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Production, Purification and Characterization of Bacteriocin Produced by *L. Pentosus* MW857478 for Enhancement of Food Saftey and Shelf-Life of Paneer

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Research Article

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ABSTRACT

This research paper is based upon the production, purification and characterization of bacteriocin by *L. pentosus* MW857478 followed by its enhancement of food safety and shelf life of paneer. In recent year LAB (Lactic acid bacteria) produced bacteriocin attract a great attention of researcher due to their many potential applications. This paper focused on isolation, identification, evaluation of broad spectrum inhibitory activity, production and purification, characterization and evaluation of food safety and shelf life of paneer. Bcateriocin produced by *L. pentosus* showed antagonistic activity against food spoiling pathogens in broad range of. Bacteriocin production parameter was optimized with pH 5.5 incubated at 35°C for *L. pentosus*. Bacteriocin was purified by ammonium sulphate precipitation and purified bacteriocin with single band on SDS-PAGE for molecular weight. The purified bacteriocin stable at 2-10 pH and 30°C-75°C temperatures, suggesting *L. pentosus* a potent candidates for safety and extending shelf life of paneer for 15 days.

INTRODUCTION

Lactobacilli are important microorganism as they recognised as their many potent abilities in food quality as preservation and also have suitability to produce drastic changes in the taste, flavour and texture, also they show broad range of spectrum against pathogenic and spoilage microorganisms. Therefore they found naturally in fermented foods, assumed to be safe without create any health risk of consumers, and designated as GRAS

(Generally Recognized as Safe) organisms because of producing various compounds such as organic acids, hydrogen peroxide and bacteriocin.

Bacteriocins are extracellular peptides or proteins that exhibiting bactericidal activity against species and closely related species. Although they may be found in many Gram positive and Gram negative bacteria, those produced by LAB have received particular attention of consumer in recent year due to their potential application in the food industry as natural preservatives. They have been shown very important role in improving microbiological quality and shelf life of many fermented food products and set very excellent examples of bio-preservation. They are mostly heat stable and responsive for protelolytic inactivation so there role as novel food preservatives have received great attention towards the bacteriocin producing lactic acid bacteriaspecies *Lactococcus*, *Lactobacillus, Streptomyces*, *Staphylococcus, Bacillus, Pediococcus* and *Carnobacterium* as reported till now [1].

METHODOLOGY

Isolation, phenotypic and molecular characterization of L.pentosus

L. pentosus was isolated from raw milk samples from cows and goats were collected from different localities of Roorkee, Uttrakhand state of India. Milk sample was utilized for making initial dilution (10^{-1}). Further serial dilution up to 10^{-6}, 1 ml of first dilution was transferred into 9 ml of sterile peptone water. 1ml of this dilution was transferred in sterilized petriplates and poured then 15 ml melted and cooled de Man Rogosa (MRS) agar and mixed properly and allowed to cool. Plates were incubated at 37°C/48 hours. After incubation, different types of colonies appeared on plate with different morphology such as colour, shape and size were picked with the help of sterilized tooth picks into sterile MRS agar plates. This process was repeated 2-3 times on fresh MRS agar media by incubating at 37°C/24 hours to have pure cultures. The purified cultures were preliminary screened for catalase test. The pure culture were further characterized by Gram's staining and, and other biochemical tests. The cultures were characterized based on cell morphology and biochemical tests. The selected strains were identified for molecular characterization of lactobacilli isolates was performed by 16S rRNA gene sequencing.

