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RESEARCH PAPER

Molecular Characterization of Bacteriocin Producing Lactic Acid Bacteria From Fermented food "*Idli*"

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ABSTRACT

Bacteriocins are the metabolites produced by lactic acid bacteria having antimicrobial activity thus, with a tremendous potential as a biopreservative agent for processed food. In this study, bacteriocinogenic LAB were isolated from the fermented food, "*Idli*". Morphological and biochemical analyses of these isolates were done by the traditional methods. The isolates (10) were able to produce bacteriocin whose antibacterial activity was analyzed by agar well diffusion assay test against indicator organisms. The antibacterial protein (bacteriocin) was characterized based on the sensitivity to heat, pH, chloroform, NaCl and incubation time. It was observed that 1% NaCl enhanced the bacteriocin production of all the bacteriocin positive isolates. The bacteriocins produced by the lactic acid bacteria isolated in this work could act as a good source to inhibit the growth of spoilage bacteria and foodborne pathogens.

Keyword: Lactobacilli, bcteriocin, biopreservative

Microorganisms are responsible for most of the spoilage of food. Preservation of fermented food by lactic acid fermentation is an age old practice involving lactic acid bacteria (LAB). Lactbacilli produce different metabolites such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin during fermentations (Lindgren and Dobrogosz, 1990). Lactic acid bacteria have long been used in fermented food and beverages for their contribution to flavor and aroma development, and spoilage retardation (Gilliland, 1986). The strains of lactic acid bacteria are potential candidate as a bio preservative or natural food preservative (Joshi, *et al.*, 2005). The antimicrobial activity of bacteriocin produced by LAB has been detected in foods such as dairy products, meat, barley, sourdough, fermented vegetables, etc. During the last few decades considerable research has been focused on the detection, characterization and purification of bacteriocins, which are considered as safe biopreservatives, as they are degraded by the proteases in gastrointestinal tract (Holzapfel *et al.*, 1995).

During fermentation, the LAB break down sugars and carbohydrates to produce alcohol, carbon dioxide and lactic acid (Vela, 1997). By-products generated during this process are responsible for the unique taste of fermented foods and help preserve and increase palatability. There are several previous reports about the isolation of bacteriocin from LAB of *appam* batter and pickle. Bola and Damsa (2011) studied

the antifungal activity of Lactic Acid Bacteria from salad vegetables. Nithya, *et al.* (2012) characterize the bacteriocin producing *Lactobacillus fermentum* from whey. *Lactobacillus delbrueckii* and other lactic acid bacteria are important in the fermentation of many foods from dairy products to fruits and vegetables (Joshi *et al.,* 2016). *Idli* is a traditional breakfast in south Indian households. *L. delbrueckii* is naturally found in many fermented food like *Idli (*Joshi, 2006). In this paper, we report on characterization and molecular analysis of bacteriocins from strains of *L. delbrueckii* isolated during *Idli* processing.

MATERIALS AND METHODS

Isolation and Screening of *Lactobacilli*

Samples of fermented food '*idli*' were collected from Krishna Foods, local market of Nashik. The *idli* batter is prepared from overnight fermentation of rice (*Oryza sativa*) and black gram (*Phaseolus mungo*), a legume. Further, serial saline dilution was made from fermented '*idli*' to obtain purified bacterial colonies on agar plates incubated anaerobically at 37 °C for 24-48 h. Pure culture of the organisms was streaked centrally on sterilized solidified plate count agar plates. Plates were incubated at 37°C for 24 hours. The pure cultures thus obtained, were overlaid with glycerol and preserved for further study.

Pure Cultures and the LAB Isolates

Pure cultures of *Bacillus licheniformis* (NCIM 2051), *Streptococcus thermophillus* (NCIM 2904), *Zymomons anerobica* (NCIM 2427), *Streptococcus acidophilus* (NCIM), *Eschereschia coli* (NCIM) were obtained from National chemical laboratory, Pune. These strains were sub-cultured on Nutrient agar slants and cultures was stored in the refrigerator. The lactobacilli isolates were propagated in MRS broth and its cell free supernatant (CFS) from 48 h culture was collected and pellet was discarded.

