Production Optimization and Characterization of a Bacteriocin Produced by *Lactobacillus casei* Isolated from Milk

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Abstract

Bacteriocin producing *Lactobacillus casei* LBM1 strain isolated from milk sample, showed broad range of antibacterial activity against some major food borne bacterial pathogens. In the present study, we have optimized the growth of *Lactobacillus casei* LBM1 for the bacteriocin production. Growth kinetics of *Lactobacillus casei* was studied in accordance to time, pH and temperature. Optimum growth as well as optimum bacteriocin activity was obtained after 18 h at pH 5.0 and temperature 35°C. Bacteriocin activity was produced on optimum conditions and were further characterized and purified. The extracted crude bacteriocin was also tested for some physiological conditions heat, pH and enzymes. The crude bacteriocin of *Lactobacillus casei* LBM1 exhibited no effect on the proteinase-K and Trypsin. And in active at 121°C. The crude proteins showed the residual activity remain at pH 3.0 to 9.0 ranges.

Keywords: *Lactobacillus casei*, lactic acid bacteria, milk, bacteriocin.

1.INTRODUCTION

Lactic acid bacteria are widely used as starter cultures and play an important role in food preservation, microbiological stability and production of aroma compounds. Many of these lactic acid bacteria produce bacteriocins (Degnan *et al*.,1992). Lactic acid bacteria are traditionally used as starters for food fermentations. Since they have a capacity to Some bacteriocins have been used inhibit spoilage and pathogenic bacteria (Guessas *et al.,* 2005), they are important in food preservation and intestinal prophylaxis. Lactic acid bacteria are the most important groups for industrial purposes, since their fermentative activity involves a notable preservative capacity because of the drop in the pH and the antimicrobial activity of their metabolites such as lactic and acetic acid, diacetyl or bacteriocins. Bacteriocins of lactic acid bacteria (LAB) have been classified into four structural classes, namely I, II, III and IV (Nes *et al*.,1996). They have been classified based on their size, chemical properties, mode of action and mechanism of export. Some of these inhibitory substances are active against food borne pathogens and they become the focus of research interest concerning their potential role as food preservatives. Use of either bacteriocin-producing LAB strains, which are generally regarded as safe (GRAS), or their bacteriocins in food production could have a positive effect on food preservation and safety. Many bacteriocins are capable of resisting inactivation at the high temperatures used in food processing and can remain functional within a broad pH range. Bacteriocins are usually inactivated by proteolytic

enzymes in the human digestive tract and would be digested just like any other protein in the diet. These natural metabolites could replace the use of chemical additives such as sorbic acid, sulphur dioxide, nitrite, nitrate, and others are used as bio-preservatives. The lactic acid bacterial bacteriocins in appropriate concentrations may be used as an additional factor for increasing the shelf life of minimal processed foods as well as in veterinary medicine and animal growth promoter instead of antibiotics. The present study deals with the possibility of developing a bio-preservative by investigating the bacteriocin production by lactobacilli. In the Present investigation reports on the Production optimization and characterization of a bacteriocin produced by *Lactobacillus casei* isolated from milk.

2.MATERIALS AND METHODS

Isolation and identification:

Homogenized ten ml of milk sample was transferred to 90 ml alkaline peptone water and the samples were serially diluted up to 10^{-6} dilution. About 1 ml of appropriate dilution of the sample was pipette into sterile Petri dishes. MRS agar media were poured and incubate at room temperature for 48 hrs. The LAB was identified based on growth on selective MRS agar (pH 5.2), cell morphology, gram staining, catalase activity and biochemical identification of LAB. Further identification of the species of this LAB was performed according to carbohydrate fermentation patterns and growth on MRS broth (HI Media) as described in Bergey's manual of systematic bacteriology. The isolated LAB was sub cultured and the purified cultures maintained at MRS agar slants

Test microorganisms

Nutrient broth was prepared and sterilized. Four pathogenic organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *P. aeruginosa* and *E. coli*, were inoculated separately and kept for incubation for further use.

