ISSN : 0974 - 7435 Volume 7 Issue 11

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 7(11), 2013 [480-487]

Study on the anti-quorum sensing activity of a marine bacterium *Staphylococcus saprophyticus* 108

Miao Li, Zhou Huiru, Ding Biting, Jiang Yun, Jiang Wei, Dong Kunming*
School of Environmental Science and Engineering, Yangzhou University, Yangzhou 225127, Jiangsu, (CHINA)

ABSTRACT

Quorum sensing inhibitors (QSI) may provide new research direction for the defense of bacterial diseases and biofilm related marine biofouling. QSI screening and development from natural source have received great interest in recent years. In this study, 17 marine bacterial strains, which were found exhibiting anti-quorum sensing activity in previous study, were secondary screened using pigment inhibition assay. One active bacterium 108 was identified as *Staphylococcus saprophyticus* based on the 16S rDNA sequence analysis and morphological observation. The culture condition of bacterium 108 was optimized. The results showed that moderate pH (6.0-7.0) and temperature (25°C) favored the bacterial growth and bioactive metabolites production. Yeast extracts content was the most important medium component affecting the bacterial growth and bioactive metabolites production. By culturing under the optimized condition, the anti-quorum sensing activity of *S. saprophyticus* 108 and the yield of crude extracts was increased about 50% and 1.5 times, respectively, compared to the bacterial cultures before optimization. The active metabolites in *S. saprophyticus* 108 cultures were further extracted, isolated and identified. Cyclo (Pro-Leu), was identified from the active subfraction of the bacterium by GC-MS analysis. It was found for first time that Cyclo (Pro-Leu) exhibited moderate anti-quorum sensing activity.
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KEYWORDS

Anti-quorum sensing activity;
Marine bacteria;
Culture conditions;
Staphylococcus saprophyticus.

INTRODUCTION

Quorum sensing (QS) is communication system in bacteria which involves the regulation of gene expression and behaviors in response to the bacterial cell density through the production and sense of some small signal molecules[21]. This bacterial communication is existed not only in the same species but also among different species, which could regulate the relationship among different bacteria. Therefore, quorum sensing is also called the language of bacteria[16].

Many important bacterial physiological functions, including bioluminescence, virulence factor production and release, antibiotic biosynthesis, bacterial aggregation, biofilm formation, extrapolysaccaride production and secretion, plasmid transduction, etc, are regulated by quorum sensing system[10,18]. For example, *Pseudomonas aeruginosa* causes life-threatening infection in cystic fibrosis patients. *P. aeruginosa* forms biofilm in host body, which makes them 1000-times more resistant to conventional antibiotics than planktonic bacteria do[2]. Quorum sensing system play important role in regulation

of the biofilm formation and maturation of *P. aeruginosa*[15]. The National Institutes of Health (NIH) estimate that 80% of all bacterial infections occurring in the human body are biofilm related[5].

With the gradually recognition on the importance of quorum sensing system in regulating microbial physiological activities, there is growing interest in quorum sensing inhibitors (QSIs) research. QSIs may block the QS and in turn to prevent the synthesis of virulence factor and formation of biofilm. Since QSI is not directly target on the significant growth process of pathogen, and will not provide fatal selection pressure to the bacteria like common antibiotics, which may limit the generation of resistant mutants[14]. Therefore, blocking quorum sensing has being recognized as a viable approach for the development of novel therapeutics in treating bacterial infections and avoiding biofouling in engineering system.

Recently, many studies have been carried out to screen for the quorum sensing inhibitive activity from plants and microbes. Some potent species and compounds with remarkable anti-quorum sensing activity have been figured out[19]. Marine bacteria have proved to be a rich resource for the discovery of bioactive secondary metabolites with unique structure[3]. Complex diversity of marine ecological environment gives marine bacteria the intense microbial competition for space and nutrients resources. It is probable that many excreted metabolites help mediate microbe- microbe interaction[20]. Therefore, marine bacteria should exhibit the ability in production of quorum sensing related substances.

