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Screening and partial characterization of natural isolates of lactic acid bacteria for bacteriocin production

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Abstract

Different food items were screened for bacteriocin producing microbial strains. Out of fifty isolates, two best strains DB1 and TB7 were selected and further tested for bacteriocin production. The bacteriocin activity was measured by agar well diffusion method against *Staphylococcus aureus* and *Bacillus coagulans*. Bacteriocin produced by these two strains were heat stable upto 80˚C and at pH range of 6-9. Because of their higher thermo-stability, wide pH tolerance and bactericidal mode of action, they could be used as natural preservative to enhance shelf life of the different processed food products.

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Introduction

Technologies of processing and preservation of food products, which helps in maintaining its nutritional values, besides ensuring safety issues, are the area of current food research. Many chemicals are being used for the inactivation of food borne pathogens to preserve food products for longer duration. However, their use is being declined due to undesirable side effects like alteration in the constituents, nutritional and organoleptic properties of food and their toxic effect on human health (Sharma *et al*., 2006). Ever increasing demand of consumers for faster, healthier and ready-to-eat products without use of chemical preservatives have evolved new techniques of using preservatives of biological origin, specifically termed as "Bio preservatives" (Oguntoyinbo *et al*., 2007; Stoyanova *et al.*, 2007). Many bacteria of different taxonomic branches and residing in various habitats produce antimicrobial substances that are active against other bacteria. Bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides (De Vuyst and Vandamme, 1994). In general, these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane, in most cases, is the target for their activity. The bacteriocins are peptides of proteins having an antimicrobial activity against closely related microorganisms and hence could be used as natural food bio-preservative. As for example lactic acid bacteria (LAB), commonly used as starter cultures in foods, are known to produce antimicrobial substances such as "Bacteriocins", having great potential as food bio-preservatives. Depending on the producer organism and classification criteria i.e. according to size, mode of action and structural characteristics, bacteriocins can be classified into several groups (Ennahar *et al*., 2000; Jack and Jung, 2000; Cleveland *et al*., 2001; McAuliffe *et al*., 2001) in which classes I and II are the most thoroughly studied. The potential application of bacteriocins will be to use as a consumer friendly biopreservative holds a great promise. Review of literature indicated that there are limited studies on isolates from fermented foods, hence current studies are highly promising and underline the important role that bacteriocinogenic strains of LAB may play in the food industry as biopreservative or starter cultures. In this study Lactic acid Bacteria were isolated from fermented food samples and were screened for their bacteriocin producing potential against common food spoiling bacteria: *Bacillus subtilts, Bacillus pumulis, Staphylococcus aureus, Lactococcus lactis, Lactobacillus fermentum, Bacillus coagulans* and *Salmonella typhimurium.* The bacteriocins were further evaluated for their stability profile under different temperature and pH conditions.

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Materials and Methods

Isolation and screening of LAB for bacteriocin assay

In the present study, different samples of various fermented foods were collected from the local market in Chandigarh and screened for bacteriocin producing organisms using lactobacilli De Mann Rogosa and Sharpe (MRS) media. Fifty randomly picked colonies were tested for antimicrobial activity against six indicator strains *Bacillus subtilts* MTCC 441, *Bacillus pumulis* MTCC 1607, *Staphylococcus aureus* MTCC 737, *Lactococcus lactis* MTCC 3038, *Lactobacillus fermentum* MTCC 903, *Bacillus coagulans* 3244 and *Salmonella typhimurium* MTCC 98 obtained from IMTECH, Chandigarh, India. Out of five food samples, two were found to contain isolates having antagonistic activity against indicator strains. Initial screening for bacteriocin activity in food samples was carried out using cell free supernatant to perform agar well diffusion assay as per the modified method by (Aslim *et al.*, 2004). Clearance zone diameter was measured in millimeter and the organisms were selected on the basis of the diameter of the zone of inhibition. Morphological analysis, gram staining and biochemical characterization were carried out applying standard protocols (Kandler and Weiss, 1986).

Optimization of culture conditions

The selected strains of *Lactobacillus* species, identified by the diameter of zone of inhibition were subjected to different culture conditions like temperature, pH and its ability to grow in the presence of Sodium Chloride by varying concentration, to derive the optimum conditions for bacteriocin production (Lade *et al*., 2006). Growth pattern of isolates was estimated at various temperatures: 15°C, 28°C, 37°C and 45°C, pH: 4.3, 6.5, 8.5 and Sodium Chloride concentrations: 6.5%, 10% and 15% after 24 and 48 hours.

