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Analysis of Antimicrobial Activity of *Lactobacillus paracasei* ssp. *paracasei-1* Isolated from Regional Yogurt

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Abstract

Lactic acid bacteria are very significant to human health due to the production of some antimicrobial substances and ability to inhibit pathogenic bacteria. In this study, *Lactobacillus* strains were isolated from yogurts of Khulna region, Bangladesh and identified as *Lactobacillus paracasei* ssp. *paracasei-1*. The antimicrobial activity of cell free supernatant was tested against both gram positive and gram negative pathogenic bacteria and found to be sensitive. The supernatant exhibited tolerance to extreme pH and was heat stable as well as sensitive to proteolytic treatment. The latter property could be attributed to a bacteriocin like inhibitory substance (BLIS) secreted from *L. paracasei* ssp. *paracasei-1*. In addition, the isolate showed good survival in presence of a number of bile salts indicating its potential application as probiotic supplement.

Keyword: Bacteriocin like inhibitory substance, *Lactobacillus*, Yogurt, Probiotics, Antimicrobial.

Introduction

Isolation and identification of microorganisms from natural resources provide a unique source for acquiring pure cultures with potential commercial utility. One of the best examples comes from Lactic Acid bacteria (LAB), which are extensively used for manufacturing a wide variety of fermented foods. As the lactic acids produced from these bacteria do not pose any health risks to mankind, they are Generally Recognized as Safe (GRAS) organisms. Apart from lactic acids, these bacteria also produce various types of compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins during lactic acid fermentations [1-3]. Bacteriocins are antimicrobial proteinaceous compounds

which exert inhibitory activities towards a broad spectrum of pathogenic microorganisms including food spoilage and/or food-borne pathogenic bacteria and are produced by both Gram-positive and Gram negative bacteria [4]. In recent times, the bacteriocins from the GRAS lactic acid bacteria have enormous scientific interest to control pathogens in foods. Therefore, LAB have been reported to be used as biopreservative agents with great economic importance [2, 5-6]. Antimicrobial activity and tolerance to bile salts are two important tests to screen potential probiotic bacteria that could also be used as human medicine [7]. Probiotic can be defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" [3].

In the present study, efforts were undertaken to identify and characterize a *Lactobacillus spp*. isolated from yogurts of Khulna region, Bangladesh in order to determine its efficacy as probiotic bacteria. For this purpose, a bacteriocin like inhibitory substance (BLIS) from the culture supernatant of the isolate was also identified and partially characterized. Antimicrobial activity of the BLIS was also determined against a number of Gram positive and Gram negative pathogenic bacteria. In addition, the isolate was tested for its ability to grow and multiply in bile salts.

Related Literature

A number of papers have been published on bacteriocinogenic *Lactobacillus spp.* [8-10]. Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes that have a bactericidal or bacteriostatic effect on other (usually closely related) species [11]. It is reported to be the most significant antimicrobial substance produced by LAB [12]. In the last few decades there has been an increasing interest in bacteriocin as possible preservative for food and potential supplements or replacements for currently used antibiotics. The ribosomal production of these small (2–6 kDa) antimicrobial peptides by LAB, as a defense mechanism against other organisms is well documented and represents an intensive area of research [13-14].

Essentially two attributes are considered for establishing an inhibitory substance as a bacteriocin. Firstly, the inhibitor factor should display a bacteriocidal mode of action and secondly, it should be proteinaceous in nature [4]. Without characterization of bacteriocin for amino acid and nucleotide sequence it is preferable to designate the inhibitory substance as bacteriocin-like inhibitory substances (BLIS) [15].

Materials and Methods

Microorganisms

A reference strain of *Lactobacillus paracasei* was obtained as a pure culture from USA Labs, Eden Prairie, Minneapolis USA for control experiments. All the LAB cultures were maintained at 4°C in MRS broth. Six pathogenic bacterial strains namely *Escherichia coli, Vibrio cholerae, Shigella dyssenteriae, Salmonella paratyphi, Staphylococcus epidermis* and *Streptococcus pyogenes* were collected from Microbiology Laboratory of Biotechnology & Genetic Engineering Discipline, Khulna University for conducting antimicrobial activity. They were cultured in nutrient broth.

Isolation and identification of Lactobacillus spp.

