

Full Length Research Paper

Isolation and evaluation of antibacterial activity of bacteriocin produced by *Lactobacillus bulgaricus* from yogurt

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Lactic acid bacteria (LAB) commonly used in food, as starter cultures are known to produce antimicrobial substances such as bacteriocins and have great potential as food bio-preservatives. Yogurt is a probiotic dairy product having *Lactobacillus bulgaricus* as natural source for fermentation. The aim of this study was to determine the antimicrobial activity of bacteriocin, produced by *Lactobacillus bulgaricus* isolated from yogurt and tested against pathogenic organisms. The identification of the culture was based on characteristics of the strains of *Lactobacillus* spp. on the basis of microscopy (morphology), Gram staining, growth at 37 and 45°C, fermentation of different carbohydrate sources and growth in MRS broth. On the basis of all of the identification tests, one strain isolated from the yogurt was identified as *L. bulgaricus*. Culture supernatants were obtained from the sixty out of hundred isolates of *Lactobacillus* spp. exhibited varying degrees of inhibitory activity against strains of *Bacillus subtilis*, ATCC #6633, *Escherichia coli*, ATCC, #10536, *Salmonella typhi*, ATCC# 19430, *Staphylococcus aureus*, ATCC #6538, *Vibrio cholerae*, ATCC #25870. Specifically, *Vibrio cholerae* was found most sensitive to bacteriocin, exhibiting maximum zone of inhibition 18.3 mm.

Key words: Lactic acid bacteria (LAB), *Lactobacillus bulgaricus*, bacteriocin, yogurt, antimicrobial activity, Pakistan.

INTRODUCTION

In spite of modern technologies and safety concepts such as hazard analysis and critical control point (HACCP) system, the reported numbers of food-borne illnesses and intoxications are still on the increase. According to the Council for Agricultural Science and Technology, microbial pathogens in food cause an estimated 6.5 to 33 million cases of human illness and up to 9000 deaths annually, with the main foods implicated including meat, poultry, eggs, sea food and dairy products. The bacterial pathogens that account for much of these cases include

Salmonella, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* (Buzby et al., 1996).

Probiotics confer a health benefit on the host. A number of human studies have clearly demonstrated that yoghurt containing viable bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* sp. *bulgaricus*) improves lactose digestion and eliminates symptoms of lactose intolerance so clearly implement the current concept of probiotics. The probiotic bacteria as a component of "thermophilic" starter cultures, used in commercial products today, are mainly members of the genera *Lactobacillus* and *Bifidobacterium* (Reuter, 1997; Bonaparte, 1997; Guarner et al., 2005). Genus

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Lactobacillus consists of beneficial micro-bacteria that are usually present in the gastrointestinal tracts. *Lactobacillus bulgaricus* (binomial name *Lactobacillus delbrueckii* subsp. *bulgaricus*) is one of several bacteria used for the production of yogurt. *L. bulgaricus* are Gram-positive, acid tolerant (relatively low pH 5.4-4.6), facultatively anaerobic, non-motile and non-spore-forming, rod-shaped members of the industrially important lactic acid bacteria (Metchnikoff, 1908; Axelsson, 1998; Hammes and Vogel, 1995; Kandler and Weiss, 1986). Cultures contain beneficial micro-bacteria *Streptococcus thermophilus* with *L. delbrueckii* subsp. *bulgaricus* and/or *L. helveticus*. Since they are involved in numerous food fermentations, hence known to man for millennia, are designated as generally recognized as safe (GRAS) and also considered as 'food grade' organisms (Siitonen et al., 1990; Saavedra et al., 1994; Biller et al., 1995).

Different antimicrobials, such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins, produced by lactic acid bacteria (LAB) can inhibit pathogenic and spoilage microorganisms, therefore extending the shelf-life and enhancing the safety of food products (Modler et al., 1990; Aymerich et al., 2000). According to Mathur and Singh (2005) antibiotics are a major tool utilized by the health care industry to fight bacterial infections; however, bacteria are highly adaptable creatures and are capable of developing resistance to antibiotics. However, recently many investigators have speculated that commensal bacteria including LAB may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens (Ammor et al., 2007). Research work has focused more, on the use of bacteriocin-producing LAB (bio preservatives for food improvement) as bacteriocin-producing cultures with inhibitory activity against Gram-positive pathogens such as *L. monocytogenes* while still no bacteriocin produced by a Gram-positive organism with activity against Gram-negative has been characterized. The potential of bacteriocin-producing starters or starter adjuncts, especially lactococci, pediococci and lactobacilli to control undesirable microbes in food has been evaluated by a number of research groups in recent years (Bredholt et al., 1999; Vignolo et al., 1996). Bacteriocins are proteinaceous substances that display antimicrobial activity against species closely related to the producer strain and/or other bacteria (Bredholt et al., 1999; Vignolo et al., 1996). Bacteriocins produced by LAB are a heterogeneous group of peptide inhibitors, which include lantibiotics (class I, e.g. nisin), small heat-stable peptides (class II, e.g. pediocin AcH/PA1) and large heat-labile proteins (class III, e.g. helveticin J) (Parente and Ricciardi, 1999). Many bacteriocins belonging to the first two groups can be successfully used to inhibit undesirable microorganisms in foods, but only nisin is produced industrially and is licensed for use as a food preservative in a partially purified form. Bacteriocins production by

