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Isolation, Characterization and Identification of Probiotic Lactic Acid Bacteria and Bacteriocin Production

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Abstract

The increasing consumer awareness towards diet and health are linked for stimulating innovative development of novel products by the food industry. Probiotic activity of lactic acid bacteria has received much attention over recent decades due to the health promoting properties of certain strains comes in light due to its better effect. The aim of experiment is the awareness about probiotic properties & safety aspects of different food available that made up from lactic acid bacteria. The present investigation highlights the isolation, characterization and production of bacteriocin by lactic acid bacteria from the curd samples which was brought from local market. The isolated cultures were screened for tolerance to acid, bile salt, NaCl and hydrogen peroxide. Antibacterial activity of probiotic cultures was studied using disc diffusion assay. The production and partially purification of Bacteriocin were carried and antibacterial activity of partially purified bacteriocin was tested against different pathogens. The curd was prepared using isolates and their viable cell count was estimated by standard plate count. The isolated were identified on the basis of morphological and biochemical characters. These isolates were *Streptococcus lactis* (TC-LAB-1), *Lactobacillus delbrueckii* (TC-LAB-2) and *Lactobacillus acidophilus* (TC-LAB-3). The three isolated lactic acid bacteria were found to show an antibacterial activity against *Staphylococcus* sp, *Klebsiella* sp, *Pseudomonas* sp, *Bacillus* sp and *Salmonella* sp. The isolate 3 was produced higher amount of bacteriocin in MRS broth (pH 6) at 30ºC with 1.5 % NaCl. On the basis of viable cell count of prepared curd, it concluded that the *Lactobacillus acidophilus* is having probiotic characters & safe to use in food preparation.

Keywords

Probiotics; Lactic acid bacteria; Bacteriocin; Antibacterial activity

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INTRODUCTION

The word 'probiotic' comes from Greek language 'pro bios' which means 'for Life' opposed to 'antibiotics' which means 'against life'. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much. In 1908 a Russian researcher Ellie Metchnikoff, who has a Nobel Prize, firstly proposed

the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long-lived people because of the consumption of fermented milk products which consists of rod-shaped bacteria [1].

The increasing consumer awareness that diet and health are linked is stimulating innovative development of novel products by the food industry [2]. Lactic acid bacteria have received much attention over recent decades due to the health-promoting properties of certain strains, called probiotic. The probiotic food comes in light due to its better effect. The awareness about probiotic properties & safety aspects of different food available that made up from Lactic acid bacteria are made attention towards these probiotic studies [3] The India is having large production of milk & milk products that consumed in large population. The large part of milk products is made at home from starter culture available locally [4, 5]. The curd production & other fermented milk products are mainly prepared using lactic acid bacteria [6, 7].

When selecting a probiotic strain, a number of aspects should be considered, and the theoretical basis for selection should involve safety, functional as well as technological. When selecting a preferable probiotic strain, several aspects of functionality have to be considered [8]. These functions may be the strains for human use are preferably of human origin and isolated from a healthy human GIT and non-pathogenic. Must survive through upper GIT and arrive alive at its site of action and able to function in the gut environment. Adhere to the intestinal epithelium cell lining and colonize the lumen of the intestinal tract. Strains should not carry transmissible antibiotic resistance genes [9]. Probiotic bacteria must be able to survive GI transit (acid and bile salt tolerant). These probiotic organisms have good technological properties so that it can be manufactured and incorporated into food products without losing viability and functionality or creating unpleasant flavors or textures. Functional aspects include viability and genetic stability [10, 11]. On the basis of above literature, following objectives were set for the present investigation. These objectives are isolation, characterization of lactic acid bacteria from the curd samples which was brought from local market. The isolated cultures were screened for tolerance to acid, bile salt, NaCl and hydrogen peroxide. Antibacterial activity of probiotic cultures was studied using disc diffusion assay. The production and partially purification of Bacteriocin were carried and antibacterial activity of partially purified bacteriocin was tested against different pathogens. The curd was prepared using isolates and their viable cell count was estimated by standard plate count

MATERIAL AND METHODS:

Collection of samples: Samples were collected for isolation of Lactic acid bacteria is based on products which are available in market & prepared at home.

