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Recovery and Screening of Bacteriocin Producing Probiotic Lactic Acid Bacteria from Dairy Products

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ABSTRACT

The lactic acid bacteria are the group of gram positive, low GC, cocci or rod, non spore former bacteria. Due to their friendly nature and other health benefits they are generally regarded as safe (GRAS). Currently, a lot of attention given in the field due to nobility of LABs. The LAB has a wide verity of health benefits viz. improve gut health, boost immunity, kill intestinal pathogens, lower cholesterol etc. In the present study the work was focused with the isolation of bacteriocin producing probiotic lactic acid bacteria from different dairy products and the characterization for different probiotic attributes. Morphologically five distinct LABs recovered from different dairy products. All the isolates were morphologically and biochemically characterized. The antimicrobial activity (bacteriocin production activity) was evaluated by agar well diffusion method against five human pathogens. All the 5 isolates were positive for bacteriocin production and shown different degree of antibacterial activity. The isolates further screened for probiotics attributes viz. temperature tolerance, ph tolerance, cell surface hydrophobicity and bile salt tolerance test. It was found that all the isolates shown varied range of value in these tests. But isolate LC shown maximum probiotic potential can tolerate a pH of 2.5, with cell surface hydrophobicity 53.8% and bile salt tolerance upto 0.8%. Therefore the isolate LC fulfill the criteria to be considered as probiotic isolate.

Keywords: *Bacteriocin; Bile salt; Dairy; Hydrophobicity; LAB; Probiotics*

1. INTRODUCTION

Lactic Acid Bacteria (LAB) are belongs to a group of gram positive, low-GC, acid-tolerant, generally non-sporulating, non-respiring rod or cocci, catalase negative, fastidious microorganisms. Lactic acid bacteria are widely used in the food industry for the development of various products as well as for the preservation of food items. It has been already reported that some lactic acid bacteria produced a number of antimicrobial agents including lactic acid, hydrogen peroxide, diacetyles and bacteriocin. The LAB are extensively used in food industry as biopreservative due to their antimicrobial potential (Savadogo et al., 2006; Mirzaei et al., 2021). The bacteriocin produced by Lactic acid bacteria are exteracellular and protein in nature. *Lactobacillus* and fermented products with Lactic Acid Bacteria has therapeutic effect upon various human and animal gastrointestinal disorders (Rodriguez et al., 2000; Asto et al., 2019). Natural microflora of human intestine is normally prevailed by *Lactobacillus* and *Bifidobacillus* sp. can generate healthy equilibrium between beneficial and potentially harmful micro-flora in gut. Due to their health benefits and friendly nature LAB are significantly used in fermented and non fermented dairy products commonly termed probiotics. When ingested in sufficient quantities many Lactic Acid Bacteria function as probiotics are beneficial to host health. Several strains of LAB have been reorted for bactericin production isolated from raw and fermented products provide protection of food from spoilage and pathogenic microorganisms (Messens and De Vugst, 2002; Eisenhofer et al., 2019). Therefore, Lactic Acid Bacteria has remarkable potential as therapeutics for medical uses as they inhibit the growth of pathogens present in intestine. Keeping these developments in mind the study was planned with the isolation, characterization and bacteriocin producing probiotics LAB from locally available dairy products.

2. MATERIALS AND METHODS

Materials used

The media used were procured from Hi-media (India) and were used as per manufacturer's direction. All the reagents used were of AR grade. All the glasswares were used of borosilicate.

Procurement of cultures
The lab isolates of SUS College of Research and Technology viz. *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Enterococcus faecalis* were used in the present study. Nutrient agar slants were made for maintenance of cultures and stored at 4ºC with periodic revival after every 15 days.

Collection of Samples
Different dairy samples were collected for isolation of lactic acid bacteria. Raw milk samples of sheep and buffalo, curd and lassi were collected from different dairy shops nearby Mohali and Chandigarh (India).

