

Full Length Research Paper

Characterization of bacteriocin producing lactic acid bacteria and its application as a food preservative

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Lactic acid producing bacteria was isolated from Whey. The isolated strain *Lactobacillus fermentum* KN02 was potent producer of bacteriocins. Bacteriocin produced by the isolate KN02 had a large spectrum of inhibition against food spoilage microorganisms (*Escherchia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Proteus mirabilis*, *Pseudomonas aeuroginosa*, *Klebsiella pneumonia*, *Bacillus cereus*). The bacteriocin inhibited the growth of pathogens but it showed high activity against *Bacillus*. The bacteriocins were found to be heat stable at 100°C. The maximum antimicrobial activity was retained within the pH range of 2, and completely affected by proteolytic enzyme (papain). Bacteriocins are used to control the frequent development of pathogens and spoiling microorganism in food and feed. Growth of *L. fermentum* KN02 was inhibited only by few antibiotics suggesting that the strain may be used as a probiotic by the individual receiving medical treatment. The new bacteriocin producing *L. fermentum* KN02 has been selected for identification and application of bacteriocins to food products (Milk and Mushroom) which reduces the growth of pathogens. The bacteriocins producers *L. fermentum* KN02 was identified on the basis of phenotypic analysis and 16S rRNA sequences.

Key words: Lactic acid bacteria, *Lactobacillus fermentum*, bacteriocin, food spoilage microorganisms.

INTRODUCTION

The term Probiotic means 'for life' is derived from the Greek language. A probiotic is a live microbial feed supplement, which beneficially affects in the host animal by improving its intestinal microbial balance.

Lactic acid bacteria (LAB) are ubiquitous in nature and as a consequence are present as natural contaminants on a variety of food (Axelsson, 1998). Genera belong to the LAB family include *Lactococcus* sp., *Lactobacillu* sp., *Leuconostoc* sp., *Weissela* sp. and *Pediococcus* sp., as well as *Streptococcus* sp. and *Enterococcus* sp., They are responsible for the contribution for the variety of the organoleptic properties characteristic of fermented food

such as meat (Fontana et al., 2005), vegetables (Randazzo et al., 2004) and dairy products (Marilley and Casey, 2004). The use of Lactic acid bacteria and their metabolites to improve microbiological safety and extend the shelf life of foods is defined as Biopreservative (De Martinis et al., 2001). Antagonistic properties of LAB allied to their safe history of use in traditional food fermented products make them very attractive to be used as biopreservatives (Parada, 1984; Caplice and Fitzgerald, 1999). The LAB, generally considered as "food grade" organisms, show special promise for selection and implementation as protective cultures. In addition, some LAB exhibit potent antimicrobial activities in the form of small, heat-stable, antimicrobial peptides called bacteriocins (Riley and Wertz, 2002; Sablon et al., 2000).

Bacteriocins are extra-cellularly released peptides or

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protein molecules, with a bactericidal or bacteriostatic mode of action against closely related species. Although bacteriocins may be found in many Gram positive and Gram negative bacteria, those produced by LAB has received particular attention in recent years due to their potential application in the food industry as natural preservatives. Several types of bacteriocins from food associated LAB have been identified and characterized, of which the important ones are Nisin, Diplococcin, Acidophilin, Bulgarican, Helveticins, Lactacins and Plantaricins (Nettles and Barefoot, 1993). The bactericidal activity of bacteriocin is attributable to destabilization of the function of the cytoplasmic membrane of the target cells, and altering the permeability properties of the membrane.

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. To inhibit pathogenic or spoilage microorganisms, bacteriocinogenic strains or partially purified bacteriocins can be added to foods (Muriana, 1996). However, the effectiveness of bacteriocins in foods may be reduced by different factors (Hanlin et al., 1993; Muriana, 1996). First, the Minimum Inhibitory Concentration (MIC) differs widely among bacteriocins and sensitive strains. Secondly, the activity spectrum of bacteriocins produced by Gram positive bacteria is usually limited and does not include Gram negative bacteria. Harris et al. (1992) also demonstrated that bacteriocin resistant variants may appear and grow in the presence of a bacteriocin, and therefore limits its efficacy (Gaenzle et al., 1999).

In recent years, the use of rRNA sequences for identification and phylogenetic analysis has been generally accepted. DNA probes based on highly variable rRNA regions have been applied successfully for the identification and detection of microorganisms in soil, intestinal tract, and clinical samples (Barry et al., 1990).

