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ISOLATION, IDENTIFICATION AND RAPID SCREENING OF BACTERIOCIN PRODUCING STRAIN FROM FRESH FOOD AND ANIMAL SAMPLES

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Abstract – The demand of bacteriocins has been increased due to their strong antimicrobial activity against various food spoiling and food-borne pathogens. The present research work has focused upon the isolation, identification and rapid screening of potent bacteriocin producing microorganism from different fruits, vegetables and animal samples. Collected samples were screened for bacteriocin producing strains. Isolates were analyzed for its antimicrobial potential and identified by biochemical characterization. The most potent isolate was then subjected to rapid identification of bacteriocin by LCMS analysis.The strain isolated from Mango, chick intestine and rectum of goat were selected as the potent bacteriocin producing isolates. Among them the strain isolated from the source of mango showed highest zone of inhibition against both gram positive and gram negative food spoilage pathogens, i.e. *Shigella, Salmonella* and *Listeria.* The strain was identifiedas *Bacillus* sp.through biochemical characterization. Crude extract of production medium inoculated with *Bacillus* sp. was then subjected to LC-MS analysis for rapid identificationof bacteriocin where it showed the presence of partial sequence of GPGTSAVGTGHVQK which was found to be overlapping with Linocin M18.LCMS was used as a rapid identification tool for the presence of bacteriocins. The bacteriocin produced by *Bacillus* sp. can be exploited by various food industries considering its worthy characteristics.

INTRODUCTION

The trade of healthy food and its demand for human welfare endorse the researchers to find novel and potent antimicrobial compounds for food preservation. Because the production of antimicrobial compounds/peptides are the primarily stage of defense mechanism and are formed in all domains of life including Bacteria, Archaea and Eukaryotes. In human beings these peptides or proteins play a very significant role in innate immunity system where they protect against various predominant microorganisms i.e. bacteria, fungi, yeast and viruses (Reddy *et al*., 2004). The antimicrobial peptides produced by bacteria are referred as bacteriocins, which serves as a reservoir of natural biopreservatives with various food applications as they increase the shelf-life and nutritional value of many food product like cheese, bread, meat and meat products etc. (Juodeikiene *et al*., 2013; Bartkiene *et al*., 2014; Stimbirys *et al*., 2015; Mozuriene *et al*., 2016). Bacteriocins are ribosomally synthesized extracellularly released bioactive antimicrobial peptides that inhibit closely related bacterial species through various mechanisms i.e. permeabilizing the target cell membrane, inhibiting the cell wall synthesis or through inhibiting the RNAse or DNAse activity (Cotter *et al*., 2005). Gratia in 1925 firstly characterized these bacteriocins in Gram negative bacteria, *Escherichia coli* V as a heat-labile product that inhibited *Escherichia coli* S and termed these peptides as colicin that created a diverse group of antimicrobial proteins. They show ample diversity related to their size, structure, immunity mechanisms, inhibitory spectrum, target cell receptor and mechanism of action. It has been reported that almost all bacteria have potential to produce bacteriocin but still many have not been studied yet. Bacteriocins are proteinaceous metabolites produced by a large number of bacterial

species including Gram positive as well as Gram negative bacteria (Galvez *et al*., 2007). Bacteriocin production by LAB Bacteriocins has many antibiotic properties but they are not regarded as antibiotics that are generally used for the controlling pathogens (Cleveland *et al*., 2001).Bacteriocin potentially inhibits the growth of antibiotic-resistant bacterial strains (Riley and Wertz, 2002. These peptides have antibacterial activity that is immediately degraded with the help of proteases in human digestive tract (Joerger *et al*., 2000). Bacteriocins are heterogeneous group which inhibits pathogenic microorganisms, however their efficacy in food preservation are limited for numerous reasons and among those reasons cost remains an issue which retard the broader use of bacteriocins in food processing industries. Therefore research is still continuing for novel bacteriocin isolation and effective improvement of already existing bacteriocin. Nisin, a bacteriocin is used as a food preservative in almost 50 countries all over the world (Field *et al*., 2012). It is active against predominant food spoilage microorganisms including *Listeria* sp., also prevents the germination of *Bacillus* and *Clostridium* spores (Ramu *et al*., 2015). This revived interest in this area has driven the discovery of novel bacteriocins with their potential food application. In this study we have isolated the potent bacteriocin producing strains, their identification, production and partial characterization from natural sources.A unique method developed in this study for rapid screening to check the presence of bacteriocin in the early stage with the help of LCMS before the involvement of chromatographic techniques which generally are very costly as well as laborious to carry out.

