

## ISOLATION OF BACTERIOCIN PRODUCING LACTIC ACID BACTERIA FROM BUFFALO MILK

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### ABSTRACT

The purpose of the study have to establish the antimicrobial activity of bacteriocin producing lactic acid bacteria is isolated from raw milk of buffalo. Fifteen lactic acid bacteria isolates based upon the distinct morphology were isolated from the samples and identified as Lactic acid bacteria according to phenotypic characteristics. Bacteriocin producing organisms were screened by Agar well diffusion assay test. There is 9 isolates were able to produce bacteriocin whose antibacterial activity was analyzed by agar well diffusion assay test against indicator organisms. This indicator bacterium included *Bacillus mycoides*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris* were inhibited by the isolates. The

bacteriocin producing lactic acid bacteria have probiotic properties like antimicrobial activity, gelatinase activity, blood hemolysis, antibiotic sensitivity and bile salts hydrolases activity (BSH). Lactic acid bacteria from raw milk samples that inhibited certain pathogenic organisms by producing bacteriocin may be beneficial for a probiotic culture to be successful in colonizing and to challenge with pathogens.

**KEYWORDS:** *Bacillus mycoides*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris*, Bacteriocin, Isolates.

## 1. INTRODUCTION

Buffalo milk and its resulting products have high nutritional value, to increase their production, it is necessary to increase food security, to improve their aroma, and to potentiate their significance. The LAB originated from milk is also potentially developed into probiotic for useful food development (Cakir 2003). LABS have an extended account of use in fermented foods, improving their defensive, industrial, and useful properties. Defensive properties refer to the production of organic acids, diacetyl, hydrogen peroxide, and bacteriocins. Acidifying activity and diacetyl, exopolysaccharide (EPS), and enzyme production are desirable scientific properties. The acidity of the stomach maintains a low concentration of harmful bacteria in the upper part of the digestive tract and destroys pathogens. The Interaction that occurs between various bacterial species is also important in maintaining the equilibrium of the intestinal microflora (Olanrewaju, 2007). Milk and milk products are usually associated with LAB, which provide supplements in maintaining beneficial intestinal balance (Isolauri 2001). To obtain all these benefits, the choice of LAB strain is crucial, as select LAB have all of these properties. Lactic acid bacteria exert a strong antagonistic activity against many food-contaminating microorganisms as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocin (Piard and Desmazeaud 1991). Bacteriocinogenic lactic acid bacteria are generally considered safe additives (GRAS), useful to control the frequent development of pathogens and spoiling microorganisms in foods and feed (Cintas *et al.* 2001). Bacteriocin is antimicrobial peptides, synthesized by ribosome and widely distributed in nature. This peptide biodiversity is supported by several differences in their structures (Heng *et al.* 2007) All constitutively synthesized peptides, in spite of sub-classification, contributes to a net positive charge which causes them to fold into an amphiphilic conformation upon interaction with bacterial membranes (Drider *et al.* 2006). Bacteriocins mainly target the cell wall and pore formation occurs. Most of the bacteriocins are bactericidal or bacteriostatic in nature. The main driving force in the mechanism of action of bacterions, is generally electrostatic action between the bacteriocins and the target cell envelop. Primary metabolites are produced in response of log or exponential phase when there is immense quantity of nutrients are available (Diep 2002). Microbes compete for the restricted space and nutrients present in natural ecological niches, then they have developed several strategies in order to survive: production of antimicrobial substance such as bacteriocin is one of them. Gram-positive bacteria, and mainly lactic acid bacteria (LAB), are now being increasingly studied for their production of bacteriocin-like substances. Most characterized bacteriocins are heat-stable,

nontoxic, and susceptible to degradation by proteolytic enzymes present in the gastrointestinal tract (Abriouel et al. 2003). The increased utilization of foods containing additives formulated with chemical preservatives and buyer concerns have created a higher demand for more natural and minimally processed foods, hence, there is a high attention in naturally formed antimicrobial agents that do not produce adverse effects.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

A total of five buffalo milk samples were randomly collected in presterilized glass vials from farmers directly.

### 2.2 Enumeration and isolation of lactic acid bacteria

Milk was serially diluted to  $10^{-4}$  to  $10^{-2}$  using sterile peptone water and 0.1mL plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar. The MRS plates were maintained in microaerophilic condition and incubated at  $35^{\circ}\text{C}\pm 2$  for 48h. After incubation, 15 well isolated typical colonies were picked up and transferred to MRS broth and incubated at  $37^{\circ}\text{C}$  for 48h. The strains were stored at  $-4^{\circ}\text{C}$  in MRS broth plus 25% (v/v) glycerol. The isolated colonies were screened for antagonistic activity. The screened isolates were identified using standard morphological, cultural and biochemical reactions (Howells 1992).

