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ORIGINAL ARTICLE

ISOLATION AND EXTRACTION OF BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA ISOLATED FROM RAW MILK SAMPLES

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Abstract:

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes.Lactic acid bacteria commonly used as a natural food preservative, which produces certain antimicrobial substances such as Bacteriocin. The present study deals with isolation and characterization of Bacteriocin producing Lactobacillus sp., from a raw unpasteurized milk sample. Screening and isolation was done on MRS agar, and isolates were identified using biochemical methods. Bacteriocin was produced from promising isolate. Bacteriocin from fermented broth was extracted by solvent extraction and antibacterial activity was determined against selected test microorganisms of both Gram positive and Gram negative groups.This study revealed the possibility of using Bacteriocin as food biopreservative to control food spoilage and pathogenic bacteria.

KEYWORDS:

Lactobacillus, Bacteriocin, Solvent Extraction, Food preservation, Antimicrobial activity.

INTRODUCTION :

Bacteriocins are protein or protein complexes produced by bacteria and have antimicrobial activity against closely related species and various Gram positive and Gram negative bacteria including food spoilage bacteria and pathogens. (Gaeng,et. al., 2000, Meghrouse,et.al., 1999,Ra, et al., 1999). The bacteriocins from lactic acid bacteria (LAB), generally recognized as safe (GRAS) and have arisen a great deal of attention as a novel approach to control pathogens in food-stuffs. (Savadogo,et al., 2004).MRS medium supportto luxuriant growth of all lactobacilli fromoral, fecal, dairy and other sources lactobacilli. MRSmedium contains peptones and dextrose which supply nitrogen and carbon. Tween 80, acetate, magnesiumand manganese provide growth factors for culturing avariety of lactobacilli. These ingredients may inhibit the growth of some organisms other than lactobacilli. Lactobacilli produce special antimicrobial compounds such as bacteriocins which are a highly specific antibacterial protein (Sowani et al., 2012) and prevent food spoilage and provide additional protection against Bacillus, Staphylococcus aureus and Clostridial spores in canned foods.Lactic acid bacteria particularly those belonging to beneficial and non – pathogenic genera (Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Leuconostoc) are widely used in various fermentations.

In a variety of ecological niches, microorganisms compete with each other for survival and through evolution form unique flora. In some food ecosystems, lactic acid bacteria constitutes the dominant microflora these organisms are able to produce antimicrobial compounds against competing flora,

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including food borne spoilage and pathogenic bacteria (Bettache,et al., 2007). Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. The antimicrobial effect of lactic acid bacteria has been appreciated by man for more than 10000 years and has enabled him to extend the shelf life of many foods through fermentation processes. Raw milk represents an ideal growth medium for microorganisms. The types of microorganisms found in milk vary considerably, and are dependent upon the specific conditions associated with a batch of milk. Bacteria (genera such as streptococci, Lactobacillus, Micrococci, Microbacteria, Coliform, Bacillus --- etc.) Yeasts (genera such as Torula), and moulds (genera such as Pencillium) are commonly encountered (Powar,et al., 2008). Raw milk play important role in the diet of low income and the majority of people living in the rural areas (Savadogo,et al., 2004).

This present study was conducted to evaluate the antibacterial property of bacteriocin producing Lactobacillus sp. isolated from raw milk samples.

MATERIALS AND METHODS

Chemicals and Media

Analytical grade chemicals were obtained from Qualigenes, Thomas baker and SD fine, India, while media were obtained from Hi-media, India.

Test Microorganisms

The test microorganisms, viz. Bacillus coagulans, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Proteus vulgariswere isolated and cultured at Dept. of Microbiology and Research Center, Shri Shivaji Mahavidyalaya Barshi, and confirmed on the basis of Morphological and Biochemical characteristics.

Screening and isolation of Lactic Acid Bacillus for Antimicrobial activity

Ten Raw unpasteurized milk samples of cows, buffaloes and goats were collected from local area of Barshi during lactation period under aseptic condition and were processed within three hours and used for further studies.

Collected milk samples were serially diluted in sterile distilled water and plated on sterile MRS agar (de Mann Rogosa Sharpe) plate and incubated in microaerophilic condition at 37° for 48 – 72 hours. The well isolated colonies were selected randomly and transferred in MRS broth. They were streaked on MRS agar to check the purity of the isolates and then stored in MRS soft agar (0.5%) overlaid with Glycerol at -20°C. The isolates were differentiated on the basis of morphological, and biochemical characteristics.

