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ISOLATION, PURIFICATION AND CHARACTERIZATION OF ANTIBACTERIAL PEPTIDE FROM SOIL IN PANVEL REGION

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Abstract:

Microbes contend for the limited space and nutrients present in natural ecological niches, thus they've developed several strategies in order to survive. Production of 'antimicrobial peptide' best known as 'Bacteriocin' is one of them. Bacteriocins extensively distributed in nature which are synthesized by bacteria. The present study was carried out to estimate naturally synthesized antimicrobial peptides and their capacities to inhibit Gram-positive pathogenic organisms. Ten bacterial spp. were isolated and screened to produce antimicrobial peptide. Antimicrobial peptide produced by two of bacterial strains showed the most significant antimicrobial activity against Staphylococcus aureus. Bacteriocins have shown significant activity at 370 with pH 7 Proteolytic enzymes verified the proteinaceous nature of antimicrobial peptides. These partially purified bacteriocins can be further used as antimicrobial agent or it can be used as food complements to control food borne pathogens.

Keywords: *Antimicrobial peptides, Bacteriocins, S. aureus, food complements*

1. Introduction

Microbes struggle for the limited space and nutrients present in natural environment, thus they have developed several strategies in order to survive. Production of 'antimicrobial peptides' is one of them. These peptides are known as 'Bacteriocins'. Gram-positive bacteria, and mainly lactic acid bacteria (LAB), are now being extensively studied for their production of Bacteriocins [1].

Bacteriocins are synthesized antimicrobial bacteria are extensively distributed and diverse in nature. This peptide diversity is supported by several differences in their structures. All constitutively synthesized peptides share a net positive charge which causes them to fold into an amphiphilic conformation upon interaction with bacterial membranes [2].

Bacteriocins generally have low molecular weight; they undergo post translational modification and can be easily degraded by proteolytic enzymes especially by the proteases of the mammalian gastrointestinal tract, which makes them safe for human consumption. Bacteriocins are in general cationic, amphipathic molecules as they contain an excess of lysyl and arginyl residues [8,9].

The increased consumption of foods containing additives formulated with chemical preservatives and consumer concerns have created a better demand for more natural and minimally processed foods, therefore there is a high interest in naturally produced antimicrobial agents that do not produce adverse effects. It have

also potential applications in health care sectors have attracted the interest of academia and industry resulting in increased research on antimicrobial peptide production, purification, genetics, and applications.

The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient [10].

The present study was undertaken to evaluate naturally occurring antimicrobial peptides for their abilities to inhibit Gram-positive test organisms.

Significance of the present study

The increased demand for natural and minimally processed foods generates high interest in naturally produced antimicrobial agents that do not produce side effects. Bacteriocins also have the applications in health care sectors.

Despite tremendous progress in medicine, infectious diseases caused by bacteria, fungi, and viruses are still a serious threat to public health. Their impact is particularly large in developing countries thanks to the shortage of access to medicines and therefore the emergence of widespread drug resistance. Increasing numbers of microbial pathogens that have acquired antibiotic-resistance have resulted in the increased demand for novel and effective antimicrobial compounds. In particular, studies evaluating the anti-microbial actions of marine peptides are attracting increased researchers' interest, and antibacterial peptides have been increasingly considered as anti-microbial drugs. The diversity of the marine environment has provided a unique source of bioactive chemical compounds that could lead to potential new drug candidates.

Knowledge of antimicrobial peptide producing bacteria is of significant importance with respect to its food preservative and antimicrobial properties. The antimicrobial peptides are a promising source for brand spanking new generation antibiotics. Present study is an attempt to find unique antimicrobial peptide from bacteria. This could be used as an antimicrobial agent or it can be used as food additives to control food borne pathogens.

2. Materials and Methods:

2.1. Collection of Soil Samples

A total of 10 soil samples were collected from different areas from Panvel, Mumbai, and Maharashtra, India.

2.2. Screening of bacterial isolates

Bacterial strains were isolated from soil samples using the standard protocol. These bacterial strains were screened for antimicrobial peptide production by crowded plate technique. In this technique, soil samples were suspended and serially diluted in sterile saline solution (0.9% NaCl). Tubes containing 0.1 ml of appropriately diluted solution were spread on nutrient agar plates and were incubated at room temperature for 24 hrs. After incubation 0.1 ml of overnight culture of *S.aureus* was mixed with Wilkins agar which was overlaid on spotted plate and incubated at RT for 24 hr. The antimicrobial peptide producer shows clear zones of inhibition in an opaque background.