The most recent food applications of bacteriocins encompass their binding to polymeric packaging, a technology referred as active packaging. Bacteriocins can be used to confer a rudimentary form of innate immunity to foodstuffs (Cotter et al., 2005). Lot of studies have focused on the inhibition of spoilage and/ or human pathogen bacteria with vegetable foods and beverages by bacteriocins and their application appeared as a good alternative to chemical compounds and antibiotics.

MATERIALS AND METHODS

Sample collection

Whey sample was collected from Salem (Tamil Nadu, India) and immediately transported to laboratory under cool condition for bacterial isolation.

Isolation and identification of bacteriocin producing bacteria

The bacteriocin produced from naturally fermented Whey was

obtained and inoculated into MRS (de Man, Rogosa and Sharpe) broth at 37°C for 48 h. After incubation, it was streaked in MRS agar and incubated at 37°C for 48 h. Cell morphology, Gram staining, Oxidase and Catalase tests were performed as a preliminary screening for Lactic acid bacteria. Gram positive, non-spore forming and Catalase negative strain was selected for further studies. The isolated strains were inoculated into sugar broth tubes containing Durham's tube to find out the fermentation capability of the organisms in different sugars.

Indicator pathogens

E. coli (MTCC 1687), *S. typhi* (MTCC 531), *B. cereus* (MTCC 1272), *S. aureus* (MTCC 96), *P. mirabilis* (MTCC 425), *K. pneumonia* (MTCC 530) and *P. aeuroginosa* (MTCC 1688) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. These pathogens were grown in nutrient broth and incubated at 37°C for 24 h.

Screening of Lactic acid bacteria for antimicrobial activity

An overnight culture of isolate was grown in MRS broth at 37°C for 48 h. After incubation, the broth was adjusted to pH 2.4 and the cell was removed by centrifugation at 5000 × g for 10 min. Supernatant was used as crude bacteriocin to evaluate antimicrobial activity using agar well diffusion method.

Detection of antimicrobial activity by agar well diffusion method

Antimicrobial activity of bacteriocin against all pathogenic microorganisms was determined by well diffusion method (Kimura et al., 1998) under aerobic conditions. Agar plates were inoculated with 500 µl of each indicator microorganisms after growing them in a nutrient broth and diluting appropriately. The inhibitory activity against all pathogenic microorganisms was tested on Muller-Hinton agar. Wells (6 mm) were cut in Muller-Hinton agar plate and 150 µl of cell free culture supernatant (crude Bacteriocin) of the isolated strain was added into each well. After incubation at 37°C for 24 h, the diameter (mm) of the inhibition zone around the well was measured.

Characterization of crude bacteriocin

Heat stability

About 5 ml of crude bacteriocin in different test tubes was taken and then heated at 37, 50, 75, 90 and 100°C for 15 min under pressure. The heat treated bacteriocin samples were then assayed for antimicrobial activity.

Effect of pH

Five ml aliquot of crude bacteriocin was taken in test tubes and the pH of the contents were adjusted to pH 2, 4, 5, 7 and 9 separately, using either diluted NaOH or HCl (1 M NaOH or 1 M HCl solution). After allowing the samples to stand at room temperature for 2 h, the antimicrobial activity was assayed.

Effect of proteolytic enzyme (Papain)

Five ml aliquot of bacteriocin was taken in test tubes and treated

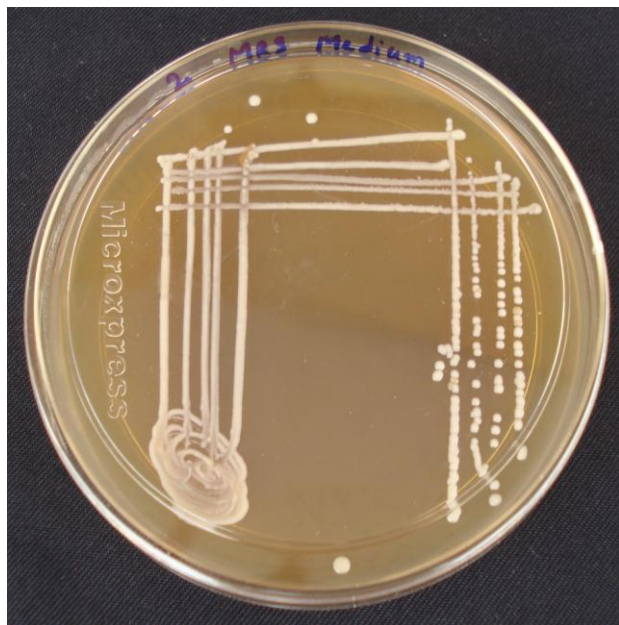


Figure 1. *L. fermentum* KN02.

with papain (1 mg/ml) at pH 7. The test tubes with and without the enzyme (control) were incubated at 37°C for 2 h and heated at 100°C for 3 min to denature the enzyme. Both the control and samples were assayed for antimicrobial activity by using well diffusion method.

