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Research Article

EVALUATION OF BACTERIOCIN PURIFIED FROM POTENT *LYSINIBACILLUSSPHAERICUS* MK788143 ISOLATED FROM INFANT FAECAL

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ABSTRACT

Numerous bacteriocins purified from *Lactobacilli* have been studied as anticancerous, biocontroling, preservatives, and immunostimulatory. But, antimicrobial potential of *Lysinibacillus* is not much reported. In this study, a focus was made to isolate and explore therapeutic potential of *Lysinibacillus* from infant fecal. A total 5 isolates were selected for their antagonistic nature against human pathogen. Amid them, one *Bacillus* sp. was phenotypically, and molecularly using 16S rRNA gene based identified as *Lysinibacillus sphaericus* which given with accession numbers MK788143 in NCBI. The strain MK788143 has revealed the 99.89% identity with the *Lysinibacillus sphaericus*, 99.77% with *Lysinibacillus fusiformis* and 99.55% *Lysinibacillus parviboronicapiens*. The isolate showed broad-spectrum antibacterial activity against gram positive (10-14 mm) and gram-negative (9-10 mm) bacteria. Active bacteriocin was extracted at 40% ammonium sulfate precipitation afterward dialysis and further purification was done by gel filtration chromatography. The molecular weight of bacteriocin was obtained nearly 14 kDa by SDS-PAGE. According to physico-biochemical properties, the purified bacteriocin belongs to class I bacteriocin. The bacteriocin was enormously heat stable in the range of 15 to 100°C temperature for 1 hr and stable within the pH range 2-9. In addition, bactericidal activity of purified protein was decreased when treated with proteolytic enzymes *i.e.* trypsin, proteinase K and pepsin, these results confirmed the peptide nature of isolated bacteriocin. As a reflection of results, the broad-spectrumantagonistic potential and physical stability of bacteriocinmight be applied as an alternative therapeutic as well as food preservative agent to control pathogenic bacterial abundance.

Keywords: Infant fecal, Probiotics, Bacteriocin, Antimicrobial activity, *Lysinibacillus* sp.

1. INTRODUCTION

The application of probiotics to augment human health has been projected for several years [1]. The term probiotics was redefined as "Live bacterial strain that are present in adequate amounts and confer a health benefit effect on the host"[2]. A numerous studies have revealed the therapeutic potential of probiotics which are used for the treatment of diverse gastrointestinal microfloral disorders like antibiotic-associated diarrhea, traveler's diarrhea, and acute diarrhea [3-5]. A few reports on the breast milk microbiome suggested that breast milk has both type of microbiota, human friendly and opportunistic pathogenic, which may cause infectious disease to mother and its neonates [6, 7]. Routinely, infections of pathogenic microorganisms are treated with various antibiotics. However, multidrug resistance in microorganisms is key base of research in novel drug discovery. Reason to the treatment failure is owing to indiscriminate use of antibiotics without testing it's *in vitro* sensitivity to the responsible microorganism [8]. The rapid rise and growth of multi-drug resistant pathogenic bacteria have forced to develop new substitute methods for fighting against infection diseases [9]. Earlier, the bacteriocins have four major classes *i.e.* Class -I bacteriocins also known as lantibiotics or post translationally modified bacteriocins. A class II bacteriocins are small peptide along with 10kd in size, non-lanthionine and mostly heat stable unmodified peptides that don't required post-translational modification for their antagonistic activity, The class II bacteriocins are further subdivided into *Listeria* active peptides along with the N-terminal consensus sequence

of YGNGVXC (class IIa).The class III bacteriocins are large (>30 kDa) in size and heat labile proteins. Conversely, class IV bacteriocins are quite complex bacteriocins which have carbohydrate or lipid moieties [10, 11]. Bacteriocin kills micro-organisms by different mechanisms *i.e.* adsorption, makes leakage of ions via pores from cell membranes and translocation [12, 13].

