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Purification and Characterization of a Bacteriocin Produced by *Lactobacillus lactis* Isolated from Marine Environment

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Abstract: Bacteriocin producing *Lactobacillus lactis* strain isolated from marine environment, showed broad range of antibacterial activity against some major food borne pathogens. Maximum bacteriocin production was observed at 30°C, pH 6.0 and 1.5% sodium chloride solution. In addition of enzymes, α-amylase, DNase, RNase and lipase were slightly positive effect bacteriocin production. Proteinase K and pepsin were strongly inhibited bacteriocin production. Among detergents, Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 stimulated bacteriocin production and strongly inhibited by EDTA and urea. The bacteriocin has purified by ammonium sulphate precipitate and ion exchange (DEAE cellulose) chromatography. Biochemically it was pure protein moiety and the molecular weight was 94 kDa. The study revealed the possibility of using bacteriocin as a food preservative and the *L. lactis* strain as probiotic.

Key words: Antagonistic activity, bacteriocin, indicator organisms, lactic acid bacteria, *Lactobacillus lactis*

INTRODUCTION

Bacteriocins from lactic acid bacteria (LAB) are natural antimicrobial peptides or proteins with interesting potential applications in food preservation and health care. Nisin, the best-known LAB bacteriocin, has been repeatedly shown to be safe and effective for use in foods over the past 30 years (Delves-Broughton, 1990; Janes *et al*., 1999). Pediocin is another well-studied bacteriocin that will likely be the second LAB bacteriocin to be widely used in the food industry (Venema *et al*., 1995a, b; Chikindas *et al*., 1995). A large number of other LAB bacteriocins have been identified and the list is still growing (De Vuyst and Vandamme, 1994). Research on LAB bacteriocin production, purification, genetics and applications is burgeoning (Turcotte *et al*., 2004).

Lactic acid bacteria produce a variety of antibacterial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocin or bactericidal proteins during lactic fermentations (Holzapfel *et al*., 2001; Hirano *et al*., 2003).Most of bacteriocins produced by gram-positive bacteria are from lactic acid bacteria (Ennahar *et al*. 2000; Garneau *et al*., 2002).

Lactobacillus bacteriocins are found within each of the four major classes of antimicrobial proteins produced by lactic acid bacteria. Class I bacteriocins (antibiotics) were discovered in the *lactobacillaceae* by Mortvedt *et al*. (1991). These bacteriocins are small membrane-active peptids (<5 kDa) containing an unusual amino acids, lanthionine. The class II bacteriocins are small heat-stable, non-lanthionine containing and membrane-active peptides (<10kDa). The class III bacteriocins, have been found in *Lactobacillus,* include heat labile proteins of large molecular mass. The class IV bacteriocins are a group of complex proteins, associated with other lipid or carbohydrate moieties, which appear to be required for activity. They are relatively hydrophobic and heat stable (Alpay *et al*., 2003). Marine environment is rich in nutrient and organic matter. Hence the strain *Lactobacillus lactis* was isolated from marine environment. In this paper, we characterized the bacteriocins produced by *Lactobacillus lactis*, which was isolated from the marine environment, determined the antibacterial spectrum, the optimum condition for bacteriocin production, and estimate the molecular weight of the bacteriocin.

MATERIALS AND METHODS

Sample collection: Sediment samples were collected during February, 2008 while cruising in the Sagar Paschimi coastal research vessel from depths of 20 m at Chennai harbour (Lat. 13º7′ N and Long. 80º23′ E) in the Bay of Bengal (Tamilnadu). The sediment samples were stored in the laboratory at 4ºC in sterile specimen cups until they were used to isolate the *Lactobacillus* spp. All the experiment was conducted by CAS in Marine Biology, Annamalai University.

