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Purification and characterization of natural food biopreservative produced from *Bacillus subtilis* R1 isolated from a refreshing drink whey

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ABSTRACT

Bacteriocin is a safe and natural food preservative produced from bacteria. Hyper bacteriocin producing bacterial strain R1 isolated from fermented milk drink- whey has been identified as *Bacillus subtilis* by 16S rRNA gene technique. Bacteriocin produced from *B.subtilis* R1 showed strong inhibition against many of the food borne serious pathogens. Bacteriocin produced from *B.subtilis* R1 was purified by gel exclusion chromatography. Molecular weight of bacteriocin was found to be 13KDa. Purified bacteriocin withstood temperature up to 121°C, found active at wider pH range and was sensitive to trypsin. This antibacterial substance showed bactericidal effect against its sensitive indicators which are main food borne / spoilage causing microorganisms.

KEYWORDS

Bacteriocin purification;
Listeria monocytogenes;
SDS PAGE.

INTRODUCTION

Foodborne pathogens are the leading causes of illness and death in less developed countries killing approximately 1.8 million people annually. In developed countries foodborne pathogens are responsible for millions of cases of infectious gastrointestinal diseases each year, costing billions of dollars in medical care and lost productivity. Among food borne pathogens *Listeria monocytogenes* is the major culprit causing serious pathogenicity i.e. human listeriosis. *Listeriae* are acquired primarily through the consumption of contaminated foods including soft cheese, raw milk, deli salads, and ready-to-eat foods such as luncheon meats and frankfurters and these are found active even refrigerated food. As a result, tremendous effort has been made in developing methods for the isolation, detection and control of *L. monocytogenes* in foods[1]. Historically, chemical preservatives and other traditional barriers have been used in food products to inhibit food borne pathogens. These chemicals not only affect natural properties of food but also causes side effects in human beings. Chemical preservatives may affect body's immune system and disturb biochemical reactions that are taking place inside our body. When some chemical is introduced into the body, natural defense system get affected and as a result the body happens to absorb these foreign substances, causing illness and breakdown of vital organs. Many diseases like chronic fatique syndrome, irritable bowl syndrome and acid

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stock are because of chemical food intake. Thus food safety has become a prime concern globally. The burgeoning consumer demand for faster, healthier and ready to eat products has largely stimulated research interests in finding natural but effective preservatives free of potential health risks. Mankind has used microorganisms for thousand of years in obscurity principally for preserving fermented food. In recent years, numerous food poisoning outbreaks involving various pathogens, along with increasing concern about the preservation of minimally processed foods, have spurred more awareness in the importance of food safety[2]. This has prompted new approach for inhibiting food borne pathogens for prolonging the shelf life of food products. In the light of above, there is an increased tendency to use biopresevatives. Food preservation is carried out to maintain the quality of raw material and physicochemical properties as well as functional quality of the product[3]. Thus, biological preservation implies a novel scientific approach to improve the microbial safety of foods. Many *Bacilli* and *Lactobacilli* are known to secrete small ribosomally synthesized antimicrobial peptides called bacteriocins. Bacteriocin have been defined as extracellularly released primary or modified products of bacterial ribosomal synthesis which can have a relatively narrow inhibitory spectrum[4]. These peptides have received considerable attention in recent years due to their potential application in food industry as natural food preservatives. The application of bacteriocin in different food preservatives to combat undesirable food spoilage and pathogenic organisms can become a remarkable breakthrough. Thus, there is a quench to explore rare and less revealed sources to isolate various novel and effective bacteriocin producing strains, their characterization and application for controlling microbial spoilage and for preventing food borne pathogens growing in food products. So, far, only one bacteriocin- nisin has been granted the status of food preservative by food and drug administration of USA and it is commercially used worldwide by different food industries[5]. In the present study, whey, a refreshing fermented milk drink of India has been chosen to isolate new strains of food grade bacteria capable of producing unique bacteriocin with desirable attributes for preservation.

EXPERIMENTAL

Isolation, screening and identification of bacteriocin producing strain

Isolation

Bacteriocin producing strain was isolated from whey-a fermented milk product. In total 4 bacterial strains were isolated on nutrient agar plates following the serial dilution method (10^2-10^8) and incubation at 37^0C for 72 h.

