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Original Article

IN VITRO STUDY ON RELEASE OF BIOACTIVE ANTIMICROBIAL COMPOUNDS FROM DAIRY PRODUCTS BY CERTAIN PROMISING PROBIOTIC *LACTOBACILLUS* STRAINS

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ABSTRACT

Objective: The antimicrobial activity and Probiotic properties of *Lactobacillus* species were evaluated. The antimicrobial compound of potent antimicrobial probiotic *Lactobacillus* was purified by column chromatography and its nature and stability were determined.

Methods: This investigation was performed with few *Lactobacillus* strains of ATCC and MTCC along with certain strains isolated from different dairy sources. They were evaluated for their probiotics properties (acid tolerance, bile tolerance, bile salt hydrolase activity, cell autoaggregation, cell surface hydrophobicity and haemolytic behaviour). Agar well diffusion method was used to screen their potency to release bioactive compound against several pathogens. This potent antimicrobial compound was purified by chromatography as well as its molecular mass was estimated by following SDS-PAGE. Finally, the stability of the compound was determined against various ranges of temperature and pH.

Results: Among all *Lactobacillus* strains, R1 was found to be a potent probiotic strain as well as cell free supernatant (CFS) of R1 showed more strong antagonistic effect against most of the pathogens. Carbohydrate fermentation and physiological characterization of strain R1 matched with *Lactobacillus fermentum* as per Bergey's manual of systematic bacteriology. The molecular mass of the purified fraction was estimated at approximately 25 kDa and could be stable after heat treatment of 100 °C for 30 min and pH range of 4.5-7.0

Conclusion: R1 showed highest antimicrobial activity while it has been found as *Lactobacillus fermentum.* It is due to the release of a bioactive compound having a molecular mass of 25 kDa.

Keywords: *Lactobacillus* species, Probiotic properties, Antimicrobial compound, Purification

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INTRODUCTION

Probiotic can be defined as living microorganisms that bring nutraceutical benefits to the consumer's health apart from their whole nutritional value after being consumed in a certain number. They have been found to be clinically effective in maintaining the balance of gastrointestinal microbiota to improve health conditions [1]. As per the guideline established by the Joint FAO/WHO (2002) expert panel, the basic parameters for the screening of potentially valuable probiotic strains mainly include functional, safety as well as physiological criteria [2]. A list of characteristics such as tolerance to low pH, bile salt, should have the ability of adherence to human intestinal lining and colonization on mucosal surfaces are explained as functional criteria whereas the ability to inhibit wide range of pathogens by producing antimicrobial substances; immunomodulation; antioxidant; cholesterol metabolism are documented as physiological criteria for screening a potential probiotic strain [3].

Lactobacillus species are also widely used as potential probiotic strains and have a long history of safe use. They are regarded as non-pathogenic and safe microorganisms. Lactic acid bacteria (LAB) also produce antimicrobial substances, such as organic acids, fatty free acids, ammonia, hydrogen peroxide, bio surfactant and low-molecular-weight antibacterial peptides known to be bacteriocins that inhibit both gram-positive and gram-negative enteric pathogens [4, 5]. Bacteriocins generally exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death. The incorporation of bacteriocins as a bio-preservative ingredient into model food systems has been studied extensively and has been shown to be effective in the control of pathogenic microorganisms [6, 7]. It can be considered as an alternative approach to medical therapy. Here, in our study excellent probiotic *Lactobacillus fermentum* has been isolated which showed an antimicrobial effect against certain pathogenic strains. The antimicrobial characteristics of *Lactobacillus fermentum* R1 indicates its possible use in starter cultures for traditional fermented foods as a means of improving the hygiene and safety of the food products. The partially purified inhibiting substance is most active at low pH values and retains its activity with an increase in temperature. This suits various food and pharmaceutical production processes.

