ORIGINAL RESEARCH PAPER

Microbiology

DETECTION, OPTIMIZATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS FERMENTUM* A STRAIN ISOLATED FROM HOME MADE CURD- INDIAN TRADITIONAL FOOD

KEY WORDS: Bacteriocin, optimization, production, *Lactobacillus fermentum*, traditional food.

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ABSTRACT

Lactic acid bacteria display numerous antimicrobial activities mainly due to the production of bacteriocins and antifungal peptides. Bacteriocins are known for anti-microbial properties against various pathogens. The aim of this work is to investigate the effect of growth conditions on the maximum production of bacteriocin by *Lactobacillus fermentum* isolated from home made curd. Bacteriocin produced by *Lactobacillus fermentum*, inhibited the growth of *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Proteus mirabilis.* The exponential phase of the growth was started at 4h from the time of incubation. The stationary phase begins at the 12h from the time of incubation. Maximum bacteriocin production of 12650 AU/mL with more biomass was obtained in presence of glucose. Yeast extract as sole nitrogen source, in MRS broth, stimulated bacteriocin production upto 2400 AU/mL. The maximum bacteriocin production of 12200 AU/mL was obtained with 2 % of NaCl. The optimum pH for bacterial growth and bacteriocin production was identified as pH 5. The highest bacteriocin activity of 7250 AU/ml and maximum growth of 1.90 was recorded at pH 5. Bacteriocin production was found to be highest at 40°C temperature (8100AU/ml). Optimization of bacteriocin production with the modification of environmental growth conditions will greatly benefit efficient commercial applications. The stability of the bacteriocin with respect to pH, temperature, enzyme sensitivity and organic solvents also studied.

INTRODUCTION

Recent explorations of the human gut microbiota suggest that perturbations of microbial communities may increase predisposition to different disease phenotypes. Dietary nutrients may be converted into metabolites by intestinal microbes that serve as biologically active molecules affecting regulatory functions in the host. Probiotics may restore the composition of the gut microbiome and introduce beneficial functions to gut microbial communities, resulting in prevention of gut inflammation and other intestinal diseases. Lactic acid bacteria (LAB) are known for the production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides. Bacteriocins of LAB are defined as ribosomally synthesized proteins usually antagonistic against pathogenic organisms. They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism varies and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis[1].

Bacteriocin production does not always correlate with the increase in cell mass or growth rate of the producer strain[2]. Higher bacteriocin levels are often recorded in the absence of growth stimulating nutrients, or at temperatures and pH conditions lower than required for optimal growth[3]. Optimal bacteriocin production is often recorded in medium with limiting concentrations of sugars, nitrogen sources, vitamins and potassium-phosphate, or when the medium pH is regulated[4]. Bacteriocin production changes dramatically upon altering of environmental conditions and optimum production may require a specific combination of environmental parameters. Studies conducted on bacteriocins from other lactic acid bacteria, e.g. pediocin AcH[5], pediocin PD-1[6], enterocin 1146[7], enterocin AS-48[8], enterocin P[9], sakP[10] and bacteriocins produced by *Leuconostoc mesenteroides* L124[11] have shown that production is often regulated by growth pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions[12-15].

Because of the increasing demand for more natural and microbiologically safe food products, there is a need for biopreservation techniques. Bacteriocins have considerable potential for food preservation, as well as for human therapy as potential supplements or replacements for currently used antibiotics. This study was focused on isolation, screening and characterization of bacteriocin producing *Lactobacillus fermentum* from curd and its inhibitory activity against pathogens with broad inhibition spectra and optimization of nutrients, medium pH and temperature on the activity levels of bacteriocin production and also stability of the bacteriocin under different conditions.

MATERIALS AND METHODS

Isolation, Identification and Screening of bacterio cinogenic *Lactobacillus fermentum*

Curd samples were collected under aseptic conditions in sterile containers for the isolation of *Lactobacillus* strains on MRS agar at 37°C for 48 h. Colonies were taken from the MRS plates, sub cultured and maintained on MRS agar medium[16].

