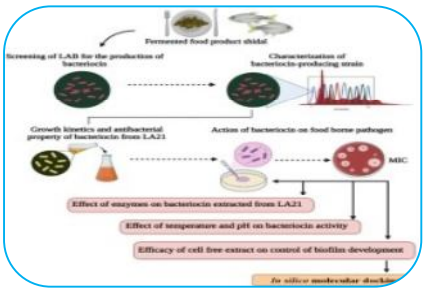
INDIAN STREAMS RESEARCH JOURNAL
ISSN NO : 2230-7850
IMPACT FACTOR : 5.1651 (UIF)
VOLUME - 12 | ISSUE - 9 | OCTOBER - 2022

BACTERIAL PRODUCTION OF BACTERIOCIN: A HEALTH VALUABLE GREEN APPROACH

Dr. Suman Lokhande-Jadhav
Research Scholar, Associate Professor and Head,
Department of Microbiology , Shri Shivaji Mahavidyalaya, Barshi,
Dist – Solapur, Maharashtra, India.

ABSTRACT



Lactic acid bacteria (LAB) commonly used as a natural food preservative to improve the food safety and stability. These organisms produce certain antimicrobial substance such as bacteriocins. The present study deals with isolation and characterization of Bacteriocin producing Lactobacillus sp., from a raw unpasteurized buffalo milk sample. The isolate was identified, based on characteristics of the strains of Lactobacillus sp., as present in Bergey's manual of determinative bacteriology and Screening and isolation was done on MRS agar, and isolates were identified using biochemical methods. The antibacterial activity of produced Bacteriocin was tested against Salmonella typhi and the zone of growth inhibition was measured.

KEYWORDS : *Lactobacillus, Bacteriocin, Antibacterial Activity, Agar Well Diffusion, Agar Disc method.*

INTRODUCTION

Milk is a highly nutritious medium with almost a neutral pH (6.6-6.7) and therefore many bacteria including spoilage and pathogenic bacteria can grow and propagate in it. Milk products made from raw milk are equally nutritious. So these milk products also make home to a complex microbial ecosystem; these bacteria are responsible for broad diversity, taste, aromas and texture of milk and milk products. The alarming increase in inappropriate antibiotic use along with bacterial resistance has lead to renewed interest in ecological methods to prevent infections which make probiotics a very interesting field for research. LAB strains are potentially promising because they generate bactericidal bioactive pep tides (bacteriocins) and enzymes that are able to control biofilm formation and the growth of the pathogens. Starter cultures are now being used in the lyophilized form for mixing in the milk components. Lactic acid bacteria (LAB) have been used in the preparation of variety of cheeses (MCSweeney, 2007) where they convert lactose to lactic acid (Azarnia *et al.,* 2006), hence they increase the acidity of the cheese and contribute to a preservative effect with the result that many pathogenic and spoilage bacteria are inhibited. The texture and the flavor of the cheese and curd are greatly influenced by the starter culture (Banks, 2004).

The most important inllibitory compounds of LAB are bacteriocins. These are ribosomally synthesized toxins with proteinaceous nature, excreted out of the cell. The genes responsible for the

production of bacteriocins are localized on the bacterial chromosome, on plasmids and on transposons (both plasmid and chromosome carried). In most cases it is localized on the plasmid (Dimov *et al.,* 2005). Bacteriocins intrigue the food industry because they are produced by many daity starter culture strains and have the capability to repress the development of pathogenic microflora in dairy products.

Lactic acid bacteria (LAB) have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic, and shelf-life characteristics (Leroy and Vuyst, 1999). They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acid. In addition, their production of acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes is of importance. Whereas a food fermentation process with LAB is traditionally based on spontaneous fermentation or back slopping, industrial food fermentation is nowadays performed by the deliberate addition of LAB as starter cultures to the food matrix. This has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end products. Recently, the use of functional starter cultures, a novel generation of starter cultures that offers functionalities beyond acidification, is being explored.