MATERIALS AND METHODS

Bacterial strains and culture condition

This study was performed with certain *Lactobacillus* strains (*L. planterum* MTCC 1407, *L. rhamnosus* MTCC 1408, *L. casei* MTCC 1423, *L. delbrucii lectis* MTCC 911, *L. casei* ATCC 9595) directly collected from both ATCC (USA) and MTCC (Chandigarh, India). Again, several dairy products (milk, yogurt, cheese) were also collected from rural regions of Odisha to isolate more *Lactobacillus* strains. One ml of sample was tenfold serially diluted in 0.89% of the normal saline solution and pour plated on MRS agar (Hi-media, India Pvt. Ltd.). For preliminary identification of *Lactobacillus* species, the pure cultures were again screened as per Bergey's Manual. It mainly includes Gram's reaction, spore formation, catalase.

Screening of *Lactobacillus* species for their probiotic properties

A. Acid and bile salt tolerance

Acid and bile tolerance of all *Lactobacillus* species were evaluated in MRS broth by adjusting its pH at different values (2 and 3) and supplementing bile salts at the concentrations of 0.3% and 0.6% (w/v) (HiMedia, India Pvt. Ltd.) in a separate experiment. Then 1 ml of inoculums were taken from all the samples at 0 h, 1 h and 2 h and serially diluted up to 10^{-7} and poured in MRS agar plates and incubated for 24-48 h at 37 °C. The colonies appeared on plates after incubation were considered as acid tolerant and bile tolerant and were counted to determine the percentage of acid and bile tolerance [8, 9]. Each experiment was carried out in triplicate.

***B.* Bile salt hydrolase activity of sample**

A direct plate assay method was employed for detection of bile salt hydrolase (Bsh) activity. Bsh activity was examined by streaking overnight grown a culture of strain on MRS agar containing 0.5% (w/v) bile (sodium cholate) (Hi-media, India Pvt. Ltd.) and 0.37g/l of $CaCl_2$. Then the plates were sealed with parafilm and incubated anaerobically at 37 °C for 3-4 d. Bsh activity was indicated by deposition of hydrolyzed product in and around the colonies [8].

***C.* Cell aggregation assay**

The freshly grown cultures were harvested by centrifugation at 8000 rpm for 10 min to collect their supernatants (step1). The pellet was washed and re-suspended in equal volume of PBS (phosphate buffer saline) to an absorbance of 0.5 at 600 nm (A_1). The suspensions were distributed in 4 microcentrifuge tubes as 2 ml in each and centrifuged at 8000 rpm for 10 min, and the pellets were resuspended in 2 ml of broth removed at step 1 and incubated at 37 °C for 2 h. 1 ml of the upper part was removed after incubation time, and absorbance was taken at 600 nm (A_0) [9]. The auto aggregation percentage was calculated as:

$$\text{Autoaggregation (\%)} = (1-A_1/A_0) \times 100$$

***D.* Cell surface hydrophobicity assay**

The bacterial cells grown in MRS broth at 37 °C for 18 h were centrifuged, and the cell pellets were washed and resuspended with equal volume of phosphate urea magnesium (PUM) buffer, and the absorbance was adjusted to OD 0.7 at 600 nm (A_1). *Lactobacilli* cell suspension (3.0 ml) and xylene (1.0 ml) were mixed by using vortex and incubated at 37 °C for 10 min for temperature equilibration. The mixture was mixed well again and kept at 37 °C for 1 h for phase separations. The aqueous phase was removed gently to measure its absorbance (A_0) at 600 nm [9]. The hydrophobicity percentage was calculated as:

$$\text{Hydrophobicity (\%)} = (1-A_1/A_0) \times 100$$

***E.* Haemolysis activity**

In haemolysis activity, all strains were spot-inoculated into sterile blood agar by adding 7% sheep blood into sterile blood agar base and incubated at 37 °C for 48 h [10]. The absence of clear zone surrounding the colonies indicates the good result for safety consumption of probiotic strains.