The isolated Lactobacillus strains were identified based on colony morphology, cell morphology and biochemical tests described by Oyeleke and Manga[17]. Identification of the producer strain to species level was done by 16 S r RNA sequence analysis, then sequenced and compared the sequences in GenBank using BLAST, Basic Local Alignment Search Tool and was identified as *Lactobacillus fermentum*.

Culture of *Lactobacillus fermentum* was grown in MRS broth at 37° C for 48 h. After incubation, the bacterial cells were removed by centrifugation at 10,000 x *g* for 5 min at 4°C. The supernatant was adjusted to pH 6 with 1 N NaOH to eliminate inhibitory activity from acid. The supernatant was used as pretreated extract of *Lactobacillus fermentum* and stored at -20° C until analysis. The different target organisms used to demonstrate antimicrobial activity are *Escherichia coli, Enterococcus faecalis, Pseudomonas fluorescence, Pseudomonas auregenosa, Staphylococcus aureus, Salmonella typhimurium, Proteus mirabilis* and *Bacillus megaterium* and were grown at 37°C for 24 h in luria broth medium.

Detection of Antimicrobial Activity and assay

The antimicrobial activity of bacteriocin producing *Lactobacillus fermentum* was studied against enteric pathogens using the agar well diffusion method[18]. Indicator lawns were prepared by spreading 0.5 ml of each target strain

grown in luria broth overnight at 37° C, over the surface of prepoured luria agar plates. Wells were cut with a sterile 5-mm cork borer and 50 µl of pretreated extract of *Lactobacillus fermentum* were transferred to the wells in preseeded agar plates and incubated at 37° C for 24 - 48 h. All antagonistic activity assays were conducted in duplicates. Finally, the plates were examined for the presence of inhibition zones and antimicrobial activity is expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

Optimization of culture conditions for the production of bacteriocin

To determine the effect of incubation time on bacteriocin production *Lactobacillus fermentum* was grown in MRS broth in 500 ml Erlenmeyer flask. 0.1 ml of overnight culture was inoculated in to the flask and the flask was incubated at 37°C for 18 h with constant shaking at 150 rpm. Sample of 5 ml was withdrawn from the flask for every 2 h time interval and the bacterial cell density was measured at 600 nm and bacteriocin activity was determined as arbitrary units/ ml (AU/ml) [19].

The effect of carbon sources on bacteriocin production by *Lactobacillus fermentum* was studied with different carbon sources (glucose, lactose, sucrose, fructose and maltose) added to MRS broth at 2 % (w/v) level and inoculated with an overnight culture of (1 % v/v) bacteriocin producing *Lactobacillus fermentum*. MRS broth without carbon source was used as a control. The influence of different nitrogen sources on bacteriocin production was determined, by adding 2 % level of beef extract, yeast extract, peptone, tryptone and ammonium chloride separately to MRS broth and inoculated with an overnight culture of (1% v/v) bacteriocin producing *Lactobacillus fermentum* then incubated at 37° C for 24 h [20]. The cell free supernatant obtained after centrifugation at 10,000 rpm was used for bacteriocin assay. Bacterial growth was monitored as optical density at 600 nm and bacteriocin activity was determined as arbitrary units/ ml (AU/ml). Bacteriocin production was also investigated with different concentrations of NaCl like 1 %, 2 %, 3 %, 4 % and 5 % at 37° C for 24 h[21]. According to the method of Ennahar *et al*., 1999, the effect of pH on production of bacteriocin was determined, 100 ml of each medium adjusted with different pH was inoculated with an overnight culture of (1% v/v) bacteriocin producing *Lactobacillus fermentum*. To select the suitable temperature for the bacteriocin production, the *Lactobacillus fermentum* was cultivated with varying temperatures like 30°C, 40°C, 50°C and 60° C[22]. Bacterial growth was determined by measuring optical density at 600 nm and bacteriocin activity was determined as arbitrary units/ ml (AU/ml). The cell free supernatant obtained after centrifugation at 10,000 rpm was used for bacteriocin assay.