For instance, LAB is capable of inhibiting various microorganisms in a food environment and display crucial antimicrobial properties with respect to food preservation and safety. In addition, it has been shown that some strains of LAB possess interesting health- promoting properties; one of the characteristics of these probiotics is the potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *Escherichia coli*, and *Salmonella sp*. This paper focuses on the role of bacteriocins as fast-acting, antibacterial peptides in both food safety and gastrointestinal health.

In the present research work the isolation and characterizations of Bacteriocin producing organisms from buffalo milk sample and detection of antibacterial potential of the extracted bacteriocin was studied.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of highest grade and obtained from Department of Microbiology, while media were procured from Hi-media, India.

Test organisms

The test microorganism like *Salmonella typhi* was obtained from Department of Microbiology, Shri Shivaji Mahavidyalaya, Barshi, Dist: Solapur.

Sample Collection

Milk sample of Buffaloes (Raw unpasteurized) were collected in sterile plastic bottles from local areas of Barshi and were processed within 4-5 hours and for further studies.

Enrichment of Culture

For Enrichment of bacteria, 1ml of milk sample was inoculated in 100ml of MRS broth and incubated at 37^0C for 5-6 days.

Isolation of Bacteriocins Producing Bacteria

For isolation of promising organism, individual milk sample was streaked on sterile MRS agar plates. Plate was incubated at 37^0C for 48-72 hours. The well grown colonies with distinct morphology were selected and maintained on sterile nutrient agar slants at 4^0C and used for further studies.

Screening of Bacteriocins producing bacteria

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 -4^{0}C. The isolates were differentiated on the basis of morphological and biochemical characteristics.

Identification of Bacterial isolates

Identification of bacterial isolates was performed by morphological and biochemical methods.

Morphological Characterization

Morphological characterization was done by observing the colony characteristics of selected colony like size, shape, color, elevation, margin, consistency, opacity, motility and Gram staining were observed. These colonies characters were used for identification of isolates.

Biochemical identification

The biochemical characterization was performed as per Bergey's Manual as described below

Sugar Fermentation:

Sugar which is to be tested was added in to peptone water containing Phenol Red indicator with Durham's tubes. After inoculation and incubation at 37^{0}C for 24 hours. Tubes were checked for color change and gas production. Glucose, lactose, fructose sugar was used.

Detection of Antibacterial activity of Bacterial Isolate

For the detection of antibacterial activity, the bacterial isolate was cultured on MRS broth and incubated at 37^{0}C for 24 hours. Aliquot culture was spotted on sterile MRS agar plate and incubated at 37^{0}C for 24 hours.

After incubation the plate was overlaid with soft nutrient agar with culture of test organism and plates were incubated at 37^{0}C for 24hrs. After incubation zone of growth inhibition of test organisms were recorded.

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

Production of Bacteriocins From selected isolate

Promising culture was inoculated in MRS broth and incubated at 37^{0}C for 24–48 hours. After fermentation, broth was centrifuged at 12000rpm for 15 min. and supernatant was collected. The pH of supernatant was adjusted to 7.0 with 2N NaOH. Precipitation was carried out with Ammonium sulphate at 40% and 70% saturation level at 4^{0}C. After precipitation, the broth was centrifuged at 15,000rpm for 15 min. Then after, precipitate was collected and stored in 0.2M Sodium phosphate buffer (pH 6.9) and labelled as crude bacteriocin preparation. With the help of agar well diffusion and paper disc methods antibacterial activity of the collected precipitate was checked for to confirm the presence of antibacterial substances in it.

Crude Bacteriocin Preparation

Precipitate was stored in 0.2M Sodium Phosphate buffer (pH6.9) and labelled as crude bacteriocin.

Extraction of Bacteriocin

The Lactobacillus isolate was propagated each in 250ml MRS broth (pH 6.8) for extraction of bacteriocin, a culture supernatant was obtained by centrifuging (6.000 rpm for 30min at 4^{0}C). The cell free solution was precipitated with ammonium sulphate (40% saturation). The mixture was rotated for 2 hours at 4^{0}C and later centrifuged at (10.000rpm for 20 min). The precipitate was obtained and resuspended in 10ml of 0.05M potassium phosphate buffer (pH 7.0).

