Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb



Document heading

Production of bacteriocin and their application in food products

Narayanapillai Udhayashree[1], Duraisamy Senbagam[2], Balakrishnan Senthilkumar[1*], Kanagaraj Nithya[1], Ramasamy Gurusamy[2]

[1] *Centre for Biotechnology, Muthayammal College of Arts & Science Rasipuram -637 408, Tamil Nadu, India.*
[2] *Research Department of Biotechnology, Vivekanandha College of Engineering for Women, Tiruchengode - 637 205. Tamil Nadu, India.*

ARTICLE INFO

Article history:
Received 3 January 2012
Received in revised form 12 January 2012
Accepted 13 March 2012
Available online 28 April 2012

Keywords:
Lactic acid bacteria
Lactobacillus fermentum
Bacteriocin
Biopreservatives

ABSTRACT

Objective: To isolate bacteriocin- producing lactic acid bacteria (LAB) and to asses its preservative effects on food products. **Methods:** *Lactobacillus* strain was isolated from chicken intestine using MRS broth (Hi-Media, India) at 37 ℃ for 48 h. The isolate was identified by 16s rRNA sequencing and phylogenetic analysis. Bacteriocin was produced, purified by ammonium sulphate precipitation and dialyzed. Antagonistic characteristics of bacteriocin were studied by agar well diffusion method. Bacteriocin stability was studied in different temperature, pH and enzymes. Biopreservative efficacy was determined for fish and fresh juice. **Results:** *Lactobacillus fermentum(L. fermentum)* strain isolated from chicken intestine was capable of producing bacteriocins, inhibiting the widest range of pathogenic bacteria. The bacteriocin was stable at 37 ℃ and acidic pH and proteinaceous in nature. Their biopreservative effect on refrigerated food products (apple juice and fish) show low count of colonies. Lactobacillus isolate was sequenced which shows high similarity with reference strain *L. fermentum* CECT 5716. **Conclusions:** Bacteriocin producing *L. fermentum* UN01 could be successfully used as biopreservative and to increase the shelf life of the food products.

1. Introduction

Technologies on processing and preservation of food products, which help in maintaining its nutritional values besides ensuring safety issues, are the area of current food research. Many chemicals are being used for inactivation of food borne pathogens so as to preserve food products for long duration [1].

Numerous strains of Lactic Acid Bacteria (LAB) associated with food systems produce bacteriocin defined as proteinaceous substance that exhibit bactericidal activity against closely related organisms [2]. Considering that bacteriocin producing LAB are mostly isolated from foods like dairy and meat products, these antimicrobial substances have been consumed for a long time. Several bacteriocins from Gram positive bacteria are very effective, have broad inhibitory spectra and may be used as antimicrobial agents for various practical applications [3]. The antimicrobial properties of LAB have enabled the extension of the shelf life of many foods through fermentation products [4].

Antagonistic effects produced by LAB towards other organisms may play an important role in maintaining a proper microbial balance in intestinal tract and preserving certain foods. Some bacteria produce bacteriocins which are protein and protein related complexes that inhibit other bacteria that are usually closely related [5]. Most bacteriocins are heat stable and sensitive to certain proteolytic enzymes. LABS are widely used as starter culture and play an important role in food preservation, microbiological stability and production of aroma compounds. Bacteriocins produced by LAB have received considerable attention during recent years for their possible as biopreservative in foods, with a resultant reduction in the use of chemical preservatives [6].

LAB was realized as a group of biopreservative bacteria in the beginning of 1900's, based on their interaction in foods. The preservation factors elaborated by LAB have been identified as "peptides", generally known as "bacteriocins". LAB play an important role in food fermentation process. Raw foods such as milk, fruits, vegetables or meat are often preserved by lactic acid fermentation [7]. In such food products LAB have the capacity to perform fermentive activities, which may result in active inhibition of pathogenic bacteria.

2. Materials and methods

*Corresponding author: Centre for Biotechnology, Muthayammal College of Arts & Science Rasipuram -637 408, Tamil Nadu, India.
Tel: +91-94432 86292
E-mail: senthilmicro@yahoo.co.in

2.1. Sample collection

Chick Intestine was collected from Salem and immediately transported to laboratory at 4 ℃ for bacterial isolation [8].