In our previous study, 272 marine bacterial isolates had been screened for their anti-quorum sensing activity against the violacein production in the reporter strain *Chromobacterium violaceum* by disc diffusion assay and double soft agar assay. 17 bacteria were found exhibiting higher anti-quorum sensing activity[6]. In this study, these active bacteria were secondary screened using pigment inhibition assay and one highly active bacterial strain 108 was identified. The culture condition of this bacterium was optimized and the active metabolites were further isolated and identified.

MATERIALS AND METHODS

Bacterial culture and the crude extract preparation

Bacterial strains, which was provided by Prof. Peiyuan Qian's research group, Hong Kong University of Science and Technology, were recovered from the frozen stock in marine broth (MB) medium, which contained 5 g/L peptone, 3g/L yeast extracts, 0.1 g/L ferric citrate, 19.45 g/L NaCl, 5.9 g/L $MgCl_2$, 3.24 g/L $MgSO_4$, 1.8 g/L $CaCl_2$ÿ0.55 g/L KCl with pH 7.4-7.6. After cultured for 36 h at 30°C, the bacterial cultures were re-inoculated into 250 mL culture flask containing 50 mL fresh MB medium, and continuously cultured for 60 h. The cultures were then extracted with equal volume of ethyl acetate (EtOAC) containing 5% acetone. The organic fractions were collected and concentrated under decreased pressure with rotor vac (Shanghai Yarong, RE52CS). The residues were weighed and dissolved in methanol (MeOH) or dimethyl sulfoxide (DMSO) to a concentration of 50 mg/mL for further analysis or bioassay.

Measurement of the bacterial growth

Bacterial growth was determined according to bacterial cell density (OD_{570}) which was measured using a spectrophotometer.

Measurement of the bacterial quorum sensing inhibitive activity

Disc diffusion assay and pigment inhibition assay were used to measure the bacterial quorum sensing inhibitive activity. In disc diffusion assay, reporter strain *Chromobacterium violaceum* 12472 was subcultured in LB liquid medium (0.5% yeast extract, 1% typtone, 1% NaCl, pH 7.0-7.2) overnight. 250 μg/mL or 500 μg bacterial extracts were loaded onto the sterile paper disc (ô 6mm). After the solvent was evaporated, the paper disc was placed onto the LB agar plate which was spread with 100 μL *C. violaceum* in advance. The inhibition zone (in diameter) of violet pigment production of the reporter strain was observed after 24 h incubation at 30°C.

In pigment inhibition assay, overnight cultured *C. violaceum* was adjusted to OD_{570} = 0.1 using fresh LB liquid medium. This solution was transferred to small test tube (2 mL/tube). Bacterial extracts was added into the solution to the test concentration (250 μg/mL or 500 μg/mL). Same volume of the solvent DMSO was served as negative control. All samples were cultured at 30°C with rotation of 150 rpm. After 24 h

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cultivation, the cultures were centrifuged at 14000 rpm for 30 min. The supernatants were removed and 1 mL DMSO was added into the cell pellet, vortex vigorously to completely dissolve the violet pigment from the bacterial cells. The resulted solutions were centrifuged at 14000 rpm for 10 min to remove the cell debris. OD_{570} of the solution was measured and the inhibition rate of violet pigment production was calculated based on the following formula.

$$\text{Inhibition rate}(\%)=\frac{OD_{570control}-OD_{570sample}}{OD_{570control}}\times 100\%$$

Bacterial genomic DNA extraction, PCR amplification and 16s rDNA sequencing

Bacterium 108 was cultured in MB liquid medium and the cells were collected by centrifugation (10000 rpm, 10 min). Bacterial DNA extraction was using the SK1201-UNIQ-10 DNA extraction kit (Sangon). The 16S rRNA genes (rDNA) in the genomic DNA were PCR amplified using the primers 7F (5'-CAGAGTTTGATCCTGGCT- 3') and 1540R (5'-AGGAGGTGATCCAGCCGCA- 3'). Each PCR mixture contained 10 pM of purified DNA, 1.25 U of Taq polymerase (TaKaRa, Dalian, China), 0.2 mM of dNTPs, 0.2 mM of each primer and 5 ml of 10 × PCR buffer in a total volume of 50 mL. PCR was performed in the following thermal cycles: initial denaturation at 98°C for 5 min; 35 cycles of denaturation at 95°C for 35 s, annealing at 55°C for 35 s and an extension at 72°C for 90 s; and final extension at 72°C for 8 min. 16s rDNA sequencing was performed by Shanghai Sangon Biotech Company. Bacterium 108 was identified by comparison with sequences available in Genbank databases using the Ribosomal Database Project (RDP). Similar sequences were aligned using multiple sequence alignment program MEGA version 5.05. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using the neighbor-joining methods.