Antimicrobial activity

Antimicrobial spectrum was carried out against various indicator LAB and pathogens by agar well diffusion method (Jamuna and Jeevaratnam, 2004). The bacterial cultures were grown in Brain Heart infusion broth and diluted appropriately. The lawn of pathogens was prepared on the nutrient agar plates. The wells were prepared and the bacteriocin obtained was added in well and were kept for incubation under aerobic conditions. The antimicrobial activity was determined by measuring the diameter, of the inhibition zone produced around the well.

Biochemical Tests

Isolated strains were Gram stained and examined microscopically to check for morphology and Gram-stain phenotype. By using 3 % hydrogen peroxide catalase activity of the selected isolates was performed. Production of acid and CO_2 from glucose was tested in MRS broth, containing Durhams tube. Homo and heterofermentative differentiation test was also carried out. Methyl Red test, citrate utilization, Indole Production test, Voges–Proskauer (VP) test, citrate utilization test, starch hydrolysis test, mannitol test were performed on selected isolates as per the standard procedure (Harrigam and McCamce, 1966)

Characterization of bacteriocinogenic isolates

About 100 µl of culture supernatant was used for agar spot assay to detect residual activity against indicator pathogens. The resistant cultural supernatants were heated for 10, 30 and 60 min at 100 °C (Larsen *et al.,* 1993). Similarly the pH of supernatant was adjusted to 2.0, 4.5, 7.0 and 9.0 for checking optimal activity. To check for the chloroform sensitivity, supernatant was mixed with an equal volume of chloroform at room temperature for 4 hrs before testing antimicrobial activity (Diaz *et al.,* 1993). MRS broth along with 1%, 3%, 4% NaCl and without NaCl were inoculated with 10% of the overnight grown culture and incubated for 24 hrs at 37°C. Effect of different incubation period was studied at 37^0 C for 15, 24, 48 and 72 hrs and bacteriocin activity was observed by inoculating supernatant against indicator organism (Lade *et al.,* 2006).

The carbon sources studied were glucose (1% – 3%) and lactose (1% – 3%) while nitrogen sources were used to study the effect of different concentrations of carbon and nitrogen source on bacteriocin production (Lade *et al.,* 2006).

Molecular analysis

Genomic DNA was isolated by the procedure as described by CTAB method. Bacteria from saturated liquid culture were lysed and the proteins were removed by digestion with proteinase K. The pure DNA obtained by thus procedure was used as a template for PCR amplification. Universal oligonucleotide primers were used for amplification purpose 27 F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3'). DNA amplification was conducted in a DNA thermal cycler to determine the species affiliation of the tested isolates. 20 µl of reaction volume was used: 10X *Taq* buffer, dNTPs mix 5.0 µl, Primer 2 µl, Taq DNA polymerase 0.5 µl, Autoclaved distilled water 8.0 µl. The amplification profile was 94°C for 5 min, 94°C for 1 min, 53°C for 1 min., 72°C for 2 min and 72°C for 7 min, 30 cycles. SDS - PAGE was performed for protein profiling of bacteriocin obtained from *idli*. In the SDS PAGE, separating gel prepared of 12% and stacking gel 5% was conducted as described (Davis, 1964).

RESULTS AND DISCUSSION

The results show that variation in the concentration of constituents has an influence on the amount of a bacteriocin produced. The results of the Agar spot assay showed that a total of ten isolates were able to inhibit the indicator organism by producing bacteriocin (Table 1). Using this method, by inoculating the wells with broth cultures from various indicator microorganisms, comparisons were made between the bacteriocin productions of different strains growing under identical conditions as shown in Table 1.

The bacteriocin is of particular importance to the food industry and also to other pathogens like *Escherichia coli, Bacillus lichenformis, Streptococcus thermophillus, Sterptococcus acidophillus, and Z. anarobica,* which are common food spoiling pathogens (Luo *et al.,* 2011). Broad spectrum of antimicrobial activity of the bacteriocin may have its potential use as a food additive/preservative in food industry, particularly refrigerated foods. These results are in compliance with the previous studies done by El-Shafei *et al.* (2000) for the isolation, screening and characterization of about hundred strains of bacteriocin-producing lactic acid bacteria from traditional fermented foods. The lactic acid bacteria from fermented vegetable bacteriocin, having antimicrobial activity (Joshi *et al.,* 2016).

