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# Isolation, Identification and Optimization of Bacteriocin Production by *Lactobacillus casei* for their Bio Prospective Applications

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**Bacteriocins are a special interest due to their potential value as natural preservative. The present study is a factual production of such bacteriocin from marine *Lactobacillus* sp. Lactic acid bacteria (LBA) was isolated from fish gut of the *Sardinella longiceps* and their density was found to be 4.8 x $10^7$ respectively. Various pathogens were also isolated from pickle. LAB strains were optimized at different parameters and maximum bacteriocin production was at pH 5, temperature of 35°C, 3.5% of salt concentration with 24$^{th}$ hour of incubation period. *Lactobacillus casei* was screened as the most potential strain for both bacteriocin production as well as antimicrobial activity. FT-IR spectrum also confirmed the obtained protein as a bacteriocin. The present study has revealed that *Lactobacillus* strains from marine origin are having the potential properties as a biopreservatives especially in seafood industries with large scale production for commercial utilization.**

**Key words:** Antimicrobial activity, Bacteriocin, Lactic acid bacteria, *Lactobacillus* sp, FT-IR.

Lactic acid bacteria (LAB) are microorganisms widely used in food industry in a variety of fermentations such as the development of meat products, vegetables and many dairy products including fermented milk, cheese, yogurt and butter [1,2]. LAB produce organic acids such as lactic, acetic acid and hydrogen peroxide which possess antimicrobial activity against several pathogenic and spoilage microorganisms[3]. LAB represent a major class which produces bacteriocins that become a current subject for several researches. These bacteriocins are now being explored for their potential utility in human and animal health applications, food biopreservation and agricultural uses[4,5].

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action[4]. In additions, it has been shown that some strains of LAB possess interesting health-promoting properties; one of the characteristics of these properties is the potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori, E. coli* and *Salmonella*. The antimicrobial spectrum frequently includes spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and

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*Staphylococcus*. The activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or a chelating agent, or after pulsed an electric field or high-pressure treatment. An experimental focus on bacteriocin production by probiotics LAB strains has indicated that this potential might play a considerable role during in vivo interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori*[6,7].

Several authors have recommended the use of bacteriocins combined with other preservation methods to create a series of hurdles during the manufacturing process to reduce food spoilage by microorganisms. In fact, it has been proven that application of chemical preservatives, physical treatments (heat), or new mild non thermal physical methods (pulsed electric field, HHP, vacuum, or modified atmosphere packaging), which increase the permeability of cell membranes, positively affects the activity of many bacteriocins. Notably, combined treatments of bacteriocins with selected hurdles affecting outer-membrane permeability increase the effectiveness of some LAB bacteriocins against Gram-negative cells, which are generally resistant[8,10].

The main purpose of our work was to select bacteriocinogenic strains from a group of LAB with antimicrobial activity isolated from different origins, to characterize the produced bacteriocin and to determine the antimicrobial spectrum of this bacteriocin produced by the selected isolate.

## MATERIALS AND METHODS

The fin fish samples were collected from the muddasalodai landing centre (Lat 11 ° 29 'N; 79° 46' E) for the isolation of lactic acid bacteria. The pickle samples were collected from Pondicherry. Tuna and Prawn pickles were analyzed in the present study. Homogenized extract of the fish sample were serially diluted up to 10-5 dilutions and were plated on MRS agar (HI-MEDIA) using spread plate technique and incubated at 28 ± 2°C for 24h. The isolated potential strains were identified up to species level based on Bergey's manual of systematic bacteriology[11]. Approximately 10g of sample (Pickle) was homogenized in a sterile mortar and pestle using 90ml of sterile 50% aged sea water and then serially diluted using the same diluents. 0.1ml of the serially diluted sample was inoculated in to the selective media to isolate the specific pathogens. The isolated pathogens were identified up to species level based on Bergey's manual of systematic bacteriology[11].

**Primary screening for antibacterial activity**

A preliminary test for inhibitory assay of LAB against food borne pathogens, isolated from the seafood pickles was done.

**Well diffusion method [12]**

After swabbing the pathogens on the Muller Hinton agar plates, 0.1 ml of cell free culture broth of Lactic acid bacteria (LAB) centrifuged at 10,000 rpm for 20 min, poured into the wells and plates were incubated at 37°C for 24 h. The bacterial culture filtrate inhibiting the growth of pathogen was assessed based on the inhibition zone around the well and the results were recorded.

**Screening of bacteriocin producing bacteria**

Screening was done by well diffusion method using the crude extract. The inhibitory activity was tested against the seafood borne pathogens.

