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Isolation of an Antimicrobial Marine Strain HS-A38 and Purification of its Bioactive Substances

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A marine strain HS-A38 with potent antimicrobial activity was isolated from the intestine of wild sea cucumber in Dalian sea area. Based on the analysis of morphological, physiological and 16S rDNA sequence, the strain HS-A38 was identified as *Bacillus subtilis*. Two bioactive substances (1 and 2) were purified from the fermented broth of the strain HS-A38 with the methods of fractional sedimentation with ammonium sulfate, CM-52 ion-exchange chromatography and Sephadex G-75 gel filtration. The SDS-PAGE analysis indicated that the relative molecular weight of both bioactive substances 1 and 2 were 41 kDa and 28 kDa, respectively. The antibacterial spectra showed that substance 1 could only inhibit Gram positive bacteria, whereas substance 2 could significantly inhibit both the Gram positive and negative bacteria.

Key words: Marine microorganisms, antibacterial activities, bioactive substance.

Bioactive substances are believed to play a key role in microbial interactions by mediating antagonistic activity and intercellular communication (Hibbing *et al.,* 2010). In addition, many microbial natural products have biotechnological potential as antibiotics, biosurfactants, antifungal, or anticancer agents (Demain *et al.,* 2009). Sequences of microbial genomes revealed that only a small fraction of the natural product diversity is known, highlighting the potential for finding novel bioactive compounds in environmental microorganisms (Fischbach, 2009). The need for novel antimicrobials to combat increasing antibiotic resistances in pathogenic bacteria has stimulated the exploration of other than the traditional sources, such as asterrestrial actinomycetes or fungi (Berdy, 2005).

The marine environment harbors bacteria with antagonistic traits (Nair, 1987; Long *et al.,* 2001) and marine microorganisms are a potential source of novel antimicrobials (Zhang *et al.,* 2005). Antagonistic marine bacteria have been isolated from surface (Gram *et al.,* 2010) and deep waters[1] (Hohmann *et al.,* 2009), but the majority originated from biotic surfaces such as sponges (Taylor *et al.,* 2007), zooplankton and macroalgae (Wiese *et al.,* 2009), corals (Rypien *et al.,* 2010), and bryozoans (Herndl *et al.,* 2010). Bioactive bacterial strains predominantly belong to Pseudoalteromonas spp. (Bowman, 2007), the Roseobacter clade (Martens *et al.,* 2007), and Actinobacteria (Bull *et al.,* 2007). A number of marine-derived antimicrobials have been characterized in greater detail, including halogenated (Andersen *et al.,* 1974) and sulfuric (Geng *et al.,* 2008) compounds, depsipeptides (Romanenko *et al.,* 2008) and lipopeptides (Das *et al.,* 2008), glycolipids (Kiran *et al.,* 2010), as well as

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high molecular weight structures such as amino acid oxidases (Gomez *et al.,* 2008).

An emerging source of new bioactives may result from the many recent studies of microbial diversity in the marine environment, particularly those microbes associated with marine plants and animals (Penesyan *et al.,* 2009; Hentschel *et al.,* 2001). Several studies have demonstrated that "living surfaces" represent an environment rich in epibiotic microorgansims that produce bioactives (Muscholl-Silberhorn *et al.,* 2008; Egan *et al.,* 2008). Nevertheless, the vast biotechnological potential of marine epibiotic microorgansims remains mostly unexplored. This study exlpores new sources potentially rich in bioactives from marine microorganism-host and the targeted isolation of bioactive producing microorganisms.

MATERIALS AND METHODS

Isolation of bacterial strains from sea cucumber intestine

Fresh sea cucumber intestines were grinded under the condition of a sterile environment. 5 mL sterile water was added, shaked and mixed thoroughly, and stand settlement. The supernatants of the sample were progressively diluted with sterilized deionized water with a series of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Then take 0.2 mL every gradient diluents and spread each onto marine agar 2216 media. After incubating at 25°C for 2 days, and the single colonies were picked up according to the differences of the sample and the morphological of the strains.