Evaluation of antimicrobial activity of bioactive compound

Antibacterial properties of both whole cell and their cell-free supernatant (CFSs) of probiotic *Lactobacillus* species have been evaluated by well diffusion method with slight modification. Overnight grown culture of all strains was inoculated in MRS broth and incubated for 18-20 h at 37 °C. The grown cultures were harvested by centrifugation at 10000 g for 10 min at 4 °C and 200 µl cell-free supernatants were used for the assay. Antagonistic profile of bacterial isolates was studied in order to observe the inhibitory activity against standard pathogens such as *E. coli* MTCC82, *Bacillus cerus* ATCC10702, *S. aureus* MTCC96, *S. typhyi* MTCC3216, *Aeromonas hydrophila* ATCC7966 and *Klebsiella pneumonia* MTCC 109. Indicator strains of 300 µl were mixed properly in 10 ml of Luria Broth (LB) soft agar and overlaid on LB-base plate and leaving for solidification. After solidification, the holes were bored on a plate with the help of pipette tips (200 µl, diameter 0.7 cm). Then the holes were sealed by adding 1-2 drops of soft agar to avoid leakage of the supernatant. Then 100 µl of prepared supernatant was added into each well and was kept in the fridge for 1-2 h for diffusion of the supernatant. The assay plates were incubated for 6-7 h at 37 °C. The zone of inhibition was measured with the help of zone measuring scale [8]. The same experiment was repeated three times.

Physiological and biochemical identification of potent antimicrobial strains

The most useful test for the determination of strain differences is carbohydrate fermentation. Eighteen different carbohydrates were used for identification. Again, gas production from glucose was assessed by inoculating the isolated strains in MRS broth containing glucose (1%) containing Durham tube in inverted condition and incubated at 37 °C for 48-72 h. The upward movement of inverted Durham tube indicates positive reaction (gas production). Arginine hydrolysis test was another step to follow the identification procedure. The isolates which gave the bright orange were found to produce ammonia from arginine. The yellow color indicated negative arginine hydrolysis. Another criterion for the identification the isolates was the ability of growth at different temperatures and NaCl concentrations [11].

Nature of antimicrobial compound

Lactobacillus species produce lactic acid, proteins and H_2O_2 as an antimicrobial compound which can prevent the overgrowth of entering invasive pathogens. 100 µl of supernatants buffered with NaOH (pH 7.0) to neutralize acid effect while in a separate experiment, 75 µl were mixed with catalase (1.0%) to negate the effect H_2O_2. Then such prepared samples were placed into the well for evaluating nature of the compound. In other hand, culture supernatants were again treated with proteinase K to determine its proteinacious nature [12]. All samples were assayed for antimicrobial activity against same pathogens.

Purification of antibacterial protein by column chromatography

For purification of the antibacterial peptide, the collected supernatant was precipitated overnight with ammonium sulfate (40%–95%). The precipitate was collected by centrifugation at 12,000 rpm for 20 min at 4 °C. The precipitate was dissolved in a minimum volume of 50 mmol PBS buffer and dialysed against the same buffer at 4 °C. 5 ml of the prepared peptide sample was loaded in a Sephadex (Sigma Aldrich, Germany) column (1.0 cm x 20 cm) equilibrated with 1X PBS (pH 7.0) and eluted with a linear gradient of NaCl (0-1 M) in the same buffer at a flow rate of 60 ml/h. All the fractions were collected and again subjected to antibacterial peptide assay [12, 13]. The protein concentration (mg/ml) of the fraction showing antibacterial activity was estimated by using bovine serum albumin as the standard Lowry method [14] where its molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Stability of antimicrobial compound

The stability of the purified antimicrobial compound was determined by exposing it to different ranges of temperature (60-100 °C) and pH (2-10). To test heat sensitivity, the sample was treated to boiling temperature (100 °C, 30 min) in a water bath and evaluated its antimicrobial activity. In a separate experiment, it was re-suspended in 1:1 ratio of different buffer solutions ranging from pH 2.0–8.0 to determine pH resistance capability of the purified antimicrobial compound [12].

