INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

ISOLATION AND CHARACTERIZATION OF LACTOBACILLI FROM CAECUM OF RAT FED WITH FRUCTOOLIGOSACCHARIDES

Jayashri G. Mahore *, Satish V. Shirolkar
Department of Pharmaceutics, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, Maharashtra, India
*Corresponding Author Email: jayashri_mahore@rediffmail.com

Article Received on: 05/06/18 Approved for publication: 30/06/18

DOI: 10.7897/2230-8407.096119

ABSTRACT

Aim: Administration of fructooligosaccharide supports the growth of beneficial bacteria. This study aims to isolate and characterize *Lactobacilli* from caecum of rat fed with FOS. Materials and Methods: *Lactobacilli* were isolated from the caecum of the rat fed with fructooligosaccharide for one month. Isolated *Lactobacilli* were characterized to study the probiotic potential and effectiveness of antimicrobial activity against *S. aureus* and *E. coli*, common pathogens of large intestine. Agar well diffusion method was used to study the antimicrobial activity. Growth conditions (temperature, pH and incubation conditions) for isolate were optimized using full 3^3 factorial design. Results and Discussion: The isolates were found to be facultative anaerobes, Gram positive, non-spore forming and non motile forming either *bacilli* or *coccobacilli*. Optimization study revealed when isolates grown at 37°C, pH 7 and anaerobic conditions, maximum growth was obtained. Conclusion: Isolated *Lactobacilli* showed acid and bile resistance. Bacteriocin produced by the isolated *Lactobacilli* (RCL3) from rat caecum showed antimicrobial activity against *S. aureus* and *E. coli.*

Keywords: Lactobacilli, Fructooligosaccharides, Bacteriocin, Rat caecum

INTRODUCTION

Healthy gut flora is very important for wellbeing of human which assimilates the nutrients, synthesize vitamins[1] and ferment carbohydrates to produce short chain fatty acids[2] Gut flora gets disturbed because of chemotherapy, antibiotic associated diarrhea,[3] colon cancer[4] and certain diseases such as irritable bowel disease.[5] Dysbiosis, disturbed gut flora can be restored by administration of probiotics and prebiotics. Probiotics are live microorganisms which when administered in adequate amounts confer health benefits.[6] Probiotics are susceptible to external environment and changing environment of GI tract. So prebiotic was come into existence to restore the disturbed microorganisms. Prebiotics are the substances when administered reach the large intestine virtually intact. Fermentation of FOS in large intestine supports the growth of beneficial microorganisms. FOS is extensively studied prebiotic which reaches the colon and acts as fertilizer for the growth of beneficial microorganism.[7] These beneficial microorganisms include lactic acid bacteria (*Lactobacilli and Bifidobacteria*). *Lactobacilli* produce organic acids, hydrogen peroxides, diacetyl and bacteriocin and use all this inhibitory compound as weapons for its survival. Microbiologists have shown special interest in bacteriocin because its ability to control the growth of pathogenic bacteria.

Though discovery of more than 100 antibiotics increased the average life span and decreased the mortality rate significantly but non-judicious use of antibiotics throughout the world contributed for the development of high antibiotic resistance. Because of overuse and inappropriate prescribing, antibiotic resistance is life threatening problem and infections may become non-curable owing to high level of multiple drug resistant pathogens.[8] If the pathogens are resistant to first line of antibiotics, then second line of antibiotic is used. Some second line drugs are more expensive and also have severe side effects. Due to antibiotic resistance, it was necessary to find out some solution which would help to fight with the antibiotic resistance. Bacteriocin produced by the *Lactobacilli* has given relief to some extent to fight against antibiotic resistance. Bacteriocin has antimicrobial activity and extensively studied against pathogens.

The *Lactobacilli* has been isolated from raw cattle milk sample[9], vagina[10] traditionally homemade white pickledcheese,[11] Meat,[12] fermented materials[13] and effect of bacteriocin produced by *Lactobacilli* isolated from different source was studied against pathogens.[14,15.] The present research work aimed to isolate *Lactobacilli* from caecum of rat fed with FOS.

MATERIALS AND METHODS

FOS was purchased from Himedia, Mumbai, India. deMan-Rogasa - Sharpe medium (MRS), other media and ingredients to prepare media were procured from Himedia, Mumbai, India. All the other chemicals used were of analytical grade. All reagents and media were prepared in sterile distilled water.

The animal protocol was sanctioned by the Animal Ethical Committee of the Institute. (DYPIPSR/IAEC/17-18/P-18).

Collection of caecum content

The animal protocol was sanctioned by the Animal Ethical Committee of the Institute. 10 weeks old healthy male Wistar rats (body weight of 150-180 g) were housed in stainless steel wire bottom cages in a temperature controlled room and 12 h light and 12 h dark cycle. Rats were fed with animal diet and water *ad*

libitum for 4 weeks. Animals were divided into two groups (n=6). Group I control: no treatment was given to this group. Group II was fed with FOS (200 mg/day/rat) suspended in water for 4 weeks. After 4 weeks the rats were fasted for 18 h before dissection. Rats were killed by cervical dislocation and digestive tract was excised. Both ends of the caecum were tied with string to ensure that the cecal contents were not exposed to the air, and the caecum was cut from the digestive tract. Immediately after removal, the surface of the caecum was sterilized by soaking it in 70% ethanol for one or two seconds. Ceca were collected aseptically, and content was squeezed into an empty sterile plastic container on dry ice. Caecum Prepared smear was taken on a clean grease free slide. Smear was allowed to dry then content was processed immediately using phosphate buffered saline and then homogenized. The homogenized sample was inoculated onto MRS and incubated at 37ºC in a candle jar for 48 h. Log cfu/g was calculated for the control and treated group. Log cfu/g of treated group (FOS) was significantly higher than log cfu/g of control group. Ashara *et al*. (2001)[7]studied *in vitro* prebiotic potential of FOS and found significant decrease in animal and human pathogens and increase in beneficial one.

