International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680, Print ISSN: 2664-7672
Received: 24-01-2022, Accepted: 08-02-2022, Published: 24-02-2022
Volume 4, Issue 1, 2022, Page No. 11-15

Optimization of bacteriocin production by *Lactobacillus fermentum* CM 36 and *Lactobacillus plantarum* CM 114 isolated from camel milk samples

P Upadhyay, Harshada Joshi*
Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India
* Corresponding Author: hjbiotech@gmail.com

Abstract
Lactic acid bacteria such as lactobacilli are well known to produce a large number of metabolic by-products such as organic acids (like lactic acid, acetic acids etc), H_2O_2, diacetyl and bacteriocins. Bacteriocins are proteinaceous in nature and play important role in combating pathogenic micro organisms. Present investigation is focused to study the effect of different parameters such as pH, incubation temperature and initial NaCl concentration on bacteriocin production. For this purpose previously isolated and identified camel milk lactobacilli isolates *Lactobacillus fermentum* CM 36 (LTZ95042) and *Lactobacillus plantarum* CM 114 (LS992102) were utilized. Production of bacteriocin and effect of parameters such as incubation temperature (30°C, 37°C and 45°C), initial pH (4.0, 7.0 and 9.0) and varying salt (NaCl) concentration (1%, 2% and 3%) on bacteriocin production was determined by using micro dilution method (Daba *et al*., 1991). Bacteriocin production was measured and represented in the form of arbitrary unit per ml (AU/ml). *L. fermentum* CM 36 showed maximum bacteriocin titre in MRS broth with incubation temperature 37°C, initial pH 7 without addition of NaCl. On the contrary, *L. plantarum* CM 114 showed optimum bacteriocin titre in MRS broth at incubation temperature 37°C with initial pH 4 and 7, and 1 and 2% NaCl supplemented in MRS broth. Above mentioned camel milk isolates were proven to have a potential probiotic qualities and study of optimum conditions for enhanced bacteriocin production can play essential role in large scale/ commercial level production.

Keywords: *Lactobacillus fermentum*, *Lactobacillus plantarum*, pH, temperature, bacteriocin

Introduction
Lactic acid bacteria (LAB) are prominent microflora utilized in fermented food products. LAB such as lactobacilli produces bacteriocins- are a group of proteinaceous antimicrobial substance inhibits the growth of similar or closely related bacterial strains either in the same species or across genera. Bacteriocins produced by LAB mostly inhibit bacteria by pore forming in cell membrane and by disrupting the proton motive force. Mode of action for different types of bacteriocin [1, 2]. Bacteriocins produced by LAB possess great potential in biotechnological applications as they are easy to produce, non-toxic to humans, shows stability at low pH and shows sensitivity to proteases [3]. The bacteriocin producing bacteria are considered as a promising natural food preservative, regarding the great distrust of the consumer against food additives such as chemical preservatives used to increase the shelf life of certain food and the use of heat treatment which deteriorate the nutritional and organoleptic properties of heat sensitive food. For enhancing the food safety and extend shelf life, use of antagonistic microorganisms or their products produced during metabolism to destroy or inhibit the growth of undesirable microorganisms is referred as preservation [4-6]. Various factors can influence bacteriocin production such as pH, temperature and presence of different salts in the growth medium. Effect of initial pH and incubation temperature are vastly covered. Camel milk possess large number of beneficial lactobacilli and the selection of strain from dairy ecosystem other than cow milk may provide a chance to obtain strains with unique characteristics to be used as bio preservative. Present investigation is focused on finding the adequate conditions for bacteriocin production by promising lactobacilli isolate with potent antibacterial activity and probiotic potential.

Materials and Methods
1. Test cultures
To study the production and effect of different parameters on bacteriocin production, two previously isolated and identified camel milk lactobacilli isolates *Lactobacillus fermentum* CM 36 (LTZ95042) and *Lactobacillus plantarum* CM 114 (LS992102) were utilized.