Screening

Screening of isolates was done by bit/disc and well diffusion assay[6,7]. Zones of clearance against the maximum number of indicators were recorded to select the best strain. Out of all isolates, *B.subtilis* R1 was selected for further studies depending upon the widest zones of inhibitions against maximum number of test indicators. The serious food borne pathogens and spoilage causing microorganisms like *Listeria monocytogenes* MTCC 839 *Staphylococcus aureus, Leuconostoc mesenteroides* MTCC 107 *Escherichia coli, Enterococcus faecalis* MTCC 2729, *Lactobacillus plantarum, Bacillius subtilis* MTCC 121, *Bacillus cereus* MTCC 1272 and *Clostridium perfringes* MTCC 450 were chosen as test indicators to study the antagonistic pattern of isolates.

Molecular Identification

The bacteriocin producing isolate was identified at the genomic level on the basis of 16S rRNA gene technique.

Isolation of genomic DNA

Genomic DNA was isolated by following standard procedure[8].

PCR amplification of 16SrRNA region

PCR amplification was done to confirm the identity of the bacterial strains, the small sub-unit 16S rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGCTCAG3') and 16SR (5'TACCTTGTTACGAC TT3') primers to get an amplicon size of 1500bp. Amplifications were carried out in 50µl reaction volume consisting of 10x buffer, 5.0µl; 2mM dNTPs, 5.0µl; 3U/µl *Taq* DNA polymerase, 0.33 µl; 100ng/µl of each primer, 2 µl; 50-100ng tem-

plate DNA, 1µl and H_2O 34.67µl in a Biorad (USA) thermocycler using the PCR conditions 92º C for 1 min (denaturation), 45º C for 1 min (annealing) and 72º C for 1 min (extension). The total number of cycles was 35, with the final extension of 72º C for 10 minutes. The amplified products (50 µl) were size separated on 1.0 % agarose gel prepared in 1% TAE buffer containing 0.5 μgml^{-1} ethidium bromide and photographed with the gel documentation system. A 1000bp DNA ladder was used as molecular weight size markers.

Purification of the PCR product

The PCR product (1500bp) was purified from contaminating products by electroelution of the gel slice containing the excised desired fragment with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 30µl of nuclease free water.

Nucleotide sequencing

Sequencing Preparation –The PCR amplicons obtained by amplifying PCR products was diluted in Tris buffer (10mM,pH 8.5). Dilutions used was 1:1000. In order to obtain the DNA concentration required for sequencing (30ng/µL), The sequencing reaction required 8µL DNA. The primer used in all sequencing reactions was 16SF (5'AGAGTTTGATCCTGGCTCAG3') and 16SR (5'TACCTTGTTACGAC TT3') at a concentration of 3µM.Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA

BLASTN analysis

Translated nucleotide sequence was then analyzed for similarities by using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLAST).The Bacterial isolate was identified as *B.subtilis*

Production and purification of bacteriocin

The nutrient broth medium containing (5g peptone; 3g beef extract; 5gNaCl in 1000 ml of H_2O) was modified by adding 3g yeast extract and 1g glucose to increase bacteriocin production. 10%inoculum of *B.subtilis*(1.0) O.D. was inoculated and incubated at 30^0C for 72 h at 150 rpm. Partial purification of the sample was done by standard salt saturation method adding ammonium sulfate at 70%saturatiomn level and kept overnight[9]. The pellet was collected after centrifugation at 20,000xg at 4^0C for 30 min[10]. The pellet was dissolved in phosphate buffer (0.1 M, pH 7.0) and stored at 4^0C. The column was packed according to the recommended procedure[11]. The sephadex G-100 (5 g) was weighed and suspended in 500 ml of phosphate buffer for overnight. It was swollen for 5 h in boiling water bath, deareated next day for 1 h and brought back to room temperature before packing the column. The size of the column used had dimensions of (75 x 1.5 cm). The packing was done to avoid entrapment of any air bubbles in the gel bed. The column was then eluted with phosphate buffer (pH 7.0, 0.1 M). The protein sample (5 ml) was loaded on the sephadex G-100 column. The column was eluted with phosphate buffer (pH 7.0) and 3 ml fractions of sample was collected in each of total 30 tubes. A flow rate of the sample of 15 ml/h was maintained. The fractions were analyzed for protein content by taking OD at 280 nm and of proteins were estimated by Lowry's method[12]. Purity and molecular weight of bacteriocin was determine by SDS PAGE.