Selective Isolation of Lactic acid bacteria
For isolation of Lactic Acid Bacteria 1ml Samples of each raw milk, lassi and curd were homogenized with 9 ml sterile distilled water, then 0.1ml of diluted samples were inoculated on MRS (Mann Rogosa Sharpe) agar plates. The MRS plates were incubated at 35±2º C for 48 hrs. Well isolated colonies were transferred to MRS broth for overnight and purified them by streaking on MRS agar to obtained pure culture.

Maintenance of isolates
Purified isolates were maintained on MRS agar slants. Slants were stored at 4ºC with periodic revival after every 15 days.

Morphological characterization of isolates
Isolates were identified using morphological and biochemical characteristics (Howells, 1992).

SCREENING OF ISOLATES FOR BACTERIOCIN PRODUCTION

Screening for antibacterial activity
The isolated Lactobacillus spp. was screened for antimicrobial activity against enteropathogens, using agar well diffusion assay. The isolates of LAB were grown in MRS broth for 24 hrs at 35±2º C in rotary shaker. After incubation broth was centrifuged at 10,000 rpm for 15 min. Cell free supernatant was collected and adjusted to pH 6.5-7.0 with 1 N NaOH and 1N HCl to nullify the lactic acid activity (Ivanova et al., 2000).

Biochemical Characterization
The various Biochemical tests viz. Catalase test, Nitrate reduction test, Sugar fermentation, Temperature tolerance test were performed according to the methods described by Aneja. (2003).

SPECIFIC TEST FOR LACTIC ACID BACTERIA FOR PROBIOTIC PROPERTIES

Temperature tolerance
Growth pattern of all the five isolates were observed at 15, 27, 40, 45ºC after 24 hrs of incubation.

Cell surface hydrophobicity
Cell surface hydrophobicity is an important characteristic, which is widely considered in the bacterial adhesion. Bacterial cells were grown in MRS agar for 24-48 hrs. Harvested cells were centrifuged at 10,000 rpm for 10 min. Supernatant was kept aside and cell pellet was washed twice with PBS buffer and resuspended in buffer. In acid washed test tubes 3 ml of bacterial suspension was added, after that 1 ml hexadecane was added, test tubes were incubated at 37ºC for 10 min. After 10 min of incubation two phases were separated, mix well the phases. For phase separation, tubes were again kept at 37ºC but for 1 hr of incubation. Absorbance was adjusted to 0.8 at 600 nm of in spectrophotometer. After phase separation aqueous phases were taken out and the absorbance was taken of aqueous phase at 600 nm. Percent hydrophobicity was calculated as (Maldonado et al., 2012):

$$\% \text{ Surface Hydrophobicity} = (Abs_{Final})/(Abs_{Initial}) \text{ X } 100$$

pH tolerance
pH tolerance of isolates MRS broth was prepared, Broth was adjusted to different pH 2, 4, 6, 8, 10 with 1 N HCl and 1N NaOH and inoculated with cultures of LAB, incubated at 35±2º C and observed for growth of isolates.

Bile salt Tolerance
Bile salt tolerance of isolates, MRS broth was supplemented with different concentrations 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0% of bile salts (Sodium deoxycholic acid and Sodium taurocholate each) incubated at 35±2º C for 24 hrs and growth of isolates were observed.

3. RESULTS

Isolation of Lactic Acid Bacteria
All samples were diluted in distilled water for isolation of LAB spreaded on Mann Rogosa Sharp agar (MRS) and incubated at 35±2º C for 48 hr. A total five morphologically distinct isolates of LAB were recovered from different sources. The result is depicted in table 1.

Morphological Characterization
Morphological characterization of isolated was studied by observing by cultural characterization on MRS. The cell morphology and arrangements was done by gram staining. The morphologically distinct isolates were shown different morphological pattern. The results are ducted in table 2. The colony morphology of RS is shown in fig.1.