Characterization of bacteriocin

The stability of the bacteriocin was characterized at different pH values, temperatures, susceptibility to denaturation by enzymes and various organic solvents. The bacterio cinogenic *Lactobacillus fermentum* was cultivated in MRS broth for 24 h at 37°C, cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C, cell free supernatant (CFS) was used as a crude extract of bacteriocin sample. The antibacterial activity of the CFS was tested against *Salmonella typhi* and *Staphylococcus aureus* and measured as AU/ml.

The stability of the bacteriocin was determined at different pH values by adjusting to a pH range of 3, 4, 5, 6, 7, 8, 9 and 10 with 1 M HCl and 1M NaOH. All the samples were incubated at 37° C for 2 h. After incubation, the samples were then readjusted to neutral pH7.0 with the same reagents (Ivanova *et al*., 2000). Thermal stability was tested by incubating the cell free supernatant at different temperatures 30°C, 40°C, 50° C, 60°C, 70°C, 80°C, 90°C, 100°C for 10 min. After incubation the residual activity was tested by agar well diffusion assay[23].

The sensitivity of the active substance to proteolytic enzymes was assessed by treating CFS with trypsin, proteinase k and α-chymotrypsin. Each enzyme was prepared at a concentration of 1mg/ml. Samples were incubated at 37° C for 2 h and the residual enzyme activity was finally stopped by heating at 95-97° C for 5 min [24]. Stability of the bacteriocin in different organic solvents like acetone, chloroform, acetic acid, ethanol, toluene, ethyl acetate, dimethylsulphoxide (DMSO) and methanol was investigated, 10 % solution of each solvent was prepared and 1 ml of cell free supernatant was added, mixed vigorously and incubated for 30 min at 37° C. Thereafter, 10 µl of each mixture was used for the detection of residual activity of the bacteriocin [25].

RESULTS AND DISCUSSION

The strain of *Lactobacillus* isolated from curd using MRS media was found to be Gram positive, rod-shaped and catalase negative. The strain was identified by physiological and biochemical characteristics. Amplification of genomic DNA with genus-specific primers by 16 S rRNA sequence analysis revealed homology with *Lactobacillus fermentum*. The antimicrobial substance (bacteriocin) produced by *Lactobacillus fermentum* was found to be active with wide inhibitory spectrum against *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Proteus mirabilis* (Fig 1).

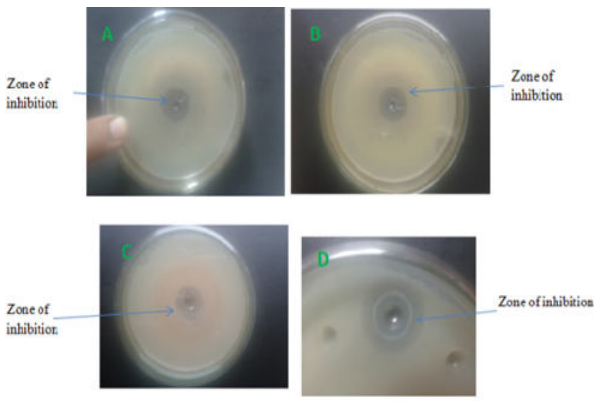

Fig 1. Antimicrobial activity of *Lactobacillus fermentum* against enteropathogens
A-*Salmonella typhimurium*, B: *Staphylococcus aureus*, C- *E. coli*; D- *Enterococcus faecalis*

Effect of incubation time, carbon and nitrogen sources on bacteriocin production

The present study was primarily aimed at determining cultural conditions for obtaining maximum and stable bacteriocin production by *Lactobacillus fermentum*. The exponential phase of bacterial growth was started at 4h from the time of incubation and the bacteriocin production starts at 6h of incubation. There is an increase in the optical density from 0.30 to 0.94 from 4 h to 6 h. Bacteriocin production was found to be stable at 10 to 18 h of incubation with the activity of 6400 AU/ml. The results obtained were then used to plot a growth curve as represented in fig 2.

Among the different carbon sources tested, the highest bacteriocin production of 12650 AU/mL was obtained in presence of glucose (5650 AU/ml) and lactose enhances more biomass (fig 3). Glucose was observed to be the best medium component for bacteriocin production. Maximum amount of bacteriocin production was observed in the medium supplemented with yeast extract (2400 AU/mL) in which the bacterial growth was 1.79 (OD_{600}) (fig 4). Yeast extract is best among all the nitrogen sources tested. The medium supplemented with 2 % of NaCl concentration yielded maximum bacteriocin production of (12200 AU/mL) (fig 5) in which highest bacterial growth of 2.25 (OD_{600}) also recorded.