Screening of strains with antibacterial activity

Screening of strains with antibacterial activity was conducted by the method of agar well diffusion. All isolated strains were respectively inoculated in 5 mL LB liquid medium and cultured on a rotary shaker (150 rpm) at 28°C for 2 days. The fermented broth was spun down at 8000 g for 10 min, and the supernatant was passed through a 0.45 mm Millipore filter. The indicator strains used in this test are *Micrococcus lysodeikticus*, *Staphylococcus aureus*, *Vibrio parahemolyticus* and *Pseudomonas aeruginosa*. Wells of 5 mm diameter were punched in Brain Heart Infusion agar plates seeded with indicator organisms. The fermented broth (200 mL) was added in the wells. Plates were then incubated overnight at 30°C for 48 hours. Where upon inhibitory activity was detected as a zone of clearing in the turbid agar around the wells containing antibacterial activity (positive samples). The diameter of the clearing zones was measured in (mm) to obtain a semi quantitative determination of the concentration of the antibacterial compound.

Identification of the strain HS-A38

A marine bacterial strain (designated as HS-A38) with higher antibacterial activity than others was isolated from the intestine of sea cucumber. The strain was identified by chemotaxonomic and morphological characters and its 16S rDNA gene sequence. The sequence was initially analyzed on the NCBI server (http://www.ncbi.nlm.nih.gov/) using the BLAST tool, and corresponding sequences were retrieved. A similarity matrix was prepared using Dnadist program in PHYLIP analysis package using Jukes-Cantor corrections. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA software package.

Preparation of bioactive substance suspension

The strain HS-A38 was incubated in 2216E broth for 16 h at 28°C and at 150 rpm. Then 2% of the seed culture was inoculated into fermentation broth containing soluble starch 1.25%, beef extract 2.5%, corn steep liquor 1%, Tween-80 0.01%, and was cultured on a rotary shaker (150 rpm) at 28°C for 2 days. The cultured bacteria were centrifuged at 8000 rpm for 10 min and the supernatants were reserved.

Preliminary authentication of bioactive substances

In order to indentify the composition of bioactive substances, 20 mL Protease k was mixed with 500 mL fermentation supernatant and kept at temperature 50°C for 1 h. The supernatant without Protease k was used as a control. The antibacterial activity of the two supernatant samples was tested by the method of ager well diffusion.

Ammonium sulfate precipitation

Crude proteins were precipitated by adding ammonium sulfate to the final concentration of 60% (w/v) and proteins were harvested by centrifugation (12,000 rpm for 30 min at 4°C). The pellet was dissolved in sterilized water and dialyzed

in order to desalt crude protein using dialysis membrane (MWCO: 12-14,000). Antimicrobial activity of the crude proteins was assayed by the method of ager well diffusion.

Ion-exchange chromatography and gel filtration

The clear supernatant was subjected to purify by CM-52 ion-exchange chromatography pre-equilibrated with 20 mM PBS (pH 5.0). The proteins were fractionated with a 0.1 - 0.6 M step gradient of NaCl in 20 mM PBS buffer and flow rate of 1 mL/min. Eluted fractions (2 mL) were collected and the OD_{280} was measured for every fraction. All fractions were used in the bioassay to check the antimicrobial activity, and then the fractions with bioactivity were dried with a vacuum concentrator and weighed.

The above dried samples were diluted and the fractions were subjected to purify by Sephadex G-75 column pre-equilibrated with 10 mM PBS (pH 7.0). The column was eluted with 10 mM PBS at a flow rate of 0.6 mL/min. Eluted fractions (2 mL) were collected and the OD_{280} was measured for every fraction. All the fractions were used in the bioassay to check the antimicrobial activity and the fractions were dried and weighed.

Protein assay and analysis of SDS-polyacrylamide gel electrophoresis

The protein concentration was measured by following the method of Bradford using bovine serum albumin as the standard. 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect the purified bioactive substances.

