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PURIFICATION AND PARTIAL CHARACTERIZATION OF A BACTERIOCIN PRODUCED BY LACTOBACILLUS REUTERI ISOLATED FROM HUMAN MILK

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ABSTRACT

Bacteriocin producing *Lactobacillus reuteri* strain isolated from human breast milk, showed broad spectrum of antimicrobial activity against clinical pathogens. Maximum bacteriocin production was observed at 37^0C and pH 7. In addition of enzymes such as α-amylase, proteinase K, Lysozyme, methanol, chloroform, ethanol, acetone and benzene were strongly inhibited bacteriocin production. The bacteriocin has purified by ammonium sulphate precipitation, dialysis and Ion exchange chromatography. Biochemically it was pure protein and the molecular weight was 10kDa. The study revealed that bacteriocin producing *Lactobacillus reuteri* may be used as probiotic to inhibit pathogens.

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KEY WORDS

Probiotics, Antimicrobial activity, Arbitory unit, Medicine.

INTRODUCTION

Human milk is a complex species-specific biological fluid perfectly adapted to satisfy the nutritional and immunological needs of neonates. Breast milk is an important source of bacteria to the infant gut where they play a key role in the initiation and development of the gut micro biota. It has been demonstrated that breast milk confers protection against different infectious diseases since the incidence of these disorders is lower in breast-fed than in formula-fed infants. This anti-infective effect is due to several bioactive compounds present in colostrums.[5]

Lactobacillus is important organism recognized for their fermentative ability as well as their health and nutritional benefits. The genus *Lactobacillus* is quite diverse and consists of a number of different species with little commonality. They are Gram positive and non sporing and typically rod shaped with a size range of about 0.5-1.2 X 1-10 µm. They are facultative anaerobes that, in general, grow poorly in air, but they are sometimes enhanced by 5% CO2. Because of the reason for being an auxotroph for a number of different nutrients, they grow best in rich complex media. Their optimum growth temperature is 30° to 40° C, but they can grow over a range of 5° to 53° C. They are also aciduric with an optimum growth pH of 5.5 to 5.8.But in general they can grow at a pH <5 [11].

Probiotic cultures have been associated historically with cultured of milks and dairy products, from which there is substantial evidence for positive effects on human health and general well being. The increasing demand for high quality and safe, processed food has created a niche for good quality of probiotic food. "A 'probiotic' is defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"[14].

Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides having a bactericidal or bacteriostatic activity[18]. Replacing the friendly intestinal bacteria destroyed by antibiotics for preventing and treating diarrhea, including infectious diarrhea, particularly from rotavirus, treating overgrowth of pathogenic organisms in the gastrointestinal tract; alleviating symptoms of irritable bowel syndrome and possibly inflammatory bowel disease; preventing and reducing the recurrence of vaginal yeast infection, urinary tract infections, and cystitis; improving lactose adsorption and digestion, in people who are lactose intolerant. Studies have suggested that the consumption of the Human breast milk *Lactobacillus reuteri* may improve natural immune response; aid the treatment of respiratory infections such as sinusitis, bronchitis, and pneumonia, and reduce the risk of recurring bladder tumors once the cancer and UTI has been treated.

MATERIALS AND METHODS

(i) Isolation and Identification [16]

Two milliliter samples of human breast milk, collected aseptically from a 35 year-old woman (15 days after delivery), was able to fulfill the defined criteria. The samples were serially diluted in peptonated saline solution and plated out onto MRS agar plates, supplemented with 50mg/litre Natamycin to rule out yeast contamination. The plates were

incubated at 37ºC for 48 hours. The culture was purified by repeated streaking. The following tests were performed with a view to pointing out the morphological, physiological and biochemical characteristics

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(ii) Production of Crude Bacteriocin Sample [2]

Lactobacillus reuteri was propagated in 100 ml MRS broth for 72 hours at 37ºC. For extraction of bacteriocin, a cell free solution was obtained by centrifuging the culture at 10,000 rpm for 20 minutes at 4ºC. It was adjusted to pH 7.0 by means of 1M NaOH to rule out the antimicrobial effect of organic acid, followed by filtration of the supernatant.