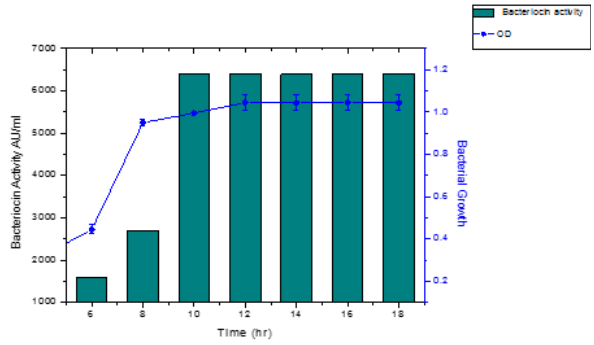

Fig 2. Effect of incubation time on cell density and bacteriocin production

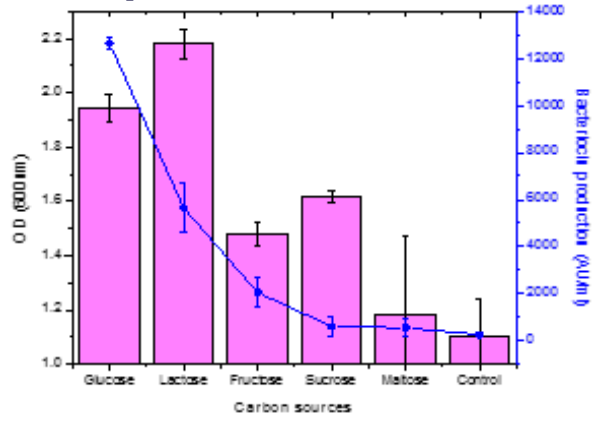

Fig 3. Effect of carbon sources on bacteriocin production

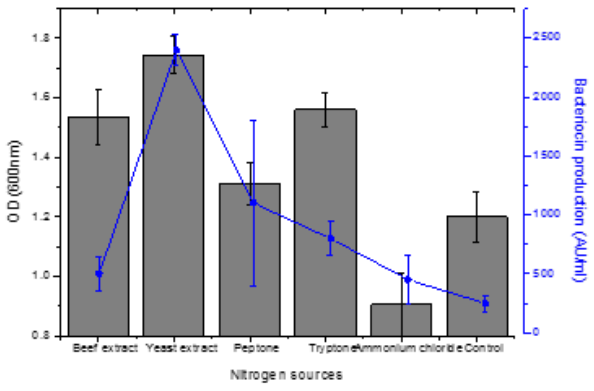

Fig 4. Effect of nitrogen sources on bacteriocin production

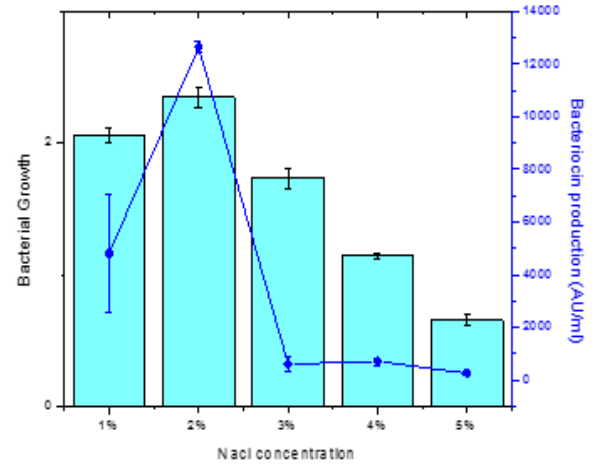

Fig 5. Effect of NaCl concentration on bacteriocin production Effect of pH and temperature on bacteriocin production