Screening for bacteriocin activity

All the selected isolates were further subjected for their their antimicrobial activity. For this purpose the isolates were screened for thir antibacterial activity against lab isolates such as *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis*. The antimicrobial test was performed with agar well diffusion method and considered positive for bacteriocin production by a clear zone of inhibition around the well. The results are shown in figure 2, 3 and 4. The diameter of zone inhibition of all the isolates is shown in table 3. For the accuracy the test was conducted in triplicate and the average of resltus was taken. All the isolated were found to be positive for the production of antimicrobial compound. All the 5 isolates (RS, RB,

LS, CH, CM) of lactic acid bacteria showed antimicrobial activity against *Escherichia coli*, 3 isolates (RB, CH, CM) showed activity against *Staphylococcus aureus*, 2 isolates (RS, LS) showed against *Salmonella typhimurium*, and 4 isolates (RS, RB, LS, CM) against *E. faecalis.*

Biochemical Characterization

Different biochemical tests like catalase test, nitrate reduction test and sugar fermentation tests were performed for the characterization of isolated LAB. Catalase is an enzyme produced by microorganisms that splits hydrogen peroxide into water and oxygen and results in gas bubbles. Presence of catalase enzyme is signified by formation of gas bubbles**.** Nitrate reduction test is done for recognition of anaerobic bacteria which uses nitrate as final electron acceptor in the respiratory chain and reduces nitrate to nitrite. Nitrite products reacts with sulfanilic acid (Reagent A) to form complex that is nitrite sulfanilic acid, which then reacts with α naphthylamine (Reagent B) to give a red precipitate (prontosil). If red colour appears that indicates nitrite is present and test is positive. All isolates were found to be catalase negative and nitrate positive. The test of nitrate production is shown in figure 5.

Isolates were also tested for acid and gas production. Galactose, sucrose, dextrose and lactose were the sugar used for sugar fermentation. After incubation at 35±2^{O}C for 24hrs change in colour in tubes shows acid production and formation of gas bubble in Durham's tube shows gas production. All isolates fermented galactose expect isolate CH. Sucrose was fermented by isolates RS, RB, CH expect isolates LS and CM. Dextrose and Lactose were fermented by all isolates. The results are depicted in table 4.

SPECIFIC TEST FOR LACTIC ACID BACTERIA FOR PROBIOTIC PROPERTIES

The LAB isolates found positive for bacteriocin production were further subjected to screened for various probiotic attributes viz. temperature tolerance test, cell surface hydrophobicity, bile salt tolerance, pH tolerance.

Temperature tolerance

Growth of isolates were tested at 15, 27, 40, 45ºC. The result is depicted in table 5. Temperature played a key role in cell growth as well as bacteriocin production. In present study isolates RB and CM has shown their growth at different temperatures at 15ºC, 27ºC, 37ºC, and 40C º. Growth of isolates RS and CH were observed at 27ºC and 37ºC while isolate LC grew at 15ºC, 27ºC and 37ºC.

Cell surface hydrophobicity

Hydrophobicity percentage of isolates observed in range between of 12.2- 53.8%. Therefore it is clear that only the isolate LC (53.8%) fulfill the criteria of probiotic. The results are depicted in figure 6. Isolate LC showed maximum cell surface hydrophobicity and isolate RB showed minimum hydrophobicity percentage. Isolate RS showed hydrophobicity of 15.9%, isolate RB showed 12.2%, isolate LC 53.8%, isolate CH 12.8% and isolate CM showed 39.2%.

pH tolerance

All the five isolates RS, RB, LC, CH, CM were subjected to pH 2.0, 2.5. 3.0. 3.5 and 4.0 each and growth pattern of isolates at different pH observed. The result is depicted in table 6. pH is another factor which play a significant role in cell growth as well as bacteriocin production. Isolates LC, and CM have shown their growth at pH 2.5. In present study growth of Lactic Acid Bacteria isolated from different samples grew maximally at pH 4.0.

