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RESEARCH ARTICLE

PRODUCTION OF A NISIN-LIKE BACTERIOCIN FROM *Lactococcus lactis* subsp. *lactis* STRAIN CCSU 1011 ISOLATED FROM MILK.

Dr. Mukta Sharma.
Prof. and Head, Department of Microbiology, Shree Bankey Bihari Dental College & Research Centre, Ghaziabad, (UP) India.

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Abstract

A total of 104 milk samples were collected from different dairies in Meerut region from which 120 isolates of lactic acid bacteria (LAB) were isolated. Of these, 68 strains showed antibacterial activity against the sensitive test organism *Lactococcus lactis* subsp. *lactis* MTCC 3038. The isolate which showed strongest activity was identified as *Lactococcus lactis* subsp. *lactis* using the characters of cell morphology, biochemical tests and carbohydrate fermentation profile. It was given the strain number as *Lactococcus lactis* subsp. *lactis* CCSU 1011. It produced bacteriocin which was active against closely related lactic acid bacteria as well as a wide range of food pathogens including *Salmonella typhi*, *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Shigella flexineri*, *Bacillus cereus*, and *Enterococcus faecalis*. The antimicrobial spectrum was nearly identical to that of nisin. Optimum bacteriocin was produced at the initial pH of 7.0 and at 35 °C temperature after 24 h of incubation period in MRS broth with 1.5% Tween-80. Bacteriocin activity was not destroyed by exposure to elevated temperatures at low pH values, but the activity was lost at high pH values. This bacteriocin was inactivated by proteases and pronases but not by trypsin, pepsin and α- amylase. The bacteriocin was purified using ammonium sulphate precipitation, cation exchange chromatography (CM-cellulose) and gel filtration (Sephadex G-50). The apparent molecular weight of the purified bacteriocin was calibrated as approximately 3.5 kDa on SDS-PAGE similar to that of nisin. The bacteriocin exhibited bactericidal mode of action against *Lactococcus lactis* subsp. *lactis* MTCC 3038. These results indicate that bacteriocin produced by *Lactococcus lactis* subsp. l*actis* CCSU 1011 is a nisin or nisin-like bacteriocin.

Introduction:-

Lactic acid bacteria (LAB) produce a wide variety of antagonistic compounds (De-Vuyst and Vandamme, 1994a) including lactic and acetic acids (Ariyapitipum et al., 1999), diacetyl (Jay, 1982), hydrogen peroxide (Chang et al., 1997), carbon dioxide, alcohol, aldehyde and bacteriocin (Klaenhammer, 1988; Scott et al., 1997), that can

Corresponding Author:- Mukta Sharma.
Address:- Prof. and Head, Department of Microbiology, Shree Bankey Bihari Dental College & Research Centre, Ghaziabad, (UP) India.

antagonize the growth of spoilage and pathogenic bacteria in foods. The bacteriocins have attracted world wide interest for many potential applications because of their antibacterial properties (Ray et al., 2001).

Bacteriocins are proteinaceous bacterial products which have bactericidal activity. They are produced by various LABs including *Lactococci*, *Lactobacilli* and *Pediococci* (Jack et al., 1995). Many bacteriocins produced by LAB inhibited not only species closely related to the producer strain (Tagg et al., 1976), but also the growth of food borne pathogens such as *Listeria monocytogenes* and spoilage bacteria (Cleveland et al., 2001; Villamil et al.,2014). Thus, these compounds have attracted increasing interest for use as natural food preservatives (Khay et al., 2011). Among the bacteriocins produced by LAB, member of the lantibiotic class are unique because of their efficacy and the established safety record of nisin. Nisin has been the most extensively studied bacteriocin. It is active against Gram - positive bacteria and can appear as two types (nisin A or Z) that differ in both amini acid composition and biological activity (de Vos et al., 1995). Nisin A is a 34 amino acid peptide containing lanthionine and β-methyllanthionine residue (Gross and Morell 1971).

Nisin's sensitivity to α-chymotrypsin, heat stability at low pH and non- toxic nature has promoted its widespread use. Nisin has thus far been the only bacteriocin to find widespread application in the food industry. It is used as a GRAS food preservative in pasteurized process cheese to prevent *Clostridial* growth (U.S. Food and Drug Administration,). It is permitted as a food additive in at least 46 countries, particularly for inhibition of *Clostridium* species in processed cheese, dairy products and canned foods (Park et al., 2003).

Lactococcus lactis is catalase negative, Gram positive, non-motile, non-spore forming mesophilic and microaerophilic bacterium, that produce lactic acid from lactose. This organism causes natural souring in milk. It occurs in milk as ellipsoidal or elliptical or oval diplococci arranged in aires or short chains (Ko and Ahn, 2000). *Lactococcus lactis* is a model LAB, many generic tools have been developed and its complete genome was also sequenced (Bolotin et al., 2001).