Biolog test

The isolated strains were characterized by determining their substrate utilization pattern with Biolog General III (Biolog Inc., Hayward, USA).
For determination, the bacterial cells were grown on nutrient agar media. Bacterial cells were harvested using sterile swab and dissolved in inoculation fluid provided by the manufacturer. The Biolog plates were inoculated by 100 µl of the cell suspension that has been adjusted by comparison with the turbidity standard supplied by the manufacturer. These plates were inoculated at 35^{o}C for 36hr. The color development in the micro plate wells was interpreted as positive, negative and borderline, in case it is not possible to differentiate positive from negative. The reading was also entered in the Biolog microlog General III databases to provide identification.

Isolation and characterization of crude bacteriocin

The selected isolates showing good antagonistic activities, based on the diameter of zone of inhibition, were grown on MRS broth and incubated at 37°C for 24 hrs. The grown culture was centrifuged at 10,000 rpm for 10 minutes at room temperature in the centrifuge tubes. Cell free supernatant was passed through 0.22 µ memberane filter and evaluated for antimicrobial activity by agar well diffusion method (Aslim et al., 2004). The bacteriocin samples were characterized with respect to heat and pH stability (Brink *et al.*, 1994). The supernatant isolated by centrifugation and filtration was used as sample of bacteriocin.

Heat resistance

Samples of bacteriocin were exposed to various heat treatments: 40°C, 60°C, 80°C and 100°C. Aliquot volumes were then removed after 0, 30, 60 and 90 minutes and assayed for bacteriocin activity using well diffusion method (Brink *et al*., 1994). Aliquots of the supernatant were placed in wells that had been cut in MRS agar plates previously seeded with the indicator strains *S.aureus* and *B.coagulans*. The diameters of the zones of growth inhibition were measured after incubation of 24 hrs.

pH sensitivity

Samples of bacteriocin were adjusted to pH 2, 6, 9 and 12 with sterile solutions of 1N NaOH or 1N HCl. After incubating for 4 hours at room temperature the bacteriocin samples were adjusted to pH 6.5 and assayed for bacteriocin activity using well diffusion method (Brink *et al*., 1994). Aliquots of the sterile supernatant were placed in wells that had been made in MRS agar plates previously seeded with the indicator strains *S. aureus* and *B. coagulans*. The diameters of the zones of growth inhibition were measured after incubation of 24 hrs.

Results and Discussions

Morphological and biochemical characteristics

A total of 50 bacterial strains isolated from five different food samples were screened for bacteriocin producing micro organisms and selected isolates were tested for antimicrobial activity against six indicator strains *Staphylococcus aureus, Bacillus subtilis, Lactobacillus fermentum, Bacillus pumilus, Lactococcus lactis* and *Bacillus coagulans*. Isolates

from only two food samples i.e Dosa batter and Tofu exhibited good antimicrobial activity. Cell free supernatant was taken to perform agar well diffusion assay. Eight best isolates were identified and selected on the basis of their antimicrobial activities against indicator strains and were designated as DB1, DB8, TB1, TB2, TB5, TB7, TB8 and TB10 on the basis of source of their isolation. The isolates were identified on the basis of morphological, cultural and biochemical characteristics. On the basis of phenotypic characterization, DB1, TB1 and TB7 were identified as *Leuconostoc* sp., TB5 as *Lactobacillus* sp. and DB8 and TB8 as *Lactococcus* sp. and TB2 and TB10 closely resembled *Bacillus megatarium*. All the strains were gram positive and rod shaped. A number of earlier reports have shown the production of some bacteriocin by gram-positive bacteria having a broad spectrum of activity (Sanni *et al*., 1999).

Determination of inhibitory spectrum

The bacteriocin activity was checked by incubating isolates for 24, 48 and 72 hours on plates inoculated with indicator strains. Clearance zone diameter was measured in millimeter and the organisms were selected on the basis of the diameter of the zone of inhibition. After 24 hours of incubation, TB7 showed maximum antibacterial activity against *Bacillus coagulans* and *Bacillus subtilis* and TB8 against *Staphylococcus aureus* and *Bacillus pumulis.* Out of these strains, six strains (DB1, TB1, TB2, TB5, TB7 and TB10) produced bacteriocin as observed after 48 hours of incubation against *Staphylococcus aureus, Bacillus coagulans* and *Bacillus pumulis.* Bacteriocins have been reported to be inhibitory against a number of other bacteria (Ogunbanwo *et al.*, 2003; Karthikeyan and Santosh, 2009). All strains except DB8 and TB8 showed bacteriocin activity against *Staphylococcus aureus.* TB1, TB2, TB5 and TB7 showed effective bacteriocin activity against *Bacillus subtilis* after 48 hours of incubation. The observations showed that the isolates showed maximum bacteriocin production at 48 hours represented by complete inhibition (no growth of test isolate was observed) by most of isolates and bacteriocin production declined at 72 hours. Hence bacteriocin production was growth associated, showing maximum production at 48 hours samples. Bacteriocin production assay revealed that the antimicrobial activity against two out of four indicator strains was found in selected strains. These results have generated much interest in the use of antimicrobial metabolites from lactic acid bacteria used in food fermentation. Raja *et al.* (2009) reported the production of bacteriocin of *Lactobacillus lactis cremoris* from kefir and controlled the food spoilage bacteria. The potential of these bacteriocins to inhibit food pathogens such as *S.aureus* and *Bacillus subtilis* makes them suitable for preservation of food items especially processed foods where the risk of contamination is present.

