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Optimization and antibacterial activity of Bacteriocin produced by *Lactococcus lactis* isolates from dairy products against Foodborne Pathogens

Armita Azad[1], Nafieseh Sadat Naghavi[1] and Vajihe Karbasizade[2*]

1. Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran.
2. Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

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ABSTRACT

Among lactic acid bacteria (LABs), *Lactococcus lactis* has been much concerned because of its acceptable stability and bacteriocin (nisin) production. This study was aimed to investigate the antibacterial effects of bacteriocin produced by *Lactococcus lactis* isolated from native dairy products against some foodborne pathogenic bacteria and to optimize its production conditions. First, 15 strains of *Lactococcus lactis* were isolated from sheep and goat milk samples in M17 culture medium and identified by biochemical and molecular methods. Then, the optimal conditions for bacteriocin production by *Lactococcus lactis* were determined using Taguchi statistical method in terms of pH, temperature and incubation time. Evaluation of the antimicrobial activity of bacteriocin produced against standard strains of foodborne pathogens was evaluated by well diffusion method. Cell-free supernatants were obtained from *Lactococcus lactis* I8 from goat milk showed antimicrobial activity against *Shigella flexneri*, *Salmonella typhimurium*, and *Staphylococcus aureus* strains; While *Escherichia coli* showed resistance to the bacteriocin. Optimal bacteriocin production by *Lactococcus lactis* was done at pH = 7, temperature of 26 °C and 22 h incubation period. The high antibacterial activity of this bacteriocin against putative pathogens such as *Salmonella typhimurium* and *Shigella flexneri* would make it a good candidate as a probiotic strain to be used for treatment of infections caused by these bacteria, and also the application possibility of the produced bacteriocin as a suitable food preservative is proposed.

1. Introduction

Todays, increasing social consumer demand for products without chemical additives has led the food industries to find new and alternative technologies for food preservation with the aim of improving the quality and safety of food products (Silva et al., 2018). The growing demand for less processed, more natural and healthier foods has led to the use of natural antimicrobials as food preservative (Nagalakshmi et al., 2013; Rattanachaikunsopon et al., 2010). The use of microorganisms or their metabolites has emerged as a new tool in this area. In this regard, lactic acid bacteria (LABs) have gained significant concern in food industries related to public health. Lactic acid bacteria are a group of prokaryotes with specific morphological, metabolic, and physiological characteristics (Axelsson, 2004).

These bacteria are classified in the order of Lactobacilalles, which includes 11 important

*Corresponding author: Vajihe Karbasizade
Tel: +983137929069
E-mail address: v.karbasi@med.mui.ac.ir

genera: *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Carnobactrium*, *Vagococcus*, W*eissella*, *Oenococcus*, *Enterococcus*, and *Tetragenococcus* (Bintsis, 2018, Todar, 2006). LABs produce antimicrobial compounds to survive in the natural environment and compete for resources with other microorganisms, which inhibits or kills competing strains (Reis et al., 2012). Antimicrobial peptides are a diverse group of antimicrobial compounds, specifically include two classes of bacteriocins and peptide antibiotics. The basis of this classification is on their biosynthesis mechanism (Pometto et al., 2005). Nisin is the most common bacteriocin produced by lactic acid bacteria and has been used as a food preservative for more than half a century (de Almeida et al., 2020). Although nisin has been approved as a preservative, the low rate of its production by bacteria has limited its use. Therefore, finding new strains with high nisin production capacity is a major issue. The first nisin producing isolates were isolated from fermented milk products; since then various other strains have been isolated from dairy products, vegetables, fermented meat products, fresh water and human milk (Mitra et al., 2005).

Many studies have been conducted on the effects of environmental factors such as pH, temperature and incubation time on bacteriocin production by LABs (Iyapparaj et al., 2013), but few studies have been performed on the simultaneous effects of these three environmental factors on nisin production by native strains of *Lactococcus lactis*. On the other hand, *Shigella flexneri*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* are the most important foodborne pathogens that always endanger food safety and health leading to a growing demand for food preservation against these bacteria especially by the use of natural preservatives (Varman et al., 1991). The present study aimed to isolation of *Lactococcus lactis* strains from different milk samples with high bacteriocin production; and determining of its antibacterial activity against foodborne pathogens.

