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Journal of Chemical and Pharmaceutical Research, 2013, 5(2):176-180

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Bactericidal activity of the lactic acid bacteria *Lactobacillus delbreukii*

Morami Devi[1], L. Jeyanthi Rebecca[1*] and S. Sumathy[2]

[1]*Department of Industrial Biotechnology, Bharath University, Agaram Road, Selaiyur, Chennai, Tamilnadu*
[2]*SSS Bioprime Technologies Pvt. Ltd, Chennai, Tamilnadu*

ABSTRACT

The present study was aimed at isolating Lactobacillus sp. from yoghurt and to determine its bactericidal activity. The strain was identified by using morphological, physiological, staining and biochemical methods. The isolate of LAB was identified as Lactobacillus delbreukii as it showed growth at 45°C and no growth at 15°C, was fermentative positive, gram-positive, rod-shaped, non-spore forming, catalase negative etc. Live culture broth were screened for their antibacterial activity. Bactericidal activity was analyzed by agar well diffusion assay against the pathogenic organisms. Pathogenic bacteria such as Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi and E.coli were used as targets. The live culture broth inhibited the growth of a range of gram positive and gram negative microorganisms.

Key Words: Lactic acid bacteria, *Lactobacillus delbreukii*, bactericidal activity, gram positive bacteria, gram negative bacteria.

INTRODUCTION

Lactic Acid Bacteria (LAB) are Gram-positive, non-spore forming cocci, coccobacilli or rods with a DNA base composition of less than 53mol% G+C. They generally are non respiratory and lack catalase. Lactic acid bacteria are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid. As agents of fermentation LAB are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut, but some species may spoil beer, wine and processed meats[1,2]. Lactic acid bacteria are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid. As agents of fermentation LAB are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut, but some species may spoil beer, wine and processed meats. LAB are capable of inhibiting various microorganisms in a food environment and display crucial antimicrobial properties with respect to food preservation and safety [3]. LAB, particularly *Lactobacilli* have been isolated from several food products of dairy industry, vegetables and fruit pulps; are also present as a normal flora in human being; possessing antimicrobial activity against a wide range of pathogenic microbes. [4-12].

L. delbrueckii are generally found in dairy products such as yogurt, milk, and cheese with the exception of *L. delbrueckii* subsp. delbruecki which reside in vegetable sources. The prtB gene encodes proteases which are found anchored along the cell wall of *L. delbrueckii subsp. bulgaricus* and *L. delbrueckii lactis*. Enzymatic activity that takes place in the casein allows these subspecies to grow in dairy products. *L. delbrueckii subsp. bulgaricus* is a component of thermophillic starter cultures used to manufacture a number of fermented dairy products. These

cultures contain Streptococcus thermophilus with *L. delbrueckii subsp. bulgaricus* and are utilized in yogurt, Swiss-type and Italian-type cheese varieties. *L. delbrueckii* is non-pathogenic. *L. delbrueckii* subsp. *bulgaricus* has been proven to have probiotic effects on human and animals which include improved lactose tolerance and its ability to stimulate immune responses. A phosphopolysaccharide produced by *L. delbrueckii* subsp. *bulgaricus* has the capability to enhance phagocytosis of macrophages in mice. Research has been carried out by many institutes worldwide that have proved that Bulgarian yogurt helps in the treatment of various diseases and conditions like infections, tuberculosis, stomach and intestine conditions, ulcers, and many other diseases. [13-16].

The objectives of this study were to isolate *Lactobacillus delbreukii* from yoghurt and to determine their bactericidal activity against some gram-positive and gram-negative bacteria, and their stability at different pH and temperature.

EXPERIMENTAL SECTION

Isolation of lactic acid bacteria:
The samples of fresh yoghurt (curd) were collected from commercial market and were stored under refrigeration conditions for subsequent experiments. Yoghurt samples were serially diluted in peptone medium [Dilutions (10^{-1} – 10^{-6})] and incubated at 23ºC for 30 min before plating by which 50% of recovery of LAB was increased. Diluted samples (0.1 ml) were plated (spread plate technique) onto De Man Rogosa Sharpe (MRS) medium for *Lactobacillus* isolation and incubated at 37ºC for 24-72 hrs. Isolated colonies with typical characteristics namely off white, raised, spherical small with entire margins were picked from each plate and transferred to MRS broth for further analysis.

Identification of the bacterial strains:
Further identification of the *Lactobacilli* was performed according to their morphological, cultural, physiological, staining and biochemical characteristics [17-19]. Gram staining endospore staining, production of catalase, carbohydrate fermentation patterns, growth at 15°C and 45°C in the lactobacilli De Mann Rogosa and Sharpe (MRS) broth as described by Bergey's Manual of Systematic Bacteriology [17, 20], methyl red and Voges-Proskauer test in MR-VP medium, nitrate reduction in nitrate broth, indole production in Tryptone broth. Purified cultures were maintained at -20°C in MRS broth with 10% glycerol and enriched in MRS broth incubated at 37°C for 24 hrs. The identified genus *Lactobacillus* was further classified to the species level based on their ability to ferment sugars.