(iii) Partial Purification of Bacteriocin: Ammonium Sulphate Precipitation [6]***:***

Ammonium sulphate competes with the protein present in the solution against the water gradient and gradually precipitates the protein. Higher the level of ammonium sulphate, higher is the precipitation of the protein. The crude bacteriocin was treated with different concentration of ammonium sulphate; 10,20,30,40,50,60,70 and 80%. After 3 hrs, the suspension was centrifuged at 10,000 rpm for 30 mins. The supernatant from each concentration was dialyzed (Cut off 1000) against demonized water with four changes over 3 days and tested for antimicrobial activity.

(iv) Determination of The Bacteriocin Titre [13]***:***

The bacteriocin titre was defined as the reciprocal of the highest dilution of the bacteriocin showing the definite inhibition of the indicator lawn and was expressed as the arbitrary units (AU ml^{-1}). The bacteriocin was diluted two fold with saline and about 50 µL of each aliquot was dispensed on to the agar plates with indicator strains as described above in the well diffusion method. The inoculum chosen for the titre was chosen in such a way that it contained 10^7 cells per mL. The plates were incubated at 37°C for 24 hrs in an upright position and the zone of inhibition corresponding to the titre value was calculated. The last dilution showing the highest zone diameter was taken as the bacteriocin titre.

(v) Antimicrobial Activity by Agar Well Diffusion Method [17]***:***

Antimicrobial activity was confirmed by using the agar well diffusion method. 5mm-dia. wells were made on pathogen spread MHA agar plates and then aliquots of 50µl of cell free supernatants were added to the wells. After drying, the dishes were kept for 2 hrs in a refrigerator to facilitate diffusion of bacteriocin in agar. The inoculated plates were then incubated for 24 hrs at 37°C and the diameter of the zone of inhibition in millimeters was measured and recorded.

(vi) Detection of Physio-Chemical Stability Of Bacteriocin [4]***:***

Temperature: The bacteriocin samples were exposed to temperatures such as 60, 70, 80, 90, and 100°C, for a time of 30 minutes and its bacteriocin activity was assessed against various pathogens using the agar well diffusion method.

pH: The bacteriocin samples were exposed to different pH such as 2, 3, 4, 5, 6, 7, and 8,9 and 10, and its bacteriocin activity was assessed against various pathogens using the agar well diffusion method.

Effect of enzyme treatment on bacteriocin activity: Bacteriocin samples were mixed with the following enzyme solutions a final concentration of 1mg/mL. Lysozyme (dissolved in 50mM Tris-HCL, pH 8.0). Proteinase K (dissolved in 50mM Tris-HCL, pH 7.5). α amylase (dissolved in 50mM sodium acetate, pH 6.0). The bacteriocin

activity was assessed against various pathogens using the agar well diffusion method.

***Effect of the organic solvents on the bacteriocin stability*:** Bacteriocin samples were mixed with the 50% of the organic solvents such as methanol, chloroform, ethanol, acetone and benzene in the ratio 1:1. After leaving at 4ºC for 2 hours, the organic solvents were allowed to separate into aqueous and organic phase and the bacteriocin activity of each phase was tested against the indicator organisms using agar well diffusion method.

Effect of UV light on the bacteriocin activity: A 10 ml aliquot of bacteriocin producing isolate was placed in a sterile petridish and exposed to short wave UV light at a distance of 30cm for 5 minutes. The bacteriocin activity was then analyzed using the agar well diffusion method [8].

(vii)Tricine-SDS Page [7]:

To estimate the molecular weight of the bacteriocin was carried out, according to the method of SDS PAGE**.** Samples in a ratio of 1:2 were diluted with samples buffer (MEDOX biochemical's) containing 2-mercaptoethonal and denatured by boiling for 2-5 min**.** Poly acrylamide concentrations in the stacking gel and separating gel were 9.6% and 16.0% respectively. Electrophoresis was conducted at the constant voltage of 30V for 18 hrs. After the run, the gel was washed during 5 hrs with sterile water that was replaced every hour. Protein bands were visualized UV transilluminator according to the manufacturer's manual. The gel was stained with Coomassie Brilliant Blue and the molecular weight of the Bacteriocin was determined in comparison with marker protein standards (low molecular weight MEDOX marker protein standards)

(viii) Estimation of Protein By Lowry's Method [9]:

Protein concentration of the bacteriocin in supernatant was determined by the Lowry's method, using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