2.2. Isolation and identification of bacteriocin producing bacteria

Chick Intestine was crushed into small pieces and inoculated into MRS broth (Hi-Media, India) and incubated at 37 ℃ for 48 h. After incubation, pure culture was made on MRS agar and tested for bacteriocin production [9]. Then the strains was Gram stained and examined microscopically. The strain isolated was inoculated into sugar broth tubes to find out the fermentation capability of the organisms in different sugars. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide and oxidase test was performed using oxidase disc.

2.3. Production of bacteriocin

The strain was grown in MRS broth at 37 ℃ for 48 h. After incubation, the broth was centrifuged at 5 000 X g for 10 min and the cells were separated out. The cell free supernatant was used as crude bacteriocin.

2.4. Antimicrobial activity againt pathogens

Escherichia coli (MTCC 1687) (*E. coli*), *Salmonella typhi* (MTCC 531) (*S. typhi*), *Bacillus cereus* (MTCC 1272) (*B. cereus*), *Staphylococcus aureus* (MTCC 96) (*S. aureus*), *Proteus mirabilis* (MTCC 425) (*P. mirabilis*), *Klebsiella pneumonia* (MTCC 530) (*K. pneumonia*) and *Pseudomonas aeurogenosa* (MTCC 1688) (*P. aeurogenosa*) were obtained from Microbial Type *Culture Collection* (MTCC), Chandigarh, India.

2.5. Screening of lactic acid bacteria for antimicrobial activity

Antimicrobial activity of bacteriocin against pathogenic microorganisms was determined by well diffusion method under aerobic condition. 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 (6mm) 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. Plates were incubated at 37 ℃ for 24 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

2.6. Partial purification of bacteriocin

Different concentration of ammonium sulphate were added to the crude bacteriocins and kept for stirring. After stirring on a magnetic stirrer, it was kept undisturbed at 4 ℃ overnight. Precipitate formed were collected by centrifugation at 10 000 X g for 10 min and dissolved in 20Mm potassium phosphate buffer (pH 7). The dialyzed sample was used to find the concentration of protein.

2.7. Estimation of total protein

The protein content of the dialysed Bacteriocin was determined according to Lowry *et al.* (1951).

2.8. Characterization of bacteriocin

2.8.1. Heat stability

A volume of 5ml of bacteriocin in different test tubes was taken and then heated at 37, 50, 75, 90, 100 ℃ for 15 min under pressure. The heat treated bacteriocin samples were then assayed for antimicrobial activity [10].

2.8.2. Effect of pH

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

2.8.3. Effect of proteolytic enzyme (Papain)

About 5 mL of aliquot of bacteriocin preparation was taken in test tubes and treated with papain (1mg/ml) at pH 7. The test tubes with and without the enzyme (control) were incubated at 37 ℃ for 2 h and heated at 100 ℃ for 3 min to denature the enzyme. Both the control and sample were assayed for antimicrobial activity by using well diffusion method.

2.8.4. Biopreservative efficiency of bacterioicn in apple juice and fish

The food products such as apple juice and fish were added with 5% of bacteriocin and refrigerated. Initial plate count of samples was serial diluted at 106 and the plates were incubated at 37 ℃ for 24 h. The colony count was recorded and compared with the control (without bacteriocin).

2.9. Effect of viable antibiotics on the growth of UN01 isolate

An overnight culture of the isolates from UN01 was swabbed in MRS soft agar plates. Antibiotic discs *viz.* Ampicillin (10 μg), Streptomycin (10 μg), Erythromycin (15 μg), Rifampicin (5 μg), Penicillin (10U), Ciproflaxacin (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 ℃ for 24 h. Growth inhibition was recorded by measuring the diameter of the inhibition zones and compared with standard antibiotic sensitivity chart (Hi Media, India).

2.10. 16S rRNA sequencing and phylogenetic analysis

Almost complete 16S RNA 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 perfectprep gel clean kit. The purified product were checked by

electrophoresis (Agarose Gel Electrophoresis) on a 1% agarose gel (Sigma, India), stained with ethidium bromide, visualized and the gel was observed over UV-Transilluminator for specific bands. The 16S RNA gene was identified according to the size by comparing with the molecular weight marker (10 kb DNA ladder).

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 and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1528 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [11]. Tree phylogram was evaluated by bootstrap analyses with 1000 replicates.