**Optimization of cultural condition for bacteriocin production**

The effect of incubation period on growth and bacteriocin production was studied for 0-52h with 12h intervals. pH ranges (3, 4, 5, 6 and 7) were tested. 20°C, 25 °C, 30°C, 35°C and 40°C were tested to find the optimum temperature. The ranges of 3-5 % of salt concentration were tested. Based on the results obtained from the optimization mass scale culture was done. Based on the results observed in the optimization the mass scale culture was carried out with the parameters pH 6, Temperature 35°C, Salt concentration 3.5%, Incubation period 24h.

**Protein Estimation**

The Folin-Ciocalteu phenol method[13] was used for the estimation of the total protein in the sample.

**FTIR (Fourier Transform- Infra Red Spectrum) analysis**

The crude sample of bacteriocin (10mg) was mixed with 100mg of dried potassium bromide

(Kbr) and compressed to prepare as a salt disc. The disc was then read spectro photometerically (Bio-Rad FTIR-40- model, USA). The frequencies of different components present in each sample were analyzed.

## RESULTS

The total heterotrophic and lactic acid bacterial count in fish gut was found to be 2.35 x 109 and 4.8 x 107 CFU/g respectively. A total of 20 morphologically distinct strains were isolated from the serially diluted fish gut sample. 3 strains from fish gut sample were identified as potent strains for bacteriocin production. After the screening procedures the potential strains were identified. They are LAB1 *Lactobacillus* animalis, LAB2 *Micrococcus varians* and LAB3 *Lactobacillus casei*.

Selective media used for the isolation of seafood pathogens are EMB agar, MRS agar, Listeria isolation agar, SS agar, TCBS agar, Yersinia identification agar where in the density of *E. coli*, *Lactobacillus* spp, *Listeria* sp, *Salmonella, Vibrio* spp and *Yersinia* spp were enumerated to be in the fish pickle 1.8x104, 1.7x104, 2.6x104, 2.8x104, 1.5x104 CFU/g. Isolated pathogens were identified as *Shigella* spp, *Vibrio cholera, Vibrio parahaemolyticus, Salmonella* spp, *E.coli, Yersinia* spp. *Staphylococcus aureus, Klebsilla* spp, *Bacillus subtilis*, *Lactobacillus vulgaris*.

The bacteriocin obtained from LAB3 showed the maximum zone of inhibition compared to all the other strains. It showed a zone of inhibition of 11 mm against *Salmonella* spp (Fig. 2), 8 mm against *S. aureus, Klebsiella* spp. 7 mm, *Bacillus subtilis* and *Shigella* spp 6mm. Whereas the supernatant of the same strain showed zone of inhibition of 9 mm against *Salmonella* spp (Fig. 1), 7 mm against *S. aureus*, 6 mm against *Shigella* spp and *Klebsiella* spp. These results are given in Fig. 1 respectively.

The bacteriocin production was found maximum at an incubation period of 24 h. The results were depicted in (Fig. 3). Among the various pH ranges studied, the growth of the potential strain was found to be highest at pH 5 (Fig. 4). The optimum temperature for bacteriocin production was found to be 35°C (Fig. 5). The optimum salt concentration for bacteriocin production was found to be 3.5% salt concentration (Fig. 6). The amount of protein was estimated to be 0.52 mg/ml. Fourier transform - infra red FTIR spectrum IR spectrum of the crude lyophilized protein sample analyze the IR-spectrum of the four major peaks at 2130.51, 2074.41, 981.39 and 451.59 $cm^{-1}$(Fig. 7). Whereas the remaining spectrum of very close values at