Bacteriocins are highly specialized deference weapon of microorganism in terms of its potency against target bacteria, higher dosage of these antibiotics is required to kill the target bacteria [14]. Though gram-positive *Bacillus* species are well-known efficient biocontrol agent, its acknowledgement is compromised for its potential use in various fields [15, 16]. Especially, *Bacillus* strains are widely known as thermo-tolerant and mainly spore-forming species which show rapid growth in liquid culture and widely distributed in soils [17]. Different *Bacillus* species are studied for bacteriocins production and in spite of their auspicious characteristics as potential antibacterial agents. A very limited bacteriocins purified from the genus *Bacillus* have been fully studied [17]. Some reported bacteriocins or bacteriocin-like inhibitory substances (BLIS) from the genus *Bacillus* are cerein, produced by *B. cereus* Gn105 which has a molecular mass of 9 kDa and displays antagonistic activity against a wide range of Gram positive opportunistic pathogen [18] and cerein 7 produced by *B. cereus* Bc7 [19] which belongs to a class II bacteriocin with a molecular weight of 3.94 kDa and prevents a broad range of unwanted Gram-positive bacteria [20]. In 1998, Hyronimus *et al.* [21] had reported coagulin which has a bacteriocin like properties was secreted by *B. coagulans* I4 strain. It exhibits a broad spectrum antibacterial inhibiting same species also. Moreover, a *B. brevis* strain which was isolated from kimchi extracellular secreted a bacteriocin which inhibits broad spectrum of bacterias, including food spoilage pathogens and some yeast strains [22]. Present study is focused on evaluation and purification of bioactive antibacterial peptide from probiotic *Bacillus* sp. We have further explored inhibitory spectrum and its antagonistic effects on gram-positive and gram-negative bacteria.

2. MATERIAL AND METHODS

2.1. Isolation of *Bacillus* spp.

Total five infants who were delivered naturally were selected for studies. All volunteer infants were healthy and prenatal problems were not recorded. A feces sample was collected with the help of sterile swabs. The samples were kept at 4°C until delivery to the laboratory and then immediately processed for microbial isolation [23]. All fecal samples were spreaded on de Man, Ragosa Sharpe (MRS) medium (HiMedia, Mumbai, India) containing plates and incubated at 37°C for 24-48 h under aerobic conditions. All isolated strains were further screened for bacteriocin production against indicator bacteria through well diffusion method. The isolates were maintained as long term frozen stock at -20°C in nutrient broth containing 40% (v/v) glycerol. Before experimental uses, the isolated cultures were propagated twofold in MRS medium at 37°C for 18 h with transferred inoculum at 1% (v/v) in glycrol.[24].

2.2. Indicator bacteria

The serious mastitis causing pathogen and food-borne pathogens like *Escherichia coli* (MTCC 10312), *Bacillus cereus* (MTCC 9762), *Bacillus subtilus* (MTCC 1789), *Pseudomonas aeruginosa* (MTCC 8076), *Staphylococcus epidermidis* (ATCC12228) and *Staphylococcus aureus* (MTCC 9542) were bring from Microbial Type Culture Collection, Chandigarh, India, to explore the antagonistic pattern of the isolates.

2.3. Screening of potential bacterial strain

All isolated strains were screened through well diffusion assay as specified in next section and size of inhibition zones were documented against the maximum number of indicator bacteria to select best strain.

2.4. Antagonistic activity against indicator microbes

Antibacterial activity of isolates was explored by agar well diffusion method [25]. Briefly, overnight grown bacterial cultures were inoculated in MRS broth at 37°C for 72 h at 120 rpm. In next phase, the culture was centrifuged at 10,000rpm for 10min and supernatant was collected to check the antimicrobial activity of the isolates. The pH of the collected supernatant was adjusted to 7.0 with 1M NaOH. A volume of 1mL of inoculum of each indicator organisms were added to slightly warm nutrient agar, mixed well then poured into petri plates separately and allowed to solidify. The wells of 7mm in diameter deep were cut and 300µl of culture supernatant was poured into each well. The plates were incubated for 30min at 15°C for uniform diffusion of supernatant then after transferred at 37°C for 24 h and the clear zones formed around the wells were measured. A bacterial strain showed largest inhibition zone against

the maximum number of indicator bacteria was selected for bacteriocin production.

2.5. Identification of the screened bacterial strain

The strain was characterized with gram's staining, colony morphology, physiological and biochemical analysis [26, 27]. The 16S rRNA gene amplification was carried out by polymerase chain reaction (PCR) through universal primer 8F (5′AGAGTTTGATCCTGGCTCAG3′) and 926R (5′CCGTCAATTYYTTTRAGTTT3′), in which Y indicates pyrimidine (C or T) and R represent purine (G or A) [28]. Purified 16S rRNA gene amplicons were sequenced and further identified by analysis of alignment and homology of the partial nucleotide sequence of *Bacillus sp*. using the basic local alignment search tool (BLAST). The multiple distance matrix was developed and further used for construct of phylogenetic trees with the help of Neighbor-Joining method in Mega 6 software [26].

