M1 was used in the subsequent investigations.

Identification of the bacterial strain M1

Figure 1a showed that the colonies of the bacterial strain M1 were white, and their morphology was flat and irregular. Our results also showed that the bacterial strain had high ability to hydrolyze starch and milk proteins (data not shown). In order to certify the taxonomic status of the isolate and the inter- and intra-specific relationships, 16S rDNA of the bacterial strain M1 was sequenced, and the sequence obtained in this study and those of 16S rDNAs of other bacterial species downloaded from NCBI was aligned, and the phylogenetic tree was constructed. The

Table 1 Drug resistance of different pathogenic *Vibrio* spp. and *S. aquimarina*

Bacterial strains	1	2	3	4	5	6	7	8	9	10	11	12	13
V. anguillarum	−	−	+	+	−	+	−	+	−	+	+	+	−
V. septicus	−	−	+	+	−	+	−	+	+	−	−	+	+
V. harveyi	−	−	+	+	+	+	−	−	+	+	+	−	−
V. parahaemolyticus	−	+	+	+	+	+	+	+	+	−	+	−	−
S. aquimarina	−	−	+	+	−	−	−	+	−	+	−	−	−
V. salmonicida	−	−	+	+	−	−	−	+	−	+	−	+	−
V. splendidus	−	−	+	+	−	+	−	+	−	+	+	+	−
V. ichthyoenteri	+	−	+	+	−	+	−	+	−	+	−	−	−
V. fischeri	−	−	+	+	−	+	−	−	−	+	+	+	−
V. vulnificus	−	−	+	+	−	+	+	−	−	−	−	+	−

The concentration of each antibiotic was 100 μg/ml

1 Chloroamphenicol, *2* ampicillin, *3* tetracycline, *4* colistin sulfate, *5* neomycin sulfate, *6* doxycycline hydrochloride, *7* levofloxacin hydrochloride, *8* kanamycin monosulfate, *9* amoxicillin, *10* fosfomvcin sodium, *11* lincomycin hydrochloride, *12* streptomycin sulfate, *13* oxytetracycline; + resistance to the antibiotic, − sensitive to the antibiotic

topology of the phylogram in Fig. 1b demonstrated that the bacterial strain M1 obtained in this study was closely related to the type strain *B. amyloliquefaciens* ATCC23350T, and the similarity between the 16S rDNAs from the bacterial strain M1 and the type strain *B. amyloliquefaciens* ATCC23350T was 98.0 %. Therefore, the bacterial strain M1 belonged to one member of *B. amyloliquefaciens*.

Optimization of medium for production of the bioactive substance against the multidrug-resistant *Vibrio* spp.

As mentioned above, the bacterial strain M1 could produce high level of amylase and proteinase. Therefore, various starchy materials and soybean mill were used as the components of the bioactive substance production medium. The results in Fig. 2a showed that 1.0 % of soybean mill was the most suitable for production of the bioactive substance by the bacterial strain M1. Among the starchy materials, we found that 1.5 % wheat flour in the medium was the most suitable for production of the bioactive substance by the bacterial strain M1 (Fig. 2b). We also found that the optimal pH in the medium was 7.0 and the optimal temperature was 28 °C (data not shown). After the fermentation was scaled up to 10 l using the medium (pH 7.0) containing 1.0 % soybean mill and 1.5 % wheat flour, it was found that within 18 h and at 28 °C, the bacterium M1 produced a high level of the bioactive substance (diameter of inhibition zone was 32 mm), amylase activity (40.2 U/ml), protease activity (1.9 U/ml), and cell mass (13×10^8 cells/ml; Fig. 3). This meant that the bacterium indeed could grow well in the cheap and agro-based raw materials as substrates and produced high level of the bioactive substance within the short period.