Bile salt tolerance

All the five isolates were subjected to different bile salt concentrations 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1 % to check bile salt tolerance capacity. In present study growth of isolates was observed at different concentrations. Growth of isolates RB and LC was observed at diverse bile salt concentrations that are at 0.1, 0.2, 0.3, 0.4, 0.6, and 0. 8, %, while Isolates CM and CH showed their growth only at 0.1, 0.2% and isolate RS tolerated 0.1, 0.2, and 0.3% of bile salts. The results are depicted in table 7.

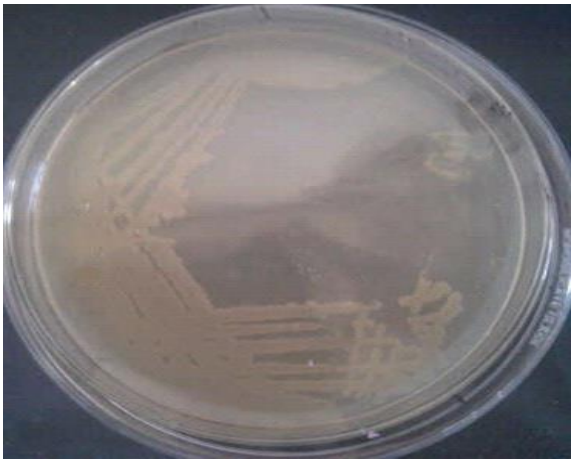

Fig: 1: Colony morphology of RC on MRS agar

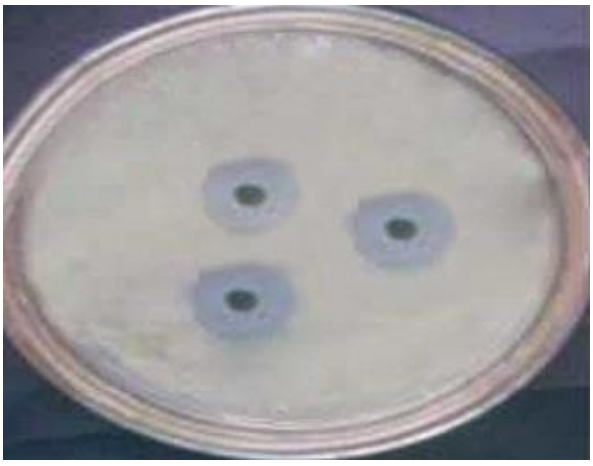

Fig 2: Antimicrobial activity of RC against *S. typhimurium*

Fig 3: Antimicrobial activity of RB against *Staphylococcus aureus*

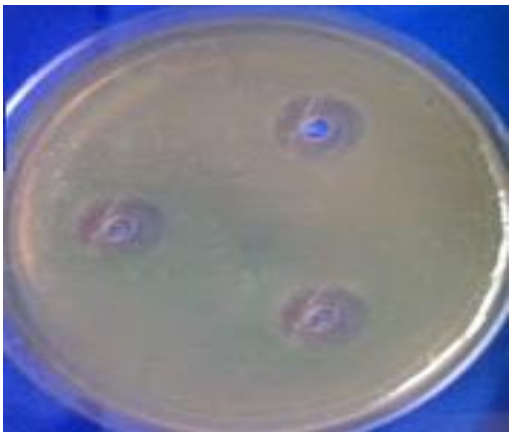

Fig 4: Antimicrobial activity of RS against *Enterococcus faecalis*

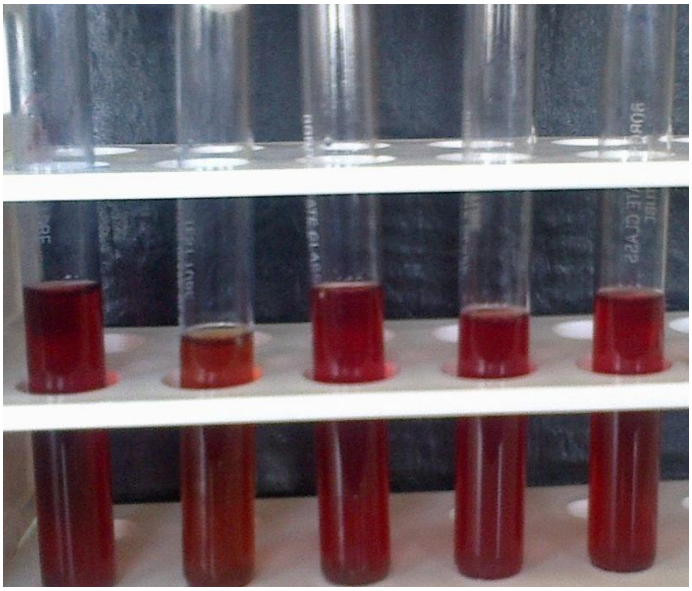

Fig 5: Nitrate reduction test