## 3. Antagonistic Activity

### 3.1 Anti-Microbial Activity

Modified agar well diffusion method was used to detect antimicrobial activity of the isolate. Antibacterial activity was determined against *Bacillus mycoides*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris*. These indicator organisms were swabbed onto the sterile NA (Nutrient Agar) plate using spreader. Using sterile tip, the broth culture of LAB was poured into the well of about 50 $\mu\text{l}$  and kept for incubation at  $37^{\circ}\text{C}$  for 24hrs. Anti-microbial activity was evaluated by measuring the zone of inhibition against the indicator organism.

### 3.2 Bacteriocin Production Assay

The bacteriocin production assay of isolated lactic acid bacteria was performed by Agar well diffusion method was used to detect antibacterial activity (Mishra and Prasad 2005, Balcazar et al. 2006, and Van Hai et al. 2007). The indicator pathogen strains employed in this experiment were: *Bacillus mycoides*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*,

*Klebsiella pneumoniae* and *Proteus vulgaris* inoculated in Nutrient Broth at 35°C for 24 h. The LAB isolates were grown in 20 mL of MRS Broth of pH 5.6 for 48 h at 35°C. The supernatants were obtained by centrifugation at 15000 x g for 20 min at 4°C and filtered through a 0.45 µm pore-size filter (Millipore, USA). Each cell free supernatant containing bacteriocin was precipitated by addition of 40% ammonium sulphate and stored at 4°C for 24 hrs, next day addition of 70% ammonium sulphate in same tube precipitate was observed. The precipitate contain Bacteriocin was collected by centrifugation at 15000 xg for 20 min. at 4°C. The pellet was dissolved in 40µl of 0.5 M potassium phosphate buffer (solution must be in concentrated form) and stored the solution at 4°C for further antimicrobial activity assay. The indicator bacteria swab over the surface nutrient agar media using L shape spreader, rest for 15 min, prepare well using 6mm size borer and then fill the wells with 40µl bacteriocin containing solution.

The plates were placed at 4°C overnight to allow the diffusion of this solution in the agar and then incubated at 35°C for 48 h. Subsequently, the diameter of the clear zone around each well was recorded in millimeters with a Vernier caliper.

#### **4. Biochemical Characterization**

Different biochemical tests like gram staining, carbohydrate fermentation test, indole test, methyl red, voges-prausker, citrate utilization, nitrate reduction, urease, oxidase, triple sugar iron agar test were carried out and the species biochemical activity was measured using Bergey's manual (Holt 1984).

#### **5. Enumeration of Probiotic Property**

##### **5.1 Gelatinase Activity**

A heavy inoculum of 18-24hr old culture of bacteria is inoculated using a stab into tubes containing nutrient gelatin. The tubes were incubated at 35°C for one week and check liquefaction every day by placing them at 2-8°C for 30 minute. A tube inoculated with *staphylococcus aureus* was kept as positive control.

##### **5.2 Blood Hemolysis**

This test is performed to check whether the organism is hemolytic or not. The hemolytic activity was determined according to (Guttmann and Ellar 2000, Gerhardt et al. 1981). Isolates were screened on freshly prepared sterile blood agar plates containing 5% blood and

plates were streaked with 16-18hrs old culture and incubated at 37<sup>0</sup>c for 24-48hrs. They were observed for clear zones surrounding the colonies (positive reaction for  $\beta$ -hemolysis).

### 5.3 Antibiotic Sensitivity

The antibiotic resistance of the isolate was assessed using antibiotic discs (Himedia HX002IPK) on Mueller Hinton agar plates. The organism was swabbed on the plate and discs were placed on the surface of the agar. The plates were incubated at 37<sup>0</sup>C for 24hours (Halami et al. 1999, Vlkova et al. 2006, Coppola et al. 2005).

### 5.4 Screening of probiotic LAB for bile salts hydrolases activity (BSH)

Qualitative determination of bile salts hydrolases activity. The BSH activity was determined as described by (Du Toit et al. 2003) (Kumar et al., 2014; Pelinescu et al., 2009),. The LAB isolates were grown on MRS agar plates containing 0.3% (w/v) taurodeoxycholic acid sodium salt (TDCA; Sigma, USA) and 0.037% calcium chloride. Plates were incubated under anaerobic conditions at 37<sup>0</sup>C for 72 h. The precipitation zone surrounding colonies indicated the bile salt hydrolase activity of bacteria.