Field emission scanning electron microscopy observation

To study the microscopic morphological features of strain 108, the bacterial cells were fixed in 2.5% glutaraldehyde in phosphate buffer saline for 2 h at 4°C and post-fixed in 1% osmium tetroxide in the same buffer for 1h. Samples were dehydrated in a gradient ethyl alcohol series (50%, 70%, 80%, 90%, 100% ethanol dissolved in ultrapure water), critical point-dried, and coated for field emission scanning electron microscopy (FESEM) with Pt/Pd and examined with Hitachi S-4800 (Tokyo, Japan) at 15.0 kV.

Optimization of the culture condition of bacterial strain

Optimization of culture temperature and pH

Single factor test was performed to study the effects of different temperature and pH on the bacterial growth, metabolites production and bioactivity. Bacterium 108 was grown on 100 mL MB liquid medium at 7 different pH values from 5.0 to 9.0 at 30°C, or grown on MB liquid medium at 15, 20, 25, 30, 35°C with pH 7.0.

Optimization of medium components

Single factor test was also performed to study the effects of different peptone (0, 2, 5, 10, 15 g/L) and yeast extract (0, 1, 3, 5, 8 g/L) concentrations on the bacterial growth, metabolites production and bioactivity. After cultured for 2 d, bacterial cell density was measured, and the bacterial cultures were extracted and tested for their anit-quorum sensing activities.

Bacterial mass culture, extraction and compound isolation

Bacterium 108 was mass cultured in MB liquid medium (45L) under optimized condition, the bacterial cultures were extracted with equal volume of EtOAC containing 5% acetone. and the solvent was evaporated. The residue was suspended in MeOH/H_2O 9:1 and extracted with equal volume of Hexane for 3 times. MeOH/H_2O layer was added with some H_2O to MeOH/H_2O 6.5:1 and was extracted again with equal volume of Dichloromethane (DCM) for 3 times. Hexane layer, DCM layer and MeOH/H_2O layer were concentrated individually and tested for their quorum sensing inhibitive activity. The active fractions were further isolated and identified using column chromatography, nuclear magnetic resonance (NMR, AVANCE 600, Bruker) and gas chromatography-mass spectrometer (GC-MS, Trace DSQ, Thermo).

GC-MS was incorporated with a relatively nonpo-

lar capillary column (DB-VRX, 60.0 m × 250 μm × 1.40 μm). The injection port was held at 280°C. The temperature was first hold at 50°C for 3 min and programmed from 50 to 330°C at 20°C per minute, and hold at 330°C for 3 min with helium as the carrier gas. EI iron source was used and the iron source temperature was 200°C, the MS scanning range was 50 to 650 amu.

RESULTS AND DISCUSSION

Secondary screen for quorum sensing inhibitive activity

In the previous study, 17 bacteria were found exhibiting anti-quorum sensing activity. These bacteria were secondary screen for their quorum sensing inhibitive activity using pigment inhibition assay. All the 17 bacteria showed some inhibitive activity (with the inhibition rate of 6% to 30%) on violet pigment production of reporter strain (Figure 1). Among them, bacteria 108, 305, 552 exhibited highly inhibition rate, especially for the bacterium 108. It was also found that the activity of strain 108 was more stable Therefore; strain 108 was selected for further identification, culture condition optimization and active compounds isolation.