This study aimed to isolate and identify bacteriocin-producing LAB from milk and to characterize their bacteriocins. In this report, isolation and characterization of nisin-like bacteriocin- producing *Lactococcus lactis* subsp. *lactis* CCSU 1011 from milk is described.

Materials and methods:-

Bacterial strains and culture conditions:-

Bacterial strains used in this study are listed in Table.1. *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* were grown in de Man Rogosa Sharpe (MRS) agar medium (Difco) at 37 °C. *Listeria monocytogenes* was grown in Trypticase soya yeast extract (TSYE) agar medium (Difco) at 30 °C. *Escherichia coli* and all other strains were grown in Nutrient agar (NAM) medium (Difco) at 37 °C. All cultures were maintained as frozen stocks held at -20°C in appropriate broth containing 50 % glycerol (w/v). Throughout the experiments strains were sub cultured every two weeks on agar plates and kept at 4°C. Before use in experiments, cultures were propagated twice in broth overnight.

Isolation of a bacteriocin –producing lactic acid bacteria:-

Milk was used as a source of lactic acid bacteria. 1 ml of milk sample was serially diluted with sterile 0.9% Nacl and spread on MRS agar plates. After incubation at 30 °C for 24 h, the colonies were picked off and streaked onto fresh MRS agar medium plates. The fresh plates were incubated at 30°C for 16-18 h to allow the colonies to appear and then overlaid with 5 ml of soft MRS agar medium containing 5 x 10^6 CFU/ml of sensitive strain, *Lactococcus lactis* subsp. *lactis* MTCC 3038. After additional overnight incubation at 37°C, formation of clear zones of inhibition around the colonies were examined (Fig 1). To determine bacteriocin production in liquid media, isolates showing antimicrobial activity were grown in MRS broth with 1% Tween 80 at 30°C until stationary phase. The cultures were centrifuged at 15,000g for 30 min at 4°C. To eliminate growth inhibition caused by organic acid, the resulting cell free filtrates (CFF) were adjusted to pH 6.5 with 1 NaoH and filtered through 0.2 µm pore size membrane filters. The samples were stored at -20°C until further use.

Identification of the bacteriocin producing strain:-

Bacteriocin producing strain was identified by the criteria based upon carbohydrate fermentation patterns, and the procedures as described by (Schillinger, 1999). The patterns were compared with those of reference lactic acid bacteria procured by IMTECH Chandigarh.

Optimization for production of bacteriocin:-
To find the optimal conditions for the maximal production of bacteriocin, the strain *Lactococcus lactis* subsp. *lactis* CCSU 1011 was inoculated in Erlenmeyer flask (250 ml) containing 40 ml MRS broth with % Tween 80.

For optimization of pH for the production of bacteriocin, MRS broth was adjusted to pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 & 9.0 with 1 N HCl and 1 N NaOH and the strain CCSU 1011 was inoculated aseptically in each of the pH sets separately in triplicate. Cultures were harvested after 24 h, growth was monitored spectrophotometrically (OD at 620 nm) and bacteriocin activity was assayed in AU/ml. After the optimization of pH the experiments were set to optimize the temperature and the cultures were incubated at 25°C, 30°C, 35°C, 37°C, 40°C, 45°C & 50°C temperatures separately. Growth and activity were monitored similarly. For optimization of incubation period, the cultures were harvested at suitable intervals from 2 to 96 h.

Bacteriocin detection and assay:-
Antimicrobial activity secreted into liquid media was detected by agar-well diffusion method (Tagg and Mc Given 1971). MRS soft agar (5ml) inoculated with 1% (v/v) of an indicator sensitive strain overnight culture was overlaid on an agar plate. After cooling, wells (6 mm diameter) were punched in the agar plates and filled with 50 µl of test samples. After incubation overnight, the antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Bacteriocin activity was assayed by two fold dilution of crude bacteriocin in terms of arbitrary unit (AU). Arbitrary unit was defined as the reciprocal of the highest dilution which showed a clear zone of inhibition (Yang et al., 1992).

Partial purification of bacteriocin:-
The bacteriocin producing strain *Lactococcus lactis* subsp. *lactis* CCSU 1011 was grown under optimized conditions. Cells were removed by centrifugation at 5,000 x g for 30 min at 4°C temperature, and the supernatant was filtered through a 0.45 µm pore size membrane filter. It was defined as cell free filterate (CFF). Ammonium sulphate was added to achieve 90 % saturation and allowed to precipitate for 24 h at 4°C. Precipitate was collected by centrifugation at 10,000 x g for 20 min and dialyzed on magnetic stirrer using a membrane with a molecular cut-off of 10,000 Dalton against 10 mM phosphate buffer (pH 7.0) for 72 h at 4°C temperature with frequent changes of fresh buffer. The bacteriocin was then freezed and stored at -20°C for further use.