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Isolation and identification: Dilutions (10^{-1} - 10^{-6}) of one gram of sediments in sterile 50% aged seawater were prepared and plated on de Man Rogosa agar (MRS agar) medium (Hi Media Laboratory Pvt. Ltd. Mumbai, India) to isolate the *Lactobacillus* (De Man *et al*., 1960). The strains were sub-cultured onto MRS agar slant (medium with 50% sea water), incubated at 30 ºC for 24 h and were preserved in 20% glycerol at -80 ºC. One of the isolate was selected for further studies which exhibited strong inhibitory activity against indicator strains and identified on the basis of growth, cell morphology, gram staining and catalase activity. Further, identification of the species of these *Lactobacilli* was performed according to carbohydrate fermentation patterns and growth at 15 and 45ºC in the de Man Rogosa Sharpe (MRS) broth as described in Bergey's Manual of systematic Bacteriology. (Holt *et al*., 1994).

Production of crude bacteriocin: The isolated strain was grown in MRS broth (Hi Media Laboratory Pvt Ltd. India) (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 30ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4ºC). The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin (Ogunbanwo *et al*., 2003).

Bacteriocin assay: The antibacterial spectrum of the bacteriocin from *Lactobacillus lactis* was determined using the well diffusion method. The supernatant from a 48-h culture of *Lactobacillus lactis* was filter sterilized by passage through a 0.45 µm pore size membrane filter (PALL Corporation, Mumbai). Aliquots (50 µl) of the sterile supernatant were placed in 4-mm-diameter wells that had been cut in Mueller-Hinton agar plates previously seeded with the indicator bacteria. After 12-18 h of incubation, the diameters of the zones of growth inhibition were measured. Antimicrobial activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Ivanova *et al*., 2000) (i.e. turbidity $\leq$50% of the turbidity of control culture grown without *L. lactis* supernatant).

Optimization of culture conditions: The selected strain *Lactobacillus lactis* was subjected to different culture conditions to derive the optimum conditions for bacteriocin production. Growth and bacteriocin production were estimated at various temperatures (20, 25, 30, 35, 40 and 45ºC), pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) and incubation time (6, 12, 18, 24, 30, 36, 42 and 48 h). Samples were collected after 48 h (expect for incubation time effect) and examined for bacteriocin production (AU/ml) as described earlier.

Effect of enzymes and detergents: The sensitivity of the active substance to enzymes was tested on cell-free supernatant (pH 6.0) of 24 h cultures incubated at 30 ºC and were treated for 2 h with 0.1 mg ml^{-1} and 1.0 mg ml^{-1} final concentration of the following enzymes: proteinase K, α-amylase, DNase, RNase, pepsin and lipase (all from Hi Media Laboratory Pvt Ltd. India).

The surfactants tested were sodium dodecyl sulphate (SDS), Tween 80, Tritone X-100, EDTA and urea at final concentration 0.1, 1, 2 or 5% (all from Hi Media Laboratory Pvt. Ltd. India). Controls, consisted of either active supernatant or detergents used. All samples and controls were incubated at 30ºC for 5 h and tested for activity.

Purification of bacteriocin: The crude bacteriocin was precipitated with 80% ammonium sulphate (Ranbaxy, New Delhi) saturation. The precipitate was dialysed against 20 mM potassium phosphate buffer (pH 7.0) for 12 h at 4ºC. Further purification was carried out in ion exchange chromatography (DEAE-Cellulose, Hi Media Laboratory Pvt Ltd. India). The dialyzed protein was applied to a DEAE- Cellulose A-50 column (20 mm diameter×60 mm long), pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 vol. of equilibration buffer, bound proteins were eluted stepwise using phosphate buffers of increasing molarity and decreasing pH values at room temperature (approx. 25 ºC). The flow rate was adjusted to 24 ml h^{-1} and fractions (1 ml each) were collected. The fractions showing high bacteriocin activity were pooled and concentrated in lyophilizer.

Determination of protein: Protein concentration of the bacteriocin in supernatant was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Molecular weight determination in SDS-PAGE: The molecular weight of the bacteriocin was determined by 15% Sodium dodecylsulfate polyacrylamide gel electrophoresis, (Laemmli, 1970) in LKB Bromma 2050 Midget electrophoresis units (Pharmacia Amersham Co). After electrophoresis, the gel was stained with Comassie Brilliant Blue R-250. Range moleculer markers (29-200 kDa) with five polypeptides were used as a marker.