The *Lactobacillus* isolate was obtained from previously isolated culture maintained by Biochemistry and Molecular Biology Laboratory of Biotechnology & Genetic Engineering Discipline, Khulna University. This *Lactobacillus spp*. was previously isolated from regional yogurt of Khulna, Bangladesh [16]. These isolates were cultured twice in MRS broth media for 16 hours at 37^{o} C [17] to exclude growth of other opportunistic microorganisms.

Lactobacillus spp. was subsequently subjected to Gram staining and other tests for phenotypic identification. These tests included the study on shape and colony morphology as well as tests for catalase, oxidase, endospore and motility [18-19]. Oxidase test was carried out with OXItest stripes of MIKRO-LA-TEST **(**Erba Lachema s.r.o.**,** Czech Republic) according to manufacturer's instruction. Sugar/ carbohydrate fermentation pattern was determined by API 50 CH stripes system (bioMe´rieux, Lyeon, France) according to instruction manual. The results were then interpreted using APIWEB identification software with database (V5.1).

Antimicrobial activity

The antimicrobial activity of *L. paracasei* spp. *paracasei-1* was assessed by a slightly modified method reported by Cuozzo et al. [20]. *L. paracasei* spp. *paracasei-1* was used at a dilution of 10^{-7} in place of 10^{-6} described by Cuozzo et al [20]. Zone of inhibition was recorded against six pathogenic bacteria. Seventy µL of a 16 hr incubated culture of these strains was gently mixed with 5 mL of molten MRS top agar (0.7% agar) at 45°C and poured into a petri dish containing MRS base agar (1.5% agar) and allowed to solidify. The plates were then incubated for 16 hr at 30°C. Plates were observed for the growth of pathogenic bacteria with and without *L. paracasei* ssp. *paracasei-1*.

Characterization of Culture supernatant

L. paracasei ssp. *paracasei-1* was grown in MRS broth over night at 37^{o}C. Cell-free supernatant was obtained by centrifugation of the bacterial culture at 13,000 rpm for 30 min. The pH was adjusted to 6.5

using 1N NaOH to neutralize the inhibitory effect of lactic acid. Culture supernatant was then sterilized using filter of 0.22µl pore size (MF-Millipore membrane filters, EMD Millipore Corporation, MA, USA). Three different methods (agar spot, well diffusion and blank disk method) were carried out for detection of antimicrobial activity of the culture supernatant as described by Nowroozi et al. [21]. The procedure described by Oh et al. [3] and [21] were followed for the detection of pH and heat sensitivity of *L. paracasei* ssp. *Paracasei-1* culture supernatant. For proteolytic treatment, method described by Barefoot and Klaenhammer [17] was considered. Culture supernatants of *L. paracasei* ssp. *paracasei-1*which retained antimicrobial activity were treated for 1h at 37^{o}C with Proteinase K (Invitrogen, California, USA) and Pepsin (Sigma-Aldrich Biochemie GmBH, Hamburg, Germany) separately at a concentration of 1mg/mL for the experiment.

Bile salt tolerance test

Growth of *L. paracasei* ssp. *paracasei-1* was determined in MRS broth containing 0.3% bile salt. Sodium taurocholate, sodium glycolate, sodium taurodeoxycholate and oxbile (Hi-Media, India) were separately used as bile salt. Positive control was MRS broth medium without bile salt. After inoculation of *L. paracasei* ssp. *Paracasei-1* in MRS broth containing bile acid, the optical density was measured at 620nm using a UV-Vis spectrophotometer (HACH, Model No: DR-5000, USA) at 620 nm after 0h, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h and 24h.

Results

Phenotypic identification

For presumptive phenotypic identification, the isolates were cultured on MRS agar media. MRS is generally used as selective media for lactobacilli [22]. Cystein, a reducing agent, is used at a concentration of 0.05% to MRS in order to improve the specificity of this medium for *Lactobacillus* isolation [23]. The results based on the biochemical and morphological tests indicated that the isolates grown on the above-mentioned media were *Lactobacillus spp.* (Table 1). Several commercially available identification kits such as API50 CH, LRA Zym and API Zym are commonly used for rapid and reproducible phenotypic identification of pure cultures. They have been reported for the characterization and identification of lactobacilli in milk [24], yogurts and other fermented milk [25] and in cheese [25-27]. Therefore, the isolates were subsequently subjected to carbohydrate fermentation using API carbohydrate kit and were identified with 99.9% confidence as *L. paracasei* ssp. *paracasei-1* based on the fermentation patterns. For control experiments, pure intact culture of *L. paracasei* (reference strain) was considered.