Screening of antagonistic activity of bacteriocin producing *L. pentosus*

L. pentosus exert antagonistic activity against indicator microorganisms viz., E. coli (ATCC 25922), B. cereus (ATCC 14579), S. aureus (ATCC 25923) was performed by disc diffusion method. The isolated L.pentosus was inoculated in 5 ml MRS broth and incubated 37°C/18-24 hrs. Cell-Free Supernatant (CFS) by centrifugation of this culture at 10000×g for 10 min at 4°C. To rule out any possibility of antimicrobial activity due to organic acid (H_2O_2), CFS was adjusted to pH 7.0 by adding 1N NaOH. The CFS also treated with catalase to eliminate the inhibitory effect of H2O2 produced by lactobacilli isolate. The discs were prepared from Whattman filter paper and autoclaved at 121°C/15 min. Culture free MRS broth disc were used as negative control. The discs were placed on Muller-Hinton agar (MHA) seeded with 18 hrs active culture of indicator microorganism. Plates were incubated at 37°C/24 hrs for clear zone of inhibition around the discs, used to determine bacteriocin activity [2].

Production and purification of bacteriocin

24 hr old culture of *L. pentosus* was propagated by 10% of inoculum on MRS broth and incubated for 48 h at 120 rpm at 37°C. The whole broth centrifuged for 1180g for 15 min and CFS used as crude bacteriocin. The bacteriocin sample protein concentration determined and Bovine Serum Albumin (BSA) used as standard. For purification of CFS of bacteriocin was saturated with 60-70% ammonium sulphate and stored at 4°C to precipitate out the proteins, pellets were collected after centrifugation at 1180 g at 4°C for 30 minutes.

Molecular weight determination

The molecular weight of purified bacteriocin was detected by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Characterization of bacteriocin activity on the basis of effect of enzymes, pH, temperature: The bacteriocin of isolate L.pentosus showing clear zone of inhibition was characterized for temperature, pH and enzymes. Enzyme (proteinase K, trpsin or pepsin) was tested on the antagonistic activity of crude bacteriocin preparation at a final concentration of 1mg/ml and incubated for 2hr/30ºC,wheras effect of temperature (heat resistance) was tested at different temperatures 30ºC, 45ºC, 60ºC, 75ºC and 100ºC, bacteriocin activity was detected against selected pathogenic bacteria for 30, 45 minutes, Effect of pH on bacteriocin activity was tested at various pH 2.0 to 10.0 adjusting through sterile 1mol/l NaOH or 1 mol/l HCl.

Shelf life studies of paneer

Preparation of samples: Freshly prepared paneer sample that was prepared in Kanya Gurukul campus, department of microbiology lab the pieces 5gm 3 cm × 3 cm and 0.5 mm thickness both the samples was sprayed equally by partially purified bacteriocin 5 µg/g on the surface of the piece with the help of hand operated sterile spray bottles and kept and stored at under refrigerated conditions (4°C) and observe at every 3 days interval until spoilage for the parameter via microbiological analysis test like Total Plate Count (TPC), coliform count.

Microbiological analysis

The paneer samples were taken out at different time intervals until spoilage. For plate count of total aerobic bacteria, serial dilutions were prepared with normal saline (0.85% NaCl). 1 g of sample was taken and added into 9ml of normal saline solution and termed as 10-1 dilution. After mixing it homogenously, 1 ml of sample was taken from 10^{-1} dilution and added in 9 mL of normal saline solution (10^{-2} dilution). Similarly, further dilutions were prepared. From appropriate dilution, 0.1 mL sample was taken and plated on solidified and dried (one day at 37°C) standard plate count agar. Plates were incubated at 37°/48hr and colonies were counted through Darkfield Quebec Colony counter.

RESULTS

Identification of bacteriocin producing LAB

Out of 56 isolates, a total of 18 isolates were analysed for the potential of bacteriocin producing lactobacilli from different milk samples whereas on the basis of morphological, and biochemical characterization of Cm12 were confirmed to be lactobacilli [3]. The data has been summarising in Table 1 and Figures 1 and 2.

Table 1: Morphological and biochemical characterization of *L. pentosus* Cm12.