Dialysate was applied to the carboxymethyl- celluolose column which had been equilibrated with 10 mM glycine NaOH buffer (pH 8.0). After the column was washed in 10 mM glycine NaOH buffer (pH 8.0), the bacteriocin was eluted by a step gradient of 50, 100, 200, 400 mM NaCl in the same buffer. Fractions of 2ml were collected at a flow rate of 0.2 ml/min. The resulting protein was concentrated and then loaded onto Sephadex G-50 column which was equilibrated with 10 mM phosphate buffer (pH 7.0). The sample was eluted with the same buffer at the flow rate of 0.05 ml/min. The active fractions were stored at -20°C.

Sensitivity of bacteriocin to degraditive enzymes and organic solvents:-
Partially purified bacteriocin (51200 AU/ml) was treated with various enzymes including trypsin, pepsin, pronase E, α- amylase, β- amylase, lipase, lysozyme, α-chymotrypsin, and β- chymotrypsin to final concentration of 1mg/ ml in sterile distilled water. Partially purified bacteriocin was also treated with 50% organic solvents such as ethanol, methanol, toluene, acetone and ethylacetate. Enzyme treated samples were incubated at 37°C for 1 h and solvent treated samples were incubated at 25°C for 1 h. At the end of incubation, the residual activity of bacteriocin was assayed using agar well diffusion method.

Antibacterial spectrum assay:-
Agar well diffusion assay (Tagg and Mc Given 1971) was made for partially purified bacteriocin against Gram +*ve* and Gram – *ve* pathogenic bacteria.

Mode of action:-
The mode of action of partially purified bacteriocin was studied against *Lactococcus lactis* subsp. *lactis* MTCC 3038. 2 ml of CFF was added to 10 ml of growing cells of sensitive strain in MRS broth in early exponential phase. The absorbance was determined at 620 nm after every 4 h up to 36 h and the culture was plated onto agar plates and examined after incubation of 24 h.

Determination of molecular weight of bacteriocin:-

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) in a 15 % discontinuous gel was performed using the method of Laemmli *et al*., 1970. 50µl of partially purified bacteriocin was mixed in 1:1 ratio with sample buffer (4.6% SDS, 10% β mercaptoethanol, 20% glycerol, 1.5% tris base, 1% bromophenol blue) and heated at 100°C for 4 min. Molecular weight standard (5 µl GENEI lower range) and 10µl of sample was applied to the gel. The sample was electrophoresed at 90 V, the gel was removed and stained with Coomassie brilliant blue R-250, destained in the solvent and molecular weight was determined using standards as marker.

Results and discussion:-

Isolation of bacteriocin producing lactic acid bacteria:-

A total of 120 isolates of LAB were screened for their antagonistic activity against *Lactococcus lactis* subsp. *lactis* MTCC 3038 an indicator sensitive strain. Only 68 of the 120 isolates, showed antibacterial activity against the indicator strain (data not shown). Isolate CCSU 1011 had the broadest spectrum of inhibitory activity and was used in further studies. The antibacterial compound produced by the strain CCSU 1011 was inactivated on treatment with proteases which suggests that the produced active biomolecule is a protein.

Identification of strain CCSU 1011:-

Strain CCSU 1011 was Gram positive cocci, in pairs or in small chains, catalase and oxidase negative. It was non motile and non endospore forming. It did not produce gas from lactose and sucrose, but produced ammonia from arginine. It showed growth at 40°C temperature and with 4% NaCl concentration. Strain CCSU 1011 showed neither gelatin liquefaction nor nitrate reduction. Based on carbohydrate fermentation profile and other physiological tests as suggested by Schillinger 2001, the strain CCSU 1011 was identified and classified as *Lactococcus lactis* subsp. *lactis.* It was named as *Lactococcus lactis* subsp. *lactis* CCSU 1011.

