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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN FROM *Lactobacillus casei* NCIM NO.2732

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ABSTRACT

Bacteriocins from lactic acid bacteria are considered as safe additives to control the frequent development of pathogens and spoiling microbes in food and feed. The present study deals with the screening of three standard strains of lactobacilli like *Lactobacillus casei* (NCIM No. 2732), *Lactobacillus fermentum* (NCIM No. 2166) and *Lactobacillus plantarum* (NCIM No. 2373) for bacteriocin production. The antibacterial activity was analyzed against two food borne pathogens, *E.coli* and *Pseudomonas aeruginosa*. Among the three strains, *L.casei* was found to be the most potent based on the zones of inhibition recorded against both the test organisms. The *L.casei* bacteriocin was partially purified by ammonium sulphate precipitation. Physico-chemical characterization of the partially purified bacteriocin revealed that the compound was relatively stable up to 70^0C for 30 min. Further heat treatment led to complete loss of activity. The pH stability studies of bacteriocin showed that there was a significant increase in the antibacterial activity from a pH of 3-7; no activity was recorded beyond pH 11. Evaluation of antibacterial activity at different storage periods at 4^0C revealed that up to 7 days the activity was stable and thereafter there was a gradual decrease in the activity. The proteinaceous nature of bacteriocin was confirmed by treatment with trypsin.

KEY WORDS: Bacteriocin, *Lactobacillus casei*, Bio-preservation, Antibacterial activity

INTRODUCTION

Lactic acid bacteria are widely used as starter cultures and play an important role in food preservation, microbiological stability and production of aroma compounds. Many of these lactic acid bacteria produce bacteriocins (1). By definition, bacteriocins are small proteins with bactericidal or bacteriostatic activity against genetically closely related species (2). Bacteriocin production seems to be aimed to compete against other bacteria which are present in the same ecological niche (3). Some bacteriocins are also active against Gram-positive food-borne pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilis* and spores of *Clostridium perfringens*. For this reason, they have received much attention for use as natural or so-called 'biopreservatives' in foods in recent years (4). Bacteriocins of lactic acid bacteria (LAB) have been classified into four structural classes, namely I, II, III and IV (5). They have been classified on the basis of their size, chemical properties, mode of action and mechanism of export. Some of these inhibitory substances are active against food borne pathogens and they become the focus of research interest concerning their potential role as food preservatives. Use of either bacteriocin-producing LAB strains, which are generally regarded as safe (GRAS), or their bacteriocins in food production could have a positive effect on food preservation and safety. Many bacteriocins are capable of resisting inactivation at the high temperatures used in food processing and can remain functional within a broad pH range. Bacteriocins are usually inactivated by proteolytic enzymes in the human digestive tract and would be digested just like any other protein in the diet. These natural metabolites could replace the use of chemical additives such as sorbic acid, sulfur dioxide, nitrite, nitrate, and others are used as bio-preservatives.

The spreading of bacterial antibiotic resistance and the demand for products with fewer chemicals create the necessity of exploring new alternatives in order to reduce the abusive use of therapeutic antibiotics. In this context bacteriocins are indicated to prevent the growth of undesirable bacteria in a food grade and a more natural way, which is convenient for health and accepted by the community. These substances in appropriate concentrations may be used as an additional factor for increasing the shelf life of minimal processed foods as well as in veterinary medicine and animal growth promoter instead of antibiotics.

The present study deals with the possibility of developing a bio-preservative by investigating the bacteriocin production by lactobacilli. The purpose of this study was to analyze the bacteriocin production by the standard strains of lactic acid bacteria and determination of antibacterial activity against food borne pathogens like *E.coli* and *Pseudomonas aeruginosa*. We also report the partial purification of *L.casei* bacteriocin and evaluation of antibacterial activity of partially purified bacteriocin under different physico-chemical conditions.