2. Materials and Methods

2.1. Isolation of Lactococcus lactis strains

Sampling was done in summer from sheep and goat herds in Isfahan. First, using an alcohol-soaked cotton swab, the animal's breast was cleaned, then the first few drops of milk was discard, and animals were milked into a sterile container. Then, serial dilutions were prepared from milk samples and 100 µl of each dilution inoculated into M17 agar medium. The culture media were incubated under anaerobic condition using gas pack model A for 24 h at 37°C. The plates containing 30-300 colonies were separated and 5 to 6 colonies that were visually different from each other were randomly selected and streaked on fresh culture media. The media were again incubated under anaerobic condition. The procedure repeated 2-3 times to reach purified colonies (Nagalakshmi et al., 2013). Then, the isolates were analyzed morphologically by Gram staining. Finally, the isolates were further identified through biochemical tests such as and catalase test, growth at 10 and 45°C, gas production in Durham tube, growth in the presence of 6.5% NaCl, and the ability to hydrolyze arginine.

2.2. PCR for amplification of 16S rDNA

To confirm the final identity of *Lactococcus lactis* strain, the 16S rDNA region in the bacterial chromosome was amplified by PCR method using specific primer pairs (forward sequence: GTA CTT GTA CCG ACT GGA T, and reverse sequence: GGG ATC ATC TTT GAG TGA T). The thermal cycle that used for amplification was consisted an initial denaturation (94°C, 60 s), followed by 35 times repeated cycles including denaturation (94°C, 30 s), primer annealing (58°C, 30 s) and extension (72°C, 30 s), and ended by a final extension (72°C, 120 s) (Buyukyoruk et al., 2010).

2.3. Production and determination of bacteriocin antibacterial activity

The overnight culture of *Lactococcus lactis* isolates in M17 broth medium (1 ml) was inoculated into 100 ml M17 broth medium containing 0.5% lactose and incubated for 24 h at 30°C under anaerobic condition. The cultured media were then centrifuged (8000 g for 15 min at 20°C). Then the cell- free supernatants were sterilized using 0.22 µm syringe filters and kept in sterile tubes at 4°C (Nagalakshmi et al., 2013). Antimicrobial activity was assessed by agar well diffusion assays. Briefly, a 24-h

culture of *Micrococcus luteus* PTCC1110 with turbidity equal to 0.5 McFarland standard was prepared in M17 broth, and then cultured on the surface of TSB agar. A circular well was cut with diameters of 6 mm and filled with 125 µl supernatant. The culture medium was first kept at 4°C for 2 h to allow the supernatant to diffuse into the medium and then incubated for 16 h at 30°C. After this time, the culture medium was checked for the formation of the growth inhibition zone. The bacteriocin-producing isolate with the highest antibacterial activity was a candidate for optimizing bacteriocin production conditions (Taheri et al., 2012).

2.4. Optimization of bacteriocin production conditions

Among non-nutritional factors, pH, temperature and incubation time had previously shown the greatest impact on bacteriocin production by *Lactococcus lactis* (Nagalakshmi et al., 2013). Therefore, the effects of these three factors were first tested individually through one-factor at a time design, and after obtaining the suitable ranges for each of the factors, experiments were designed by Taguchi software to examine the effects of all three factors in combination. Regarding pH optimization for bacteriocin production, pH values equal to 5, 5.5, 6, 6.5, 7 and 7.5 were selected and studied. *Lactococcus lactis* was cultured at each of these pH values in M17 broth and each time the supernatant was extracted in the same way as before and examined on *Micrococcus luteus*. The results were recorded based on the growth inhibition zone diameters. To determine the temperature levels for bacteriocin production, the temperature ranges as 23, 26, 29, 32, 35 and 38°C was considered, and like the previous method, the bacteriocin produced at each temperature was extracted and the growth inhibition zones were measured. Regarding the optimization of *Lactococcus lactis* incubation time, the time levels of 18, 22, 26, 30, 34 and 38 h were considered and again bacteriocin was extracted at each level and the diameter of the growth inhibition zone created by it was measured. The optimum values that obtained based on one-factor at a time experiments and the values near them were entered to Taguchi (design expert) software to detect the effect of each factor in combination to other factors (Table 1).