2.3. Agar well diffusion assay

Antibacterial activity was confirmed by agar well diffusion assay. After the incubation time period, the bacterial colonies exhibiting clear zones around them were selected for further investigations. The strain which was selected as potential antimicrobial peptide producers were grown in nutrient broth at 37ºC for 24

hrs. Cells were separated by centrifugation at 10,000 rpm for 10 min at room temperature. Around 6 mm diameter wells were made on pre inoculated Mueller-Hinton agar media and each well was 100 µl of culture supernatant added. Inhibitory activity was performed against *Staphylococcus aureus*. Inhibition zones around the wells were measured and recorded. The bacterial growth and the extent of clearing zone was measured in millimeters.

2.4. Identification of antimicrobial peptide producing bacteria

The Selected bacterial isolates were purified and plated on NB agar plates. Morphological characteristics were studied for pure single colonies for their characterization and biochemical tests were performed as per procedures outlined in Bergey's Manual of Systematic Bacteriology. The purified antibacterial peptide producing bacteria were identified up to the level of genus by means of morphological and biochemical test like sugar utilization test, Catalase test, Sugar fermentation test, Indole test, Citrate test, Urease test, methyl red test, Voges–Proskauer test.

2.5. Effect of growth period

Mueller-Hinton broth was inoculated with 1 % of an overnight culture of Isolate 3 and isolate 9. The cultures were incubated at 37^0c in static condition. At appropriate intervals samples were removed for measurement of biomass by absorbance at 600 nm. The antibacterial activity was estimated by spot lawn assay method

2.6 Effect of temperature and pH

Effect of pH and temperature were studied on antibacterial peptide production. This was performed with 100 ml MH broth. The mixture of isolate(1 % v/v) and MH broth was inoculated with an overnight culture and incubated at different temperatures(25,37 and 45^0 C) and pH(3,5,6,7and 9) and incubated. Samples collected after 24 hr were examined for antibacterial activity.

2.7 Effect of proteinase K

1 ml culture supernatants were mixed with 0.5 ml of proteinase K and incubated at 37°C for 3 h. After enzyme treatment, antimicrobial activity of the supernatant was checked with *S.aureus* in comparison with the untreated sample.

2.6 Partial Purification of antimicrobial peptides

The isolated cultures were grown in NB broth for 30 hours until turbidity was observed. Cell free supernatant was obtained by centrifuging the broth in a cooling centrifuge for 10 minutes at 6000 rpm. The Cell free supernatant was purified using ammonium sulphate precipitation. The cell free supernatants were saturated with 80% and 100% ammonium sulphate solution and the suspended protein was obtained as a pellet. Finally, the pellet was dissolved in phosphate buffered saline (pH 7). And it was dialysed in a magnetic stirrer in Phosphate buffered saline (PBS) for 12 hours changing the buffer for every 3 hours.

3. Results and Discussions:

3.1. Collection of Soil Samples

Soil samples were collected during Jan to June 2019 from different habitats in Panvel region including Uran, Panvel, Nevale, Shivkar, Nere and Wakadi. Samples were collected from crop fields. A total number of 15 soil samples were collected from different areas in and around Panvel region. The samples were screened to isolate for antibacterial peptide producing abilities.

3.2. Screening of bacterial isolates

A total of 9 bacterial isolates were isolated from these soil samples and maintained as pure cultures. Two bacterial isolates that showed antibacterial activity were selected for further studies. The isolates 3 and 9 showed a greater clear zone around the colony on Mueller Hinton agar plate. The appearance of clearing zones around the colonies of the organism is the indication of the presence of antibacterial producing organisms.

3.3. Agar well diffusion assay

Antibacterial activity was confirmed by agar well diffusion assay. Two out of 9 isolates show greater zones of inhibition against *S.aureus*. The isolates 3 and 9 showed a clear zone around the colony on Mueller Hinton agar plate. The appearance of clearing zones around the colonies of the organism is the indication of the presence of antibacterial producing organisms. Isolate 3 and Isolate 9 show inhibition of around 13mm and 12 mm against *Staphylococcus aureus* respectively.

3.4. Identification of antimicrobial peptide producing bacteria

Characterization of antimicrobial peptide producing bacteria on the basis of cultural, morphological and biochemical characteristics along with sugar utilizing ability was determined according to Bergey's manual. The soil isolate 3 was identified as Gram positive *Streptococci* while the soil isolate 9 was identified as Gram negative *Escherichia coli.*

3.6 Effect of temperature and pH

Influence of growth conditions on antimicrobial peptide studies revealed that incubation temperature and pH plays an important role in their cell growth and antimicrobial production.