Isolation and identification of Lactic acid bacteria: Curd were serially diluted and plated on de Man Rogosa agar (MRS agar) medium to isolate the *Lactobacillus* [12]. The strains were sub-cultured onto MRS agar slant, incubated at 30ºC for 24 hr and were preserved. The isolates were selected for further studies which exhibited strong inhibitory activity against indicator strains and identified on the basis of growth, cell morphology, and gram staining and catalase activity. Further, identification of the species of these *Lactobacilli* was performed according to carbohydrate fermentation patterns and growth at 15 and 45ºC in the de Man Rogosa Sharpe (MRS) broth as described in Bergey's Manual of systematic Bacteriology [13].

Antibacterial activity of isolated LAB: The cultures of the indicator strains (*Staphylococcus auerus, Klebsiella* sp.*, Pseudomonas* sp.*, Salmonella* sp. and *Bacillus* sp.) were prepared by pouring 2ml of the inoculum onto MRS plates to completely cover the surface of the agar. Using the disc diffusion method, Probiotic inoculum was carefully diffused on plate using the disc. The diameter of the inhibition zones around the disc were observed after incubating the plates for 48 hr at 37°C.

Selection of potential probiotic:

Acid tolerance: The acid tolerance of LAB was studied in different pH. The solutions were prepared by adjusting the hydrochloric acid (HCl) solution to pH levels of 2, 3, 4 and 5 in distilled water. Sterile distilled water (pH 6.4) served as a control. Solutions were prepared in 100ml volume and sterilized at 121ºC for 20 min and stored at room temperature until used. After thorough mixing, 10ml of each pH solution was taken in sterilized test tubes. A cell suspension of selected LAB cultures containing about 1010cells/ml was added to each pH solution of 2, 3 and 4 and control

(pH 6.4) and mixed. The samples were poured on MRS agar plate and incubated aerobically at 37ºC for 72 hr [2, 14].

Bile tolerance: The bile salt solutions were prepared using Ox-gall powder. The powder was rehydrated by preparing 10g dry powder base in 90ml distilled water. From this solution, concentrations of 0.5%, 1%, 1.5% and 2% were prepared. Sterile distilled water without Ox-gall (pH 6.4) was used as control. All solutions were autoclaved and stored at room temperature until used. 10ml of each solution was transferred into sterile test tubes. Cell suspensions containing about 104cells/ml was added to each solution, i.e., 1%, 2% and control and incubated at 37ºC aerobically. The samples were poured on MRS agar plate and incubated aerobically at 37ºC for 48 hr [2, 14].

Tolerance to NaCl concentration: For the determination of NaCl tolerance of isolated Lactic acid bacteria, 4 test tube containing MRS broth were adjusted with different concentrations (1, 2, 3, 4) of NaCl. After sterilization each test tube was inoculated with fresh overnight culture of *Lactobacillus* and incubated at 37°C for 24 hr. After 24hr of incubation their growth were determined by plating 1ml of broth onto MRS agar incubating at 37ºC for 48 hrs [2, 15].

Determination of bile salt hydrolase activity: Melted MRS agar poured separately into sterile Petri dishes. Once plates solidify, the plates place in an anaerobic chamber for at least 48 hr before using. Overnight cultures were inoculated on each plate. Plates were incubated at 37°C in anaerobic jars for 48 hr. The bile salt hydrolase activity of the cultures is evidenced by the formation of a white precipitate around the colonies grown in MRS agar. This precipitate is not observed in MRS agar (control) without bile salts, where colonies are translucent.