RESULTS

Screening of antimicrobial marine microorganisms

The isolated 200 marine bacteria strains were tested for their antimicrobial activities against *S. aureus*, *M. lysodeikticus*, *V. parahemolyticus* and *P. aeruginosa*. The results showed that 13 of the screened strains possessed antimicrobial activities, accounting for 6.5% of total isolated strains. As shown in Table 1, the strain HS-A38 exhibited the most inhibitory effects against the four indicator strains.

Identification of the strain HS-A38

The strain HS-A38 isolated from sea cumber intestine was identified through morphological and molecular tools. The strain HS-A38 was strictly aerobic, motile, Gram-positive, spore forming and rod-shaped (Fig. 1). Further characterization was confirmed with its 16S rDNA gene sequence. The result showed that an approximately 1648 bp of the 16S rDNA gene sequence in the strain HS-A38 possesses 100% similarity with *Bacillus subtilis* (AB195282). The phylogenetic analysis showed that the isolated strain HS-A38 stood in the identified strains of *Bacillus subtilis* clade (Fig. 2). Hence, the isolated strain in the present study was identified as *Bacillus subtilis* HS-A38. The 16S rDNA gene sequence of the strain was submitted to GenBank under the accession number GQ 466597.

Table 1. Strains with strongest antibacterial activities

Strain no	Inhibition zone (mm)			
	ML	*SA*	*VP*	*PA*
A9-4	22.6	26.6	26.8	-
A8-5	26.6	30.0	29.7	15.0
A1-15	23.0	22.0	28.1	-
A8-3	25.4	30.4	17.8	-
A8-20	25.4	28.0	18.0	-
A8-4	23.4	32.0	18.0	-
C6-7	23.2	26.0	27.0	-
C8-11	24.0	27.0	27.0	-
C7-17	21.5	26.7	26.2	-
C7-21a	22.0	24.7	28.5	-
C7-21b	20.0	25.3	31.2	-
C7-18	21.5	27.0	27.7	-
HS-A38	24.6	32.4	36.0	18.0

ML: *M. Lysodeikticus* *SA*: *S. aureus*
VP: *V. parahemolyticus* *PA*: *P. aeruginosa*

Table 2. Antibacterial spectra of bioactive substances

Substance no	Inhibition zone (mm)			
	ML	*SA*	*VP*	*PA*
1	14.0	17.2		-
2	23.1		33.1	17.0

ML: *M. Lysodeikticus* *SA*: *S. aureus*
VP: *V. parahemolyticus* *PA*: *P. aeruginosa*

Preliminary authentication of bioactive substances

Compared to the supernatant of the strain HS-A38 fermented broth without the treatment of Protease k, the bacteria inhibition zone of the

