

Isolation and characterization of bacteriocin produced by locally isolated lactic acid bacteria from drainage of various dairies

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ABSTRACT

Lactic acid bacteria (LAB) commonly used as starter cultures in food is known to produce antimicrobial substances such as bacteriocins, having great potential as food biopreservatives. The present study was aimed at isolating bacteriocin producing LAB from drainage of dairy. About thirteen colonies of LAB were isolated and screened for bacteriocin production potential. Three isolates showed good antimicrobial activity such as *Bacillus cereus*, *Staphylococcus aureus*, and *Bacillus subtilis*. One of the bacilli has been reported as low producer of bacteriocin. Therefore, in this study characterization of 3 isolates through morphological, physiological, biochemical and carbohydrate fermentation tests.

Keywords: bacteriocin, lactic acid bacteria, antibacterial activity, SDS-PAGE

INTRODUCTION

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. This certainly holds true for lactic acid bacteria (LAB), which are used throughout the world for manufacture of a wide variety of traditional fermented foods. Since they are involved in numerous food fermentations, known to man for millennia, it is assumed that most representatives of this group do not pose any health risk to man. The LAB, generally considered as “food grade” organisms, show special promise for selection and implementation as protective cultures. There are many potential applications of protective cultures in various food systems [10]. Such organisms have been isolated from grains, dairy products, dairy wastes, meat products, fermenting vegetables, and the mucosal surfaces of animals. During lactic acid fermentation these bacteria not only have their effect on food and flavour but they are also known to produce and excrete compounds with antimicrobial activity such as bacteriocins. Bacteriocins of LAB are considered as safe natural preservatives or biopreservatives, as it is assumed that they are degraded by the proteases in gastrointestinal tract [5].

Bacteriocins are extracellularly released peptides or protein molecules, with a bactericidal or bacteriostatic mode of action against closely related species. The inhibitory spectrum of some bacteriocins also includes food spoilage and food-borne pathogenic microorganisms [11]. The discovery of nisin, the first bacteriocin used on a commercial scale as a food preservative dates back to the first half of last century but research on bacteriocins of LAB has expanded in the last two

decades, searching for novel bacteriocin producing strains from dairy, dairy waste, meat and plant products, as well as traditional fermented products. Many bacteriocins have been isolated and well characterized [12]. We have reported the characterization of LAB isolates, from drainage of dairy and characterization of the partially purified bacteriocins derived from the same [13]. Present investigation reports on the isolation and characterization of the other bacteriocin producing LAB.

MATERIALS AND METHODS

Isolation and screening of LAB

Three samples from three dairies (20 ml each) were separately inoculated in 80 ml sterile MRS broth containing casein peptone (tryptic digest) 10 gm, meat extract 10 gm, yeast extract 5 gm, glucose 2 gm, tween 80 1 gm, potassium dihydrogen phosphate 2 gm, sodium acetate 5 gm, magnesium sulphate 0.2 gm, manganese sulphate 0.05gm, diammonium citrate 2 gm with pH 6.2 to 6.5 and incubated at room temperature in anaerobic condition for 24 hrs. After incubation 0.1 ml of each sample was sprayed over MRS agar medium and incubated anaerobically to get microbial colonies. Five test strains were spotted on surface of MRS agar plates. Plates were incubated overnight at anaerobic conditions. Plates were overlaid with soft agar containing target strains. After incubation three different clear zones were observed. Three strains 1, 2 and 3 which were giving clear zones selected for further study [4]. All three strains were designated as strains B₀, B₂ and B₃ for 1, 2 and 3 respectively and maintained on sterile MRS agar stab at 4°C.

Production of bacteriocin from isolated strains

Three enrichment media were inoculated with three isolated strains (B₀, B₂ and B₃) and incubated overnight anaerobically. Production medium (sterile MRS broth 100 ml each) was prepared and inoculated with enriched pure isolates. Inoculated flasks were incubated anaerobically by jar method. The protein concentration and pH of broth checked at particular interval. After 18 hrs the fermentation was stopped [2].

Characterization and differentiation

Bacteriocins producing bacterial strains were Gram stained and examined microscopically for cellular morphology and Gram-stain phenotype. Catalase activity was tested by spotting colonies with 3% H₂O₂. Growth was assayed in MRS broth at 10, 15, 37 and 45°C and at pH of 4.4, 5.0, 8.6 and 9.0. It was found that better growth was obtained at 37°C and pH 6. Salt tolerance was tested with 6.5, 10 and 15% (w/v) NaCl maintained in MRS broth. Acid and CO₂ production from glucose was tested in MRS broth containing Durhams tube [10]. Ammonia production in MRS broth without glucose and meat extract, containing 0.3% arginine and 0.2% sodium citrate was monitored using Nessler's reagent. Evaluation of the fermentation ability of various carbohydrates was done using MRS broth supplemented with filter sterilized sugar solutions of a final concentration of 1% w/v and 0.004% chlorophenol red without glucose and meat extract [8].

Extraction of adsorbed bacteriocin from cells

The incubated broths were adjusted at pH 6.5. Each flask was heated at 60°C for 10 min to kill the cells. The cells were harvested by centrifugation at 12,000 rpm for 15 min. Then the cells washed with 5 mM sodium phosphate and suspended in 5 ml of 100 mM NaCl at pH 2 (Adjusted with 5% phosphoric acid). Cell suspensions were centrifuged at 18,500 rpm for 20 min. Cell mass again

resuspended in 5 mM sodium phosphate. Cell mass and supernatant were separated. Crude bacteriocins were precipitated by mixing the supernatant with an equal volume of cold (-20°C) 96% ethanol [13]. After 3 hrs at -20°C, the precipitate was collected by centrifugation (10,000 rpm for 30 min.).