Table 1: Screening of Isolates for Antimicrobial Activity

Species name	Zone of inhibition (mm)
E.coli	3.0 cm
Bacillus licheniformis	2.0 cm
Sterptococcus acidophillus	2.3 cm
Streptococcus thermophillus	2.4 cm
Zymomons anerobica	2.2 cm

Table 2: Various biochemical and phyio-chemical characteristics of selected isolates

Test Name	Different bacterial isolated form							
	IL1	IL2	IL3	IL4	IL5	IL6	IL7	IL8
Cell form	*Rod*	*Rod*	*Rod*	*Rod*	*Rod*	*Rod*	*Rod*	*Rod*
Homo-hetero fermentation	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho
Catalase production	–	–	–	–	–	–	–	–
Gram's Reaction	+	+	+	+	+	+	+	+
Motility	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	+
MR	–	–	–	–	–	–	–	–
nitrate	–	–	–	–	–	–	–	–
citrate	–	–	–	–	–	–	–	–
VP	–	–	–	–	–	–	–	–

The selected isolates were characterized by classical methods. These isolates were shown to be Gram positive. Results of various other physiological and chemical tests which were carried for these selected isolates as shown in Table 2. The maximal bacteriocin production is known to be associated with several factors such as pH, temperature, composition of the culture medium, on maximal bacteriocin production (Aymerich, *et al.,* 2000; Biswas *et al.,* 1991; Vignolo *et al.,* 1995). Temperature and pH is known to play an important role in cell growth as well as bacteriocin production. There was no change in antimicrobial activity when bacteriocin was heated at 60, 70, 80, 90 and 100 °C and antimicrobial activity was considered to be heat resistant. It is one of the very useful characteristic for the bacteriocin to be used as a food preservative, because many food-processing procedures involves heating step.

The activity of bacteriocin seems to have been lost at higher alkaline pH which might be ascribed to proteolytic degradation, instability of proteins at this extreme pH (Aasen *et al.,* 2000; Parente & Riccardi, 1994).

The effect of NaCl on the production of bacteriocin from these selected isolates indicated that 1% NaCl increased the production of bacteriocins in almost all the strains. Two strains showed superior activity in presence of 3% NaCl when compared to other isolates, but this activity was lost at 4% NaCl. Larsen *et al.* (1993) also detected that bavaricin A from *L. bavaricus* production was inhibited with increasing amount of NaCl. The effect of incubation period at 37° C on bacteriocin production proved that at the end of 24 hrs the activity was found to be the maximum, while at 48 and 72 hrs no inhibitory action was found.

The biochemical properties such as catalase production, citrate, nitrate were utilization negative for all the selected isolates. All the isolates were homo-feremetative. There are several reports of studies conducted on the effect of nitrogen and carbon sources on the production of bacteriocins (Enan *et al.,* 1996; Todorov, 2008 and Daeschel *et al.,* 1990).

The bacteriocin production in this study require tryptone as the major nitrogen source. Similarly, bacteriocin production was enhanced in the case of glucose as a carbon source. The conventional characterization of the isolates showed that the isolates could be *Lactobacillus delburckii*, but this characterization was not found sufficient to distinguish sub-species.

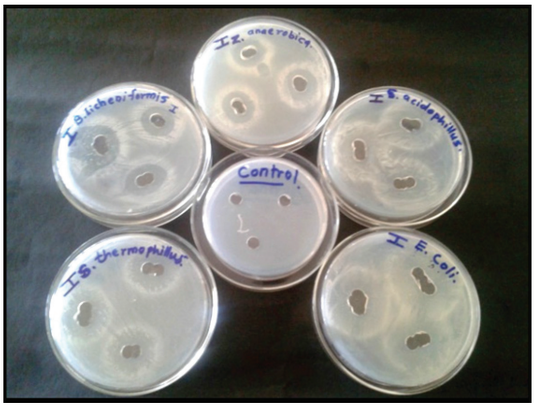


Fig. 1: Antimicrobial activity by Bacteriocin obtained from LAB isolated from ***Idli*** sample IL1

Thereafter, PCR based molecular tools were employed to identify the sub-species of lactobacillus. After amplification 850 bp band was clearly found by using 1.2 % agarose gel as shown in Fig. 2.