MATERIALS AND METHODS

Microorganisms

The standard Lactobacilli cultures such as *Lactobacillus casei* (NCIM NO. 2732), *Lactobacillus fermentum* (NCIM NO. 2166) and *Lactobacillus plantarum* (NCIM NO. 2373), obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune were used in the present study. The cultures were maintained on MRS (deMan, Rogosa and Sharpe) medium (pH 6.5) containing (g/L) Proteose peptone 10, Beef Extract 10,Yeast extract 5, Dextrose 20, Ammonium citrate 2, Sodium acetate 5, Magnesium sulphate 0.1,Manganese sulphate 0.05, Dipotassium phosphate 2, Tween 80 1 and Agar 15. Two pathogenic bacteria; *E.coli* and *Pseudomonas aeruginosa*, collected from the local diagnostic laboratory were used as the test organisms and were maintained on Nutrient agar (pH 7.0) containing (g/L) Peptone 5, Beef extract 3, Sodium chloride 5 and Agar 20 at a temperature of 4^0C. Regular sub culturing of the cultures was performed at an interval of every 4 weeks.

Preparation of culture- free supernatants

The standard cultures of Lactobacilli were grown in the MRS broth at a temperature of 37^0C for 18-20 hrs. The growth was determined in terms of optical density at 600nm (OD 0.2-0.3). This inoculated broth was used for the preparation of bacteriocin sample or culture free supernatant for assay. The overnight cultures were centrifuged at 12,000g for 10 min and then the supernatant was adjusted to pH 6.5-7.0 with 1N NaOH to avoid interference of antimicrobial effects of organic acids (6).

Bioassay of bacteriocins

The bioassay of bacteriocin was performed by disc diffusion procedure (7). The test microorganism was inoculated by swabbing over the entire surface of the pre-set nutrient agar plates. Care was taken to evenly distribute the test pathogens throughout the entire surface of the plates. Sterile filter paper discs of 6 mm diameter were prepared from Whatman filter paper. No 1. Each disc was impregnated with the 100 µl of respective culture supernatant, air dried and placed on the plates. After 18-24 hrs of incubation at 37^0C each plate was examined for the zones of inhibition and their diameters were measured.

Acid neutralization test

This test was performed by agar well diffusion assay as per the method of Karaoglu *et al* (2003). In addition to 100 µl of supernatants buffered with NaOH to 7.0, 75 µl of Lactobacilli suspension and 25 µl of 10% $CaCO_3$ solution were mixed and placed into the well. The original culture supernatants were used as control samples.

Confirmation of Bacteriocin activity (Sensitivity to catalase)

A bacteriocin assay was performed to confirm that the antimicrobial activity was due to the extraneous secretion of bacteriocin into the medium and not due to hydrogen peroxide (H_2O_2). Eighteen-hour cultures of strains showing antimicrobial activity were diluted at 1:10 ratio in 10 mM Tris HCl (pH 7.0) and 2 µl of the suspension was inoculated on Rogosa SL agar in a culture plate and incubated. Eight-hour growing cultures of indicator strains were diluted at a 1:10 ratio in 10 mM Tris HCl (pH 7.0) and mixed with Rogosa SL soft agar (48^0C). Catalase enzyme was then added at a final concentration of 0.5 mg/ml. The mixture was poured onto the plate wells. One well having no catalase enzyme was used as the control. The plates were examined after 18-24 h of incubation. The presence of an inhibition zone around wells both with and without catalase was determined to be the effect of bacteriocin (8).

Partial purification of bacteriocin

Ammonium sulphate precipitation at 0-80% saturation was carried out for the partial purification of *Lactobacillus casie* bacteriocin. The partially purified bacteriocin was again checked for the antibacterial activity (9).

Physico-chemical characterization of the partially purified bacteriocin

Effect of temperature on stability of bacteriocin

The partially purified bacteriocin was subjected to various heat treatments (6).The sample was incubated for 30 min at temperatures ranging from 30-90^0C at an interval of 20^0C. A control was maintained by incubating the bacteriocin sample at 37^0C. The antibacterial activity was then determined as described earlier.

Effect of pH on stability of bacteriocin

The sensitivity of the active substance to different pH was estimated by adjusting the pH of the partially purified sample to pH ranging from 3-12 with 1M NaOH or 1M HCl. Each pH treated bacteriocin was then assayed against the test organisms as described earlier.

Evaluation of antibacterial activity during storage

The test sample was stored at 4^0C for different time intervals of 7, 15, and 30 days to assess the stability of the antibacterial compound under shelf life condition (10).