Table 2 Antibacterial activity of the crude bioactive substance produced by bacterial strain M1

No.	Sensitive strains	Antibacterial activity (diameter of inhibition zone/mm)
1	*V. anguillarum*	20±0.2
2	*V. septicus*	21±0.1
3	*V. harveyi*	12±0.3
4	*V. parahaemolyticus*	17±0.1
5	*S. aquimarina*	14±0.2
6	*V. salmonicida*	27±0.3
7	*V. splendidus*	17 ± 0.1
8	*V. ichthyoenteri*	28±0.4
9	*V. fischeri*	15±0.1
10	*V. vulnificus*	19±0.2

Data are given as means±SD, $n=3$

Purification and identification of the bioactive substance

After the crude bioactive substances were prepared and treated as described in the "Materials and methods" section, they were separated on the TLC plate. The results in Fig. 4a indicated that four bands (N1, N2, N3, and N4) appeared on the TLC plate. After extraction of the bioactive substance from each band, it was found that only the bioactive substances from the bands N1 and N3 had killing activity against the pathogenic *V. anguillarum*, and the band N3 had the highest activity against the pathogen (Fig. 4b). Therefore, the bioactive substance from the band N3 was further isolated and purified using semi-preparative HPLC as described in the "Materials and methods" section. The purified fraction with anti-vibrio activity was analyzed using ESIMS. The data of the molecular mass of the anti-vibrio compound N3 (Fig. 5)

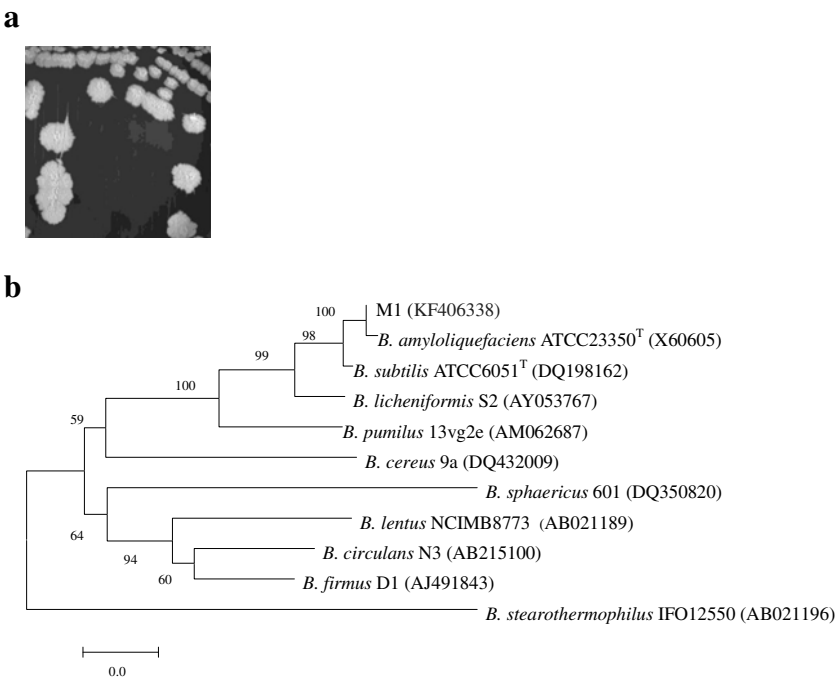

Fig. 1 The morphology (**a**) of the colonies of the bacterial strain M1 and the consensus tree (**b**) of the bacterial strain based on 16S rRNA gene sequences obtained in this study and those downloaded from GenBank at NCBI. The type strains were selected from ATCC and other databases

