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Study on bacteriocin producing lactic acid bacteria from traditional South Indian foods

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Abstract
Lactic acid bacteria (LAB) are getting more attention now, since they are capable of producing bacteriocins with broad spectrum antimicrobial activity. Two common fermented traditional food in all south Indian food schedule are curd and idly mix (i.e. batter) were used as a source for LAB isolation on DeManRogosa Sharpe (MRS) agar. Crude bacteriocin Production by spot on lawn method and pure bacteriocin was extracted from the culture by solvent extraction method using chloroform. Bacteriocin assay was performed by with bacterial strains *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus.* with crude bacteriocin, crude extract adjusted pH6, crude bacteriocin mixed with tween -80 for efficient diffusion, Solvent extracted bacteriocin and bacteriocin heat treated at 70 ^{0}C. Solvent extracted bacteriocin has higher antimicrobial activity of 6.83 ±0.17, 5.67 ± 0.31, 6.17 ± 0.31, 6.83 ± 0.31 and 6.40 ± 0.26 respectively. Potent antimicrobial activity of this bacteriocin against all the five food borne organisms ensures the betterment of food industry.

Keywords: Bacteriocin, LAB, food borne pathogen

Introduction
Bacteriocins are ribosomally synthesized peptides or proteins produced by all bacteria as their natural weapon against closely related bacteria to make their survival fit in their environment. Each and every bacterium has unique bacteriocin producing genes that can be modified to produce stronger bacteriocins when they are given more pressure from its competitor [1]. Lactic acid bacteria (LAB) are getting more attention now, since they are capable of producing bacteriocins with broad spectrum antimicrobial activity. Traditional fermented foods like curd and batter will have safe, stable and more powerful LAB cultures as they occur in natural process and these cultures will be more useful in scientific research and commercial production of bacteriocins. Lactobacilli produce special antimicrobial compounds such as bacteriocin which are species specific and prevent food spoilage and provide additional protection against Bacillus, Staphylococcus and clostridium spores [2]. Traditional fermented products are not only unique in their flavor and taste they also have unique strain of LAB. Based on the pH, temperature, humidity employed in the process and ingredients used bacteriocin production differs. Curd and batter are the most common fermented foods stored in room temperature. To understand and study the complete potential of these bacteriocin correct and efficient extraction method should be employed. Use of chemical preservatives to control food borne zoonotic organisms extend greater threat to achieve food safety. Under this backdrop, the present study was conducted with the aim of isolating LAB from traditional foods like curd and batter samples using traditional as well as solvent extraction methods and also to check its antibacterial activity against common foodborne pathogens.

Materials and Methods
Source and Isolation of Lactic Acid Bacteria
Two common fermented traditional food in all south Indian food schedule are curd and idly mix (i.e. batter). These two fermented food items were used as a source for LAB isolation. Curd and Batter samples were collected from domestic sources and transported on ice to the laboratory. One ml of the sample was diluted with 9 ml of 0.85% normal saline, inoculated on DeManRogosa Sharpe (MRS) agar by spread plate method and incubated at 37 ^{0}C for 48 hrs. The individual colony from the MRS plate were tested and confirmed as LAB biochemically [3].

Screening of the LAB isolates for Bacteriocin Production

The confirmed LAB isolates were screened for Bacteriocin production by Spot on Lawn method which is the conventional well as a reliable method for bacteriocin monitoring [4]. Listeria monocytogenes was used as an indicator bacteria for a spot on lawn method. *L. monocytogenes* was revived in Brain Heart Infusion Broth and 30µl of overnight culture was poured on the TGE agar plates. Each of the individual colony LAB isolates were spotted on the lawn i.e. agar plate with indicator bacteria and incubated for overnight at 30 °C.