Statistical analysis

The data recorded during the course of the investigation were subjected to significance testing using mean±standard deviation (SD) analysis. Statistical significance was set at $P<0.05$. Results were denoted as mean±standard deviation (SD) of triplicate experiments

RESULTS AND ANALYSIS

Identification of collected bacterial strains

The primary identification (as per Bergey's Manual) of *Lactobacillus* species from collected dairy samples was mainly based on characteristics such as a gram positive rod, negative catalase, positive oxidase and non-spore formation. Gram staining followed by microscopic observation indicated that out of all bacterial isolates only 30 strains were Gram-positive, very small rods (cocobacilli) occurred singly or in pairs, non-spore forming and also catalase negative. They were again selected for probiotic activity evaluation along with *Lactobacillus* species directly collected from ATCC and MTCC.

Probiotic evaluation

***A.* Acid and bile salt tolerance**

In general, acid and bile salt tolerance tests mainly indicate the survival potency of the strains in the gastrointestinal tract (GIT). In the present study, the viability of *L. rhamnosus* MTCC 1408, *L. casei*

MTCC 1423 along with more four isolated *Lactobacillus* species (named as R1, R4, R7 and R18) in MRS broth have been observed in different pH values (2 and 3) and bile salt concentration (0.3% and 0.6%) till 2 h. All the values were measured in the form of log cfu/ml (fig. 1). The marginal reduction in the viability of the isolates indicated the excellent survival potency of the strain against physiological harsh conditions prevalent in the stomach like high acid and bile salt percentages. ANOVA test revealed that a highly significant variation of log CFU/ml values was observed among all the isolates (P<0.05).

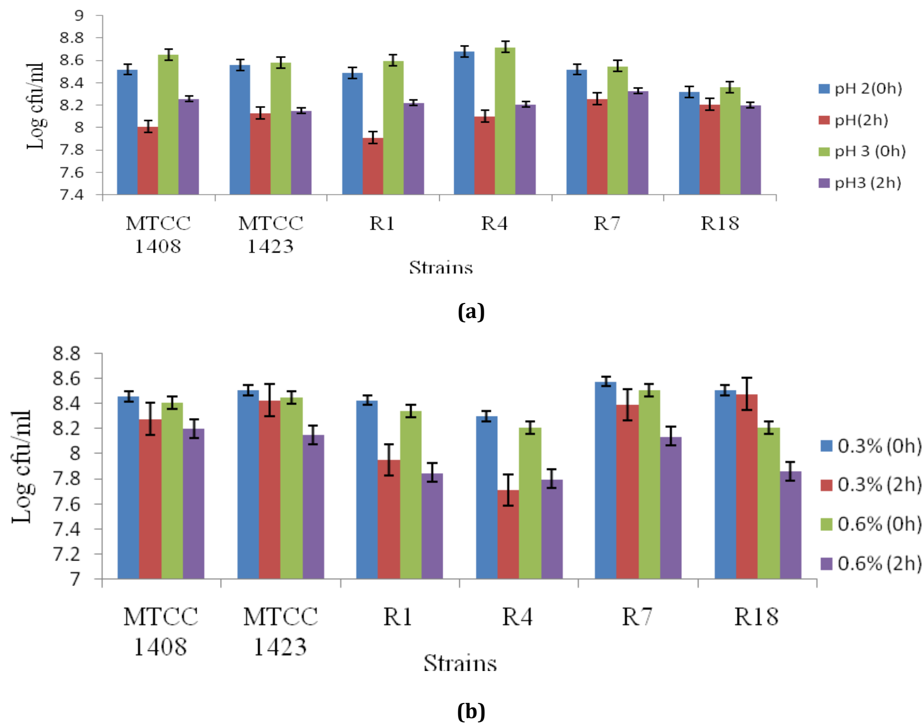

Fig. 1: (a) Viability (log cfu/ml) of all probiotic strains with respect to pH 2-3 till 2 h, (b) viability (log cfu/ml) of same probiotic strains with respect to various bile salt conc. (0.3, 0.6%). The results are the means of 3 independent experiments and the bars correspond to standard deviations (P<0.05)