Calculation of activity units (Arbitrary units – AU/ml)

Activity units in terms of arbitrary units were calculated by serial two fold dilution method. The activity units of culture supernatant, partially purified and purified bacteriocin of *B.subtilis* RI were calculated by serial two fold dilution method[6]. The culture supernatant, partially purified and purified bacteriocin was diluted in the range of10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12}. The reciprocal of smallest detectable zone of inhibition was marked for calculation of AU/ml.

Characterization of purified bacteriocin

Effect of pH

0.5 ml of aliquot of purified bacteriocin was added to 4.5 ml nutrient broth of different pH, viz.3.0 to11.0 with an increment of 1, and incubated for 30 min at 37^0C. Each pH treated bacteriocin was assayed by well diffusion method against the test indicators[13].

Effect of temperature

0.5 ml of bacteriocin preparations were added into 4.5 ml nutrient broth in sterilized test tubes. Each test tube was then overlaid with paraffin oil to prevent evaporation and then treated at different temperatures of 40^o,50^o,60^o,70^o, 80^o,90^o,100^o and 121^{o}C each for 10

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min. Each temperature treated bacteriocin was assayed by well diffusion assay[14].

Effect of proteolytic enzymes[15]

Lawns of test indicators were prepared and effect of proteolytic enzyme on activity of purified bacteriocin was studied by following method.

Enzyme activity

I. Enzyme control. I (EC_I)	0.3 ml phosphate buffer
II. Enzyme control II (EC_{II})	0.15 ml bacteriocin isolate + 0.15 ml of phosphate buffer
III. Enzyme reaction (E_R)	0.25 mg of enzyme trypsin (sigma chemicals) was dissolved in 1 ml of 0.5 M phosphate buffer and then added to bacteriocin of *B.subtilis* in the ratio 1 : 1.

The preparations EC_I, EC_{II}, E_R were then incubated for 1 h at 37°C. The enzyme reaction and both enzyme controls were assayed by well diffusion method against the lawns of indicators.

Mode of action of purified bacteriocin of *Bacillus subtilis* RI against its respective indicators

To determine the mode of action, bacteriocin of *B.subtilis* was mixed with its respective indicators in the ratio 1:1(1ml bacteriocin+1 ml indicator).The preparation was kept for incubation at 30^0C for different time intervals viz 1 to 10 h with hourly intervals. Control with out indicator was run in parallel. After each time interval 0.1 ml of the preparation was mounted on nutrient agar plate by spread plate method. After incubating petriplates at 30^0 C, observations were made by counting CFU of bacteriocin treated and untreated indicators (CFU/ml) on the plates.

RESULT AND DISCUSSION

The bacteriocin producing strain isolated from whey showed antagonism against number of pathogens like *L.monocytogenes* MTCC 839, *L.plantarum, S.aureus, B.subtilis* MTCC 121, *C.perfringenes* MTCC 450, *B.cereus* MTCC 1272 *E.coli* and *L.mesenteroides* MTCC 107. The zones of clearance were formed on the lawns of selected indicators in the range of 2-4mm by crude bacteriocin of *B.subtilis* RI in the Bit disc/ well diffusion assay (Plate 1). Its inhibitory activity was found to be 20×10^5AU/ml The broad range antagonistic spectrum and higher activity units made this isolate interesting for bacteriocin production. It was identified as *Bacillus subtilis* R1 at genomic level by using 16S rRNA PCR technique (Plate 2)