Bacteriocin Extraction

a. Crude bacteriocin Production

A single colony of the LAB positive for bacteriocin production by spot on lawn method was inoculated into 10 ml of MRS broth and incubated at 37°C for 24 hrs. MRS broth with LAB culture was centrifuged at 10,000rpm for 10 minutes. The cell free supernatant fluid (CFSF) was used as Crude bacteriocin. pH of the CFSF was adjusted to 6 and its anti- bacterial activity was evaluated.10 ml of the 24 hr LAB culture was added with 1ml of 0.5% Tween 80, mixed well and then centrifuged at 10,000rpm for 10 minutes. The CFSF treated with tween 80 was tested for its efficiency.

b. Solvent extraction of bacteriocin using Chloroform

Pure bacteriocin was extracted from the culture by solvent extraction method using chloroform as it has a polarity index (PI) of 4.1 and its solubility in water is only 0.815% [5]. Suitability of chloroform for bacteriocin extraction is attributed to properties like intermediate polarity immisciblity with water and amphiphilic nature of bacteriocin peptides [6]. So, 100ml of culture supernatant was mixed with equal volume of Chloroform and stirred vigorously using a magnetic stirrer for 20 min, then centrifuged at 10400 g (12 ^{0}C) for 20 min. After centrifugation three layers were separated viz. top aqueous layer, cloudy solid floating intermediate layer and bottom sediment. Once again culture supernatant was mixed to transfer bacteriocins from the aqueous medium to the interfacial layer. The floating interfacial layer was removed without disturbing aqueous layer. Bacteriocin rich interfacial layer were resuspended in 1 to 2 ml buffer (0.1 molTris, pH 7.0) [7]. Heat stability of the bacteriocin @ 70 ^{o}C for 10 minutes in water bath was also assessed.

c. Bacteriocin Assay

Bacteriocin assay was performed by testing the extracted bacteriocins against bacterial strains *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* obtained from the repository of Department of Veterinary Public Health and Epidemiology, Madras Veterinary College by agar well diffusion assay. Each standard test bacterial culture was revived in their respective selected broth and 5 µl of each indicator bacteria was poured separately in Muller Hinton agar plates in duplicates, after that wells were punched out in the agar plates, and the wells were filled with 100 µl of each of five types of bacteriocins i.e. crude bacteriocin, crude bacteriocin adjusted to pH6, crude bacteriocin mixed with Tween-80, solvent extracted bacteriocin and solvent extracted bacteriocin treated @ 70 ^{0}C. For better diffusion of bacteriocin in agar, the plates were pre-incubated at room temperature for 3hrs and then incubated at 37 ^{0}C. After 24 hrs, zone of inhibition against all the five bacteria was recorded [8]. Diameters of the zones of inhibition produced by different strains were compared.

Results and Discussion

Screening LAB isolates for Bacteriocin production

Of 40 samples analysed, twenty LAB isolated were recovered. Out of this twenty LAB isolates only five isolates (LAB C2, LAB C15, LAB B6, LAB B7, LAB 18) showed bacteriocin production based on the spot on lawn method. Bacteriocin produced by one isolate (LABB6) from batter sample which had greater antibacterial activity against all five indicator bacteria used in this study. The antimicrobial activity of bacteriocin is the reciprocal of the highest dilution showing at least 2mm zone of inhibition in the indicator lawn and it is expressed as Activity units AU/ml [9]. The negative results of the remaining thirty five isolates could be due to the fact that bacteriocin production is not highly conserved in these strains. Some of the bacteriocins are plasmid-mediated proteins, so one should also consider the possibility of some cultures losing their plasmids after consecutive transfers during purification [10]. Among the five samples, the one with powerful bacteriocin (greater AU/ml) was success fully identified based on crude bacteriocin activity and the zone of inhibition against the five bacterial genera used in this study. Bacteriocin producing ability of LAB from traditional fermented foods was already reported [11].

Effect of crude bacteriocin and pH adjusted bacteriocin

Crude bacteriocin from the identified batter sample was further used in this study. The crude bacteriocin thus obtained was adjusted to pH 6 by using NaCl in order to avoid the inhibiting action of organic acids produced during the bacterial growth. From the results (Table 1), it is evident that *Staphylococcus aureus* being most sensitive (p) and *C. jejuni* (4.67 ± 0.2) least. Whereas sensitivity of crude bacteriocin adjusted to pH6 was lesser than that of crude bacteriocin for the bacterial strains *C. jejuni*, *E. coli*, and *B. cereus* whereas *L. monocytogenes* show no change in inhibitory pattern and *S. aureus* (5.83 ± 0.17, 5.50 ± 0.34) was affected very little by pH adjustments. The reduction in the sensitivity may be due to the neutralization of H_2O_2 and other excreted product of bacterial replication [12].