RESULTS

Isolation and identification of bacterial strain: The bacteriocin producing strain was isolated from the marine environment of the Chennai harbour and the selected strain was identified as *L. lactis* based on its physiological and biochemical characteristic (Table 1).

Table 1: Physiological and biochemical characteristic

Physiological and biochemical characteristic	Result
Colony morphology	Creamy, little sticks and smooth round colonies
Gram staining	Gram positive, rod
Growth in MRS broth	uniform turbidity
Type of fermentation	Homofermentative
Galactose, glucose, fructose, mannitol, lactose, sucrose and maltose	Fermentation positive
Catalase, oxidase, indole and amylase production	Negative
Growth in Bilary salt	Resistant
H2S production	positive

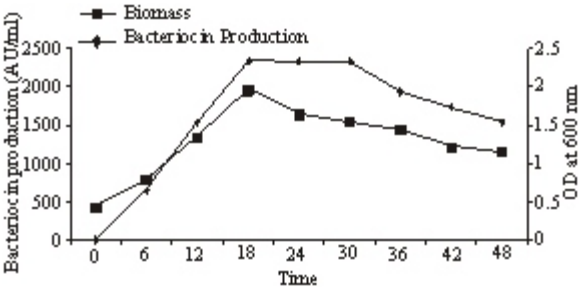

Fig. 1: Biomass and bacteriocin production

Biomass and bacteriocin production: Measurements of biomass and bacteriocin production are shown in Fig. 1. Results showed that *L. lactis* produced bacteriocin in MRS broth. The strain *L. lactis* exhibited a good bacteriocin activity of 2344 AU/ml, at pH 6.0, sodium chloride 1.5% and 30 ºC. The bacteriocin production was higher during the stationary phase of the growth of the organism, whereas maximum biomass occurred at 18 h.

Determination of inhibitory spectrum: The susceptibilities of various Gram-positive and Gram-negative bacteria to growth inhibition by the supernatant of *L. lactis* are presented in Table 2. It shows inhibitory activity against *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella shiga* and *Shigella boydii*. Among these, maximum activity observed against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* and minimum activity observed against *Shigella boydii* (Fig. 2 and 3).

Effect of temperature, pH and salt concentration on bacteriocin activity: Temperature and pH played an important role in cell growth as well as bacteriocin production. The bacteriocin activity was tested with different temperatures (20, 25, 30, 35, 40 and 45ºC). Furthermore, the maximum arbitrary unit was measured as 2647 AU/ml at 30ºC and minimum levels were recorded as 1876 AU/ml at 20ºC (Fig. 4 and 5). Regarding pH the maximum arbitrary unit was measured as 2847 AU/ml at

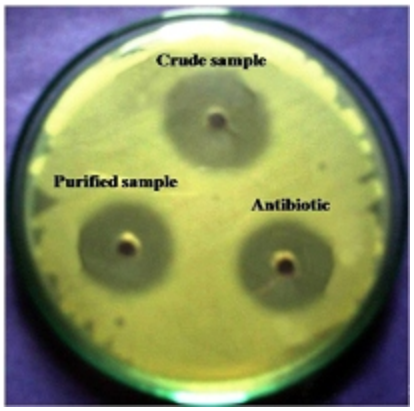

Fig. 2: *Lactobacillus lactis* showing bacteriocin activity by agar-well diffusion method against *Enterococcus faecalis* as sensitive culture

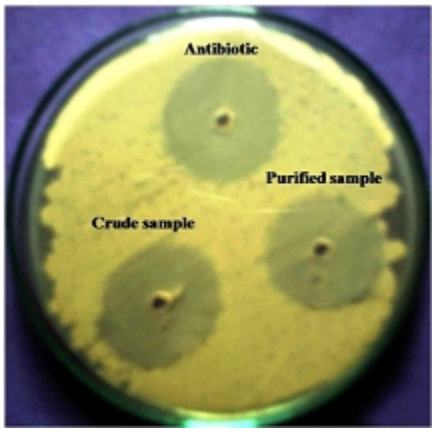

Fig. 3: *Lactobacillus lactis* showing bacteriocin activity by agar-well diffusion method against *Staphylococcus aureus* as sensitive culture

