Effect of pH, heat treatments and proteinase K enzyme on the activity of *Lactobacillus Acidophilus* bacteriocin

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ABSTRACT

Bacteriocins are natural metabolites produced by many strains of Lactic acid bacteria that used in food preservation. They have potential healthy role in suppressing the growth of some spoilage and pathogenic bacteria. The aim of the current study was to demonstrate the stability of the bacteriocin extracted from *Lactobacillus acidophilus* at different pH values, heat treatments and proteinase K enzyme. The obtained results revealed that the inhibitory effectiveness of bacteriocins was higher on Gram-positive bacteria than Gram-negative bacteria. Further, the largest inhibition zone was obtained by *L. acidophilus* bacteriocin against *Bacillus subtilis* while the smallest one was against *E.coli.* The extracted bacteriocin exhibited broad spectrum of inhibition at concentration 6400AU/ml against *Staph. aureus*, *Bacillus subtilis* and *E.coli.* The antimicrobial activity of crude supernatant fluid was stable after heating at 100°C for 30 min and declined thereafter. Stability of antimicrobial activity was observed at pH ranged from 2.0 to 8.0. Its active principle was proteinaceous in nature since the bacteriocin was inactivated by proteinase K enzyme.

Keywords: Bio preservation, Lactic acid bacteria, Lactobacillus acidophilus, Bacteriocin and Antimicrobial activity.

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1. INTRODUCTION

Increasing demands by consumers for natural and chemical-free food products have led the food industry to search for a new novel and alternative strategies for food biopreservation (Cosentino et al., 2012). Among these alternatives, the bacteriocins which are defined as ribosomally-synthesized peptides or proteins with antimicrobial activity against many food-borne pathogens and spoilage bacteria in foods (Muñoz et al., 2007). They are produced by different strains of lactic acid bacteria (LAB) such as *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Enterococcus* and *Lactobacillus* (Mc Auliffe et al., 2001). Bacteriocins have several potential characteristics rendering them useful natural food preservatives as they are small hydrophobic cationic peptides, stable at a wide range of pH, temperature and considered as Generally Recognized as Safe (GRAS) by FDA (Cleveland et al., 2001). *Lactobacillus acidophilus* is the most important LAB used for production of bacteriocin (Bogovic Matijasic et al., 1998). It is widely used for production of fermented dairy products in the world and it is one of probiotic bacteria. Probiotic microorganisms are thought to counteract disturbances in the normal microflora and thereby reduce the risk of colonisation by pathogenic bacteria (Sullivan et al., 2001). Several theories have been proposed to explain the antimicrobial and beneficial effects of probiotics, including their capacity to compete for nutrients and their

ability to secrete antimicrobial substances such as organic acids, bacteriocins and peptides (Ljungh and Wadström, 2006). Previous studies of probiotics have been demonstrated an impressive increase in the interest of *L. acidophilus* as a probiotic agent, and have contributed to its application in functional food and supplements in a worldwide market (Naidu et al., 1999; Kitazawa et al., 2002). *Lactobacillus acidophilus* DSM 20079 produces a heat-stable peptide bacteriocin named acidocin D20079, active over a wide range of pH, with suitable properties for use in the food industry (Deraz et al., 2005). The addition of bacteriocin of *L. acidophilus* DSM 20079 to plain yoghurt can improve organoleptic characters, enhance microbial safety and prolonge the shelf life of plain yoghurt up to 28 days (Awad, 2011). In the present study, *L. acidophilus* was activated and its bacteriocin was extracted to detect its stability at different heat treatments, pH values and proteinase K enzyme.

2. MATERIAL AND METHODS

2.1. Activation of Lactobacillus acidophilus:

Lyophilized strain of *L. acidophilus* DSM 20079 was obtained from MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *Lactobacillus acidophilus* strain was activated on MRS broth (De Man, Rogosa and Sharp which obtained from Biolife, Italy) at 37^0C for 24 hours then 3 transfers were performed to activate this culture. Decimal serial dilutions were prepared till obtaining the concentrations of 10^7- 10^9 CFU/mL in order to meet the required recommended level for active probiotic (IDF, 1988).

2.2. Extraction of bacteriocin:

Ten mL of activated culture of *L. acidophilus* was separately inoculated into one liter of MRS broth under aseptic conditions (Chumchalova et al., 2004). Bacteriocin producing culture was adjusted to pH 2.0 by adding HCl 1N then culture was heated in a water bath at 100 ^{0}C for 5 min. The cells were harvested by centrifugation at 10,000 rpm for 20 min at 4^0C. The cell-free supernatant containing bacteriocin extract was adjusted to pH 6.0 using NaOH 1M to exclude the effect of organic acids. The bacteriocin extract was sterilized by using Seitz filter to eliminate the probability of presence of viable cells (Simova et al., 2009).

