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Research Article

Bacteriocin Profiling of Probiotic *Lactobacillus* spp. Isolated from Yoghurt

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Abstract

Bacteriocin producing *Lactobacillus* spp. isolated from yoghurt, showed wide range of antimicrobial activity against some major food borne pathogens. Antimicrobial assay was conducted by modified agar overlay method and well diffusion method. Isolate 3 showed highest (42 mm) antimicrobial activities and isolate 4 showed lowest (15 mm) antimicrobial activities against test organisms. *Bacillus subtilis* showed the highest and *Staphylococcus aureus* showed the least susceptibility to the bacteriocins of isolated *Lactobacillus* spp. Cell extract & culture supernatant of isolates also showed antimicrobial activity. Bacteriocins were isolated from cell lysate of *Lactobacillus* spp. & separated by SDS-PAGE. All the isolates produced bacteriocins of 94 kDa, 60 kDa & 45 kDa molecular weight intracellularly& crude extracts of these bacteriocins showed considerable antimicrobial activity against test pathogens. This study revealed the possibility of bacteriocin as bio-preservatives for food stuffs.

Key words: Probiotic bacteria, *Lactobacillus* spp., Bacteriocin, SDS-PAGE

1. Introduction

LAB including *Lactobacillus spp.* has gained a considerable industrial and medical interest because of their important role in human health and nutrition by its influence on the intestinal flora. LAB is an abundant source of diverse antibacterial substances including bacteriocin[1]. They occur naturally as indigenous microflora in fermented milk products such as yoghurt[2]. Probiotics such as *Lactobacillus* spp. are reported to have inhibitory activity against common human pathogens[3,4,5,6]. They are able to produce antimicrobial substances such as bacteriocins which have great potential to be used in therapeutics and as food bio-preservatives[7]. Lactic acid bacteria including *Lactobacillus* spp. are gaining increasing interests worldwide to be used in the prevention, control and treatment of diseases and health maintenance[8]. There is a growing interest in the use of *Lactobacillus* spp. as probiotics due to the increasing emergence of antibiotic resistance[9]. Increased antibiotic usage is a key factor in the emergence of antibiotic resistant pathogens. Thus there is an urgent need to develop alternatives to antibiotics[8].

Bacteriocins are biologically active peptides produced by several bacterial species especially by *Lactobacillus* spp. and are active against both gram positive and gram negative pathogenic bacteria[10,11]. Bacteriocin of Lactobacillus spp. are considered as ideal bio-preservative as they have proven to be non –toxic to humans, do not alter the nutritional properties of the food, effective at very low concentration, active under refrigerated storage conditions[12]. On the other hand, novel

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applications of bacteriocins are emerging such as use in functional foods (prebiotics, synbiotics & probiotics or neutraceuticals) & use in human therapy as an alternative to antibiotics[13]. Bacteriocins antimicrobial properties are aimed at stimulating the immune system & can be used as an aid treatment of gastrointestinal & urinary tract diseases[14]. Bacteriocin production may also facilitate the establishment of a probiotic strain such as Lactobacillus spp. in the competitive environment of the gut[7]. Research on bacteriocin has attracted considerable attention now a day as their use in extending shelf life of vegetables and milk or its products has proven to be promising[11].

Considering the above facts in mind, the present study was undertaken to isolate and characterize bacteriocins from indigenous *Lactobacillus* spp. from traditional Bangladeshi yoghurt and to assess their anti-microbial activity against some common human pathogens in vitro.

2. Materials & Methods

2.1 Culture Collection and Maintenance

Four *Lactobacillus* spp. previously isolated from Yoghurt & antimicrobial activity of those have already been reported[15] were obtained from culture collection pool of Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh. The strains were cultured on MRS agar and maintained on Nutrient agar as working cultures.

2.2 Test Organisms

Nine different human pathogens belonging to both gram-positive and gram negative groups such as *Bacillus subtilis* (laboratory strain), *Bacillus megaterium* (laboratory strain), *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 65154, *Salmonella paratyphi* (laboratory strain) and *Vibrio parahaemolyticus* ATCC 17802 were used in this study as test pathogens.

2.3 Antibacterial Activity of Whole cell

Agar overlay method was used to determine the antimicrobial activities of the isolated *Lactobacillus* spp.[16] Antibacterial activity was further characterized by determining whether bacteriostatic or bactericidal. The test was performed by swabbing of the growth inhibition zone of the plate and then swab was streaked onto nutrient agar plate and incubated aerobically at 37°C for 48 hours.The presence of growth in nutrient agar plate was interpreted as an inhibitory activity i.e. bacteriostatic, while no growth was interpreted as bactericidal.