Antibacterial spectrum:-

Antibacterial spectrum of crude CFF and partially purified bacteriocin on agar plates against various indicator/test strains/species is presented in Table 1. Antibacterial activity of partially purified bacteriocin using agar well diffusion method revealed that activity of partially purified bacteriocin by and large remained the same, however, on partial purification it lost the activity against *Salmonella paratyphi* and *Shigella dysenteriae* against which CFF was also showing minimum inhibition of 5 mm. Nagalakshmi (2013) have also reported the loss or decrease in the bacteriocin activity on partial purification of the bacteriocin. Thus, the antimicrobial spectrum of the strain CCSU 1011 suggested that it has a broad range; effective against LAB including genus *Lactococcus, Lactobacillus, Leuconostoc* and *Pediococcus,* and other Gram positive and Gram negative pathogenic bacteria including *Salmonella typhi, Listeria monocytogenes, Clostridium perfringens, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Streptococcus sp., Micrococcus luteus* and *Enterococcus* sp. Nisin producing *Lactococcus lactis* subsp. *lactis* MTCC 440 used as an experimental control, showed a nearly identical inhibitory spectrum to that of *Lactococcus lactis* subsp. *lactis* CCSU 1011 . Of the tested strains, only two *Salmonella typhi* and *Shigella flexineri* showed different sensitivity. On the basis of these observations it appeared that *Lactococcus lactis* subsp. *lactis* CCSU 1011 produced a nisin like bacteriocin. **Our findings thus, confirm the previous reports of Daeschal, 1989 and Muriana, 1996. Most of the food microbiologists have reported the antimicrobial activity of nisin mainly against Gram +ve bacteria but to the knowledge of the authors none has reported its activity against *Salmonella typhi* against which bacteriocin produced by CCSU 1011 strain was highly effective**. It suggests that our producer strain *Lactococcus lactis* subsp. *lactis* CCSU 1011 is unique than those reported in scientific literature and further studies are in process. On the basis of these observations it appeared the *Lactococcus lactis* subsp. *lactis* CCSU 1011 produced a nisin-like bacteriocin.

Production of bacteriocin:-

Bacteriocin activity in *Lactococcus lactis* subsp. *lactis* CCSU 1011 was detected after 1 h of growth and reached maximum (12,800 AU/ml) at the stationary phase after 24 h (Fig.1) of incubation at 35°C temperature (Fig.2) and pH 7.0 (Fig.3). Several other LAB have been reported to produce highest amount of bacteriocin during log phase of growth. (Nagalakshmi *et al*., 2013: Loh et al., 2017). Microbial biomass in terms of O.D. revealed that bacteriocin activity increased with microbial biomass upto 24 h period of incubation (Fig. 1) and further prolonged incubation did not enhance bacteriocin activity rather it was reduced. (Fig. 1).The incubation temperature in the range of 25°C to 50°C revealed that though, the temperature did not significantly influence the growth in terms of biomass (O.D. at 620 nm) but it showed remarkable effect on the activity of bacteriocin which was maximum at 35°C temperature showing 5 fold increase than that of 30°C (Fig.2). Optimum activity of bacteriocin by CCSU 1011 was produced

when initial pH of the culture medium was 7.0. (Fig.3). An early production of bacteriocin just after 1 h of incubation by the strain *Lactococcus lactis* subsp. *lactis* CCSU 1011 was unique as Batish, *et al*., (1990) have reported that *Lactococcus diacetylactis* showed bacteriocin production only after 18 h of incubation. It suggests that CCSU 1011 is a better producer strain which has potential for commercial exploitation.

Partially purified bacteriocin:-

The bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011 was precipitated using ammonium sulphate (90% saturation) followed by dialysis. The dialyzed fraction yielded 11.76% (approx) protein which showed two fold increase in bacteriocin activity (Table.2). Resuspended bacteriocin was applied to a carboxymethyl cellulose cation exchange column (equilibrated with glycine NaOH buffer, pH 8.0) and eluted by a step salt gradient 50,100,200,400 mM NaCl (Fig.5) where the purity increased 68 fold while the bacteriocin activity enhanced only 4 fold. The fractions which showed bacteriocin activity were collected and then applied onto a Sephadex G-50 column (Fig.6). Active fractions were collected again and defined as partially purified bacteriocin. After gel filtration, the yield of bacteriocin was about 2.35% (85 fold purity) but the specific activity was enhanced to 128000 AU/mg from the initial activity of 1505 AU/mg.

Sensitivity of bacteriocin to degradative enzymes and organic solvents:-

Protease sensitivity assays demonstrated that the antimicrobial substance produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011 was bacteriocin as the inhibitory activity was completely eliminated by the treatment with pronase and partially eliminated with α-chymotrypsin, and β- chymotrypsin (Table 2). However, it was neither affected by the treatment of lysozyme, lipase, trypsin, pepsin, α-amylase and β-amylase nor was it inactivated by the organic solvents as shown in Table 2.

Estimation of molecular weight by SDS- PAGE:-

The molecular weight of the partially purified bacteriocin was determined by SDS- PAGE (Fig.7). The gel stained with coomassie brilliant blue showed protein bands of approximately 3.5 kDa in the partially purified bacteriocin samples. Based on antibacterial spectrum and molecular weight, the bacteriocin was identified as nisin.

Mode of action:-

The mode of action of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011 was studied against *Lactococcus lactis* subsp. *lactis* MTCC 3038, a bacteriocin sensitive strain. (Fig.8). where the growth of the later was initially inhibited during first four hour period of incubation and thereafter it decreased and stopped growing further after 24 h period of incubation. To confirm it further the culture after 24 h of period of incubation was plated onto MRS agar and on incubation it was found that no colony of sensitive strain was developed both bacteriostatic as well as bactericidal.