Dialysis

Dialysis tube of suitable diameter was selected and cut into suitable length to contain the volume required. Prior to use, dialysis tube was rinsed inside and outside with distilled water. One end of the tube was sealed with a dialysis clip and solution to be dialyzed was poured in the bag. The air from the bag was expelled and sealed the top end with a dialysis clip. The bags containing bacteriocins (B₀, B₂ and B₃) were placed in a large volume of distilled water and agitated gently with a magnetic bar and stirrer motor. It was ensured the bags were not knocked by the magnetic bar to prevent rupture. The assembly was left to reach equilibrium in overnight [9].

Finally, the antimicrobial activity was tested by performing agar well diffusion assay. Bacterial were isolated from the drainage of dairy by appropriate dilutions with saline, plated on MRS agar and incubated anaerobically at 37°C for 2-3 days. Five well isolated colonies were picked up and transferred to MRS broth. They were propagated twice and streaked on MRS agar to check the purity of the isolates and then stored in MRS soft agar (0.5%) overlaid with 50% glycerol at -20°C. These 5 isolates from drainage of dairy were tested for their ability to produce bacteriocins. The isolates maintained in frozen stocks were propagated twice in MRS broth and used for further study. These were inoculated into MRS broth and incubated at 37°C for 48 hrs. Cell free supernatants adjusted to pH 5.0 with 2 N NaOH, were concentrated to one tenth of the original volume by flash evaporator, sterilized by passing through 0.22 µm membrane filter (Millipore, India) and evaluated for antimicrobial activity by agar well diffusion method [12] against *B. cereus*, *S. aureus*, *B. subtilis*.

Sterile nutrient agar plate was overlaid with soft agar containing test microorganisms. Three wells per plate with the help of sterile cork borer (6 mm). Bacteriocin isolated from three samples poured in wells. Inoculated plates were kept in freeze for diffusion for 20 min. Plates were incubated for 24 h at 37°C. After incubation, plates were observed for zone of inhibition and zone diameter for each plate was measured [12]. Cell wall proteins were extracted from final pellets with 0.5 ml of 0.01 M Tris-HCl, 0.01 M EDTA, 0.01 M NaCl, 2% SDS, pH 8.0, at 100°C for 5 min. After treatment suspensions were centrifuged at 11600 g for 10 min and supernatant fluids were examined using Tris-Glycine SDS-PAGE [14] with stacking gel of 5% and separating gel of 20% acrylamide-bisacrylamide [8].

RESULTS AND DISCUSSION

Three out of 5 selected bacterial colonies exhibited good antimicrobial activities against gram positive bacteria e.g. *B. subtilis* and *S. aureus*. Three strains were found to be gram positive and named as B₀, B₂ and B₃. Morphologically B₀ was short rods, while B₂ and B₃ were cocci. These strains were catalase negative and did not produce CO₂ from glucose. Luxurious growth was observed for both B₂ and B₃ strains at 37°C while it was weak or no growth at 10°C and 45°C. Carbohydrate fermentation pattern showed that both B₂ and B₃ strains were able to ferment maltose. B₀ was found to be gram positive, catalase negative bacilli. Its growth was weak at 15°C and at pH 4, while grew luxuriously at 37°C and pH 8.5. It also ferments manitol and sucrose (Table 1).

Sugar concentration in the production medium (0.2%) was minimized as compared to inoculum medium (2%) to enhance bacteriocin production. The bacteriocin was produced during logarithmic growth and highest activity was found in early stationary phase. After that activity was declined

possibly by re-adsorption of bacteriocin to producer cells. No production was observed on a chemically defined medium containing glucose. Different isolates as B₀, B₂ and B₃ showed different antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis*. Cell extract of B₀ and B₃ showed more activity than B₂ (Table 2). Antibacterial activity of bacteriocin was more when direct cell biomass was used. But it was less observed when supernatant was used due to loss of bacteriocin; whereas, dialyzed supernatant improved antibacterial activity of bacteriocin. Dialyzed sample of 3 isolates increased the antimicrobial activity against target organisms showed that dialysis have improved quality of purification. After dialysis B₃ was found to have highest antimicrobial activity against target organisms than B₂ and B₀.

Table 1. Screening of bacteriocin producing isolates at different growth parameters.

Growth parameters	B ₀	B ₂	B ₃
Growth at (°C)			
10	-	-	-
15	+	+	+
37	++	++	++
45	-	-	-
Growth at pH			
4	+	+	+
5	+	+	+
8	++	++	++
9	+	+	+

Note: +: Growth; ++: Luxurious growth; -: No growth

Table 2. Antimicrobial activity of bacteriocin against three pathogenic bacterial strains.

Test bacteria	B ₀	B ₂	B ₃
<i>S. aureus</i>	+	+	++
<i>B. subtilis</i>	+	+	++

Note: +: Antimicrobial activity, ++: Highest antimicrobial activity

A coomassie blue stained SDS-PAGE gel revealed a sharp band from B₃ preparation especially when they are tested within 2 weeks of preparation. Three potential bacteriocin producing strains were screened out of 15 isolated strains the bacteriocin activity confirm after partial purification. The test organism *S. aureus* and *B. subtilis* were proved sensitive to product bacteriocin. The molecular weight of bacteriocin of B₃ was 12 KD. The bacteriocin can be used in food application and further application studies required for optimization of product.

Thus, as per defined objectives three potential bacteriocin producing strains were screened out of 15 isolated strains. The bacteriocin activity confirm after partial purification. The test organism *Staphylococcus aureus* and *Bacillus subtilis* were proved sensitive to produce bacteriocin. The molecular weight of bacteriocin of B₃ was approx.12 KD. The bacteriocin can be used in food preservatives.

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