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Antibacterial Activity of Lactic Acid Bacteria and Extraction of Bacteriocin Protein

Rabiul Islam[1], Md. Nur Hossain[2], Md. Khasrul Alam[3], Md. Ekhlas Uddin[4], Mehadi Hasan Rony[3], Md. Abu Sayeed Imran[3], Md. Firoz Alam[3]

[1]Divisional DNA Screening Laboratory, Faridpur Medical College Hospital, Ministry of Women & Children Affairs, Faridpur, Bangladesh
[2]Industrial Microbiology Laboratory, Institute of Food Science & Technology, Bangladesh Council of Scientific & Industrial research, Dhaka, Bangladesh
[3]Department of Biotechnology & Genetic Engineering, Islamic University, Kushtia, Bangladesh
[4]Department of Biochemistry and Molecular Biology, Gono Bishwabidyalay, Dhaka, Bangladesh
Email: rabiulbtge@gmail.com

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Abstract

Biopreservation systems in foods are of increasing interest for industry and consumers. Bacteriocin producing *Lactobacillus spp.* is considered Generally Recognize as Safe (GRAS), useful to control the fast development of pathogens and spoiling microbes in food and feed. *Lactobacillus spp.* was isolated from traditional winter fermented vegetable cucumber & carrot by the use of selective media. Especially De Man, Rogosa and Sharpe (MRS) Agar media were used to isolate the *Lactobacillus* species. Morphologically identified by gram staining & colony morphology. Biochemically recognized by catalase, oxidase, MRVP & carbohydrate fermentation test. Antimicrobial activity of *Lactobacillus spp.* was confirmed by Well Diffusion Method. Molecular characterization of bacteriocin protein and molecular weight determined by SDS PAGE method. The isolate was found to be facultative anaerobic, Gram positive, and catalase negative. The result of antimicrobial activity measured by the Arbitrary Unit (AU/ml) of zone of the inhibition. Six isolates found from the sample but most activities exhibited isolate 4 against *Bacillus megaterium* (55 mm) zone of diameter. The molecular weight of the washed bacteriocin was calculated to be about 40 kDa (Isolate 1) and 15 kDa & 30 kDa (Isolate 5). Bacteriocin protein reduces chemical preservatives and uses in future as biopreservative in food industry.

Keywords

Lactobacillus spp., Bacteriocin, Antimicrobial Activity, SDS PAGE, Biopreservative

1. Introduction

Control of both pathogenic and spoilage microbe in a variety of foods is important to guarantee food quality and safety. Recently, biopreservation has arrived a topic of interest [1]. This technique is used as an alternative to chemical additives for rising self-life storage and augment safety of food by using native microflora and their antimicrobial products [2]. Lactic acid bacteria are trusted to be safe because they have been long effective as the normal flora in fermented food; thus, they have great potential for use in biopreservation. The conserving effects of lactic acid bacteria are due to the production of antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocin or related substances [3] [4].

Modern food processing is currently facing a challenge in that it aims to extend shelf-life and safety of foods and beverages by chemical means, while on the other hand consumers prefer foods that are minimally processed and free from chemical preservatives. This has aroused great interest in so-called "green technologies" including new strategies to minimally process and exploit microbial metabolites for biopreservation [5] [6] [7]. Lactic acid bacteria (LAB) are generally recognized as safe and play a significant role in food and feed fermentation and preservation either as the native microflora or as starter cultures added under controlled conditions. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid) which result in lowered pH [8].

Lactic acid bacteria (LAB) have been extensively studied for their commercial potential [9], food preservation and health benefits. They are industrially significant microorganisms used worldwide primely in the dairy industry for manufacturing fermented milk products and cheese. Industrial importance of LAB is based on their capability to ferment sugars readily into different metabolites and provide an efficient method for preserving fermented food products. These bacteria are gram positive, non-spore forming and naturally present in media affluent in organic products such as food products [10].

2. Materials & Methods

2.1. Collection of Samples

Winter vegetables (carrot, and cucumber) were collected from the retail market locations in Azimpur bazar in Dhaka city of Bangladesh. About 250 g each of carrot, and cucumber was obtained. The samples were wrapped separately in sterile polyethylene bags, and transported off the Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka-1205, Bangladesh for further analysis.

2.2. Preparation of Sample

Sample kept at room temperature before analysis. There are many ways to carry out natural fermentation process: Ten grams each, of fresh vegetables samples

were soaked in 90 ml of normal saline solution (8.5 g NaCl/L), homogenized for 20 min, appropriately diluted in normal saline. From each sample, serial dilutions were made by following the method of Harrigan (1998) [11].