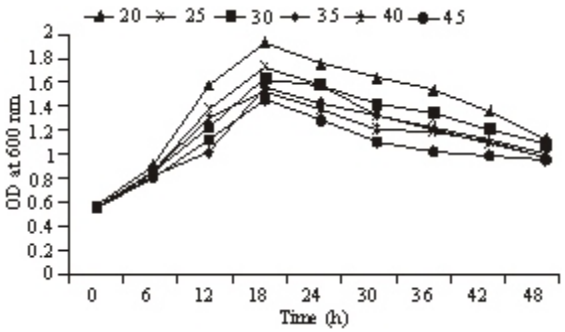

Fig. 4: Effect of temperature on growth kinetics

pH 6.0 (Fig. 6 and 7) and minimum in 1969 AU/ml at pH 9.0. Regarding various concentration of sodium chloride (NaCl %) tested from 0.5 to 3% NaCl, the high level of bacteriocin production was recorded as 2447 AU/ml at 1.5% and minimum in 1879 AU/ml at 3% (Fig. 8 and 9).

Table 2: Inhibition of various indicator organisms by bacteriocin produced by *Lactobacillus lactis*

Indicator organisms	Strain No.	Diameter of zone of inhibition (mm)		
		Crude sample (50 µl)	Purified sample (50 µl)	Ampicillin (30 µl)
Gram positive				
Bacillus subtilis	QL-166	23	15	25
Bacillus megaterium	QL-38	12	07	17
Bacillus cereus	QL-29	-	-	16
Staphylococcus aureus	ATCC-259233	25	16	21
Enterococcus faecalis	NBIMCC 6783	27	19	29
Gram negative				
Escherichia coli	ATCC 10536	13	06	17
Pseudomonas aeruginosa	CRL	17	13	19
Shigella shiga	ATCC-26107	11	06	15
Shigella dysenteriae	AL-35587	-	-	14
Shigella boydii	AL-17313	10	04	11

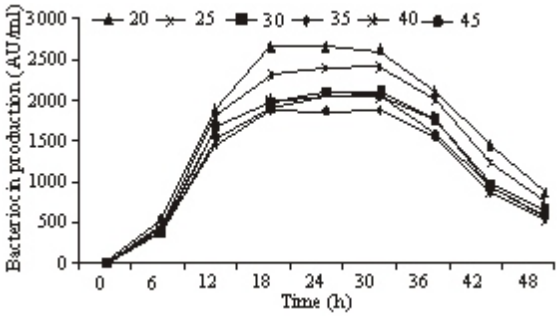

Fig. 5: Effect of temperature on bacteriocin production

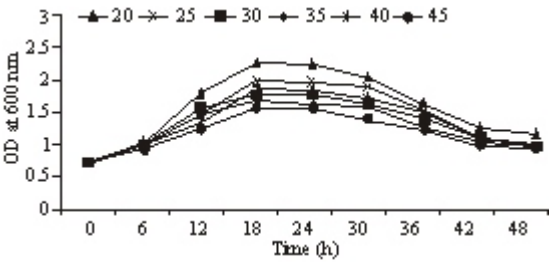

Fig. 6: Effect of pH on growth kinetics

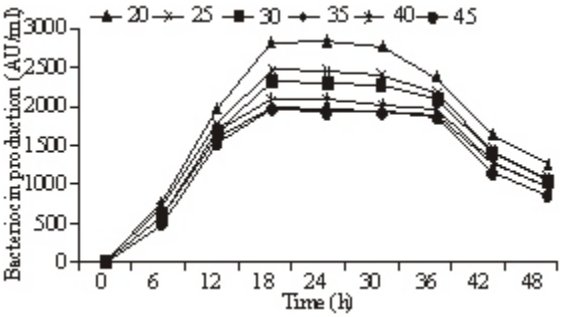

Fig. 7: Effect of pH on bacteriocin production

Effect of enzymes and detergents: The effect of enzymes used were: proteinase K, α-amylase, DNase, RNase, pepsin and lipase. In the presence of α-amylase,