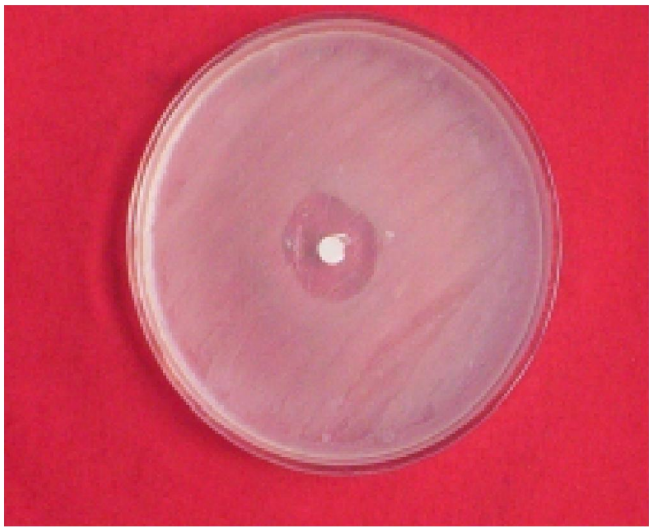
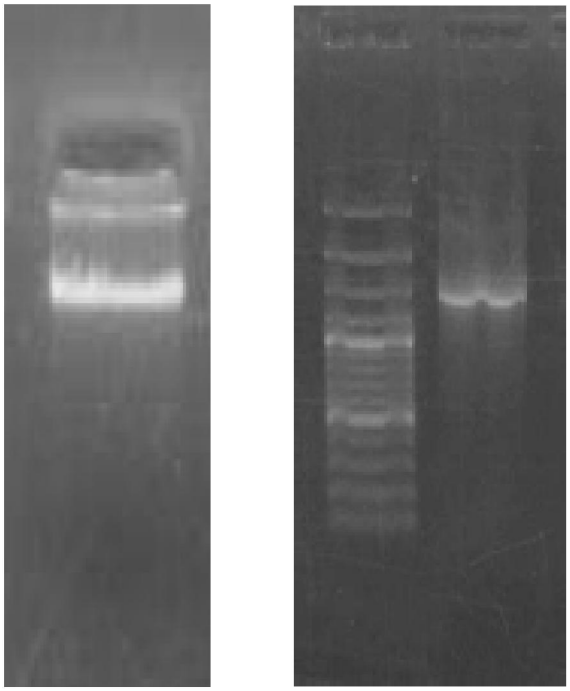


Plate 1 : Inhibitory spectrum of bacteriocin producing bacteria by Bit/disc method against *L.monocytogenes*



Genomic DNA *Bacillus subtilis* **PCR product *Bacillus subtilis***

Plate 2 : Molecular characterization of *Bacillus subtilis R1* using 16S rRNA

Partial purification of bacteriocin was achieved by precipitating it by adding ammonium sulfate at 70% level of saturation. Bacteriocin after precipitation resulted in an increase in size of inhibition zones against

its test sensitive strains indicating its concentrated effect on them. The partially purified bacteriocin expressed higher activity units of 30×10^5AU/ml. The complete purification of bacteriocin sample of *B.subtilis* R1 was achieved following column chromatography. Different steps to purify bacteriocin are exhibited in TABLE 1. Fraction no 51-55 were pooled together based upon highest antagonistic activity Figure 1. The halos of clearance widest i.e. 11mm, 14mm and 12mm against *L.monocytogenes* MTCC 839, *L.mesenteroides* MTCC 107 and *L.plantarum* respectively. The activity units of purified bacteriocin were escalated to 5×10^6AU/ml. Homogenity of pure bacteriocin sample was checked by single band obtained after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of bacteriocin was found to be 13KDa.To confirm the nature of purified single band of *Bacillus subtilis* R1.It was cut from the gel and its antimicrobial activity against standard indicators i.e. *L.monocytogenes* MTCC 839 and *L.mesenteroides* MTCC 107 was recorded. The zone size of 10mm and 14mm respectively ascertain the true status of bacteriocin. As already reported, low molecular weight obtained in the present investigation is a characteristic feature of most of the bacteriocins. Warnerin 20,a bacteriocin produced by *S.warneri* FM 20 purified by SDS-PAGE has been found to have a molecular mass of 6KDa[16]. Another bacteriocin produced from strain *Lactobacillus plantarum* isolated from nham (Traditional fermented pork) also showed molecular weight of 8 KDa based on gel filteration analysis[17].