Determination of hemolytic activity of organism: Actively growing culture of test organisms is prepared by growing them in MRS broth. A filter paper disc dipped in culture of respective organism is kept on blood agar plate aseptically. The plates incubate at 37^0C in anaerobic condition for 24 hours. Check hemolytic activity by observing zone hemolysis around filter paper disc. Non hemolytic organism shows no zone of hemolysis around colony.

Determination of antibiotic resistance of Lactic acid bacteria Antibiotics powder must be accurately weighed and dissolved in the appropriate diluents to yield the required concentration. Stock solutions were prepared using the formula (1000/P) X V X C=W, where P= potency of the antibiotic base, V=volume in ml required, C=final concentration of solution and W=weight of the antimicrobial to be dissolved in V. Antibiotic resistance was determined using the disc diffusion method [2].

Production of bacteriocin: The isolated strain was grown in MRS broth (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 42ºC for 48 hr. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4ºC). The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin [16, 17].

Partial purification of bacteriocin: After incubation broth is removed & centrifuged at 1000 rpm for 10 minutes to remove cell. Supernatant is collected and precipitated at 80 % ammonium salt. These are kept overnight at 4°C for precipitation of protein. Next day precipitated protein collected by centrifugation at 1000 rpm for 15 minutes. Precipitate then dissolved into Phosphate buffer. Presence of bacteriocin in precipitate was confirming by Folin Lowry method.

Antibacterial activity of precipitated bacteriocin: Using disc diffusion method antibacterial activity of probiotic cultures were studied. *Staphylococcus auerus, Klebsiella* sp.*, Pseudomonas* sp.*, Salmonella* sp. and *Bacillus* sp. cultures were used for antibacterial activity. Sterile plates of MRS were prepared by autoclaving at 121^0C, 15 psi for 20 minutes. Suspensions of 24-hour old culture of pathogen are prepared. The suspension of respective organism is spread plated on sterile nutrient agar plate. Filter paper disc dipped in suspension of Lactic acid bacteria is kept on plate of pathogen aseptically and plates were kept at 4°C for diffusion for 20 min. Each plates of pathogen then incubated at 37 ° C for 24 hours. The above zone of inhibition around filter paper disc is observed.

Effect of pepsin on precipitated bacteriocin: 1 % pepsin solution is prepared using distilled water. Then Precipitate dissolved into Phosphate buffer 10 ml is taken into sterile test tube. To this 1 ml of 1% pepsin is added & kept for incubation at 37 ° C for 2 hours.

Preparation of Curd: The pasteurized milk was aseptically transferred into test tube. Aseptically 1 % of probiotic culture was added to above milk. These

were incubated at 42°C for 6 hours. After completion of incubation period curd is formed. This curd was kept for at 4°C for 30 days in refrigerator. After completion of 30 days the viability & cell number of probiotic organisms is determined.

RESULTS AND DISCUSSION:

Isolation and identification of bacterial strain: The curd samples which were brought from local market were used for isolation of lactic acid bacteria. Bacteriocins produced by probiotic Gram-positive bacteria are protein in nature. They have considerable attention as food preservatives and as potential replacement of antibiotics [18]. These strains were identified as *Streptococcus* sp. (TC-LAB-1), *Lactobacillus* sp. (TC-LAB-2) *and Lactobacillus* sp. (TC-LAB-3) based on its physiological and biochemical characteristic.