Sensitivity‡					
Indicator strain* MTCC 440	CFF		partially purified		
	(CCSU 1011)	Bacteriocin (CCSU 1011)			
Lactococcus lactis subsp. *lactis* MTCC 3038	+++		+++		+++
Lactococcus lactis subsp. *chacetylactis* MTCC 3042	++		++		++
Lactococcus lactis subspcremoris MTCC 1484		++		+++	+++
Lactobacillus acidophilus MTCC 447		+++		+++	+++
Lactobacillus brevis MTCC 1750	+		+		+
Lactobacillus acidophilus MTCC 447		+		++	++
Lactobacillus plantarum CCSU 2007		++		++	++
Lactobacillus sake CCSU 2014		+++		+++	+++

Lactobacillus casei MTCC 1432		++		++		++		
Lactobacillus brevis MTCC 1750		+++		+++		+++		
Leuconostoc sp. CCSU 3005		+++		+++		+++		
Leuconostoc cremoris CCSU 3018	+++		+++		+++			
Leuconostoc mesenteroides MTCC 107		++		++		++		
Leuconostoc carnosum CCSU 3020	++		++		++			
Pediococcus acidilactici CCSU 4012		++		++		++		
Pediococcus pentosaceus CCSU 4004		+++		+++		+++		
Listeria monocytogenes MTCC 657	+++		+++		+++			
Bacillus subtilis MTCC 441		+++		+++		+++		
Bacillus polymyxa MTCC 122		++		++		++		
Bacillus cereus MTCC 430		+++		+++		+++		
Clostridium perfringens MTCC 450		+++		+++		+++		
Escherichia coli CCSUB 80		NZ		NZ		NZ		
Enterococcus faecalis MTCC 439		+++		+++		+++		
Enterococcus faecium CCSUB 28		+		+		+		
Micrococcus luteus CCSU B11		++		++		++		
Pseudomonas aeruginosa MTCC 2581		NZ		NZ		NZ		
Salmonella typhi MTCC 734		+		+		+		
Salmonella typhimurium MTCC 98		++		++		++		
Salmonella paratyphi CCSUB16		+		NZ		NZ		
Shigella dysenteriae CCSUB 20		+		NZ		NZ		
Shigella flexineri MTCC1457		+		+		NZ		
Staphylococcus aureus MTCC 96		+++		+++		+++		
Staphylococcus epidermidis MTCC 435		++		++		NZ		
Streptococcus sp. CCSUB 15		+		+		+		

Table 1:- Antimicrobial spectrum of the crude and partially purified bacteriocin produced by strain *Lactococcus lactis* subsp. *lactis* CCSU 1011 and *Lactococcus lactis* subsp. *lactis* MTCC 440.
*Abbreviations used: MTCC-(Microbial Type culture collection, IMTECH Chandigarh), CCSU (Chaudhary Charan Singh Univesity, Meerut);
‡ NZ, no inhibition zone, +++ Inhibitory activity (10mm), ++ Inhibitory activity (8mm), + Inhibitory activity (5mm),

Treatment		Activity (AU/ml)[3]
Enzymes[1]	Pronase	0
Lysozyme		51,200
	α-amylase	51,200
	β-amylase	51,200
	Lipase	51,200
	α-chymotrypsin	0
	β-chymotrypsin	12,800
	Pepsin	51,200
Trypsin		51,200
Solvents[2]	Ethanol	51,200
	Methanol	51,200
	Acetone	51,200
	Toluene	51,200
	Ethyl acetate	51,200

Table2:- Effect of various enzymes and organic solvent treatment on the stability of the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011
1). Final concentration of enzymes is 1mg/ml.
2). 50% concentration was used
3). Bacteriocin activity of untreated bacteriocin was 51,200 AU/ml

Purification step	Protein[a] conc. (mg/ml)	Activity[b] (AU/ml)	Specific Activity[c] (AU/mg)	Fold of purity	Yield (%)
Culture supernatant	8.5	12800	1505	01	100
Ammonium sulphate precipitate	1.0	25600	25600	17	11.76
CM-Cellulose column	0.5	51200	102400	68	5.88
SephadexG-50 column	0.2	51200	128000	85	2.35

Table.3:- Partial purification of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011
a. Estimation by the Lowry method (Lowry, *et. al.*, 1951).
b. The highest dilution that gave a definite zone of growth inhibition was used to calculate AU/ml.
c. Specific activity is total activity in AU divided by the protein conc.