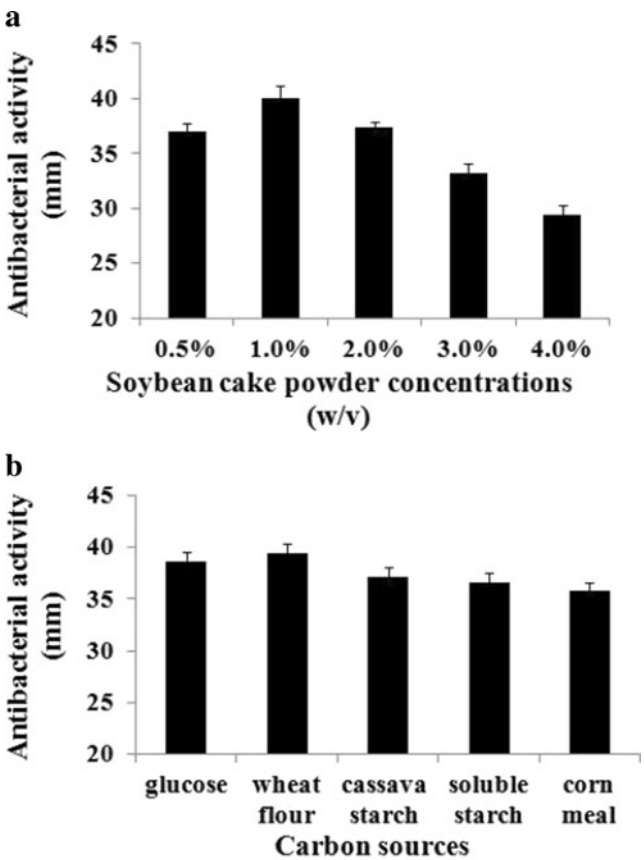

Fig. 2 Effects of different concentrations of soybean mill (**a**) and different carbon sources (1.5 %; **b**) on the bioactive substance production. Data are given as means±SD, $n=3$

were very close to those of a cyclic lipopeptide produced by *Bacillus subtilis* CSY191 (Lee et al. 2012). The cyclic lipopeptide corresponded to the well-known heptapeptide moiety of surfactin, Glu-Leu-Leu-Val-Asp-Leu-Leu, linked to C12, C13, C14, and C15 hydroxy fatty acids, respectively (Chen and Juang 2008; Fig. 6). Figure 6 showed that the N-terminal of L-Glu was *N*-acylated by the carboxyl of the fatty acids, and the carboxyl of L-Leu was esterified by 3-OH of the fatty acids. So, the cyclic lipopeptides were identified as the surfactins and designated as the lipopeptide N3 according to characteristics of the surfactins (Fernandes et al. 2007). The minimal inhibitory concentration of the purified lipopeptide N3 against *V. anguillarum* was 1.5 μg/ml (data not shown).

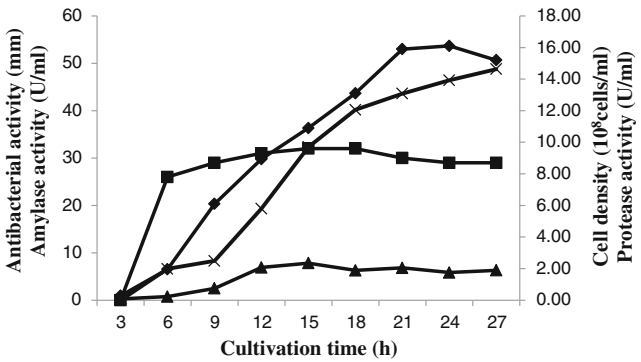

Fig. 3 Time course of the bioactive substance production (*black square*), amylase production (*multiplication sign*), protease production (*black triangle*), and cell growth (*black diamond*) during the 10-l fermentation. The medium contained 1.5 % wheat flour, 1.0 % soybean meal, pH 7.0. The cultivation temperature was 28 °C. Data are given as means±SD, $n=3$

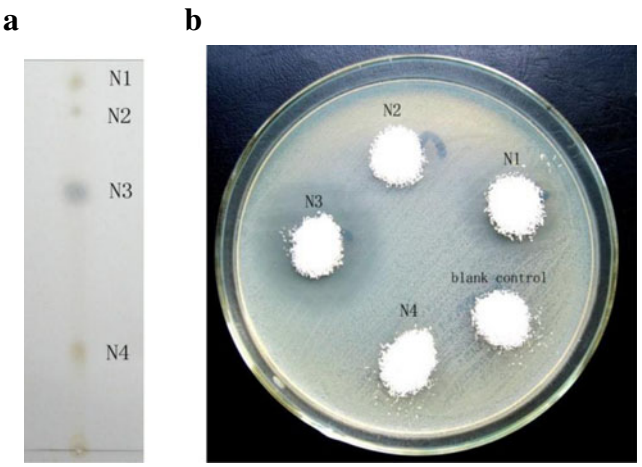

Fig. 4 Separation of the bioactive substances on TLC (**a**) and the antibacterial activity (**b**) of each component