Effect of crude bacteriocin treated with Tween 80

Tween 80 is a non-ionic detergent, and increased diffusion of bacteriocin when mixed with Tween80. Results are in line with Wolf and Gibbons tween treated bacteriocin showed increased zone of inhibition with all test bacterial strains which evidence the possible role of tween 80 in releasing bacteriocin molecules from the LAB cell wall [13]

Effect of solvent extracted bacteriocin

Solvent extracted Bacteriocins had greater antimicrobial activity when compared to all other bacteriocins [7]. Although bacteriocins are very tightly attached to the cell wall of the LAB, they are amphiphillic and get easily attracted towards the solvent chloroform thus resulting in greater recovery. This is well documented by the greater antimicrobial activity of solvent extracted bacteriocin against all the five bacterial strains used in this study. Solvent extracted bacteriocin has higher antimicrobial activity of 6.83 ±0.17, 5.67 ± 0.31, 6.17 ± 0.31, 6.83 ± 0.31 and 6.40 ± 0.26 respectively. Deshmukh and Thorat reported maximum zone of inhibition against food borne pathogens from 00mm to 28.00mm [14], whereas Enan *et*

al., (1996) reported 0.5 – 13.00mm against indicator organisms [15] and 15mm against *Staphylococcus* [16].

Effect of solvent extracted bacteriocin heated treated @ 70 ºC

For the large scale and commercial use, these bacteriocins should have a thermal stability which was tested at 70 ^{0}C in water bath. After the heat treatment all the isolates showed decrease in the antimicrobial activity which is not significant where was retained but with reduced potency against all bacterial strains studied

Table 1: Anti-microbial activity of Bacteriocins

	Zone of inhibition (mm)				
Bacterial cultures	***Bacillus cereus***	***Campylobacter jejuni***	***Escherichia coli***	***Listeria monocytogenes***	***Staphylococcus aureus***
Crude bacteriocin	5.17±0.34	4.33± 0.33	4.67 ± 0.210	5.33 ± 0.33	5.83 ± 0.17
Crude bacteriocin pH6	5.00± 0.34	4.67 ± 0.21	4.67 ± 0.210	5.17 ± 0.31	5.50 ± 0.34
Crude bacteriocin+ Tween-80	5.83± 0.17	4.50 ± 0.22	5.50 ± 0.00	5.83 ± 0.40	6.10 ± 0.43
Solvent extracted bacteriocin	6.83 ±0.17	5.67 ± 0.31	6.17 ± 0.31	6.83 ± 0.31	6.40 ± 0.26
Bacteriocin heat treated at 70 ^{0}C	6.00 ± 0.00	5.17 ±0.21	6.00 ± 0.00	6.20 ± 0.00	6.03 ± 0.34

Conclusion

Bacteriocins have potent antimicrobial activity against food borne organisms and the method of extraction of bacteriocin causes greater variation in their antimicrobial activity. The bacteria used in this study are closely related to the food industry as they cause food borne infections which are major block stones in food safety. Potent antimicrobial activity of this bacteriocin against all the five food borne organisms ensures the betterment of food industry. Bacteriocins usually have low molecular weight which undergo post translational modification and can easily be degraded by proteolytic enzymes especially by the proteases of the mammalian GI tract thus making them safe for human consumption. Use of bacteriocins in the food industry can reduce the addition of chemical preservatives as well as the intensity of heat treatment, resulting in foods which are more naturally preserved and rich in organoleptic and nutritional properties. Immobilized bacteriocins can be used for bioactive food packaging. Thus more studies for commercial exploitation of these bacteriocins can help the food industry.

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