Table 1. The experiments designed by Taguchi software

Experiment number	pH	Incubation time (hr)	Temperature (°C)
1	5.5	18	**26**
2	5.5	22	**29**
3	5.5	26	**32**
4	5.5	30	**35**
5	6	22	**26**
6	6	18	**29**
7	6	30	**32**
8	6	26	**35**
9	6.5	26	**26**
10	6.5	30	**29**
11	6.5	18	**32**
12	6.5	22	**35**
13	7	30	**26**
14	7	26	**29**
15	7	22	**32**
16	7	18	**35**

2.5. Evaluation of proteinaceous nature of bacteriocin

Each of the three enzymes proteinase K, pepsin and trypsin (2 mg each) was added to 2 ml of *Lactococcus lactis* supernatant. Then the tubes were incubated at 37°C for 2 h for the enzymatic activity. Then the tubes were subjected to steam (100°C) for 3 minutes to stop the reaction and the content was passed through 0.22 nm syringe filter. Finally, the antibacterial activity of supernatant was examined against *Micrococcus luteus* as previously described (Nagalakshmi et al., 2013).

2.6. Evaluation of antibacterial activity of bacteriocin

The well-diffusion assay was used for investigating the antibacterial activities. Briefly, the supernatant was obtained from24 h grown M17 broth culture of *Lactococcus lactis* and treated with 1 mg/ml bovine catalase; then the pH was adjusted to 6.5 and kept at 4°C after passing through a 0.22 µm syringe filter. On the other hand, 24h cultured *Staphylococcus aureus* PTCC1112, *Escherichia coli* PTCC1392, *Salmonella typhimurium* PTCC 14028, and *Shigella flexneri* PTCC 1234 with the turbidity equal to 0.5 McFarland standard were prepared and 0.2 ml of each was inoculated separately

into 19.8 ml BHI agar media containing 7% agar via pour plate method. After cooling and solidification of the media, one well was cut into each agar and 100 µL of the cell-free supernatant was placed into well. The culture media was first kept at 4°C for 2 hours and then incubated for 16 hours at 30°C. Then the growth inhibition zone diameters were measured. The assays were done in triplicate (Mitra et al., 2005).

3. Results

Among the isolates initially identified as *lactococcus lactis* by biochemical tests, 15 isolates that had the ability to grow at the temperature of 10°C, and in the presence of 6.5% NaCl and had no growth at 45°C were selected. These isolate were also able to hydrolyze arginine. PCR reaction was performed to molecularly confirm the selected strains. The strains that showed a 163 bp band in agarose gel electrophoresis were definitely identified as *Lactococcus lactis* (figure 1).

3.1. Antimicrobial activity of bacteriocin produced by Lactococcus lactis

Among the *Lactococcus lactis* isolates, bacteriocin produced by strain I8 showed the highest antimicrobial activity, so this strain was selected for further studies.

3.2. Assay of proteinaceous nature of bacteriocin produced by Lactococcus lactis I8

In figure 2, the lack of growth inhibition zone in the well containing the enzyme shows the proteinaceous nature of the extracted bacteriocin.

3.3. Optimized conditions for bacteriocin production by Lactococcus lactis I8

After calculating the effect of factors, the analysis of variance (ANOVA) was performed using Taguchi software and the percentage of the studied factors effectiveness on bacteriocin production was determined. ANOVA showed that the most significant effect on bacteriocin production was related to pH with 74.113% effectiveness and then to temperature with 22.695% effectiveness. Incubation time in the studied ranges had the lowest effect (0.531%) on the production of bacteriocin by *Lactococcus lactis*. Regarding the interaction between the factors, the highest effect was seen by the interaction between incubation time and temperature (23.33%) and the lowest effect was seen by the interaction between pH and temperature (3.33%). Finally, the optimal condition proposed to produce the highest amount of bacteriocin which created a growth inhibition zone of 26 mm on the foodborne pathogenic bacteria was at pH 7, temperature of 26°C and incubation time for 22 h (figure3).

