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Original Research Paper

Isolation and Identification of *Staphylococcus epidermidis* S14 Screening Extracellular Antimicrobial Metabolites

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Abstract: Total 24 isolates with antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Shigella castellani*, out of 259 strains, were isolated from the internal of seven marine fishes. The extracellular metabolites of strain S14, isolated from the internal of shark, showed stronger inhibition against the four tested bacteria, including G- and G+ bacteria. Based on the 16S rDNA the sequencing analysis, the strain S14 belonged to a member of *Staphylococcus epidermidis* with 99% DNA similarity and named to *Staphylococcus epidermidis* S14. The *Staphylococcus epidermidis* S14 secreted rarely antibacterial substance in the stabilization period and the antibacterial active substances might be the secondary metabolites. The antibacterial metabolites were not hydrolyzed by pepsin, trypsin and lysozyme. The antibacterial metabolites were stable for 30 min to high temperature treatment at 100°C and had higher antibacterial activity at neutral pH.

Keywords: *Staphylococcus epidermidis*, Antimicrobial Activity, Food Pathogens, Marine Fish, Shark

Introduction

The marine environment, with extensive biological and chemical diversity, was rather complicated and possessed many life forms (Abdessamad *et al*., 2010). The complex interactions between biological and chemical factors stimulated marine organisms to produce different biological active compounds (Zheng *et al*., 2011). Associated microorganisms utilized their host's nutrients and in return protected themselves to avoid damage by secreting bioactive compounds. Marine fishes were known as microbial accumulators because of their extreme survival conditions. These marine fishes had abundant nutrient compounds in their enteric canal, tissues and organs and these adhesive nutrients were considered to provide auspicious conditions for growth, propagation and exist of many microorganisms, predominantly including bacteria, fungi, viruses and spores (Singh *et al*., 2015). The enteric bacteria attached of marine organism had been proven to be a source of producing bioactive molecules with antimicrobial, cytotoxic or antineoplastic abilities, which had huge potential applications in biotechnological and pharmaceutical industry (Rajeev and Xu, 2004; Felício *et al*., 2015).

During the past 30 to 40 years, a lot of new compounds were isolated or purified from marine animal tissues and some of these had strong bioactivity, such as antimicrobial, antioxidative, antiviral, antitumor, anticancer, lowering blood lipid/sugar and anti-obesity activity. Some of these compounds were of interesting for their potential drug development. The cephalosporin was one of antibacterial drugs from marine fungus source (Burkhard, 2003). Didemnin, one of the cyclic depsipeptides, was isolated primitively from *Tridemnum*, which was one of marine ascidian with antiviral and antitumor activity (Schwartsmann *et al*., 2001). Other two antiviral compounds, avarol and avarone, were firstly purified from a sponge, *Disidea avara*. These antiviral compounds had high therapeutic index, crossed the blood-brain barrier easily and then inhibited the immunodeficiency virus (Sarin *et al*., 1987; Harnedy and FitzGerald, 2012). Bryostatins, as one kind of the most famous anticancer drugs, were the macrolides and isolated primarily from the bryozoans.

The bryostatin stimulated peripheral blood cells from lymphocytic leukaemia patients and sensitized protein kinase C, which would released arachidonic acid metabolites. During the past ten years, a lot of novel bioactive compounds had been isolated or purified from marine organisms. However, only very few of these bioactive compounds had been successfully industrialized to pharmaceutical products. At the same time, the diversity of marine microorganisms and their bioactivity, inter relationship between these microorganisms, or the relationship between the microorganism and host organism, were still not very clear. Therefore, it was very important to study the known or unknown microbial diversity of marine products and to search potential microorganisms with ability of secreting bioactive metabolites (Romanenko *et al*., 2008).