Effect of proteolytic enzyme on bacteriocin

Lawns of test indicators were prepared and effect of proteolytic enzyme on activity of purified bacteriocin was studied by the following method. The enzyme reaction consisted of partially purified bacteriocin mixed in 1:1 (v/v) ratio with enzyme preparation (0.25

mg of enzyme trypsin dissolved in 1 ml of 0.5 M phosphate buffer). A control was maintained by mixing the bacteriocin with 0.5 M phosphate buffer. The preparations were then incubated for 1 hr at 37^0C. The enzyme reaction and control were then assayed against the lawns of test indicators (11).

RESULTS

Antibacterial activity and bioassay of bacteriocins

The antibacterial activity of the three *Lactobacilli* cultures i.e. *Lactobacillus casei, L. fermentum* and *L. plantarum* and their degree of inhibition against the test pathogens was studied. Screening of cultures showed that all the three strains have antibacterial activity against *E.coli* and *P. aeruginosa* but with varying zones of inhibition. The results are depicted in plate 1. The diameters of the zones of inhibition ranged from 0.8-2.5 cm. The highest zone of inhibition was recorded for the culture supernatant of *L. casie* against *P. aeruginosa* (2.5 cm) and the lowest inhibition was recorded for the culture supernatant of *L. fermentum* against *E.coli* (0.8 cm)

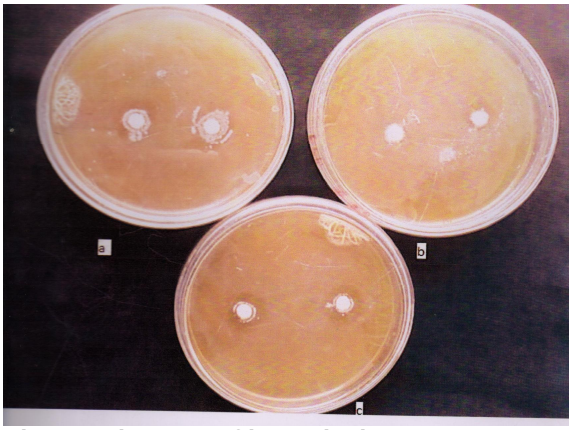

Plate 1: Bioassay of bacteriocins
a. L. fermentum* b. *L. plantarum* c. *L casei

Plate 2 depicts the results of acid neutralization test. When the inhibition zone was determined around the wells of the control and buffered samples there was no change, thus the inhibitory effect was found to be due to bacteriocin or H_2O_2.

Sensitivity to catalase

The activity of all the bacteriocins was maintained after catalase treatment, indicating that the antibacterial activity was due to bacteriocin and not due to hydrogen peroxide. The results are shown in plate 3.

Partial purification of bacteriocin

In the present study, *L. casei* bacteriocin reported the highest inhibitory effect on both the test indicators; hence it was selected for further studies. Ammonium sulphate precipitation at 0-80% saturation was carried out. The maximum antibacterial activity was retained with 80 % fraction. Table 1 shows the results of the antibacterial activity of the precipitated fractions.

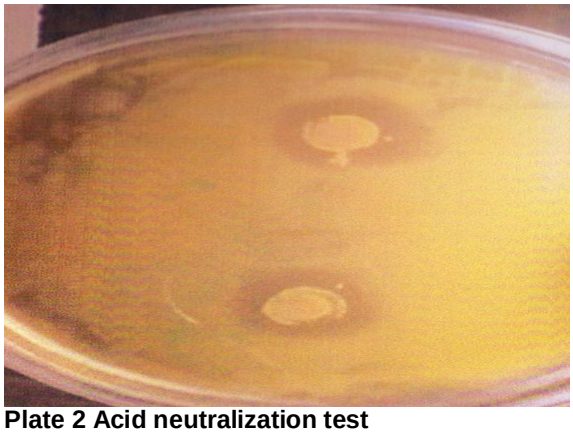

Plate 2 Acid neutralization test

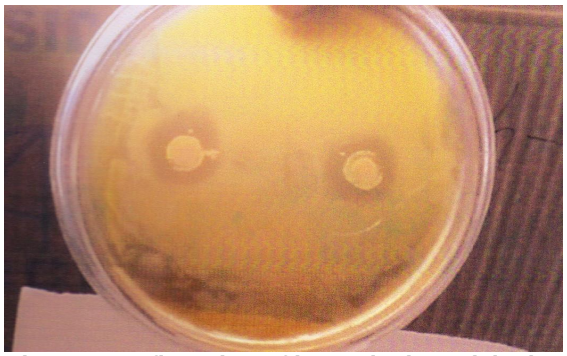

Plate 3: Confirmation of bacteriocin activity by Catalase test

Physico-chemical characterization of the partially purified bacteriocin

Effect of temperature on stability of bacteriocin

The effect of different temperatures on the stability of bacteriocins produced by *L. casei* has been outlined in the figure 1.The activity was found to gradually decrease from 30-90^0C, the antibacterial compound was stable up to 70^0C. The bacteriocin was found to be heat labile and it was completely inactivated at 90^0C.