MATERIALS AND METHODS

Collection of Indigenous microorganisms

Different samples of fruit, vegetables (i.e. mango, banana, coconut, lemon, onion, tomato, chili, and carrot), animal samples, i.e. intestine and rectum of chick, goat, pig and sheep and fermented idli batter were collected from relevant trading centres of Jaipur city and were stored at 4 °C.

Isolation of bacteriocin producing strains

10g of each collected sample was weighed and homogenized in 90 mL normal saline for 5 minutes. Serially diluted samples were plated on tryptic soy agar with 1% yeast extract and incubated at 37 °C for 48 hours. After incubation all different type of colonies were picked and purified. The purified bacterial colonies were kept at -20 °C in 20% glycerol stock solution for further use.

Indicator Bacterial cultures

Salmonella typhi (MTCC 3224), *Shigella boydii* (MTCC 11947), *Pseudomonas aeruginosa* (MTCC 1688), *Listeria monocytogenes* (MTCC 657), and *Vibrio parahaemolyticus* (MTCC 451) were purchased from Microbial Type Culture Collection (MTCC) Chandigarh, to study the antimicrobial activity of bacterial isolates.

Screening of positive isolates for bacteriocin activity

The bacteriocin activity was determined by agar well diffusion method by using the above mentioned indicator bacterial strains. The isolates were grown in 50 mL tryptic soy broth with 1% yeast extract and then centrifuged at 10,000 rpm for 15 minute at 4 °C. The supernatant was filtered by 0.22μm size membrane (Millipore, India) and pH of supernatant was adjusted to 6.5. Aliquots (50μL) of sterile supernatant were placed in 6 mm diameter wells that have been made on Mueller-Hinton agar plates previously seeded with indicator strains and incubated at 37 °C for 24h. After incubation the zone of inhibition were measured. The bacteriocin activity assay was expressed in arbitrary unit per mL (AU/mL) (Jadhav *et al*., 2010).

Biochemical identification

After the antimicrobial activity the most potent isolates were identified according to Bergey's manual of Systematic Bacteriology on the basis of cultural, morphological and Biochemical characteristics.

Cultural and morphological characteristics

The most potent isolates were grown in nutrient agar and were preliminary identified on the basis of colony morphology, shape, size, color, elevation, margin, texture and gram staining (Abbasiliasi *et al*., 2012).

Biochemical characteristics

(i) Catalase Test

This test was performed to determine whether the isolates were able to produce catalase enzyme or not. Nutrient agar slants were inoculated with potent isolates and incubated at 37 °C for 24 h. After

incubation 3-4 drops of hydrogen peroxide (H_2O_2) was added to the inoculated culture. Presence of gas bubbles indicates the production of catalase.

(ii) Oxidase Test

A loopfull of bacterial culture was taken on a filter paper and added 2 drops of fresh 1% tetramethyl-p-phenylene-diamne (TMPD) dihydrochloride on it. Presence of deep blue color indicates positive result.

(iii) Indole production

Tryptone broth was inoculated with selected potent isolates and incubated at 37 °C for 48 h. After incubation 1 mL of Kovac's reagent was added to the inoculated medium and presence of red ring at the top of broth indicated the positive result.

(iv) Methyl-Red and Voges-Proskauer Test (MRVP)

MRVP broth was prepared and inoculated with selected potent bacteriocin producing strains. Take 5 mL of inoculated medium into test tubes and incubated at 25 °C for 48 h. Add one drop of methyl red indicator to one set of test tubes and presence of magenta red color indicates positive result. Then add 1 mL of VP reagent to another set of test tubes and presence of red pink color indicates the positive result.

(v) Citrate utilization

Microorganisms used citrate as their carbon source. Simmons citrate agar slants were prepared and inoculated with bacteriocin producing strain. The slants were incubated at 37°C for 48 h. If the color changes from green to blue, it indicates positive result.

(vi) Carbohydrate fermentation

When microorganisms ferment carbohydrate they produce an acid or acid with gas, and this production of acid lower down the pH of the medium, which is detected by color change of pH indicator. Basal medium containing any carbohydrate (glucose, lactose, sucrose etc) and a pH indicator (phenol red) was prepared and inoculated with 24 h old culture of isolates. Inverted Durham's tubes were inserted in the test tubes containing basal medium. The change in color indicates acid and a bubble in the Durham's tube indicates the production of acid and gas by the fermentation of carbohydrate.