Antibacterial activity of isolated LAB: The antibacterial activity of isolated lactic acid bacteria from was determined using disc diffusion inhibition method. The three Lactic acid bacteria *Streptococcus* sp. (TC-LAB-1), *Lactococcus* sp. (TC-LAB-2) *and Lactococcus* sp. (TC-LAB-3) were found to show an antibacterial activity in the disc diffusion assay. The antibacterial substance produced by Lactic acid bacteria inhibited pathogen such as *Staphylococcus auerus, Klebsiella* sp, *Pseudomonas* sp, *Bacillus* sp and *Salmonella* sp. TC-LAB-3 showed the inhibition of all tested organisms; than the TC-LAB-2 and TC-LAB-1. The inhibitory effects were significantly seen against *Staphylococcus sp, Klebsiella* sp, *Pseudomonas* sp, *Bacillus* sp and *Salmonella* sp. by TC-LAB-3. Results also revealed the presence of the compound bacteriocin in the test organisms. Bacteriocins have been reported to be inhibitory against several other bacteria. Antibacterial activity of CFC from normal yoghurt, Acidophilus milk, Bifidus milk and probiotic Acido-bifido-yoghurt was studied using cup-well assay against enterotoxigenic pathogen [16, 19].

Potentials of Probiotics:

Acid tolerance: The survival of Lactic acid bacteria strains at pH 2, 3, 4 and 5 was observed under 42˚C incubation. Only TC-LAB-3 was survived at pH 2, 3, 4 and 5. TC-LAB-1were survived at pH 3, 4 and 5 whereas growth was recorded. TC-LAB-2 wasn't survived at pH 2 and 3. Strains showed consistency in terms of tolerance to pH 4 and 5. Survival at pH 4 and 5 was promising for all strains (Table-1). The residual counts were 10^7cfu/ml even after 3 h of incubation. Survival at pH 3 was promising for all strains but not at pH 2. Survival at pH 3 is significant as ingestion with food or dairy products raises the pH in stomach to 3.0 or higher. Some lactic acid bacteria used as starter cultures in milk and fermentation, and probiotic bacteria such as *L. acidophilus* and B. bifidum produce b-d-galactosidase. This enzyme hydrolyses lactose, which results in increased tolerance for dairy products [2, 6].

Table 1- Potential of Probiotics

Acid tolerance			
pH	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**
2	-	-	+
3	+	-	+
4	+	+	+
5	+	+	+

NaCl tolerance			
NaCl %	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**
1.0	+	+	+
2.0	+	+	+
3.0	-	-	+
4.0	-	-	+

(+) Growth, (-) No growth

Bile tolerance			
Bile %	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**
0.5	+	+	+
1.0	+	+	+
1.5	-	-	+
2.0	-	-	+

Bile salt hydrolase activity			
Activity	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**
Bile salt hydrolase activity	+	-	+

Hemolytic activity			
Activity	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**
Blood agar plate	No hemolysis	No hemolysis	No hemolysis

Bile tolerance: The bile tolerance efficiency of all isolates was resistant at 1% bile concentration under 12 hr incubation periods. All isolated strains showed better tolerance at 0.5 and 1% bile concentration. Whereas, TC-LAB-1 and 2 was sensitive at 1.5 and 2% bile concentration. Among the three isolated strains, TC-LAB-3 showed comparatively better tolerance at higher concentrations (2%) (Table-1). *L. acidophilus* survives better than the traditional yogurt culture organisms in MRS broth with 0.5 to 1% bile salt concentration [9, 15].

NaCl Tolerance: Sodium Chloride is an important physicochemical factor for bacteriocin production. The NaCl effect on growth of all isolated strains in the medium with various NaCl concentrations was studied (1 to 4%). The all isolated organisms were tolerated NaCl at 2%. Whereas TC-LAB-3 were tolerate higher concentration of NaCl (Table-1). The inhibitory compound was found to be less salinity concentration, losing activity when NaCl concentration was increased above 5%. The inhibitory activity was seen only in a narrow NaCl volume, when added NaCl volume to lower than 0.5% NaCl or 2% NaCl all inhibitory activity was lost. The relative salt concentration tends support to the assertion that the inhibitory compound. The sodium chloride is an important physiochemical factor for any marine and estuarine animal to maintain the osmoregulation. It has been reported that *L. acidophilus* survives better than the traditional yogurt culture organisms, *L. bulgaricus* and *S. thermophilus*, in yogurt under 2% NaCl Concentration [9, 15].