Biopreservative efficiency of crude bacteriocin in milk and mushroom

The food products such as Milk and Edible Button Mushroom were added with 5% of bacteriocin and refrigerated. Initial plate count of samples was serially diluted at 10^{-6} and the plates were incubated at 37°C for 24 h. The colony count was recorded and compared with the control (without bacteriocin) (Vinod Kumar et al., 2006).

Effect of viable antibiotics on the growth of isolate KN02

An overnight culture of the isolate KN02 was swabbed in MRS soft agar plates. Antibiotic discs (Ampicillin (10 µg), Streptomycin (10 µg), Erythromycin (15 µg), Rifampicin (5 µg), Penicillin (10 U), Ciprofloxacin (5 µg), Vancomycin (30 µg), Ofloxacin (5 µg), Tetracyclin (30 µg), Co-trimoxazole (1.25 µg), Chloramphenicol (30 µg) and Amoxycillin (20 µg)] were placed on the agar surface and incubated at 37°C for 24 h. Growth inhibition was recorded by measuring the diameter of the zones and compared with standard antibiotic sensitivity chart (Hi Media, India).

16S rRNA sequencing and phylogenetic analysis

Almost complete 16S rRNA sequence was amplified using universal M13 primer 16S F (5'-AGAGTTGATCCCTGGCTCAG-3') and 16S R (5'-GTACGGCTACCTTGTTACGAC-3') purchased from MWG Biotech Private Limited, Bangalore. After amplification the PCR products were purified using Eppendorf perfect prep clean kit. The purified product were checked by electrophoresis (Agarose Gel Electrophoresis) on a 1% agarose gel (Sigma, India), stained with ethidium bromide, visualized using UV-Transilluminator (Biometra,

India) for specific bands. The DNA bands were identified according to the size by comparing with the molecular weight marker (10 kb DNA ladder) loaded in separate lane.

Sequence homology and analysis were performed using the BLAST program available online at the National Center for Biotechnology Information, NCBI. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2007). Phylogenetic analyses were conducted in MEGA4. Tree phylogram was evaluated by bootstrap analyses with 500 replicates.

RESULTS

Isolation and identification of bacterial strain

Bacteria isolated from the Whey was identified as *L. fermentum* KN02 (Figure 1) based on physiological and biochemical characteristics (Table 1) and 16s RNA gene (Figure 2) sequenced (Gene Bank Accession No. HQ650232) and BLAST.

The isolate KN02 was Gram positive, rod shaped and negative for catalase test having smooth round colonies on the MRS media. The strain was capable of fermenting sugars, namely Galactose, Glucose, Xylose, Ribose, Cellobiose, Fructose, Arabinose, Lactose, Sucrose, and Maltose (Table 2).

Screening of lactic acid bacteria for antimicrobial activity

An overnight culture of isolated strain was grown in MRS broth at 37°C for 48 h. After incubation, the broth was adjusted to pH 2.4 and the cell was removed by centrifugation at 5000 × g for 10 min. Supernatant was used as crude bacteriocin to evaluate antimicrobial activity using agar well diffusion method.

Detection of antimicrobial activity by agar well diffusion method

An agar well diffusion method was used to assess the production of antimicrobial compounds by the selected isolates KN02 against Food spoilage pathogens. The susceptibilities of Gram-positive and Gram negative bacteria to growth inhibition by the crude bacteriocin of isolate *L. fermentum* KN02 (Figures 3 and 4) showed inhibitory activity against *B. cereus* (MTCC 1272), *S. aureus* (MTCC 96), *P. mirabilis* (MTCC 425), *K. pneumonia* (MTCC 530), *P. aeuroginosa* (MTCC 1688) *E. coli* (MTCC 1687), *S. typhi* (MTCC 531), (Table 3).