Total 150 colonies were counted from FOS treated group. Out of these, 50 colonies were randomly selected and streaked onto MRS agar plates. Typical colonies were subjected to morphological and biochemical characteristics. After the incubation, colonies were restreaked on the MRS agar petri plate for the formation of isolated colonies. Then from these plates isolated colonies were restreaked on MRS agar slants and stored at 4°C.

Gram staining test

Prepared smear was taken on a clean grease free slide. Smear was allowed to dry then heat fixed. Then smear was flooded with crystal violet (primary strain) and kept for 1 minute before washing with water and then covered with Gram's iodine (mordant). The smear was again washed with water followed by decolorization with 95% ethyl alcohol. Safranin was added as counter stain and kept for 1 min before washing. The smear was washed with water, air dried and observed under oil- immersion objective.[16]

Motility test

Vaseline or petroleum jelly was spread on the four corner of a clean cover slip, with the help of toothpick. A small drop of the bacterial suspension was kept in the centre of a cover slip. The hanging drop slide was turn over and place on the stage of the microscope so that the drop was over the light hole. The drop was first locating by its edge under low power and then focused on the drop. The high-power objective (40 x) was used to increase the contrast and observe the motility of bacteria.[17]

Catalase test

A single isolated colony was streaked on a glass slide and one drop of 3% hydrogen peroxide was added on it. The effervescence of oxygen indicated the positive response of the bacteria to catalase test.

Sugar fermentation test

Glucose (100 µl) of the glucose solution was added to previously sterilized 5 mL of nutrient broth containing 1 mL phenol red. Phenol red is reddish orange at pH 7.4 and turns yellow at pH 6.4. Fermentation of glucose produces lactic acid which decreases pH and causes change in color. All the tubes were inoculated with 18 h freshly grown bacterial culture and incubated at 37ºC for 48 h. One tube was maintained without inoculation as control, to check the sterility of the medium.

Survival of lactobacillus spp. under acidic conditions

Orally administered *Lactobacilli* should survive the acidic condition of GI tract. To evaluate the ability of *Lactobacilli* to survive in acidic condition, freshly grown culture was mixed with simulated gastric acid for specific time and then aliquots were spread on MRS agar to check the viability and result calculated as log cfu/mL.

Survival of lactobacillus spp. in the presence of bile salts

Freshly grown *Lactobacilli* were grown in MRS broth containing 0.3% oxgall. Aliquots were taken initially and after 24 h of incubation and spread on MRS agar to check the viability of the isolates. Viable counts were determined by pour plate method of all the samples using 10-fold serial dilutions.

Bacterial adhesion to hydrocarbons test (BATH)

Overnight cultures were harvested by centrifugation at 3000×g (Cooling centrifuge, C24, Remi Electro Tech, India) for 15 min at 4ºC and washed with PBS and resuspended in the same buffer, after which the absorbance (A_{600}) of the cell suspension was measured. Equal proportions of viable bacterial culture and xylene were then mixed by vortexing for 2 min.

A two-phase system developed and the aqueous phase was removed for determination of absorbance after 30 min at room temperature. Affinity to hydrocarbons (hydrophobicity) was reported as the average percentage of 3 replicates, according to the formula

$$BATH\% = [(A_0\text{-}A)/A_0] \times 100$$

where, A_0 and A are the absorbance before and after mixing with xylene, respectively.

Auto-aggregation analysis

Aggregation of the bacterial cultures was screened using spectrophotometric assays, as described by Collado *et al*. (2008).[18] Overnight cultures were centrifuged and washed twice with PBS buffer and suspended in the same buffer. Equal volumes of each of the microbial suspensions were combined in sterile test tubes and mixed for 10s on a vortex mixer, and then incubated at 37ºC for 24 h without agitation, anaerobically.

$$\% \text{ Aggregation} = A_{initial} - A_{final} / A_{initial} \times 100$$

Where, A_{final} represents the absorbance of the culture after incubation

The auto-aggregation properties of *L. acidophilus* and pathogen strains were measured over 24 h at 37°C using absorbance measurements at 600 nm.

Co-aggregation analysis

Overnight cultures of *Lactobacilli*, *S. aureus* and *E. coli* were centrifuged. All the cultures were washed once with sterile water and suspended in PBS. The test was scored positive if sedimentation of the cells was visible within 2 h at room temperature. Appropriate controls containing *Lactobacilli* and

indicator strain (*E. coli* and *S.aureus*) suspensions in PBS were used.

$$\% \text{ Co-aggregation} = [(A_{pat} + A_{probio}) - (A_{mix})/(A_{pat} + A_{probio})] \times 100,$$

Where, A_{pat} and A_{probio} represent A_{600} of the pathogen and probiotics suspensions at time 0 min and Amix represents A_{600} of the mixed bacterial suspension.

Aggregation (auto- and co-aggregation) abilities of micro-organisms were screened by visual observation, and the degree of aggregation was recorded on a scale 0 to 4+ as follows: a score of 0 for no visible aggregates in the cell suspension, 1+ for small uniform aggregates in the suspension, 2+ for aggregates that were easily seen but did not settle, 3+ for large aggregates which settled and left some turbidity in the supernatant fluid, and 4+ for large aggregates which settled and left clear supernatant fluid. Co-aggregation was considered to occur when the score in the reaction mixtures was greater than the auto-aggregation score of either strain in monoculture.

Determination of zone of inhibition

Preparation of culture supernatants

The bacteriocin-producing strains were grown in MRS broth at 37°C for 18-20 h. The *Lactobacilli* culture was centrifuged at 10,000 rpm for 5 min, and then the supernatant was adjusted to pH 6.5-7.0 with 1N NaOH.