2.4 Antibacterial Activity of Cell Culture Supernatant

The isolated strain was grown in MRS broth (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 37ºC for 48 hrs. 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. Antibacterial activity of the culture supernatant of the isolates was determined by Well Diffusion Assay[17].

2.5 Antibacterial Activity of Cell Lysate

48h old 10 ml MRS broth was taken to centrifuge at 10000rpm for 20 min at 4°C. The supernatant were discarded. The pellet was re-suspended in 3ml distilled water. 1ml of solution were taken in eppendorf tube and centrifuged at 10000 rpm for 10 min. Then 0.01M 0.5ml Tris-HCl, 0.01M 0.5ml EDTA, 0.01M 0.5ml NaCl and 2% 0.5ml SDS were added. The suspension was boiled for 5 min at 100°C. Then the suspension was centrifuged at 11,500 rpm for 10 min. Antibacterial activity of the cell lysates of the isolates was determined by Well Diffusion Assay[17].

2.6 Preparation of Crude Bacteriocin Extract

Lactobacillus isolates were grown in 10 ml MRS agar at 37°C for 48 hrs in anaerobic condition. The cultures were then collected into centrifuge tube and centrifuged at 10000 rpm for 15 mins at 4°C. The supernatant which mainly contain medium were discarded. And then the pellet was washed with 3 ml distilled water. 1ml was transferred into sterile eppendorf tube and centrifuged at 10000 rpm for 10 mins. The supernatant were discarded. After washing the pellets were suspending in 0.5ml of 0.01M Tris-HCl, 0.5ml of 0.01M EDTA, 0.5ml of 0.01M NaCl, and 0.5ml of 2% SDS and boiled at

100°C for 8 mins. After boiling, centrifugation was done at 10000 rpm for 10 mins. This whole cell protein extract was stored at 4°C until use[18].

2.7 Molecular Weight Determination of Bacteriocins

Whole cell protein extract was mixed with an equal volume of concentrated sample loading buffer and was heated at 80 ºC for 10 min. The processed samples were then loaded onto 7.5% SDS-polyacrylamide gel wells as previously described by Laemmli[19]. Electrophoresis was carried out at 30mA until the tracker dye (Bromophenol blue) reached the bottom of the gel. Gels were stained for protein with Coomassie Brilliant Blue (CBB) destaining with a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) methanol. The molecular weights were estimated using SDS-PAGE protein molecular weight pre-stained markers (Promega, USA).

3. Results

3.1 Antibacterial Activity of Whole cell

Antimicrobial activities of whole cell of *Lactobacillus* spp. were screened for 8 test organisms and the result recorded in table 1.

Table 1: Antimicrobial activity of *Lactobacillus* spp.isolates against test organisms by modified agar overlay method

Test organism	Isolate 1		Isolate 2		Isolate 3		Isolate 4	
	Zone diameter (mm)	Mode of Action	Zone diameter (mm)	Mode of Action	Zone of diameter (mm)	Mode of Action	Zone of diameter (mm)	Mode of Action
Salmonella typhi	32	Bs	37	Bc	40	Bc	20	Bc
Staphylococcus aureus	20	Bs	23	Bs	35	Bs	15	Bc
Salmonella paratyphi	35	Bs	37	Bs	40	Bs	18	Bc
Escherichia coli	34	Bs	34	Bs	38	Bs	21	Bs
Pseudomonas aeruginosa	27	Bc	35	Bs	35	Bs	27	Bs
Bacillus subtilis	42	Bs	43	Bs	46	Bs	35	Bc
Vibrio parahaemolyticus	34	Bc	28	Bc	47	Bs	30	Bs
Bacillus cereus	41	Bc	37	Bc	42	Bs	30	Bs

(Bc= Bactericidal; Bs= Bacteriostatic)

3.2 Antibacterial Activity of Cell Culture Supernatant

Crude bacteriocins (100µl) were placed in 6mm in diameter well on MHA to determine the antimicrobial spectrum of bacteriocin. After 24 hrs of incubation results were observed and recorded in table 2.