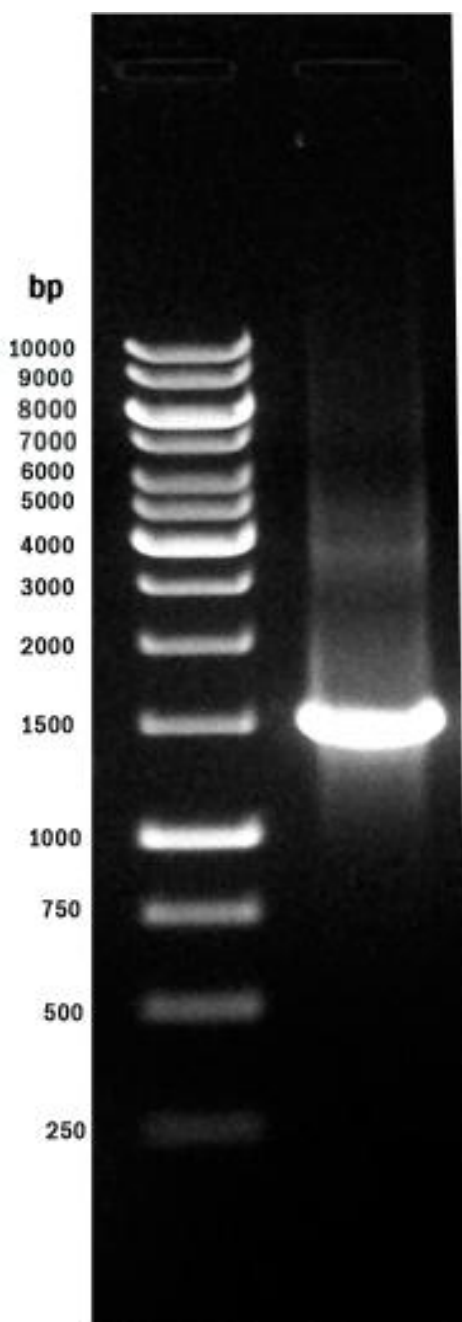
Characterization of crude bacteriocin

Effect of temperature and pH on bacteriocin activity

Temperature and pH played an important role in cell growth as well as bacteriocin production. The bacteriocin activity from the isolate KN02 was tested at different

Table 1. Physiological and biochemical characteristic of isolate KN02.

Test	KN02
Colony morphology	Creamy, little sticks and Smooth round colonies.
Gram staining	Gram positive, rod
Growth in MRS broth	Uniform turbidity
Catalase	Negative
Oxidase	Positive

**Figure 2.** Amplified 16S rRNA of *L. fermentum* KN02.

Lane 1- Molecular Weight Marker - 10000 bp
Lane 2- Amplified 16S rRNA gene product

Table 2. Biochemical characteristics of isolate KN02.

Carbohydrates	KN02
Glucose	A + G
Galactose	A
Sucrose	A +G
Lactose	A
Maltose	-
Cellobiose	A
Fructose	A
Xylose	A
Arabinose	A
Ribose	A

A: Acid production; G: Gas production; - : No acid or Gas production.

temperatures (37, 50, 75, 90 and 100°C) and the inhibition zone was observed up to 100°C (Figure 5). Regarding pH (2, 4, 5, 7, 9), the inhibition zone was observed upto pH 2 (Figure 5).

Effect of enzyme on bacteriocin activity

The antibacterial activity of bacteriocin from isolate KN02 was completely inhibited by proteolytic enzyme (papain) indicated that isolated bacteriocin is proteinaceous in nature.

Biopreservative efficiency of crude bacteriocin in milk and edible button mushroom

The partially purified bacteriocin from isolate KN02 was tested for preservative effect. Maximum reduction of bacterial population was observed in Milk when compared to Edible Button Mushroom at the concentration of 5%, and in control (without bacteriocin), no reduction of population was observed. The results further revealed that microbial count drastically decreased in Milk and edible Mushroom (Figures 6).

