

Antimicrobial Activity of Bacteriocins Isolated from Lactic Acid Bacteria Against Resistant Pathogenic Strains

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Abstract: This study was carried out to treat the microbial diseases in natural way and reduced the antibiotic resistance pressure. Five species of Lactic acid bacteria namely, *L. acidophilus*, *L. bulgaricus*, *L. dulbrueckii*, *L. plantarum*, and *L. fermentum*, were isolated from locally produced yogurt. Bacteriocins were isolated from MRS broth culture of these Lactic acid bacteria through the ammonium sulphate precipitation method. Extracted bacteriocins were tested against test strain i.e. *E. coli* ATCC 25922 to obtain optimum pH, storage temperature and heating temperature for maximum antibacterial activity. For this (2-12) pH range, storage temperatures (-20 °C, 4 °C and 37 °C for 1 month) and heating temperatures (60 °C, 100 °C and 121 °C for 15 minutes) were tested. Later the antibacterial activity of these bacteriocins were tested at concluded optimum conditions against each other as well as against different locally isolated pathogens viz. *Methicillin-Resistant-Staphylococcus-aureus*, *E. coli*, *Salmonella* and *Staphylococcus aureus* by using well diffusion agar method. It was found that bacteriocins activities were maximum at pH 6 and 4 °C storage temperature. Maximum activity was found after heating bacteriocins at 60 °C as compared to 100 °C and 121 °C. Bacteriocins of lactobacilli were harmless against each other at any pH, heat temperatures and storage temperatures. Antibacterial activity of bacteriocins extracted from *L. acidophilus* showed maximum activity against pathogenic bacteria as compared to others. The present study has highlighted the antibacterial role of bacteriocins isolated from Lactic acid bacteria by indicating their potential to treat a variety of human and animal diseases.

Keywords: Lactic Acid Bacteria (LAB), Antibiotic Resistant Pathogens, Optimum pH, Heat and Storage Temperature

1. Introduction

Continuously increasing level of resistance against antibiotics is the highlight aspect of 21st century [1]. Microbial diseases have been treated with antibiotics just after their introduction. Since then, antibiotic resistance is the problem and threat in the successful antibiotic treatment of bacterial infections [2].

Microbes become antibiotic resistant due to partial exposure to one or more antibiotics. Incomplete therapy, gene mutations as well as vertical and horizontal gene transfer among bacteria are also important factors for the development of resistance. [2,3]

Choice of antibiotics against multi drug resistant (MDR) bacteria is limiting continuously so the discovery of new antimicrobial tools or antibiotics is the solution of this problem [4]

Antagonist microbial interference has been reported from last century of microbiology [5]. This cross interference has become more valuable and alternative method to treat and kill the pathogens [6].

Bacteriocins producing Lactic acid bacteria are the best alternatives to conventional antibiotics [7]. So lactic acid bacteria (LAB) are helpful in treatment without raising the antibiotic resistance level [6]. Lactic acid bacteria are capable to generate diverse antimicrobial

composites in the course of lactic acid fermentation as bacteriocins, hydrogen peroxide (H₂O₂), organic acids and diacetyl, [8]. Bacteriocins are helpful in treatment without raising the antibiotic resistance level [6].

Bacteriocins are released extracellular low weight peptides that may be bacteriostatic but surely bactericidal [9]. Primary tool of *Lactobacilli* to kill the pathogens is the reduction of pH through the production of lactic acid [10].

Bacteriocins are actually ribosomally synthesized peptides, consisting of 30-60 amino acids and they show effective antimicrobial activity against certain other pathogens [11]. These bacteriocins are harmless against its own producers because the secretory bacterial strains have genetically pre determined genes of immunity [12,13]

Bacteriocins of *Clostridia*, *Staphylococcal* and *Lactobacillus* may be the combination of carbohydrate, lipid and protein or purely proteinaceous. Beneficially protenatious bacteriocins are easily get digested in human gastrointestinal (GI) tract [13].