Effects of the lipopeptide N3 on the whole cells and protoplasts of *V. anguillarum*

In order to know how the purified lipopeptide N3 kills *V. anguillarum*, the whole cells of *V. anguillarum* were treated with the purified lipopeptide N3 and observed under scanning electronic microscope. It could be observed from Fig. 7 that the whole cells were broken, and integrity of the cell wall disappeared. The data in Fig. 8a, b revealed that only the lipopeptide N3-treated cells were stained by PI, indicating the plasma membrane of the sensitive bacterial cells was damaged by the lipopeptide N3. However, their nucleic acid molecules in the intact bacterial cells could not be stained by the dye (Fig. 8c, d). After the protoplasts were treated using the purified lipopeptide N3 and SDS, they were lysed and the suspensions became clear, while the protoplasts in the control were not affected (Fig. 9).

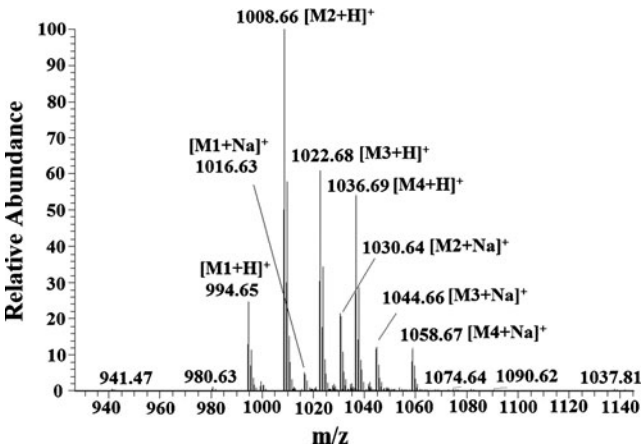

Fig. 5 Positive ESI-MS spectra of the cyclic lipopeptide N3 produced by *B. amyloliquefaciens* M1 on a Q-TOF Ultima Global GAA076 LC mass spectrometer

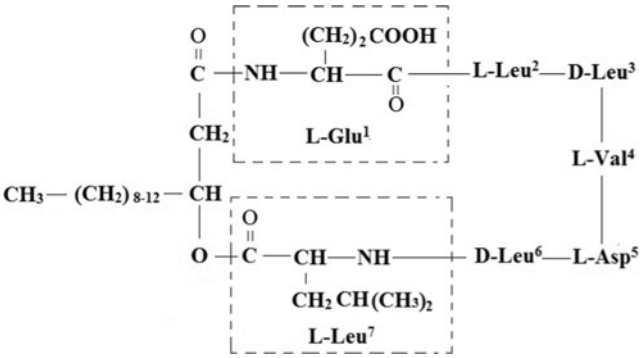

Fig. 6 The chemical structure of the cyclic lipopeptide N3 produced by *B. amyloliquefaciens* M1

Discussion

The results in Table 1 showed that all the bacterial pathogens isolated from the diseased marine animals were the multidrug-resistant strains. It has been reported that within the Vibrionaceae, the species causing the most economically serious diseases in marine culture are *V. anguillarum*, *Vibrio ordalii*, *V. salmonicida*, and *V. vulnificus* (ToranzoT et al. 2005), and out of the 134 isolates examined, 10 showed multidrug resistance to several different antibiotics including chloramphenicol.