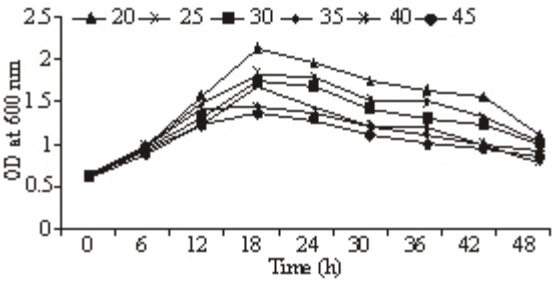

Fig. 8: Effect of sodium chloride on growth kinetics

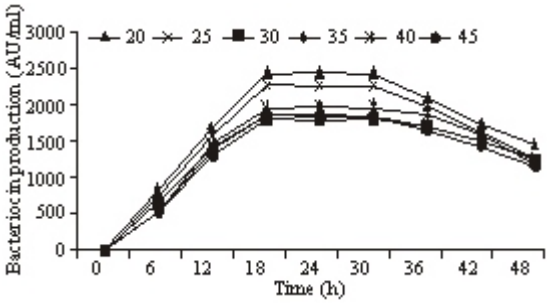

Fig. 9: Effect of sodium chloride on bacteriocin production

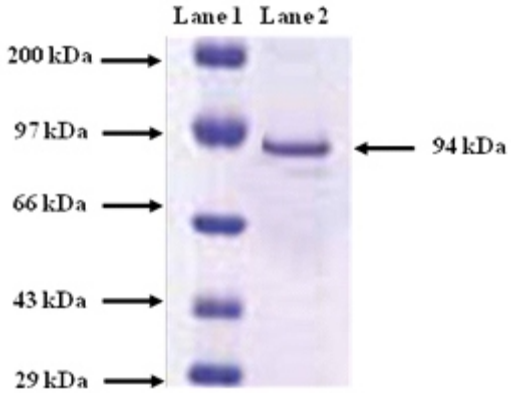

Fig. 10: SDS-PAGE of the purified enzymes: Lane 1, molecular weight markers; lane 2, Bacteriocin

Table 3: Effect of enzymes on bacteriocin production

Treatment	Bacteriocin activity	
	Concentration (mg/ml)	AU/ml
Enzymes		
Control*	-	7350
Proteinase K	0.1	-
α-amylase	0.1	6540
Dnase	0.1	6210
Rnase	1.0	5430
Pepsin	0.1	-

*without addition of enzymes.

Table 4: Effect of detergents on bacteriocin production

Treatment	Bacteriocin activity	
	Concentration (mg/ml)	AU/ml
Detergents		
Control	-	7350
SDS	1	5340
Tween 80	2	4370
Tritone X-100	0.1	1240
EDTA	5	-
Urea	1	-

*without addition of detergents.

Table 5: Summary of the purification steps of bacteriocin from the culture supernatant of *Lactobacillus lactis*

Purification Stage	Volume (ml)	Total activity (AU/ml)	Total protein (mg)	Specific activity (AU/mg)	Purification (fold)	Recovery (%)
Culture supernatant	100	7350	225.2	32.64	0.0	100
Ammonium sulphate precipitation, (80% saturation) and dialysis	25	2680	28.7	105.92	3.25	41.36
DEAE-Cellulose chromatography	5	800	0.68	1176.47	11.11	26.32

DNase, RNase and lipase were positive effect of bacteriocin production. Proteinase K and pepsin were strongly inhibited bacteriocin production (Table 3).

Effect of detergents used were: sodium dodecyl sulphate (SDS), Tween 80, Tritone X-100, EDTA and urea. Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 were could stimulate the bacteriocin production. In contrast, it was strongly inhibited by EDTA and urea (Table 4).