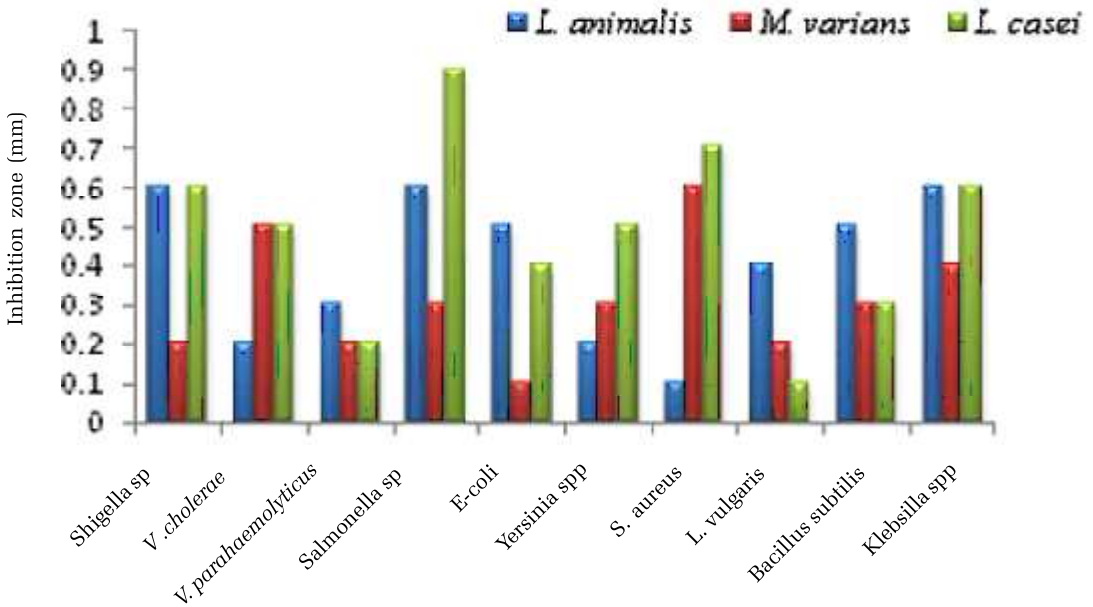


**Fig. 1.** Antibacterial activity of LAB cell free extracts (Zone of clearance in mm)

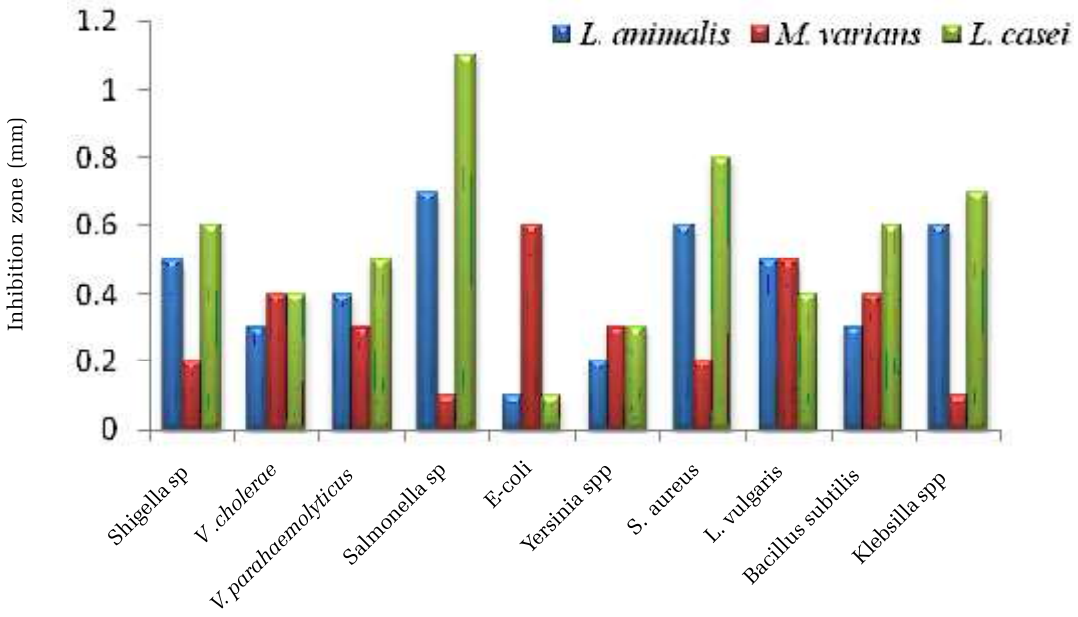


**Fig. 2**. Activity of bacteriocins against pathogens (Zone of clearance in mm)

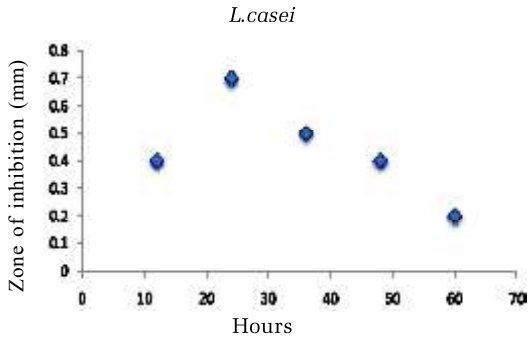


**Fig.3.** Bacteriocin activity at 12, 24, 36, 48, 60 hrs of incubation period

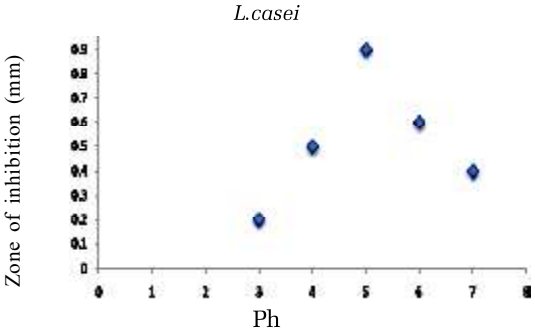


**Fig.4.** Bacteriocin activity between pH range of 3, 4, 5, 6 and 7

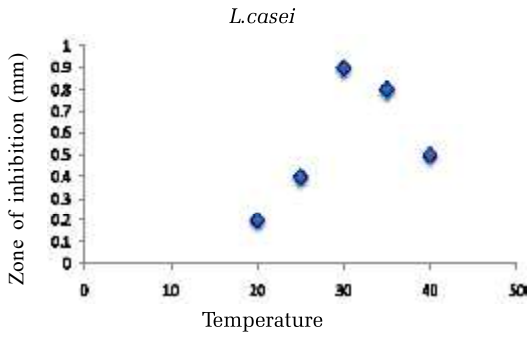


**Fig.5.** Bacteriocin activity between 20°C, 25°C, 30°C, 35C and 40°C of temperature ranges

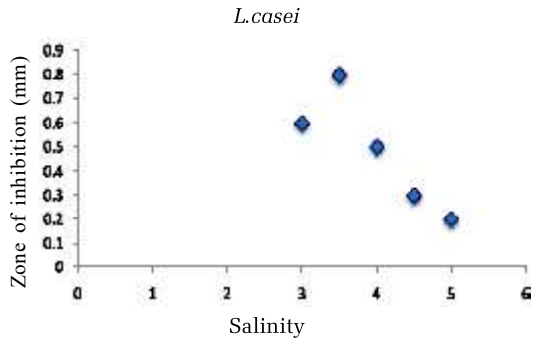


**Fig. 6.** Bacteriocin activity between 3%, 3.5%, 4%, 4.5% and 5% of salt concentration

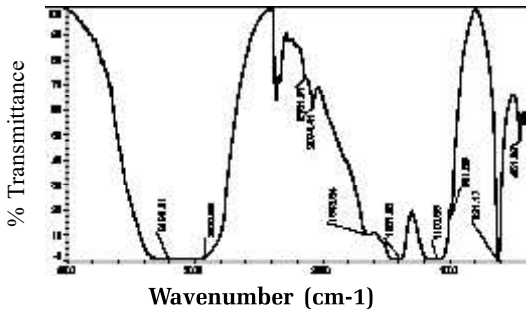


**Fig. 7.** Showing the FTIR spectrum of lyophilized bacteriocin sample

3198.81, 2920.55, 1643.84, 1381.58, 1100.36 and 621.17 $cm^{-1}$.

## DISCUSSION

In the present study the isolation, partial characterization and activity of bacteriocin produced by *L. casei* was done. The bacteriocin producing lactic acid bacteria (LAB) were isolated from the fresh meat of marine fin fish. It is interesting to note that majority of the *Lactobacillus* spp. that have been isolated from fresh and frozen fish/prawns were those species which were commonly found in animals and human beings[14]. There are only a few reports available on isolation of LAB from fresh and seawater fish[15-16]. The LAB strains were evaluated for the production of inhibitory substances against various food borne pathogens. The pathogens used in the present were isolated from fish pickle. All the LAB strains showed a moderate inhibitory activity against the pathogens isolated from pickle samples. The use of bacteriocinogenic starter/protective cultures