Detection of Antibacterial activity

Twenty Five bacterial isolates were cultured in MRS broth and incubated at 37°C for 20 hours under anaerobic condition. Aliquots of culture were spotted on the sterile MRS agar plate and incubated at 37°C for 24 hours under microaerophilic condition. After incubation the plates were overlaid with soft nutrient agar inoculated with culture of test microorganisms and plates were incubated at 37°C for 24 hours under aerobic condition.After incubation zone of growth inhibition of test microorganisms were recorded.

The isolates showing zone of growth inhibition of test microorganisms were selected and preserved on MRS agar slants for further studies.

Production of Bacteriocin from selected isolates

Selected cultures were inoculated into MRS broth and incubated at 37°c for 24–48 hours under microaerophilic condition. After fermentation, broth was centrifuged at 12000 rpm for 15 min. and supernatant was collected. The pH of supernatant was adjusted to 7.0 with 2N NaOH (Vijai,et al., 2004). Precipitation was done with Ammonium sulphate at 40% and 70% saturation level at 4°C.

After precipitation, the broth was centrifuged at 15,000 rpm for 15 min. precipitate was collected and stored in 0.2M Sodium phosphate buffer (pH 6.9) and labeled as crude bacteriocin preparation.(Navarro,et al., 2000).With the help of agar well diffusion and paper disc methods antimicrobial activity of the collected precipitate was checked for to confirm the presence of antimicrobial substances in it.

Extraction of Bacteriocins

Chloroform – Methanol (2:1 v/v) was used for crude Bacteriocin extraction.However produced precipitate at Solvent-Aqueous interphase was collected aseptically, solvent was evaporated and precipitate was kept in buffer which was used for antimicrobial study (Burianek, et al., 2000).

Antimicrobial activity of extracted bacteriocin

In this study two techniques were used for to obtain reliable technique for the detection of antimicrobial activity.

1. Agar well diffusion
2. Paper disc assay.

Agar well diffusion technique was performed as described by (Ohmomo, et al., 1998). 0.1ml culture of indicator microorganisms is spreaded on sterile nutrient agar and wells are prepared on the same nutrient medium. Extracted bacteriocin preparation was added in that wells and plates were incubated at 37°C for 24 hours under aerobic condition.

Paper disc assay was done (Martirani, et al., 2002), paper discswere prepared from bacteriocin preparation. 0.1ml culture of indicator microorganism was spreaded on nutrient agar plate and disc was placed on the same. Plates were incubated at 37°C for 24 hours under aerobic condition. The zones of growth inhibition of test microorganisms were recorded.

Results

In this study Lactobacillus sp. isolated from different unpasteurized milk samples were characterized and identified on the basis of morphological and biochemical characteristics. Out of 25 isolatesselected, only 10 were belonged to the genus Lactobacillus(Table.1) which showed promising antibacterial activity against test microorganisms.

Morphological and biochemical studies (Table. 1)were showed that, all the 10 isolates were Gram positive and Catalase Negative. Out of 10 isolates two were cocobacilli and eight isolates were rod shaped. All the isolates were able to produce acid fromglucose and sucrose.

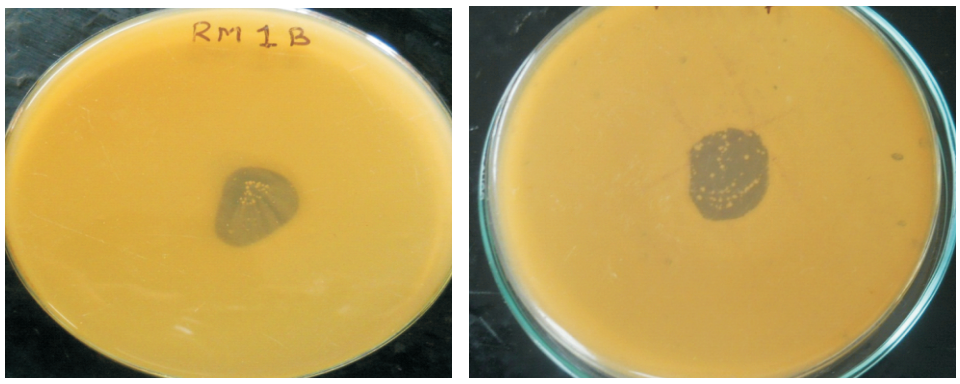
Table 1. Morphological and Biochemical Characteristics of the promising isolates.