Purification of bacteriocin: In the purification of filtrate culture, was removed by centrifugation, and the proteins were concentrated by 80% ammonium sulphate precipitation and dialysis. The recovered proteins were then fractionated by ion-exchange chromatography, using DEAE- Cellulose. All procedures were done in cold room. Extracellular bacteriocin was purified up to 11.11 fold from culture supernatant. The overall yield and activity are summarized in Table 5.

Molecular weight determination in SDS-PAGE: Molecular weight of the bacteriocin was determined by SDS-PAGE gel electrophoresis (Fig. 10). Single protein band was observed when stained with Comassie blue and it clearly indicated the purity of the protein. The molecular weight of the purified bacteriocin was calculated to be about 94 kDa.

DISCUSSION

The present investigation highlights the isolation, characterization and activity of bacteriocin produced by *L. lactis* from marine environment. It is rich in nutrient and organic matter. To state that the isolate *L. lactis* was tested for antibacterial activity against gram-positive and gram-negative bacteria such as *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella shiga*, *Shigella dysenteriae* and *Shigella boydii* associated with food borne illnesses. The highest inhibitory activity was demonstrated against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* while the least activity was demonstrated against *Shigella boydii*. The inhibitory effect demonstrated by *L. lactis* against these bacteria is an indication of possession of antibacterial activity.

Results also revealed the presence of the compound bacteriocin in the test organisms. Bacteriocins have been reported to be inhibitory against several other bacteria (Ogunbanwo *et al*., 2003; Flythe *et al*., 2004; Moghaddam *et al*., 2006; Ogunshe *et al*., 2007; Karthikeyan and Santosh, 2009). Possession of bacteriocin by *L. lactis* is an indication that the bacteria can be used as probiotic and as biopreservative.

Bacteriocin production was strongly dependent on pH, nutrients source and temperature as claimed by Todorov and Dicks (2004). Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity was noted at pH 6.0, temperature 30°C and 1.5% NaCl. From the results proved that it could be used in acidic foods like pickle or yogurt. It might be secondary metabolites. MRs seemed to be more suitable medium for the bacteriocin production. Similar results were observed by Karthikeyan and Santosh, 2009; Ogunshe *et al*., 2007; Ivanova *et al*., 2000).

Bacteriocin production was influenced when incubated in different enzymes α-amylase; DNase, RNase and lipase resulted in greater Bacteriocin production. . Proteinase K and pepsin were strongly inhibited Bacteriocin production. This is in contrast to results obtained by Ivanova *et al*. (2000) and Ogunbanwo *et al*. 2003.

Among the detergents, Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 stimulated

bacteriocin production, which was strongly inhibited by EDTA and urea. Similar results were observed by Ivanova *et al*. (2000) and Ogunbanwo *et al*. (2003). But, stimulatory effect of Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 on bacteriocin in playing that the detergents act as co-factors, which are required to increase the bacteriocin production.

During purification several different protocols were applied. Optimal recovery was achieved by including ammonium sulphate precipitation and ion-exchange chromatography. The used protocol resulted in increase in the specific activity and 26.32% recovery.

Purified bacteriocin from *L. lactis* revealed homogeneity of a single protein band on 15% native PAGE. Its molecular weight was estimated at 94 kDa by SDS-PAGE. Similar results were recorded by Ivanova *et al*. (2000), Karthikeyan and Santosh (2009) and Ogunshe *et al*. (2007). In conclusion therefore, the peculiar antimicrobial characteristics of *L. lactis* can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of the food products so produced.

CONCLUSION

The bacteriocin produced by *Lactobacillus lactis* was assayed by agar well diffusion method and bacteriocin activity was measured in terms of AU ml^{-1}. The highest dilution that gave a define zone of growth inhibition was used to calculate AU ml^{-1}. Mode of action of bacteriocin produced by *Lactobacillus lactis* was tested and the behavior of the bacteriocin produced by isolated strain was considered as bactericidal.

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