2. Production of bacteriocin
Bacteriocin activity was determined by dilution method [7] with some modifications. The isolated strains were propagated in MRS broth (10ml) seeded with 10% inoculum (10^8 CFU/ml) of overnight culture and incubated at

37°C. After centrifugation (8,000 g) of samples at 4°C for 15 min, the supernatant was filter-sterilized (0.22 µm, Millex-GV filter, Millipore).
Bacteriocin activity was calculated as follows: serial two-fold dilutions of the supernatant were made in 125 µl volumes of nutrient broth in a 96-well micro titer plate. Well was then inoculated with 50 µl of 100-fold diluted culture of the indicator strain grown overnight. The micro titer plate was then incubated at 37°C for 16 h. Bacteriocin activity was expressed as arbitrary unit per ml (AU/ml) and calculated using following formula:

$$\text{AU/ml} = \frac{1000}{125} \times \frac{1}{\text{HD}}$$

HD here represents the highest dilution that allowed no growth of the indicator strain. Optical density (OD) was take at 600nm using a spectrophotometer for the determination of bacterial growth

3. Effect of various factors on bacteriocin production by lactobacilli
The factors like incubation temperature, initial pH and saline conditions which were expected to influence the production of bacteriocin by the selected strains were optimized by selecting one parameter at a time. Experiment was performed in duplicate sets and readings were taken using Microtitre plate reader spectrophotometer at 600nm.

Temperature: The effect of different temperatures (30°C, 37°C and 45°C) on bacteriocin production was tested for 24 h using MRS broth at an initial pH of 6.8 without agitation. Bacteriocin production (AU/ml) was calculated using the formula as described in previous section.

pH: The effect of initial pH of medium on bacteriocin production was tested at 37°C for 24 h. MRS broth were adjusted to three different pH i.e. 4.0, 7.0 and 9.0 by using 6M HCl or 1M NaOH and then autoclaved. Bacteriocin production (AU/ml) was calculated using the formula as described in previous section.

Salt concentration: NaCl at various concentrations 1%, 2% and 3% were added to MRS broth at an initial pH of 7.0 and then autoclaved. Bacteriocin production (AU/ml) was calculated using the formula as described in previous section.

Results
Production of bacteriocin and selection of indicator organism
Selection of indicator organism was done by determining the bacteriocin titre of the cell free supernatant neutralized with 1 N NaOH of all *Lactobacillus* isolates against indicator organisms.
Lactobacillus fermentum CM 36 and *Lactobacillus plantarum* CM 114 produced maximum bacteriocin activity 2048 and 512 AU/ml against *B. cereus* respectively. *L. fermentum* CM 36 and *L. plantarum* CM 114 produced maximum bacteriocin activity 4096 and 1024 AU/ml against *B. subtilis* respectively. *L. fermentum* CM 36 and *L. plantarum* CM 114 produced maximum bacteriocin activity 4096, 512 AU/ml against *E. coli* respectively.
On the basis of maximum sensitivity against *Lactobacillus* isolates, *E. coli* was found to be most effective test organism and was further used as test organism for studying the effect of pH, temperature and salt on bacteriocin production.

Factors affecting the production of bacteriocin
The effect of various factors such as incubation temperature, initial pH and salt concentration on bacteriocin production by *Lactobacillus* isolates namely *Lactobacillus fermentum* CM 36 and *Lactobacillus plantarum* CM 114 was studied. In each set up, effect of incubation temperature, initial pH and NaCl concentration was determined on bacteriocin titer in terms of activity unit (AU/ml) was measured after 16 h of incubation. For each experiment two replicates (microtitre plates) were prepared.
Three different incubation temperatures i.e. 30°C, 37°C and 45°C were used in the study and bacteriocin titre in terms of AU/ml was determined. The bacteriocin titre produced by lactobacilli isolates against *E.coli* varied from 64 to 4096 AU/ml. At 30°C, maximum bacteriocin titre was observed for *L. fermentum* CM 36 was 512 AU/ml followed by *L. plantarum* CM 114 with 256AU/ml. At 37°C incubation temperature, higher bacteriocin titre then 30°C was observed for the lactobacilli isolates. Highest bacteriocin titre was observed in *L. fermentum* CM 36 i.e. 4096 AU/ml followed by 512 AU/ml for *L. plantarum* CM 114 respectively. At 45°C incubation temperature, sharp decline in bacteriocin titre of all the lactobacilli was observed. Highest bacteriocin titre was observed for *L. fermentum* CM 36 i.e. 512AU/ml, followed by 128 AU/ml for *L. plantarum* CM 114 The data for the same is presented in Fig. 1. Among the three incubation temperatures, best bacteriocin titre was observed at incubation temperature 37°C therefore for further experiments 37°C was used.
Three different initial pH i.e. 4, 7 and 9 were maintained and bacteriocin production in the terms of bacteriocin titre was determined. At pH 4, 512 AU/ml bacteriocin titre was observed for both *L. fermentum* CM 36 and *L. plantarum* CM 114. At pH 7, maximum bacteriocin titre 4096 AU/ml was observed for *L. fermentum* CM 36, 512 AU/ml for *L. plantarum* CM 114. At initial pH 9, sharp decline was observed, 128 AU/ml bacteriocin titre for *L. fermentum* CM 36 and 64 AU/ml was observed for *L. plantarum* CM 114. The data for the same is

presented in Fig. 2. Among the three pH, best bacteriocin titre was observed at pH 7 therefore for further experiments pH 7 was used.