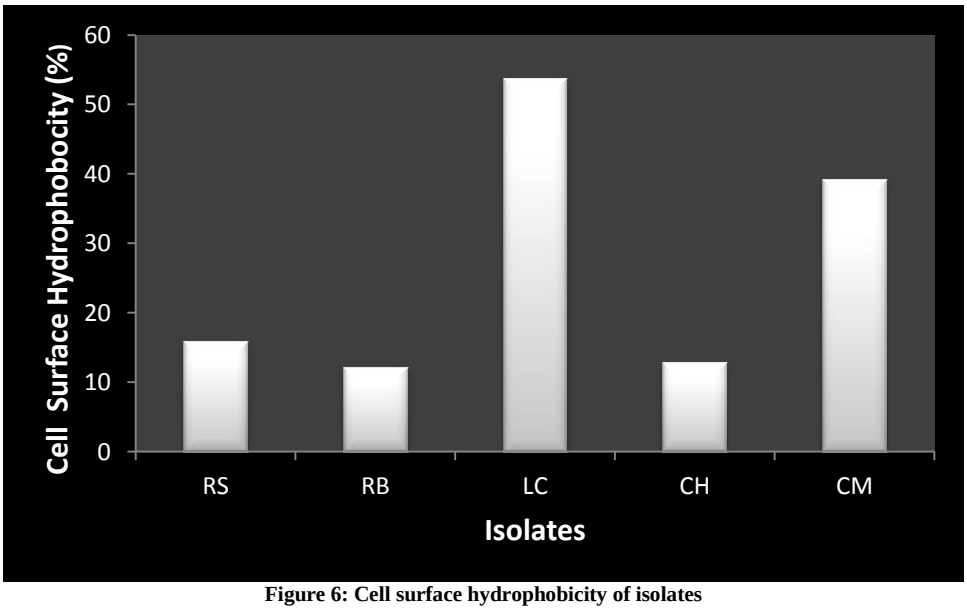

Figure 6: Cell surface hydrophobicity of isolates

Table 1: Isolation of Lactic Acid Bacteria

Sample	No. of isolates	Designation
Raw milk-Sheep, Buffalo	2	RS, RB
Lassi- Cow milk	1	LC
Curd- Home, Market	2	CH, CM

Table 2: Colony Morphology

Isolate	Colony Morphology	Gram Reaction	Cell Morphology and Arrangement
RS	Whitish, glistering colonies	G+	Rods in clusters
RB	Single and some in clusters	G+	Cocci present singly
LC	Whitish, single and spiral	G+	Rods present singly
CH	Large, Whitish colonies, single and some in clusters	G+	Rods in clusters
CM	Single and some in clusters	G+	Rods in clusters

Table 3: Bacteriocin Assay

Enteropathogens	RS	RB	LS	CH	CM
	Diameter of inhibition zone (mm)				
Escherichia coli	+	+	+	+	+
Zone of inhibition (mm)	11	12	10	11	11
Staphylococcus aureus	-	+	-	+	+
Zone of inhibition	-	12	-	11	12
Salmonella typhimurium	+	-	+	-	-
Zone of inhibition	14	-	13	-	-
Enterococcus faecalis	+	-	+	+	+
Zone of inhibition	14	-	13	14	14