At this moment, there are significant environmental concerns with widespread use of antibiotics. They increase the possibility of selection of antibiotic-resistant determinants and bacteria that may affect animals and humans. Resistance to antibiotics in the aquatic environment results from selection of spontaneous mutants by antibiotics in the environment and by horizontal gene transfer and its stimulation between different species and genera including marine bacteria, human, and fish pathogens. In general, the more an antibiotic is used, the greater the risk of emergence and spread of resistance against it as a result of increased selective pressure, thus rendering the drug increasingly useless. The most severe consequence is the emergence of new bacterial strains that are resistant to several antibiotics simultaneously (Burridge et al. 2010). Antibiotics used in aquaculture can also reach wild fish and shellfish surrounding aquaculture sites and are collected for human consumption, therefore potentially affecting food safety. Furthermore, the application of antibiotics still remains the main method for disease control in marine animals (Burridge et al. 2010). In order to find new bioactive substances from *Bacillus* spp. isolated from the mangrove systems, the antibacterial activity of the bioactive substances produced by the bacterial strain M1 against the multidrug-resistant *Vibrio* spp. and *S. aquimarina* was examined. The results in Table 2 indicated that the bioactive substances from the bacterial strain M1 had high killing activity and a wide spectrum of killing activity.

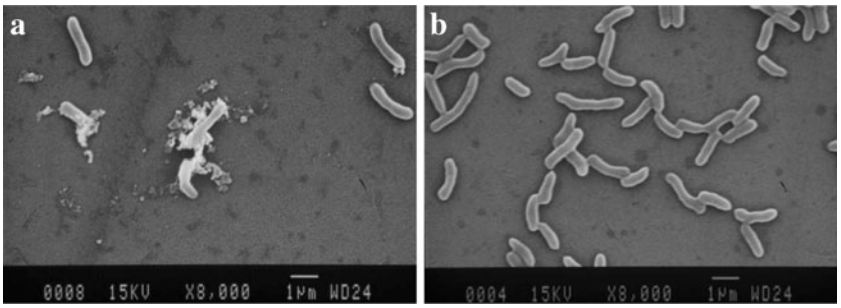

Fig. 7 Morphological changes in the bacterial cells of *V. anguillarum* (**a**) treated with the purified lipopeptide N3 and observed with a scanning electron microscope and no morphological changes in the bacterial cells (**b**) untreated with the purified lipopeptide N3. The concentration of the purified lipopeptide N3 was 200 μg/ml

After identification of the bacterial strain M1, it was found that the bacterial strain M1 was one strain of *B. amyloliquefaciens* (Fig. 1). It has been reported that *Lactobacillus plantarum* and *Lactobacillus casei* were the most effective in inhibiting the growth of *Vibrio* species, and inhibition of the *Vibrio* species was probably due to the production of organic acids by the *Lactobacillus* species (Koga et al. 1998). In our previous studies, the recombinant antibacterial peptides produced by *Yarrowia lipolytica* have high anti-vibrio activity (Zhao et al. 2013). The bioactive substances produced by *Pseudomonas* I-2 strain (Chythanya et al. 2002), *Pseudomonas* PS-102 (Vijayan et al. 2006), the red seaweed *Gracilaria fisheri* (Kanjana et al. 2011), *Pseudoalteromonas* sp. (Morya et al. 2013), a marine bacterium strain S2V2 (Isnansetyo et al. 2009), *Pseudoalteromonas* sp. strains DIT09, DIT44, and DIT46 (Aranda et al. 2012), *Pseudoalteromonas* sp. A1-J11 (del Castillo et al. 2008), and *Pseudomonas* sp. W3 (Rattanachuay et al. 2010) had high anti-vibrio activity. Therefore, this is the first time to report that the bioactive substances produced by *B. amyloliquefaciens* M1 isolated from the mangrove systems has high antibacterial activity against the multidrug-resistant and pathogenic *Vibrio* spp. and *S. aquimarina.*

The results in Figs. 2 and 3 revealed that *B. amyloliquefaciens* M1 indeed could grow well in the cheap and agro-based raw materials as substrates and produced high level of the bioactive substance within the short period. In spite of the immense