Since quorum sensing system control many bacterial physiological behaviors especially affecting pathogenicity, more and more researchers started to focus on the study of interruption of quorum sensing system, such as quorum sensing inhibitors screening. To screen for the QSI, many kinds of screening methods have been developed. The violacein production in *Chromobacterium violaceum*, β-galactosidase production in *Agrobacterium tumefaciens* and bioluminescence in *Vibrio harveyi* are found to be critically regulated by QS system. The variation of these characteristics in these three bacteria could be easily detected in the bioassays. Wild type or mutants of the Gram negative strains *C. violaceum*, *A. tunefaciens* and *V. harveyi* have been usually used as AHL relevant QS reporter strain, Cross cultivation agar plate assay, overlaid soft agar assay and disc diffusion assay have been developed to screen for the QSI activity from target bacteria or bacterial/plant extracts. In this study, pigment inhibition assay which was conducted in liquid medium had been used as primary measuring method. The pigment inhibition rate in this method could be precisely quantified. However, this assay needs extracts sample which takes more efforts in the sample pretreatment. This method should be more suitable used in secondary screening of bacterial samples. High amount of DMSO in the liquid medium (larger than 5 μL/mL) could also affect the growth of reporter strain. Therefore, the addition of DMSO in the assay system should be less than 5 μL/mL.

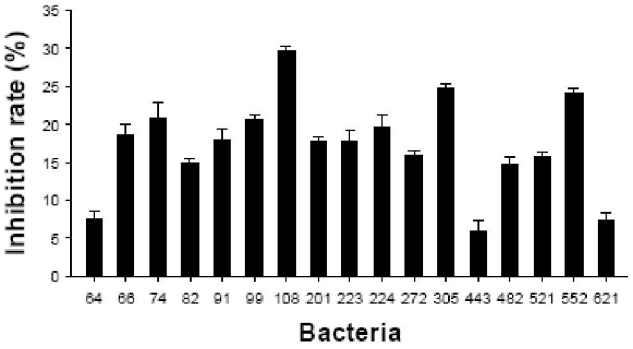

Figure 1: The anti-quorum sensing activity of the bacterial extracts in pigment inhibition assay.

Identification of strain 108

Genomic DNA of strain 108 was extracted, the 16s rDNA was PCR amplified and sequenced. The nearly complete 16S rRNA gene sequence of strain 108 (1437 bp) was obtained. Comparative analysis of the 16S rRNA gene sequence with sequences deposited in GenBank using BLAST indicated that the strain fell within the *Staphylococcus* clade and shared the highest sequence similarities, 99 %, with *S. saprophyticus*. Based on the 16S rDNA sequence analysis, the phylogenic tree of the strain 108 and its related species was constructed as shown in Figure 2a. FESEM was also carried out to observe the bacterial cell structure (Figure 2b), which showed that 108 was a round-shaped bacterium with smooth surface and cells were 0.7–0.8 μm in diameter. Based on the sequence analysis and morphological observation, strain 108 was identified as *S. saprophyticus*.

Quorum sensing signaling molecules are usually different in Gram negative and Gram positive bacteria. Gram negative bacteria commonly use N-acyl homoserine lactones (AHLs) as signaling molecules, while gram positive bacteria generally using modified oligopeptide as autoinducers, which is called

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autoinduced peptides (AIPs)[1]. Some gram negative bacteria belonging to different species have been found exhibiting AHL relevant QSI activity, most commonly in *Vibrio* and *Pseudomonas* group[11]. In this study, the active bacterium was identified as *S. saprophyticus*, which was a gram positive bacterium. Ribeiro et al. (2002) found that some amino acids produced by *S. saprophyticus* could inhibit RNAIII synthesis and then interfere with quorum sensing RNAIII-dependent expression of virulence of *S. aureus*. In this study, *S. saprophyticus* was the fist time to be reported for their anti-quorum sensing activity against Gram negative bacteria. The results indicated that Gram positive bacteria may also produce AHL relevant quorum sensing inhibitive or inductive that affect the quorum sensing system in Gram negative bacteria. Some studies also suggested that *Bacillus* species could secrete lactonase to degrade Gram negative quorum sensing chemicals by hydrolysing the AHL ring. In contrast, Everts reported that *P. aeruginosa* could produce an AHL autoinducer and later degrade into tetramic acid to kill gram positive competitors[7]. Quorum sensing communications not only exist within G- or G+ bacteria, but also exist among the bacteria of these two groups.