Over 30 000 compounds had been isolated from marine sources. In recent years, lots of useful bioactive compounds, possessing antibacterial, antifungal and antimycobacterial activities, were secreted from various marine bacteria, fungi and cyanobacteria (Habbu *et al*., 2016), including *Streptomyces* sp. (Arasu *et al*., 2013; Rashad *et al*., 2015; Selvin *et al*., 2004). However, this is the first report about to obtain a bacteria, identified to be *Streptomyces epidermidis,* from the interal of marine shark with secreting extracellular active substances to inhibit intensively the food pathogens, including G+ bacteria and G- bacteria. The aim of our study was to definite the growth characteristics, identified the strain and evaluate the potential antimicrobial metabolites.

Materials and Methods

Isolation and Screening of Bacteria

The strains were isolated from marine fishes internal organs collected from the Bay of the Wenzhou, the coastal of South Zhejiang province, in June and July 2015. The fishes were transported to lab at ice temperature within 4 h and immediately cut with a scalpel aseptically at super-clean bench. Small amounts of internal organs were take out and put in a sterile bag, homogenate in Man Rogossa Sharp (MRS) agar by refiner for 300 s (Ghosh *et al*., 2013). These samples were serially diluted for appropriate multiple and coated onto agar medium plates. Media for isolation were MRS, Marine 2216E Agar (MA) and Tryptic Soya Agar (TSA). The coated plates were incubated at 28, 30 and 37^{o}C, respectively. Different colonies were selected after incubation of 2, 3, 4 and 6 days and recoated on other agar plates to obtain pure cultures. The pure culture strains were kept in MRS broth with 30% (*v*/*v*) glycerol at -20°C.

All isolates were examined for antibacterial activity against of commonly food pathogen. The *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Shigella castellani* were used as indicators, which were obtained from China Center for Type Culture Collection (CCTCC).

Antimicrobial Activity Assay

Antimicrobial activity was assayed by using the Oxford cup assay method (Wang *et al*., 2010) to test inhibitory activity against indicator strains. This isolate strain was grown at 37°C for 48 h in MRS medium. The indicator strains of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Shigella castellani* was cultivated on the medium of Lysogeny Broth (LB) at 37°C. Also, sterilized MRS medium was used to be a blank for antimicrobial activity analysis.

Preparation of the Cell-Free Supernatants

The isolated strains were cultured in the culture fluid of MRS media for 48h at 37°C and 160 rpm. The culture solutions were centrifuged at 10,000 rpm for 10 min to obtain the Cells Free Supernatants (CFS) and then adjusted with 1N NaOH to pH 6.5 before filtered by 0.22 μm membrane (Furutani *et al*., 2014). About 100 μL indicator strains after overnight incubation at the appropriate temperatures was spread on the plates. The Oxford cup (Φ10 mm) was put on the plates and about 120 μL CFS was added in cup hole. Every sample was analyzed in triplicates. The Oxford cup plates were incubated at the optimal growth temperature of various indicator strains for 22-24 h to form the inhibit-zone surrounding the cups. The inhibited cycle diameter (mm) was used for evaluating CFS's antimicrobial effects. The MRS broth solution supernatant was used for control instead of CFS.

16S rDNA Gene Sequence Analyses

The genome was extracted by DNeasy Blood and Tissue kit according the manufacturer protocol (Axygen, USA) after the tested strain incubation overnight at 37°C in 5 mL MRS broth. A pair primer of 16S rRNA was designed and synthesized by Shanghai Songong Bioteck Company (Shanghai, China) based on the common 16S rRNA sequence of the test strain. The amplifications were conducted as following conditions: Initial denaturation at 95°C for 5 min; 35 cycles at 95°C for 30 s; annealing at 58°C for 30 s; extension at 72°C for 90 s. Finally, the amplified Polymerase Chain Reaction (PCR) products were analyzed in 1× TAE buffer at 90 V for 65 min; and gels were visualized by using gel documentation system (Alpha Imager) and recovery by use the agarose gel reclaim kit. The sequences homology were analyzed by the ABI3730-XL sequenator and compared with the Genbank database at National Center for Biotechnology Information (NCBI) accessible on-line.