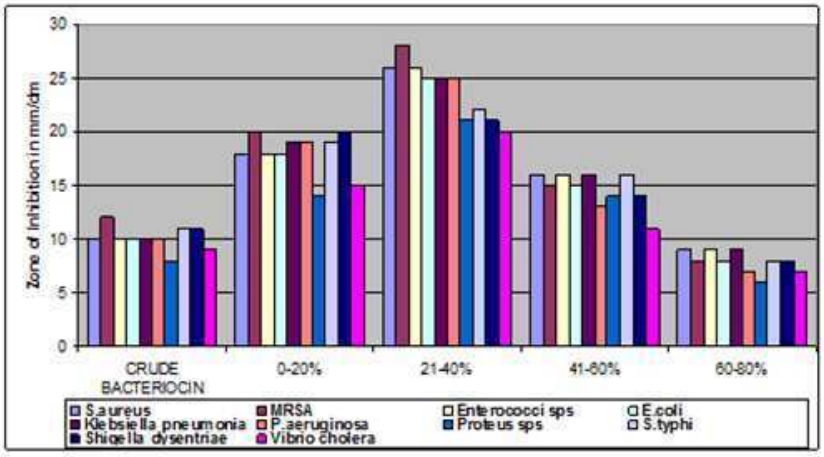
The present investigation highlighted the isolation, partial purification and physio-chemical stability analysis of bacteriocin, produced by *Lactobacillus reuteri* from breast milk

Breast milk samples were obtained from 150 lactating women aged 19 to 36 years old. The samples were collected 6 to 32 days after delivery. The organism was isolated from Human breast milk sample and cultured on MRS agar plates. *Lactobacillus reuteri* was identified on the basis of conventional techniques. Identification was carried out on physiological and biochemical characteristics. Gram positive, catalase negative, cocci, coccobacilli or rod shaped, non branching, non capsulated, non sporing isolates with characteristic cell arrangement when grown on MRS growth media were identified as lactic acid bacteria. 69 *Lactobacillus reuteri* were identified to the species level according to Bergey's manual of determinative Bacteriology.

The crude bacteriocin samples were partially purified by treating with the ammonium sulphate at 60% saturation and then centrifuged at 10000 rpm for 10 mins. The black colored pellets obtained were subjected to dialysis using 1200 KDa value of dialysis membrane and was partially purified.

Figure 1

Comparison of Antimicrobial Activity in crufe and Ammonium Sulphate Precipitated Bactericin



The antimicrobial activity of bacteriocin of Lactobacillus reuteri and degree of inhibition is shown in Fig. 1. The antibacterial spectrum exhibited the activity against Staph aureus, MRSA, Enterococcus spp, E.coli, Klebsiella spp, Pseudomonas aeroginosa, Proteus spp, Salmonella typhi, Shigella dysentriae and Vibrio cholera showed sensitivity towards the bacteriocin. The antibacterial titers for the Bacteriocin produced by the Lactobacillus reuteri after the two fold serial dilution.

Bacteriocin are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer strain.[2] It was tested against 7 different bacterial pathogens isolated from clinical specimen. The bacteria selected were *Staphylococcus aureus, MRSA (Methicillin Resistant Staphylococcus Aureus), and Enterococcus sps, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeroginosa and Proteus sps,*) [5]. The result indicated the present strains seemed to have antimicrobial activity against *Staphylococcus aureus, MRSA (Methicillin Resistant Staphylococcus Aureus) Enterococcus sps, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeroginosa, Proteus sps, Salmonella typhi, Shigella dysentriae, and Vibrio cholera* (Fig.2). The study had proved the possibility of using these strains as a probiotics or medicine.