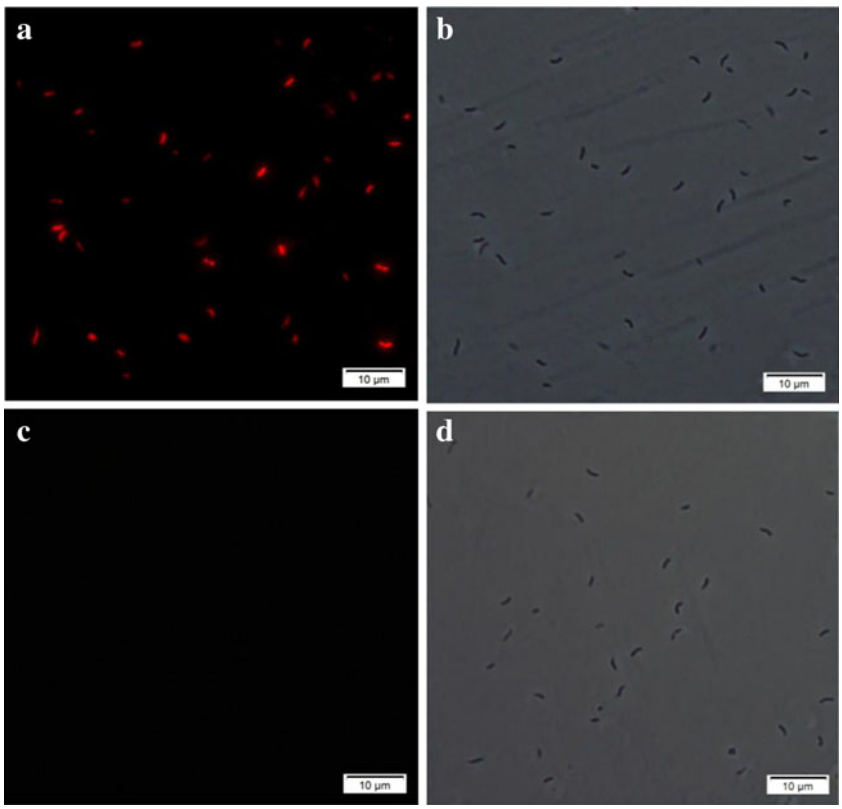

Fig. 8 PI staining of the lipopeptide N3-treated sensitive bacterial cells of *V. anguillarum* (**a**, **b**) and the lipopeptide N3-untreated sensitive bacterial cells (**c**, **d**). The cells in **a** and **c** were observed under a fluorescent microscope, and the cells in **b** and **d** were observed under a light microscope. The concentration of the purified lipopeptide N3 was 200 μg/ml

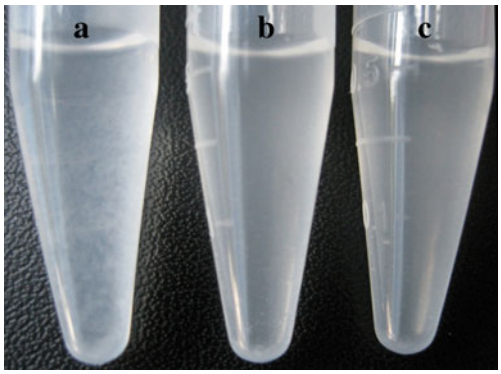

Fig. 9 The sensitivity of the protoplasts of *V. anguillarum* to the purified lipopeptide N3 (**b**) and SDS (**c**). **a** The control, the protoplasts were intact. The concentrations of the purified lipopeptide N3 and SDS were 200 μg/ml

potential for commercial, therapeutic, and environmental applications, the use of lipopeptide is still limited due to its high cost of production and recovery (Fernandes et al. 2007). Often the amount and type of a raw material can contribute considerably to the production cost; it is estimated that raw materials account for 10–30 % of the total production cost in most biotechnological processes. Thus, to reduce this cost it is desirable to use low-cost raw materials for the production of lipopeptides. One possibility explored extensively is the use of cheap and agro-based raw materials as substrates for lipopeptide production. A variety of cheap raw materials, including plant-derived oils, oil wastes, starchy substances, lactic whey, and distillery wastes, have been reported to support lipopeptide production (Fernandes et al. 2007). An efficient and economical bioprocess is the foundation for every profit-making biotechnology industry. Hence, bioprocess development is the primary step towards commercialization of all biotechnological products, including lipopeptides (Koglin et al. 2010). Therefore, the medium used in this study may be suitable for large-scale production of lipopeptides by the bacterial strain M1. It has been reported that batch fermentation for surfactin must be terminated at an appropriate time in order to avoid the utilization of lipopeptide as a carbon source (Koglin et al. 2010). The results in Fig. 3 indeed confirmed this because the amount of the bioactive substance in the culture was decreased after 18 h of the fermentation.