2.3. Characterization of Bacteriocin:

The crude bacteriocin extracted from *L. acidophilus* DSM 20079 was examined for stability to pH, heat treatments and proteolytic enzyme as follows:

2.3.1. Effect of pH values:

Accurately, 5ml of crude bacteriocin was taken into different test tubes and the pH values were adjusted to pH 2, 4, 6, 8, 10 and 12, using either 1N HCl or 1M NaOH. The samples were incubated at 28^0C for 16 hours and then the bacteriocin activity was determined by using agar well diffusion method (Ogunbanwo et al., 2003).

2.3.2 Effect of heat:

A volume of 5 ml from crude bacteriocin was exposed to various heat treatments (40, 60, 80, 100^0C for 30 min) in thermostatic water bath and 121^0C for 15 min in autoclave. The bacteriocin activity was then assayed by agar well diffusion method (Ogunbanwo et al., 2003, Rajaram et al., 2010).

2.3.3 Effect of Proteinase K enzyme:

To test the sensitivity to proteinase K enzyme, aliquots of 1ml of crude bacteriocin was treated with proteinase K (Sigma, St. Louis, USA) at a concentration of 1mg/ml at pH 7. The test tubes with and without the enzyme (control) were incubated at 37^0C for 2 hours and then heated at 100^0C for 3 min to denature the enzyme. Both control and treated samples

were assayed for antimicrobial activity by using agar well diffusion method (Neha and Nivedita, 2009).

2.4 Statistical analysis:

Statistical comparisons were made by using one-way analysis of variance (ANOVA). The results were considered significantly different with $P < 0.05$ as described by Clarke and Kempson (1997).

3. RESULTS

Bacteriocins are generally of low molecular weight proteins with bactericidal effect on Gram-positive bacteria and bacteriostatic action on Gram-negative bacteria (O'Sullivan et al., 2002).

3.1. Effect of pH:

The effect of different pH values (2-12) on the activity of crude bacteriocin of *L. acidophilus* were presented in Table (1). The sensitivity of crude bacteriocin to different pH values was examined and it was very stable over a wide pH range (2, 4, 6 and 8). The inhibition zones of indicator bacteria *Staph. aureus*, *Bacillus subtilis* and *E.coli* measured by agar well diffusion method.

3.2. Effect of heat:

The effect of different temperature on the activity of *L. acidophilus* DSM 20079 is presented in fig (1). Regarding to the effect of temperature on the stability, *Lb. acidophilus* bacteriocin was heat stable to heat treatment up to 80^0C for 30 min. The activity of the bacteriocin was completely disappeared by heating at 121^0C for 15 min.

3.3. Effect of proteolytic enzyme:

Table (2) showed the effect of proteinase K on the activity of crude bacteriocin of *L. acidophilus* DSM 20079. The activity was completely disappeared by the treatment with proteinase K.

Table (1): Effect of pH values on the stability of bacteriocin extracted from L. acidophilus DSM 20079.

PH values	Diameter of inhibition zone (mm)		
	Staph. Aureus	*Bacillus subtilis*	*E.coli*
Control (6.6)	12 ±0.39^b	14±0.57 ^{a}	10±0.24^c
2	12±0.39^b	14±0.57 ^{a}	10±0.24^c
4	12±0.39^b	14±0.57 ^{a}	10±0.24^c
6	12±0.39^b	14±0.57 ^{a}	10±0.24^c
8	12±0.39^b	14±0.57 ^{a}	10±0.24^c
10	7.34±0.18^b	9.5±0.25 ^{a}	4.35±0.14^c
12	ND	ND	ND

a, b, c mean values in the same raw having different superscripts letters are significantly different ($P<0.05$). Control: bacteriocin without treatment. ND not detected. Results are mean of three determination (n=3).