2.6. Production and purification of bacteriocin

Extraction of bacteriocin

Cell-free neutralize supernatant (CFNS) was used for bacteriocin production. The CFNS (crude bacteriocin) was saturated with 40% ammonium sulfate (HiMedia, Mumbai, India) and stored at 4°C for proteins precipitation. The pellet was collected later by centrifugation at 10,000×g at 4°C for 20 min. The pellet was suspended in phosphate buffer saline(0.1M, pH 7.0) and then dialyzed against the same buffer at 4°C overnight with 10kD cutoff membrane (Sigma, Aldrich, USA).

2.7. Column purification of dialyzed protein

Eventually, the active fraction was injected on to a sephadex column (2×150 cm) which was pre-equilibrated with 50mM of phosphate buffer (pH7.0), eluted with buffer containing 0.4M NaCl at a flow rate of 2 ml/10min. Absorbance of protein fractions was analyzed at 280nm. Fractions with high antagonistic activity were used for further study. Antagonistic effect was represented as arbitrary units (AU) per ml which was termed as the reciprocal of the highest dilution which showing a clear zone of growth inhibition [29].

Arbitrary activity units (AU)= (Reciprocal of the highest dilution/Volume of bacteriocin)x 100

2.8. Determination of protein molecular weight

Molecular weight of purified bacteriocin was determined using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[30]. Gel was stained with Commassie Brilliant Blue R-250 and destained by combination of Water: Methanol: Acetic acid (5:4:1).

2.9. Physiochemical characterization of bacteriocin

2.9.1. Sensitivity of purified bacteriocin at broad pH range

Sensitivity of purified bacteriocin was studied with 0.1mg/ml final concentration. The pure bacteriocin was dissolved in different buffer solutions (*i.e.* acetate for pH 2-6; phosphate for pH 7; borax and hydrochloric acid for pH 8-9, glycine-NaOH for pH 9-11)with 50mM concentration along with varied pH ranging from 2, 4, 6, 7, 8, 9, 10 and 11 then incubated for 30min at 37°C. Antibacterial activity of treated bacteriocin and untreated bacteriocin (as a control) were analyzed against indicator bacteria using well diffusion method [31]. The antimicrobial activity of bacteriocin was expressed in terms of percent residual activity when compared with the untreated controls.

Residual Activity= (Zone of inhibition treated/ Zone of inhibition untreated) x 100

2.9.2. Sensitivity of purified bacteriocin at different temperatures

The thermal stability of bacteriocin was studied at different temperatures. Bacteriocin was resuspended in 25mM Tris-HCl buffer (pH 8.0) and subjected to different temperature like 15°C, 28°C, 37°C, 50°C, 70°C, 80°C, 90°C and 100°C for 30min. Subsequently effect of temperature on antagonistic activity was explored by residual activity of treated bacteriocin [32, 33].

2.9.3. Stability of purified bacteriocin against proteolytic enzyme

Influence of proteolytic enzyme like protease, proteienase k and trypsin on the activity of purified bacteriocin was examined as described by Paik *et al.* in 1997 [19]. Briefly, three different vials were taken. One vial contained 0.3mL of phosphate buffer as control 1, second vial contained 0.1mL of bacteriocin and 0.1mL of phosphate buffer which called as enzyme control 2, while third vial contained enzyme reaction mixture in which 0.25mg of each trypsin and pepsin and proteinase k in separate tubes (1mL in 0.5M phosphate buffer), and purified bacteriocin (in a ratio of 1:1). The effect of

proteolytic enzyme on antagonistic activity was checked by residual activity of treated bacteriocin.

2.10. Statistical analysis

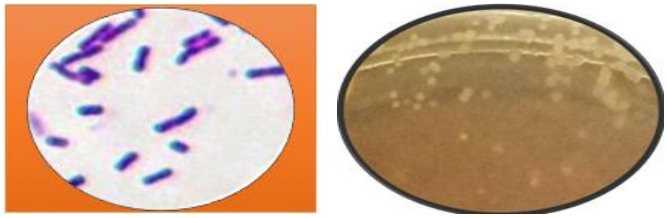
All the experiments were performed in triplicate, and the results were represented in form of mean± standard deviations (SD).