This strategy has been evaluated by many researchers. The production of bacteriocins up to 10-100 different types has been reported even by a single species. So this diversity of bacteriocins have made them best candidate as an alternative to conventional antibiotics [14,15]

Lactobacilli belong to lactic acid bacteria and are generally recognized as safe (GRAS) because they are the habitant of normal microflora of Gastric Intestinal (GI) tract. Some members of LAB are considered as probiotics [16,17]. *Lactobacilli* are being used frequently as probiotics among *Enterococcus*, *Bifidobacterium*, *Bacillus*, as well as *Saccharomyces* strains [18,19]. So now a day's probiotics are medically great important, in the treatment of infections and their control [20].

Probiotics have valuable contribution in physiology and in the maintenance of fitness of human beings. So they have high rank for their use in such practices that are being carried out to get benefits of livestock and human. Probiotics have antimicrobial substances and bacteriocins like lethal tools for different pathogens that help in maintaining the fitness of human beings without using antibiotics. Treatment through this substitutional technique is being practices on large scale [12,21].

The aim of study was extraction of bacteriocins from Lactic acid bacteria isolated from locally produced yogurt and determination of antibacterial effect of bacteriocins against different antibiotic resistant pathogenic bacterial strains.

2. Materials and Methods

2.1. Collection and Isolation of Lactic Acid Bacteria from Yogurt

30 yogurt samples collected from the local market of Faisalabad were spreaded on de Man Rogosa Sharp (MRS) agar medium and incubated at 37 °C for 24-48 hours anaerobically. The isolated organisms from the sample

were screened on the basis of their morphological and biochemical characteristics as described in Bergey's Manual of systematic bacteriology [22].

2.2. Extraction of Bacteriocins

Cell free supernatants of Lactic acid bacteria were obtained after centrifugation at 7100 rpm for 15 min at 12 °C. Cell free supernatant was heated at 90 °C for 12 minutes. The obtained cell free supernatant was then precipitated with 40% solution of ammonium sulphate. The mixture was stirred for 24 hours at 12 °C and then centrifuged at 10000 rpm for 20 minutes at 7 °C. The pellets were collected in Tris-buffer having 7 pH [23,24].

2.3. Confirmation of Bacteriocin

Bacteriocin was confirmed through proteolytic enzymes i.e protease, proteinase K, and trypsin with concentration 0.25mg mL⁻¹.

2.4. Stability of Isolated Bacteriocins

Stability as well as antimicrobial potential of isolated bacteriocins were checked at (2-12) pH range, at different heat temperatures (60 °C, 100 °C and 121 °C for 15 min) and at different storage temperatures (-20 °C, 4 °C and 37 °C).

2.5. Cross Sensitivity

All the bacteriocins producing isolates were challenged against each other to check the cross sensitivity.

2.6. Pathogenic Bacterial Strains

The cultures of pathogenic strains (*Methicillin-Resistant-Staphylococcus-aureus*, *E.coli*, *Salmonella*, *Staphylococcus aureus*) were obtained from stock culture of the Department of Clinical, Medicine and surgery, Agriculture University, Faisalabad. Cultures used in this study were transferred twice in to the blood agar medium before use.

2.7. Determination of Antimicrobial Activity

The antimicrobial activity was checked by the well diffusion assay [25]. Aliquot of 50 µl from each extract were added to the well of size 6 mm. Then indicator strains of bacteria (5x10⁸ CFU/ml) were spread on Mueller-Hinton agar (MH) plates. Plates were incubated at 37 °C for 24 hrs to check pathogenic indicator bacterial growth. The diameters of the zones of inhibition were measured as recommended by [26].

2.8. Statistical Analysis

Data were analyzed by applying Standard Error (Mean ± SE) and analysis of variance (ANOVA) under completely randomized design. Mean of triplet values having p<0.05 are significant.