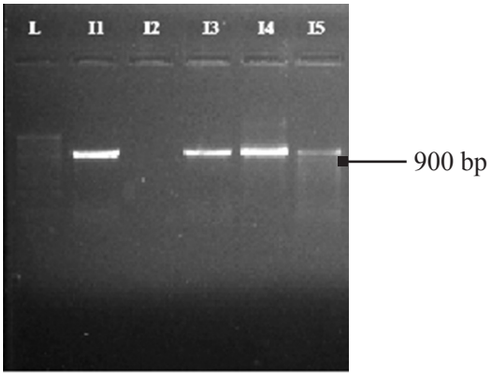
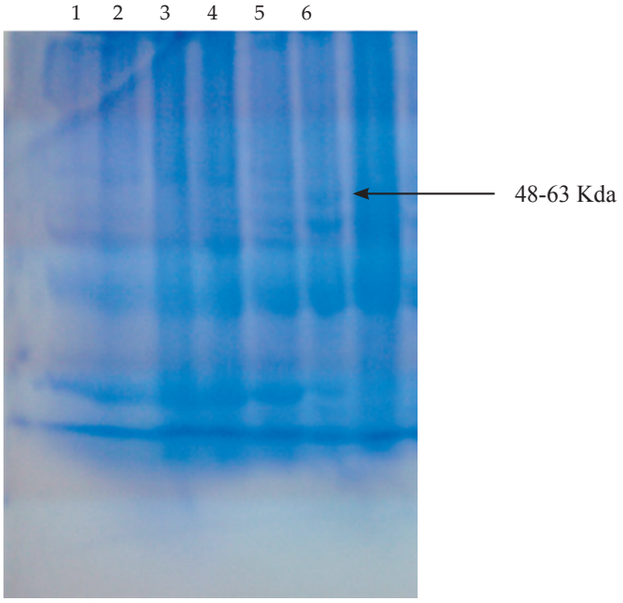


Fig 2: PCR of isolated genomic DNA from *L. delbrueckii*

The rate of migration of proteins depends on the charge on the molecule, its molecular mass, size and the strength of the electric field. The protein profiles for bacteriocin was observed in the range of

48 – 63 kDa, as depicted in Fig. 3. Bacteriocinogenic lactic acid bacteria and their isolated bacteriocins are considered safe additives (GRAS), useful to control the frequent development of pathogens and spoiling microorganisms in foods and feed.



Lane 1: MW marker
Lane 2: Protein isolated from bacteriocin 1
Lane 3: Protein isolated from bacteriocin 2
Lane 4: Protein isolated from bacteriocin 3
Lane 5: Protein isolated from bacteriocin 4
Lane 6: Protein isolated from bacteriocin 5
Lane 7: Protein isolated from bacteriocin 6

Fig. 3 : Molecular characterization by SDS PAGE

The bacteriocins are shown to prevent the growth of undesirable bacteria in a food-grade and more natural way, which is convenient for health and accepted by the community.

CONCLUSION

The study revealed that bacteriocin from *Lactobacillus* sp. isolated from naturally fermented food possesses a wide spectrum of inhibitory activity against *Escherichia coli*, *Streptococcus thermophillus* and *Bacillus lichenformis*. Thus, it has a potential for application as a biopreservative in different food products as such or in combination with other preservation methods. Since lactic acid fermentation is employed mostly for development of products, especially for flavor 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 product.

REFERENCES

Aasen I.M., Moreto T., Katla T., Axelsson L., Storro I. 2000. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Appl. Microbiol. Biotechnol.* **53:** 159-166.

Arokiyamary, A. and Sivakumar P.K. 2012. Antibacterial spectrum, and mode of action of bacteriocin produced by *lactobacillus* sp., isolated from traditional dairy products. *Int. Journal of PharmTech Research.,* **4(1):** 315-320.

Aymerich, T., Artigas, M.G., Monfort, J.M. and Hugas, M. 2000. Effect of sausage ingredients and additives on the production of enterocins A and B by *Enterococcus faecium* CTC492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. *Journal of Appl. Microb.* **88**: 686–694.

Biswas S.R., Ray P., Johnson, M.C. and Ray, B. 1991. Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. *Appl. Environ. Microbiol.* **57:** 1265- 1267.

Bola, A. and Damsa, I. 2011. Antifungal capacity of Lactic Acid Bacteria Isolated from Salad Vegetables. *Afr .J. Biomed Res.,* **14**: 137-141.