## 6. RESULT AND DISCUSSION

### 6.1 Isolation and development of pure culture

The Serially diluted suspension was used for isolation on selective MRS agar media. The plates were incubated at 37<sup>0</sup>C for 24-72 h. (Howells, 1992). After incubation pin point colonies of 2-5 micrometers, convex, entire, opaque, and without pigment was isolated. On the basis of colony characteristics 15 colonies was used to develop a pure culture by repetitive subculture on MRS agar media.

### 6.2 Antagonistic activity

The isolated lactic acid bacteria were screened using Antimicrobial activity and Bacteriocin production assay.

#### 6.2.1 Antimicrobial activity

Lactic acid bacteria have potential to produces some organic acids such as lactic acid, hydrogen peroxide, diacetyl and bacteriocin etc. against pathogenic microorganisms.

There was 9 isolates out of 15 demonstrate a zone of inhibition against indicator organisms such as *Bacillus mycoides*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella*

*pneumonia* and *Proteus vulgaris*. According to the supreme antimicrobial activity 4 out of 9 isolates was selected for further bacteriocin production assay.

### 6.2.2 Bacteriocin production assay

The antimicrobial active bacterial isolates were selected for bacteriocin production assay. The precipitated bacteriocin exclude of other organic acid was used for detection of antagonistic activity against indicator organisms via the agar well diffusion method (Arokiyamary and Sivakumar 2011). The photograph was describes a bacteriocin production against *Staphylococcus aureus*. The Inhibition against indicator organism and bacteriocin production activity was evaluated by measuring the zone of inhibition.

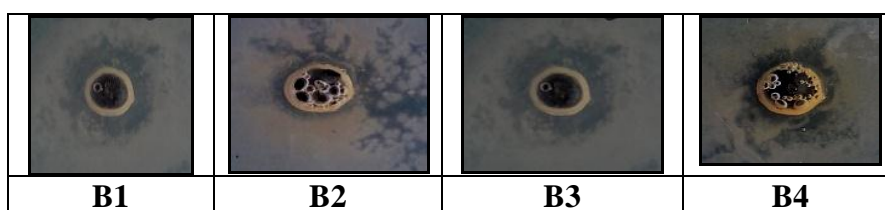


Table 6.2.2 was view a millimeter of zone of inhibition against indicator microorganisms. The antagonistic active isolates were selected for further biochemical characterization.

| Sr. No. | Indicator Bacteria            | Control (mm) | B 1(mm) | B 2(mm) | B 3(mm) | B 4(mm) |
|---------|-------------------------------|--------------|---------|---------|---------|---------|
| 1       | <i>Bacillus mycoides</i>      | 0            | 12      | 11      | 10      | 10      |
| 2       | <i>Staphylococcus aureus</i>  | 0            | 11      | 9       | 11      | 10      |
| 3       | <i>Pseudomonas aeruginosa</i> | 0            | 11      | 10      | 11      | 9       |
| 4       | <i>Klebsiella pneumoniae</i>  | 0            | 13      | 11      | 15      | 12      |
| 5       | <i>Proteus vulgaris</i>       | 0            | 12      | 11      | 12      | 9       |

### 6.3 Biochemical characterization

The screened isolates was biochemical tested using gram staining, carbohydrate fermentation test, indole test, methyl red, voges-prausker, citrate utilization, nitrate reduction, urease, oxidase, triple sugar iron agar test were carried out and the species of these lactic acid bacterial isolates was measured using Bergeys manual . The table 6.3.1 and 6.3.2 was show a biochemical profile of lactic acid bacterial isolates from buffalo milk. In table 6.3.1 lactic acid bacterial isolates was biochemically tested using methyl red test, voges proskaure's test and indole production test.



| 6.3.1 : Biochemical tests of isolated lactic acid bacteria |                           |         |      |     |     |     |
|------------------------------------------------------------|---------------------------|---------|------|-----|-----|-----|
| Sr. No.                                                    | Test                      | Control | B 1  | B 2 | B 3 | B 4 |
| 1                                                          | Methyl red test           | +++++   | ++++ | +++ | +   | ++  |
| 2                                                          | voges<br>Proskaure's test | +       | -    | -   | -   | -   |
| 3                                                          | Indole production test    | -       | +    | +   | +   | +   |

(+): Indicates presence of microbial colonies, (-): Indicates Absence of microbial colonies.