3.4. Antimicrobial activity of bacteriocin against foodborne pathogenic

The culture supernatant obtained from *Lactococcus lactis* I8 from goat milk showed the greatest growth inhibitory zones on *Shigella flexneri*, *Salmonella typhimurium* and *Staphylococcus aureus* strains although had no antibacterial effect on *Escherichia coli* (Table 2).

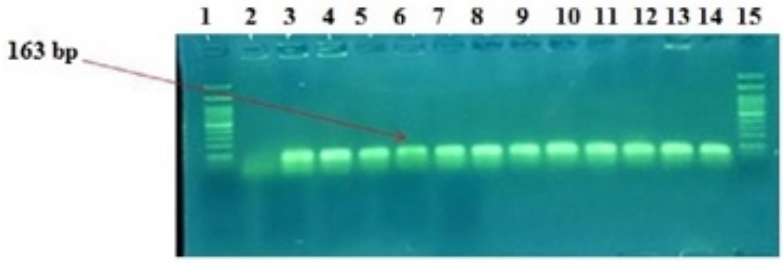

Figure 1. Agarose gel electrophoresis of the amplified 16S rDNA fragments by PCR. Molecular weight marker (lanes 1 and 15), negative control (lane 2), bacterial isolates (lanes 4-13) and *Lactococcus lactis* PTCC 1336 (lane 14).

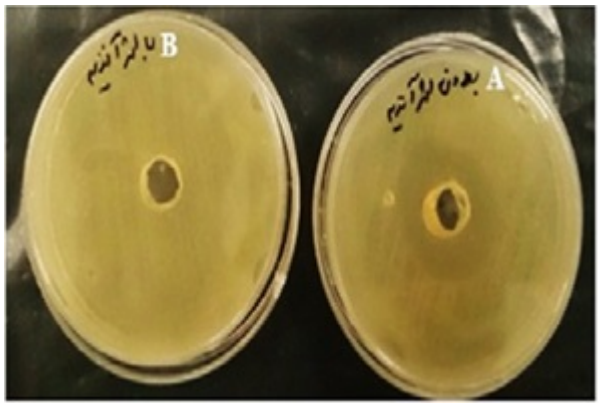

Figure 2. Verification of the proteinceous nature of *Lactococcus lactis* I8 bacteriocin. A: the well containing bacteriocin untreated by proteolytic enzymes. B: well containing bacteriocin treated with the enzymes

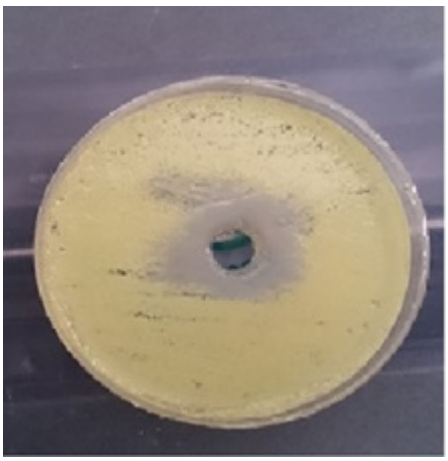

Figure 3. Antimicrobial activity of bacteriocin against Micrococcus luteus PTCC1110 in optimized conditions

Table 2. Antimicrobial activity of bacteriocin against standard foodborne pathogenic strains

Bacteria	**Inhibition Zone (mm)**
Shigella flexneri PTCC 1234	24
Salmonella typhimurium PTCC 14028	26
Staphylococcus aureus PTCC 1112	25
Escherichia coli PTCC 1392	0

4. Discussion

The results of this study showed that bacteriocin produced by *Lactococcus lactis* isolated from native dairy products had the highest bactericidal effect on *Salmonella typhimurium*, *Shigella flexneri* and *Staphylococcus aureus*, while no antibacterial effect was observed on *Escherichia coli*. However, this study had some limitations. First, we did not completely purify the bacteriocin produced. Second, we were unable to characterize bacteriocin.