S No	Test performed	*L. pentosus*
1	Gram's Reaction	+
2	Shape	Rods
3	Catalase	+
4	Glucose	+
5	Arbinose	+
6	Lactose	+
7	Galactose	+
8	Maltose	+
9	Ribose	+
10	Manitol	-
11	Gas formation	+

Molecular characterization and submission of sequence to NCBI

Lactobacillus isolates Cm12 was identified by 16S rRNA sequence homology as a strain of *Lactobacillus pentosus* .The 16S rRNA sequence of the isolate Cm12 showed 99.64% identity to *Lactobacillus pentosus* strain LMEM1001. The phylogenetic tree for the isolate was constructed and has been depicted in Figure 3. The 16S rRNA gene of isolate GM 6 was successfully sequenced and deposited to gene bank with accession number MW857478 was obtained. BLAST homology search showed 100% sequence similarity with *Lactobacillus pentosus* strain JCM 1149.

Figure 1: Colony morphology of L. pentosus Cm12 on MRS agar.

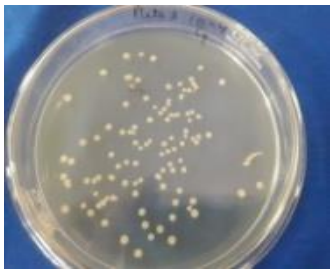


Figure 2: Microscopic view (1000x) of Lactobacillus pentosus.

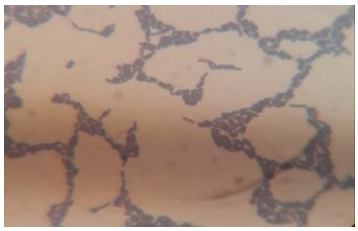


Figure 3: Phylogenetic tree of L. pentosus Cm12

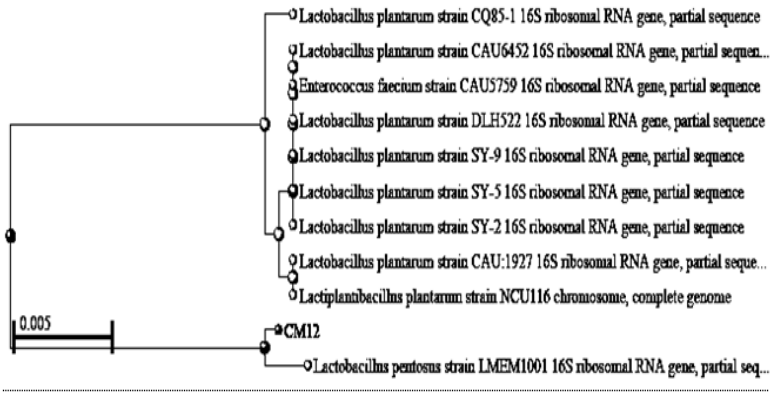
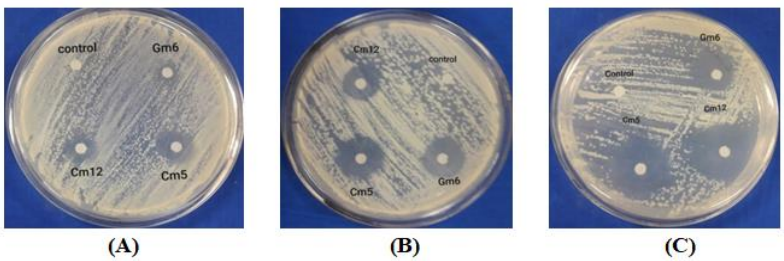


Figure 4: Antagonistic activity against selected food borne pathogens (A) *B. cereus* ATCC14579 (B) *S. aureus* ATCC 25923 (C) *E. coli* ATCC 25922.



(A) (B) (C)

Antagonistic activity of bacteriocin producing LAB

Observations on antagonistic activity of identified lactobacilli isolates obtained from cow and goat milk have been presented in Table 2 and Figure 3. Antagonistic activity of lactobacilli strains was determined by disc diffusion method. The cell free extract was neutralized with 1 M NaOH to eliminate acid effect that could inhibit growth of indicator bacteria. *L. pentosus* Cm12 out of these demonstrated highest antagonistic activity i.e. >15 mm zone of inhibition against three tested indicator *S. aureus* ATCC 25923, *B. cereus* ATCC14579 and *E. coli* ATCC 25922 indicative of broad spectrum inhibition. Our observations are in corroboration with also showing inhibitory effect of LAB against *S. aureus* and *B. cereus* to the tune of 8 and 9 mm zone of inhibition. Inhibitory activity of Lactobacillus can be enhanced selectively and confirmed that *L. cornyimis* XN8 exhibited broad spectrum antimicrobial effect against *S. aureus*.