Screening for bactericidal activity of the strain:
Bactericidal activity were screened against the test pathogens *E.coli, Staphyloccus aureus, Bacillus subtilis, Klebsiella pneumoneae,* and *Salmonella typhi* by well diffusion assay according to the method of [21]. Aliquots (50 µl) of the cell culture were placed in 4-mm-diameter wells that had been cut in nutrient agar plates previously swabbed with the test pathogens. After 12-18 h of incubation, the diameters of the zones of growth inhibition were measured. Antimicrobial activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition.

Screening the stability of the strain at different pH and temperature:
The strains were inoculated into MRS broth and adjusted to different pH (3,5,7,9,) and temperature (30, 40, 50 °C) and the activity of the strains were tested by well diffusion assay. Zone of inhibition was observed and measured.

RESULTS AND DISCUSSION

The present investigation highlights the isolation and bactericidal activity of *L. delbreukii* from yoghurt samples. It is a starter culture in yoghurt production along with *Streptococcus thermophilus*. Colonies of LAB (*Lactobacillus sp*.) appeared on MRS medium after 48 hrs of incubation. A total of thirty colonies were isolated from the MRS medium plates of different dilutions, and sub-cultured by streaking on MRS medium plates.

The colonies formed were mostly pale-yellow/ off-white, raised, spherical small with entire margins. The off-white colonies were selected for further identification. Out of the thirty colonies, six isolates showed growth at 45° C and no growth at 15°C. (Table-1), which suggested that they belonged to the *Lactobacillus sp* .Six of the isolates that showed growth at 45°c and no growth at 15°c were subjected to Gram staining and they were examined under light microscope. All the strains gave blue- purple color with staining; hence they all were Gram positive bacteria (Table-1). The isolates were all bacilli with long and rounded ends. They appeared mostly as a chain of 3-4 cells or single. The six isolates subjected endospore staining appeared pink in color after the counter staining as they did not retain the primary strain and hence concluded that they are non-endospore forming (Table-1).

All of the six isolates were subjected to IMViC, catalase test, oxidase test, nitrate reduction test, urease test, motility test and casein hydrolysis test. None of them showed positive activity except for casein hydrolysis (Table-2). All of the isolates were also subjected to the test for gas production from glucose and the test tubes were observed for five days. There was no gas accumulation in durham tubes, although the cultures were well grown. This result confirmed the homo-fermentative behavior of the isolates (Table.1). All the six isolates, out of thirty, after identification were designated as LB-01 to LB-06. *Lactobacillus* isolates from temperate regions has been classified according to their morphology, physiology and molecular characteristics [22]. The morphological characters of raw milk, natural whey starter and cheese were also analyzed [23]. Lactobacilli are generally isolated on rich media such as MRS which is routinely used for the isolation and counting of Lactobacilli for most fermented food products [17]. The addition of the reducing agent such as cysteine (0.05%) to MRS improved the specificity of the medium for *Lactobacillus* isolation [24, 25].

Out of the six selected strains screened for bactericidal activity (Fig-1), all the strains showed bactericidal activity against *E.coli*, with LB-03 showing the highest zone of inhibition of 2.2 cm. Five strains showed activity against *S. aureus*, with LB-03 showing the highest zone of inhibition of 1.8 cm. Two strains showed activity against *B. subtiis*, five strains showed activity against *K. pneumoneae* and two strains showed activity against *S. typhi* (Table-3)

The six isolates were screened for their stability at different pH and temperature (Fig-2,3). Out of them two strains LB-03, LB-06 showed maximum bactericidal activity, and LB-03 was comparatively stable in selected pH. The optimized conditions for the strains have been established as pH 5 and temperature 40°C. Among the three strains, it is seen that against every pathogen and at all temperatures and pH, the strain LB-03 has consistently maintained more prominent bactericidal activity in comparison with the others. Further, identification of the species of these *Lactobacilli* was performed according to carbohydrate fermentation patterns and growth at 15°C and 45ºC (Table-1) in the MRS broth as described in Bergey's Manual of systematic Bacteriology. Based on the results the species was identified as *Lactobacillus delbreukii*. The studies have shown the isolates are defensive and they are been labeled as exceptional bacteria as they have shown their constructive role on human pathogens by inhibiting their growth for which they are said to be the second immune system of the body [26].