The antagonistic effects of culture supernatants of bacteriocin producing Lactobacillus strains on Gram positive and Gram-negative organisms were tested by the agar-well-diffusion assay. In brief, 6 mm-diameter wells were made on pre-inoculated agar media and each well was filled with culture supernatant of bacteriocin producing Lactobacillus strains after neutralization with NaOH. *S. aureus and E. coli* was grown on Nutrient agar and MacConkey agar respectively. All cultures were grown aerobically at 37°C for 48 h. Inhibition zones around the wells were measured and recorded. Bacteriocin producing strains were further characterized for bacteriocin assay. Sensitivity of Bacteriocin to heat and pH was checked

To test heat sensitivity, 100 µl of culture supernatant was heated for 10 min at 40°C, 50°C, 60 °C and 70°C. The resistant culture supernatants were further heated for 10, 20, 30 and 40 min at 40°C. To test pH sensitivity, the pH of culture supernatants was adjusted to 3.0, 4.5, 7.0 and 9.0 and then kept at room temperature for 4 h. The agar well diffusion test was performed to detect residual activity.

Optimization of growth condition of isolates

Briefly, 100µL of overnight grown isolate was added in 10 mL MRS broth, pH was adjusted to 1.5, 3 and 7.2 using HCl/NaOH. This 10 mL broth was grown at different incubation conditions (aerobic, anaerobic and microaerophilic) for 48 h at 37°C. After 48 h, OD_{600nm} was measured and noted to determine the effect of condition on the growth of isolated *Lactobacilli*.

Optimum condition for the growth of isolates was determined by response surface methodology. Design Expert version 10 Software (Stat-Ease, Inc., Minneapolis, MN) was used to perform response surface methodology. Effect of three independent variables (temperature, pH and incubation condition) on dependent variable (OD) was evaluated by 3^3 full factorial designs. Analysis of variance generated by software representing coefficient of determination (R^2) and the Fisher test value (F-value) was used to evaluate adequacy of model. Statistical significance of the model and model variables was determined at the 5% probability level ($p < 0.05$). Software used quadratic model equation to build response surface. Two-dimensional contour plot and three dimensional response surface was generated by the software for each incubation condition. The coded values and corresponding actual values of the optimization parameters used in the Response surface analysis are given in table 1. Complete design using three independent variables at three different levels consisted of 27 experimental points.

Table 1: The Coded Values and Corresponding Actual Values of the Optimization Parameters Used in the Response Surface Analysis

Code	Temperature (°C) A	pH B	Incubation Condition C
-1	30	1.5	Aerobic
0	37	3	Microaerophilic
+1	45	7.2	Anaerobic

Table 2: Colony characteristics of isolated strain

Isolate	Types of colony	Gram staining	Microscopic morphology	Spore testing	Motility test	Catalase test	Fermentation test
RCL1	Small with rough surfaces	+	Short rods	-	-	-	Acid
RCL2	Large with rough surfaces	+	Short rods	-	-	-	Acid
RCL3	Small with smooth surfaces and convex colonies	+	Short rods	-	-	-	Acid
RCL4	Small with smooth surface, flat colonies	+	Cocci and bacilli	-	-	-	Acid and Gas
RCL5	Large with smooth surfaces	+	Mixture of long and short rods	-	-	-	Acid and Gas

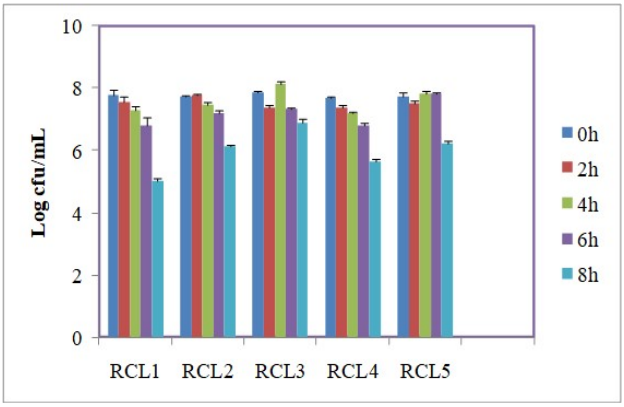

Figure 1: Acid tolerance ability of isolated strains of *Lactobacilli* spp. over 8 h

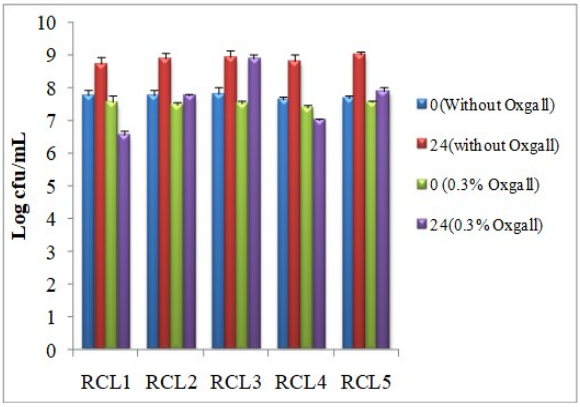

Figure 2: Bile tolerance ability of isolates

Table 3: Hydrophobicity shown by isolated colonies

Strain	% BATH
RCL1	49.4±6.2
RCL2	39.85±0.81
RCL3	60.85±0.73
RCL4	51.22±1.35
RCL5	56.7±0.65
E.coli	41.6±3.4
S.aureus	21.2±4.1