Effect of Antibiotics on the Growth of Isolate KN02

Growth of isolate KN02 was not inhibited by

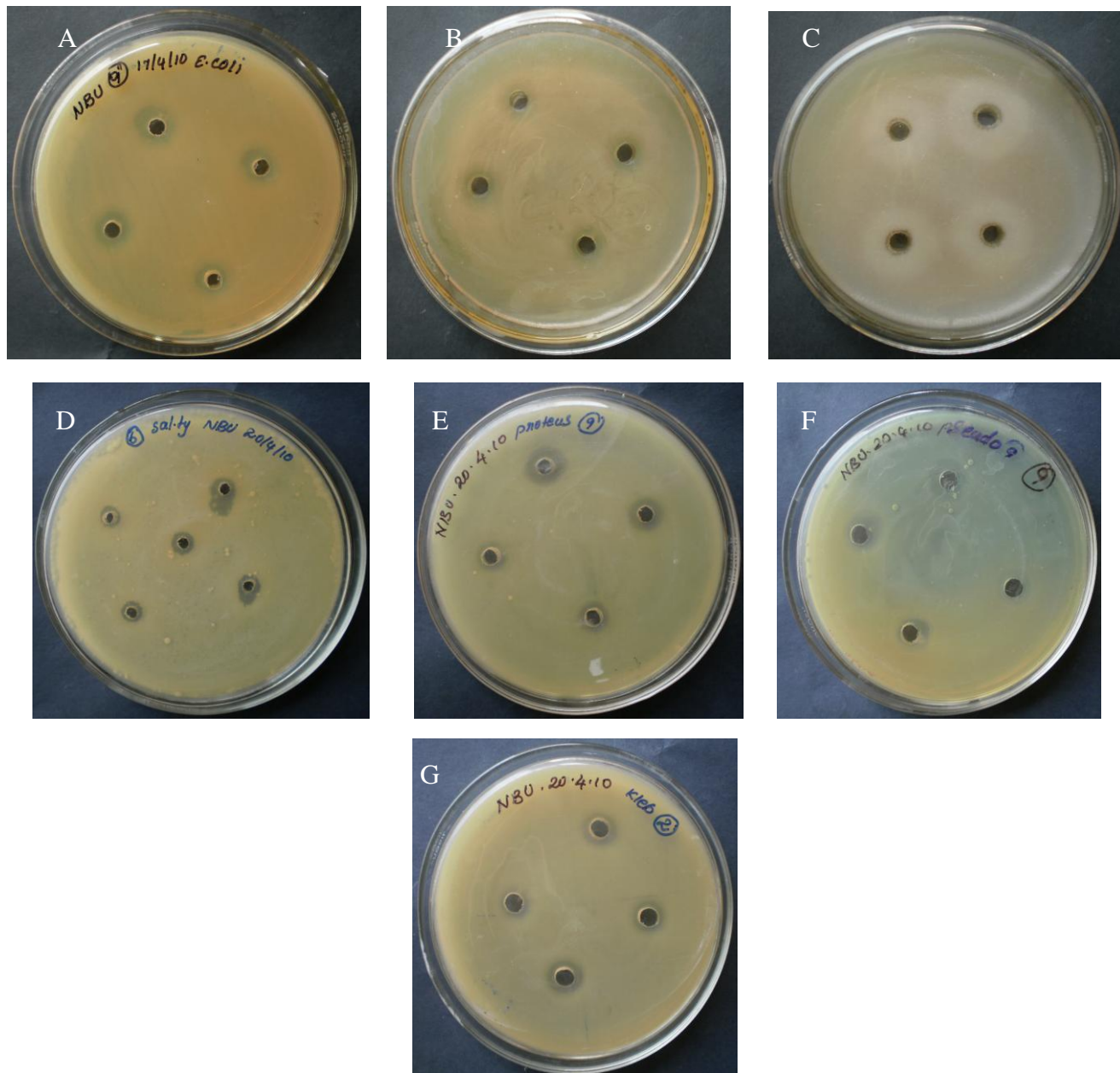


Figure 3. Antimicrobial Activity of Bacteriocin (KN02) against (A): *E. coli* (B): *S. aureus* (C): *B. cereus* (D): *S. typhi*. (E): *P. mirabilis* (F): *P. aeruginosa* (G): *K. pneumoniae*.

Ciprofloxacin, Co-trimoxazole, Vancomycin. Zone of inhibition was sensitive to Ofloxacin, Tetracycline, Amoxicillin and resistance to Streptomycin, Erythromycin, Penicillin and Chloramphenicol (Table 4).