Table2:Antibacterial activity by Cell Culture supernatant

Test organism	Zone Diameter (mm)			
	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Bacillus cereus	13	12.5	13	11.5
Escherichia coli	10	10	11	10
Vibrio parahaemolyticus	10.5	10.5	11	9
Bacillus subtilis	13	13	14	12
Pseudomonas aeruginosa	9	10	11	9
Staphylococcus aureus	10	10	11	9
Salmonella typhi	9	9	10	8

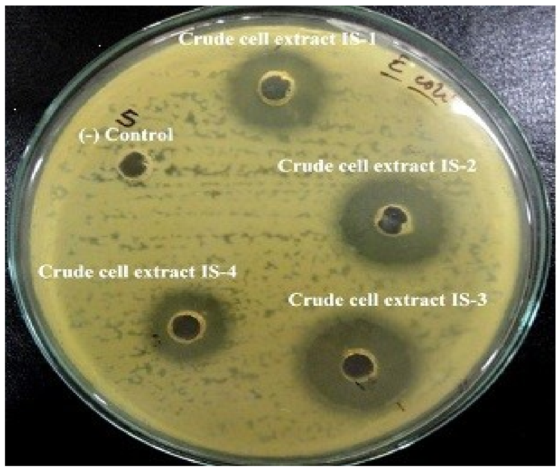
3.3 Antibacterial Activity of Cell Lysate

Cell extract were prepared from 48 hrs old *Lactobacillus* cell grown in MRS broth were subjected to antimicrobial activity against a gram positive and a gram negative organism. 40 µl of cell extract were placed in 6mm in diameter well on MHA. The results were recorded in the table 3 and figure 1.

Table 3: Antibacterial activity of Cell Lysate

Test organism	Zone diameter for Isolate 1	Zone diameter for Isolate 2	Zone diameter for Isolate 3	Zone diameter for Isolate 4	Negative control
Bacillus cereus	13	15	15	14	10
Escherichia coli	14.5	17	18	13	12

Fig 1: Antibacterial activity of Cell Lysate against *E. coli*



3.4 Molecular Weight Determination of Bacteriocins

After SDS-PAGE electrophoresis followed by staining and destaining, several distinct bands of proteins were visible which are almost similar for all isolates. These proteins are considered as bacteriocins. Molecular weight of the bacteriocins were determined comparing them to molecular weight marker obtained from Promega, USA. The SDS-PAGE of bacteriocins are shown in figure 2 and table 4.

Fig 2: Molecular masses obtained from SDS-PAGE for whole cell protein extraction

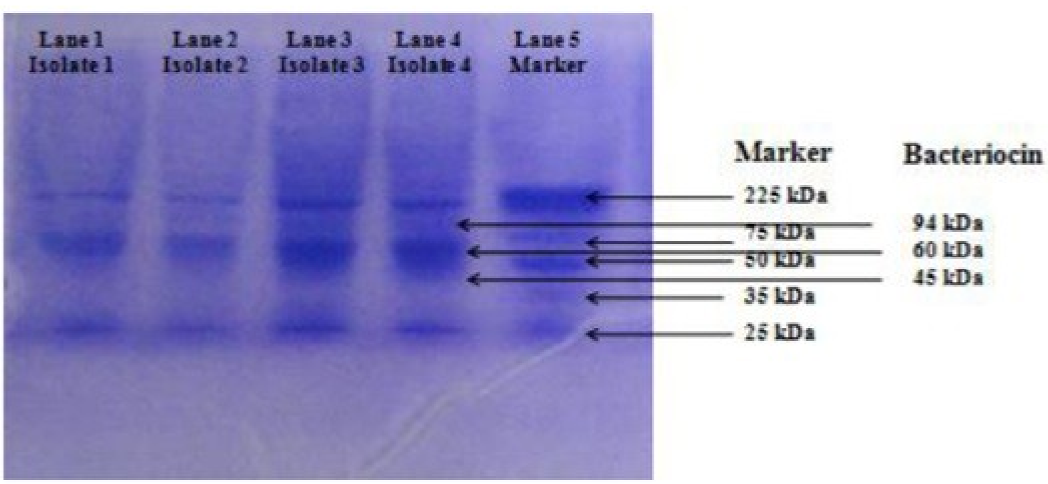


Table 4: Molecular masses obtained from SDS-PAGE for whole cell protein extraction

Molecular weight obtained (kDa)	Isolates
150	1, 2, 3, 4
94	1, 2, 3, 4
60	1, 2, 3, 4
45	3, 4
25	1, 2, 3, 4

4. Discussion

The aim of this study was to isolate and identify antimicrobial compounds (bacteriocin) from four *Lactobacillus* spp. isolated from yogurt and to determine the antimicrobial activity pattern of the bacteriocins.

To determine antimicrobial activity modified overlay method was used in this study and antimicrobial activity was determined against eight pathogenic organisms; *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Vibrio parahaemolyticus*, *Salmonella typhi*, *Salmonella paratyphi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. The highest zones of inhibition were observed against *Bacillus subtilis* in case of all four isolate and lowest zones were found against *Staphylococcus aureus*. The diameter of zone of inhibition against test organisms was always larger by Isolate 3 in comparison to the other 3 isolates. Isolate 2 produced smaller zone of inhibition than isolate 3 against all test pathogens. Isolate 4 produced lowest zone of inhibition against all the pathogens.