TABLE 1 : Partial purification and purification of bacteriocin produced by *Bacillus subtilis* R1

	Volume (ml)	Activity unit (AU/ml)	Total Activity	Protein (mg/ml)	Specific activity (10^5)	Purification fold	Recovery %
Crude (culture supernatant)	1000	10×10^5	1×10^9	4.186	2.38	1.00	100.00
Partially purified bacteriocin (Amm. Sulphate ppt)	20	30×10^5	6×10^7	2.558	11.72	4.97	61.1
Purified bacteriocin (washing + SDS)	10	4×10^6	4×10^7	1.012	39.5	16.5	24.1

Total activity was determined by the multiplication of volume/activity
- **Protein concentration was determined by lowry's method**
- **Specific activity is the activity unit/protein concentration**
- **Purification fold is increase in the specific activity**
- **Recovery % is remaining protein concentration as % of the initial protein concentration**

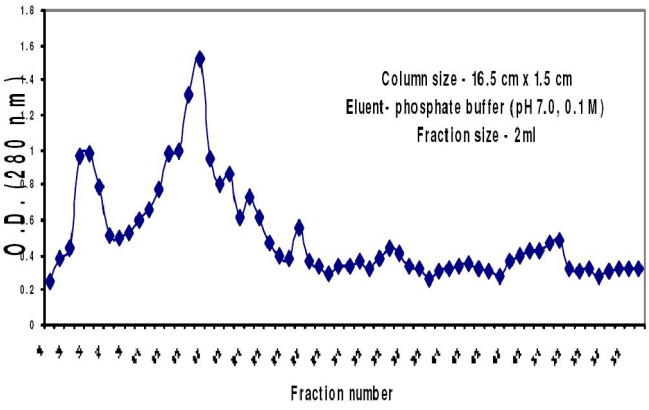


Figure 1 : Elution profile of purified bacteriocin of *B.subtilis* RI on sephadex G-75 column

Pure bacteriocin of *Bacillus subtilis* was characterized to assess its capability to work in different environmental conditions viz temperature, pH, salt concentration etc. from food preservation point of view. Bacteriocin showed thermo stability against *Listeria monocytogenes* MTCC 839 upto100^{0}C for 10 min and against *Leuconostoc mesenteroides* MTCC 107 upto 121^{0}C, although there was a partial loss in activity with a continuous increase in temperature (Figure 2). Purified bacteriocin of *Bacillus subtilis* R1has worked well in a wide pH range. Bacteriocin showed resistance against *Listeria monocytogenes* MTCC 839 in the pH range 4.0 to 11.0 and against *Leuconostoc mesenteroides* MTCC 107 between 5.0 to 10.0 but significant effect was observed in pH range 6.0 to 7.0 (Figure 3). If we compare it with bacteriocins cited in the literature, other bacteriocins have ex-

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pressed activities in a limited pH range viz., bacteriocin of *L.lactis* was found stable in acidic to neutral range pH (4.0-7.0) while that of *L.plantarum* retained activity from pH 3.0-6.0 only[18]. Stability at high temperature and at wider pH range are desirable characters for using bacteriocin of *B. subtilis* R1 in a heat processed, acidic /alkali /neutral food items. Bacteriocin of *B.subtilis* R1 showed sensitivity to proteolytic enzyme trypsin, proteinase k and pepsin. When it was treated with these enzymes in the ratio of 1:1 and welled into lawns of indicators, there was no zone formation in the plates (Plate 3) indicting nil activity of bacteriocin strongly proving its proteinaceous nature. This further adds to the fact that if once administered it will be broken down by gastric juices of digestive tract thus rendering it completely safe for human consumption[19]. There is no toxicity reported so far by use of bacteriocins. Toxicological studies have confirmed that nisin is not toxic at all even at much higher concentration than used in food[20]. The bactericidal effect of purified bacteriocin of *B. subtilis* on its sensitive strains *L. monocytogenes* MTCC 839, *L. mesenteroides* MTCC 107 and *L. plantarum* have been exhibited in Figure 4. When indicators were treated with purified bacteriocin of *B. subtilis* in the ratio 1:1 (bacteriocin : indicator cells) in the time interval of 1, 2, 3 .....9, 10 h., with increase in time, there was a decrease in CFU/ml of respective indicators corresponding to their controls. At 1 h, *L. monocytogenes* MTCC 839 treated with purified bacteriocin had 40 x10^6 CFU/ml. After 5 h decrease was observed to 37x10^6 CFU/ml while at 10 h it reached to 26x10^6 CFU/ ml. Similarly, reduction in CFU/ml was observed with other two indica-