Molecular phylogeny

The resulted 16 rRNA sequences were aligned with available, almost complete sequence of strains of *Lactobacilli* family. Then, corresponding sequences of representative *Lactobacillus* species, in each case, the

reference sequences were retrieved from the Gene Bank Databases. The phylogenetic tree (Figure 7) revealed that the bacterial strain is closely related to *L. fermentum* with similarity matrix of 99%. The 16S rRNA sequences of isolate KN02 bacteriocin-producing strain have been registered in the Gene Bank Database under the accession number (HQ650232). The phylogenetic data described were obtained by using MEGA4 package using neighbour-joining, minimum evolution, maximum parsimony and bootstrapping methods. The evolutionary history was inferred using the UPGMA method (Tamura et al., 2007). The optimal tree for isolate KN02 with the

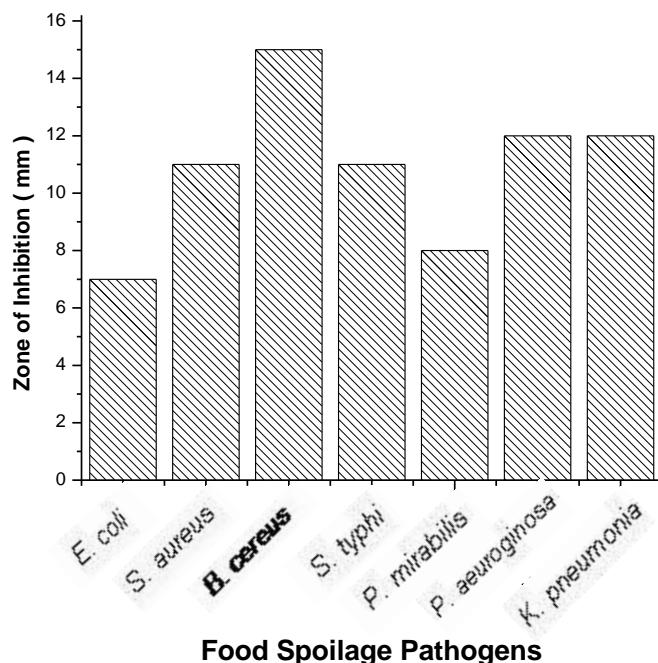


Figure 4. Antimicrobial activity of Bacteriocin from KN02.

Table 3. Inhibition activity of bacteriocin from isolate KN02 against food spoilage bacteria.

Test organisms	Zone of inhibition (mm)
<i>E. coli</i>	7
<i>S. aureus</i>	11
<i>B. cereus</i>	15
<i>S. typhi</i>	11
<i>P. mirabilis</i>	8
<i>P. aeruginosa</i>	12
<i>K. pneumonia</i>	12

sum of branch length = 0.01912580.

DISCUSSION

The present investigation highlights the isolation, characterization and activity of bacteriocin produced by *L. fermentum* KN02 from Whey. Similarly, a *L. plantarum* SIK- 83 strain, which was later thought to be a *L. lactis* strain, which produced a nisin like bacteriocin, was isolated from fermented carrots (Andersson et al., 1988).

Well diffusion assay performed to assess the antagonistic activity of bacteriocin produced by *L. fermentum* KN02 was tested for antimicrobial activity against Gram positive and Gram negative bacteria such as, *S. aureus*, *B. cereus*, *E. coli*, *S. typhi*, *P. mirabilis*, *P. aeruginosa*, *K. pneumonia* associated with food borne illness. The highest inhibitory activity was

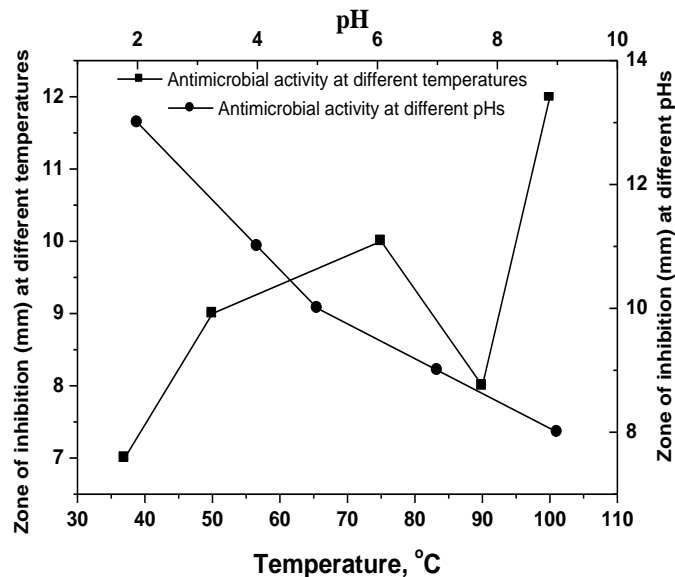


Figure 5. Activity of Bacteriocin from KN02 against *E. coli* at different temperature and pH.

demonstrated against *B. cereus*, while the least activity was demonstrated against *E. coli*. The inhibitory effect demonstrated by *L. fermentum* KN02 against these bacteria is an indication of possessing of antibacterial activity.