2.3. Purification of Culture Isolates

The typical colonies on MRS agar were sub-cultured on the same medium again and again until a pure growth for each culture isolate was obtained. Four or five times subcultured in this procedure on MRS Agar media.

2.4. Identification of Bacteria from Pure Culture

2.4.1. Identification of *Lactobacillus spp*

The bacteriocin producers from naturally fermented carrot, and cucumber were isolated by streak plate method technique as per the traditional method [12] using MRS agar. After incubation for 48 hour at 32˚C, typical colonies were isolated and exonerated. The isolates were differentiated on the base of their morphological, cultural and physiological feature such as oxidase test, utilization of citrate as a sole carbon source and catalase test [13], and accordingly were tentatively recognize up to the genus level [14].

2.4.2. Morphological Examination

The colony characteristics on solid medium and cellular morphology of culture isolate after Gram's staining were examined at each step of incubation according to the methods of Collins *et al.* [15] for identification. The Gram's staining after slide preparation and smear fixation was performed by the following procedure:

2.4.3. Biochemical and Enzymatic Tests

The following biochemical tests were carried out according to the methods described by Harrigan (1998).

1) Procedure of Catalase test

At first fresh glass slide were taken. Then one drop of 3% hydrogen peroxide taken on the glass slide. After the use of loop pick up test organism and touch on the 3% hydrogen peroxide solution. Then waited 2 minutes for observation of bubble. *Salmonella* used positive control in this procedure.

2) Procedure of Oxidase Test

At first taken Whatman filter paper on the experimental table. Then it places one drop oxidase reagent (P-aminomethyl aniline oxalate). After the place of isolated colony by the use of loop. Waited 10 - 30 second to observe the color and *Pseudomonas* used positive control in this procedure.

3) Procedure of Methyl red test

This test was conducted to detect the organisms, which produced acid during the fermentation of glucose. The ingredients were dissolved in distilled water. The pH was adjusted to 7.5 and the medium was distributed in 5 mL amounts in test tubes. Then these test tubes were sterilized at 115˚C for 20 minutes. The medium was inoculated with the specific/test organisms and incubated at 37˚C for 48 - 72 hours using 5 drops of methyl red indicator (0.1g of methyl red in 300

mL of 95% ethanol, made up to 500 mL in distilled water). *E. coli* used positive control and *Pseudomonas* used as a negative control. The results were recorded for acid production with change in color.

4) Procedure of VP (Voges Proskauer) Test

Isolated test organism incubated 37˚C at 48 h. 40% KOH (Baritts reagent B) and 5% sodium of alpha naphthol (Baritt's reagent A) were added. After the tube was shaken vigorously and allowed to stand for 20 minutes. *E. coli* used negative control and *Bacillus cereus* used as a positive control.

5) Carbohydrate fermentation test

Phenol red broth base medium was used as a medium for this experiment. Various sugar substrates namely, sucrose, maltose, lactose, sorbitol, and glucose were used. 0.1 gram (0.1% w/v) of each sugar substrate was added to 100 ml of the medium. 5 ml of each mixture was transferred into each tube. For gas identify, Durham tube was added into the test tube containing glucose. All the tubes were sterilized for 15 minutes at 121˚C. The tubes were inoculated with a single colony of the bacteria under study. The positive reaction of the bacteria was reported by the changes in the colour of the medium [16].

2.5. Maintenance of Pure Culture

The pure cultures were maintained in Industrial Microbiology Laboratory of Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhanmondi, Dhaka, at refrigeration temperature with a continuous sub culturing techniques on MRS agar after every 10 days in order to avoid any contamination and other problems.

2.6. Screening of Isolates for Antimicrobial Activity

Well Diffusion Method

Antimicrobial activity of the bacterial isolates against all the pathogenic microbes was ascertained by well diffusion method [17] under aerobic conditions. Agar plates were inoculated with 100 mL of each target microbes after growing them in a broth and diluting favourably. Wells (3 mm) were excise into the plates and 100 mL of cell-free culture supernatant fluid of the different strain was placed into every well. The inhibitory activity against *Escherichia coli*, *E. coli* 12079, *E. coli fecalis*, Acetobacter, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella typhimoreum*, *Staphylococcus spp*., *Staphylococcus aureus* was grown nutrient agar. Plates were kept at a cool temperature for 2 h and then incubated at 37˚C for 24 hours. The antimicrobial activity was identified by measuring the diameter of the inhibition zone around the wells.

2.7. Purification of Bacteriocin from *Lactobacillus spp*

The cells were harvested and the bacteriocin isolated from the cell free supernatant fluid as described by Green *et al.* (1997) [18]. Purification of bacteriocin from *Lactobacillus spp.* was given in the following.