LAB has been an important subject of research work in recent years because of their potential use as novel, natural food preservatives as well as medical purposes (Abee et al., 1995; Enan et al., 1996; Todorov and Dicks, 2006). The objectives of this study were isolation of *L. bulgaricus* from yogurt, production of bacteriocin from *L. bulgaricus*, isolation of bacteriocin from *L. bulgaricus* and evaluation of the antibacterial effect of bacteriocin on the pathogenic bacteria.

MATERIALS AND METHODS

Isolation of bacterial strains from yogurt samples and culture conditions

A total of hundred samples of yogurt were collected from the local market during the month of January to February, 2010 from Islamabad and its peripheral areas. Each sample was diluted in the distilled water and mixed by stirrer. 1 ml of each diluted sample was then inoculated in MRS Broth tube, prepared according to the methods described by De Man et al. (1960) under aseptic condition. A control was also run with each batch of samples by pouring distilled water instead of sample. The inoculated MRS Broth tubes were incubated under anaerobic condition at 37°C for 48 h. MRS broth and agar culture were subjected to different parameters which affect growth such as temperature, pH, anaerobic conditions and incubation time periods. Tubes were then observed and bacterial growth was sub-cultured on Petri dishes containing MRS Agar and incubated anaerobically for 48 h at 37°C and then stored.

Morphological, biochemical characterization and identification of *L. bulgaricus*

The bacterial cultures were examined for identification of bacteriocins producer strains, by using various morphological, cultural and biochemical testing methodologies according to the Bergey's manual (Kandler and Regular, 1986). Morphological identification was carried out by using simple staining, Gram staining and cell morphology tests. Biochemical testing includes catalase, carbohydrate fermentation and Bacteriocin production tests (James et al., 1991).

Extraction of bacteriocins from cultures of *L. bulgaricus*

Extraction of bacteriocins was carried out from *L. bulgaricus* cultures, according to the reported methodology (Schillinger et al., 1989). Ten milliliters of broth was inoculated with strain of *Lactobacillus* spp. and were incubated at 35°C for 48 h. After incubation, a cell-free solution was obtained by centrifuging (6000 × g for 15 min) the culture, followed by filtration (cellulose acetate filter, 0.2 µm pore size). Some supernatants were neutralized with 1 N NaOH to pH 6.5, and the inhibitory effect of the hydrogen peroxide was eliminated by the addition of catalase (5 mg/ml). Neutralized (bacteriocin and bacteriocin-like metabolites) supernatants of *Lactobacillus* spp. were checked for antibacterial activity against pathogenic bacteria in inoculated nutrient agar.

Detection of antibacterial activity

Antibacterial activity of presumptive strains of *L. bulgaricus* on *Bacillus subtilis*, ATCC #6633, *Escherichia coli*, ATCC, #10536,



Figure 1. Growth of *L. bulgaricus* in MRS broth.



Figure 2. Growth of *L. bulgaricus* on MRS plates.

Salmonella typhi, ATCC# 19430, *Staphylococcus aureus*, ATCC #6538, *Vibrio cholerae*, ATCC # 25870 were determined by the agar diffusion method according to Tag and Mc Given. The test organisms were obtained from Microbiology Section of Drugs Control and Traditional Medicines Division, National Institute of Health, Islamabad, Pakistan. Fresh indicator organisms of ATCC cultures were prepared by spreading each target organism on nutrient agar plates. Bacteriocins extracted were inoculated in the wells in pre-seeded agar plates. Reference standard of Ofloxacin (100 µg/ml) was inoculated in the same Petri plates as a positive control. The plates were kept undisturbed for 2 to 3 h and then incubated for 24 h at 37°C aerobically. Finally, the plates were examined for the presence of inhibition of zones by the help of vernier calliper in mm (Reinheimer et al., 1990).