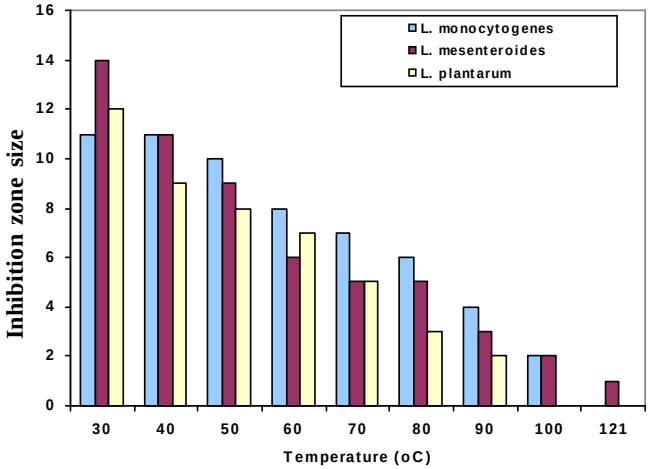


Figure 2 : Effect of temperature on activity of purified bacteriocin of *Bacillus subtilis* R1 (in terms of inhibition zone at 10 min.)

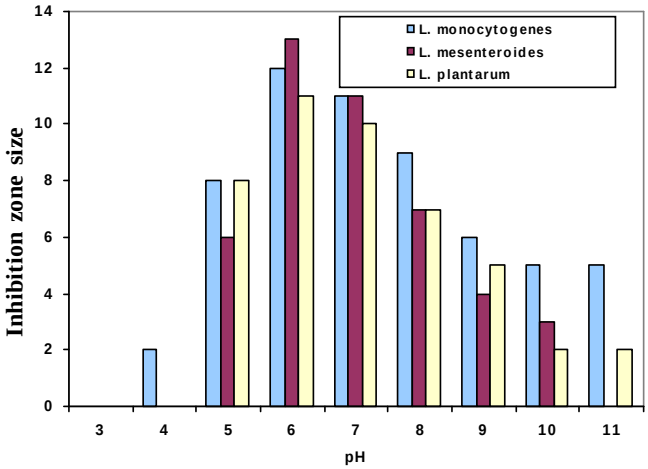
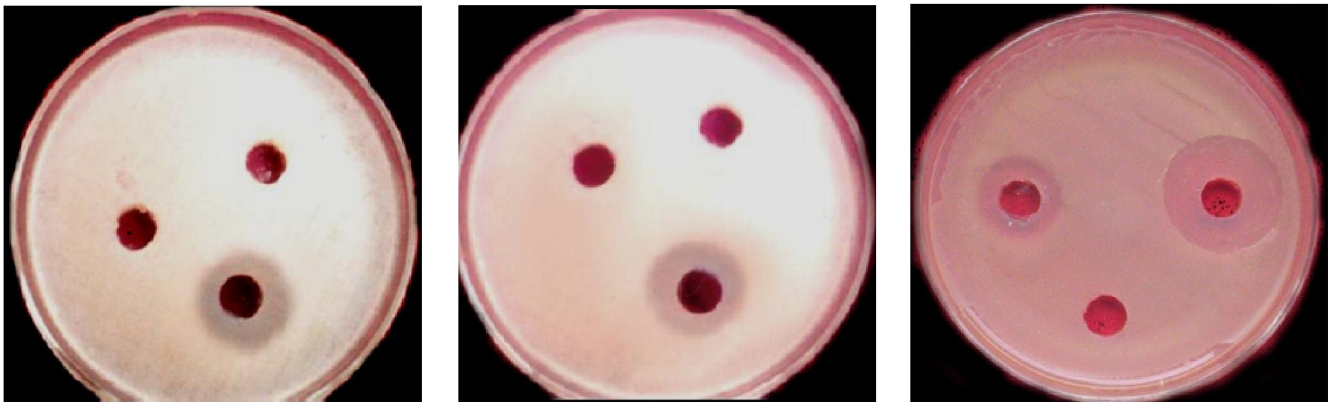