Optimization of culture conditions

The effect of different physiological conditions i.e temperature, pH and salt concentration was checked on the growth of the strains showing bacteriocin activity. Temperature and pH played an important role in cell growth as well as bacteriocin production.

Four temperatures (15, 28, 37 and 45°C) and three pH (4.3, 6.5 and 8.5) were chosen to test the growth pattern of bacterial isolates. Six bacterial isolates TB1, TB2, TB5, TB7, TB8 and TB10 had shown growth at all temperature ranges with luxurious growth at 37°C which was similar to observations of *Lactobacillus* species isolated from fermented foods by Jagadeeswari *et al.* (2010). Mohankumar and Murugalatha (2011) also observed growth at 15°C but not at 45°C for *Lactobacillus* isolated from cow milk. The isolates showed growth in the range of 15-37°C but the optimum temperature for all the strains was reported to be 37°C. All the bacterial isolates showed growth at pH 6.5 and 8.5 but growth was declined at pH 4.5. Jagadeeswari *et al.* (2010) reported weak growth at pH 4 and luxurious growth at pH 8.6 for his isolates. From the above results, it was found that isolates were able to survive in acidic pH range of 6.5 and basic range of 8.5; hence they would be suitable to use in slightly acidic and would have good activity in alkaline range products. NaCl is an inhibitory substance, which may inhibit growth of certain types of bacteria. When subjected to salinity test, none of the isolate was able to grow in 6.5%, 10% and 15% amended media. Isolates from traditional fermented foods exhibited similar declining pattern of growth at similar concentration range of NaCl (Yamazaki *et al*., 2005; Jagadeeswari *et al*., 2010). Characterization of bacteriocin and Biolog test was performed for two best isolates (TB7 and DB1) showing promising activity against indicator strains and broad range of activity in terms of physiological parameters.

Biolog test

Biolog (Biolog Inc., Hayward, USA) test was also performed for two best isolates (TB7 and DB1) and different carbon substrate utilization patterns of isolates were observed and matched with the BIOLOG database. Isolates were able to utilize number of varied carbon substrates, which ranged from 17 to 19. Maximum number of

19-carbon substrates was utilized by TB7 and 17 were utilized by DB1. Homology searching of the Biolog database revealed that TB7 and DB1 had similarity with *Leuconostoc* sp. Similarities varied from 0 to 1% with no probability with the existing database of the Biolog. Similarity percentage of the DB1 isolate was 67.4 % with *Leuconostoc citreum* and TB7 showed 54% similarity with *Leuconostoc mesenteroides* clearly indicating that these bacterial isolates were different from the reported bacteria. The metabolic diversities of the isolates were shown by the ability of isolates to metabolize any substrate, including amino acids and carboxylic acids on the basis of color development in different wells on Biolog plates. Carbohydrate fermentation pattern of these strains showed that DB1 and TB7 were able to ferment glucose, mannose, maltose,fructose, trehalose, sucrose and D-turanose . On the basis of all these properties, the two strains were identified as *Leuconostoc* sp.; however, they showed different characteristics in terms of distinct sugar fermentation pattern in addition to wide resistance and sensitivity towards various antibiotics.