3. Results

Isolated colonies of Lactic acid bacteria appeared on MRS agar were circular, convex, smooth and white to creamy. Isolates were Gram positive, acid fast negative, catalase negative, indole negative and oxidase negative. These isolates were identified as *L. acidophilus*, *L. bulgaricus* and *L. delbrueckii* on the basis of sugar fermentation tests [23,27].

In this study bacteriocins were subjected to different pH conditions against test strain i.e *E.coli* ATCC 25922 and the results obtained showed that maximum activity of bacteriocins were at pH 6. The activity was lower at pH 4 while the activity was even more reduced at pH 8. There was no significant activity at pH 2, 10 and 12 (Table 2).

Different heating temperatures i.e. 60 °C, 100 °C and 121 °C were employed for 15 min to detect the stability of bacteriocins at these temperatures against test strain i.e *E.coli* ATCC 25922. At 60 °C significant activity was obtained but this activity was lower as compared to activity of bacteriocins at normal room temperature. The bacteriocin extracted from *L.acidophilus* showed higher stability as compared to bacteriocins extracted

from other lactobacilli. Non significant activity was detected at 100 °C and 121 °C among all lactobacilli bacteriocins except bacteriocin of *L. acidophilus* (Table 3).

Different storage temperatures were evaluated to conclude best storage temperature suitable for bacteriocins. The overall activity of bacteriocins after storing them at 4 °C for 30 days was maximum against test strain i.e *E.coli* ATCC 25922 but slightly reduced when bacteriocins were stored at -20 °C and 37 °C. No significant change in activity was observed in case of *L.acidophilus* at any storage temperature (Table 4).

Different pre-isolated pathogens were used to determine the activity of different bacteriocins extracted from different lactobacilli species. Antimicrobial activity of bacteriocins were observed at 6 pH, 60 °C heat temperature and 4 °C storage temperature where its activity was maximum against test strain. *L.acidophilus* showed highly significant activity against MRSA, *Staphylococcus aureus* and *E.coli* while non-significant activity was observed against *Salmonella typhi*. Non-significant activity was observed in rest of bacteriocins against all pathogens (Table 5).

Table 1. Pathogenic bacteria resistant against couple of antibiotics.

Pathogens	Resistant against antibiotics
Methicillin-Resistant-Staphylococcus-aureus	Methicillin, Amoxicillin-clavulanic acid, Amoxicillin, Ampicillin
<i>E.coli</i>	Amoxicillin-clavulanic acid, Ceftriaxone, Ceftazidime
<i>Salmonella</i>	Ceftazidime, Amoxicillin-clavulanic acid
<i>Staphylococcus aureus</i>	Amoxicillin, Ampicillin

Table 2. Zones of inhibition of LAB at different pH against *E.coli* ATCC 25922.

pH level	Bacteriocin of differents LAB					Mean
	<i>L. acidophilus</i>	<i>L. bulgaricus</i>	<i>L. delbrueckii</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	
2	10±0.58cd	0±0.00f	0±0.00f	0±0.00f	0±0.00f	2±1.07 E
4	19±1.15b	9±0.58cde	9±0.58cde	8±0.58de	9±0.58cde	10.8±1.13 B
6	26±0.58a	9±0.58cde	10±0.58cd	9±0.58cde	9±0.58cde	12.6±1.81 A
8	27±1.00a	8±0.58de	7±1.15de	0±0.00f	0±0.00f	8.4±2.66 C
10	18±1.73b	0±0.00f	0±0.00f	0±0.00f	6±1.15e	4.8±1.90 D
12	12±0.58c	0±0.00f	0±0.00f	0±0.00f	0±0.00f	2.4±1.29 E
Mean	18.67±1.58 A	4.33±1.06 B	4.33±1.09 B	2.83±0.98 C	4±1.02 B	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 3. Zones of inhibition of LAB after different heat treatments against *E.coli* ATCC 25922.