In table 6.3.2 lactic acid bacteria was identified according to their substrate utilization.

| Table 6.3.2: biochemical characterization of screened bacterial isolates. |                               |     |     |     |     |
|---------------------------------------------------------------------------|-------------------------------|-----|-----|-----|-----|
| Sr. No.                                                                   | Carbohydrates                 | B 1 | B 2 | B 3 | B 4 |
| 1                                                                         | Lactose                       | +   | +   | +   | +   |
| 2                                                                         | Xylose                        | +   | +   | +   | +   |
| 3                                                                         | Maltose                       | +   | +   | +   | +   |
| 4                                                                         | Fructose                      | +   | +   | +   | +   |
| 5                                                                         | Dextrose                      | +   | +   | +   | +   |
| 6                                                                         | Galactose                     | +   | +   | +   | +   |
| 7                                                                         | Raffinose                     | +   | +   | -   | -   |
| 8                                                                         | Trehalose                     | +   | +   | +   | +   |
| 9                                                                         | Melitose                      | +   | +   | -   | -   |
| 10                                                                        | Sucrose                       | +   | +   | +   | +   |
| 11                                                                        | L-Arabinose                   | +   | +   | +   | +   |
| 12                                                                        | Mannose                       | +   | +   | +   | +   |
| 13                                                                        | Inuline                       | +   | +   | -   | -   |
| 14                                                                        | Sodium Gluconate              | +   | +   | -   | -   |
| 15                                                                        | Glycerol                      | +   | +   | -   | -   |
| 16                                                                        | Salicin                       | +   | +   | +   | +   |
| 17                                                                        | Dulcitol                      | +   | +   | -   | -   |
| 18                                                                        | Inositol                      | +   | +   | -   | -   |
| 19                                                                        | Sorbitol                      | +   | +   | -   | -   |
| 20                                                                        | Mannitol                      | +   | +   | +   | +   |
| 21                                                                        | Adonitol                      | +   | +   | -   | -   |
| 22                                                                        | Arabitol                      | +   | +   | -   | -   |
| 23                                                                        | Erithritol                    | +   | +   | -   | -   |
| 24                                                                        | $\alpha$ Methyl D Glucoside   | +   | +   | -   | -   |
| 25                                                                        | Rhamnose                      | +   | +   | -   | -   |
| 26                                                                        | Cellobiose                    | +   | +   | +   | +   |
| 27                                                                        | Melezitose                    | +   | +   | +   | -   |
| 28                                                                        | $\alpha$ Methyl D Mannosidase | +   | +   | -   | -   |
| 29                                                                        | Xylitol                       | +   | +   | -   | -   |
| 30                                                                        | ONPG                          | +   | -   | +   | +   |
| 31                                                                        | Esculin                       | +   | +   | +   | +   |
| 32                                                                        | D Arabinose                   | +   | +   | +   | -   |
| 33                                                                        | Citrate                       | +   | -   | +   | +   |
| 34                                                                        | Malonate                      | +   | -   | +   | -   |
| 35                                                                        | Sorbose                       | +   | +   | -   | -   |
| 36                                                                        | Control                       | +   | +   | -   | -   |

(+): Indicates presence of microbial colonies, (-): Indicates Absence of microbial colonies.

#### 6.4 Enumeration of Probiotic Property

A diversity of lactic acid bacteria are typically food grade and evaluated for their probiotic potential and were applied as accessory cultures in a variety of types of foods. The bacteriocin produced lactic acid bacterial isolates were having some probiotic properties. These are the probiotic properties: gelatin hydrolysis test, haemolysis test, antibiotic

resistance and bile salt hydrolysis test was performed for the enumeration of probiotic properties of selected isolates. This isolate does not have activity of gelatin liquefaction and hemolysis of blood agar media. The isolates were resistant to high concentration of bile salt and antibiotic. The resistance was assessed against penicillin G, Erythromycin, vancomycin, ampicillin and cotrimoxazole.

## CONCLUSION

The present study was conducted to isolate, screen and identify bacteriocin producing LAB present in the buffalo milk samples and also to evaluate its inhibitory activity against selected pathogens. The results thus obtained revealed that LAB which are generally used as Starter culture for manufacturing the fermented food products, also possess some additional beneficial effects by producing bacteriocin which inhibits the growth of pathogens that renders the food unsafe for human consumption. Thus, the isolates of *Lactobacillus* spp. found effective against the test microorganisms can be further studied for their probiotic activity as well as inhibitory activity that could be utilized in therapeutic and food applications.

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