B. Bile salt hydrolase activity analysis

Out all isolated and collected strains, the growth of only R1 and R7 along with a zone of salt precipitation around the colonies at the concentration of 5% could be observed on plates supplemented with sodium cholate. It suggested that R1 and R7 produced Bsh activity specific to bile salt (sodium cholate) hydrolysis.

C. Cellular autoaggregation and cell surface hydrophobicity

The auto-aggregation ability of the strains is one of the proposed mechanisms to explain the protective role of *Lactobacilli* where hydrophobicity may be playing a role in the cellular interaction. To evaluate the capability of strains to self-aggregate, the cellular autoaggregation were measured and under identical conditions only two strains, namely, *L. casei* MTCC 1423 (38.7%) and R1 (46.5%) showed maximum cell auto aggregation ability. Further study on cell surface hydrophobicity, adherence values of same two strains found to be highest out of all. Cell surface hydrophobicity of strains R1 and has been reported *L. casei* MTCC 1423 as high 75.25 % and 65.853% respectively.

D. Haemolysis activity for safety consumption

No strain tested in this work exhibited α-and β-hemolytic activity. The absence of clear zone around the strains indicates the safety consumption of probiotic.

Antagonistic profile of bacterial strains

After getting two potential probiotic strains (R1 and *L. casei* MTCC 1423), they were again screened for the release of antimicrobial bioactive compound that can inhibit the growth of large numbers of pathogenic bacteria like *E. coli* MTCC82, *Bacillus cereus* ATCC10702, *S. aureus* MTCC96, *S. typhyi* MTCC3216, *Aeromonas hydrophila* ATCC7966 and *Klebsiella pneumonia* MTCC 109. The culture supernatant prepared by using R1 showed highest antimicrobial activity against all pathogens except *Aeromonas hydrophila* ATCC7966. The diameters of inhibition zones observed in well diffusion method were greater than 10 mm (fig. 2). ANOVA test revealed that a highly significant variation of the zone of inhibition values was observed among all the isolates (P<0.05) against pathonens.

Biochemical identification of *Lactobacillus* R1

The potent probiotic *Lactobacillus* R1 gave positive test results with sugars, glucose, ribose, arabinose, trehalose, melibiose, raffinose, galactose, maltose, sucrose, fructose and lactose produced both gas from glucose and ammonia from arginine. It was resistant to 2% salt concentrations and grew at 45 °C. When these biochemical test results are compared with the literature information [11], it seems that R1 is like to be *Lactobacillus fermentum*.

Nature of antimicrobial compound

The antimicrobial activity of culture supernatant of R1 was completely destroyed by proteinase K treatment. However, it was maintained after neutralization of acid and H_2O_2 which indicated that antibacterial activity was due to the activity of bacteriocin protein.

Purification of antimicrobial milk bioactive peptide released by *Lactobacillus* R1

All seven fractions (at different concentration of NaCl) collected by performing column chromatography were checked for an antimicrobial activity where Elute 1 (by 0.01N NaCl) demonstrated activity against indicator strains. Peptide concentration of the active fraction of R1 was found as 1.5 micrograms/ml by Lowry method. The molecular mass of the purified peptide fraction estimated from

SDS-PAGE was approximately 25 kDa which show a beneficial antagonistic effect against all tested pathogens.

Effect of heat and pH treatment on stability

The purified antimicrobial protein was considered to be heat stable, as there was no reduction in activity after heat treatment from 60 to 100 °C for 15 and 30 min. Again, it was also found stable in the pH range of 4.5-7.0.