Bile salt hydrolase activity: The bile salt hydrolase activity of the cultures is evidenced by the formation of a white precipitate around the colonies grown in MRSBA agar. This precipitate is not observed in MRS agar (control) without bile salts, where colonies are translucent. Whereas the organism which does not showing bile salt hydrolase activity is not grows on MRSBA agar (Table-1).

Hemolytic activity: Hemolytic activity checked by observing zone hemolysis around filter paper disc. Organism shows no zone of hemolysis around colony (Table-1).

Antibiotic resistance of Lactic acid bacteria: TC-LAB-3 was sensitive towards the all used antibiotics. Whereas TC-LAB-1 and 2 were sensitive towards higher concentration of antibiotics.

Table 2-Antibiotic resistance of Lactic acid bacteria

Ampicillin dilution (µg/ml)	TC-LAB-1	TC-LAB-2	TC-LAB-3	Penicillin dilution (µg/ml)	TC-LAB-1	TC-LAB-2	TC-LAB-3
0.5	R	R	S	**0.5**	R	R	S
1.0	R	S	S	**1.0**	R	R	S
1.5	S	S	S	**1.5**	R	S	S
2.0	S	S	S	**2.0**	S	S	S
2.5	S	S	S	**2.5**	S	S	S
3.0	S	S	S	**3.0**	S	S	S
3.5	S	S	S	**3.5**	S	S	S
4.0	S	S	S	**4.0**	S	S	S
4.5	S	S	S	**4.5**	S	S	S
5.0	S	S	S	**5.0**	S	S	S
Tetracycline dilution (µg/ml)	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**	**Amoxicillin dilution (µg/ml)**	**TC-LAB-1**	**TC-LAB-2**	**TC-LAB-3**
0.5	R	R	S	**0.5**	S	R	S
1.0	R	S	S	**1.0**	S	S	S
1.5	R	S	S	**1.5**	S	S	S
2.0	S	S	S	**2.0**	S	S	S
2.5	S	S	S	**2.5**	S	S	S
3.0	S	S	S	**3.0**	S	S	S
3.5	S	S	S	**3.5**	S	S	S
4.0	S	S	S	**4.0**	S	S	S

4.5	S	S	S	**4.5**	S	S	S
5.0	S	S	S	**5.0**	S	S	S

S- Sensitive; R-Resistance

Cephaloxin dilution (µg/ml)	TC-LAB-1	TC-LAB-2	TC-LAB-3
0.5	R	R	S
1.0	R	R	S
1.5	R	S	S
2.0	R	S	S
2.5	S	S	S
3.0	S	S	S
3.5	S	S	S
4.0	S	S	S
4.5	S	S	S
5.0	S	S	S

Production of Bacteriocin: TC-LAB-3 was produced bacteriocin in MRS broth. The TC-LAB-3 was exhibited a good bacteriocin production at pH 6.0, sodium chloride 1.5% and 30 ºC. The bacteriocin production was higher during the stationary phase of the growth of the organism. Bacteriocin production was strongly dependent on pH, nutrients source and temperature. Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity was noted at pH 6.0, temperature 30°C and 1.5% NaCl. From the results proved that it could be used in acidic foods like pickle or yogurt. It might be secondary metabolites. MRs seemed to be more suitable medium for the bacteriocin production. Similar results were observed [19, 20].

Partial purification of bacteriocin: In the purification of filtrate culture, was removed by centrifugation, and the proteins were concentrated by 80% ammonium sulphate precipitation. Precipitate then dissolved into Phosphate buffer. This precipitate indicates the presence of protein which confirmed by Folin Lowry method. During purification several different protocols were applied. Optimal recovery was achieved by including ammonium sulphate precipitation.