Figure 2
Zone of Inhibition Against Clinical Pathogens

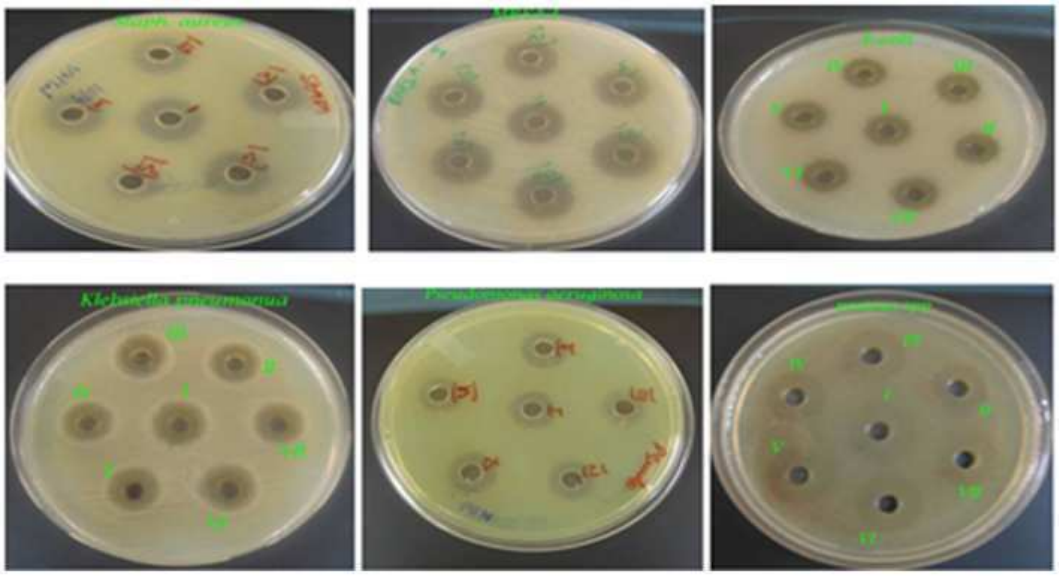


Table.1
Physiochemical Characterization of Bacteriocin Production Lactobacillus reuteri By Different pH, Temp., and Organic Solvents.

S.No	Indicator Organisms	Effect Of Different pH									Temperature (°C)				
		2	3	4	5	6	7	8	9	10	60	70	80	90	100
1)	*Staph. aureus*	R	R	S	S	S	S	S	S	R	S	S	S	S	S
2)	*MRSA*	R	R	S	S	S	S	S	S	R	S	S	S	S	S
3)	*Enterococcus spp*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
4)	*Escherichia coli*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
5)	*Klebsiella spp*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
6)	*Pseudo. aeroginosa*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
7)	*Proteus vulgaris*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
8)	*Salmonella typhi*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
9)	*Shigella dysentriae*	R	R	S	S	S	S	S	S	R	S	S	S	S	R
10)	*Vibrio cholera*	R	R	S	S	S	S	S	S	R	S	S	S	S	R

Table 1 show that the zone of inhibition was greater than 2mm and so the organism is said to be sensitive. S* = sensitive, R = resistant, AQ = aqueous phase, OR = organic phase. A standard was maintained were the bacteriocin is added as such, without the addition of the organic solvents.

The bacteriocin extract of the cultured *Lactobacillus reuteri* when subjected to temperature of 100°C, for a time of 30 minutes lost its bacteriocin activity which was assessed against various pathogens using the agar well diffusion method. The bacteriocin samples were exposed to different pH. It was found that the bacteriocin activity was lost at a highly alkaline pH greater than 9 and also at a high acidic pH 3, which was confirmed from the bacteriocin activity using the agar well diffusion method against the pathogens. However the highest activity of the bacteriocin was determined at an optimum pH of 7.0 to 8.0. (Table.1)

Table.2

***Physiochemical Characterization of Bacteriocin Production Lactobacillus reuteri By Organic Solvents*.**

S.No	Indicator Organisms	Chloroform		Benzene		Ethanol		Methanol		Acetone	
		OR	AQ	OR	AQ	OR	AQ	OR	AQ	OR	AQ
1)	*Staph. aureus*	R	R	10	9	11	12	9	10	10	11
2)	*MRSA*	R	R	9	10	10	11	11	11	11	11
3)	*Enterococcus spp*	R	R	10	11	9	10	10	10	9	10
4)	*Escherichia coli*	R	R	11	12	11	12	12	12	10	9
5)	*Klebsiella spp*	R	R	10	11	10	11	10	9	10	11
6)	*Pseudo. aeroginosa*	R	R	8	11	11	12	8	11	10	10
7)	*Proteus vulgaris*	R	R	10	9	10	11	8	11	10	9
8)	*Salmonella typhi*	R	R	10	10	9	11	10	11	10	10
9)	*Shigella dysentriae*	R	R	9	12	10	10	10	10	9	12
10)	*Vibrio cholera*	R	R	10	9	11	12	9	9	12	9

Table 2. show that the zone of inhibition was greater than 2mm and so the organism is said to be sensitive. S* = sensitive, R = resistant, AQ = aqueous phase, OR = organic phase. A standard was maintained were the bacteriocin is added as such, without the addition of the organic solvents.