DISCUSSION

Several lactobacilli are known to have potential application as starters in food fermentation and have also been shown to exert a variety of positive health and nutritional effects [8, 9, 13]. The present investigation highlights the isolation, characterization and activity of the antimicrobial compound produced by *L. fermentum* R1 isolated from the dairy products to assess their anti-bacterial activity against some common pathogenic bacteria.

This study was performed with certain *Lactobacillus* strains directly collected from ATCC and MTCC and again, several dairy products (milk, yogurt, cheese) were collected from rural regions of Odisha for preliminary screening of more *Lactobacillus* strains. After gram staining the isolated bacteria were rod-shaped, smooth, shiny, irregular, circular, gram-positive, facultatively anaerobic, nonspore forming which indicate them to be the member of *Lactobacillus*.

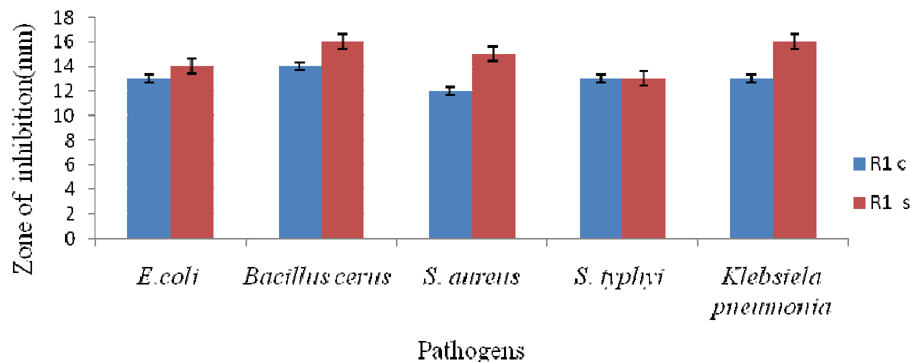

Fig. 2: Zones of inhibition shown by R1 culture (c) and its supernatant (s) against several pathogens. The results are the means of 3 independent experiments and the bars correspond to standard deviations (P<0.05)

The result matched with Bergey's manual of systematic bacteriology. A similar result was also observed by [13, 15 16] who isolated lactic acid bacteria (LAB) from dairy products. Probiotic microorganisms need to resist the adverse factors in the gastrointestinal tract, like the stomach acidity and bile salts excreted in the duodenum [17]. Here, probiotic attributes were evaluated based on two aspects, such as safety and functional. Again, functional criteria studies for successful probiotics mainly included survival, adherence, and colonisation while *in vitro* safety screenings of all isolated *Lactobaciilus* included haemolytic and gelatinase activities of the strains. Highly potent probiotic strains would have the ability to resist passage through the stomach in the presence of acid (pH ranges from 1.5 to 3) and most of the examined strains *L. rhamnosus* MTCC 1408, *L. casei* MTCC 1423 along with more four isolated *Lactobacillus* species (named as R1, R4, R7 and R18) were resistant to pH 3.0 even after 2 h of exposure. These results are consistent with reports of the ability of lactobacilli to retain their viability when exposed to pH values of 1.5-3.0 [8, 9].