A review on similar studies reveals that different results have been obtained regarding the effect of *Lactococcus lactis*-produced bacteriocin on Gram-negative and Gram-positive bacteria. In a study by Goyal et al., the

bacteriocin produced by a selected strain of *Lactococcus lactis* had inhibitory activity on Gram-positive bacteria but did not show inhibitory effect on any of the tested Gram-negative bacteria (Goyal et al., 2018). In a study conducted by Taheri et al., the antibacterial effect of bacteriocin produced by *Lactococcus lactis* ST1 isolated from goat's milk was investigated on *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* isolates (Taheri et al., 2012). The bacteriocin showed the greatest antibacterial activity against *Bacillus cereus*, then *Staphylococcus aureus* and had little effect on *Escherichia coli*. In another study by Mirdamadi., the antibacterial effect of bacteriocin nisin extracted from *Lactococcus lactis* was investigated on *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* presented in Iranian feta cheese (Mirdamadi et al., 2015). This bacteriocin had the most inhibitory effect on *Listeria monocytogenes* and then on *Staphylococcus aureus*, but had no antibacterial effect on *Escherichia coli*. After further analysis, they concluded that nisin would be able to eradicate *Escherichia coli* only if it has used in a synergistic effect with other metabolites produced by *lactococcus lactis*. Kiaie et al. investigated, the antagonistic effect of lactic acid bacteria isolated from yogurt against gastrointestinal pathogens such as *Shigella dysentery*, *Yersinia enterocolitica*, *Escherichia coli* and *Salmonella typhimurium*. The highest inhibitory effect was belonged to *Lactobacillus* spp. and *Lactococcus* isolates on *Yersina enterocolitica* and lower effects was seen on other strains (Kiaie E, 2006). The results from the present study along with the results from other studies shows that bacteriocins produced by *Lactococcus lactis* have antibacterial activities on Gram-positive bacteria and some Gram-negative bacteria. Because the mechanisms of bactericidal activities of bacteriocins similar to nisin is to affect the cell membrane, the resistance of Gram-negative bacteria may be due to the presence of the outer membrane that makes it difficult for bacteriocins to reach the cell membrane.

Process optimization is one of the most important duties of biotechnology today. Because this process requires a lot of time due to the repetition of experiments as well as high costs, it is important to find laboratory methods that save some time and money and on the other hand provide accurate results. In this study, Taguchi statistical method was used to optimize the production conditions of bacteriocin by *Lactococcus lactis*. This rerearch shows the best incubation temperature for bacteriocin production was 26°C. This result was consistent with the study of Absalan et al., in which the optimum temperature for bacteriocin production was determined to be 27°C (Absalan et al., 2012).

In the present research, after optimizing the production of bacteriocin by *Lactococcus lactis* with different pH values, the highest production was assigned to pH 7. In Nagalakshmi study, reported the highest bacteriocin production was produced by *Lactococcus lactis* at pH = 6.5 (Nagalakshmi et al., 2013). The difference in results may have been due to the type of strain isolated and uncontrollable laboratory conditions.

Among three interactions that were created, the highest yield was obtained by the interaction between the incubation time and the incubation temperature (23.33%) although incubation time alone had little effect and temperature alone had a significant effect on bacteriocin production. On the other hand, the least effect on bacteriocin production was related to the interaction between pH and incubation time, while pH alone had a large effect on the production of bacteriocin by *Lactococcus lactis*. Taken together, the results indicate that the effect of each factor on bacteriocin production by *Lactococcus lactis* depends on the conditions create by other factors.