3. RESULTS

3.1. Screening and identification of bacteriocinogenic strain

In present study, 5 MRS grown bacterial isolates from infant fecal, were screened for antagonistic activity against bacterial indicators. Approximately 20% isolates were able to give significant antagonistic activity against human pathogenic bacteria. One isolate, MK788143, showed strong inhabitation against human pathogens that was characterized as a gram positive, rod shape, facultative aerobic and identified as *Lysini bacillus* according its phenotypic and molecular characteristics.

Lysini bacilli are gram-positive rods, aerobic, endospore forming bacterium which belongs in family Bacillaceae of phylum firmicutes and main habitat is plants, soil, animals mainly puffer fish liver specimens and human. Subsequently, identified isolate *Lysinibacillus sphaericus* given with accession numbers MK788143, and its inhibitory spectrum was shown in table 1 & fig.1, thus, MK788143 strain was carefully chosen for baceriocin purification.



[A] **[B]**

Fig. 1: Morphological characterization of *Lysinibacillus sphaericus* MK788143 strain: [A] Gram staining (100X) [B] Colony characteristics

Table 1: A biochemical profiling of *Lysinibacillus sphaericus* MK788143 strain

Biochemical Test	MK788143	Biochemical Test	MK788143
Methyl Red Test	-	Nitrate Reduction Test	-
Voges Proskauer Test	+	Ammonia Production Test	+
Citrate Utilization Test	-	Casein Hydrolysis Test	-
Indole Production Test	-	Starch Hydrolysis Test	-
Hydrogen Sulphide Production Test	-	Gelatin Hydrolysis Test	-
Urea Hydrolysis Test	+	Catalase Test	+
Deamination test	+	Oxidation fermentation test	facultative aerobic

+: Positive test; -: Negative test

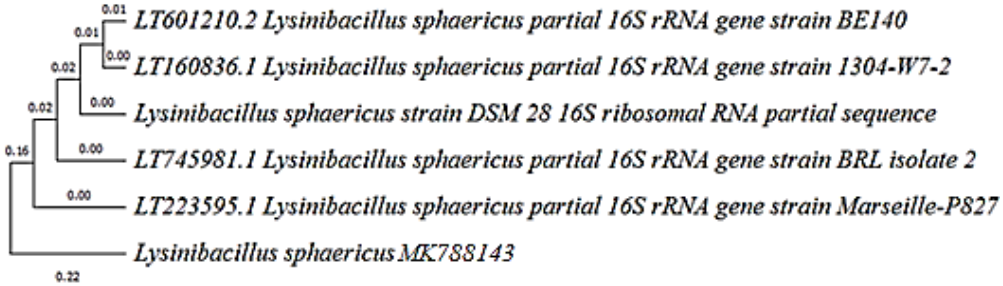


Fig. 2: Phylogenetic analysis of the *Lysinibacillussphaericus*MK788143 strain

Table 2: An Antimicrobial activity of CFNS against indicating microbes

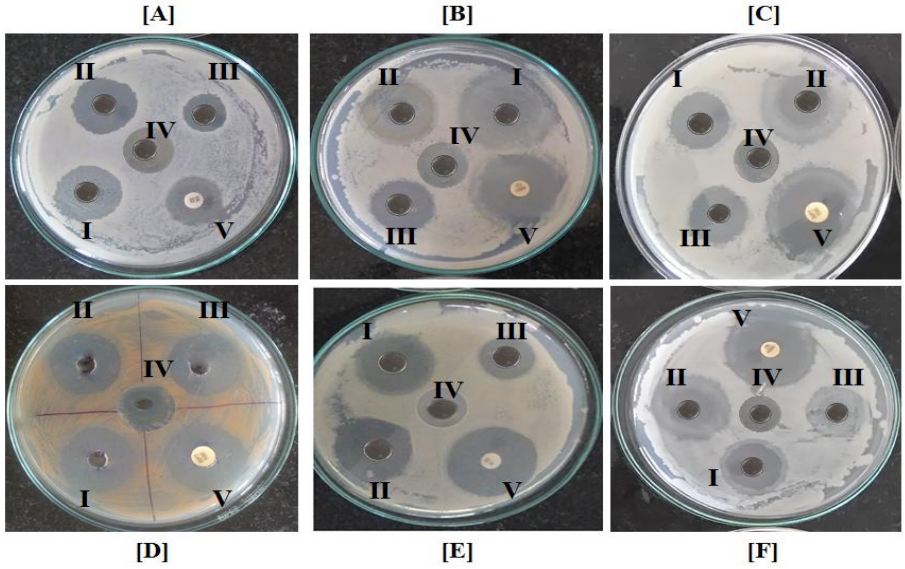
Isolate No.	Indicator Microorganisms [Diameter of inhibition zone(mm)]					
	E. coli	*P. aeruginosa*	*B. subtilus*	*B. cereus*	*S. aureus.*	*S. epidermidis*
BFI1	-	-	5	3	-	3
BFI2	5	-	-	-	-	2
BFI3	**9**	**10**	**10**	**11**	**12**	**14**
BFI4	5	4	6	3	6	2
BFI5	-	7	-	5	-	