Growth Curvature Determination

According to the linear relation between the microbial concentration and absorbance, the absorption value at 600 nm was used to describe the growth condition of the isolate strain. The isolate strain was inoculated in the test tube which contain 5 mL MRS culture fluid with a percentage of 2% stains culture and cultured at 37°C, 160 rpm and then the absorption value at 600 nm was measured at intervals of 6 h. The test was performed in triplicates. At the same time, the antimicrobial activity against Escherichia coli of the cell-free supernatants was analyzed by Oxford cup method described above.

Effects of Hydrolysis Enzymes, pH and Temperature on Antibacterial Activity

The Cell-free supernatants at pH 6.5 were treated with the following enzymes (1 mg mL^{-1}): Proteinase K (pH 7.0, Aladdin China), trypsin (pH 8.2, Merck, Germany), pepsin (pH 2., Aladdin China) and lysozyme (pH 6.5, Aladdin China). All these solutions were filter-sterilized and then added to supernatants (*v*/*v*, 1/1). Untreated CFS and enzyme solutions were used as controls. All samples were kept at 37°C for 30 min to test the antimicrobial activity against *Escherichia coli* by Oxford cup method described above.

To determine the activity at different pH levels, the CFS was adjusted with sterile 1N NaOH or HCl to different pH (from 2.0 to 10.0) and was kept at 37°C for 30 min. The pH-treated sample was neutralized to initial pH 5.5 and then tested for antibacterial activity.

To evaluate thermostability, aliquots of sterile cell-free supernatant were incubated at -20°C, 4°C, 30°C and heated to 60°C, 100°C for 30 min, immediately revert to room temperature and tested for antibacterial activity.

The residual antibacterial activities against the indicator bacteria for treated or untreated samples were determined by using agar well diffusion method described above. All experiments were performed in triplicate.

Results and Discussion

Isolation and Identification of Bacteria

A total of 259 single clones were obtained from the internal of seven marine fishes, including yellow croaker, bass, octopus, shark, pomfret, cuttlefish and sea eel. By Oxford cup diffusion assay, out of the 259 isolates, 24 isolates possessed antimicrobial activity and the result was shown as the Table 1. The S14 strain isolated from the internal of shark, showed stronger inhibition against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*, shown in Fig. 1. The inhibitory effect, which was observed clear and distinct zones around the Oxford cup, might be due to the production of several antibacterial compounds.

Until now, many bacteria isolated from the intestinal tract had been reported, most of these researches were about the bacteriocin from *Lactobacillus* which isolated from human or animal gastrointestinal tract and it had wide spectrum of inhibition against human pathogen (Sahnouni *et al*., 2015).

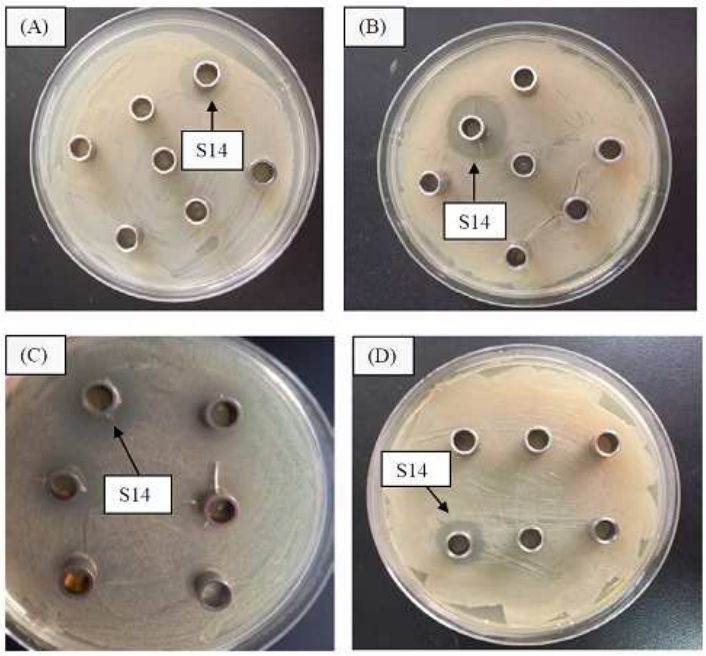

Fig. 1. The effects of *Staphylococcus epidermidis* S14 extracellular metabolites on several food pathogens. (A) *Staphylococcus aureus*; (B) *Escherichia coli*; (C) *Shigella Castellani*; (D) *Bacillus Cereus*