3. Results

3.1. Isolation and identification of bacterial strain

The bacteriocin producing strain was isolated from the source of Chick intestine and the selected strain was identified as *L. fermentum* UN01 based on its physiological and biochemical characteristic. The isolate UN01 was gram positive, rod shaped and positive for oxidase and catalase test having smooth round colonies on the MRS agar media. The strain was capable of fermenting sugar viz. Galactose, Glucose, Xylose, Ribose, Cellobiose, Fructose, Arabinose, Lactose, Sucrose and Maltose.

3.2. Production of bacteriocin

The isolated strain was grown in MRS broth at 37 ℃ for 48 h. After incubation, the broth was adjusted to pH 2.4 and centrifuged at 5 000 X g for 10 min and the cells were separated out. Supernatant was used as crude bacteriocin. This crude bacteriocin was used to determine the antimicrobial activity.

3.3. Determination of inhibitory spectrum

An agar well diffusion method was used to access the production of antimicrobial compounds by the selected isolates from chick intestine against 7 pathogens. The susceptibilities of various Gram - positive and Gram - negative bacteria to growth inhibition by the crude bacteriocin isolated from *L. fermentum* UN02 (Figure 1) showed inhibitory activity against *E. coli*, *S. typhi*, *B. cereus*, *S. aureus*, *P. mirabilis*, *K. pneumonia* and *P. aeurogenosa*.

3.4. Estimation of protein by Lowry's method

The concentration of protein present in bacteriocin produced by the isolate UN01 was found to be 160 μg/mL.

3.5. Characterization of bacteriocin

3.5.1. 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 UN01 isolate of was tested with different temperature (37, 50, 75, 90 and 100 ℃) the maximum inhibition zone was measured at 37 ℃. Regarding pH (2, 4, 5, 7, 9) the maximum inhibition zone was measured at pH 2.