The highest bacteriocin activity of (7250 AU/ml) and maximum growth of 1.90 (OD_{600}) was recorded at pH 5 (fig 6). Bacteriocin activity of (2400 AU/ml) was recorded in MRS broth adjusted to pH 3.0. The results are in agreement with reports of bacteriocin production by *Lactobacillus plantarum* (Daeschel *et al*., 1990, Kelly *et al*., 1996). Fig 7 shows that the production of bacteriocin was found to be highest at 40° C temperature (8100 AU/ml). Many studies have reported that MRS medium is a better medium for bacterial growth and bacteriocin production than other media (Daba *et al.* 1993, Todorov *et al*. 2004, Todorov and Dicks 2006, 2007a). A difference between optimal cultivation temperature for bacteriocin production and bacterial growth has also been reported by Rajaram *et al*. (2010). Therefore, a temperature of 30°C was used for incubation in subsequent experiments.

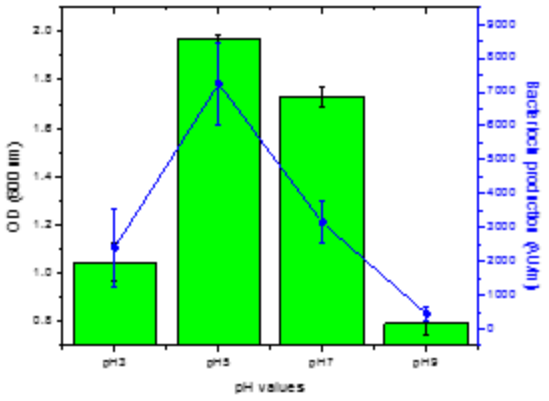

Fig 6. Effect of pH on bacteriocin production

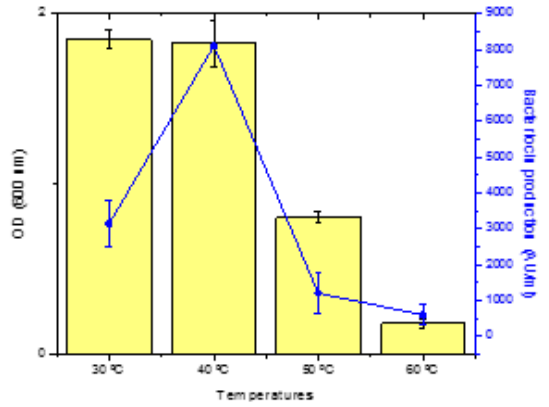

Fig 7. Effect of temperature on bacteriocin production
Note: Values are the means ± standard deviations of duplicate experiments

Characterization of bacteriocin

Bacteriocin produced by *Lactobacillus fermentum* was characterized by evaluating the stability of the bacteriocin by studying various parameters like effect of pH, temperature, ezymes and various organic solvents. Then the antibacterial activity of the bacteriocin was evaluated against *Salmonella typhi* and *Staphylococcus aureus* and measured as AU/ml.

The pH stability of the bacteriocin was studied in the range from 3 to 12. Activity was found to be stable from pH 4 to 7 with 2700 AU/ml against *Salmonella typhi* and 1600 AU/ml against *Staphylococcus aureus*. The bacteriocin activity was progressively inactivated at alkaline conditions may be due to alkali lysis (fig 8). The antibacterial activity of the bacteriocin was stable at 40°C to 50°C with 640 AU/ml against both *Salmonella typhi* and *Staphylococcus aureus*. However, when the temperature increased subsequently the antibacterial activity was decreased to half of its initial activity (fig 9). Studies conducted on the effect of various enzymes on the inhibitory activity of the bacteriocin revealed that the

complete inactivation of the cell free supernatant with trypsin, proteinase k and □- chymotrypsin treatment which indicated proteinaceous nature of the active substance. No activity was found against *Salmonella typhi* and *Staphylococcus aureus* after the enzyme treatment indicating active substance is the bacteriocin (fig 10). As shown in fig 11, the organic solvents acetone, chloroform, acetic acid, ethanol, toluene and ethyl acetate did not affect the activity of the bacteriocin in the concentration used, having activity of 3200 AU/ml against *Staphylococcus aureus* and 2700 AU/ml of activity against *Salmonella typhi*. The antibacterial activity is ineffective when the bacteriocin substance was treated with hexane and diethyl ether and there was a decrease in the activity with DMSO and methanol.