50 ml of de Man, Rogosa and Sharpe (MRS) broth and 2 ml of *Lactobacillus* culture incubated at 37˚C for 14 hours in anaerobic condition. Then 100 ml MRS broth and 1% of *Lactobacillus* culture incubated at 37˚C for 24 hours in anaerobic condition. Centrifuged at 6000 rpm for 15 minutes at 4˚C. Cell free supernatant was concentrated to 100 ml in a rotary vacuum evaporator. Ammonium sulfate was added at 4˚C with stirring to 70% saturation. Centrifuged at 1000 rpm for 20 min at 4˚C. Precipitate was collected and resuspended in 3 ml distilled water at a centrifuge tube.

2.8. Molecular Characterization of Bacteriocin by SDS PAGE Technique

For the determination of protein profile of the lactic acid bacteria, strains previously identified by their phenotypic characteristics were submitted to SDS-PAGE as described by Laemmli [19].

Preparation of Reagent for SDS PAGE Method

1) Prepare in the following reagent

30% acrylamide-bisacrylamide solution, 10% ammonium per sulfate (APS) [Aliquots of 200 µl should be stored at −20˚C for single time use. APS decomposes slowly, therefore fresh solution should be prepared weekly.], 0.1% BMB (Bromophenol blue solution) or tracking dye, Destaining solution, Staining solution (Coomassie blue gel stain), Sample loading buffer, Electrophoresis buffer (pH 8.3), Stacking/Upper gel buffer (pH 6.8), Separating/Lower gel buffer (pH 8.8).

2) Preparation of separating gel (10%)

At first, clean and fresh glass plates were assembled in the gel casting chamber. A 10% separating gel was constructed by gently mixing the distilled water, lower gel buffer along with 10% SDS, 30% acrylamide-bisacrylamide solution, 10% SDS, 10% freshly prepared Ammonium per sulphate (APS) and TEMED according to given below. As soon as APS and TEMED were added to the mixture the freshly mixed solution was carefully poured immediately into the glass plate chamber with a sterile pasteur pipette without wasting a few seconds.

3) Preparation of stacking gel (5%)

After the polymerization of the separating gel has been completed, a 5% stacking gel mixture was prepared according to given below. Just like the separating gel, stacking gel mixture was also prepared by primarily mixing the distilled water, upper gel buffer along with 10% SDS and 30% acrylamide-bisacrylamide solution, 10% APS and TEMED. The gel mixture was then rapidly poured above the previously constructed separating gel.

4) Sample preparation

The protein sample (10 µL) was mixed with sample loading buffer at a ratio of 1:1 and was then boiled in a water bath for 3 minutes. During boiling precaution was taken so that the sample does not bump up. 5 µL tracking dye (0.1% Bromophenol blue) was then added to the boiled mixture.

5) Sample loading

After the polymerization of stacking gel has been completed, the comb was removed from the glass chamber with a soft hand so that the well divider did not crack. The glass chamber was then fixed in the electrophoresis unit and was placed gently in the buffer reservoir. The wells were filled with the running buffer by pouring running buffer inside two glass chambers of the electrophoresis unit up to the top edge of the glasses. Running buffer was also poured into the main buffer reservoir up to 1/3rd of the height of the reservoir. 20 µL of sample was then added to the wells from the right side of the gel. 10 µL of molecular weight standard (BIO-RAD) was used as a marker.

6) Running the gel

After the loading process was done carefully, the electrophoresis unit was connected with the power pack adjusting at 20 mA current keeping the voltage supply free. The power supply was kept on for 1 - 1.5 h. As soon as the tracking dye reached the bottom level of the gel, the power supply was turned off.

7) Staining and destaining of the gel

After the run had been completed, the gel was released from the glass plates and immersed in a fresh staining solution (0.1% Coomassie Brilliant blue R 250). The gel was then shaken on a shaker (Memmert, USA) for approximately 2 h. Then the gel was taken out of the staining solution and flooded with destaining solution (10% Acetic acid) and placed on a rotary shaker for more 2 h. When the gel background became transparent, it was taken out of the destaining solution and immersed in distilled water.

3. Results

3.1. Isolation & Identification of Bacteria

Bacteria isolated from fermented vegetable cucumber & carrot was identified as *Lactobacillus* spp. by observing their colony morphology, physiological and as well as some biochemical characteristics. All isolates showed white and creamy colour on the MRS Agar plate (Figure 1). Colony morphology was observed creamy, little sticks and smooth round colonies.