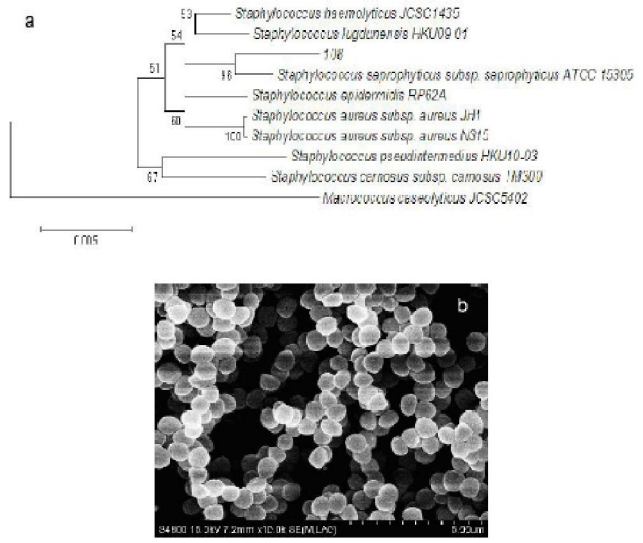

Figure 2 : Phylogenetic tree based on its 16S rDNA sequences (a) and scanning electron microscope photograph of strain 108 (b)

Optimization of culture temperature and pH

Strain *S. saprophyticus* 108 could grow well in all tested temperature (15 to 35°C). The highest growth was found in 20°C and the OD_{570} of the bacterial culture reached 0.751 after 36 h cultivation (Figure 3a). Although strain 108 grew well at 15°C, the quantity of the extract from this culture was much lower than that of other treatment. In addition, almost no pigment inhibitive activity was found in the treatment cultured at 15°C at the test concentration of 500 μg/mL. Strain 108 could produce more metabolites in higher temperature, and the highest production (9.4 mg/100 mL) was found at 25°C (TABLE 1). The pigment inhibitive activity of the 25°C extract at the concentration of 500 μg/mL was also the strongest and the inhibition rate was about 28.9 % (Figure 3a). The optimal culture temperature of the bacterium was at 25°C.

Bacterium *S. saprophyticus* 108 grew well at the pH of 5.0 – 9.0, especially at pH 6.0 and 7.0. Along with further acidification or alkalization of the culture condition, the growth of the bacterium decreased gradually (Figure 3b). More extracts (above 8.0 mg/100 mL) was acquired from the cultures of pH 6.0 and 7.0 (TABLE 1). Compared to the negative control in pigment inhibition assay, higher anti-quorum sensing activities were found in pH 6.0 and 7.0 samples, much lower activities were detected in other pH samples. Based on the results of bacterial growth, extract amount and the quorum sensing inhibitive activity, the optimal culture pH of bacterium 108 was at 7.0 (Figure 3b).

TABLE 1: Dry weight of extracts from each bacterial culture (100 mL) at different temperature and pH

Temperature. (°C)	Weight of Extract (mg)	pH	Weight of extract (mg)
15	1.3±0.26	5	5.6±0.71
20	8.7±0.83	6	8.0±0.59
25	9.4±0.60	7	8.2±1.21
30	6.5±0.75	8	6.8±0.65
35	7.1±0.66	9	3.0±0.75

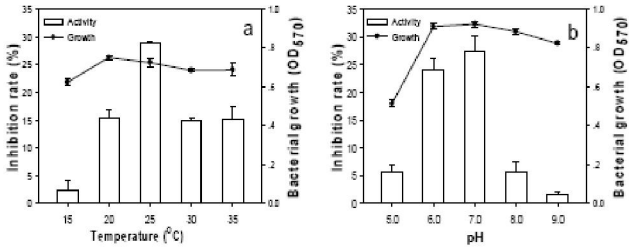

Figure 3 : The effect of temperature (a) and pH (b) on the growth and activity of the bacterium *Staphylococcus saprophyticus* 108