Heat Temp	Bacteriocin of differents LAB					Mean
	<i>L. acidophilus</i>	<i>L. bulgaricus</i>	<i>L. delbrueckii</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	
60 °C	24±2.31a	9±0.58b	9±0.58b	9±0.58b	9.00 ± 1.00b	12.00 ± 1.67 A
100 °C	20±1.00a	7±1.15b	6±1.15b	0±0.00c	0.00 ± 0.00c	6.60 ± 1.98 B
121 °C	20±0.58a	0±0.00c	0±0.00c	0±0.00c	0.00 ± 0.00c	4.00 ± 2.14 C
Mean	21.33±1.00 A	5.33±1.41 B	5±1.37 BC	3±1.51 C	3.00 ± 1.53 C	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 4. Zones of inhibition of LAB after storing at different temperatures against *E.coli* ATCC 25922.

Storage Temp	Bacteriocin of different LAB					Mean
	<i>L. acidophilus</i>	<i>L. bulgaricus</i>	<i>L. delbrückeii</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	
-20 °C	26.00 ± 1.00a	7.00 ± 1.15b	7.00 ± 1.15b	10.00 ± 0.58b	7.00 ± 0.58b	11.40 ± 2.01 B
4 °C	25.00 ± 0.58a	10.00 ± 0.58b	9.00 ± 0.58b	8.00 ± 0.58b	11.00 ± 1.15b	12.60 ± 1.70 A
37 °C	26.00 ± 1.15a	8.00 ± 0.58b	8.00 ± 0.58b	7.00 ± 1.15b	8.00 ± 0.58b	11.40 ± 1.98 B
Mean	25.67 ± 0.50 A	8.33 ± 0.60 B	8.00 ± 0.50 B	8.33 ± 0.60 B	8.67 ± 0.73 B	

Means sharing similar letter in a row or in a column are statistically non-significant ($P > 0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

4. Discussion

Considering the pace of bacteria to become multi-drug resistant there is a need of some alternative techniques for their treatment. The purpose of this study was to isolate the bacteriocin producing Lactic acid bacteria for the cure and treatment of bacterial infectious diseases. Many researchers have highlighted the lactic acid bacteria as best alternative to antibiotics and recommended them for therapeutic use due to their antimicrobial ability [23,28]. Lactic acid bacteria were isolated from locally produced yogurt as it was reported that these bacteria are inhabitant of milk and milk products [29,30].

Bacteriocins were isolated from cell free supernatant of LAB in MRS broth through ammonium sulphate precipitation method. Garneau *et al.*, (2002) also preferred this technique on the basis of short time and bacteriocin quantity [11]. Bacteriocin is an antimicrobial proteinaceous secretion of lactic acid bacteria. Abada (2008) documented that most of the bacteriocins are purely build up with peptides as well as some are the compositions of protein, carbohydrates and lipids [12].

Lactic acid bacteria can inhibit the antibiotic resistant bacterial growth through the secretion of bacteriocin, hydrogen peroxide (H_2O_2), organic acids and diacetyl [31,32]. To confirm that antimicrobial agent is extracted bacteriocin it was treated with some proteolytic enzymes. There were no zones of inhibition by bacteriocin after dealing it with proteolytic enzymes. That indicated its proteinaceous nature along with proofing it as antimicrobial agent. Same methodology was chosen by some scientists to confirm its proteinaceous nature and their results were parallel to our results [34].

Inhibitory activity against antibiotic resistant bacteria was checked through agar well diffusion method and

zone of inhibition were measured. The diameter of zone of inhibitions against pathogenic bacteria were different. Schved *et al.*, (1993) described that zone of inhibitions can describe the degree of sensitivity and resistance of bacteria [34].

After study it was concluded that bacteriocin of lactobacilli showed antimicrobial activity against *E.coli* ATCC 25922 in 4-8 pH range. Most of lactobacilli showed non-significant activity at 2,10 and 12 pH except *lactobacillus acidophilus* (Table 2). Gerez *et al.*, (2009) and Todorov *et al.*, (2006) documented that bacteriocins performed well in killing as well as limiting the growth of pathogens in acidic pH as compare to basic pH [35,36].