Table 4: Biochemical properties of the isolates

Isolates	Catalase test	Nitrate Test	Galactose	Sucrose	Glucose	Lactose
RS	-	+	A,G	A	A,G	A,G
RB	-	+	A,G	A,G	A,G	A,G
LC	-	+	A	-	A	A
CH	-	+	-	A	A,G	A,G
CM	-	+	A,G	-	A,G	A

A- Acid ; G- Gas; Negative (-); Positive (+)

Table 5: Temperature tolerance of isolates

ISOLATES	15ºC	27ºC	37ºC	40ºC	45ºC
RS	NG	GG	GG	NG	NG
RB	MG	GG	GG	MG	NG
LC	MG	GG	GG	NG	NG
CH	NG	GG	GG	NG	NG
CM	MG	GG	GG	MG	NG

NG: no growth; MG: moderate growth; GG: good growth

Table 6: pH tolerance of isolates

Sample	pH 2.0	pH 2.5	pH 3.0	pH 3.5	pH 4.0
RS	-	-	-	+	+
RB	-	-	-	+	+
LC	-	+	+	+	+
CH	-	-	+	+	+
CM	-	+	+	+	+

Table 7: Bile salt tolerance of isolates

Isolates	0.1%	0.2%	0.3%	0.4%	0.6%	0.8%	1.0%
RS	+	+	+	-	-	-	-
RB	+	+	+	+	+	+	-
LC	+	+	+	+	+	+	-
CH	+	+	-	-	-	-	-
CM	+	+	-	-	-	-	-

4. DISCUSSION

The colony morphology of the LAB varied from whitish to cream, smooth and round margin (Iniguez et al., 2007). Similarly, colonies of lactic acid bacteria were smooth and round margin and white to cream in colour that colonies were white creamy colonies (Abdi et al., 2006).

Gram staining was accomplished to find out cell morphology and gram staining characteristics of isolates. It was observed that all isolates were gram- positive, few were arranged in rods or cocci, single or in clusters. Lactic Acid Bacteria form white creamy

colonies which are non motile and gram positive (Inguez et al., 2007).

The antibacterial activity of the isolates was evaluated by agar well diffusion method. In some study it has been proved that some strain of LAB produced antibacterial compound to confer health benefits. In the present study all the five isolates has been observed positive for the production of bacteriocin like compound and evaluated against wide array of lab isolates viz *E. coli, S. aureus, E. feacalis and S. typhimurium.* It has been already reported that some lactic acid bacteria produced wide array of antimicrobial compounds including lactic acid, hydrogen peroxide, diacetyles and a protein like compound called bacteriocin. The LAB is extensively used in food industry as biopreservative due to their antimicrobial potential. LAB is natural microbes and their metabolites are generally regarded as safe. Some species of LAB is widely used as probiotics in food industry due their health benefits such as immunomodulaters, antimicrobial activity agains some pathogenic bacteria and fungi (Beristain-Bauza et al., 2016; Mirzaei et al., 2021) . In a similar study it was found that lactic acid bacteria have are not very much effective against *Salmonella typhimurium* (Meera and Devi, 2012). The capability of a strain to produce antimicrobial activity can be related to its ability to withstand unfavorable condition, environmental factors such as temperature and pH(Pennachia et al., 2004).