Table 2: Antimicrobial activity of bacteriocin producing lactobacilli isolates by disc diffusion method on Muller-Hinton agar.

Isolates	Zone of inhibition (mm)		
	E. coli ATCC 25922	*B. cereus* ATCC14579	*S. aureus* ATCC 25923
L. planatrum	20.66 ± 0.33	15.33 ± 0.33	18 ± 0.33

Growth and bacteriocin production of *L. pentosus*

The result of the study of growth and bacteriocin production revealed that there was a positive correlation existed between the growth and bacteriocin production.The growth increase was up to 24hour of incubation but bacteriocin production attained maximum at 18hour of incubation and thereafter no increase was noticed maximum bacteriocin production of L.pentosus is 6.14 mg/ml.results are summerised in Table 3.

Table 3: Total protein concentration of bacteriocin produced by *L. pentosus*.

Strain	Purification stage	Volume (ml)	Protien concentration (mg/ml)
L. pentosus	Culture supernatant (Crude)	500	21.42
	Ammonium sulphate precipitation (Partial purification)	20	6.14

Determination of molecular weight by SDS PAGE: Molecular weight of L.pentosus bacteriocin was carried out by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis). Single protein band having molecular weight ± 11 kDa after stained with coomasie brilliant blue that clearly indicated the purity of protein.

Characterization of bacteriocin

L. pentosus was stable over at 30°C-75°C and more at 60°C for 30 minutes and declined afterwards against different food borne pathogens results were showing in Figure 5, However pH on bacteriocin activity was tested by incubating at various pH at 2.0-10.0 and the stability of *L. pentosus* on bacteriocin activity is stable at 2.0-10.0 pH showing in Figure 4 and more at 6.0 pH. Whereas *L. pentosus* exhibited complete in inactivation of antimicrobial activity. After the treatment of bacteriocin with proteinase K, trypsin and pepsin which confirms its proteinaceous nature showing in Table 4.

Figure 4: Effect of pH on characterization of bacteriocin of *L. pentosus*.

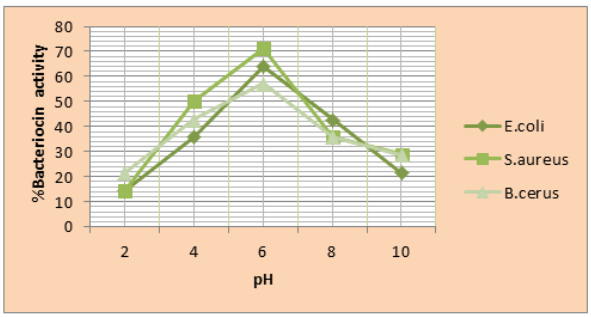


Figure 5: Effect of temperature on characterization of bacteriocin of *L. pentosus*.