Table 1. The effects of metabolites screening by isolated strains on food pathogens

	Indicator bacteria			
Isolate strains	*Bacillus cereus*	*Shigella castellani*	*Escherichia coli*	*Staphylococcus aureus*
S20	+++	-	-	++
S14	+	++	+	++
S21	-	-	++	-
S16	-	+++	-	-
S3	+	-	-	-
S22	++	-	-	-
Z28A	++	+	-	-
Z28B	++	-	-	-
Z8	++	-	-	-
Z12	+++	-	-	-
Z17	++	-	-	-
Z15	++	-	-	-
Z16	++	++	-	+
Z1	-	-	-	++
Z24	+++	-	-	-
MU2	++	-	-	-
MU5	-	-	+	-
L16.2	++	-	-	++
M19	-	-	++	-
M23	+	-	-	-
G4	+++		+++	+
W3	-	++	-	-
H5	-	-	-	+
H7	-	-	-	++

a: +, inhibition zone <12 mm; ++, inhibition zone at 12-18 mm; +++, inhibition zone > 18 mm; -, no inhibition

The bacteriocins from *Lactococcus lactis* sp. *Lactis*, isolated from gastrointestinal tract of coastal fish sardine (*Sardina pilchardus*) and bug (*Boops*), show their ability against *Listeria innocua*, *Brochothrix thermophacta*, *Salmonella* sp., *Staphylococcus aureus*, *Bacillus cereus*, *Aeromonas hydrophila*, *Pseudomonas aeuroginosa*, *Escherichia coli* and Methycilin resistant *Staphyococcus aureus* (Sahnouni *et al*., 2015). The *Enterococcus faecium* isolate from the gut of poultry expressed protease sensitive antibacterial peptides (Wang *et al*., 2010), which showed stronger inhibition against the growth of *M. flavus, E. coli, Staphylococcus aureus, Streptococcus agalactiae* and *Salmonella pullorum*. Therefore, the strain S14 had potentially appilication in aquaculture feeding (Tengku *et al*., 2012).

Strain S14 Identification

The DNA sequences of strain S14 were amplified and the product were test in 1% agarose gel electrophoresis to confirm the PCR amplification fragment, shown in Fig. 2. Then, the DNA of strain S14 was recovered and was used to analysis the sequences. The sequences of the 16S rDNA was analyzed by the ABI3730-XL sequenator, submitted the result to Genbank database at National Center for Biotechnology Information (NCBI). Partial rDNA sequences of strain S14 showed high homology with the 16S rDNA sequences from Genebank database. The phylogenetic tree was constructed, shown in Fig. 3. The strain S14 showed the highest homology (>99%) with *Staphylococcus epidermidis* strain O1.

The genus *Staphylococcus* contained more than 30 species, including *S. epidermidis* and *S. aureus* and *S. pasteuri* (Hong *et al*., 2014). *S. epidermidis* are usually found on the skin of organisms, different from the *S. aureus*. The *S. epidermidis* as an opportunistic pathogen are the normal flora and the *S. aureus* are thought to be a primary target of food contaminate. It was well known that *Staphylococci* produced a wide range of antibacterial substances including bacteriocins, bacteriolytic enzymes, peptide antibiotics and polythiazoles (Vandecandelaere *et al*., 2014). *S. epidermidis* secreted various cytotoxins and enzymes, such as staphyolysin, enterotoxins, leukocidin, Toxic Shock Syndrome Toxin (TSST-1) and coagulase. Furthermore, it also produced the *Staphylokinase, Staphylococcal fibrinolysin*, Heat-stable nuclease, hyaluronidase and lipase. So far as know, the report about *S. epidermidis* isolates from the internal organs of the fish with antimicrobial activity against human pathogenic bacteria was rarely. Though, Vandecandelaere *et al*. (2014) reported the *S. epidermidis* produced protease with obvious effects on *S. aureus* biofilms. Additional, the isolates of *S. epidermidis* TYH1 from Japanese fish-miso also produced a histidine decarboxylase, which were elucidated using native purified enzyme (Furutani *et al*., 2014).