could improve the quality and increase safety by inhibiting the food-borne pathogens and spoilage microorganisms. Recent outbreaks of emerging pathogens such as *L. monocytogenes* that has caused severe illness through food ingestion have prompted the scientific community to focus their studies on the anti-Listeria activity of bacteriocins produced by *Lactobacillus* and *Pediococcus* strains[17-19].

The inhibitory effect, which was observed by the formation of clear and distinct zones around the wells, may be due to the production of several antimicrobial compounds like organic acids, hydrogen peroxide or bacteriocins[20]. *L. casei* produced supernatant in high level and the strain showed the maximum inhibitory activity against *Salmonella* spp and *S. aureus*[21] reported that the diameters of inhibition zones of the indicator strains by the cell-free supernatant neutralized and treated with catalase are ranging from 14 to 20 mm. The highest diameter (20mm) was obtained with the cell-free supernatant of Lb. plantarum F12 and Lb. curvatus on B. subtilis, whereas the lowest diameter was obtained with the cell free supernatant of *Lactobacillus* sp. B5 against MRSA. The fact that, the cell-free supernatants (neutralized and treated with catalase) inhibited the growth of the indicator strains gives evidence that the antimicrobial activity is due to the production of bacteriocins[22]. Gram-positive indicator bacteria are much more sensitive to bacteriocin of our LAB strain than Gram-negative indicator bacteria. These results indicated that our LAB had an inhibitory spectrum towards closely related Gram-positive bacteria. The resistance of Gram-negative bacteria is attributed to the particular nature of their cell membrane; the mechanism of action described for bacteriocin involved a phenomenon of adsorption.