Results also revealed the presence of the compound bacteriocin in the test organisms. Bacteriocins have been reported to be inhibitory against several other bacteria (Karthikeyan and Santosh, 2009). Possession of bacteriocin by *L. fermentum* KN02 is an indication that the bacteria can be used as probiotic and as preservative. *L. fermentum* KN02 fermented Glucose, Xylose, Arabinose, Ribose, Cellobiose, Lactose, Galactose, Fructose, Maltose and Sucrose.

Bacteriocin production was strongly dependent on pH and temperature as claimed by Torodov and Dicks (2004). Bacteriocin like substance from *L. fermentum* KN02 isolated from whey was resistant to heat at 100°C. A similar result reported by Aslim et al. (2005) was bacteriocin – like substance from Lactic acid bacteria isolated from Turkish dairy products showed resistant to heat at 100°C. Bacteriocin produced by *L. fermentum* KN02 was noted to have the maximum activity at pH 2, so the result proved that it could be used in acidic foods like pickles. Similarly, Reinheimer et al. (1990) studied the pH dependent activity of bacteriocin elaborated by Lactic acid bacteria showed that majority of the strains, highest antimicrobial activity was exhibited in an acidic pH range of 2.0–4.0.

Bacteriocin production from *L. fermentum* KN02 was influenced when incubated in proteolytic enzyme (papain). Bacteriocin activity was completely affected by the enzyme papain, as it also was the case with nisin, the only established bacteriocin for commercial use until

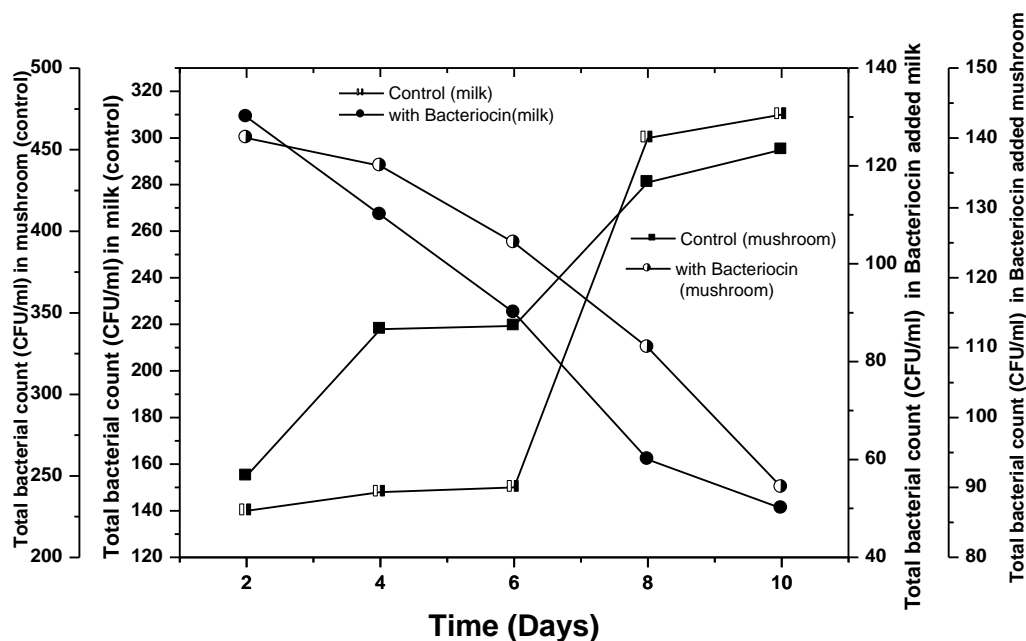


Figure 6. Biopreservative effect of Bacteriocin on Milk and edible button mushroom.

Table 4. Sensitivity of Isolate KN02 against viable antibiotics.

Antibiotics	Inhibition zone (diameter in mm)	Remarks
Ampicillin (10 µg)	14	Intermediate
Streptomycin (10 µg)	10	Resistance
Erythromycin (15 µg)	11	Resistance
Rifampicin (5 µg)	17	Intermediate
Penicillin (10 U)	12	Resistance
Ciproflaxacin (5 µg)	-	-
Vancomycin (30 µg)	-	-
Ofloxacin (5 µg)	20	Sensitive
Tetracycline (30 µg)	23	Sensitive
Co- trimoxazole (1.25 µg)	-	-
Chloramphenicol (30 µg)	24	Resistance
Amoxycillin (20 µg)	27	Sensitive

date. So the results show that the antibacterial compounds produced are inactive by the proteolytic enzyme (papain), indicating that the inhibitory compound are proteinaceous nature, a general characteristic of bacteriocin (Harris et al., 1992).