BFI3: Lysinibacillus sphaericus MK788143 strain

The serious mastitis causing pathogen and food-borne pathogens like gram negative indicators, *Escherichia coli* (MTCC 10312) and *Pseudomonas aeruginosa* (MTCC 8076), while as gram positive indicators *Bacillus cereus* (MTCC 9762), *Bacillus subtilus* (MTCC 1789), *Staphylococcus epidermidis* (ATCC12228) and *Staphylococcus aureus* (MTCC 9542) were used in this study. Based on antagonistic effect, isolate was found to be most potent against gram positive *S. epidermidis* (14 mm ZOI), *S. aureus* (12 mm ZOI) followed by *B. cereus* and *B. subtilus* (10 mm ZOI) (Table 2).

3.2. Antagonistic activity and bioassay of bacteriocin

Bacteriocin extraction and purification was carried out using cell free neutralized supernatant of *Lysinibacillus sphaericus* MK788143. The developed inhibition zone was examined after 24hours of incubation. Obtained results revealed that the column purified bacteriocin unveiled a broad inhibitory spectrum against all pathogenic indicator microbes (figure 3).



Where, [I]Column purified bacteriocin[II] Dialyzed bacteriocin [III] Ammonium sulfate extracted bacteriocin [IV] Crude bacteriocin (supernatant) [V] Antibiotic (Ceprofloxine)

Fig. 3: Antibacterial spectrum of bacteriocin produced by *Lysinibacillussphaericus*MK788143 at various purification stage against indicator microbes [A] *E. coli* [B] *B. cereus* [C] *B. subtilus* [D] *S. aureus* [E] *S. epidermidis* [F] *P. aeruginosa*

Table 3: Summary of purified antibacterial protein from the culture supernatant of *Lysinibacillus sphaericus* MK788143

Step	Concentration of proteins (mg)	Total Activity (AU)	Specific activity (AU/mg)	Recovery (%)	Purification (-fold)
Crude supernatant	1.6	66.7	41.7	100	1
Ammonium Sulfate precipitation	0.82	133.3	162.6	51.3	3.9
Dialyzed protein	0.67	200	298.5	41.9	7.2
Column purified protein	0.42	533.3	1300.7	26.3	31.2

A quantitative study was done to assess the unit activity of the bacteriocin *i.e.* 533.3 AU/mL. Purity of bacteriocin was found to be nearby 31.2-fold which was more than that of the crude bacteriocin. A purified bacteriocin also has 1.3×10^3 AU/mg specific activity against indicator microbes (Table 3).

3.3. Protein concentration and its molecular weight

Purified fraction of bacteriocin contained 0.42 mg/mL of protein. Results showed in figure 4 reveled single band of purified bacteriocin with around 10-14kDa size in SDS-PAGE.

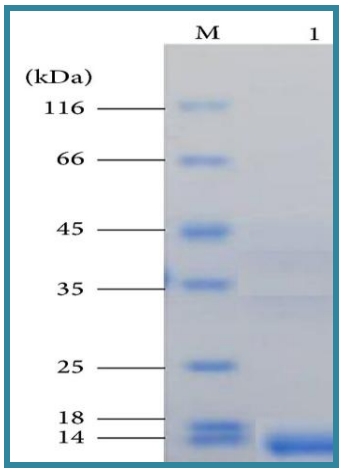


Fig. 4: SDS-polyacrylamide gel electrophoresis of purified bacteriocin from *Lysinibacillus sphaericus*MK788143

Bacteriocin molecular weight was evaluatedusing SDS-PAGE on 12% separating gel with a 5% stacking gel and stained using Coomassie brilliant blue R-250 (Himedia). Lane 1: Ultra-low molecular weight protein marker. Lane 2: Column purified bacteriocin

3.4.Sensitivity of bacteriocin against wide pH range and temperature

Lysinibacillus sphaericus MK788143 exhibited tolerance to varied range of pH (2-11) along with highest antagonistic effect at pH 7.0, substantial antagonistic activity was shown from pH 2 to 9 whereas activity was found to be decreased at pH 10 and 11(Figure 5).