Production of crude bacteriocin:

The isolated strain was grown in MRS broth (Hi Media Laboratory Pvt Ltd. India) (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 30ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4ºC). The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin (Ogunbanwo *et al*., 2003).

Bacteriocin assay

The antimicrobial activity of bacteriocin was determined by agar well diffusion method. Muller Hinton agar plates were overlaid with 10 ml Muller Hinton soft agar (0.75% agar) lawn containing an indicator bacterial strain. The indicator lawns prepared by adding 0.25 ml of a 10^{-1} dilution overnight cultures of test organisms. Wells 8 mm in diameter were cut into agar using sterile cork borer. Then 100 µl of culture supernatant fluids of *Lactobacillus casei* LBM1 was placed into each well. The plates were incubated at 37 °C for 24 h and examined for zones of inhibition. The bacteriocin activity was expressed in terms of inhibition zone (mm).

Optimization of bacteriocin production process

Determination of growth kinetics

The candidate LAB was first grown in MRS broth for 72 h, and then bacterial growths were observed after 0, 6, 12, 18, 24, 48 and 72 h to measure OD of the culture using a spectrophotometer at 600 nm wavelengths.

Determination of optimum pH and Incubation temperature

Optimum growth was measured by using the different pH medium ranging from pH 3 to 9 by measuring OD of the culture using a spectrophotometer at 600 nm wavelength. Optimal temperature for the growth was calculated using the different temperatures for bacterial candidate growth ranging from 20 to 50°C. The changes in growth of the medium were measured by measuring OD of the culture using a spectrophotometer with 600 nm wavelengths (Kanasaki *et al*., 1975). The bacteriocin activity was tested by agar well diffusion assay.

Sensitivity of crude protein to enzymes, heat and pH

The sensitivity of crude protein to proteolytic and other enzymes were tested on crude protein (pH 7.0) of 24 h cultures incubated at 37 °C. Samples of 100 µl were treated for 2 h with 1 mg ml^{-1} of Trypsin, proteinase-K, α-amylase and lipase. All the samples and controls were incubated at 37 °C for 5 h and tested for activity. The sensitivity of crude protein to different pH was estimated by adjusting the pH of crude bacteriocin of *Lactobacillus casei* LBM1 to pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 with NaOH or HCl and testing against the indicator strain after 2 h incubation. The sensitivity to heat was tested by heating crude bacteriocin of *Lactobacillus casei* LBM1 to 37, 50, 70, 90, 100 °C for 30min. and 121 °C for 10 min. and testing the residual activity after the treatment by well diffusion assay.

3.RESULT AND DISCUSSION

Isolation and Identification:

Bacteriocins are described as ribosomally synthesized small poly peptides that exert antimicrobial effects against closely or non-closely related bacteria. In this study, lactic acid bacteria were isolated from cheese. Microscopic identification of the isolate could determine the rod-shaped cells, gram positive, catalase negative, non-motile rods and oxidase negative which indicated the typical basic characteristics of Lactobacilli. Based on the carbohydrate utilization pattern of bacterial isolates were identified as *Lactobacillus casei*LBM1. The results are tabulated in Table 1. Similar characters for lactic acid bacteria observed earlier by Kandler and Weiss (1986). MajaTolinacki *et al*.,2010 in their studies isolated and characterized the *Lactobacillus paracasei* subsp. *paracasei* BGUB9 from homemade hard cheese. The lactic acid bacterial isolate was tested for their inhibitory activity over some food borne pathogens, *Staphylococcus aureus*, *Bacillus subtilis*, *P. aeruginosa* and *E. coli*. Almost all pathogens were inhibited by bacteriocin producer. The results are tabulated in Table 2.