Figure 1. *Lactobacillus spp.* (Isolate-3) on MRS agar media.

3.1.1. Gram Staining

Microscopically all isolates showed Gram-positive, rod shaped. Their morphological characteristics have shown (Table 1).

3.1.2. Biochemical Test

Result of Biochemical test (include: Catalase, Oxidase, Methyl Red & Voges-Proskauer) are given below (Table 2). Carbohydrate fermentation test result is given below (Table 3). Figure 2 showed carbohydrate test of lactose.

3.1.3. Antimicrobial Activity of Lactic Acid Bacteria Isolates against Target Bacteria by Dual Agar Overlay Method

Antimicrobial activity of *Lactobacillus* spp. (isolates 1 - 6) against test organisms' result showed (Table 4). Figure 3 showed antibacterial activity of isolate-1 against *Bacillus subtilis*.

3.1.4. Molecular Weight Determination of Bacteriocins

Then SDS-PAGE electrophoresis followed by staining & destaining, isolate 1 & 5 showed protein band was visible by naked eye. These proteins are considered as bacteriocins. Molecular weights of the bacteriocins were determined to compare them to molecular weight marker obtained from Promega, USA. The SDS-PAGE of bacteriocins is shown (Figure 4). Bacteriocin protein band was seen when stained with Coomassie blue and it cleanly indicated the purity of protein. The molecular weight of purified bacteriocin was calculated to be about isolate 1 (40 kDa) and isolate 5 (15 k Da, 30 kDa).

Table 1. Morphological characterization of bacterial isolates.

Bacterial isolates	Source	Shape	Arrangement	Gram Staining
Isolate - 1	Cucumber	Long chain	Cluster	Gram positive
Isolate - 2	Cucumber	Small rod	Single & Cluster	Gram positive
Isolate - 3	Cucumber	Small rod	Cluster	Gram positive
Isolate - 4	Carrot	Long rod	Single to cluster	Gram positive
Isolate - 5	Carrot	Medium chain	Cluster	Gram positive
Isolate - 6	Carrot	Medium rod	Single	Gram positive

Table 2. Result of Biochemical test.

Name of Isolate	Result of the Test/Appearance			
	Catalase	Oxidase	Methyl Red	Voges-Proskauer
(Positive Control)	Produce bubble	Purple Color	Red color	Red color
Isolate-1	Absence bubble	Colorless	Red color	Red color
Isolate-2	Absence bubble	Colorless	Red color	Red color
Isolate-3	Absence bubble	Colorless	Red color	Red color
Isolate-4	Absence bubble	Colorless	Red color	Red color
Isolate-5	Absence bubble	Colorless	Red color	Red color
Isolate-6	Absence bubble	Colorless	Red color	Red color
(Negative control)	Absence bubble	Colorless	Yellow color	Yellow color

Table 3. Carbohydrate fermentation test.

Name of Isolate	Name of the Carbohydrate fermentation test:			
	Glucose	Sucrose	Lactose	Sorbitol
Isolate-1	Gas & acid Production	No gas but acid Production	Gas but slightly acid Production	No gas but acid Production
Isolate-2	Gas & acid Production	Gas & acid production	Gas & acid production	Gas & acid production
Isolate-3	No gas & no acid production	Gas & acid production	Gas & acid production	Gas & acid production
Isolate-4	No gas but acid production	No gas but acid Production	Gas & slightly acid production	No gas & no acid production
Isolate-5	No gas but acid production	Gas & acid production	Gas & acid production	Gas & acid production
Isolate-6	Gas & acid Production	Gas & acid production	Gas & acid production	Gas & acid production
Control (Absent Isolate)	No gas & no acid production	No gas & no acid production	No gas & no acid production	No gas & no acid production

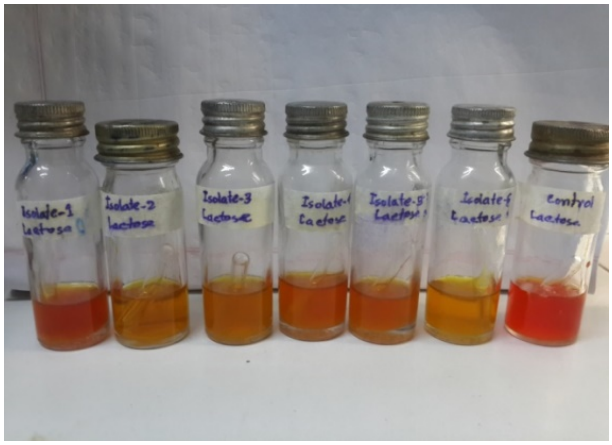

Figure 2. Carbohydrate test of Lactose.