Bacteriocin produced by *L. fermentum* KN02 from whey, when applied to Milk and edible Mushroom inhibited the multiplication of aerobic bacteria up to ten days. During storage under refrigeration, bacterial counts in treated Milk and Edible Button Mushroom presented a slow multiplication when compared to the untreated control. Under refrigeration the bacteriocin was stable within the first 24–48 h in vegetable juices, while its stability was longer (at least 15 days) in fresh juices and

mixed juices (Grande et al., 2005).

Since bacteriocin is considered as natural products, they might have good acceptance from customers who start to demand for more natural and safer food products. Some legal drawbacks that must however be considered for the application of novel bacteriocins are safety factors in foods and feeds as well as a continued research, since upto date only a few have been officially approved for use in foods (Parada et al., 2007).

In this study with antibiogram indicated that the native isolate KN02 was sensitive to Ofloxacin, Tetracycline, Amoxycillin and showed resistance to Streptomycin, Erythromycin, Penicillin and Chloramphenicol. Gupta and Malik (2007) studied the MIC values for Ampicillin,

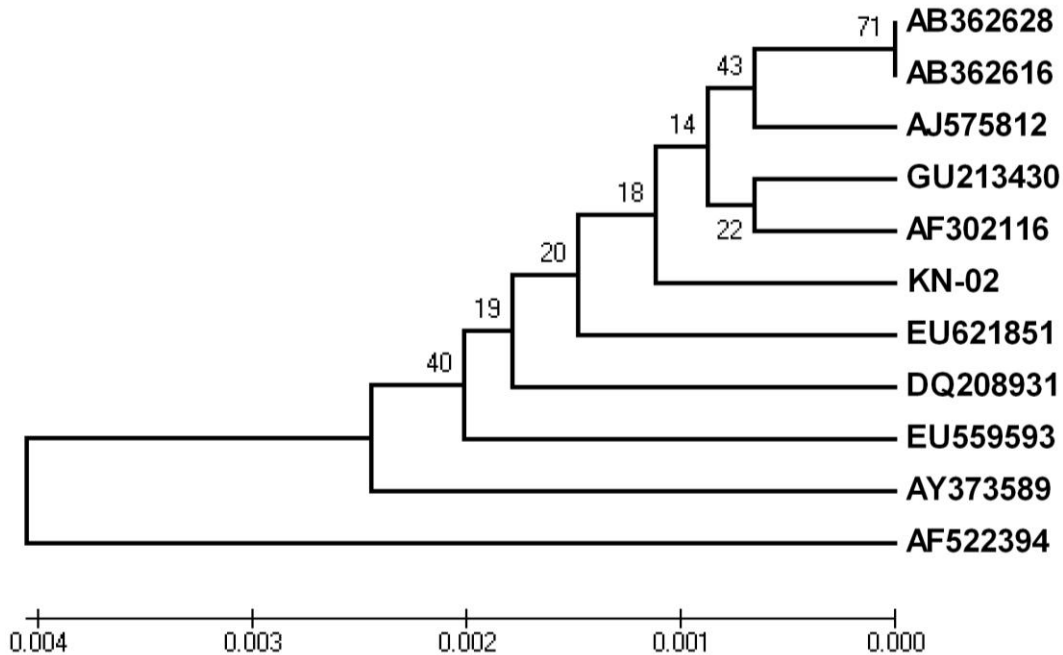


Figure 7. The phylogenetic position of the *Lactobacillus fermentum* KN02 strain among neighboring species.

Streptomycin, Tetracyclin, Chloramphenicol and Erythromycin were determined to be 1, 1.25, 0.5, 8, and 0.5 µg/ml, respectively for *Enterococcus*.

The 16S rRNA sequencing results showed that the bacteriocin – producing strains *L. fermentum* KN02 isolated in this study shared 99% sequence similarity with *Lactobacillus* sp.

This study concluded that the bacteriocin antibiotic produced by *L. fermentum* KN02 was demonstrated that inhibitory effects against bacterial pathogenic. Like nisin the bacteriocin produced by *L. fermentum* KN02 in the present study also has the enormous potential for food applications as biopreservatives and probiotics.

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