Daeschel M.A., McKenney M.C., McDonald L.C. 1990. Bacteriocidal activity of *Lactobacillus plantarum* C-11. *Food Microbiology,* **7(2):** 91-98.

Davis, B.J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci.,* **121**: 404-427.

Diaz, R., Sanchez, R.M.R., Desmazeud, M.., Ruiz-Barba, J.L. and Piard, J.C. 1993. Plantaricin S. and T, two new bacteriocins produced by *Lactobacillus plantarum* LPC010 isolated from a green olive fermentation. *Appl. Environ. Microbiol.*, **59:** 1416-1424.

El-shafei, H.A., Abd-el-sabour, J.I., Ibrahim, M. and Mostafa, Y.A. 2000. Isolation, screening and characterization of bacteriocin producing lactic acid bacteria isolated from traditional fermented food. *Microbiol. Rev.* **154 (4)**: 321-331.

Enan, G., El-Essawy, A.A., Uyttendaele, M. and Debevere, J. 1996. Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. *International Journal of Microbiology,* **30,** 189–215

Gilliland, S.E. 1986. Bacterial Starter Cultures for Foods. CRC Press, Boca Raton, Florida.

Haurigan, W.F. and McCane, E.M., Laboratory Methods in microbiology, Academic Press, London and New York, 1996.

Holzapfel, W.H., Geisen, R. and Schillinger, U. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int. J. Food. Microbiol.,* **24**: 343–362.

Jama, Y.H. and Varadaraj, M.C. 1999. Antibacterial effect of plantaricin LP84 on food borne pathogenic bacteria occurring as contaminants during *idli* batter fermentation. *World J Microbiol Biotechnol.,* **15**: 27–32.

Jamuna, M. and Jeevaratnam, K. 2004. Isolation and characterization of lactobacilli from some traditional fermented foods and evaluation of the bacteriocins. *J. Gen. Appl. Microbial.,* **50**: 70-90.

Joshi, V.K. (Ed.) 2016. Indigenous fermented foods of south Asia, CRC press, Taylor and Francis, Boca Raton, Florida.

Joshi, V.K., Sharma, S. and Rana., N.S. 2005. Production, Purification, Stability and efficacy of Bacteriocin from Isolates of Natural Lactic Acid Fermentation of Vegetables. *Biotechnol and Food Technol.,* **44(3)**: 435-43.

Lade, H.S., Chitanand, M.P., Gyananth, G. and Kadam, T.A. 2006. Studies on some properties of Bacteriocins produced by Lactobacillus species Isolated from Agro-Based waste. *Int. J. Microbiol.,* **2**: 1.

Larsen, A.G., Vogensen, F.K. and Josephsen, J. 1993. Antimicrobial activity of lactic acid bacteria isolated from sour doughs: purification and characterization of bavaricus M1401. *J. Appl. Bacteriol.,* **75**: 113-122.

Lindgren, S.W. and Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiol. Rev.,* **87:** 149-164.

Nithya, K., Senbagam, D., Senthilkumar, B., Udhayashree, N. and Gurusamy, R. 2012. Characterization of bacteriocin producing lactic acid bacteria and its application as a food preservative. *African J. of Micro. Res.,* **6(6):** 1138-1146.

Okoro, I.A., Ojimelukwe, P.C., Ekwenye, V.N., Akaeru, B., Prince-Will Ogbonna, I.L. and Offia-Olua, B. 2011. Comparative evaluation of the bacteriocin producing characteristics of lactic acid bacteria from selected traditional fermented foods. *Continental J. Food Science Biotech.,* **5(1)**: 1-5.

Parente E., Riccardi A. and Addario G. 1994. Influence of pH on growth and bacteriocins production by *Lactococcus lactis subsp. lactis* 140VWC during batch fermentation. *Appl. Microbiol. Biotechnol.,* **41:** 388- 394.

Tododrov S.D. 2008. Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of adsorption of bacteriocin AMA-K to *Listeria* spp. *Brazilian Journal of Microbiology*, **38:** 178–187.

Vela, G. 1997. Applied Food Microbiology. Star, CA.

Vignolo, G.M., Dekairuz, M.N., Holgado, A.A.P.D. and Oliver, G. 1995. Influence of growth conditions on the production of lactacin-705, a bacteriocin produced by *Lactobacillus casei* CRL-705. *J. Applied Bacteriol.,* **78:** 5-10.