Conclusion

The results of this study showed that goat and sheep milk were rich in bacteriocin producing LABs, which had antibacterial activity against foodborne pathogens. Therefore, by optimizing more environmental factors for its production, *Lactococcus lactis* can be a suitable candidate as a probiotic in the treatment of infections caused by the studied bacteria and also the possibility of using the produced bacteriocins as a suitable food preservative is proposed. Further studies on these products is necessary, and other fermentation products will be used to isolate other bacteriocin-producing bacteria.

Refereces

Absalan & Kabousi (2012). Production of nisin by Lactococcus lactis and its applications in food. First National Biotechnology Student Conference.

Axelsson, L. (2004). Lactic acid bacteria: classification and physiology. food science and technology-new york-marcel dekker. 139: 1-66.

Bintsis, T. (2018). Lactic acid bacteria: their applications in foods. J Bacteriol Mycol. 6: 89-94.

Buyukyoruk, S., Cibik, R., Cetinkaya, F., Soyutemiz, G. E., Goksoy, E. O. & Kirkan, S. (2010). Isolation, phenotypic and molecular identification of Lactococcus lactis isolates from traditionally produced village cheeses. Journal of Animal and Veterinary Advances. 9: 2154-2158.

De Almeida, G.M., Mendonça, C.M.N., Converti, A. & De Souza Oliveira, R.P. (2020). Kinetic and thermodynamic parameters of nisin thermoinactivation. Journal of Food Engineering. 280, 109986.

Goyal, C., Malik, R. & Pradhan, D. (2018). Purification and characterization of a broad spectrum bacteriocin produced by a selected Lactococcus lactis strain 63 isolated from Indian dairy products. Journal of food science and technology. 55: 3683-3692.

Iyapparaj, P., Maruthiah, T., Ramasubburayan, R., Prakash, S., Kumar, C., Immanuel, G. & Palavesam, A. (2013). Optimization of bacteriocin production by Lactobacillus sp. MSU3IR against shrimp bacterial pathogens. Aquatic biosystems. 9: 1-10.

Kiaie, E.M.N., Samioladab, H., Jandaghi, N., Ghaemi, E. (2006). Antagonistic effect of lactic acid bacteria isolated from yogurt against pathogenic bacteria. Scientific Journal of Gorgan University of Medical Sciences. 8: 28-33.

Mirdamadi, S. & Agha Ghazvini, S. (2015). A comparative study between inhibitory effect of L. lactis and nisin on important pathogenic bacteria in Iranian UF Feta cheese. Biological J Microorganism. 3: 79-92.

Mitra, S., Chakrabartty, P.K. & Biswas, S.R. (2005). Production and characterization of nisin-like peptide produced by a strain of Lactococcus lactis isolated from fermented milk. Current microbiology. 51: 183-187.

Nagalakshmi, P., Sumathi, R., Kanimozhi, K. & Sivakumar, T. (2013). Isolation of bacteriocin nisin producing Lactococcus lactis from dairy products. J. Acad. Indus. Res. 1: 627-630.

Pometto, A., Shetty, K., Paliyath, G. & Levin, R. E. (2005). Food biotechnology, CRC Press

Rattanachaikunsopon, P. & Phumkhachorn, P. (2010). Lactic acid bacteria: their antimicrobial compounds and their uses in food production. Annals of biological research. 1: 218-228.

Reis, J., Paula, A., Casarotti, S. & Penna, A. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. Food Eng Rev 4: 124-140.

Silva, C.C., Silva, S.P. & Ribeiro, S.C. (2018). Application of bacteriocins and protective cultures in dairy food preservation. Frontiers in microbiology. 9: 594.

Taheri, P., Samadi, N., Ehsani, M.R., Khoshayand, M.R. & Jamalifar, H. (2012). An evaluation and partial characterization of a bacteriocin produced by Lactococcus lactis subsp lactis ST1 isolated from goat milk. Brazilian Journal of Microbiology. 43: 1452-1462.

Todar, K. (2006). Todar's Online Textbook of Bacteriology.http://www.textbookofbacteriology.net

Varman, A. & Evans, M. (1991). Food Borne Pathogens. AIMS Microbiology. 3: 529-563.