Bacteriocins were more effective after heating at 60 °C for 15 minutes against *E.coli* ATCC 25922. Bacteriocin of few species showed positive activity even at 100 °C, 121 °C. Because bacteriocin is proteinaceous in nature so with the increase in temperature its effectiveness decrease but diminished except *L.acidophilus* (Table 3). Rowaida *et al.*, (2009); Mojgani and Amirinia, (2007) recorded antimicrobial activity of bacteriocin at 121 °C that is in accordance to our results [37,38].

Storage temperature and duration affects the antimicrobial activity of bacteriocin. 4 °C temperature was found most suitable for the storage of bacteriocin because in our study maximum zones of inhibitions were obtained against *E.coli* ATCC 25922 after storing bacteriocin at this temperature. Bacteriocin were effective also at -20 °C and 37 °C but the antimicrobial potential after storage at these temperatures were less than the antimicrobial potential after storage at 4 °C temperature (Table 4). Rowaida *et al.*, (2009) ; Malini and Savitha, (2012) concluded after their study that cold temperature is more suitable for bacteriocin storage as compare to hot temperature. Storage at -20 °C may reduce the potential due to crystallization of bacteriocin [37,38].

Table 5. Zones of inhibition of LAB against different antibiotic resistant bacteria.

Antibiotic resistant bacteria	Bacteriocin of different LAB					Mean
	<i>L.acidophilus</i>	<i>L.bulgaricus</i>	<i>L.delbrückeii</i>	<i>L. fermentum</i>	<i>L.plantarum</i>	
<i>MRSA</i>	23.00 ± 1.15a	11.00 ± 1.15b	11.00 ± 1.15b	8.00 ± 0.58b	11.00 ± 1.00b	12.80 ± 1.45 A
<i>Staphylo coccus</i>	24.00 ± 2.31a	10.00 ± 0.58b	10.00 ± 0.58b	8.00 ± 0.58b	10.00 ± 1.00b	12.40 ± 1.63 A
<i>E.coli</i>	27.00 ± 1.00a	9.00 ± 0.58b	9.00 ± 0.58b	9.00 ± 0.58b	8.00 ± 0.58b	12.60 ± 1.94 A
<i>Salmonella</i>	10.00 ± 0.58b	8.00 ± 0.58b	7.90 ± 0.58b	9.00 ± 0.58b	8.00 ± 0.58b	8.40 ± 0.31 B
Mean	21.00 ± 2.06 A	9.50 ± 0.47 B	9.49 ± 0.47 B	8.25 ± 0.25 B	9.50 ± 0.48 B	

Means sharing similar letter in a row or in a column are statistically non-significant ($P > 0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Antimicrobial activity of bacteriocin was checked in its optimum conditions (pH 6, heating at 60 °C for 15 minutes and storage at 4 °C for 30 days) against couple of antibiotic resistant bacteria. *L.acidophilus* found as most effective against all antibiotic resistant bacteria. Over all antimicrobial activity of all lactobacilli species were significant against MRS, *Staphylococcus* and *E.coli*. Results of extracted bacteriocins against *Salmonella* were non-significant (Table 5). Many scientists as Malini and Savitha, (2012); Hermine et al., (2010) recommended the LAB as best option to treat and kill resistant bacteria [33,4].

Inhibitory activity of lactic acid bacteria was checked against each other but there were no zones of inhibitions. This is due the presence of the pre-determined immunity genes against their own bacteriocin so producers are harmless against their own bacteriocin [39,40].

Conclusion of this study is that bacteriocins extracted from lactic acid bacteria have the potential to inhibit the growth of many antibiotic resistant bacteria including *Methicillin* resistant *staphylococcus aureus*, *E.coli* and *staphylococcus aureus*. So we can use them as an alternative therapeutic agent with no risk of antibiotic resistance as well as their use in the form of probiotic can reduce the risk of different infections.

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