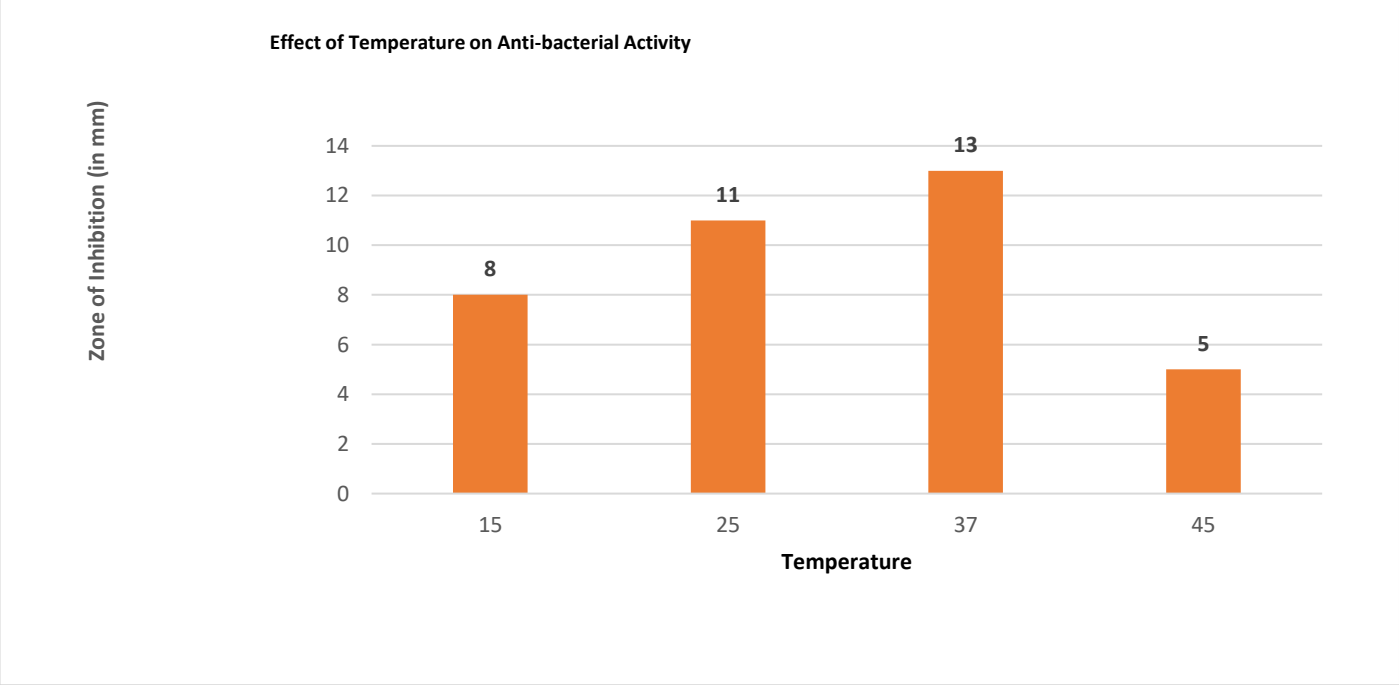

Fig 2. Effect of Temperature on Anti-bacterial Activity

The antimicrobial peptide production was found to be most at 37^0C while it shows lowest at 45^0C (Fig. 2).