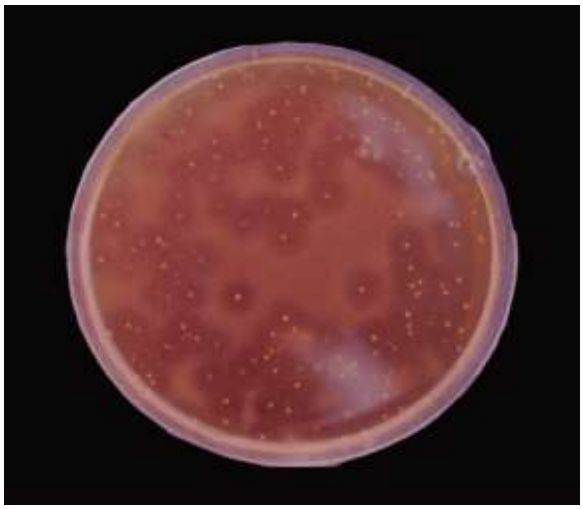


Fig. 1:- Detection of bacteriocin production from *Lactococcus lactis* subsp. *lactis* CCSU 1011 by agar overlaid method described by yang, Johnson and Ray , 1992.

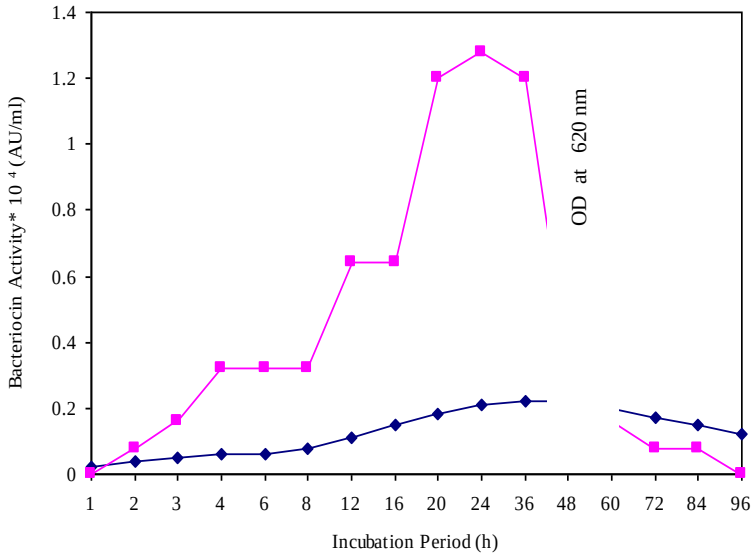


Fig 2:-Bacteriocin production by *Lactococcus lactis* subsp. *lactis* CCSU 1011 in MRS broth with 1.5 % Tween 80 at 35 ° C at different incubation periods, at pH 7.0.

■ Bacteriocin activity, ♦ OD at 620 nm.

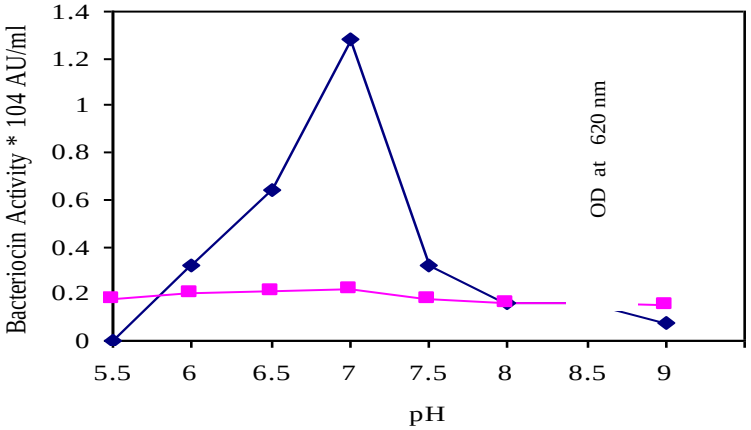


Fig 4:- Bacteriocin production from *Lactococcus lactis* subsp. *lactis* CCSU 1011 in MRS broth with 1.5% Tween 80 at 37 ºC at different pH after 24 h.

■ Bacteriocin activity, ♦ OD at 620 nm.

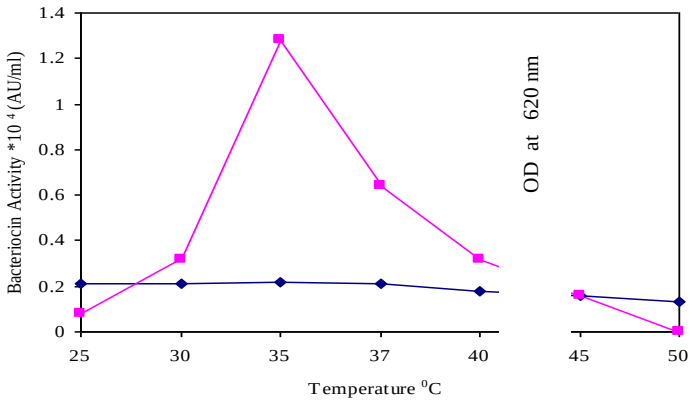


Fig 3:- Bacteriocin production from *Lactococcus lactis* subsp. *lactis* CCSU 1011 in MRS broth with 1.5 % Tween 80 at different incubation temperature after 24 h at pH 7.0.