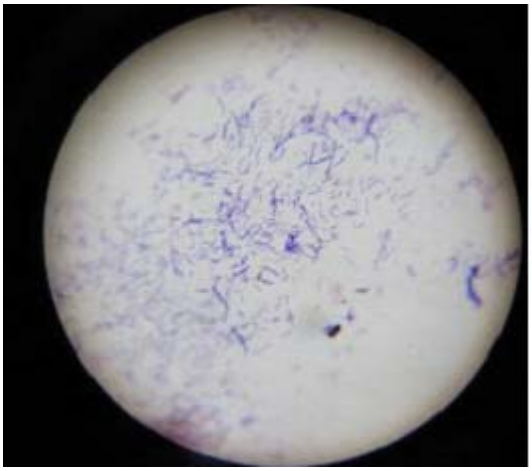

Fig. 1. Morphology of the strain HS-A3

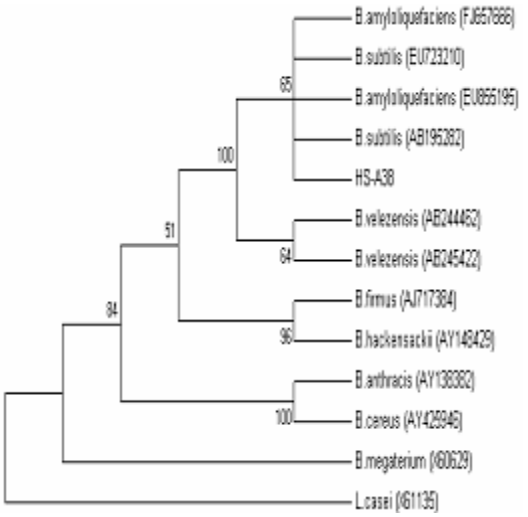

Fig. 2. Phylogenetic tree of the strain HS-A38 based on the 16S rDNA sequence

Fig. 3. Antibacterial result of the extracted liquid processed by Protease k

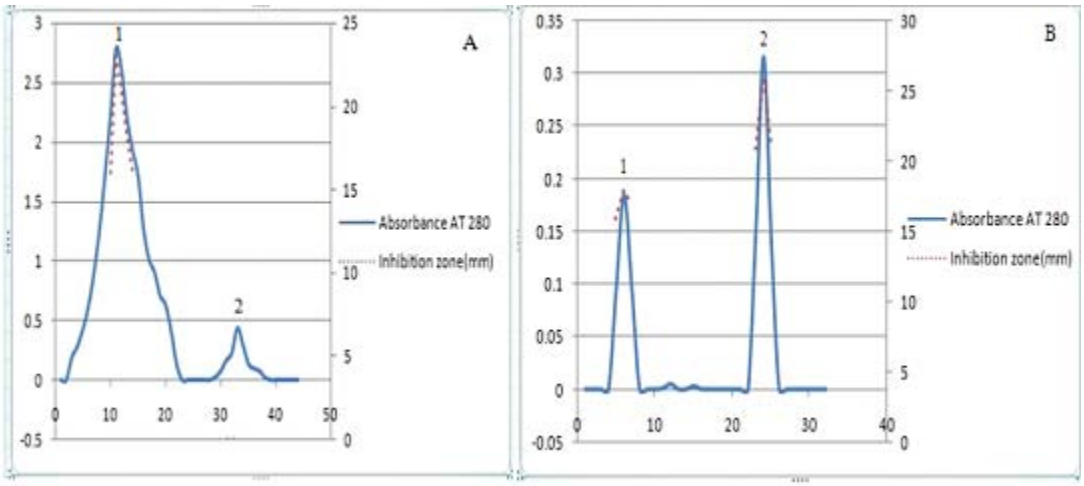

Fig. 4. The chromatography of CM-52 anion exchange chromatography (A) and Sephadex G-75 (B)

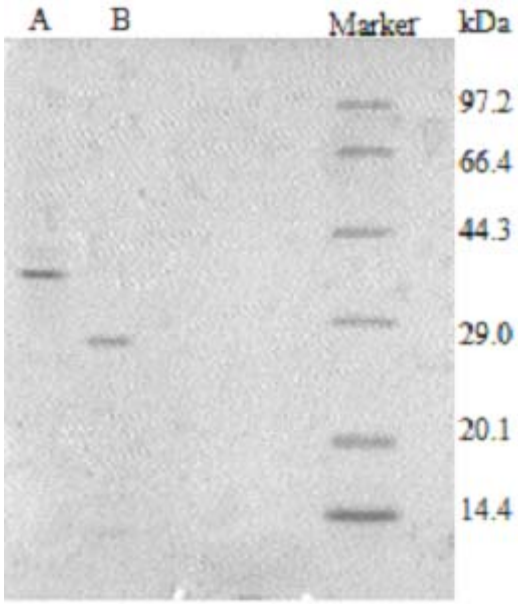

Fig. 5. SDS-PAGE assay of purified proteins after chromatography of Sephadex G-75.
A: Substance 1, B: Substance 2