3.5.2. Effect of enzymes on bacteriocin activity

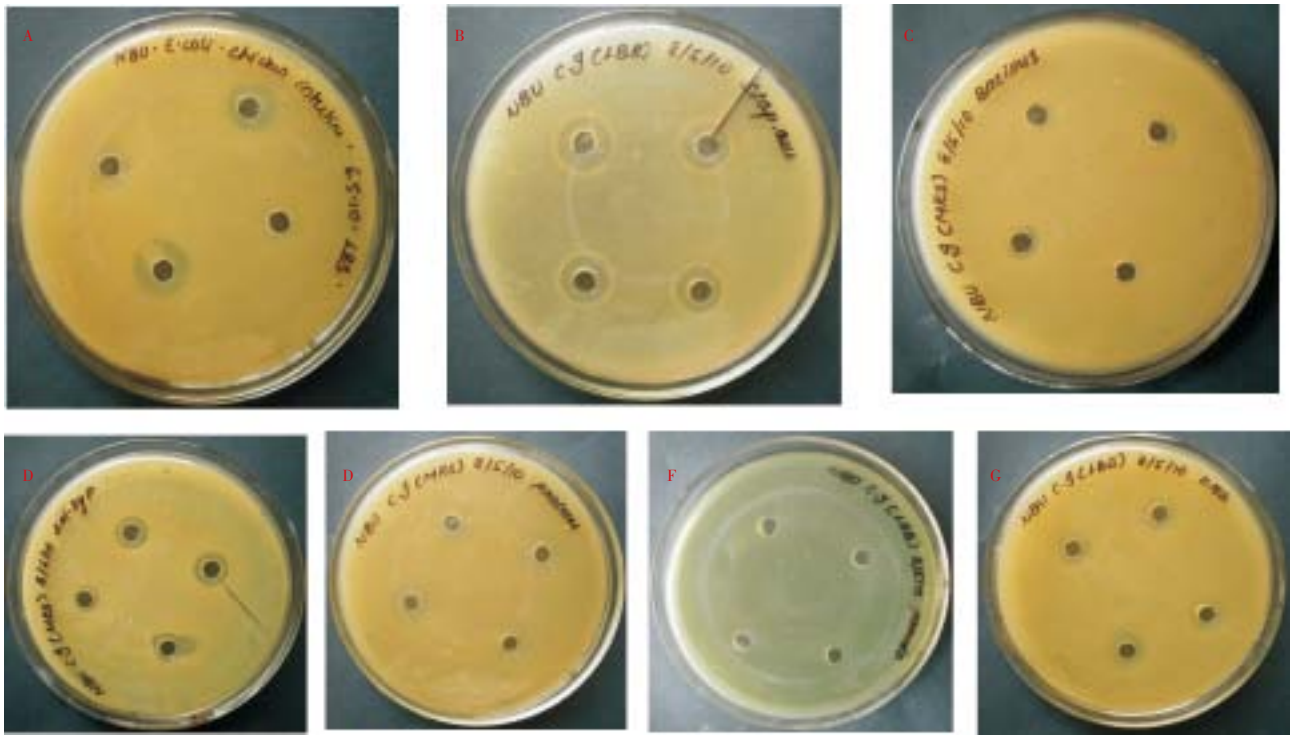


Figure 1. Antimicrobial activity of bacteriocin (UN01) against A: *E. coli* B: *S. aureus* C: *B. cereus* D: *S. typhi* E: *P.mirabilis* F: *P. aeurogenosa* G: *K. pneumonia*.

The activity of bacteriocin from isolate UN01 was completely inhibited by proteolytic enzyme (papain) indicated that isolated bacteriocin is proteinaceous in nature.

3.6. Biopreservative efficiency of bacteriocin in apple juice and fish

The partially purified bacteriocin from isolate UN01 was tested for preservative effect. Maximum reduction of population was observed in fish compared to apple juice at the concentration of 5 %; in control (without bacteriocin) no reduction of population was observed. The results further revealed that microbial count drastically decreased in apple juice and fish.

3.7. Antibiotic sensitivity of the isolate against viable antibiotics

Growth of isolate UN01 was not inhibited by Co-trimoxacole and Ofloxacin. Growth was sensitive to Erythromycin, Rifampicin and resistance to Streptomycin, Ciproflaxacin, Vancomycin, Chloramphenicol, Ampicillin and Amoxycillin.

3.8. Amplification and sequencing of 16S rRNA gene

16S rRNA was amplified using universal M13 (forward and reverse) primers. The amplified 16S rRNA (Figure 2) was identified as L. fermentum UN01 (NCBI Accession No. HQ449670).

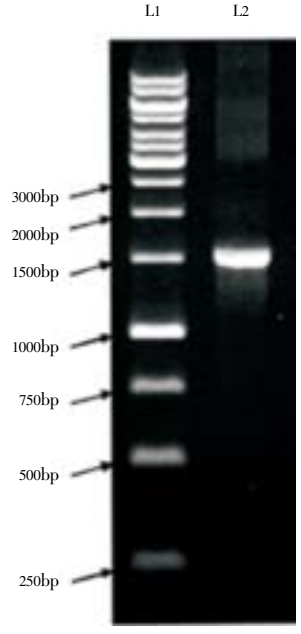


Figure 2. Amplified 16S rRNA gene of *L. fermentum* UN01 isolate

3.9. Phylogenetic Analysis

On the basis of BLAST and phylogenetic results, strain UN01 was identified as *L. fermentum* UN01. The phylogenetic data described above were obtained by using MEGA4 package using neighbour-joining, minimum evolution, maximum parsimony and bootstrapping methods. A direct analysis of the genetic distance and the phylogenetic tree (Figure 3) was determined by the 16S rRNA sequence. Various strains have been examined for the phylogenetic relationships. The evolutionary history was inferred using the UPGMA method. The optimal tree for UN01 with the sum of branch length was found to be 0.15751446. In general, 16S rRNA is most frequently used for finding out the phylogenetic relationships between closely related species. From this, the closely related UN01 suggests an evolutionary pathway for the novel form.

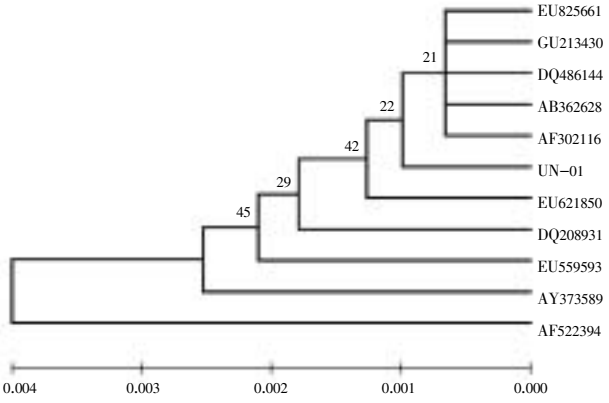


Figure 3. The phylogenetic position of the *L. fermentum* UN01 strain among neighbouring species.