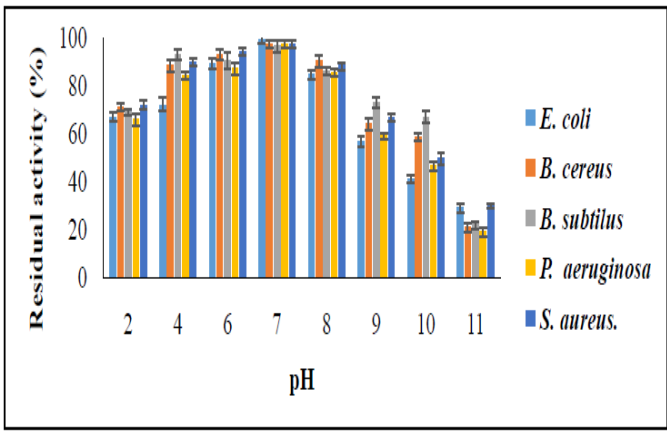


Fig. 5: Stability of purified bacteriocin against board pH range

Similarly, the bacteriocin was nearly activated at broad range of temperatures along with minimum 66% and maximum 95% residual activity against all indicator microbes but relatively maximum residual activity at 37°C was observed (Figure 6). The purified bacteriocin showed considerable antimicrobial activity upto 100°C for 30 min. Upon treatment with proteolytic enzyme, bacteriocin showed remarkable loss of activity which revealed the peptide/protein nature of purified bacteriocin (Figure 7).

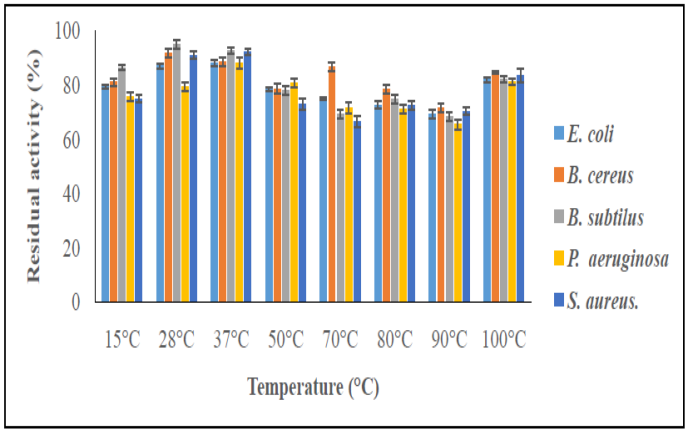


Fig. 6: Stability of purified bacteriocin against different temperature

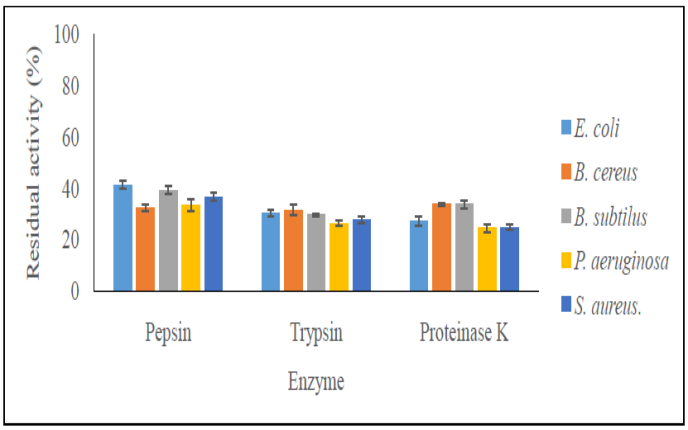


Fig. 7: Sensitivity of purified bacteriocin against different proteolytic enzyme

3.5.Statistical analysis

The obtain results after each experiment did not differ by higher than 5% and single data points are expressed in the figures as a standard deviation bar.