(vii) Temperature and pH tolerance

The most potent bacteriocin producing isolates were grown in nutrient broth and incubated at different temperature (10-60 °C) and pH ranges from 3-12. Growth of isolates was then observed.

Rapid identification of bacteriocin by Liquid Chromatography-Mass spectrometry analysis

After the antimicrobial activity of bacterial isolates, the most potent isolate that showed highest zone of inhibition against various indicator organisms was subjected to LC-MS. This technique of LC-MS was employed to check the presence of bacteriocin in the sample. As bacteria also known to produce some low-molecular weight antibiotics (e.g. tetracycline), toxins, lytic agents, bacteriolytic enzymes, metabolic by-products like organic acids, hydrogen peroxide, diacetyl that have an antimicrobial potential to inhibit the growth of microorganisms (Chen and Hoover, 2003). So in order to check whether the antimicrobial action is due to bacteriocin or other than that, LC-MS was done.

LC Separation

3 μg of each peptide sample was separated out with Eksigent C18-reverse phase column (100*0.3mm, 3μm, 120Å) by using Eksigent Micro LC 200 system (Eksigent, Dublin, CA) as described by Zendo *et al*., 2008 with some modifications. The sample material was loaded in column with 97% of mobile phase A (100% water, 0.1% FA) and 3% of mobile phase B (100% ACN, 0.1% FA) at 7 μL/min flow rate. Peptides were eluted with a 120 min with together a gradient of 3% to 50% mobile phase B. The column temperature was setup at 40 °C and auto sampler adjusts at 4 °C. The same chromatographic parameters were selected for both IDA and SWATH acquisition.

Full MS/MS2 acquisition

The sample was analyzed in high-sensitivity mode on AB-Sciex 5600 triple TOF mass-spectrometer. The dual source parameters were optimized for enhanced results: ion source gases GS1, GS2, curtain gas at 25 psi, temperature 200 °C and ion spray voltage floating (ISVF) at 5500 V. The accumulation time in full scan was 250 ms for a mass range of 350-1800 m/z. The parent ions are selected as: ions in the MS scan with intensities more than 120 counts per second, charge stage between +2 to +5 and mass tolerance 50 mDa. Ions were fragmented in collision

cell using rolling collision energy (CE) with an additional CE spread of ± 15 eV. IDA mass spectrometric files were searched in ProteinPilot software, Version 4.0.8085 (AB SCIEX, MA, USA) with the Paragon algorithm against bacteriocin protein database (from www.uniprot.org) at 1% FDR.

RESULTS AND DISCUSSION

Isolation of bacteriocin producing strain and antimicrobial activity

A total of 136 types of colonies were isolated from the collected samples. They comprising both gram positive and gram negative bacteria. On the basis of their efficiency to inhibit majority of indicator organisms, three potent bacteriocin producing isolates were selected. They give highest zone of inhibition against the indicator strains among all the isolated bacterial cultures. The most potent strains were isolated from the source of Mango, rectum of goat and Chick intestine. The zone of inhibitionof positive isolates against indicator organisms shown in Table 1. The strain VS101 was isolated from the sample of Mango at 10^{-5} dilution; VS103 was isolated from goat rectum at 10^{-4} dilution and VS128 was isolated from intestine of chick at dilution 10^{-4}. All the three strains showed inhibitory activity against both gram positive and gram negative bacteria that cause serious food spoilage. A study conducted by Sharma *et al*., 2011 isolated bacteriocin producing strain *Bacillus subtilis* R75 from mung bean which showed zone of inhibition upto 5 mm only whereas in our study the widest zone of inhibition was upto 19.5 mm. Similar results were observed in a study by Ahmad *et al*., 2019 produced bacteriocinfrom *Lysinibacillus* sp. found active against both gram positive and gram negative bacteria. In contradiction to our study, *Enterococci* producing bacteriocin showed antimicrobial activity only against gram negative bacteria (Pantev *et al*., 2003).

Biochemical Identification

In case of isolate VS101, it gave positive reaction to gram staining, as it is gram positive when observed under light compound microscope. The bacterial cells were rod shaped, motile and flagellated. The isolate was able to grow at temperature range from 10-60 °C and pH in the range 3-11. These characteristics were similar to *Bacillus* sp. The results for biochemical test performed were summarized in Table 2. Similarly isolate VS103 and VS128 were subjected for biochemical identification where both the strains gave negative reaction to gram staining when observed under the light compound microscope. They were also able to grow at temperature in the range 10-60 °C and pH 3-11. These characteristics were similar to *Acinetobacter sp.* and *Escherichia sp*. respectively.