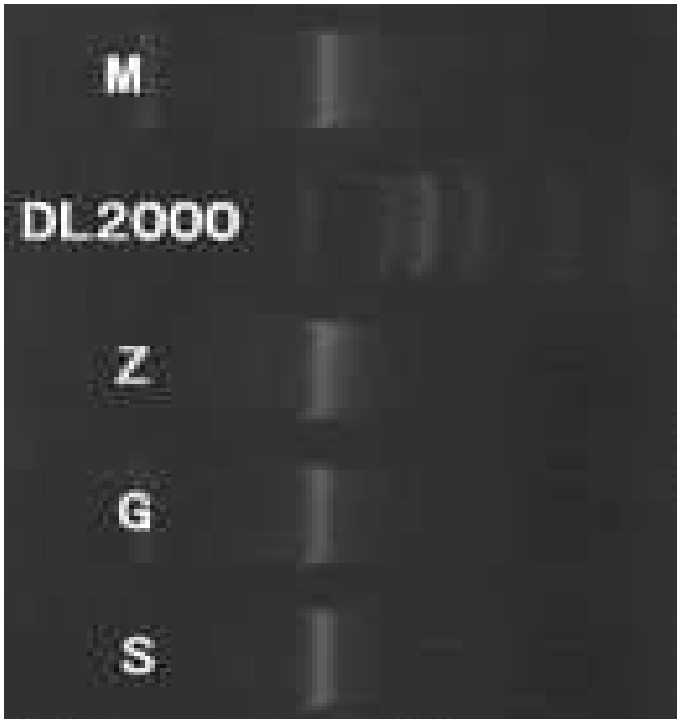

Fig. 2. The electrophoretogram of amplified DNA fragment from *Staphylococcus epidermidis* S14. Lane M: strain M19 genomic DNA; Lane DL2000: Gene Ruler™ DNA Ladder Mix (Fermentas); Lane S: strain S14 genomic DNA; Lane Z: strain Z16 genomic DNA; Lane G: strain G4 genomic DNA