Characterization of crude bacteriocin

Crude bacteriocin was isolated by a modified method of Aslim *et al.* (2004). Further characterization of bacteriocin was carried out based on effects of temperature and pH. Bacteriocin of TB7 showed high thermostability, i.e. upto 80°C for 60 minutes and 60°C for 90 minutes, but in case of DB1 resistance was observed upto 60°C for 30 minutes. However, partial loss of activity was noticed with the continuous increase in temperature. Crude bacteriocin was very stable to heat with respect to all the temperatures used and durations because the original activity of crude extracts was changed to a little extent upon all the heat treatment regimes upto 80°C. The stability of bacteriocin preparations has often been shown to decrease significantly with increased purification. Heat resistance is a major characteristic of many bacteriocins and bacteriocin-like compounds produced by lactic acid bacteria and can vary dramatically ranging from 60°C to 100°C for more than 30 min (e.g. lactocin 27, lactocin S, carnobacteriocins A and B) to autoclaving at 121°C for 15-20 min (e.g. lactacin B, lactacin F, nisin) (De Vuyst and Vandamme, 1994). Many of the bacteriocins produced by lactic acid bacteria, particularly the ones of class I and class II, are described as small hydrophobic proteins containing little tertiary structure, which explains their heat stability. Other factors contributing to heat stability of the bacteriocin of LAB are stable cross-linkages, a high glycine content and occurrence of strongly hydrophobic regions. Thermostability of bacteriocin has also been reported in bacteriocin of *L. lactis, L. plantarum* by Lade *et al.* (2006) and *Cornobacterium piscicola* C5526 (Mendoza *et al.*, 1999). Heat sensitivity of these bacteriocins may be due to their non-complex, linear structures. At pH 6, bacteriocin produced by TB7 showed more inhibition against *B. coagulans* and *S. aureus* with zones of 19.5 mm and 15 mm while there was decrease in activity of bacteriocin produced by DB8 with zones of 11 mm and 10 mm against test strains respectively. While with increase in pH that is at a range of 9, same trend was observed with increased activity of bacteriocin produced by TB7 with inhibition zones of 12 mm and 8 mm as compared to 6 mm and 5.4 mm produced by DB1 against *B. coagulans* and *S. aureus* but overall activity of bacteriocin produced by isolates declined with increase in pH. The bacteriocin was found active for pH range of (6.0 to 9.0); however the maximum activity was retained at neutral pH. Bacteriocin from both isolates (DB1 and TB7) showed sensitivity at extreme acidic (pH=3) and alkaline pH range (pH=12). The prolonged exposure to the alkali pH was found to be detrimental to the bacteriocin activity. The loss of activity at higher pH could be due to change of conformation of the molecule. This result was similar to the properties reported for bacteriocins produced by other lactic acid bacteria such as bovicin HC5 (Russell *et al.*, 2005). On the other hand, bacteriocin of *L. lactis* was found stable in acidic to neutral range pH 4.0 to 7.0 (Lade *et al.*, 2006), while bacteriocin produced by *L.plantarum* and *L.brevis* OGI retained their antimicrobial activity in an acidic range pH 2.0 to 6.0 (Ogunbanwo *et al.*, 2003). Thus, high heat tolerance and wide pH range confirmed that the antibacterial substance secreted by DB1 and TB7 is a potent bacteriocin and can be used for biological preservation of food.

Bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH and temperature. Many of the bacteriocins and bacteriocin-like substances produced by lactic acid bacteria are only stable at acid and neutral pH (De Vuyst and Vandamme, 1994) and are inactivated even at a pH above 8.0 (e.g. nisin, lactostrepcins, pediocin AcH, leucocin A-UAL 187). This can be attributed to the solubility of the bacteriocins of LAB (lactic acid bacteria) and the solubility of the bacteriocins decreased with increasing pH. In recent years, bacteriocins produced by lactic acid bacteria have attracted great attention due to their application in food processing and preservation to control undesirable organisms. Only strains of

Lactococcus lactis and and *Pediococcus*, producer strains of nisin and pediocin PA- 1, respectively have been used up to now. Nisin is the most widely used bacteriocin and it has been granted the status of GRAS (generally recognized as safe) in the United States for food. However, nisin has several deficiencies such as instability at neutral to alkaline pHs, decaying property in its antimicrobial activity when incorporated into complex foods, low solubility over the physiological pH range, and a spectrum of activity restricted to gram-positive bacteria (Mendoza *et al.*, 1999). Hence, it would be desirable to find other antimicrobial compounds, which could be successfully exploited as food preservatives.

Conclusion

The potential application of bacteriocins as consumer friendly biopreservatives either in the form of protective cultures or as additives is significant. The present study introduced such bacteriocin that can be used successfully as food preservative. In summary, strains DB1 and TB7 were isolated from dosa batter and tofu in Chandigarh province. On the basis of morphological, biochemical and Biolog test the DB1 and TB7 isolates showed closed resemblance to *Leuconostoc citreum* and *Leuconostoc mesenteroides*. The previous studies mainly aimed at isolation of *Lactococcus* sp and *Pediococcus* sp and few studies were based on characterization of bacteriocins from *Leuconostoc* sp. The bacteriocin produced by DB1 and TB7 was assayed by agar well diffusion method and bacteriocin activity was measured in terms of diameter of zone of inhibition against *Staphylococcus aureus* and *Bacillus coagulans*, food spoilage bacteria. The behaviour of the bacteriocin produced by isolated strains was considered as bactericidal. Bacteriocins produced by these two strains were heat stable upto 80°C and at alkaline pH range. Because of its higher thermostability, wide pH tolerance and bactericidal mode of action, it could be used as natural preservative to enhance shelf life of the different processed product.

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