Effect of pH on stability of bacteriocin

Figure 2 shows the effect of varying pH on bacteriocin stability. There was a significant increase in the antibacterial activity from a pH of 3-7. The highest zones of inhibition of 1.5 cm and 2.5 cm were obtained against *E.coli* and *P. aeruginosa* respectively at pH 7.0. The lowest zones of inhibition of 0.3 cm and 0.6 cm were obtained against *E.coli* and *P. aeruginosa* respectively at pH 10. No zone of inhibition was recorded at a pH beyond11. The partially purified *L. casei* bacteriocin displayed higher stability at acidic pH than at the basic pH.

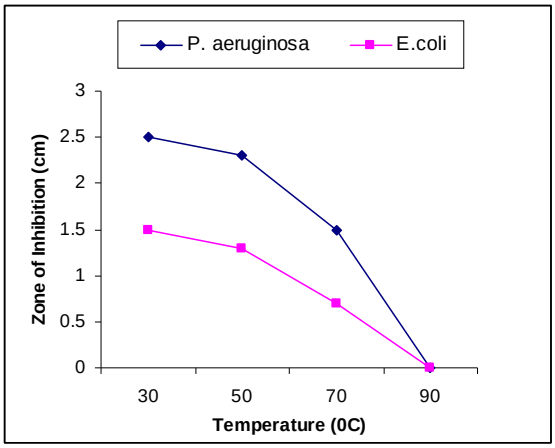

Figure 1: Effect of temperature on stability of bacteriocin

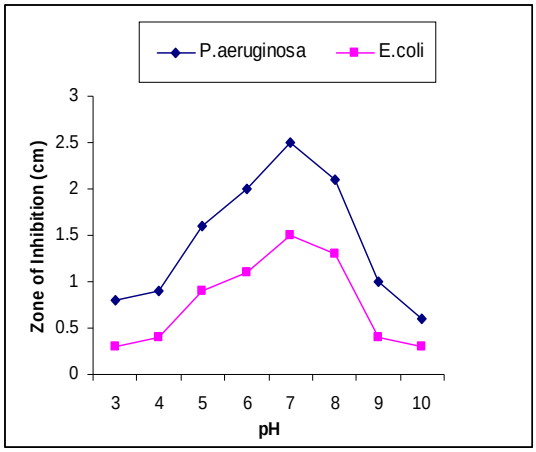

Figure 2 Effect of pH on stability of bacteriocin

Table 1: Partial purification of bacteriocin

Sl. No	Ammonium sulphate precipitation (%)	Zone of inhibition (cm)	
		E.coli	*P. eruginosa*
1.	0 (Crude extract)	1.5	2.5
2.	20	0.4	0.5
3.	40	0.8	1.1
4.	60	1.2	1.5
5.	80	2.2	3.1

Evaluation of antibacterial activity during storage

The effect of storage on the inhibitory activity of bacteriocin produced by *L. casei* has been outlined in table 2. Exposure of bacteriocin to different storage periods of 7, 15, and 30 days at 4^0C was studied. Up to 7 days the activity was stable and thereafter there was a gradual decrease in the activity.

Table 2: Evaluation of antibacterial activity during storage

Sl. No	Duration (days)	Zone of inhibition (cm)	
		E.coli	*P. aeruginosa*
1.	0	1.5	2.5
2.	7	1.5	2.5
3.	15	0.4	0.7
4.	30	0.2	0.4

Effect of proteolytic enzyme on bacteriocin

Lawns of test indicators were prepared and effect of proteolytic enzyme on activity of purified bacteriocin was studied. Absolutely no inhibition was recorded when the bacteriocin was treated with trypsin, this shows that the compound is completely inactivated by the enzyme, thus reflecting its proteinaceous nature.