Figure (1) Effect of heat treatments on the stability of bacteriocin

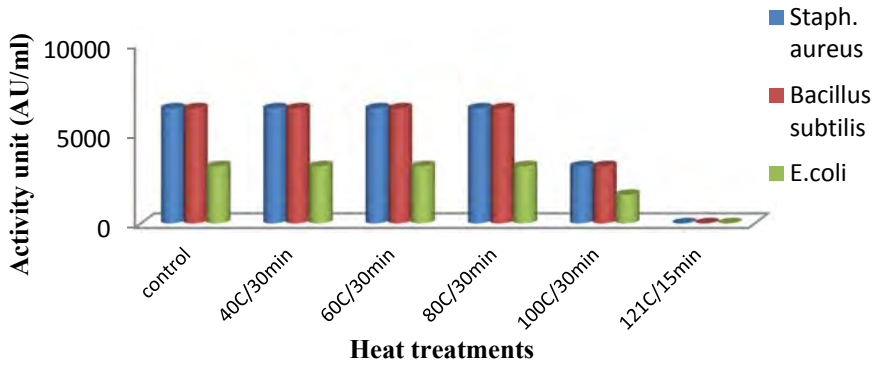

Table (2) Effect of proteinase K enzyme on the stability of bacteriocin

	Diameter of inhibition zone (mm)		
Enzyme treatment	*Staph. aureus*	*Bacillus subtilis*	*E.coli*
Control	12.56	15.46	11
Proteinase K	ND	ND	ND

Control: bacteriocin without enzyme treatment. ND not detected. Results are mean of three determination (n=3)

4. DISCUSSION

Lactic acid bacteria are used in the food industry due to their ability to ferment carbohydrates into lactic acid and, as a consequence, reduce the pH of the medium. This acidification is one of the most desirable effects of LAB fermentations because it prevents the growth of undesirable bacteria and thus prolonging the shelf life, aroma, texture and flavor of the food (Du Toit et al., 2000). Preservation of fermented foods by LAB is mainly due to the production of organic acids and other compounds such as bacteriocins which have a potential role to inhibit a variety of harmful microorganisms (Daeschel, 1989; De Vuyst and Leroy, 2007). During characterization of bacteriocin produced from *L. acidophilus* DSM 20079; the antimicrobial activity was determined using *Staph. aureus*, *Bacillus subtilis* and *E.coli* as indicator organisms. Regarding to the effect of pH values on the activity of the bacteriocins extracted from *L. acidophilus,* the activity of bacteriocins were very stable over a wide range of pH (2, 4, 6 and 8), which was recorded by the diameter of inhibition zone (mm) with mean values of 14±0.57, 12±0.39 and 10±0.24 mm against *B. subtilis, Staph. aureus* and *E. coli,* respectively. While, its activity slightly decreased by increasing pH value up to 10 with inhibition zone 9.5±0.25, 7.34±0.18 and 4.35±0.14 mm against *B. subtilis, Staph. aureus* and *E. coli,* respectively. In contrast, the activity of bacteriocins was completely disappeared at pH 12 as shown inTable (1). This bacteriocin was stable over a wide pH range, which is a common feature of many bacteriocins as reported by (Ogunbanwo et al., 2003; Corsetti et al. 2004; Rattanachaikunsopon and Phumkhachorn (2006) & Todorov and Dicks, 2006). The wide range of pH tolerance indicates that such bacteriocin may be useful in acidic as well as non-acidic foods. Temperature stability is important if the bacteriocins are to be used as a food preservative, because many procedures of food preparation involve a heating step. Fig (1) showed the activity of bacteriocin of *L. acidophilus* DSM20079 after heat treatment up to 80^0C for 30min was stable and it was 6400AU/ml, but its activity was declined after heating at100^0C for 30 min to be 3200 AU/ml. The activity of bacteriocin by autoclaving at 121^0C for 15min was disappeared. The phenomenon of heat stability of bacteriocin from different LAB at 121^0C for 15min has been reported earlier in Lactocin RN 78 (Mojgani and Amirinia, 2007) and in *L.brevis* OGI (Ogunbanwo et al., 2003) whom reported similar results with our finding loss of activity of antibacterial substances produced by Lactobacillus spp. after heat treatment at 121^0C for different time duration. In respect to the temperature stability, the examined bacteriocin exhibited strong heat stability, which means it could be placed within the heat stable low molecular weight group of bacteriocins. The antimicrobial activity of extracted bacteriocin was destroyed by treatment with proteinase K as there was no inhibition zone appeared compared with the untreated bacteriocin sample (control). Similar results were reported by Corsetti et al. (2004); Todorov and Dicks (2004); Hernandez et al. (2005); Rattanachaikunsopon and Phumkhachorn (2006). The sensitivity of the extracted bacteriocin to proteolytic enzymes is approving the proteinaceous nature of bacteriocin (Deraz et al., 2005). As conclusion, *L. acidophilus* bacteriocin was characterized by high stability at different

pH values, heat treatments, and completely inactivation by proteinase K enzyme. Accordingly, the ability of bacteriocin, produced by *L. acidophilus*, for inhibiting a wide-range of bacteria may be of potential interest for food safety and preservation providing future scope for application as food preservative.

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