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

Antimicrobial activity of extracted Bacteriocin

With help of Agar Well Diffusion and Paper Disc Assay antibacterial activity was checked.

Agar Well Diffusion method

Each well bored with the help of 5mm cork borer was filled with 40µl culture of the LAB isolate and the culture was allowed to diffuse into the agar for 2 hours at room temperature without disturbing the plate. It was then incubated at 37^0C for 24 hours and examined for clear zone of inhibition and measured in mm.

Agar Disc Assay

5mm disc of blotting paper was soaked with 40µl of fresh culture of the LAB isolate and kept over the pre-inoculated MHA plate with the pathogen and allowed to stand on the media at room temperature and then incubated at 37^0C for 24 hours. The zone of inhibition was measured in mm.

RESULTS AND DICUSSION

Isolation

In this study the bacterial isolates were isolated from different unpasteurized buffalo milk samples were characterized and identified on the basis of morphological and biochemical characteristics. A total 05 bacteria were isolated. These all isolates were transferred on sterile nutrient agar slants and stored at 4^0C and were used for further study.

Screening

The screening was performed in MRS broth and further by agar overlay method. A total of 05 isolates were screened on the basis of observation of zone of inhibition of test organisms. Those isolates were rescreened by same method. Finally total 01 bacterial isolates was found efficient bacteriocin producers as it exhibited highest antibacterial activity. This isolate was further identified by its morphological and biochemical characterization.

Identification of Bacterial isolates:

Morphological characteristics:

Total 05 bacteria were isolated out of which a single isolate was used for the morphological identification. Where LB1 were Gram positive in nature and were non motile. Where microscopic observation showed LB1 was rod in shape (Table 2).

Table 1: Colony characterizations of LB1 isolated on sterile MRS agar and incubated at 37^0C for 24 hours

Culture code	Size	Shape	Color	Margin	Consistency	Elevation	Opacity
LB1	1mm	Circular	Creamish white	Entire	Mucoid	Raised	Opaque

Table 2: Morphological characterizations of LB1 isolated on sterile MRS agar and incubated at 37^0C for 24 hours

Microscopic Observation	Gram Nature	Motility
Rod	Gram positive	Non-Motile

Gram staining

Microscopic observation for buffalo milk sample showed Gram positive bacilli in cluster. The result was interpreted by following the Bergey's manual of Bacteriology.

IMViC:

Total 05 bacteria were isolated. Of which 01 isolate was used for biochemical identification. IMViC test was negative for LB1 (Table 3).

Table 3: IMViC Test of Promising bacterium

Bacterial isolate	Indole	Methyl red	Voges proskauer	Citrate utilization
LB1	-	-	-	-

Sugar fermentation test:

Sugar fermentation test was performed as per Bergey's Manual for LB1. The given LB1 promising isolate has ability to ferment glucose, fructose and lactose (Table 4).

Table No.4: Sugar fermentation test of isolated promising bacteria

Bacterial isolate	Glucose	Fructose	Lactose
LB1	+	+	+

From the above morphological and biochemical characterizations, the promising bacterial isolate was identified as shown in (Table 5).

Table 5: Identification of potent bacterial isolate under study

Bacterial isolate	Identification
LB1	*Lactobacillus sp.*

DETECTION OF BACTERIOCIN PRODUCING BACTERIA

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

Crude Bacteriocin Preparation

Precipitate was stored in 0.2M Sodium Phosphate buffer (pH6.9) and labelled as crude bacteriocin.

Extraction of Bacteriocin:

Extraction of bacteriocin of Buffalo milk sample was done by using chloroform: methanol (2:1) and kept in buffer.

Antimicrobial activity of extracted bacteriocin preparation

01 isolate of lactic acid bacillus was selected for bacteriocin production and its antibacterial activity against test microorganisms *Viz. Salmonella typhi* was checked by agar well diffusion and paper disc method.