DISCUSSION

The present study tests antibiosis of lactobacilli against selected food borne pathogens such as *E..coli* and *P. aeruginosa*. The study also focused on the partial purification and characterization of *L.casei* bacteriocin and the data revealed the possibility of using this bacteriocin in food preservation. The results of the primary screening for bacteriocin production revealed that all the three lactobacilli were positive, however *L.casei* showed the highest inhibitory effect against both the test indicators. The bacteriocin activity was confirmed by acid neutralization and catalase-sensitivity test. Karaoglu *et al* (2003) reported that the activity of six bacteriocins from various lactobacilli strains was maintained after catalase treatment, indicating that antibacterial activity was due to bacteriocin not H_2O_2. Our results are in good agreement with those of Karaoglu *et al* (2003). In their study, six strains of lactobacilli were observed to have bacteriocin activity against eight of 10 different Lactobacillus species as well as *S. milleri, P. aeruginosa, E. coli, P. vulgaris, E. cloacea* and *G. vaginalis.*

Partial purification of the *L.casei* bacteriocin by ammonium sulphate precipitation revealed that the maximum antibacterial activity was retained with the

80% fraction, showing the highest zones of inhibition against both the test pathogens. Bhattacharya and Das (2010) reported the partial purification of bacteriocins from seven different isolates of lactic acid bacteria at 45% ammonium sulphate saturation and the apparent molecular weights of the bacteriocins were found to be in the range of 16.5 - 48 kDa.

The study on the effect of temperature on the stability of bacteriocin revealed that the compound was relatively stable up to 70^0C for 30 min. Further heat treatment led to complete loss of activity. This indicated that bacteriocin belongs to heat labile group of bacteriocins. According to Bhattacharya and Das (2010), the antibacterial substances produced by lactic acid bacteria were relatively stable up to 80^0C for 30 min; however the activity was markedly lost after autoclaving. Dicks and Todorov (2005) reported two bacteriocins, ST28MS and ST26MS, produced by *Lactobacillus plantarum* remained active after 20 min at 121 °C. According to Fira D *et a*l (2010) a bacteriocin BacUB9 from *Lactobacillus paracasei subsp. paracasei* BGUB9 is a relatively heat-stable molecule. The antimicrobial activity was not affected by treatment at 100^0C for 30 min. After heat treatment at 100^0C for 60 min the activity decreased and after heat treatment at 100^0C for 120 min, the activity of bacteriocin BacUB9 was completely abolished. Gyananath *et al* (2006) studied bacteriocins from *Lactobacillus lactis* and *Lactobacillus plantarum* and found the bacteriocins of both isolates were stable at 100°C for 10 minutes. Bacteriocin of *Lactobacillus lactis* retained its activity even at 121°C for 10 minutes. On the contrary, Itoh *et al* (1991) reported a heat-labile bacteriocin from *Lactobacillus acidophilus* LAPT 1060 sensitive to heat for 10 min at 60°C.

Further studies were carried out at different pH to test the pH stability of bacteriocin. The results showed that the compound was more stable at the acidic pH than at the basic one. Similar results are also reported by Bhattacharya and Das (2010). According to Gyananath *et al* (2006) bacteriocin of *Lactobacillus lactis* was stable in acidic to neutral range i.e. from pH 4 to 7 but it became inactive in the alkaline range whereas bacteriocins of *Lactobacillus plantarum* remained active only in the acidic range from pH 4 to 6.Our results are in close agreement with those of Gyananath *et a*l (2006). Fira D *et al* (2010) reported a bacteriocin; BacUB9 which retained its activity within the pH range from 1 to 10. Antimicrobial activity was lost at pH 11. The results of Karaoglu *et al* (2003) showed that all the bacteriocins from six different strains of lactobacilli were stable between pH 4.5 and 7.0, but sensitive to pH 9.0. Two bacteriocins isolated from *L. delbrueckii subsp.delbrueckii* TL059a and L. acidophilus TL099a were found to be active at pH 3.0.

The evaluation of antibacterial activity after different storage periods of 7, 15 and 30 days at 4^0C showed that the compound retained the activity up to 7 days , as there was no change in the zone of inhibition. However further incubation led to decrease in the activity. Similar observations have been reported by Bhattacharya and Das (2010).

Treatment of the bacteriocin with trypsin showed that there was complete loss of activity, revealing its proteinaceous nature Similar results are reported by Bhattacharya and Das (2010), Karaoglu *et a*l (2003), Gyananath *et al* (2006), Fira D *et al* (2010) etc.