Culture Codes	Morphology	Gram nature	Motility	Catalase	Utilization of -	Arabinose	Glucose	Fructose	Starch	Maltose	Sucrose	Mannitol	Lactose	Optimum Temp. (°c)		Probable identity
														15	45	
RM1	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	-	*Lactobacillus fermentum*
RM2	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	A	A	+	+	*Lactobacillus casei*
RM3	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	A	A	+	+	*Lactobacillus lactis*
RM4	Cocobacilli	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	-	*Lactobacillus lactis*
RM5	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	-	*Lactobacillus fermentum*
RM6	Rod	Gram positive	NM	NC		NA	A	A	NA	NA	A	A	A	+	-	*Lactobacillus plantarum*
RM7	Cocobacilli	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	-	*Lactobacillus lactis*
RM8	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	-	*Lactobacillus lactis*
RM9	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	-	*Lactobacillus lactis*
RM10	Rod	Gram positive	NM	NC		NA	A	A	NA	A	A	NA	A	+	+	*Lactobacillus lactis*

Notation:(NM) Non motile, (NC) Non catalase,(NA) No Acid production, (A) Acid production,
(+) Growth observed, (-) No growth observed.

Screening of Bacteriocin Producers

Screening of Bacteriocin producers werecarried out by agar overlay method as described earlier. In which the cultures or isolates showing maximum zone of growth inhibition of test microorganisms (Fig. 1) were selected and used for bacteriocin production studies.



(a) (b)

Fig.1. Zone of Growth inhibition of (a)Bacilluscoagulansand (b)Klebsiella pneumoniae by isolate RM1.

Antibacterial activity of extracted bacteriocin preparation

Ten isolates of lactic acid bacilli were selected for bacteriocin production and their antibacterial activity against test microorganisms viz.Bacillus coagulans, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Proteus vulgariswere checked by Agar well diffusion and paper disc method.The antibiogram of bacteriocin from isolated Lactobacillus sp. against test microorganisms were shown in (Table 2).

Table 2. Antibiogram of Bacteriocin from promising Lactobacillus sp. against test microorganisms

Culture Code	Zone of Growth inhibition (mm)				
	Bacillus Coagulans	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*	*Proteus vulgaris*
RM 1	15	18	17	25	20
RM 2	23	18	14	28	13
RM3	20	17	15	26	16
RM4	00	09	10	15	06
RM5	00	00	00	00	00
RM6	15	10	07	25	14
RM7	11	20	24	06	19
RM8	20	05	15	13	24
RM9	21	19	17	08	16
RM10	25	15	23	17	26

DISCUSSION

In the present study, Lactobacillus sp. was isolated from ten different unpasteurized milk samples for the production of bacteriocin. Lactobacilli have been used in many fermentation processes, which can be characterized as Gram positive, catalase negative, non sporulating, non-pigmented bacteria (Axelson,et al., 1993). The isolated Lactobacillus sp.was characterized by morphological and biochemical methods and these were identifiedbycomparing results with Bergeys Manual of Systematic Bacteriology. In our study we isolated Lactobacillus lactis, Lactobacillus casei, Lactobacillus fermentum,and Lactobacillus plantarum from milk samples.These results were similar with Mohankumaret al., (2011) who isolated the 100 Lactic acid Bacillus strains for their antimicrobial properties from raw cattle milk.The similar results were shown by Shafei et al., (2000) who reported the 100 lactic acid bacterial strains producing bacteriocins from traditional fermented foods. Antibacterial property of isolated lactobacillus species was checked against test microorganismsviz.Bacillus coagulans, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Proteus vulgaris and our isolates showed considerable zone of growth inhibition of all test microorganisms. This results supports with the results of Arokiyamary et al., (2011) in that they isolated the bacteriocin producing lactobacillus species from traditional milk products and checked the antimicrobial activity against common pathogens. In our study we observed the maximum zone of growth inhibition against test microorganismswhose diameter ranging from (0.0 mm to 28.00 mm).We got similar results like Enanet al., (1996), which showed inhibition zones between 0.5 – 13.00 mm in diameter by bacteriocin producing Lactobacillus strains against indicator organisms.

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