Table 1: Comparison of *L. paracasei* from two different origins.

Characteristics	*L. paracasei* ssp. *paracasei*-1 (Isolate under study)	*L. paracasei* (Reference strain)
Colony morphology	Spindle like round, white	Small round, white
Catalase test	-	-
Gram-staining	+	+
Oxidase test	-	-
Endospore test	-	-
Motility	Non-motile	Non-motile
Bacterial shape	Rod shaped	Rod shaped

Assay for antimicrobial activity

Antagonistic activity of *L. paracasei* ssp. *paracasei-1* was tested against six major gram positive and negative pathogenic bacteria (Table 2). All the pathogens were found to be sensitive to *L. paracasei* ssp. *paracasei-1*.

Table 2: Inhibitory activity of *L. paracasei* ssp. *paracasei-1* against six pathogenic bacteria.

Test organisms	Results
E. coli (gram –ve)	+
V. cholerae (gram –ve)	+
S. dysenteriae (gram –ve)	+
S. paratyphi (gram –ve)	+
S. epidermis (gram +ve)	+
S. pyogenes (gram +ve)	+

It could be interpreted that these *L. paracasei* ssp. *paracasei-1* possessed bactericidal properties by producing some inhibitory substances. The antimicrobial activity of LAB may be attributed to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide and bacteriocins [28].

Characterization of antimicrobial activity

Exposure of six pathogenic test organisms to active culture supernatant of *L. paracasei* ssp. *paracasei-1* resulted in a strong inhibition of growth of those test organisms, which suggested the presence of BLIS in the supernatant [6]. A crude extract of this BLIS was heat stable and retained full activity after 30 min autoclaving at 121°C. Moreover, the BLIS was stable and retained inhibitory activity at extreme pH values. Supernatants were treated with proteolytic enzymes and results indicated that the inhibition factor was proteinaceous in nature which showed bactericidal mode of action. Based on these evidences, it was interpreted that the inhibitory molecules of *L. paracasei* ssp. *paracasei-1* could be a bacteriocin. The

immediate and drastic reduction in optical density of test pathogens indicated a potent bactericidal effect by BLIS secreted from *L. paracasei* ssp. *paracasei-1*. The results of BLIS activity assay in culture supernatant are shown in the Table 3.

Table 3: Culture supernatant sensitivity test.

Parameters	Activity
pH 3.0	+
pH 12.0	+
121^{o}C, 15 min	+
Proteinase K treatment	-
Pepsin treatment	-

Bile salt tolerance test

L. paracasei ssp. *paracasei-1* survived in 0.3% bile salts containing MRS broth. Four bile salts sodium taurocholate, sodium glycolate, sodium taurodeoxycholate and oxbile (Hi-Media, India) were used. *L. paracasei* ssp. *paracasei*-1 showed good survival abilities in presence of sodium taurocholate, sodium glycolate and sodium taurodeoxycholate (Figure 1, A). However, in presence of oxbile comparatively low absorbance was recorded. In the control experiment, the survival abilities of pure culture of the reference strain were also determined in presence of abovementioned bile salts (Figure 1, B). It was found that *L. paracasei* ssp. *paracasei-1* could withstand sodium taurocholate, sodium taurodeoxycholate and oxbile more efficiently than the reference strain. On the contrary, *L. paracasei* showed better growth in sodium glycolate. Bile tolerance has been described as an important factor for the survival and growth of lactobacilli in the intestitinal tract. Additionally, this attribute has been correlated with the hypocholesteromic effect in human [29]. Survival and growth in the intestinal tract as well as reducing cholesterol level are important considerations for future use of lactobacilli isolate as dietary adjunct.