Figure 3. Antibacterial Activity of *Lactobacillus spp.* (Isolate-1) against Bacillus subtilis.

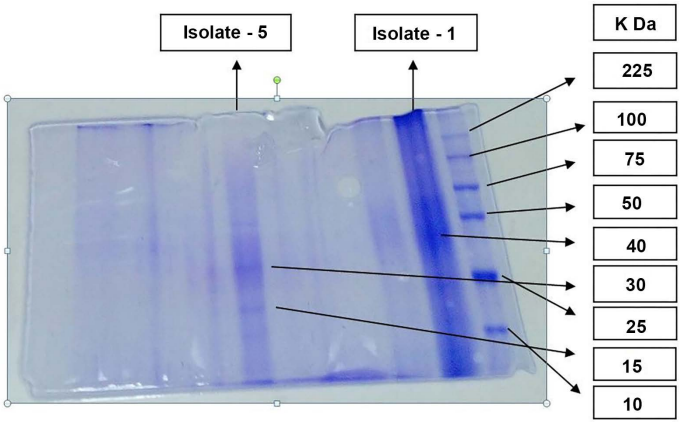

Figure 4. Molecular masses of Bacteriocin protein (40 kDa for Isolate-1, 15 kDa & 30 kDa for Isolate-5) obtained from SDS-PAGE method.

Table 4. Antimicrobial activity of *Lactobacillus* spp. (isolates 1 - 6) against test organisms on nutrient agar media at 37˚C.

Name of the Target Organism	Media/Temperature	Zone of diameter (mm)					
		Isolate-1	Isolate-2	Isolate-3	Isolate-4	Isolate-5	Isolate-6
Bacillus cereus	Nutrient Agar/37˚C	35 mm	20 mm	47 mm	40 mm	35 mm	35 mm
Bacillus subtilis	Nutrient Agar/37˚C	20 mm	25 mm	50 mm	40 mm	40 mm	40 mm
Bacillus megaterium	Nutrient Agar/37˚C	22 mm	25 mm	50 mm	55 mm	30 mm	30 mm
Escherechia coli fecalis	Nutrient Agar/37˚C	26 mm	24 mm	42 mm	34 mm	25 mm	40 mm
Escherechia coli 12079	Nutrient Agar/ 37˚C	45 mm	40 mm	43 mm	50 mm	35 mm	35 mm
Staphylococcus	Nutrient Agar/37˚C	40 mm	30 mm	45 mm	45 mm	37 mm	34 mm
Staphylococcus aureus	Nutrient Agar/37˚C	35 mm	30 mm	42 mm	47 mm	27 mm	25 mm
Salmonella typhimoreum	Nutrient Agar/37˚C	30 mm	25 mm	40 mm	42 mm	24 mm	25 mm
Salmonella typhi	Nutrient Agar/37˚C	30 mm	45 mm	40 mm	45 mm	35 mm	30 mm
Pseudomonia aeroginosa	Nutrient Agar/37˚C	35 mm	30 mm	55 mm	47 mm	40 mm	40 mm

4. Conclusions

The lactic acid fermentation with the bacteria has long been known and practical by the humans for making different food stuffs. Lactic acid bacteria (LAB) produce different compounds such as organic acids, diacetyl, hydrogen peroxide & bacteriocin or bacteriocidal proteins during lactic fermentations, in this research to isolate lactic acid bacteria that have antibacterial activity of some pathogenic bacteria that are in future use as probiotic for the food industry, finally from two isolates (Isolate-1 and Isolate-5) purify and extraction of bacteriocin protein that is in future used as biopreservative in the food industry.

During purification several different protocols were applied. Optimal recovery was achieved by including ammonium sulphate precipitation. The used protocol resulted in an increase in the specific activity and 26.32% recovery. Purified bacteriocin from *Lactobacillus spp.* revealed homogeneity of a protein band on 10% SDS PAGE. Its molecular weight was estimated at 40 kDa (Isolate-1) and 15 kDa,

30 kDa (Isolate-5) by SDS-PAGE.

The study revealed that bacteriocin from *Lactobacillus* sp. isolated from natural lactic acid fermentation of vegetables (cucumber & carrot). *Lactobacillus spp.* isolate possesses a wide spectrum of inhibitory activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*, *Bacillus subtilis*, *Acetobacter*, *E. coli fecalis*, *Salmonella typhi.* Therefore, it has a potential for application as a biopreservative in different food products as such or in combination with other preservation methods. Additionally, this study suggests the possible role of *Lactobacillus spp.* in enhancing the antibacterial activity of carrot & cucumber.

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Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the present research work.

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