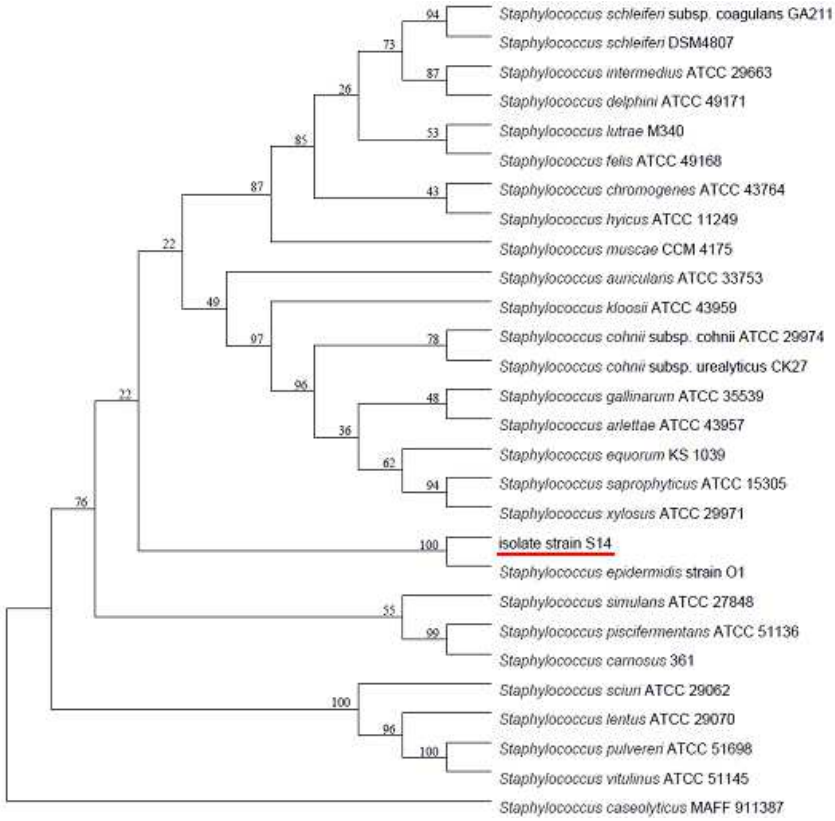

Fig. 3. Phylogenetic tree of *Staphylococcus epidermidis* S14

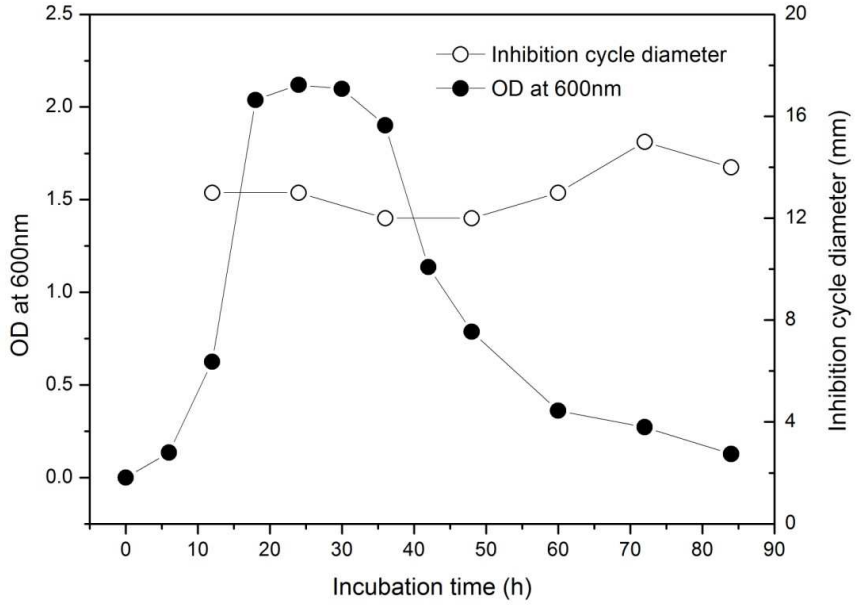

Fig. 4. The growth curvature of *Staphylococcus epidermidis* S14

Table 2. The antibacterial activity of *Staphylococcus epidermidis* S14 extracellular metabolites against *Escherichia coli* affected by digestion enzymes

Enzyme	CK	Proteinase K	Trypsin	Pepsin	Lysozyme
Inhibition diameter (mm)	12	0	14	15	12

Table 3. The antibacterial activity of *Staphylococcus epidermidis* S14 extracellular metabolites against *Escherichia coli* affected by pH

pH	2	3	4	5	6	7	8	9	10
Inhibition diameter (mm)	10	10	10	11	27	12	11	11	12

Table 4. The antibacterial activity of *Staphylococcus epidermidis* S14 extracellular metabolites against *Escherichia coli* affected by temperature

Temperature (°C)	-20	4	30	60	100
Inhibition diameter (mm)	10	10	13	16	14

Growth Curvature of Staphylococcus epidermidis S14

The *Staphylococcus epidermidis* S14 growth curvature was shown in Fig. 4. The results showed that the strain active growth period was the incubation time for 18-36h. The change in antibacterial activity in Fig. 4 also improved that the strain secreted rarely active substance in the stabilization period, mainly of the bacterial reproduction and the antibacterial active substances might be the secondary metabolite for the microorganism.