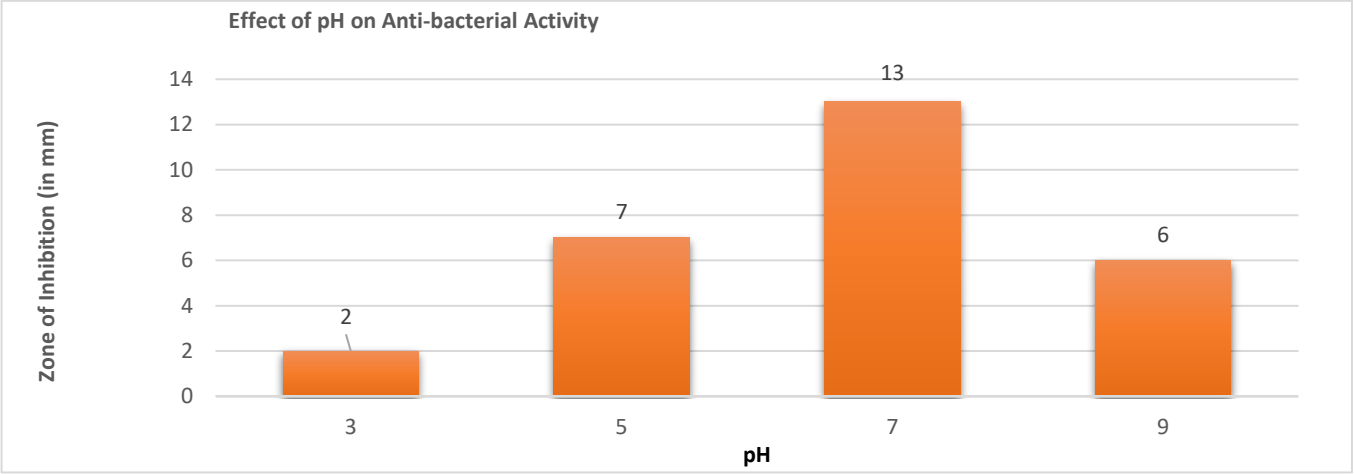

Fig 3. Effect of pH on Anti-bacterial Activity

The antimicrobial peptide production was found to be most active at pH 7.0 while it showed less activity at pH 3.0 and pH 9.0. (Fig. 3).

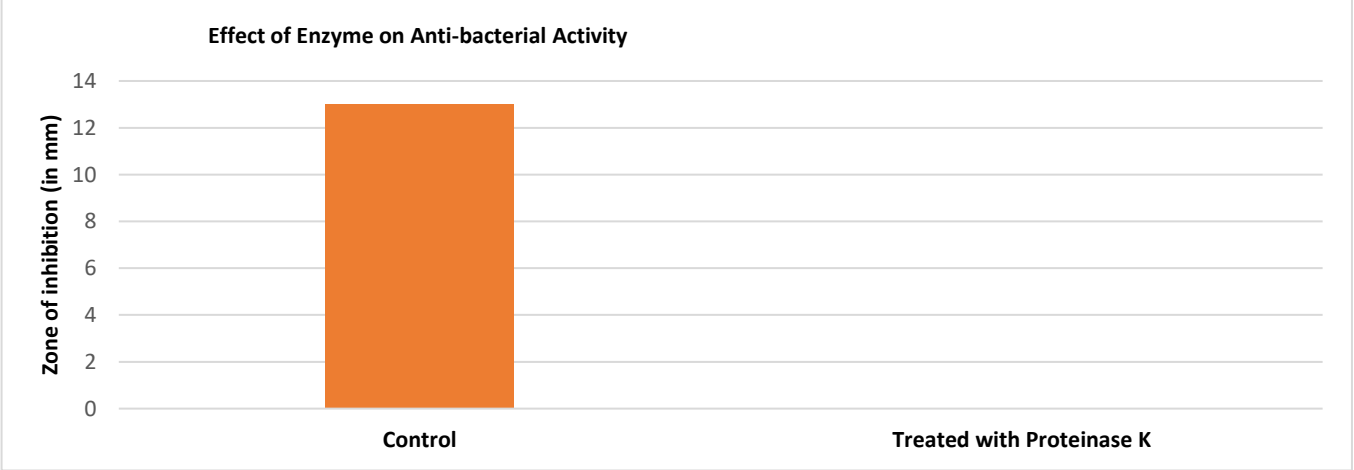

Fig 4. Effect of Enzyme on Anti-bacterial Activity

The proteinaceous nature of antimicrobial peptides was confirmed by treating their sensitivity with proteolytic enzymes. The antimicrobial peptide shows complete inactivation of antimicrobial activity after the treatment with proteinase K (Fig. 4).

2.6 Purification and characterization of antimicrobial peptides by ammonium sulfate precipitation and dialysis.

The antimicrobial peptide partially purified by ammonium sulphate precipitation was tested for antimicrobial activity by agar well diffusion assay. During this, partially purified antimicrobial peptide from isolate 3 and 9 showed antimicrobial activity against S.aureus. The antimicrobial activity was found to increase after purification.

The antimicrobial activity of supernatants by centrifuging the colonies in broth at 10000 rpm for 10 minutes. Isolate 3 and Isolate 9 shows inhibition of around 15mm and 13 mm against Staphylococcus aureus respectively

The zone of inhibition of test organisms indicates that the growth of test organisms has been halted by the supernatant containing antimicrobial compounds.

4 Conclusions:

In this study, antimicrobial peptide produced by two of bacterial strains showed the most significant antimicrobial activity against Staphylococcus aureus. These isolates can be ideal candidate for bacteriocin production or use.

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