4. Discussion

Our result suggested that bacteriocins producing *L. fermentum* UN01 naturally occurs and survive in chick intestine. Similarly [12] *Lactobacillus salivarius* K7 produce bacteriocin which was isolated from Chick intestine and[13] isolated bacteriocins producing organism, Pediococcus pentosaceous, Tetragenococcus halophilus.

In this present study, we used phenotypic characteristics for grouping the isolate and then employed 16S rRNA sequence analysis to identify the representative isolates from each group. These methods generated reliable data concerning the identity and distribution of LAB during fermentation. All the representative isolate sequence has high similarity with the reference strains, *L. fermentum* UN01.

It is likely that activity of bacteriocin elaborated by *L. fermentum* UN01 was pH dependent i.e., acidic. This point was reflected well by the fact that the pH level reached in 24 h enabled the bacteriocin to be effective against the indicator bacterium. Studies relating to the pH – dependent activity of bacteriocin elaborated by LAB have been shown that in a majority of the strains, highest antibacterial activity was exhibited in an acidic pH range of 4.0 – 5.0 [14, 15].

The antibacterial activity exhibited by *L. fermentum* UN01 was proteinaceous in nature and stable at 37 ℃. Similar result was reported that antibacterial substances produced by Lactobacillus species were heat stable and proteinaceous in nature [16]. A potentially novel pediocin NV5 was found active against some species of *Enterococcus*, *Leconostoc*, *Staphylococcus*, many of which are associated with food spoilage and food related health hazards [17].

For biopreservative effect of refrigerated food product

(apple juice and fish), the organism producing bacteriocin *L. fermentum* UN01 is probably be useful in food products and shows low count of colonies. This shows that refrigerated products containing bacteriocin extend the shelf life of the refrigerated raw material from three to nine days. It is also agreed with previous study that bacteriocins produced by other Lactic acid bacteria have been demonstrated to have application in enhancing quality and safety of meat products; where pediocin Pa -1, produced by *Pediococcus acidilactici* showed bactericide effect on *Lactobacillus monocytogenes* in fresh meat and its application of 5 000 AU/mL caused a significant reduction in the bacterial population in meat [18].

In our present study, the growth of *L. fermentum* UN01 was inhibited in the presence of Ampicillin, Streptomycin, Erythromycin, Rifampicin, Penicillin, Ciproflaxin, Vancomycin, Tetracyclin, Chloramphenicol and Amoxycillin. Patients taking probiotics are often treated for other illness. It is thus important to determine the effect of antibiotics on the growth of probiotic strains, especially if the product is considered as a possible probiotic products. Similarly, Torodov *et al.* [19] reported the growth of Enterococcus mundtii (from soya beans) was inhibited by similar antibiotics such as Ofloxacin, Amikacin, Ceftazidine, Nalidixic acid, Sulphamethoxzole, Neomycin, Tobramycin, Cefuroxime and Sulphonamide compounds.

The present study revealed that bacteriocin from *L. fermentum* UN01 isolated from natural Lactic acid fermentation of Chick intestine posses a wide spectrum of inhibitory activity against *E. coli*, *S. aureus*, *B.cereus*, *S. typhi*, *P. mirabilis*, *P. aeurogenosa* and *K. pneumonia*. Therefore, it is potential for application as a biopreservative in different food products such in a combination with other preservation method. Since Lactic acid fermentation is employed mostly for the development of products, especially for flavour and taste of the fermented products, the production of bacteriocin in such products, assumes more significance as biopreservative apart from imparting probiotic effect to the food products.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

The Authors thanks the management of Muthayammal Educational Charitable Trust to provide all the facilities to carry out this work (MCAS/BT/02/2010).

References

[1] Bali V, Panesar PS, Bera MB. Isolation, screening and evaluation of antimicrobial activity of potential bacteriocin producing lactic acid bacteria isolate.*Microbiol J* 2011; **1**(3): 113-119.