The purified lipopeptide N3 from the crude bioactive substances produced by *B. amyloliquefaciens* M1 was the surfactin isoforms (Figs. 4, 5, and 6). However, the cyclic lipopeptide produced by *B. amyloliquefaciens* Anti-CA contains a heptapeptide, L-Asp→L-Leu→L-Leu→L-Val→L-Val→L-Glu→L-Leu, in which the carboxyl of L-Leu was esterified by 3-OH of the fatty acid (15 carbons) and the N-terminal of L-Asp was *N*-acylated by the carboxyl of the fatty acid (Song et al. 2013). The purified lipopeptide N3 was found to be able to kill all the species of *Vibrio* spp. and *S. aquimarina* tested in this study (data not shown). However, the surfactin produced by *B. subtilis* CSY191 inhibited growth of MCF-7 human breast cancer cells (Lee et al. 2012). The cyclic lipopeptides iturin or surfactin and fengycin produced by *B. amyloliquefaciens* strains MEP218 and ARP23 are effective in biocontrol of sclerotinia stem rot disease by *S. clerotiorum* (Alvarez et al. 2011). An amylolysin (a protein), a novel bacteriocin from *B. amyloliquefaciens* GA1, has the antilisterial activity and can be used as a food preservative (Halimi et al. 2010). Difficidin and bacilysin produced by *B. amyloliquefaciens* possess biocontrol activity against fire blight, a serious disease of orchard trees caused by *E. amylovora* (Chen et al. 2009). Strain S2V2 produced extracellular non-proteinaceous antibacterial substances against different species of *Vibrio* spp. (Isnansetyo et al. 2009). *Pseudoalteromonas* sp. A1-J11 produced AVS-03*d*, the major anti-*Vibrio* substance which was thermostable up to 100 °C and pH stable over a pH range higher than 4.0, and showed strong inhibitory activities, specifically against *V. harveyi* strains (del Castillo et al. 2008). The major component of EtOAc-W3 produced by *Pseudomonas* sp. W3 was supposed to be 2-heptyl-4-quinolone, and it inhibited shrimp pathogenic vibrios (Rattanachuay et al. 2010). Therefore, this is the first time to report that such structure of the lipopeptide was also produced by *B. amyloliquefaciens* and the lipopeptide N3 obtained had high killing activity against the multidrug-resistant pathogenic *Vibrio* spp. and *S. aquimarina.*

It could be seen from the data in Figs. 7, 8, and 9 that the purified lipopeptide N3 had strong antibacterial action on the whole cells and cell membrane. This strong antibacterial actions of the purified lipopeptide N3 could be a consequence of its ability to form ion-conducting channels in bacterial cell membranes by exploiting its detergent-like action on cell membranes, also called membrane active properties. Unlike conventional antibiotics that penetrate into the target cells to show their actions, the antibacterial membrane active lipopeptides N3 killed target cells by destroying their membrane and whole cells(s) (Figs. 7, 8, and 9), thereby mimicking the actions of porins. This mode of action drastically reduces the chance of development of resistance in microbes and hence, offers a promising alternative in the treatment of raging multidrug-resistant infectious diseases in marine animals (Table 2). The broad-spectrum antimicrobial activities (Table 2), even against many multidrug-resistant strains make the lipopeptide N3 attractive alternatives to conventional antibiotics (Fernandes et al. 2007).

In contrast, it has been reported that surfactin displays hemolytic, antiviral, antimycoplasma, and a limited of antibacterial activities but no marked fungi toxicity (Pauli 2006), suggesting that the purified lipopeptide N3 obtained in this study was different from the common surfactin. However, the detailed membrane damage mechanism of the lipopeptide N3 is still unknown.

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