Biological preservation employs a novel scientific approach to improve the microbial safety of foods. The present study concludes that the *L casei* bacteriocin can be explored as a bio-preservative. Interesting feature of this compound is its heat stability up to 70^0C, which supports that fact that it might constitute an advantage as a food additive in processes like pasteurization. In addition, wide range of pH tolerance is an important feature. To the best of our knowledge, this is the first report on the partial purification and characterization of bacteriocin from *L casei* NCIM 2732. However, future approaches should consider the application of bacteriocin in combination with treatments enhancing its effectiveness in foods. The activity can be enhanced by using it in combination with other bacteriocins or other compounds including surfactants, chelating agents etc. Further research is required to focus on the synergistic effects of natural preservation in conjugation with advanced technologies. This could result in replacement of chemical preservatives, or could allow less severe processing treatments, while still maintaining adequate microbiological safety and quality of foods.

REFERENCES

1. Degnan AJ., Yousef AE and Luchansky JB. (1992). Use of *Pediococcus acidilacti*ci to control *Listeria monocytogenes* in temperature-abused vacuum-packaged Wieners. J Food Protect. 55: 98.
2. Klaenhammer TR. (1988) Bacteriocins of lactic acid bacteria, Biochimie, 70: 337
3. Nettles CG and Barefoot SF (1993). Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. J Food Prot. 56: 338.
4. Savadogo A., Ouattara Cheik AT., Bassole Imael HN and Traore SA. (2004). Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Burkina Faso Fermented Milk. Pak J Nutr. 3: 174
5. Nes IF., Diep DB., Halvarstein LS., Brurberg MB., Eijsink V and Holo H. (1996). Biosynthesis of bacteriocins in lactic acid

bacteria. Antonie Van Leeuwenhoek. 70: 113.
6. Sharpe ME., Fryer TF., Smith DG., (1979). Identification of Lactic Acid Bacteria. In: Identification Methods for Microbiologists, Gibbs, E.M. and F.A. Skinner (Eds.). Academic Press, London, ISBN-10: 0126477507, pp: 233.
7. Hernnadez D., Cardell E. and Zarate V., (2005). Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: Initial characterization of plantaricin TF711 a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. J. Appl Microbiol 99: 77
8. Karaoglu AS., Faruk A., Kilic SS and Kilic AO. (2003). Antimicrobial activity and characteristics of bacteriocins produced by vaginal lactobacilli. Turk. J. Med. Sci., 33: 7
9. Schöbitz RP., Bórquez PA., Costa ME., Ciampi LR and Brito CS. (2006). Bacteriocin like substance production by *Carnobacterium piscicola* in a continuous system with three culture broths. Study of antagonism against *Listeria monocytogenes* on vacuum packaged salmon Braz. J Microbiol 37: 52
10. Khalil R., El-Bahloul Y., Djadouni F and Omar S. (2009). Isolation and partial characterization of a Bacteriocin produced by a newly isolated *Bacillus megaterium* strain 19. Pak J Nutr 8: 242
11. Srikanjana K.,Helmut V., Jakkapan S and Okonogi S. (2008). Probiotic properties of Lactobacilli isolated from Thai traditional food. Sci. Pharm., 76: 485.
12. Bhattacharya S and Das A. (2010). Study of Physical and Cultural Parameters on the Bacteriocins Produced by Lactic Acid Bacteria Isolated from Traditional Indian Fermented Foods. Am J Food Technol 5: 111
13. Todorov SD and Dicks LMT. (2005).*Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. Enz Microbial Technol 36: 318
14. Tolinački M, Kojić M, Lozo J, Terzić Vidojević A, Topisirović and Fira D (2010). Characterization of the bacteriocin-producing strain *Lactobacillus paracasei subsp. paracasei* BGUB9. Arch Biol Sci 62 : 889
15. Lade HS., Chitanand MP., Gyananath G and Gyananath TA.(2006). Studies On Some Properties Of Bacteriocins Produced By Lactobacillus Species Isolated From Agro-Based Waste. The Internet J Microbiol. 2
16. Toba T., Yoshioka E and Itoh T. (1991).Acidophilucin A, a new heat-labile bacteriocin produced by *Lactobacillus acidophilus* LAPT 1060. Lett Appl Microbiol, 12: 106.