Statistical analysis

The results were presented as the mean ± S.D of four groups. Statistical analysis of data was performed by using the student's unpaired *t*-test.

RESULTS AND DISCUSSION

Hundred yogurt samples were collected from Islamabad and its adjoining areas and subjected to the isolation of experimental culture, *L. bulgaricus* by implying different morphological and biochemical testing methodologies. The culture growth was evaluated by turbidity in the nutrient broth, as compared to control shown in Figure 1. It has been observed that *L. bulgaricus* was best grown on temperature 35 to 45°C for 24 h incubation anaerobically. The selective growth was obtained by providing selective media that is, MRS broth and agar. Sub culturing on MRS agar result in the pure colonies. The turbidity in MRS broth showed the growth of *L. bulgaricus*. The result of this study revealed an increase in bacteriocin production in MRS medium. It was also revealed that strict anaerobic condition can reduce the fungal contaminations and helpful in maintaining the

selective growth of bacteria. The effect of incubation period was also studied and it was observed that at the end of 48 h, the production of bacteriocin was found maximum. Results showed that bacteriocin produced by *L. bulgaricus* was sensitive to alkaline pH while resistant to acidic pH, hence this bacteriocin can retain at stomach pH.

Morphological evaluation resulted in white colonies on MRS agar plates (Figure 2). The Gram staining showed that bacteria were Gram-positive rods with rounded ends, non-motile and non-sporing. According to Oral-Jensen (1919) and Wests et al. (1984), these bacteria are thermophilic and facultative anaerobes so the selective growth was obtained by providing anaerobic conditions. Biochemical identification consist of catalase, carbohydrate fermentation and Bacteriocin production tests. It was reported previous that *L. bulgaricus* ferment only few carbons (Oral-Jensen 1919). Similarly, results showed that *L. bulgaricus* ferment only lactose and did not ferment mannose, malibose, manitol, sucrose and galactose, which is specific characteristic of *L. bulgaricus* and is shown in Figure 3. Carbohydrate fermentation tests showed positive only for Lactose and results has been mentioned in Table 1 and maximum fermentation by *L. bulgaricus* was observed within nutrient broth culture. Results showed that all isolates were catalase negative, as using H₂O₂, no bubble production was observed. Based on all of the identification tests bacterial strains isolated from the yogurt samples were identified as *L. bulgaricus*.

One hundred samples were used for bacteriocin production; however sixty samples were screened for antibacterial activity. Other forty were discarded due to contamination. These sixty bacteriocins sample were evaluated in triplicate for antibacterial activity by well diffusion assay (Geis et al., 1983) against selected Gram positive and Gram negative pathogenic bacteria that is,

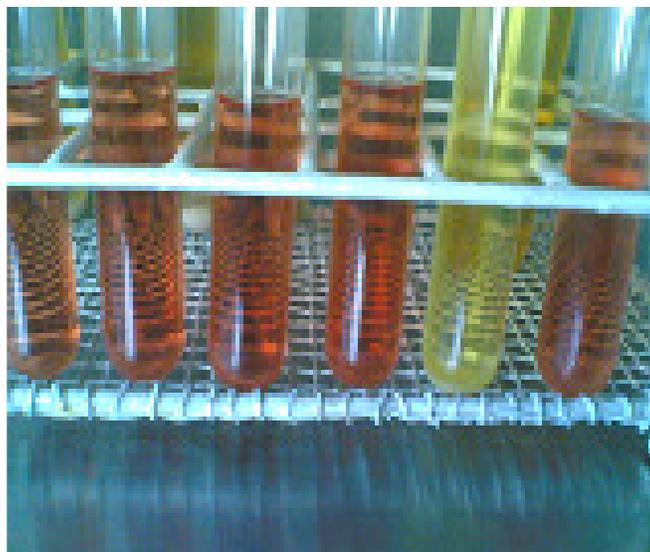


Figure 3. Carbohydrate fermentation by *L. bulgaricus*.

Table 1. Carbohydrate fermentation test.