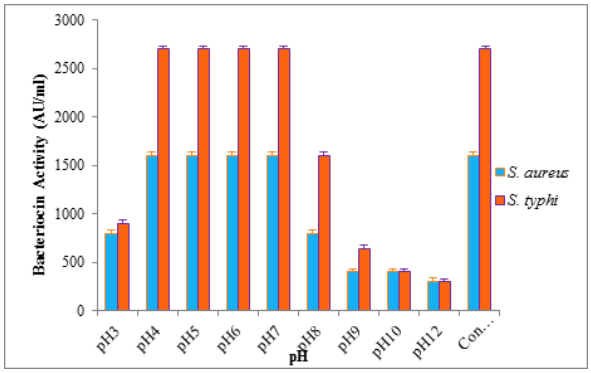

Fig 8. Effect of pH on bacteriocin stability

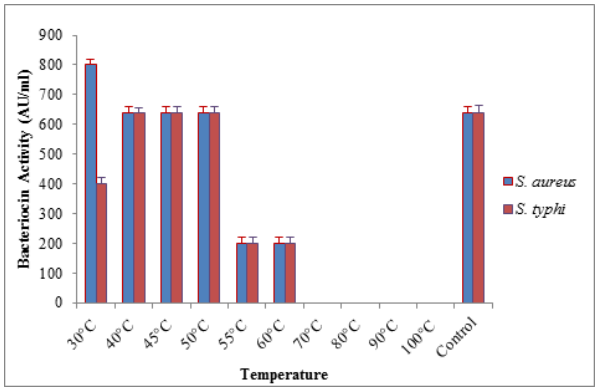

Fig 9. Effect of temperature on bacteriocin stability

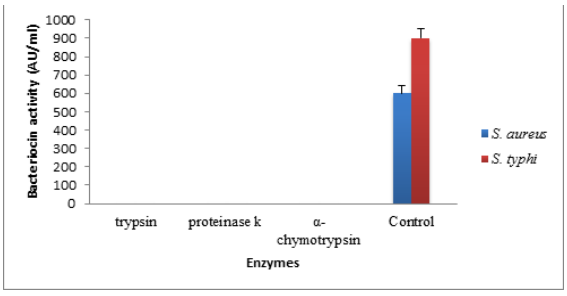

Fig 10. Effect of enzymes on bacteriocin stability

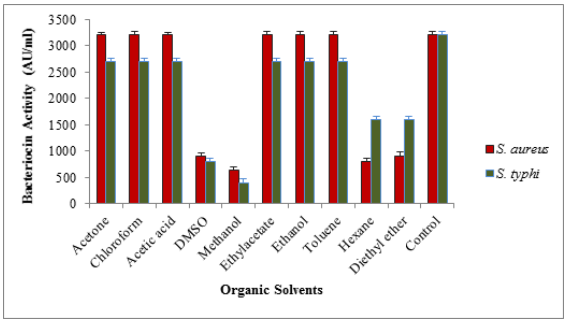

Fig 11. Effect of organic solvents on bacteriocin stability

DISCUSSION

In the current study, bacteriocinogenic Lactobacillus *fermentum* was isolated and found to inhibit *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Proteus mirabilis*. Bacteriocin activity was characterized with the stability of the cell free supernatant of *Lactobacillus fermentum* under different conditions which may provide important attributes towards its application in food preservation.

The optimization of culture medium components on the production of bacteriocin was investigated. Results showed that maximum bacteriocin was produced when nutrients were available for metabolic activity. Larger amounts of the bacteriocin were synthesized, when the medium was supplemented with glucose (2 %), yeast extract (2 %) and NaCl (2 %) at equal concentration. The effect of incubation period, temperature and initial pH of medium on production of bacteriocin was also investigated. Thus variation in the concentration of constituents/ supplementation of cultivation media might have an influence on the amount of bacteriocin produced by microorganisms. Similar observations have been made previously. Daba *et al* [26] obtained similar results in the production of mensenterocin 5. Modification of nutrients of cultivation media should be considered for maximal production of bacteriocin that has potential use as a food biopreservative[27]. The question of production cost is an important issue to be taken into account when large-scale production of the bacteriocin for use as a food preservative is considered. Our results also show that bacteriocin could be produced in a relatively inexpensive medium. The use of constituted medium at 40° C incubation temperature with initial pH 5 and for 18 h fostered the best production of bacteriocin by *Lactobacillus fermentum*. The designed optimized medium showed significant variation in bacteriocin production by submerged fermentation. The production levels were found to be dependent on the culture condition and medium component. It could be observed that there is an increased expectation in production of bacteriocin (15800 AU/ml), when compared to the initial production (12000AU/ml) with the optimum conditions. These results gave promising indication of future commercial use of *Lactobacillus* strains either in pharmaceutical or food industry.