Table 4: Percent auto-aggregation of probiotic and pathogenic strains

Bacterial strains	% Auto Aggregations		Visual Score after 24 h of incubation
	0 h	24 h	
RCL1	4.5±0.4	20.09±8	1+
RCL2	3.1±0.7	34.5±2.6	3+
RCL3	5.1±1.1	44.4±4.7	4+
RCL4	3.2±0.9	15.9±6.3	1+
RCL5	1.4±0.4	28.8±2.3	3+
E.coli	2.9±1.3	29.16±7.6	3+
S..aureus	5.7±3.7	44.09±9.1	3+

Mean ±S.D. of three separate experiments

Table 5: Percent Co-aggregation of probiotic and pathogenic strains

Isolates	*E. coli*		*S. aureus*	
	%Co-aggregations	Visual observation	% Co-aggregations	Visual observation
RCL1	30±2.6	2+	45±3.6	3+
RCL2	39±1.8	3+	57±5.7	4+
RCL3	48.3±2.3	3+	64.3±2.9	4+
RCL4	36±3.5	2+	40.9±1.0	3+
RCL5	23±1.4	2+	53.6±2.4	4+

Mean ±S.D. of three separate experiments

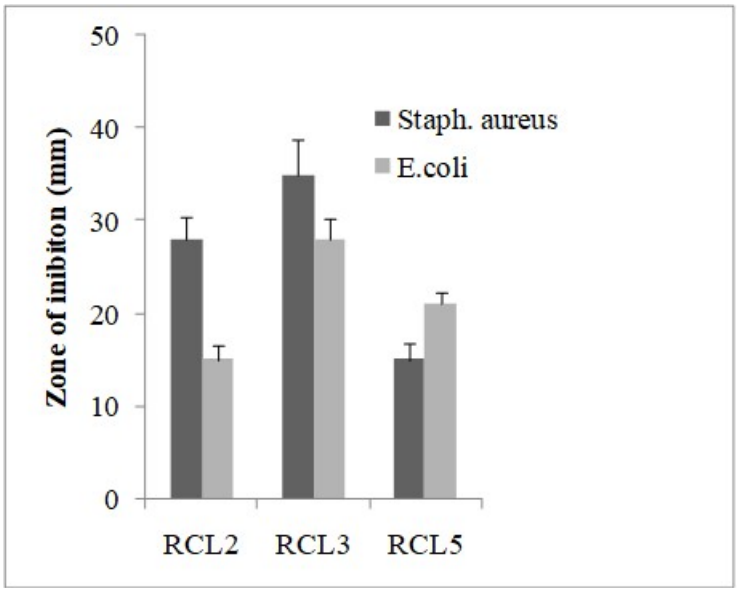

Figure 3: Antimicrobial activity of bacteriocin produced by RCL2, RCL3 and RCL5 against *S. aureus* and *E. coli*

Table 6: Characterization of Bacteriocin produced by different *Lactobacilli* colonies

Sample	Sensitivity to heating (10 min) at different temperature (°C)				Sensitivity to constant temperature (40°C) with variation in time (min)				Sensitivity to different pH			
	40	50	60	70	10	20	30	40	3	4.5	7	9
RCL2	R	R	R	S	R	R	R	S	S	R	R	S
RCL3	R	R	R	R	R	R	R	R	R	R	R	R
RCL5	R	R	S	S	R	R	S	S	R	R	R	R

R-Resistant, S- Sensitive

Table 7: Trial of 27 runs with two variables and one response; OD (600nm)

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	A:Temperature	B:pH	C:Incubation condition	OD
11	1	0	-1	Level 2 of C	0.6
2	2	0	-1	Level 1 of C	0.7
22	3	-1	0	Level 3 of C	1.2
7	4	-1	1	Level 1 of C	1.1
9	5	1	1	Level 1 of C	0.6
5	6	0	0	Level 1 of C	1
10	7	-1	-1	Level 2 of C	0.4
20	8	0	-1	Level 3 of C	0.9
6	9	1	0	Level 1 of C	0.6
15	10	1	0	Level 2 of C	0.8
26	11	0	1	Level 3 of C	1.9
4	12	-1	0	Level 1 of C	0.8
21	13	1	-1	Level 3 of C	0.6
18	14	1	1	Level 2 of C	0.7
3	15	1	-1	Level 1 of C	0.3
24	16	1	0	Level 3 of C	1.1
16	17	-1	1	Level 2 of C	0.7
8	18	0	1	Level 1 of C	1.6
12	19	1	-1	Level 2 of C	0.6
14	20	0	0	Level 2 of C	1.1
17	21	0	1	Level 2 of C	0.8
23	22	0	0	Level 3 of C	1.5
25	23	-1	1	Level 3 of C	1.5
19	24	-1	-1	Level 3 of C	0.9
27	25	1	1	Level 3 of C	0.7
1	26	-1	-1	Level 1 of C	0.5
13	27	-1	0	Level 2 of C	0.6

Table 8: Analysis of variance for the fitted quadratic polynomial model

Source	F-value	p-value	
Model	9.43	0.0001	**significant**

Table 9: Estimated regression model of relationship between dependent (OD) and independent variables (A, B and C)

Variables	F-value	P-value
A-Temperature	4.86	0.0435
B-pH	28.29	< 0.0001
C-Incubation conditions	14.82	0.0003
AB	2.52	0.1330
AC	3.65	0.0510
BC	2.24	0.1410
$\mathbf{A^2}$	23.70	0.0002
$\mathbf{B^2}$	2.97	0.1055

F-value and p-value was used to determine the significance of each term (table 9). It could be seen that response, OD was affected significantly by B ($p< 0.0001$), C ($p<0.05$), AC ($p<0.05$), and A^2 ($p<0.01$).