4. DISCUSSION

Probiotic supplements and treatment in improvement of human health is studied since long time. However, concrete base of this practice has not received remarkable attention till now. Clinical popularity and acceptance of probiotic is observing shortfall, as beneficial scientific evidences are lacking. A clinical studies have been

supported the application of some probiotic microbes for the prognosis of many diseases caused by pathogenic microbes since the last decade [34]. For instances, the antimicrobials peptide produced by *Bacillus* probiotic strains, which are present in healthy milk samples, are interfered in the growth of many human pathogenic bacteria like *staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus, E. coli* [35]. The antimicrobial activities of probiotics are attributed to the production of hydrogen peroxide and organic acids *i.e.* lactic or acetic acids which exerts bactericidal effects on most human disease causing microorganisms[36, 37].

Bacteriocins are cationic, hydrophobic and antimicrobial compounds which are extracellularly secreted by probiotic organism [38]. Bacteriocins from lactic acid bacteria (LAB) are of special interest because of their vital application in food preservation as it they exhibit antagonistic activity against food borne pathogens [39]. Bacteriocins are used in beverages, bakery items, canned foods, processed meat products, etc [40]-43]. Hence, in the present study, bioactive antibacterial compounds from *Lysinibacillus sphaericus* MK788143 was extracted, purified from Cell-Free Neutralize Supernatant (CFNS) and characterized. Furthermore, purified bacterocin was subjected for its bactericidal activity against *Escherichia coli, Bacillus cereus, Bacillus subtilus, Pseudomonas aeruginosa,* and *Staphylococcus aureus.*

Active bacteriocin having molecular weight of approximately 14 kDa was extracted at 40% ammonium sulfate precipitation followed by dialysis and gel filtration chromatography. Quantitatively, no difference in unit activity of bacteriocin was observed against all indicator pathogenic microbes. Our result was coincided with Bhattacharya *et al.,* 2010 [44, 45]. A bacteriocin was enormously stable within the pH range 2-9. Similarly, Coventry e*t al.* [41] has reported maximum activity of their purified bacteriocin over a wide range of pH (2-9). Because of this feature our bacteriocin can be used for preservation of acidic foods like pickle or yogurt. A reduction of bactericidal activity after the treatment with proteolytic enzyme(proteinase K, trypsin and pepsin) confirmed the proteinaceous or peptide nature of bacteriocin [42].The bacteriocin showed activity till 100°C for 30 min and at pH 7 [43]. Base on this classes, our purified bacteriocin may be belong to class 2 bacteriocin as class 2 bacteriocins are cationic, small, heat-stable, hydrophobic peptides [44, 45]. Probiotics may interfere with pathogen invasion by reducing or inhibiting pathogen adherence, producing antimicrobials or interfering with toxins[46]. *Bacillus*-based probiotics present an advantage because of the inherent resistance spores[47]. Although *Bacillus clausii* preparation (Enterogermina®) has been described as an antidiarrheal, it prevents antibiotic-associated diarrhea [3, 48-52]. In the past, Ghadbane *et al.,* [17] reported purified bacteriocin Bac-GM17 from *B. clausii* with 5.1 kD molecular size which displayed a bactericidal activity against *Agrobacterium tumefaciens* C58 and antifungal activity against *Candida tropicalis* R2 CIP203.

Hence, physico-chemical and biological characterization of bacteriocin from *Lysinibacillus sphaericus* MK788143 revealed interesting properties that proves its importance in food safety, hygiene and preservation. These findings will have impact on its efficacy to control food spoilaging microorganisms and multidrug resistant microbes. We have demonstrated antibacterial activity of bacteriocin *in vitro*, future experiment can be designed to explore its effect *in vivo* too. Bacteriocin from *Lysinibacillus sphaericus* MK788143 has bright prospects to be used as a probiotic bacterium in dairy products, therapeutic purpose for curing disease like mastitis, and food biopreservative as it carries desirable characteristics. Bacteriocin has strong antagonism against a broad range of serious and challenging mastitis causing pathogen, food borne pathogens and spoilage-causing microorganisms as reflected from our findings. Additionally, action of bacteriocin at higher temperature and wider pH range, imparting its stability, while degradation of bacteriocin in the presence of proteolytic enzymes makes it completely safe for human consumption. Hence, *in vivo* and *in situ* studied of bacteriocin and bacteriocin producing *Lysinibacillus sphaericus* MK788143 will conform its capability to be used for human consumption.

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