An efficient probiotic strain should be able to grow in bile salt with concentration ranging from 0.15-0.30% (w/v) [18]. The isolated strains tolerated up to 0.6% which showed a good tolerance to bile salt. Bsh activity is a relevant property for probiotic strains to survive the toxicity of conjugated bile salts in the duodenum [19]. During the study, R1 and R7 produced Bsh activity specific to bile salt (sodium cholate) hydrolysis. A similar result was also observed by Pisano *et al.* (2014) [20] and Anas *et al.* (2014) [21] where they found deposition of sodium cholate indicate a positive result for Bsh activity. *L. casei* MTCC 1423 (38.7%) and R1 (46.5%) showed maximum cell autoaggregation ability while cell surface hydrophobicity of strains R1 and *L. casei* MTCC 1423 have been reported as high 75.25 % and 65.853% respectively. In a previous study, the autoaggregation percentage of KSBT56 is 39.3% which was higher than LP9 (31.0), close to LA1 (40.4%) but lower than other *L. acidophilus* i.e. LA7 (46.5%) and LA14 (60.9%). Again, the cell surface hydrophobicity of other standard probiotic strains (37.7% to 58.3% in n-hexadecane and 37.1% to 60.8% in xylene for the standard strains) [8, 9]. All LAB tested fulfilled the safety requirement that a probiotic strain should not have α-and β-haemolytic activity. No strain tested in this work exhibited α-and β-hemolytic activity. Anas *et al.* (2014) [21] described the similar result in their study. Probiotics *Lactobacillus* spp are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. Here, the antagonistic profile of all *Lactobacillus* spp. was studied in order to observe the inhibitory activity against standard pathogens such as *E. coli* MTCC82, *Bacillus cerus* ATCC10702, *S. aureus* MTCC96, *S. typhyi* MTCC3216, *Aeromonas hydrophila* ATCC7966 and *Klebsiela pneumonia* MTCC 109. The culture supernatant prepared by using R1 showed highest antimicrobial activity against all pathogens except *Aeromonas hydrophila* ATCC7966. The diameters of inhibition zones observed in well diffusion method were greater than 10 mm. The same antimicrobial study was found in previous researches [13, 22, and 23] where *Lactobacillus* species were found to be effective against many gram-positive and negative pathogens. The probiotic isolate exhibited the highest antibacterial activity chosen for its further characterization and analysis. The potent probiotic *Lactobacillus* R1 was identified as *Lactobacillus fermentum* by standard biochemical and physical characterizations (Bergey's manual 2nd edition), in agreement with other findings [11].

In other hand, culture supernatants were again treated with proteinase K to determine its proteinacious nature and could be purified by column chromatography. The antimicrobial activity of culture supernatant of R1 was completely destroyed by proteinase K treatment indicating its antibacterial activity was due to the activity of bacteriocin protein. The same observation was obtained in the literature [12] that supports the proteinacious nature of antimicrobial compound released from *Lactobacillus fermentum* R1. The compound responsible for this activity was identified as bacteriocin protein and was purified by column chromatography. Molecular weight of this antimicrobial protein is approximately 25 kDa. Thus we believe that the compound secreted by R1 could release antimicrobial protein against a wide range of gram positive and gram negative pathogens and may be used as chemotherapeutic agent or biopreservatives. Similar studies were also observed by Ogunbanwo *et al.* (2003) [24] where the bacteriocin of *L. plantarum* F1 and *L. brevis* OG1 were recovered following the 60% saturation of the culture broths with ammonium sulphate with an increase to specific activity of 9.4 and 5.2 AU/µg protein respectively (Fraction

1). The purified antimicrobial protein was found to be stable after heat treatment from 60 to 100 °C for 15 and 30 min and in the pH range of 4.5-7.0. Many researchers who were working on the field bacteriocin characterization found the same results in their studies [12, 24].

CONCLUSION

Though all isolated and collected *Lactobacillus* strains exhibit some good probiotic properties, only *Lactobacillus fermentum* R1 fulfills all the required criteria to be effective among all. It includes high viability to harsh conditions such as low pH, high bile salt concentration, Bsh activity and a high percentage of adherence and colonisation. It can produce bacteriocin which inhibits a number of pathogenic organisms. In conclusion, the antimicrobial characteristics of *Lactobacillus fermentum* R1 indicates its possible use in starter cultures for traditional fermented foods as a means of improving the health condition. The partially purified inhibiting substance is most active at low pH values and retains its activity with an increase in temperature. This suits various food and pharmaceutical production processes.

CONFLICT OF INTERESTS

Declared none

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