■ Bacteriocin activity, ♦ OD at 620 nm

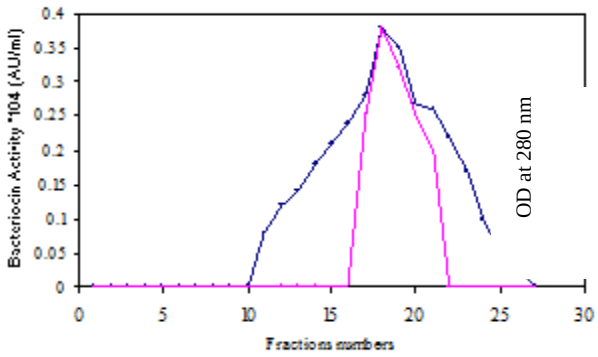


Fig. 5:- Elution profile of partially purified bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011 with gel filtration (Sephadex G-50).

The bacteriocin activity of each fraction (2ml) was assayed by the spot on lawn method. (■) A 280, (●) bacteriocin activity (AU/ml).

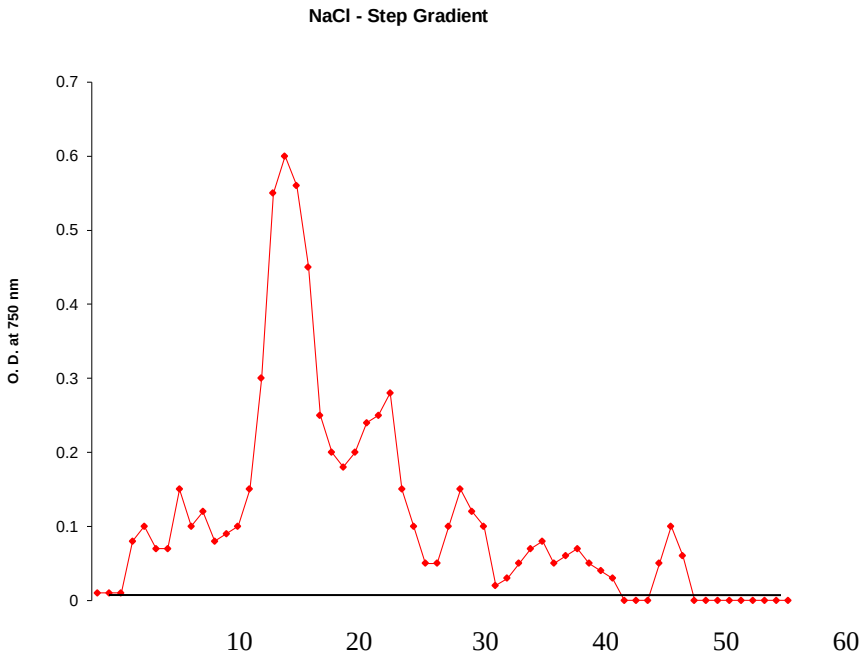


Fig. 5:- CM- cellulose chromatography of Ammonium sulphate precipitated fractions of bacteriocin produced by *Lactococcus lactis* subsp *lactis* CCSU 1011 Bacteriocin was eluted from the column with 50 mM NaCl. Step gradient in single large protein peak III comprising of fraction numbers 15 to 22.

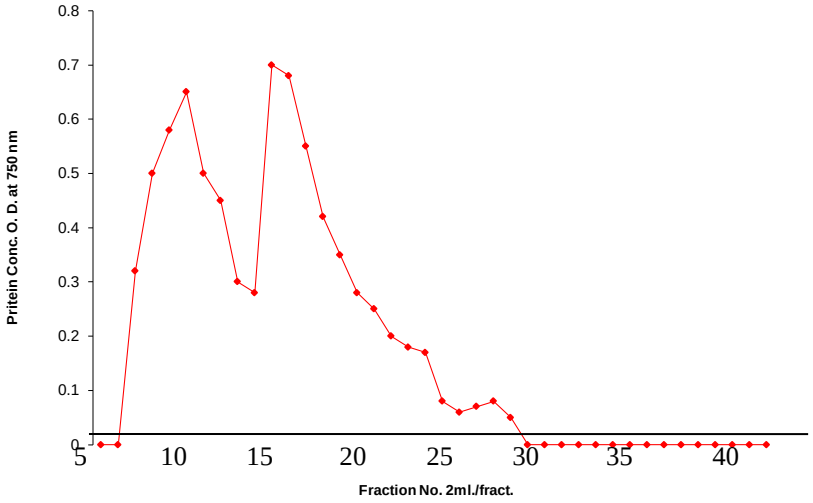


Fig. 6. Gel- filtration chromatography of bacteriocin produced by *Lactococcus lactis* subsp *lactis* CCSU 1011 C M- Cellulose purified bacteriocin fractions loaded onto the Sephadex G-50 column. Bacteriocin was eluted from both peaks (I & II) with 10mM phosphate buffer. (pH 7.0). Fraction numbers 6 and 10 show strong bacteriocin activity.