Determination of growth kinetics

L. casei LBM1recorded highest growth was achieved after 18 h. Cell free supernatant of this point showed highest bacteriocin activity. Growth and activity were maintained in 24 h culture then slightly decreases and in 72 h culture OD was declined to less than 1.0. The highest inhibition zone 18.33 mm from the culture having OD of 2.7. These results were like the finding of Sharma et al. (2010). Ivanova et al. (2000) cultivated the strains before bacteriocin extraction from the culture in deMan, Rogosa and Sharpe (MRS) medium (Figure -1).

Determination of optimum pH and temperature: *L. casei* LB M1 recorded highest growth was achieved at pH 5.0h. Cell free supernatant of this point showed highest bacteriocin activity. After the pH 7.0 The Growth and activity was slightly decreasing OD was declined to less than 1.0. (figure-2). The highest growth was measured by using the different pH media ranging from 3 to 9.0 by measuring OD of the culture using a spectrophotometer at 600 nm. Optimum growth obtained at OD 2.8 and the highest inhibition zone of bacteriocin was obtained at pH 5.0. After pH 7.0, bacteriocin activity was decreased (Figure -3). Sharma *et al*. (2010) reported the optimum pH as 7.5, while Ivanova *et al.* (2000) reported the optimum pH 5.5. Ahmed *et al*., (2006) reported the three different strains growing maximally at pH 4.88, 4.89 and 4.82. *L. lactis* exhibited a good bacteriocin activity of 2344 AU/ml, at pH 6.0 and 30°C.

L. casei LBM1optimum temperature was observed as 35°C. The highest bacteriocin activity was obtained as 18.50mm (Figure -3). These findings are in accordance with the previous reported by Aslam et al.,2012 optimum growth temperatures for *L. lactis the o*ptimum growth temperature for the *Lactococcus* is at 37 °C; they can grow as low as 20°C but not more than 45°C.

Sensitivity of crude protein to enzymes, heat and pH

The effect of various enzymes on the crude protein of *Lactobacillus casei* LBM1was studied, inhibitory activity of crude bacteriocin of *Lactobacillus casei* LBC1 was completely inactivated by proteolytic enzymes such as proteinase k and trypsin, but not affected by non-proteolytic enzymes such as α-amylase, lipase (Table-3). Thus, it can be inferred that the bacteriocin is proteinaceous in nature and does not require a carbohydrate or lipid moiety for the activity (De Martinis *et al*., 2001). According to Fricourt *et al*. (1994) lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity. Many of these agents are bacteriocins with a proteinaceous active moiety while others are non-protein agents (Piard and Desmazeaud, 1991). The bacteriocins were shown to be stable over a broad pH range with all peptides maintaining some antimicrobial activity within the pH range of pH 3 to 10. According to Tagg *et al*. (1976), bacteriocins differ greatly with respect to sensitivity to pH and Tween 80. Many of them considerably more tolerant of acid than alkaline pH values. In the present study, crude bacteriocin of *Lactobacillus casei* LBM1 exhibited inhibitory activity at pH values between 2 to 10. Highest inhibitory activity was observed at pH 6.0. These results surely support the view expressed by Ravisankar *et al*., (2012) reported that bacteriocin of *Lactobacillus plantarum* was active in a wide range of pH, but the maximum activity was observed at pH 5.0 and 6.0. Bacteriocin could retain its antimicrobial activity partially when there was a shift to acidic or basic range. stability of bacteriocin at different pH scale is a limiting factor for recommending its use in food items. The crude bacteriocin of

Lactobacillus casei LBM1was relatively stable during heat treatments at 37, 50, 70, 90, 100 °C for 30 min and 121 °C for 10 min. Among the different heat treatments, the highest inhibitory was recorded at 37 °C. Residual activity of bacteriocin did not show significant difference from the control. In the present study, the inhibitory activity of crude bacteriocin of *Lactobacillus casei* LBM1 decreased with increase in temperature levels from 37°C to 100°C for 30 min. The activity of crude bacteriocin of *Lactobacillus casei* (LBM1) was completely inactivated at 121°C for 15 min. These results were in accordance with Sivakumar *et al*.,(2010) who observed that the activity of bacteriocin produced by *Lactobacillus acidophilus, P. acidilactii* remained after heat treatment with 100°C for 10, 20 and 30 min but complete in activation occurred after 10 min exposure to 121°C. Considering the harsh conditions of food processing, high thermal and wide range of pH stability are major criteria in the selection of candidate bacteriocins for bio preservation of processed foods (Yi *et al*., 2016) .