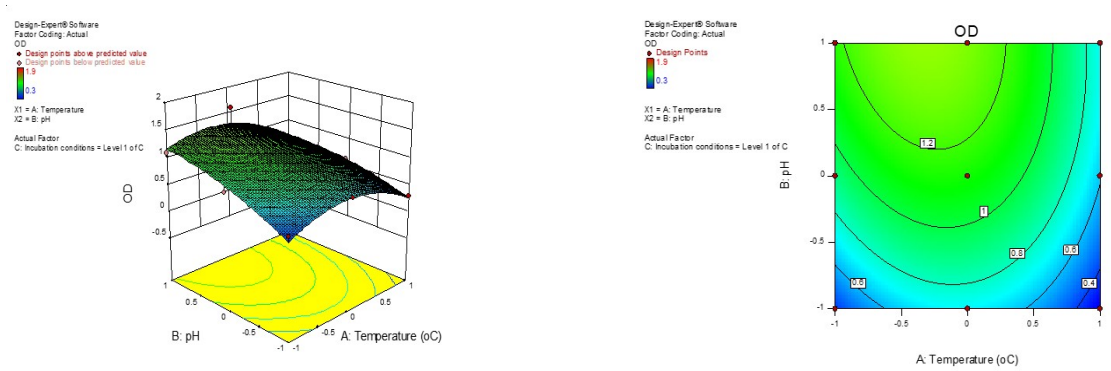

Figure 4: Response surface plot and contour plot for effect of temperature, pH and aerobic incubation temperature on OD (600nm)

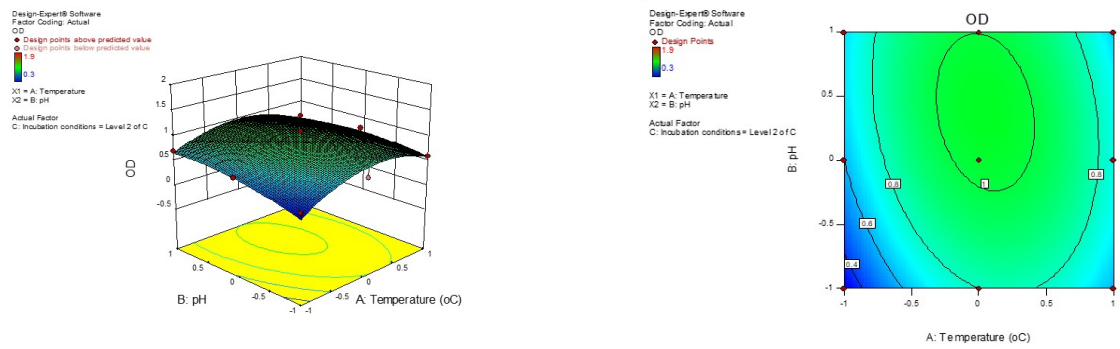

Figure 5: Response surface plot and contour plot for effect temperature, pH and microaerophilic incubation conditions on OD (600 nm)

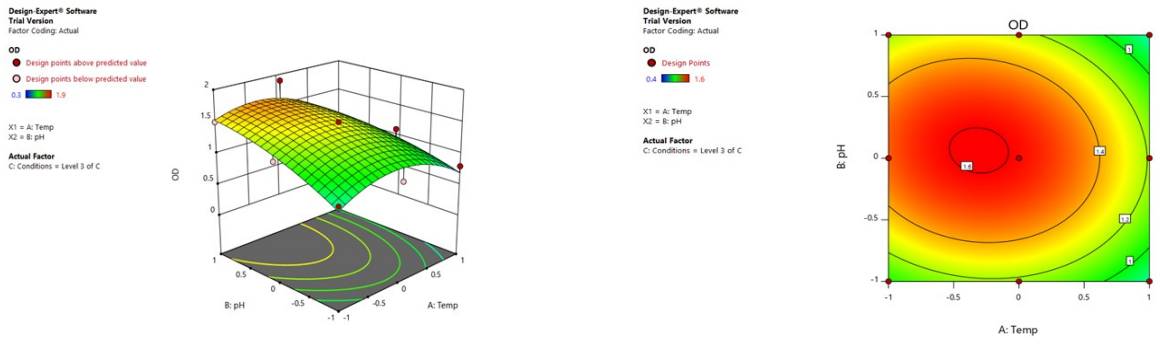

Figure 6: Response surface plot and contour plot of temperature, pH and anaerobic incubation temperature

RESULTS AND DISCUSSIONS

Colony characteristics of isolated strains

Total 150 colonies were counted from FOS treated group. Out of these, 50 colonies were randomly selected and streaked onto MRS agar plates. Typical colonies were subjected to morphological and biochemical characteristics. Only colonies showing Gram positive and catalase negative reaction were selected for further studies. Some of the isolated colonies were having smooth edge, convex, translucent appearance while other having moist and creamy, dry and granular with flat, matte surface colonies characteristics. Those colonies (RCL1-RCL5) showing Gram positive and catalase negative test were reported in table 2.

Isolates were non-spore forming, and non-motile with production of acid and/or acid and gas in Duhrams tube. Colonies with Gram positive and catalase negative reaction were picked up and further resolved for morphological, cultural and biochemical characteristics. Sugar fermentation pattern showed that some of the isolates were homofermentive and some were heterofermentive. The microorganism fermented glucose to acid which was evident by changing color of medium from red to yellow. From the morphological, cultural and biochemical characteristics, it was concluded that identified bacteria were from Lactobacillus spp.

Survival of *Lactobacillus spp.* under acidic condition

After oral administration, *Lactobacilli* are subjected to various pH of gastrointestinal tract. The ability of Lactobacillus to withstand with the changing atmosphere was checked in simulated gastric juice. Those bacteria who survived better in changing acidic condition could colonize in the gut. Survival of five strains under acidic condition is illustrated in figure 1.

RCL3 colonies showed better survival (6.87 log cfu/mL) compared to other isolates. RCL1 was unable to survive and

number of colonies dropped significantly. Marginal decrease was observed in other isolates.

