Original Article

ISOLATION AND PURIFICATION OF LOW MOLECULAR WEIGHT PEPTIDE FROM MARINE B.cereus AND IT’S ANTIMICROBIAL ACTIVITY

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Abstract

Many marine sponges contain dense, highly diverse microbial communities. In the present study, totally eight associated bacterial strains were isolated from sponge namely Hyattella cribiformis. Isolated associated bacterial strains were tested for the antibacterial activity against human bacterial pathogens such as Staphylococcus aureus, Vibrio cholerae, Salmonella paratyphi, Klebsiella pneumoniae, Salmonella typhi, Proteus mirabilis, Klebsiella oxytoca, E.coli, Pseudomonas aeruginosa and Lactobacillus bulgaricus on Muller Hinton agar plates. The most potential strain was selected based on their antibacterial activity and it was characterized by biochemical tests and 16S rDNA sequencing. Phylogenetic analysis indicated that the strain has high sequence similarity with Bacillus cereus. It was designated as Bacillus cereus SBS02. An antagonistic low molecular weight protein was purified from the culture supernatant using centrifugation filters, having cut off value of 10 kDa and gel filtration chromatography. A low molecular weight antibiotic peptide of 8KDa was obtained and analyzed by 15% SDS-PAGE. It was inhibitory to human pathogens and food-spoilage bacteria, such as C. albicans, Listeria monocytogenes and Pseudomonas aeruginosa, B. cereus, B. subtilis and other LABs viz, L. bulgaricus and L. vulgaris. The purified protein was thermostable as well as tolerant to wide range of pH. Based on the properties (inhibition of closely related spp. and stability) it was confirmed as class II bacteriocin produced by B.cereus and designated as Cerein SBS02.

Keywords: Sponge, Bacillus cereus, BLIS, antimicrobial activity.

1. INTRODUCTION

Microbial associates of sponges gained significance as source of bioactive compounds. Remarkable similarity was found between those compounds isolated predominantly from sponges and those found in terrestrial organism of entirely different taxa. It is hypothesized that symbiotic marine microorganism harbored by sponges are the original producers of these bioactive compounds (Proksch et al., 2002; Zhang et al., 2005). Bacteria produce a variety of antimicrobial substances that are able to kill or inhibit other microorganisms. Bacterial antibiotics can be subdivided into two types on the basis of their chemical nature: (i) non-peptide antibiotics and (ii) peptide antibiotics. One group comprises non-ribosomally synthesized peptides produced on large enzymatic complexes (eg: surfactin) (Carrillo et al., 2003). The second group comprises ribosomally synthesized peptides (i.e. bacteriocins). Bacteriocins produced by Gram-positive bacteria exhibit a number of characteristics that make them attractive for both the food industry and biomedical applications (Bower et al. 2001; Chen and Hoover 2003). Several bacteriocins or bacteriocin-like substances (BLIS) produced by the genus Bacillus have been reported eg., a bacteriocin of B. brevis (Hyung et al., 2001), subtilin by B. subtilis (Klein et al., 1992), thurin 7 by B. thuringiensis (Cherif et al., 2001) and many others. In the last few years, the number of scientific reports dealing with Bacillus antimicrobial peptides classifiable as bacteriocins has appreciably increased. The newly characterized bacteriocins include the lantibiotics ercin A (2986 Da) and ercin S (3342 Da) produced by B. subtilis (Stein et al., 2002); cerein 7A (3940 Da) and cerein 7B (4893 Da) from Bacillus cereus (Oscariz et al., 1999; Oskouie et al., 2006) and the subclass IIa peptide SRCAM 1580 (3486 Da) produced by Bacillus circulans (Svetoch et al., 2005).

In this research article, isolation of sponge associated bacteria, purification of low molecular weight peptide and its anti microbial potential of have been discussed.
2. MATERIALS AND METHODS

2.1. Isolation of bacteria associated with marine sponge

The sponge samples soon after collection were transferred to a sterile polyethylene bag and transported at 4°C to the laboratory for the isolation of associated microbes. On reaching the laboratory, the invertebrate was brought to room temperature and cut aseptically into small pieces (2x2cm) using a sterile scissors. The pieces were freed from adhering particles by vortexing twice for 20 sec. with 2ml of sterile seawater. The seawater was decanted, which was once again replaced with sterile seawater with continued vortexing between washings. Finally, sample in sterile seawater was homogenized using sterilized mortar and pestle in a laminar flow chamber. The homogenate was serially diluted up to 10^{-6} dilutions using sterile 50% aged sea water blanks and plated on the surface of Zobell marine agar plates. The plates were incubated at room temperature for 24-48hrs.