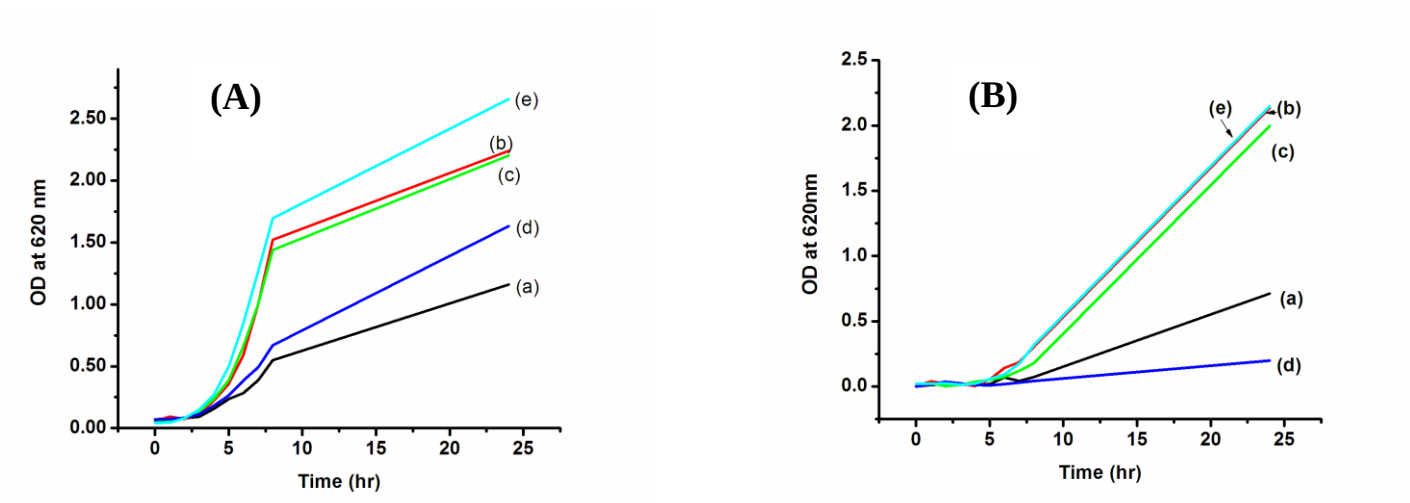

Figure 1: Bile salt tolerance of (A), *L. paracasei* ssp. *paracasei-1*, and (B) the reference strain. Here (a), oxbile; (b), sodium taurocholate; (c), sodium glycolate; (d), sodium taurodeoxycholate and (e), positive control.

Conclusion

LAB can produce antimicrobial compounds that vary in their spectra of activity. In the present study, *L. paracasei* ssp. *paracasei-1* isolated from traditional yogurts from Khulna region, Bangladesh was found to produce antimicrobial compounds. Similar observations were reported by Schillinger and Lucke [30] with lactobacilli. The antimicrobial agent from strain *L. paracasei* ssp. *paracasei-1* demonstrated a strong antimicrobial activity against both Gram-positive and Gram negative bacteria. The antimicrobial agent was heat-stable while it retained its activity even after autoclaving at 121°C for 30 min in agreement with other reports [6, 31-32]. Therefore, this agent could maintain its activity in heat processed food-stuffs.

Moreover, this antimicrobial substance was proteinaceeous nature, as it was inactivated by treatment with proteolytic enzymes. Proteolytic enzyme-mediated inactivation suggests the identity of inhibitory substance as bacteriocin. Previous studies reported that many of the antimicrobial compounds produced by LAB were bacteriocins which were proteinaceous nature, while other non-protein agents are also produced [33-34]. Furthermore, the antimicrobial agent demonstrated a bacteriolytic mode of action, as the immediate decrease in the optical density of the pathogenic bacteria.

In conclusion, the potential of *L. paracasei* ssp. *paracasei-1* to inhibit a number of pathogenic bacteria, such as *E. coli*, *V.cholerae*, *S. dysenteriae*, *S. paratyphi*, *S. epidermis*, *S. pyogenes*, is of crucial interest. Some of these bacteria can produce toxins resulting in human illness. In addition to the broad inhibition

spectrum, its technological properties and especially heat and storage stability indicated that the BLIS had potential for application as a biopreservative to control pathogens in processed foods. Furthermore, bile tolerance of *L. paracasei* ssp. *paracasei-1* renders further study on probiotic properties of this isolate.

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