Optimization of medium components

With the increasing of peptone content, the OD_{570} value and the extracts yield of the bacterial cultures increased gradually (Figure 4a). Low concentration of peptone inhibited the production of bacterial secondary metabolites. Less than 2.5 mg extracts was obtained from 0 g/L and 2 g/L peptone treatments (TABLE 2). The results of pigment inhibitive assay showed that all of the peptone treatments could inhibit the violet pigment production of the reporter strain. The highest inhibitive activity was found in the treatment containing 15 g/L of peptone, with the inhibition rate of 30.3%. The treatment containing 5 g/L of peptone also showed higher activity, with the inhibition rate of 27.7% (Figure 4a). Therefore, the best peptone content in the culture medium was 15 g/L.

With the increasing of yeast extracts content from 0 g/L to 8 g/L, the OD_{570} value of the bacterial cultures largely increased from 0.4 to 1.2 (Figure 4b). Although, the yields of extracts in different treatments were similar (TABLE 2), the inhibitive activities of different treatments were quite different. Higher activity was found in the treatments containing higher amount of yeast extract. The extract from 5 g/L yeast extract culture showed the highest pigment inhibitive activity, with the inhibition rate of 48.67% compared to the negative control, which was significantly higher than other treatments (Fig. 4b). The optimal yeast extract content in the culture medium was 5 g/L.

TABLE 2 : Dry weight of extracts from each bacterial culture (100 mL) in different medium content

Peptone (g/L)	Weight of extract (mg)	Yeast extract (g/L)	Weight of extract (mg)	NaCl (g/L)	Weight of extract (mg)
0	2.0±0.68	0	4.2±0.47	0	3.2±0.56
2	2.6±0.40	1	4.4±0.51	10	2.6±0.35
5	4.8±0.64	3	4.8±0.65	15	3.6±0.67
10	6.6±0.35	5	4.0±0.55	20	4.8±0.48
15	6.8±0.51	8	3.6±0.31	25	4.6±0.52

The effect of different yeast extracts content on the growth, metabolites production as well as the pigment inhibitive activity of *S. saprophyticus* 108 was the most important. Yeast extract content was also found important in the bioactive compounds production of other *Staphylococcus* species. Ghribi et al.[9] reported that the lipase production in *S. xylosus* was greatly varied under different concentrations of yeast extract and 6 g/L yeast extract in culture medium mostly favored the lipase production. High concentration of organic nitrogen source (both peptone and yeast extract) favored the growth of *S. saprophyticus* 108, while moderate nitrogen concentration favored active metabolites podcution, which was similar to many other studies[13]. Many quickly metabolized carbon or nitrogen substrates sustaining maximum cell growth rates can inhibit secondary metabolite production[8].

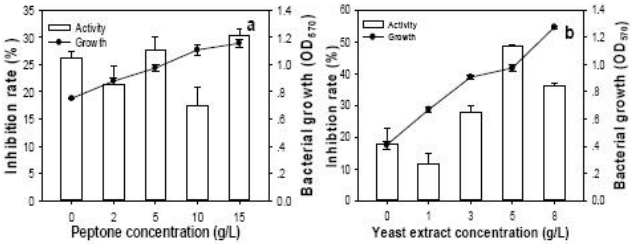

Figure 4 : The effect of peptone (a) and yeast extract (b) content on the growth and activity of the bacterium *S. saprophyticus* 108.

Comparison of the bioactivity of bacterial extracts before and after the optimization

To compare the bioactivity of bacterial extracts before and after the optimization, we cultured the bacterium *S. saprophyticus* 108 under the original fermentation condition and optimized condition, and then measured the weight as well as the anti-quorum sensing activity of bacterial extracts. It was found that both extracts showed the higher anti-quorum sensing activity in the concentrations of 500 μg/mL. The inhibition rate of bacterial cultures after optimization was significantly increased from 35.8% to 50.2%, compared to the bacterial cultures before optimization. In addition, the yield of extract after optimization (91.1 mg/L) was about 1.5 times higher than that of the original extracts (56.6 mg/L).