2.2. Primary screening for antimicrobial activity

2.2.1. Antagonistic Assay against bacterial pathogens

Antagonistic assay was done by an agar-well diffusion method in aerobic condition. Isolated bacterial strains were tested for the antibacterial activity. Human bacterial pathogens such as Staphylococcus aureus, Vibrio cholerae, Salmonella paratyphi, Klebsiella pneumoniae, Salmonella typhi, Proteus mirabilis, Klebsiella oxytoca, E.coli and Pseudomonas aeruginosa were spreaded on Muller Hinton agar plates. The pathogenic strains were obtained from CMC, Vellore. Then wells were made and 50µl cell free extract of each sponge associated bacterial strain was inoculated in to separate wells. Antagonistic activity was detected after an incubation period of 24-48 hrs at 35°C. The presence of zone of clearance on agar plates was used as an indicator for the antibacterial activity. The strain which showed the maximum zone of clearance was chosen for further study. The presence of zone of clearance on agar plates was used as an indicator of bioactive potential of the strain (Portait et al., 1999).

2.3. Identification of bacteria

Potential strain was selected based on antimicrobial activity which was identified biochemically and 16S rRNA partial sequencing. Morphological characters were observed under a microscope and all the organisms were biochemically identified up to the species level by following Bergey’s Manual of determinative bacteriology (Buchanan et al., 1974).

2.3.1. 16S rDNA based identification of bacterial spp. using PCR

DNA extraction

DNA for PCR amplification was extracted from the bacteria by placing a single colony from media plates into 50µL of sterile distilled water and heat shocking the sample for 5 min. at 95° C to lyse the cells, releasing the DNA into the water. The sample was then centrifuged to remove cellular debris. The supernatant was transferred to a new tube and stored at -20°C.

The genomic DNA extracted from bioactive compound producing potent strain was PCR amplified for 16S rRNA genes using the universal bacterial primers Eubac27F (5'-AGA GTT TGA TCM TGG CTC AG- 3') and 1492R (5'-GGT TAC CTU GTC ACG ACT T-3'). This primer combination amplifies a 1500bp 16S rDNA fragment (Weisburg et al., 1991). Amplification reaction was performed in a 0.2 mL optical-grade PCR tube (Tarsons, India). 50 nanogram of DNA extract was added to a final volume of 50µL of PCR reaction mixture containing 1.5mM MgCl_{2}, 1X Reaction buffer (without MgCl_{2}) (Fermentas), 200µM of each dNTPs (Fermentas), 100pM of each primer and 1.5U Taq DNA polymerase (Fermentas). PCR was performed in an automated thermal cycler with an initial denaturation at 95°C for 5min. followed by 30 cycles of 95°C for 30sec., (denaturation), 52°C for 45sec., (annealing), 72°C for 90 sec., (extension) and 72°C for 10min., (final extension). PCR product was run on 1% agarose in TAE buffer (40mM Tris, 20mM Acetic acid, 1mM EDTA (pH8.0) to confirm that the right product (1500bp) was formed. The PCR product was purified using the QIAGEN PCR purification kit for sequencing and further analysis (Weisburg et al., 1991).

2.4. Purification of low molecular weight peptide

Bacillus cereus SBS02 was cultivated aerobically in 500 ml Erlenmeyer flasks containing 150 ml of Zobell marine broth at 30°C, 180rpm for 48 hrs. Cells were harvested by centrifugation at 10,000 rpm for 30 min at 4°C and the resulting supernatant was separated by the centrifugation filters, centripepes (Millipore, India) having cut-off value of 10 kDa, by centrifugation for 30min. (3000xg at 4°C). After separation the filtrate protein (< 10 kDa) was checked in 15% SDS-PAGE. The filtrate was then lyophilized to store at -20°C for further use.

2.5. Gel filtration chromatography

The lyophilized powder of low molecular weight peptides was resuspended in 10 mmol/L phosphate buffer (pH 7.2) and sample was applied to a column of Sephadex G-10 (SIGMA, USA), eluted with PBS containing 1.5mol/L NaCl. Fractions were collected and absorbance (OD) was measured at 280 nm. The fractions showed same OD value was checked on 15% SDS-PAGE and the same were checked for their antimicrobial activity against C. albicans, Listeria monocyogenes and Pseudomonas aeruginosa, other B. cereus, B.subtilis and other LABs viz, L. bulgaricus and L. vulgaris. Active fractions were pooled together and lyophilized.

3. RESULTS

The sponge Hyattella cribiformis was analyzed for associated bacterial population. In Hyattella cribiformis bacterial density was in the range of 6.14x10^{7} to 1.4x10^{8} CFU/g. Out of 4 isolates subjected for primary screening process, only 2 isolates showed activity against all the test organisms. Of which, only SBS02 was found to be more inhibitory compared to the strain SBS04. Hence it was selected for further study.