Figure 3 : Effect of pH on activity of purified bacteriocin of *Bacillus subtilis* R1 (in terms of inhibition zone)



Enzyme- pepsin **Enzyme- proteinase K** **Enzyme- trypsin**

Plate 3 : Effect of proteolytic enzymes trypsin, pepsin and proteinase K on activity of purified bactereiocin of *Bacillus subtilis* R1 against *L. monocytogenes*

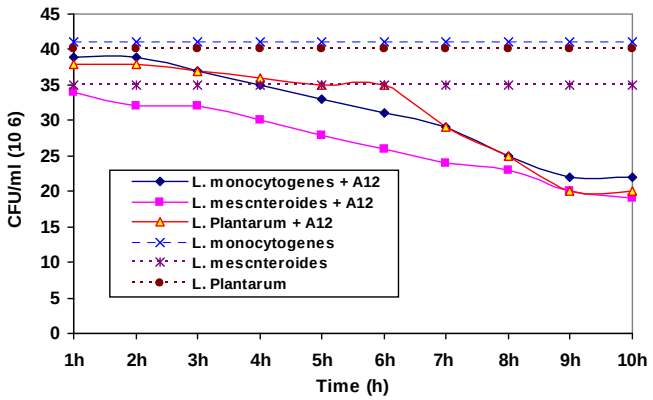


Figure 4 : Mode of action of purified bacteriocin of *B.subtilis* R1 against test indicators

tors *L. mesenteroides* MTCC 107 and *L. plantarum* having 33x10^6 CFU/ml and 38x10^6 CFU/ml at 1 h, respectively. It was 28 x 10^6 CFU/ml and 31x10^6 CFU/ml at 5 h and 17x10^6 CFU/ml and 19x10^6 CFU/ml at 10 h for *L. mesenteroides* MTCC 107 and *L. plantarum* respectively. While three controls exhibited the gradual increase in their CFU/ml ranging from 41x10^6 CFU/ml at 1 h to 46x10^6 CFU/ml at 10 h for *L. monocytogenes* MTCC 839, 35x10^6 CFU/ml at 1 h to 38x10^6 CFU/ml at 10 h for *L. mesenteroides* MTCC 107 and 40x10^6 at 1 h to 45x10^6 CFU/ml at 10 h for *L. plantarum*. Similar mode of action of bacteriocin was observed with *B.lentus* and that of *B,mycoides* against sensitive gram positive bacteria. Most of the bacteriocins reported till date are bactericidal[10,3] and thus leads to effective preservation of food by killing and eradicating the targeted pathogen populations rather than simply checking their growth.

CONCLUSION

It may be concluded from above study that bacteriocin of *B.subtilis* R1 isolated from whey has bright prospects to be used as a food biopreservatives as it poses a combination of desirable characteristics viz: bacteriocin is secreted from food grade bacteria already existing in edible food sample, thus rendering it completely safe for consumption, strong antagonism against a broad range of serious and challenging food borne pathogens/spoilage causing microorganisms, bactericidal mode of action causing death of pathogens, action of bacteriocin at higher temperature and wider pH range, imparting it stability ; degradation of bacteriocin in the presence of proteolytic enzyme making it completely safe for human consumption.

All the above mentioned attributes advocate strongly that bacteriocin of *B.subtilis* R1 isolated from whey has vulnerable potential to be used as effective food biopreservative in place of chemical preservatives thus keeping intact the nutritive properties of processed food as well as safer for consumption.

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