Three different salt (NaCl) concentrations were maintained and bacteriocin production in terms of bacteriocin titre was determined. At 1% NaCl concentration, maximum 1024AU/ml was observed for *L. fermentum* CM 36 followed by 512 AU/ml for *L. plantarum* CM 114. At 2% and 3% NaCl salt concentration bacteriocin titre seems to decline as concentration increases. At 2% NaCl concentration bacteriocin titre 512 AU/ml was showed by both lactobacilli isolates. And at 3% NaCl concentration highest bacteriocin titre 512 AU/ml was observed for *L. fermentum* CM 36, followed by 256AU/ml by *L. plantarum* CM 114.The data for the same is presented in Fig. 3.

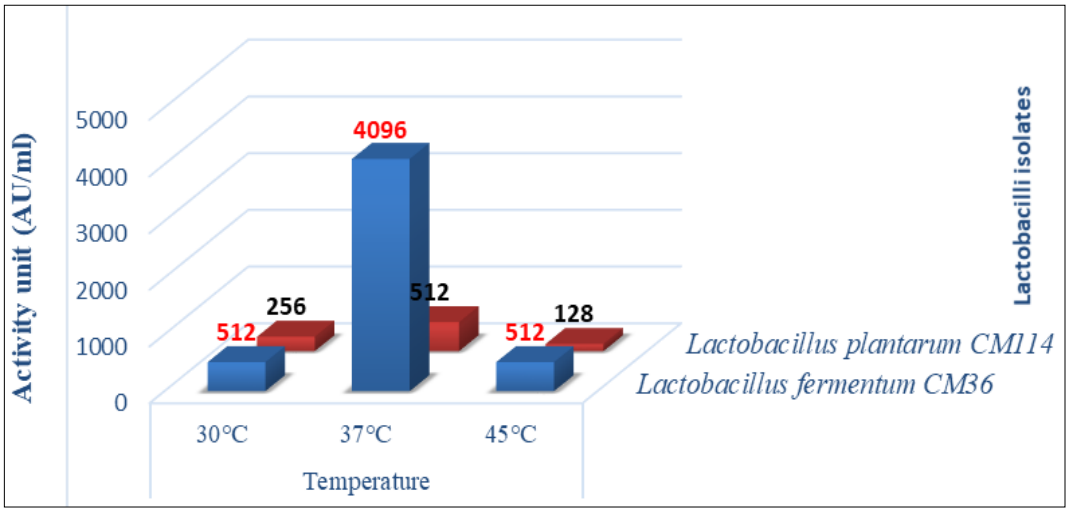

Fig 1: Effect of incubation temperature of MRS medium on bacteriocin production by *Lactobacillus* isolates after 16 h of incubation period against *E. coli*

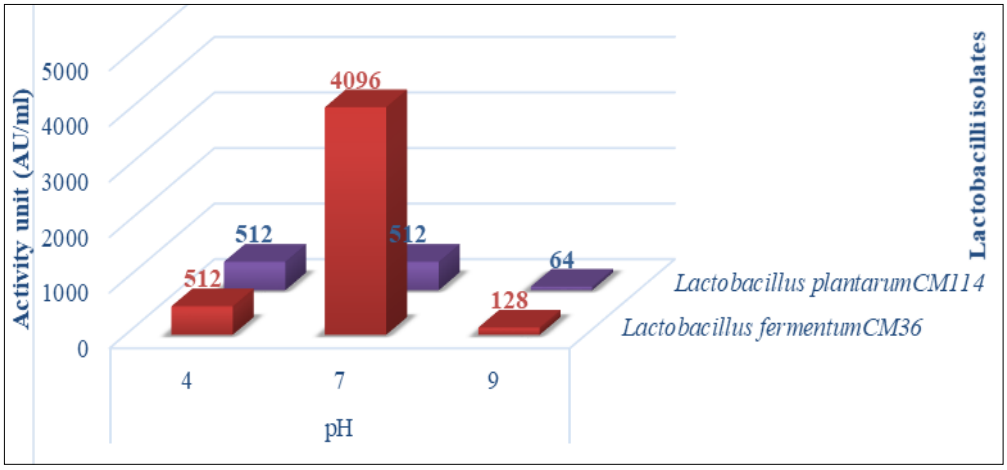

Fig 2: Effect of initial pH of MRS medium on bacteriocin production by *Lactobacillus* isolates at 37˚C after 16 h of incubation period against *E. coli*