After screening for bcateriocin production the isolates were further characterized for various biochemical test including catalase, nitrate reduction and sugar fermentation test. All isolates were found to be catalase negative and nitrate positive. Catalase is an enzyme produced by microorganisms that splits hydrogen peroxide into water and oxygen and results in gas bubbles. Presence of catalase enzyme is signified by formation of gas bubbles (Aneja, 2003)**.**

Nitrate reduction test is done for recognition of anaerobic bacteria which uses nitrate as final electron acceptor in the respiratory chain and reduces nitrate to nitrite. Nitrite products reacts with sulfanilic acid (Reagent A) to form complex that is nitrite sulfanilic acid, which then reacts with α naphthylamine (Reagent B) to give a red precipitate (prontosil). If red colour appears that indicates nitrite is present and test is positive (Aneja, 2003). In a similar study, isolated Lactic Acid Bacteria were gram positive, catalase negative (Mami et al., 2012). Lactic Acid Bacteria isolated from dairy products which were nitrate positive (Bromberg, 2004).

Isolates were tested for acid and gas production. Galactose, sucrose, dextrose and lactose were the sugar used for sugar fermentation. After incubation change in colour in tubes shows acid production and formation of gas bubble in Durham's tube shows gas production. All isolates fermented galactose expect isolate CH. Sucrose was fermented by isolates RS, RB, CH expect isolates LS and CM. Dextrose and Lactose were fermented by all isolates. All isolates of LAB generally exhibit different patterns in terms of acid production from different carbon sources (Guessas and Kihal, 2004).

The production of bacteriocin is greatly affected by temperature. The organism produce secondary metabolites in the log phase therefore the active multiplication is required. In the present study it was suggested that the lactic acid bacteria grow optimum in the temperature range between 27-37 ºC. Similarly, a researcher reported that the growth temperature seems to play a significant role in bacteriocin activity (Meera and Devi, 2012).

Cell surface hydrophobicity is another criterion for selection of probiotic strain. Hydrophobicity higher than 40%, is the minimum necessity for the isolates considering probiotics. Hydrocarbon hexadecane was used to assess the cell surface hydrophobicity. Similarly, Cell surface hydrophobicity percentage using hexadecane has been observed from 55-95% while selecting LAB as probiotics (Pinto et al., 2006).

Being resistant to low pH is one of the major selection criteria for probiotic strains (Ouwehand et al., 2001, Cotter et al., 2005). Since, the destination of the probiotics is small intestine and they have to pass through stressful condition of stomach to reach the small intestine they have to pass through from the stressful conditions of stomach (Chen and Hoover,2003;). Therefore a strains chosen for probiotic must have acid tolerance capacity of stomach acidity. In a similar study it was reported that the maximum antibacterial activity was exhibited in an acidic pH range of 2 to 6, while inactivation occurred at pH 8 to 12 (Maldonado et al., 2012). At pH 6 bacteriocin activity was highest (Meera and Devi, 2012).

Another criterion of being probiotic is the strain must have bile tolerance capacity. On increasing the concentration of Sodium deoxycholic acid and Sodium taurocholate the growth was severely hindered. The strains, resistant to low pH, were further screened for their ability to tolerate the bile salt. The isolate RB and LC shown highest bile tolerance about 0.8%. The bile salt concentration of human intestinal tract is varied but it is assumed that it is approx 0.3% w/v and with staying time 4 hrs (Prasad et al., 1998).

5. CONCLUSION

The aim of present day study was to isolate bacteriocin producing Lactic Acid Bacteria (LAB) and to determine their antimicrobial property against enteropathogens. Morphologically 5 distinct LAB were recovered from different dairy products. All the isolate were gram positive, catalase negative and nitrate positive. All the isolates were found to be positive for the production of antibacterial activity demonstrates by Agar well diffusion assay method. The bactericin producers were again tested for various probiotics attributes including temperature tolerance, ph tolerance, cell surface hydrophobicity and bile salt tolerance test. Therefore, from the above study it can be concluded that dairy products are the potential source of lactic acid bacteria as well as probiotic lactic acid bacteria. The isolate LC can tolerate a pH of 2.5, with cell surface hydrophobicity 53.8% and bile salt tolerance upto 0.8%. Therefore the isolate LC fulfill the criteria to be considered as probiotic isolate.

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7. ACKNOWLEDGEMENTS

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