Liquid Chromatography-Mass spectrometry

In most of the existing literature, once the potent microorganism is identified; the medium is optimized for maximum production of antimicrobial compound and then purification protocol is followed. As mentioned earlier, there are many more compounds involved in the inhibition of bacterial growth other than bacteriocins. As the antimicrobial activity of crude extracts not always because of bacteriocins; involvement of costly and laborious chromatographic methods is not at all appreciable without confirming the presence of bacteriocin. So, in order to confirm the presence of any bacteriocin; a new methodology employing LCMS is developed in this manuscript. LCMS is very accurate method which was earlier reported for the rapid identification of novel bacteriocins (Zendo *et al*., 2008).

After carrying out antimicrobial activity, crude extract of potent strain was subjected to LC-MS analysis in order to check the presence of bacteriocin in respective samples. Crude extract of the strain isolated from mango origin showed the presence of

Table 1. Zone of inhibition of selected potent bacteriocin producing strain against indicator organisms

Isolated Strains	Zone of Inhibition				
	Salmonella typhi	*Shigella boydi*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	*Vibrio parahaemolyticus*
VS101	13	19.5	11	5	-
VS103	14	9	12	-	3.7
VS128	7.8	6.4	6.4	-	-

('-' sign show no zone of inhibition)

Table 2. Biochemical identification of potent bacteriocin producing strain

Characteristics	VS101	VS103	VS128
Colony morphology	Fuzzy white or slightly yellow, dry, flat, irregular, opaque, medium sized, with lobate margins, in liquid medium form pellicles	Pale yellow to greyish white, smooth, medium sized, round	Large, thick, Gyeyish white, moist, smooth
Gram reaction	Gram positive	Gram negative	Gram negative
Cell morphology	Long rods with unstained endospore spaces, flagellated	Rod-shaped, non flagellated	Rod-shaped, non-sporing, flagellated
Motility	Motile	Non-motile	Motile
Catalase test	Positive	Positive	Positive
Oxidase reaction	Positive	Negative	Negative
Indole production	Negative	Negative	Positive
Methyl Red test	Negative	Negative	Positive
VogesProskauer test	Positive	Negative	Negative
Citrate utilization	Positive	Positive	Negative
Growth at 10°C	Positive	Negative	Positive
At 20°C	Positive	Negative	Positive
At 25°C	Positive	Positive	Positive
At 30°C	Positive	Positive	Positive
At 37°C	Positive	Positive	Positive
At 40°C	Positive	Positive	Positive
At 45°C	Positive	Positive	Positive
Glucose	Acid production, no gas	Negative	Acid and gas production
Lactose	Acid production, no gas	Negative	Acid and gas production
Sucrose	Acid production, no gas	Negative	Variable
Galactose	Acid production, no gas	Negative	Negative

bacteriocin. LCMS analysis resulted a presence of peptide fragment with the following amino acid sequence- GPGTSAVGTGHVQK; which coincided with that of linocin M18 when analyzed using peptide search option of UniProt database (Fig. 1). Hence the bacteriocin produced by bacteria isolated from mango might be linocin M18. Considering both the results of antimicrobial activity as well as LCMS analysis; the bacteria isolated from the source of mango was regarded as most potent one and was further studied.

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        10         20         30         40         50
ELAPISDAAW AQIEAEASRT LKQYLSARRV VDVPPPKGPG TSAVGTGHVQ
        60         70         80         90        100
KIEAPCEGAQ AVQHAARPLV ELRVPFLLSR QAIDDVERGA LDSDWTSLQD
       110        120        130        140        150
AARKLAFAED RSVFDGYAAA GIQGIRQAAS NPITALPAVV TGYPGAVAQA
       160        170        180        190        200
VNHLRLAGVN GPYALVLGAD PYTAISGVTE EGYPVQPHLE RIVEGGIVWS
       210
PAIEGGLVLS TRGG
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Fig. 1. Identified Amino acid sequence of bacteriocin that coincides with Linocin M18

CONCLUSION

In this study we have isolated three bacteriocin producing isolates from different animal, fruits and vegetable samples. Among these, *Bacillus*sp., a strain derived from the mango, could produce a bacteriocin namely linocin M18 identified by a rapid method of LCMS. *Bacillus sp.* was found to be potentially active against various pathogens like *Shigella boydii*, *Salmonella typhi*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*. Future study will be comprised of molecular identification of potent isolate by 16S rDNA analysis. Complete purification of bacteriocin, its biochemical characterization and applicatory study will also carry out.

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