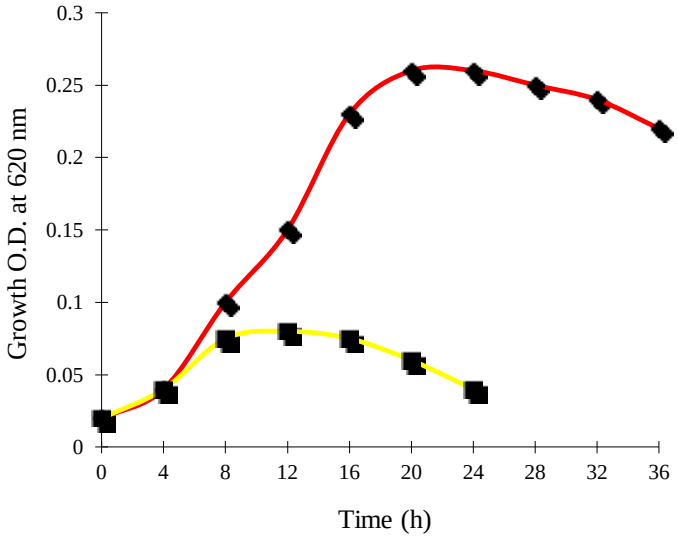


Fig. 7:- Mode of action of acteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011 against sensitive strain (*Lactococcus lactis* subsp. *lactis* MTCC 3038)

♦Sensitive strain without bacteriocin

□ Sensitive strain with bacteriocin

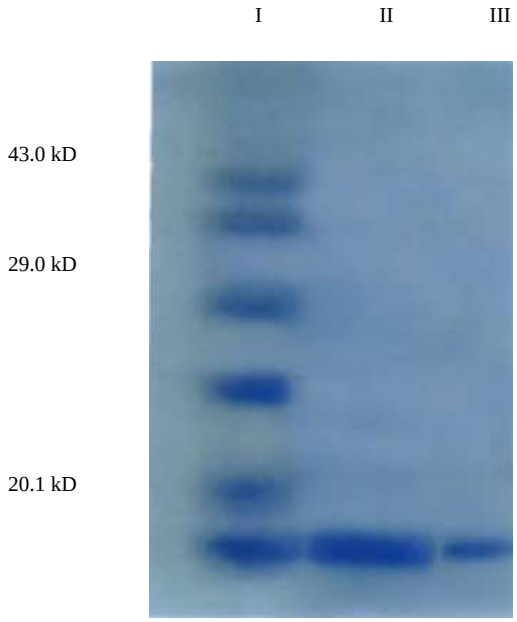
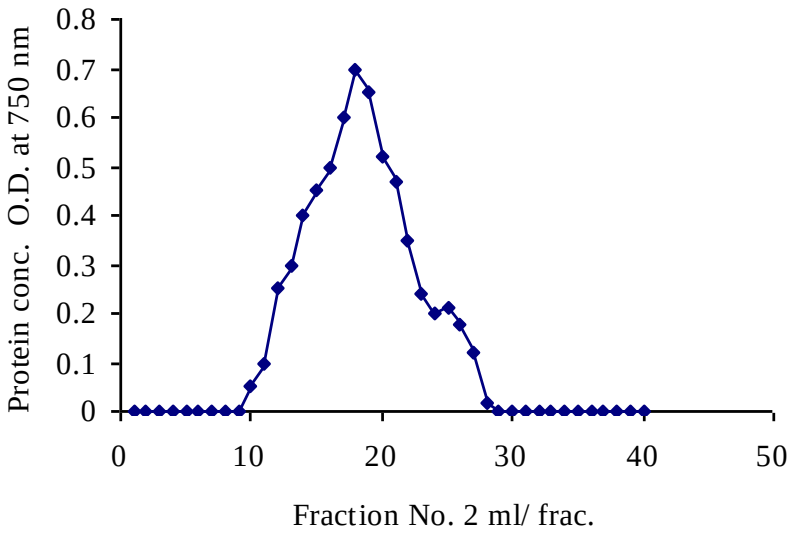


Fig 6:- SDS-PAGE (15% gel) of partially purified bacteriocin stained by comassie brilliant blue R-250
Lane I: Low molecular weight marker with 43.0, 29.0, 20.1, 14.3, 6.5 and 3.0 kD respectively (GENEI)
Lane II: Nisin produced by *Lactococcus lactis* subsp. *lactis* MTCC 440
Lane III: purified bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSU 1011



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