Among 4 strains tested strain SBS02 showed many interesting features. The cell free extract of the strain showed wide spectrum of activity. Among 10 pathogens tested most of them were inhibited. The most inhibited pathogen was Staphylococcus aureus (13mm) followed by Salmonella typhi (11mm), Vibrio cholerae (10mm), Salmonella paratyphi (10mm), Lactobacillus bulgaricus (10mm), Klebsiella oxytoca (9mm), Klebsiella pneumoniae (9mm), Pseudomonas aeruginosa (10mm), Acinetobacter baumannii (10mm), Escherichia coli (10mm), Proteus mirabilis (10mm), Staphylococcus epidermidis (10mm), Staphylococcus warneri (10mm), Salmonella enteritidis (10mm), Listeria monocytogenes (10mm), Lactobacillus rhamnosus (10mm), Lactococcus lactis (10mm) and Staphylococcus saprophilus (10mm).
(9mm), *Pseudomonas aeruginosa* (8mm), *Proteus mirabilis* (7mm) and *E.coli* (5mm) (Table:1).

The most potential strain was identified using biochemical tests as per Bergey’s Manual of Systemic Bacteriology as well as 16s rDNA sequencing. The potential strain was motile, spore forming, gram-positive, rod shaped bacterium and was identified as *Bacillus cereus* (Table:2 and Fig:1). The name of the strain was designated as *B. cereus* SBS02. In the present study a low molecular weight antibiotic peptide 8KDa was obtained and analyzed by 15% SDS-PAGE (Fig:2). It showed activity against *C. albicans* (15mm), *Listeria monocytogenes* (13mm) and *Pseudomonas aeruginosa* (7mm).

The 8KDa protein isolated in the present study inhibited other *B. cereus* (20mm), *B.subtilis* (8mm) and other LABs viz, *L. bulgaricus* (21mm) and *L. vulgaris* (8mm) (Fig:3). The result confirmed that it might be a bacteriocin compound produced by this organism.

**Fig: 1 Phylogenetic tree view**

## 4. DISCUSSION

Marine bacteria have been recognized as an important and untapped resource for novel bioactive compounds. The chemical compounds of marine microorganisms are not well known as terrestrial counterparts. However, in the last decade several bioactive compounds have been isolated from marine bacteria and are new resources for the development of medically useful compounds (Donia and Haman, 2003; Anand et al., 2006). Antibacterial activity among marine bacteria is a well-known phenomenon and has been demonstrated in a number of studies (Isnansetyo and Kamei, 2003; Uzair et al., 2006). In the present study all the sponge associated bacterial isolates from *Hyattella cribriformis* were screened for antimicrobial potential by agar-well diffusion assay. The most potential strain was selected based on their antibacterial activity against human pathogens and identified using biochemical tests as per Bergey’s Manual of Systemic Bacteriology as well as 16s rDNA sequencing. The name of the strain was designated as *B. cereus* SBS02.
It presents a broad spectrum of microorganisms. Reportedly, mersacidin, a bacteriocin produced by Bacillus sp. HIL Y-85, inhibits the growth and colonization of methicillin resistant Staphylococcus aureus and Staphylococcus epidermidis. Similarly, Osorio and Pisabarro, 2000 reported bacteriocin cerein 7 from Bacillus cereus which was found to inhibit the growth of Staphylococcus aureus. Therefore, mersacidin, a lantibiotic produced by Bacillus sp. HIL Y-85, 54728 inhibits the growth and colonization of methicillin resistant Staphylococcus aureus (Kruszewksi et al., 2004). Beke et al., 2007 also reported about bacteriocin, cerein MRX1, produced by B. cereus MRX1. The molecular mass of cerein MRX1 as determined by MS was 3.1 kDa. Similarly, Naclerio et al., 1993 reported that the production and activity of cerein produced by B. cereus was recorded at the stationary growth. Some bacteriocins from Bacillus present a narrow antimicrobial spectrum. Oscariz and Pisabarro (2000) isolated and identified cerein 7, a bacteriocin produced by B. cereus Bc7 that was inhibitory for Listeria spp. and other Gram-positive bacteria. Coagulin, a bacteriocin-like substance produced by B. coagulans 14, has been reported (Hyronimus et al., 1998). It presents a broad spectrum of antibacterial activity, inhibiting strains of the same species as the producer strain. However the peptide isolated in the present study did not inhibit the producer strain.

5. CONCLUSION

Thus the present study showed the bioactive potential of sponge associated bacteria especially that of the most potential strain Bacillus cereus SBS02. The low molecular weight peptide (i.e.) 8kDa was found to be a class II bacteriocin active against C. albicans, Listeria monocytogenes, Pseudomonas aeruginosa, B. cereus, B. subtilis, L. bulgaricus and L. vulgaris. The peptide cerein SBS02 may be used in food industries as well as a human therapeutic protein.

REFERENCES


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