Most of the active substance obtained from the microorganism were the secondary metabolites. These microorganisms biosynthesized many complex secondary metabolites for competition for space, predation, symbiosis or tide variations during thousands of years evolution, which in turn improved their adaptation ability to natural or hostile environment (Amraoui *et al*., 2014). The existing studies had shown that metabolite produced by the microorganism attach to the marine products be possessed of a great antibacterial activity against the pathogenic bacteria (Thompson *et al*., 1985).

Partial Characteristics of Antibacterial Metabolites from Staphylococcus epidermidis S14

The antibacterial activity of the antibacterial substances was lost completely when treat with proteinase K and was resistant to trypsin, pepsin and lysozyme, shown in Table 2. The control sample and enzymes sample show no loss of activity. The treatment of the antibacterial substances by proteases suggested that this compound could be a complicated mixture which might contain peptides and could be hydrolyzed by trypsin, pepsin and lysozyme or bacteriocin, like the

enterocin produced by *Enterococcus gallinarum* (Jennes *et al*., 2000) and bacteriocin produced by *Lactococcus* sp. (Yildirim and Janhson, 1998), which were sensitive proteinase K. Furthermore, the active substance may be selective to the source of the enzyme because the proteinase K extract from microorganism and others from the animals or human.

The activity of the antibacterial substances could be detected in the culture supernatant after it was adjusted to pH 2.0. The residual antibacterial activity was remained relative high level over a wide pH range from 2.0 to 10.0 (Table 3). The highest antibacterial activity against *Escherichia coli* was recorded for the pH adjust to 6.0, which indicated the antibacterial metabolites showed a strong antibacterial activity in the neutral or acidic environment. The metabolites of *Staphylococcus epidermidis* S14 with good antibacterial activity at wide pH range might be advantageous, because the pH was acidic (about pH 2.0) in stomach and pH 7.0-8.0 in the large intestine, respectively. On the point of probiotic application, the *Staphylococcus epidermidis S14* could grow and produce antibacterial metabolites at low pH, which might be the advantages in survival at intestinal tract environment (Toit *et al*., 2010).

The antibacterial activity of S14 culture was slightly lower when incubating at -20 and -4°C for 30 min comparation with the control kept at 30°C, which indicated that it kept a weaker antibacterial activity in the low temperature. The substances possessed a strong antimicrobial activity at 60°C when compared with a control, that indicating the antibacterial activity was most sensitive to heat. However, the substances maintained relative high residual antibacterial activity when heat treatment at boiling temperature for 30 min (Table 4) and could therefore be deemed to be heat stable. Accordingly, the strains isolate from marine fish may be of great interest as a probiotics for veterinary and food applications.

Conclusion

In this study, a strain S14 with antibacterial metabolites screening ability was isolated from the internal organs of shark and the fermentation cell-free supernatants of strain S14 had a visible antimicrobial activity against to test stains. The strain S14 was identified by 16S rDNA to be *Staphylococcus epidermidis* and this strain produced broad spectrum antibiotic active substance. This finding maybe the first report about the *S. epidermidis* isolate from the internal organs of the shark and have the potential to produce useful antibacterial metabolites. The antibacterial metabolite from strain S14 cultures was stable to most digestion enzymes, except for proteinase K and also it had higher activity at neutral pH and was stable to thermal till in boiling water for 30 min. These characteristic improved that the antibacterial metabolite possessed potentially important future useful for food bio-preservation. Certainly, the properties of the antibacterial metabolites in details, purification and antibacterial mechanism should be researched in the next studies.

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Author's Contributions

Jie Zhang: Performed the experiments and wrote the manuscript.

Han Jiang: Read and improved the manuscript.

Jiaxin Jiang and Guangrong Huang: Developed the idea and advised in experimental methods.

Ethics

The authors declare that they had no conflict of interest.

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