Survival of *Lactobacillus* spp. in the presence of bile

Lipophillic nature of bile makes it suitable candidate for disruption of structure envelop, affecting both cell and colony morphology.[19] Varying level of bile concentration in the intestine influences the viability of Lactobacillus in the GI tract. Davenport (1977) [20] reported that 0.5 to 2% of bile concentration was observed in first hour of digestion and then decreases subsequently. The rate of bile secretion and the concentration of food consumed mainly affect the bile concentration in the intestine. Bile tolerance ability was checked in presence and absence of bile salt at concentration of 0.3% oxgall.

The addition of bile in MRS influences the growth and growth pattern of isolates. RCL1 was more susceptible to oxgall with decrease in bacterial growth form log 7.57 to log 6.57. RCL3 showed resistance (Fig 2) with 2 log increase in bacterial growth. RCL2 and RCL5 strains showed moderate growth in presence of bile. Resistance of RCL3 to bile could be due to one of the following mechanism; extrusion of the bile acids that accumulate in the cytoplasm trough efflux pump[21], four transporters in *Lactobacillus acidophilus* NCFM, LBA0552, LBA1429, LBA1446, and LBA1679 might be participating in efflux process[22]; metabolism of unconjugated (glycine and taurine free) acid by gut bacteria produced by bile salt hydralase. From the isolates smooth colonies (RCL3) showed more resistance to bile than rough colonies (RCL1). Changes in envelop architecture making smooth colonies more resistant than rough one might be another reason for resistant power of smooth over rough one.[23] Duary *et al.* (2012)[24] found that *L. plantarum* isolates were over represented the genes coding for surface-associated proteins, such as mucus-binding protein (*mub*), or mucus adhesion promoting protein (*mapA*) when the *L. plantarum* grown in media supplemented a mixture of mucin and bile. All the isolates derived from the caecum fed with FOS showing marginal resistance to bile. Discussed the production of external exopolysacharide (EPS) layers is an extended trait among intestinal bacteria. As RCL3 was isolated caecum, formation of external exopolysachharide might be one of the reasons for resistance to bile.

Bacterial adhesion to hydrocarbons (BATH) test

Viscosity of the hydrocarbons or size of droplets formed during mixing affects the hydrophobicity of Lactobacillus.[25] Hydrophobicity study was performed to check adhesion ability of isolates to biological GI membrane. Highest hydrophobicity was shown by RCL3 > RCL2>RCL5>RCL4>RCL1 (Table 3).

Auto-aggregation analysis

Ability of beneficial microorganism to aggregate and colonize in the gut was studied by the autoaggregation ability. Aggregation is very important for colonization and longtime persistence of beneficial microorganism. Beneficial microorganism should aggregate rapidly to form biofilm which can prevent the attachment of pathogens. Highest autoaggregation was showed by RCL3>RCL2 >RCL5>RCL4 >RCL1. (Table 4)

Co-aggregation analysis

Displacement ability of pathogens by beneficial microorganisms is important property of probiotic organism and has greater advantage over non-co-aggregating pathogens. Collado *et al.* (2007)[26] suggested that the beneficial microorganism forms physicochemical barrier with pathogens and prevent colonization of pathogens. Self-aggregation may substantially increase the colonization potential of *Lactobacilli* in environments with short residence times such as GIT. Highest coaggregation was shown by RCL3 compared to other isolates. (Table 5)

Determination of zone of inhibition

From the colony characteristics it was concluded that isolates were of *Lactobacilli* species. Only isolates which has shown significant antimicrobial activity against pathogens were screened for further characteristics. Highest zone of inhibition was shown by bacteriocin produced by RCL3 isolates (35 mm against *S. aureus* and 28 mm against *E. coli*). Bacteriocin produced by RCL2 and RCL5 showed less activity with zone of inhibition of 28 mm and 15 mm against *S. aureus* and 15 and 21 mm against *E. coli* respectively (Figure 3). *Lactobacilli spp.* produces antimicrobial substances such as organic acids, fatty acids, diacetyl and acetoin. *Lactobacilli* use these antimicrobial substances as weapon to fight against pathogens. Antimicrobial action of produced bacterocin might be due to increased permeability of cytoplasmic membrane of target cell. Other mechanism might be due to production of lactic acid as end product of fermentation. Lactic acid not only decreases pH of the surrounding but undissociated form function as permeabilizer of the Gram-negative bacterial outer membrane[27] and thus lactic acid synergies action of other antimicrobial substances.[28] Effect of bacteriocin is more profound on Gram Positive than Gram negative bacteria as it mainly interacts with the cell membrane and alters its properties. Other mechanism of action of bacteriocin includes lowering of membrane potential,[29] prevention of uptake of amino acids, triggering of release from the cell[30] exclusion of potassium ions, depolarization of cytoplasmic membrane, hydrolysis and partial efflux of cellular ATP[31,32] and endonuclease activity on sensitive cell.[33]

RCL3 showed quite good resistance against heat and pH compared to RCL2 and RCL5 (Table 6).

Optimization of growth conditions

RCL3 showed most significant results in all evaluated parameters and therefore it was decided to optimize growth parameters for RCL3.