S. No	Carbohydrates	Fermentation by <i>L. bulgaricus</i>
1	Manitol	Negative
2	Mannose	Negative
3	Sucrose	Negative
4	Melibiose	Negative
5	Lactose	Positive
6	Mannose	Negative



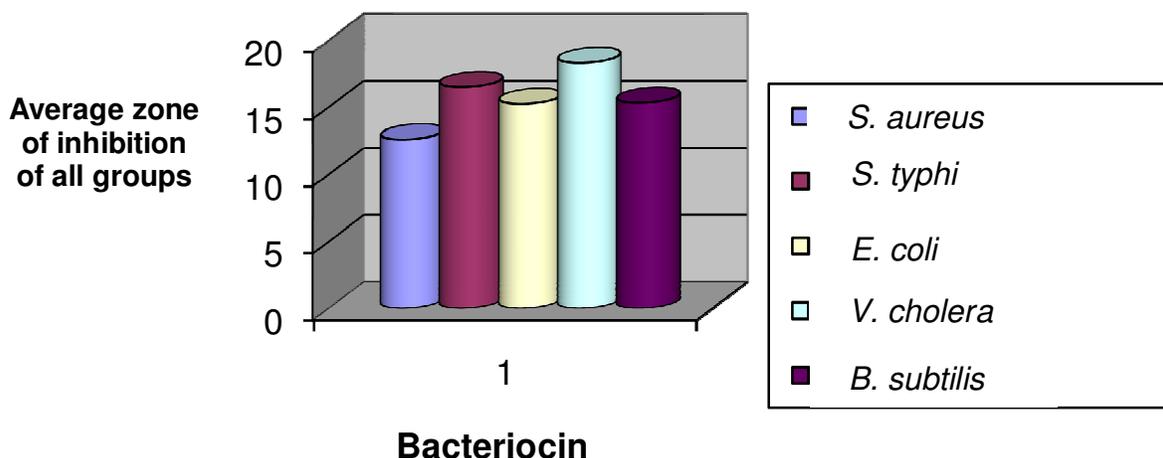
Figure 4. Growth of *L. bulgaricus* in MRS broth.

B. subtilis, ATCC #6633, *E. coli*, ATCC, #10536, *S. typhi*, ATCC# 19430, *S. aureus*, ATCC #6538, *V. cholerae*,

ATCC # 25870 (Figure 4). It has been observed that *V. cholerae* was most sensitive to bacteriocin and exhibiting

Table 2. Antibacterial activity (measurements of zones of inhibition) of bacteriocins against pathogenic gram bacteria

Bacterial extract	Measurements of zones of inhibition (mm)±S.D				
Bacteriocins	<i>S. aureus</i>	<i>S. typhi</i>	<i>E. coli</i>	<i>V. cholera</i>	<i>B. subtilis</i>
A	12±0.1	16.4±0.3	15±0.2	18.1±0.1	15.2±0.0
B	13.6±0.05	16.5±0.34	15.2±0.2	18.2±0.9	15.3±0.5
C	11.8±0.0	16.5±0.12	15.3±0.4	18.4±0.1	15.3±0.56
D	13±0.1	16.6±0.7	15.5±0.0	18.6±0.1	15.5±0.11

**Figure 5.** Comparison of overall average zone of inhibition by *L. bulgaricus* against pathogenic gram bacteria.

maximum zone of inhibition 18.32 mm, as obvious from results mentioned in Table 2. Average zone of inhibition has been shown graphically in Figure 5. Significant inhibiting potential has been observed against *S. typhi* (16.5 mm), *E. coli* (15.25 mm), and *B. subtilis* (15.32 mm). These results also are accordance with previous work carried out by Erdourul and Erbulur (2006) but on different strain types, in which supernatants (CFF) obtained from *Lactobacillus casei* and *L. bulgaricus* exhibited varying degrees of inhibitory activity against strains of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *B. subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 18833, *Salmonella typhimurium* ATCC 13311, and *Enterobacter cloacae* ATCC 13047. The probiotic potential of these bacteria is also vastly investigated (Gilliand, 1990; Cleveland et al., 2001; Mojgani et al., 2006; Diez-Gonzalez, 2007). It has been known that *L. bulgaricus* has a preservative effect on the product not only because of the production of lactic acid and hydrogen peroxide, but also by the help of the antimicrobial compounds (example, bacteriocin) it produces. The compound, namely Bulgarican, is inhibitory towards both Gram-positive and Gram-negative bacteria. Some inhibitory compounds against *Staphylococcus* and *Clostridium* species have also been found (Erkus, 2007).

Conclusion

The present investigation showed that, some *L. bulgaricus* strains which were isolated from yoghurts had antibacterial potential against some food borne pathogen and spoilage microorganisms especially *V. cholera* and *E. coli*, because of significant characteristic of bacteriocin production. Investigation revealed that incubation period (48 h) results in maximum production of bacteriocin. So, in yogurt manufacturing, there is need for considering different culturing parameters for best bacteriocin yield. There is need for more research on antibiotic resistance profiles of yoghurt bacteria.

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