[2] Cassia Regina Nespolo, Adriano Brandelli. Production of bacteriocin-like substances by lactic acid bacteria isolated from regional ovine cheese. *Braz J Microbiol* 2010;**41**: 1009-1018.

[3] Torodov SD, Dicks LMT. Bacteriocin production by Lactobacillus pentosus ST7 12BZ isolated from boza. Bra. *J Microbiol* 2007; **38**:166-172.

[4] Sarika AR, Lipton AP, Aishwarya MS. Bacteriocin Production by a new isolate of Lactobacillus rhamnosus GPI under different culture conditions. *Adv J Food Sci Technol* 2010; **2**(5): 291-297.

[5] Ravi V, Prabhu M, Subramanyam D. Isolation of bacteriocin producing bacteria from mango pulp and its antimicrobial activity. *J Microbiol Biotech Res* 2011; **1**(2): 54-63.

[6] Karthikeyan V, Santhosh SW. Study of Bacteriocin as a Food Preservative and the *L. acidophilus* strain as probiotic. *Pak J Nutr* 2009; **8**(4): 335-340.

[7] Mechal Abdelbasset, Kirane Djamila. Antimicrobial activity of autochthonous lactic aicd bacteria isolated from Algerian traditional fermented milk "Raib". *Afr J Biotechnol* 2008; **7**(16): 2908-2914.

[8] Nongpanga Khunajakr, Aporn Wongwicharn, Duangtip Moonmangmee, Sukon Tantipaiboonvut. Screeing and identification of Lactic acid bacteria producing antimicrobial compounds from pigs Gastrointestinal tracts. *KMITL Sci Tech J* 2008; **8**: 1.

[9] Atta Hm, Refaat BM, El-Waseif AA. Application of biotechnology for production, purification and characterization of peptide antibiotics produced by probiotic Lactobacills planatarum, NRRL B-227. *Global J Biotech & Biochem* 2009; **4**(2): 115-125.

[10] Rajaram G, Manivasagam P, Thilagavathi B, Saravanakumar A. Purification and Characterization of a Bacteriocin produced by Lactobacillus lactis isolated from Marine Environment. *Adv J Food Sci Technol* 2010; **2**(2): 138-144.

[11] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software version 4.0. *Mol Biol Evo* 2007; **24**: 1596 - 1599.

[12] Pilasombut K, Sakpuaram T, Wajjwalka W, Nitisinprasert S, Swetwiwathana A, Zenda T, Fujita K, Nakayama J, Sonomoto K. Purification and amino acid sequence of a bacteriocin produced by lactobacillus salivarius K7 isolated from chicken intestine .*J Sci Technol* 2006; **28**(suppl.1): 121-131.

[13] Vijai P, Marilingappa J, Kadirvelu J. Isolation and characterization of bacteriocin producing Lactic acid bacteria from south Indian special dosa (Appam) batter. *J Cul Coll* 2005; **4**: 53-60.

[14] Daeschel MA, Mc Kenney MC, Mc Donald LC. Bactericidal activity of lactobacillus plantarum C-11. *Food Microbiol* 1990; **7**: 91-98.

[15] Reinheimer JA, Mc Kenney MC, Mc Donold LC. Bactericidal activity of Lactobacillus platarum C-II. *Food Microbiol* 1990; **7**: 91-98.

[16] Nettles CG, Barefoot SF. Biochemical and genetic characteristics of bacteriocin of food-associated Lactic acid bacteria. *J Food Prot* 1993; **56**: 338-356.

[17] Vivekanandha M, Sukanta KS, Narayan CM. Isolation and characterization od pediocin NV5 producing Pediococcus acidilactici LAB5 from vaccum packed fermented meat product. *Indian J Microbiol* 2011; **51**(1):22-29.

[18] Nielsen JW, Dickson JS, Crouse JD. Use of a bacteriocin produced by pediococcus acidiLactici to inhibit listeria monocytogenes associated with fresh meat. *Appl Environ Microbiol* 1990; **56**: 2142-2145.

[19] Torodov SD, Von Mollendorff JW, Moelich E, Muller N, Corli Witthuhn R, Dicks MT. Evaluation of potential probiotic properties of Enterococcus mundtii, its survival in Boza and in situ bacteriocin production. *Food Technol Biotechnol* 2009; **47**(2): 178-191.