Table 7 shows results obtained from 3^3 full factorial design regarding studied variables: temperature (A), pH (B) and incubation condition (C) on growth of isolated *Lactobacilli spp.* The highest OD obtained achieved in the verification experiment was 1.9

Following equation demonstrated that OD was a function of test variables in coded units. Quadratic equation (1) was generated by the software which represents relationship between OD and each coded variable

$$Y(OD) = +1.21 - 0.094A + 0.23B - 0.081C[1] - 0.18\,C[2] - 0.083\,AB - 0.056\,AC[1] + 0.16\,AC[2] + 0.072\,BC[1] - 0.13\,BC[2] - 0.36A^2 - 0.13B^2 \quad \text{Equation 1}$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The high levels of the factors are coded as +1, middle levels as 0 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 8 results for OD in form of analysis of variance (ANOVA). A very low probability value ($p<0.0001$) indicated that the model is highly significant. Determination coefficient (R^2) of 0.8737 was used to determine goodness-of-fit of the model. In this case, R^2 value (0.8737) indicates that sample variation for OD of 87.37% was attributed to independent variables and 12.63% of the total variations cannot be explained by the model

Variable B (pH) had significant effect ($p< 0.001$) (table 9). A positive coefficient in (Equation 1) indicates increase in pH led to an increased OD. Change in bacterial membrane with acid stress and disruption in DNA and peptidoglycan of Gram + bacteria could be probable reason for decreased OD with decrease in pH. This results in morphological and phenotypic changes which creates unfavorable condition for further growth.[34] This result is in accordance with previous research which concluded the increased sensitivity to pH below 3.[35,36] Quadratic parameter A^2 also had significant impact ($p<0.001$) on the OD, a negative coefficient revealed decrease in OD when it increased in the system.

Graphical representation of 3D response surface and 2D contour plot gives relationship between independent and dependent variables. Graphical representation of quadratic equation was given by 3D response surface plot. 3D response surface plot helps to locate the optimum level of each variable for maximum response. The convex response surfaces suggest that there are well-defined optimal variables. If the surfaces are rather symmetrical and flat near the optimum, the optimized values may not vary widely from the single variable conditions.[37] Different interaction between variables is given by different shapes of the contour plot. Interaction between variables is given by an elliptical contour plot whereas circular contour plot indicates means otherwise. Figure 4 and figure 5 showed interaction between temperature and pH when incubated in aerobic and microaerophilic condition respectively. Both figures indicate that increase in temperature from -1 to 0 improved OD. However, after 0, there was decrease in OD. This might be because of heat stress. Heat stress not only cause protein denaturation but also causes injury to membrane and nucleic acid.[38] Heat stress also decreases intracellular pH by disturbing transcellular proton gradient.[39] also observed sensitivity of exponential phase cells of *Lactobacillus delbrueckii subsp. bulgaricus* to temperature in same temperature range. Every microorganism has optimum temperature at which maximum growth occur. Minimum and maximum temperature is respectively the temperatures below and above which growth is not possible.

Figure 6 showed interaction between temperature and pH when incubated in anaerobic condition. Response surface showed highest OD in anaerobic condition compared to aerobic and microaerophilic condition. Formation of reactive oxygen species are formed during conversion of O_2 to H_2O. These are superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO*). Ability of O_2^- to penetrate and to interact with cellular protein could be having detrimental effect on growth of *Lactobacilli* in aerobic incubation condition. HO* can target proteins, lipids and DNA to add detrimental effect of aerobic incubation of isolated *Lactobacilli*.

One can observe form the results of all run and from equation 1; temperature and pH has significant effect on growth and change in incubation did not show significant effect between anaerobic and aerobic. *Lactobacilli* used for optimization study was isolated from rat caecum, quite anaerobic conditions and therefore it might be showing high growth in anaerobic condition. *Lactobacillus* is an example of microaerophile, means that requires oxygen to survive but in low concentration than present in the atmosphere. This could be probable reason for no significant difference in OD when incubated in microaerophillic and anaerobic conditions. Desirability suggested by the software for optimum condition to get maximum viable count of *Lactobacillus* isolates was 0.55 for temperature, 0.9 for pH and level 3 of C in coded value.

CONCLUSION

Intestinal microbial ecology is not a spontaneous process; it is a result of evolutionary process. Disturbed ecology can be restored by the application of new food additives to dairy and other food products, which can stimulate growth of lactic acid bacteria and increase their antagonistic activity against pathogens. Log cfu/mL count of *Lactobacilli* of caecum of rat fed with FOS was significantly higher than control group. RCL3 showed bile resistance and the bacteriocin produced by RCL3 showed resistance to heat and pH. This new approach to restore and elevate the growth of beneficial microorganism can offer great possibility to isolate *Lactobacilli* from caecum of rat fed with FOS.

REFERENCES

1. Burkholder PR, McVeigh I. Synthesis of vitamins by intestinal bacteria. Proceedings of the National Academy of Sciences. 1942;28(7):285-9.
2. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 2016;7(3):189–200.
3. Issa I, Moucari R. Probiotics for antibiotic-associated diarrhea: do we have a verdict?. World journal of gastroenterology: WJG. 2014;20(47):17788.
4. Erdman SE, Poutahidis T. Gut bacteria and cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2015;1856(1):86-90.
5. Matsuoka K, Kanai T. The gut microbiota and inflammatory bowel disease. In Seminars in immunepathology, Springer Berlin Heidelberg. 2015;3(1)47-55.
6. Mack DR. Probiotics: Mixed messages. Canadian Family Physician. 2005;51(11):1455.
7. Asahara T, Nomoto K, Shimizu K, Watanuki M, Tanaka R. Increased resistance of mice to Salmonella enterica serovar Typhimurium infection by synbiotic administration of *Bifidobacteria* and transgalactosylated oligosaccharides. Journal of Applied Microbiology. 2001;91(6):985-96.
8. Ventola CL. The Antibiotic Resistance Crisis, Part 1: Causes and Threats. Pharmacy and therapeutics 2015;40(4):277–283.
9. Mohankumar A, Murugalatha N. Characterization and antibacterial activity of bacteriocin producing Lactobacillus isolated from raw cattle milk sample. International Journal of Biology. 2011;3(3):128.
10. Parolin C, Marangoni A, Laghi L, Foschi C, Palomino RA, Calonghi N, Cevenini R, Vitali B. Isolation of vaginal *Lactobacilli* and characterization of anti-Candida activity. PLoS One. 2015;10(6):e0131220.
11. Lozo J, Vukasinovic M, Strahinic I, Topisirovic L. Characterization and antimicrobial activity of bacteriocin 217 produced by natural isolate Lactobacillus paracasei subsp. paracasei BGBUK2-16. Journal of food protection. 2004;67(12):2727-34.
12. Schillinger U, Lücke FK. Antibacterial activity of Lactobacillus sake isolated from meat. Applied and environmental microbiology. 1989;55(8):1901-6.
13. Zhang Y, Zhang L, Du M, Yi H, Guo C, Tuo Y, Han X, Li J, Zhang L, Yang L. Antimicrobial activity against Shigella sonnei and probiotic properties of wild *Lactobacilli* from