Table- 1: Physiological, staining and sugar fermentative test

S.No	Test	Strain code					
		LB-01	**LB-02**	**LB-03**	**LB-04**	**LB-05**	**LB-06**
1	Gram staining	+, rod	+, rod	+, rod	+, rod	+, rod	+, rod
2	Endospore staining	-	-	-	-	-	-
3	Growth at 15ºC	-	-	-	-	-	-
4	Growth at 37ºC	+	+	+	+	+	+
5	Growth at 45ºC	+	+	+	+	+	+
6	Growth at pH3.4	+	+	+	+	+	+
7	Growth at pH9.6	-	-	+	-	+	+
8	Fermentative type	Homo	Homo	Homo	Homo	Homo	Homo
9	Glucose	+	+	+	+	+	+
10	Lactose	+	+	+	+	+	+
11	Sucrose	-	-	-	-	-	-

• (+): positive response
• (-): negative reaction

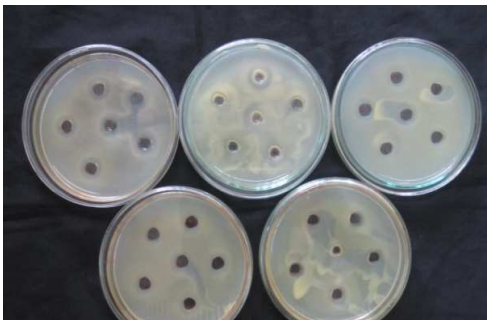

Figure-1: Bactericidal activity of the strains using live culture broth against test pathogens.

Table-2: Biochemical and enzymatic test

S. No	Biochemical and enzymatic tests	Strain code					
		LB-01	LB-02	LB-03	LB-04	LB-05	LB-06
1	Indole test	-	-	-	-	-	-
2	Methyl Red Test	-	-	-	-	-	-
3	Vogues Proskauer Test	-	-	-	-	-	-
4	Citrate Utilization test	-	-	-	-	-	-
5	Catalase Test	-	-	-	-	-	-
6	Oxidase test	-	-	-	-	-	-
7	Urease test		-	-			
8	Nitrate reduction test	-	-	-	-	-	-
9	Motility test	-	-	-	-	-	-
10	Casein hydrolysis	+	+	+	+	+	+

• (+): positive response
• (-): negative reaction

Table-3: Bactericidal activity of live culture broth against test pathogens

S. No	Strain code	Zone of inhibition (in cm)				
		E. coli	*S. aureus*	*B. subtilis*	*K. pneumonia*	*S. typhi*
1	LB-01	1.2	-	-	0.9	-
2	LB-02	1.5	1.0	-	0.8	-
3	LB-03	2.2	1.8	0.7	1.2	1.0
4	LB-04	1.5	1.2	-	-	-
5	LB-05	1.4	1.1	0.9	1.0	-
6	LB-06	1.7	1.5	-	1.4	0.8

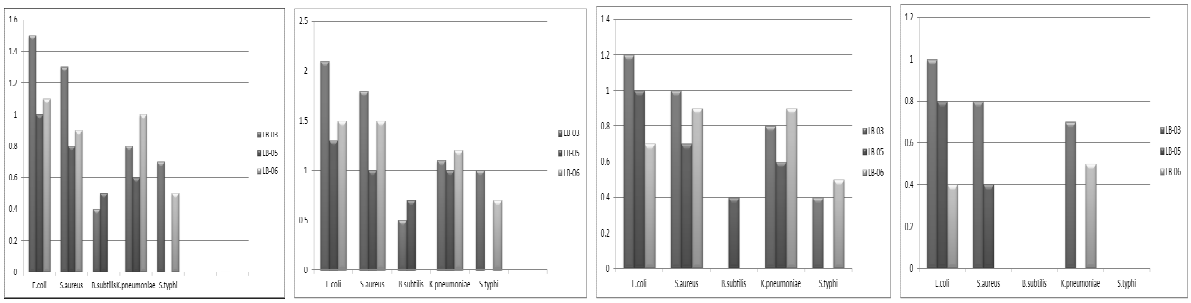

Figure-2: Bactericidal activity of the selected strains LB-03, LB-05 and LB-06 at pH 3, 5, 7, 9.

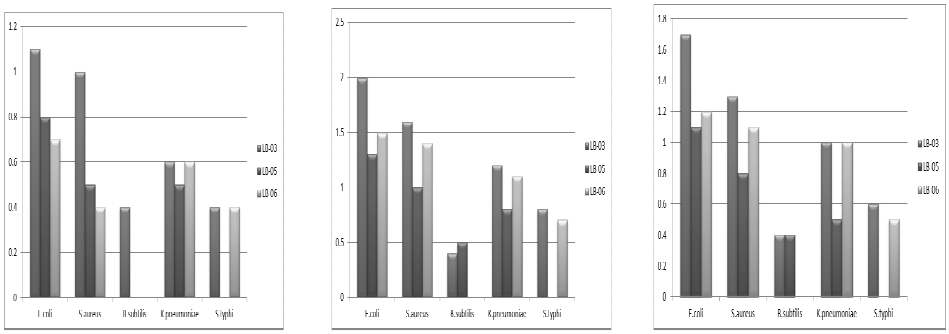

Figure-3: Bactericidal activity of the selected strains LB-03,LB-05 and LB-06 at 30^0C, 40^0C, 50^0C.

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