Conclusion

The bacteriocin produced by *Lactobacillus casei* LBM1 was assayed by agar well diffusion method and bacteriocin activity was measured in terms of millimetre. Mode of action of bacteriocin produced by *Lactobacillus casei* LBM1 was tested and the behaviour of the bacteriocin produced by isolated strain was considered as bactericidal.

Table 1: Morphological and biochemical characteristics of lactic acid bacterial strain

Morphological characters	**LBM1**
Gram reaction	+
Shape	Rods
Size	0.8 µm x2µm
Motility	-
Biochemical Characters	
Catalase test	-
Oxidase test	-
NH3 from arginine	-
Gas production from glucose +	-
Carbohydrates	
Arabinose	-
Cellobiose	+
Esculin	+
Fructose	+
Galactose	+
Glucose	+
Lactose	D
Maltose	+
Mannitol	+
Mannose	+
Melezitose	+
Melibiose	-

Raffinose	-
Rhamnose	-
Ribose	+
Salicin	+
Sorbitol	+
Sucrose	+
Trehalose	+
Xylose	-
Identified as	*Lactobacillus casei*

Table 2: Inhibitory spectrum of crude bacteriocin of *Lactobacillus casei* LBM1 against food borne pathogens

Food Borne Pathogens	**Inhibition Zone (in mm)**
Staphylococcus aureus	18.35
Bacillus subtilis	16.50
Escherichia coli	14.20
Pseudomonas aeruginosa	12.30

Table 3: Effect of enzymes, pH and heat treatment on inhibitory activity of crude bacteriocin of *Lactobacillus casei* LBM1

Treatment	**Inhibition Zone (in mm)**
Crude bacteriocin	18.30
Trypsin	-
Proteinase K	-
α- amylase	18.10
Lipase	18.30
pH	
2	6.30
3	9.00
4	14.20
5	18.10
6	17.40
7	16.30
8	12.60
9	9.40
10	7.20
Temperature	
37°C	18.30
50°C	16.50
70°C	13.40
90°C	9.30
100°C	5.60
121°C	-

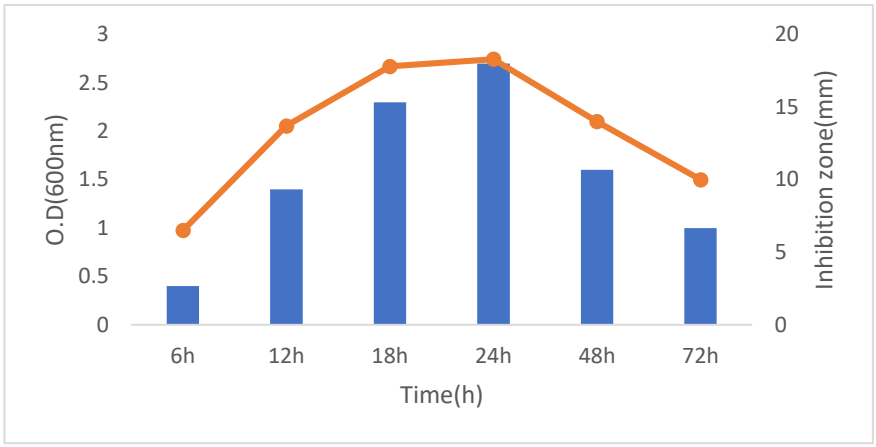

Figure1.Growth kinetics and bacteriocin activity relationship of *Lactobacillus casei* LBM1.