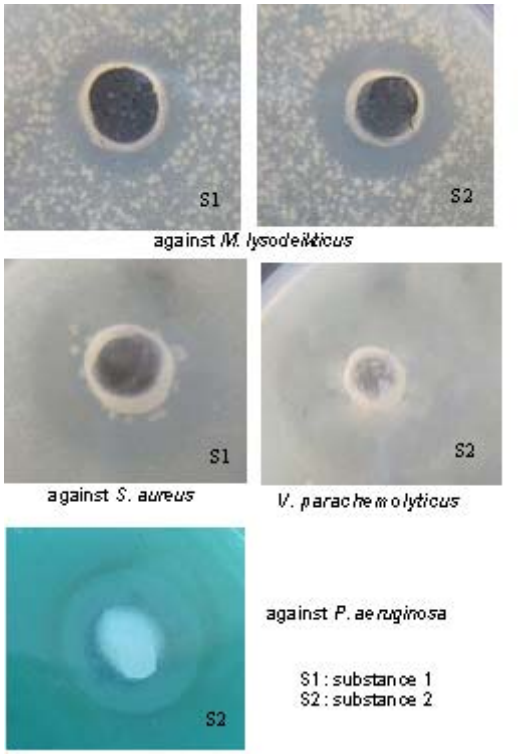

Fig. 6. Antibacterial results of bioactive substances

supernatant mixed with protease k reduces significantly (Fig. 3). From this point of view, it is concluded that the composition of the bioactive substances contains proteins.

Purification of bioactive substances

The crude extract from the supernatant of the strain HS-A38 was precipitated by 60% ammonium sulfate solution and dialysed. The dialysate was supplied to CM-52 ion-exchange chromatography for purification (Fig. 4A). The fractions possessing antimicrobial activities were collected and re-chromatographed on the Sephadex G-75 column, resulting in the appearance of two peaks of bioactive substances (Fig. 4B). Each of the fractions, designated as substances 1 and 2, was collected and independently estimated its purity by SDS-PAGE. The results showed that both of substances 1 and 2 exhibited a single band with the molecular weight of 41 kDa and 28 kDa, respectively (Fig. 5).

Antimicrobial activities assay

The antimicrobial activities of substances 1 and 2 were tested against four indicator strains (Fig. 6). As shown in Table 2, substance 1 exhibited inhibitory effects against Gram positive strains, and substance 2 possessed significantly inhibitory effects against both Gram positive and negative bacteria.

DISCUSSION

The widespread occurrence of antibiotic-resistant human pathogens and the paucity of effective antifungal drugs have created an urgent need for new antimicrobial agents. Marine microorganisms have proved to be rich sources of bioactive substances and numerous compounds with potent biological activities and unique chemical structures were isolated. The large numbers and diversity of marine microorganisms such as bacteria, fungi and cyanobacteria suggest that this resource will be of significant importance in the discovery of new antimicrobial agents and other drugs and are expected to compete with terrestrial microorganisms in this aspect in the near future. In additionÿcompared with other marine organismsÿmarine microorganisms own the superiority that is easy to realize industrialization by fermentationÿwe can optimistically predict that unique metabolites will be mass-produced by

cultivation of genetically-modified microbes. Therefore, it is no doubt that study of marine microorganisms will have a vast range of prospects. In this project, we report screening of antimicrobial marine microorganisms and purification of their bioactive substances. The strain HS-A38 with obvious antimicrobial activity was isolated from sea cumber intestine. It was identified by chemotaxonomic and morphological characters and its 16S rDNA sequence. The results showed that it belongs to *Bacillus sp*. Two bioactive substances were purified from the fermented broth of the strain HS-A38 with the methods of ammonium sulfate sedimentation, CM-52 ion-exchange chromatography and Sephadex G-75 gel filtration. The molecular mass of purified bioactive substances detected on SDS-PAGE was 41 kDa and 28 kDa, respectively. Substance 1 exhibited antibacterial activities against *M. lysodeikticus* and *S. aureus*, whereas substance 2 showed significantly inhibitory effect on *V. parahemolyticus*, *M. lysodeikticus* and *S. aureus*. The N-terminal amino acid sequence for both substances will be analyzed for further study. We are planning to conduct future studies in analyzing the gene responsible for bioactive substance 2 and to over-express the protein in a suitable recombinant expression system (Cong *et al.,* 2009).

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