The bacteriocin activity was stable to wide range of pH from 4-7 and at 30° C–60° C of temperatures respectively. The bacteriocin activity drastically reduced when the pH increased more than 8 and temperature above 60° C. Similar results were recorded for a number of bacteriocins produced by *Lactobacillus* and *Lactococcus* species [28-30]. Heat stability and protease sensitivity is a key criterion for the characterization of an inhibitory substance such as bacteriocin. Since bacteriocins are proteinaceous substances, they are inactivated by proteolytic enzymes such as trypsin, proteinase K and α-chymotrypsin. Kelly *et al* [31] reported similar results for other bacteriocins of *Lactobacillus* strains. The heat stability is a very useful character if the bacteriocin is to be used as a food preservative, because many food- processing procedures involve a heating step. Pilasombut *et al.,* reported that the bacteriocin from *Lactobacillus salivarius* K7 was found to be stable at the pH range of 4-7, and highest production was obtained at the pH range of 8-10, and the heat stability was reported to be 100°C for 5 min. Lee[32] characterized the bacteriocin from *Lactococcus lactis,* subsp. *Lactis* H-559 in which the bacteriocin sustains its activity even at 121°C for 20 min. The phenomenon of heat stability of Lactobacillus bacteriocins has been reported earlier in literature[33-35].

The effect of various organic solvents on the bacteriocin activity was determined to select the appropriate organic solvents for the purification of bacteriocin. The bacteriocin activity was remained stable with the organic solvents acetone, chloroform, acetic acid, ethanol, toluene and ethyl

acetate and was insensitive with hexane and diethyl ether. Vivekananda *et al.,* 2008 also reported that the bacteriocin retains its activity with the organic solvents like acetone, acetonitrile, chloroform, ethanol, methanol and isopropanol. The present investigation also indicated the novelty of bacteriocin in being tolerant to different organic solvents. Retention of bacteriocin activity in organic solvents suggested that the solvents had no effect on the bacteriocin and on its structure.

The properties of the bacteriocin of *Lactobacillus fermentum,* it's proteinaceous nature, the stability over a wide range of pH, resistance to heat, organic solvents indicated that the bacteriocin could preserve its structure and bactericidal functions even under extreme conditions, which is an extremely important property in view of its potential use as a biopreservative in foods. Sensitivity of some Gram-positive and the Gram-negative pathogens to bacteriocin provides another distinctive property to this bacteriocin. Further studies on food systems and more purification steps are needed for the practical application of isolated bacteriocins.

CONCLUSION

Bacteriocin produced by the *Lactobacillus fermentum* has some interesting characteristics. *Lactobacillus* are ubiquitous in nature and in humans, they are known to play a very significant role in the general health maintenance of the host. In conclusion, the potential of bacteriocin to inhibit enteropathogens is of crucial interest where some of these bacteria can produce toxins resulting in human illness. As a consequence of the diverse array of bacteriocinogenic lactic acid bacteria that are available and the fact that they can be produced as lyophilized bacteriocinogenic starter, adjunct or protective cultures, there is great potential for the use of cultures as biopreservatives in food. As there are no regulatory issues that limit the use of bacteriocionogenic lactic acid bacteria in food. Our future research with antimicrobial peptide will investigate its safety as a means of determining its potential in practical care applications and this approach may be on economical alternative to the application of chemical preservatives or purified bacteriocin preparations for controlling spoilage and pathogenic bacteria.

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