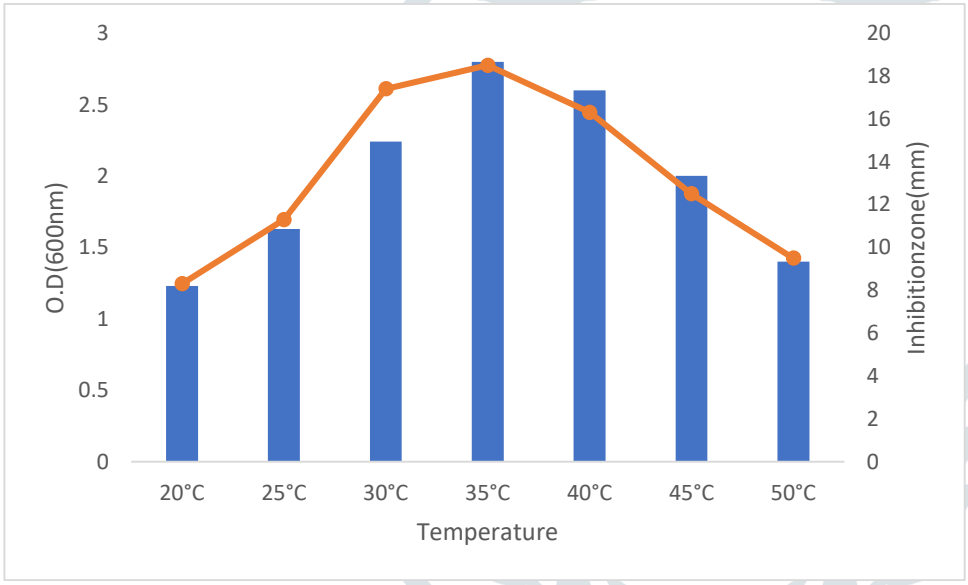

Figure .2-Growth and kinetics and bacteriocin activity of *Lactobacillus casei* LBM1 at different temperature

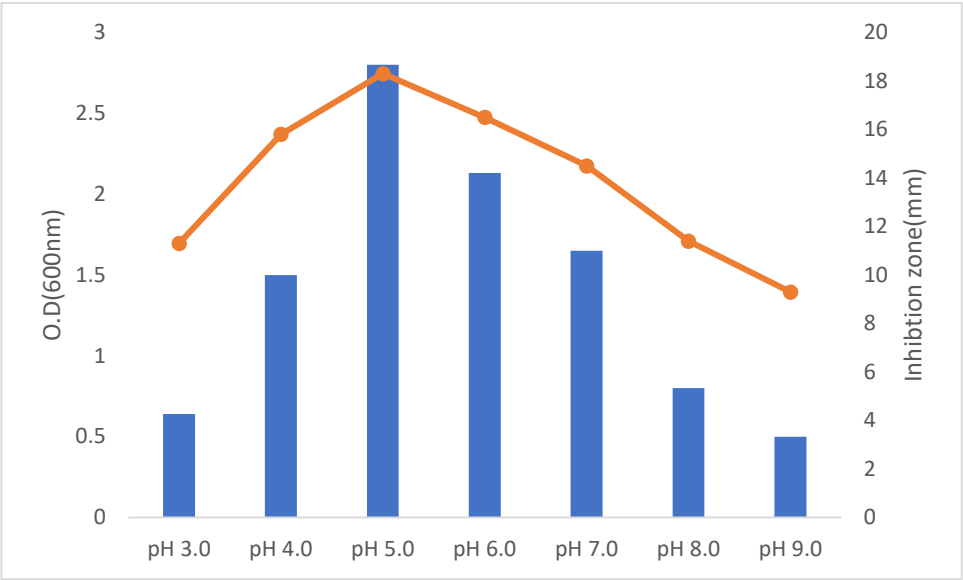

Figure-3. Growth and kinetics and bacteriocin activity of *Lactobacillus casei* LBM1 at different pH.

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