Compound isolation and identification

S. saprophyticus 108 was mass cultured for 45 L under optimized condition. In total, 2.9 g of crude extracts had been obtained from bacterial cultures by EtOAc extraction. After partition with Hexane, DCM, three fractions were obtained. The weight of Hexane fraction, DCM fraction and MeOH/H_2O fraction were 0.56 g, 1.38 g and 0.96 g, respectively. Only DCM

fraction showed higher quorum sensing inhibitive activity, with the inhibition rate of 26.3% at the concentration of 250 μg/mL. DCM fraction were isolated using reduce pressure ODS column chromatography eluted with H_2O/MeOH from 90:10 (I), 80:20 (II), 60:40 (III), 40:60 (IV), 5:95 (V) to 0:100 (VI). The anti-quorum sensing activity of each subfractions were tested by disc diffusion assay, and it was found that at the concentration of 250 μg/mL, subfraction I (30 mg), II (150 mg), III (290 mg) and IV (320 mg) showed strong activity with the violacein inhibition zone of 16 mm, 14 mm, 11 mm and 20 mm in diameter, respectively. No activity was detected in subfraction V (510 mg) and VI (80 mg). The results indicated that the active substances were in subfraction I to IV, especially in subfraction IV.

Subfraction IV was subjected to run GC-MS. Several peaks had been detected in the GC spectrum from the retention of 13.29 min to 15.49 min. Among them, one peak with the retention time of 13.38 min have been deduce as cyclo (Pro-Leu), based on the results of library search using its MS data, with the possibility of 79.49%. The anti-quorum sensing activity of pure cyclo (Pro-Leu), which was stored in our laboratory compound stock, had been further tested by using disc diffusion assay. The result showed that cyclo (pro-Leu) weakly inhibited the violacein production of *C. violaceum* with the inhibition zone of 10 mm in diameter at the concentration of 250 μg/disc.

Since the first report in 1924, a large number of bioactive diketopiperazines was discovered spanning activities as antitumor, antiviral, antimicrobial, antihyperglycemic or glycosidase inhibitor agents. Diketopiperazines, such as cyclo (l-Pro-l-Tyr), cyclo (l-Phe-l-Pro), cyclo (l-Pro- l-Val), were also reported to modulate LuxR-mediated quorum-sensing systems of bacteria, and they are considered to influence cell-cell signaling offering alternative ways of biofilm control by interfering with microbial communication[4]. In this study, we identified another anti-quorum sensing diketopiperazines, cyclo (Pro-Leu), from active subfraction IV by GC-MS analysis. Cyclo (Pro-Leu) had been reported for the inhibitive activity against the larval settlement of marine fouling organism *Balanus reticulatus,* and *Pinctada martensi* by Liu et al.[12]. The antifouling activity of this diketopiperazine may be partially contributed by its anti-quorum sensing activity. However, the anti-quorum sensing activity of pure cyclo (Pro-Leu) was found much lower than that of subfraction IV. The highly activity of subfraction IV might due to the synergy effect among cyclo (Pro-Leu) and other compounds existed in subfraction IV. There should also be other active substances in the subfraction IV which had not been identified yet. Cyclo (Pro-Leu) is a safe and non-toxic natural organic compound with remarkable antifouling activity and moderate anti-quorum sensing activity. This compound has also been found in many microbial secondary metabolites and is relatively easy to be large acquired by synthetic technology. It is believed that this compound should have potential application in biofouling controls. The producer of this compound, *S. saprophyticus* 108, might also have potential application in quorum sensing inhibition.

ACKNOWLEDGEMENTS

This study was generously supported by National Natural Science Foundation of China (No. 41006097), Natural Science Foundation for college and university of Jiangsu Province (No. 10KJB170013) and Key Laboratory of Marine Drugs (Ocean University of China), Ministry of Education (KLMD(OUC)201203). The authors would also like to thank senior engineer Zhou Weidong, Testing Center of Yangzhou University, for his contribution in scanning electron microscopy of bacterial cells.

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