fermented food. Microbiological research. 2011;167(1):27-31.
14. Dasari S, Shouri RN, Wudayagiri R, Valluru L. Antimicrobial activity of Lactobacillus against microbial flora of cervicovaginal infections. Asian Pacific journal of tropical disease. 2014;4(1):18.
15. Coconnier MH, Liévin V, Bernet-Camard MF, Hudault S, Servin AL. Antibacterial effect of the adhering human Lactobacillus acidophilus strain LB. Antimicrobial Agents and Chemotherapy. 1997;41(5):1046-52.
16. Kokare CR. Pharmaceutical Microbiology, Experiments and Techniques, Bacterial staining and motility, Grams Staining, 2nd edition, Career Publications, 2008:42-44.
17. Kokare CR. Pharmaceutical Microbiology, Experiments and Techniques, Bacterial staining and motility, Hanging Drop Technique, 2nd edition, Career Publications, 2008:54-57.
18. Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. The American journal of clinical nutrition. 2008;88(4):894-9.
19. Kurdi P, Kawanishi K, Mizutani K, Yokota A. Mechanism of growth inhibition by free bile acids in *Lactobacilli* and bifidobacteria. Journal of bacteriology. 2006;188(5):1979-86.
20. Yogeswara A, Rahayu E, Utami T. Resistance Of Lactic Acid Bacteria Isolated From Indonesian Fermented Foods In Simulated Gastric Juice And Bile Solution. Virgin: Jurnal Ilmiah Kesehatan Dan Sains. 2015;1(2).
21. Piddock LR, Margolles A, Sanchez B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Front Microbiology. 2013;4:396.
22. Ruiz L, Margolles A, Sánchez B. Bile resistance mechanisms in Lactobacillus and Bifidobacterium. Frontiers in microbiology. 2013;4:396.
23. Šušković J, Kos B, Matošić S, Besendorfer V. The effect of bile salts on survival and morphology of a potential probiotic strain Lactobacillus acidophilus M92. World Journal of Microbiology and Biotechnology. 2000;16(7):673-8.
24. Duary RK, Batish VK, Grover S. Relative gene expression of bile salt hydrolase and surface proteins in two putative indigenous Lactobacillus plantarum strains under in vitro gut conditions. Molecular biology reports. 2012;39(3):2541-52.
25. Rosenberg M. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiology Letters. 1984;22(3):289-95.
26. Collado MC, Meriluoto J, Salminen S. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Letters in applied microbiology. 2007;45(4):454-60.
27. Alakomi HL, Skyttä E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Applied and environmental microbiology. 2000;66(5):2001-5.
28. Niku-Paavola ML, Laitila A, Mattila-Sandholm T, Haikara A. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology. 1999;86(1):29-35.
29. Jack RW, Tagg JR, Ray B. Bacteriocins of gram-positive bacteria. Microbiological reviews. 1995;59(2):171-200.
30. Hugenholtz J, De Veer GJ. Application of nisin A and nisin Z in dairy technology. Nisin and novel lantibiotics. 1991;31:440-7.
31. Abee T, Krockel L, Hill C. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. International journal of food microbiology. 1995;28(2):169-85.
32. Chikindas ML, García-Garcerá MJ, Driessen AJ, Ledeboer AM, Nissen-Meyer J, Nes IF, Abee T, Konings WN, Venema G. Pediocin PA-1, a bacteriocin from Pediococcus acidilactici PAC1. 0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Applied and Environmental Microbiology. 1993;59(11):3577-84.
33. Pugsley AP. The ins and outs of colicins. Part I: Production, and translocation across membranes. Microbiological sciences. 1984;1(7):168-75.
34. De Angelis M, Gobbetti M. Environmental stress responses in Lactobacillus: a review. Proteomics. 2004;4(1):106-22.
35. Jin LZ, Ho YW, Abdullah N, Jalaludin S. Acid and bile tolerance of Lactobacillus isolated from chicken intestine. Letters in applied Microbiology. 1998;27(3):183-5.
36. Kashket ER. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiology Reviews. 1987;3(3):233-44.
37. Rao YK, Lu SC, Liu BL, Tzeng YM. Enhanced production of an extracellular protease from Beauveria bassiana by optimization of cultivation processes. Biochemical Engineering Journal. 2006;28(1):57-66.
38. Piper PW. Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. FEMS microbiology reviews. 1993;11(4):339-55.
39. Piper PW, Ortiz-Calderon C, Holyoak C, Coote P, Cole M. Hsp30, the integral plasma membrane heat shock protein of Saccharmyces cerevisiae, is a stress-inducible regulator of plasma membrane H+-ATPase. Cell stress & chaperones. 1997;2(1):12.

Cite this article as:

Jayashri G. Mahore and Satish V. Shirolkar. Isolation and characterization of lactobacilli from caecum of rat fed with fructooligosaccharides. Int. Res. J. Pharm. 2018;9(6):216-224 http://dx.doi.org/10.7897/2230-8407.096119

Source of support: Nil, Conflict of interest: None Declared