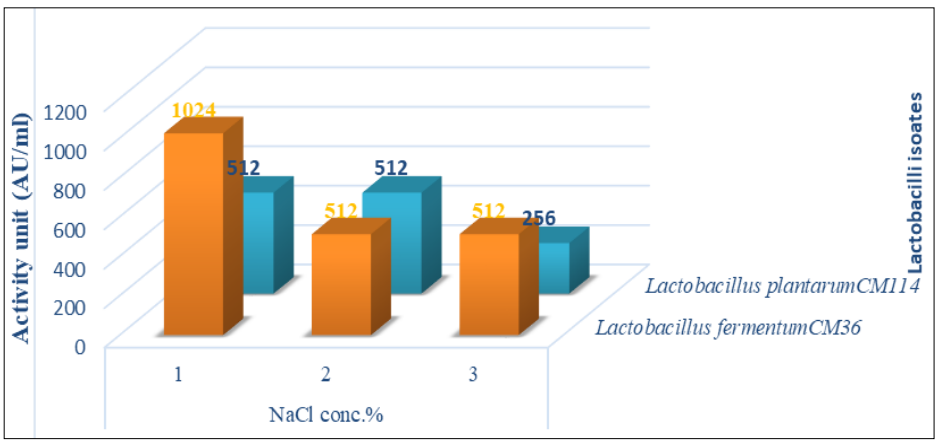

Fig 3: Effect of supplementation of sodium chloride in MRS medium on bacteriocin production by *Lactobacillus* isolates at 37˚C after 16 h of incubation period against *E. coli*

Discussion

In present investigation, the effect of various factors such as incubation temperature (30°C, 37°C and 45°C), initial pH (4, 7 and 9) and salt (NaCl) concentration (1%, 2% and 3%) on bacteriocin production by *Lactobacillus* strains namely *Lactobacillus fermentum* CM 36 and *Lactobacillus plantarum* CM 114 in MRS medium was studied against *E.coli* as indicator organism. Both the *Lactobacillus* strains, *L. fermentum* CM 36 and *L. plantarum* CM 114 showed higher bacteriocin titre in MRS medium (pH 6.8) at 37°C after 16 h of incubation against *E.coli* (Fig. 1). Mohankumar and Murugalatha [8] studied the effect of incubation temperature on bacteriocin production from lactobacilli isolated from raw cattle milk sample and reported that maximum bacteriocin production was observed 37°C after 24h of incubation period. The results of present study were in agreement with the above mentioned studies as maximum bacteriocin production by isolates was observed at37°C. Wayah and Philip [9] studied the bacteriocin production from *Lactobacillus pentosus.* They explained that *Lactobacillus sp.* grows faster at 37°C as it is the optimum temperature for growth and hence maximum bacteriocin production was observed at 37°C. This can be a possible explanation for the findings of the present investigation.

The *Lactobacillus* strains, *L. fermentum* CM 36 showed significantly higher bacteriocin titre in MRS medium with initial pH adjusted to 7 at 37°C after 16 h of incubation against *E. coli* (Fig. 2). Optimum pH for bacteriocin production is observed to be between 6.0 and 7.0 [10]. Sure *et al*. [11] studied the pH optimization for bacteriocin production by *Lactobacillus viridescence* and reported highest bacteriocin production at pH 7 and also reported considerable decrease at both acidic and alkaline pH at 37°C after 48 h of incubation period. The results of present study are in agreement with above mentioned report as maximum bacteriocin production by the isolate was observed at pH 7. Although the findings in the case of *Lactobacillus plantarum* CM114 are in contrast as 512 AU/ml bacteriocin titre was observed for both pH 4 and 7. Goncalves *et al*. [12] explained the reason for low bacteriocin production at initial pH 4 that low pH conditions induce low growth likely to be caused by limitation in cytoplasmic process (acidification of cytoplasm and collapse of motive force of cell). They further mentioned that nutrient transport is also a pH dependent process, at low pH nutrient transport gets affected which affects the growth rate hence bacteriocin production gets affected. This may be the probable reason for the low bacteriocin production by the isolates at pH 4 in the present study.