Antibacterial activity of precipitated bacteriocin: The susceptibilities of various Gram-positive and Gram-negative bacteria to growth inhibition by the supernatant of TC-LAB-3 were presented in Table 3. It shows inhibitory activity against *Staphylococcus auerus, Klebsiella* sp, *Pseudomonas* sp., *Bacillus* sp and *Salmonella* sp. Among these, maximum activity observed against *Pseudomonas* sp, *Bacillus* sp and *Salmonella* sp and activity were not observed against *Klebsiella* sp and *Staphylococcus auerus*. Production of organic acids by the probiotics lowers the pH and alters the oxidation–reduction potential in the intestine, resulting in antimicrobial action [2, 3]. Combined with the limited oxygen content in the intestine, organic acids inhibit especially pathogenic gram-negative bacteria types, e.g. coliform bacteria [9,14].

Table 3- Antibacterial activity of precipitated bacteriocin

Bacteria	Zone of inhibition (diameter in mm)
Staphylococcus auerus	12
Klebsiella sp	11
Pseudomonas sp	10
Bacillus sp	11
Salmonella sp	11

Effect of Proteolytic enzyme (pepsin) on bacteriocin: Pepsin was strongly inhibited bacteriocin production. This is in contrast to results obtained [16, 20].

Table -4 Effect of Proteolytic enzyme (pepsin) on bacteriocin

Bacteria	Zone of inhibition without Proteolytic treatment	Zone of inhibition with Proteolytic treatment
Staphylococcus auerus	+	-
Klebsiella sp	+	-
Pseudomonas sp	+	-
Bacillus sp	+	-
Salmonella sp	+	-
Bacteria	Zone of inhibition without Proteolytic (diameter in mm)	Zone of inhibition with Proteolytic treatment
Staphylococcus auerus	12	-
Klebsiella sp	11	-
Pseudomonas sp	10	-
Bacillus sp	11	-
Salmonella sp	11	-

Determination of probiotic potential of isolated Lactic acid bacteria (TC-LAB-3)

1. Probiotic potential is determined using following formula
   Probiotic potential = (observed score ÷ maximum score) × 100
2. Score are determined by following way

Table 5-Score determination and probiotic potential of isolated lactic acid bacteria

Probiotic characters	Indication (Score)	Maximum Score	Observed score (Probiotic potential)		
			TC-LAB-1	TC-LAB-2	TC-LAB-3
Acid tolerance at pH 2.0	Sensitivity (0) Resistance (1)	1	0 (0)	0 (0)	1 (100)
Bile tolerance 2.0 %	Sensitivity (0) Resistance (1)	1	0 (0)	0 (0)	1 (100)

Determination of viability of probiotic dahi:

The prepared curd was serially diluted. The dilutions were spread on sterile MRS agar plates. After incubation the following results were obtained for Lactic acid bacteria isolation from 1 gm curd sample. On 1st day 100000 cfu/gm for 10^7 diltuion viable cell count number was estimated; while we also found the same viable cell count number on 30 day.

CONCLUSION:

The three lactic acid bacteria were isolated and identified as *Streptococcus* sp. (TC-LAB-1), *Lactobacillus* sp. (TC-LAB-2) and *Lactobacillus* sp. (TC-LAB-3). These isolated lactic acid bacteria were found to showed antibacterial activity against *Staphylococcus* auerus, *Klebsiella* sp, *Pseudomonas* sp, *Bacillus* sp and *Salmonella* sp. The TC-LAB-3 was produced higher amount of bacteriocin in MRS broth (pH-6) at 30ºC with 1.5 % NaCl. *Lactobacillus* sp. (TC-LAB-3) is more acidic & bile resistant which shows antibiotic sensitivity. From sample tested the market dahi is less acidic & bile resistant, so unable to survive in the gastric juices & intestinal tract. The homemade dahi is more acidic & bile resistant but shows antibiotic resistance that may be transferred to pathogenic organism. On the basis of viable cell count of prepared curd, it concluded that the *Lactobacillus* sp. (TC-LAB-3) is having probiotic characters & safe to use in food preparation.

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