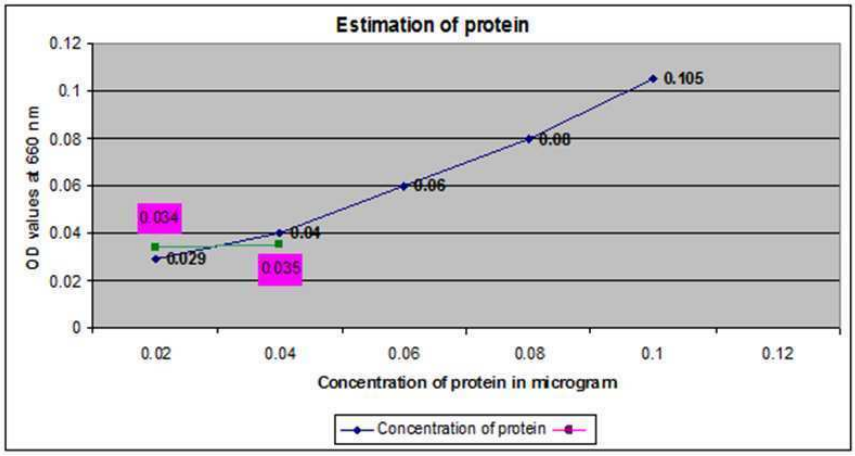
The bacteriocin samples, treated with equal volume, acetone, benzene, methanol and ethanol retained its bacteriocin stability and inhibited the pathogens. However the samples treated with the chloroform as solvent lost its bacteriocin ability and showed no inhibition against the different pathogens. The treatment of the crude bacteriocin with the proteolytic enzyme (1mg/ml) resulted in the complete loss of the bacteriocin activity and the indicator organisms showed resistance to the bacteriocin treated with the proteolytic enzymes. The 5 mins UV exposed LAB culture's bacteriocin failed to produce an antimicrobial activity against the test organisms. (Table 2).

Various physiochemical factors seemed to affect bacteriocin production as well as its activity. Being resistant to low pH and temperature is one of the major selection criteria for probiotic strains [1,4]. In our study the minimum activity was noted at pH 4, maximum activity was

noted at temperature 90^{0}C, so *L.reuteri* may be act as a probiotic.

Bacteriocins are antimicrobial agents produced by bacteria which are active against closely related bacteria.[3] They are active against many other bacteria including pathogens. Hence they may be used as probiotic or as medicine especially in human beings. The bacteriocin was tested for the sensitivity (loss of activity) to various enzymes. The antimicrobial activity was lost or unstable after treatment with all the Organic solvents like acetone, benzene, methanol and ethanol and proteolytic enzymes like proteinase K, Lysozyme and α-amylase treatment. Results from enzyme inactivation studies demonstrated that antimicrobial activity was lost or unstable after treatment with all the proteolytic enzymes[12].

Figure 3
Estimation of Proteins By Lowry's Method (L.reuteri)



The partially purified bacteriocin was subjected to Tricine SDS- PAGE and the molecular weight

was found out. The molecular weight of the bacteriocin from *Lactobacillus reuteri* was determined as 2.5 KDa similar to the low molecular weight protein marker. The protein was estimated by Lowry's method and was compared with the standard graph. The protein present in the partially purified sample was found to be 0.21mg/ml. (Fig.2). Bacteriocins produced by *Lactobacillus reuteri* were partially purified by ammonium sulphate precipitation and dialysis. The molecular weight of the plantaricin ranged from 4.5 to 8 KDa[11]. In the present study, the molecular weight of the protein was estimated using the tricine SDS-PAGE with 12% separating gel and 5% stacking gel and was identified as 2.5 KDa.

CONCLUSION

This study demonstrated that human milk *Lactobacillus reuteri* isolated from lactating women, are able inhibit the growth of pathogens by the effect of bacteriocin. The antagonistic activity on *Staph. aureus, MRSA, Enterococcus, E.coli, Klebsiella spp, P.aeroginosa*, *Proteus spp, Salmonella typhi, Shigella dysentriae and Vibrio cholera* was found to be good. The results of *Lactobacillus reuteri* can be used in probiotic products to prevent infections of the urogenital tract. In the future, influence of complex nutrients and different incubation conditions on synthesis of the bacteriocin have to be investigated along with elucidation of bactericidal action mechanism and genetic characterization of bacteriocin.

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