Agar well diffusion method

Antibacterial testing of bacteriocin preparation was performed on *Salmonella typhi* obtained from Shri Shivaji Mahavidyalaya, Department of Microbiology, Barshi, Dist- Solapur. This method was carried out by agar well diffusion method. The 24 hourss old culture of test organism was spread on

sterile Nutrient agar plate separately. Wells of 5mm were prepared on the plate and the well was filled 0.1ml of Bacteriocin preparation. The plate was kept for incubation at 37^{0}C for 24 hours. After the incubation period, plate was observed for zone of growth inhibition around the well. The zone of inhibition for buffalo milk sample is 16mm in diameter (Table 6).

Antibacterial activity of Bacteriocin produced by LAB against *Salmonella typhi*

Paper Disc Assay

Antibacterial testing of bacteriocin preparation was performed on *Salmonella typhi* obtained from Shri Shivaji Mahavidyalaya, Department of Microbiology, Barshi, Dist- Solapur. This method was carried out by paper disc assay. Paper disc was prepared from bacteriocin preparation. The 24 hours old culture of test organism was spread on sterile Nutrient agar plate. Disc was placed on same plate with the help of sterile forcep. The plate was kept for incubation at 37^{0}C for 24hrs. After the incubation period, the plate was observed for zone of inhibition around the wells. The zone of inhibition for buffalo sample is 18mm in diameter (Table 6).

Antibacterial activity of Bacteriocin produced by LAB1 against Salmonella typhi by Agar Disc method

Table 6: Zone of growth inhibition by agar well diffusion and paper disc assay of buffalo milk sample

Culture Code	Zone of Growth inhibition (mm)
LAB1 (Agar Well Diffusion)	16mm
LAB1 (Agar Disc Diffusion)	18mm

These results are in accordance with the results of Deshmukh P. V. and Thorat P. R., (2013) in that they isolated the bacteriocin producing lactobacillus species from traditional milk products and was checked the antimicrobial activity against common pathogens.

In the present study, the maximum zone of growth inhibition against test microorganism was 16mm and 18mm by Agar well diffusion assay and Agar Disc assay respectively.

Our results are also in strong agreement with the results of Arokiyamary *et al*., (2011), which showed inhibition zones between 15mm– 16 mm in diameter.

CONCLUSION

From the present study it was concluded that, the isolate *Lactobacillus spp. LB1* shows good bacteriocin production in modified MRS medium which further showed antibacterial spectrum of LB1 againstt *Salmonella typhi*.

REFERENCES

1. Arokiyamary A and Shivkumar P.K. (2011). Antibacterial activity of bacteriocin producing *Lactobacillus sp.,* isolated from traditional milk products. *Current Botany*. 2(3):5
2. Azarnia. S. Robert. N. and Lee. B. (2006). Biotechnological methods to accelerate Cheddar cheese ripening. Crit. Rev. Biotechnol. 26: 121-143 .
3. Banks. J.M. (2004). The technology of low fat cheese manufacturing. *International Journal of Dairy Technology*. 57: 199-207.
4. Deshmukh P.V. and Thorat P.R. (2013). Isolation and Antimicrobial Spectrum of New Bacteriocin From *Lactobacillus rennanquilfy WHL 3. Int. J. Pharm. Sci. Rev. Res*., 22(2), 32: 180-185
5. Dimov, S., Ivanova, P. and Harizanova, N. (2005). Genetics of Bacteriocins biosynthesis by lactic acid bacteria. *Biotechnology and Biotechnology*. 19: 4-10.
6. Leroy. F. and De Vuyst. L. (1999). The presence of salts and a curing agent reduces bacteriocin production by *Lactobacillus sakei CTC494*. A potential starter culture for fermentation sausage. *Applied Environmental Microbiology*. 65: 5350-5356.
7. McSweeney. P. L. H. (2007). Cheese Problems Solved. Woodhead Publishing Limited. Cambridge. England. 18 .