The mode of action (bacteriostatic or bactericidal) of *Lactobacillus* isolates on test organisms was also determined by inoculating from zone of inhibition on Nutrient agar. Although isolate 3 produce large zone of inhibition but it was bacteriostatic for most of the pathogen, but in case of isolate 4 it produced lowest zone of inhibition but most of them are bactericidal. From this result it was clear that all of them produce substance which was inhibitory for a wide range of bacteria, and the zone size varies with organism to organism. All of them are effective against test organisms.

Many researchers have attempted and isolated bacteriocins from *Lactobacillus* spp. Rajarum *et al*.[20] used crude and purified bacteriocin to determine their antimicrobial spectrum. Ashokkumar *et al.*[21] purified bacteriocins from *Lactobacillus paracasei* isolated from donkey milk and the crude bacteriocin was subjected to antimicrobial activity against clinical pathogens. Maximum inhibitory activity was observed against *Salmonella typhi* followed by *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.* On the other hand *Staphylococcus aureus* showed the least susceptibility to the crude bacteriocin. Several studies indicated that bacteriocin can prevent the growth of undesirable bacteria in a food-grade, which is convenient for health. These substances in appropriate concentrations may be used in veterinary medicine and as animal growth promoter instead usual antibiotics, as well as an additional factor for increasing the shelf life of minimal processed foods[22].

Well diffusion method was used for bacteriocin assay from crude bacteriocin and crude cell extract. Crude bacteriocins were prepared from 48h old broth to detect extracellular activity of 4 isolates. 100µl crude bacteriocin was placed in each well. After incubation at 37°C for 24 h the zone was observed. Zone of inhibition was produced surrounding the well. These zones of inhibition confirm the presence of bacteriocin in the extract hence confirms that the isolates are capable to produce bacteriocin. In case of crude bacteriocin and crude cell extract, zone of inhibition was also similar to the zones observed with whole cells. Crude extracts from all the isolates produce zone of inhibition though smaller possibly due to low concentration of bacteriocin in the extracts. Of all the crude extracts, extracts from Isolate 3 produce larger zones of inhibition in well diffusion method.

Cell extract was prepared by treating the pellet with EDTA, Tris-HCl, SDS, NaCl. As a result cells were lysed and intracellular bacteriocins were released in the solution. The solution (40µl) was placed in 6mm diameter well on MHA which was previously inoculated with test organism. A negative control with all other chemicals except cell lysate was used. After incubation at 37°C for 24h the plates were observed. Clear zone was produced surrounding the well as well as the control, but the zone diameter was lowest for the negative control. These results further confirms production of bacteriocins by the isolates. Zone diameter produced against *Escherichia coli* were 14.5, 17, 18, 13 mm for cell lysate of isolate 1, 2, 3 and 4 respectively and 12 mm for negative control.

There was a consistency with all the zone of inhibition found using different extracts and lysates. Zone size was always larger for isolate 3 and smaller for isolate 4.

Bacteriocin was extracted from cells of *Lactobacillus*spp by chemical lysis using 0.5ml 2% SDS, 0.5ml 0.01M Tris-HCl, 0.5ml 0.01 EDTA, 0.5ml 0.01M NaCl. To determine molecular weights, extracted bacteriocins were separated through 7.5% SDS-PAGE, stained and destained to visualize the bands of proteins.

The banding pattern was same for all isolates which also confirms the isolates to belong to the same genus. A broad range protein molecular weight marker from Promega was used as molecular weight marker which produced 225, 150, 100, 75, 50, 45, 35, 25, 15 & 10 kDa molecular weights band. All the isolates had three distinct but similar bands. In this study, all isolates had a protein band near 150 kDa, a band near about 94 kDa and a band of 60 kDa. These three proteins can be considered as bacteriocins produced by the isolated lactobacillus spp. as they cohere with the results of the previous researches. Ivanova *et al*.[23], Karthikeyan and Santosh[24], Ogunshe *et al*.[25] and Rajaram *et al*.[20] also reported a 94 kDa bacteriocin from *Lactobacillus lactis*. Asseldonk *et al.*[26] have reported bacteriocin of 60 KDa in *Lactobacillus lactis* which was extracellular. In case of Isolate 3 and isolate 4, a band around 45 kDa was found which also corresponds with the study of Asseldonk *et al.*[26] where they also found bacteriocin of similar size which also had antimicrobial activity against pathogenic organism.

5. Conclusion

The four Lactobacillus sp. included in this study showed capability to produce bacteriocins of different molecular weight those have antimicrobial potential. These strains and bacteriocins can be used as food bio-preservatives in future. In-depth research including molecular biology approaches are needed to convert these strains industrially applicable.

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