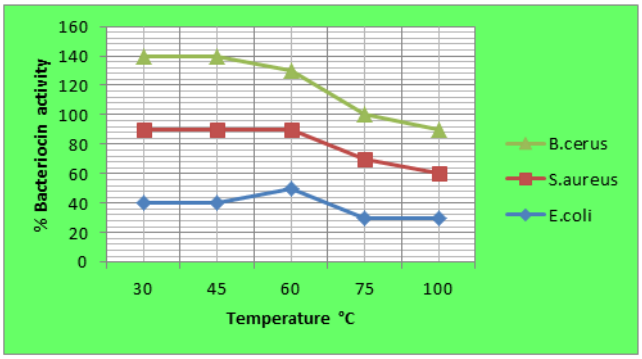


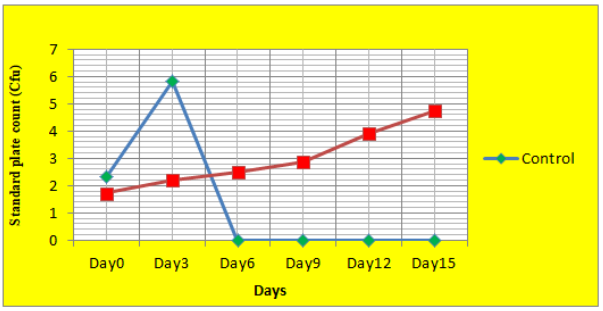
Table 4: Showing effect of enzymes on *L. pentosus*.

Enzymes	Bacteriocin activity
	L. pentosus
Protinase K	-
Trypsin	-
Pepsin	-
Catalase	+

Evaluation of shelf life extension potential of bacteriocin

Freshly panner sample was prepared in Kanya Gurukul campus, department of microbiology lab the pieces 5 gm 3 cm × 3 cm and 0.5 mm thickness both the samples was sprayed equally by partially purified bacteriocin 5µg/g on the surface of the piece with the help of hand operated sterile spray bottles and kept and stored at under refrigerated conditions (4°C) and observe at every 3 days interval until spoilage for the parameter via microbiological analysis test like Total Plate Count (TPC), coliform count, through microbiological analysis the plates for standard plate count were incubated at 37°C for 48 h and colonies were counted with the help of Quebec Colony counter [4]. Showing the microbial analysis of paneer for control and pentocin during refrigerator storage (4 ± 1°C) in which there is no coliform detected until day 15 in pentocin while TPC is 4.74 ± 0.08 cfu/g at day 15 of pentocin while control TPC at day 3 is 5.82 ± 0.08 cfu/g and Not Performed (NP), As per the Bureau of Indian standards (IS:1983), the TPC should not exceed 5 × 105 at day 6,9,12 and 15 because numbers are too high for count in control, Hence pentocin bacteriocin could be easily used as biopresevative for extending the shelf life of paneer after incorporated maximum 15days in refrigirator condition (4 ± 1ºC) .

Figure 6: Microbiological analysis of paneer with different treatments during refrigerated storage (4°C ± 1°C) until spoilage.



DISCUSSION

This study aimed to evaluate the ability of *L. pentosus* to produce bacteriocin and enhance the shelf life of paneer. The bacteriocin production and detection was formed in-vitro accompanied by the production of other metabolites like hydrogen peroxide and lactic acid. Thus neutralize the effect of other metabolites and assure that protein extract was not related to these metabolites [5]. The results suggest that a protineceous nature of bacteriocin produced by *L. pentosus*. Based on the described results we conclude that *L. pentosus* produce bacteriocin with an expected size ± 11kDa. This molecular weight is within the range of the most frequently reported. Further studied may include the enhancement of shelf-life of paneer.

CONCLUSION

In parallel we conducted antimicrobial tests of crude and partially purified bacteriocin against different food borne pathogens showing the effectiveness of bacteriocin against gram negative and gram positive bacteria, then pentocin bacteriocin produced by *L. pentosus* showing the effectiveness in enhancing the shelf life of paneer by incorporating of pentocin.

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REFERENCES

1. Elayaraja S, et al. Production, purification and characterization of bacteriocin from Lactobacillus murinus AU06 and its broad antibacterial spectrum. Asian Pacific J Trop Biomed. 2014;4:S305-S311.
2. KP S, et al. Production and characterization of bacteriocin produced by Lactobacillus viridescence (NICM 2167). Brazilian Archiv Biol Technol. 2016; 59.
3. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970;227:680-685.
4. Lowry OH, et al. Protein measurment with folin phenol reagent. J Biol Chem. 1951;193:265-275.
5. Marchesi JR, et al. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol. 1998;64:795-799.