*L. casei* produced bacteriocin in high level and the strain showed the maximum inhibitory activity against *Salmonella* spp and *S. aureus*. Besides, the productions of bacteriocins having a wide spectrum of antibacterial activity against seafood borne pathogens like Listeria even Gram-negative pathogens like *E. coli* to employ as biopreservatives. Accordingly *L. acidophilus* and *L. casei*[22] may be of great interest as probiotics strains because of their ability to adhere to intestinal epithelial cells and being of human origin. Ivanova[23] found that, the bacteriocin produced by Lactococcus lactis subsp. lactis B14 inhibited only wide range of strains from the group of closely related LAB. The known bacteriocins does not still act on the sorts taxonomic close, for example, nisin has an inhibitory effect against a wide variety of Gram-positive food-borne pathogens and spoilage microorganisms and can also act on several Gram-negative bacteria when the integrity of their outer membranes is disrupted [24]. The isolate *L. casei* was selected for further studies. *L. monocytogenes* has become one of the most significant food borne pathogens. In food industry, the control of this pathogen remains a challenge because of its

widespread occurrence and its ability to survive and persist even in hostile environment[25]. For this reason we tested the ability of bacteriocins produced by Lb. plantarum F12 to inhibit this bacterium. Hartman[25] observed that the cell-free supernatant produced by eight antagonistic bacteria strains were able to inhibit *L. monocytogenes* in different food matrices. In another study, Singh and Prakash[26] found that, several LAB strains isolated from cottage cheese are capable of inhibiting pathogenic microorganisms in the food environment and display crucial antimicrobial properties with respect to food preservation and safety. They can also be used more specifically to inhibit certain high-risk bacteria like *Listeria* sp in food. Application of bacteriocins may help reduce the use of chemical preservatives and /or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved.

The effect of incubation period, pH, temperature and salinity of medium on the production of bacteriocin was also investigated in all LAB strains. The pH 5, temperature of 30°C, salinity of 35 ppt, 24 h of incubation was found to be the optimal parameters for the most potential producers of bacteriocin (that is) *L. casei*. According to Ogunbanwo[27] the use of constituted medium at 30°C of incubation temperature, initial pH of 5.5 and 48 to 60 h fostered the best production of bacteriocin by *Lactobacillus* brevis OG1 which seemed to differ from the results of the present study[21] reported that the effect of temperature on bacteriocin production by Lb. plantarum F12 was tested in Erlenmeyer flasks cultures containing sterile MRS and maintained at different temperatures (30, 37 and 40°C). Mataragas[28] as they found that the optimum temperature for the production of bacteriocins produced by *Leuconostoc mesenteroides* L124 and Lb. curvatus L442 was 25°C and was lower than that of growth (30°C). [21]reported that the antimicrobial activity of Lb. plantarum F12 is significantly influenced by pH residual activities were significantly higher in the range of pH 6.0 to pH 10.0 then those at pH 2.0, 4.0 and 12.0; with a maximum activity at pH 6.0, suggesting that compounds other than acids inhibited growth of MRSA.

In the present study FT-IR Spectrum of the protein revealed the presence of peaks at the wave numbers of 3198.81, 2920.55, 2130.51, 2074.41, 1643.84, 1381.58, 1100.36, 981.39 and 451.59 $cm^{-1}$ which indicated the presence of NH, $NH_3$, $CH_3$, $CH_2$ and $CH_2$ groups respectively. The wave numbers 1643.84, 1381.58 and 1100.36 $cm^{-1}$ indicated the presence of their bending mode of amide, methyl, and amide groups respectively. The peak at 1546 $cm^{-1}$ indicated a secondary amide was reported[29,30]. The FT-IR spectrum offered concrete evidence that the substance contained a peptide in its structure.

The present study showed that the bacteriocin of *L. casei* effects on some clinically important food borne pathogens. This revealed the potential application of bacteriocin produced by *L. casei* as a biopreservatives for the improvement of the microbial safety of fermented foods and reduction in food contamination which causes illness to human beings. The study revealed that *Lactobacillus* strains of marine origin are having the potential to use as biopreservatives especially in sea foods. The production of bacteriocin from *L. casei*, seems to be ideal for industrial scale production and commercial utilization.

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