Addition of salt in MRS medium negatively impacted the bacteriocin production in all the *Lactobacillus* strains. All the *Lactobacillus* strains showed decreased bacteriocin titre in all concentrations of NaCl (1%, 2% and 3%) supplemented in MRS medium with initial pH 7 at 37°C after 16 h of incubation against *E. coli* (Fig. 3). Forhad *et al*. [13] studied the effect of increasing concentration of NaCl on growth and bacteriocin production by *Lactobacillus* spp. and concluded that as salt concentration increased from 1% to 10% growth and bacteriocin production was affected negatively. Verluyten *et al*. [14] studied the effect of NaCl on bacteriocin production by *Lactobacillus curvatus* and they explained that the inhibition of the bacteriocin production in presence of salt was mainly due to the role of NaCl as lowering agent, which reduces the a_w (water activity) and in turn affects the bacteriocin production. This may be the possible reason for inhibition of bacteriocin production even at low concentration.

Conclusion

Among the natural antimicrobial substances bacteriocin have gained rapid interest in recent years. The proteinaceous biomolecules bacteriocins with antimicrobial properties can act as replacements of chemical preservatives and antibiotics in near future. Both the strains showed demonstrable probiotic attributes such as antibacterial activity due to bacteriocin production, bile tolerance and antibiotic resistance and showed high bacteriocin titre in MRS medium adjusted to initial pH 7 at 37˚C after 16 h incubation period against *Escherichia coli*. It may be concluded that, bacteriocins produced by *L. fermentum* CM 36 and *L. plantarum* CM 114 isolates can be used as promising biopreservatives.

Refrences

1. Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol,2005:3:777-788.
2. Gaspar C, Donders GG, Palmeira-de-Oliveira R *et al*. Bacteriocin production of the probiotic *Lactobacillus acidophilus* KS400. AMB Express,2018:8(1):153.
3. Todorov SD. Bacteriocins from *Lactobacillus plantarum*: Production genetic organization. Braz. J. Microbiol,2009:40:209-221.
4. Schillinger U, Geisen R, Holzapfel WH. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci Technol,1996:7:158-164.
5. Chen CC, Lai CC, Huang HL *et al*. Antimicrobial activity of *Lactobacillus* species against carbapenem-resistant Enterobacteriaceae. Front Microbiol,2019:10:789.
6. Qian Z, Zhao D, Yin Y, Zhu H, Chen D. Antibacterial activity of *Lactobacillus* strains isolated from Mongolian yogurt against *Gardnerella vaginalis*. Biomed Res Int. Article ID 3548618. 2020.
7. Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C. Detection and activity of bacteriocin produced by *Leuconostoc mesenteroids*. Appl. Env. Microbiol,1991:57:3450-3455.
8. Mohankumar A, Murugalatha N. Characterization and antibacterial activity of bacteriocin producing *Lactobacillus* isolated from raw cattle milk sample. Int. J. Biol,2011:3(3):128-143.

9. Wayah SB, Philip K. Characterization, yield optimization, scale up and biopreservative potential of fermencin SA715, a novel bacteriocin from *Lactobacillus fermentum* GA715 of goat milk origin. Microb. Cell Fact,2018:17:125-143.
10. Skytta E, Haikara A, Mattila STC. Production and characterization of antibacterial compounds produced by *Pediococcus damnosus* and *Pediococcus pentosaceous*. J. Appl. Bacteriol,1993:74:134-142.
11. Sure KP, Kotnis PV, Bhagwat PK, Ranveer RC, Dandge PB, Sahoo AK. Production and characterization of bacteriocin produced by *Lactobacillus viridescence* (NICM 2167). Braz. Arch. Biol. Technol,2016:59:1-7.
12. Gonçalves LMD, Ramos A, Almeida JS, Xavier AMRB, Carrondo MJT. Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. Appl. Microbiol. Biotechnol,1997:48:346-350.
13. Forhad MH, Khaledur RSM, Md Rahman S, Saikot FK, Biswas KC. Probiotic properties analysis of isolated lactic acid bacteria from buffalo milk. Arch. Clin. Microbiol,2015:71- 5.
14. Verluyten J, Messens W, De Vuyst L. Sodium chloride reduces production of curvacin A, a bacteriocin produced by *Lactobacillus curvatus* strain LTH 1174, originating from fermented sausage. Appl. Environ. Microbiol,2004:70(4):2271-2278.