Characterization of Bacteriocin Produced by
*Lactococcus lactis* ssp. *Lactis*

Mian Anjum Murtaza, Muhammad Shahid, Iram Hafiz and Ghulam Mueen-ud-Din

**Abstract**—Bacteriocins are bioactive peptides produced by the certain bacteria that inhibit the growth of other bacteria. Bacteriocins are used as biopreservatives of food stuffs. Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention. The research work was planned to check the production of bacteriocin by lactic acid bacteria. Bacteriocin producing bacteria (*Lactococcus lactis* ssp. *Lactis*) was isolated from raw milk and sour cream. Bacteriocin was produced under optimum growth conditions and partially purified by ammonium sulphate and gel filtration. The characterization was carried out at different temperatures and pH and it was found to be active up to 100°C and in slightly acidic pH (6.8 to 5.3). But completely inactivated by heat treatment of 121°C and pH lower than 3.8. Different proteases enzymes had negative effect on antimicrobial activity while NaCl concentration to the level of 4% was acceptable for bacteriocin production.

**Keywords**— Bacteriocin, Characterization, *Lactococcus lactis*, pH, Temperature.

I. INTRODUCTION

*Lactic* acid bacteria (LAB) play an important role in food fermentation as the products obtained with their aid are characterized by hygienic safety, storage stability and attractive sensory properties. Many bacteria of different taxonomic branches and residing in various habitats produce antimicrobial substances that are active against other bacteria. Both Gram negative and Gram positive bacteria produce bacteriocins.

Bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides. In general these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity.

These compounds have received much attention mainly because of their potential use as ‘natural’ food preservatives (2). Furthermore, they show an interesting potential application as food additives in the control of food spoilage and pathogenic food born microorganisms (11).

These are bioactive peptides produced by the certain bacteria that inhibit the growth of other bacteria (8). These are the pertinacious compounds of bacterial origin that are lethal to bacteria other than the strain. Normally the cells producing the bacteriocins are immune to its antagonistic action and therefore might enjoy a competitive advantage over sensitive bacteria inhibiting the same ecological niche.

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous in native and generally possessing a narrow specificity of action against strains of same or closely selected species. Extensive use of antibiotics induces resistance against a number of pathogenic bacteria in animals and poultry consuming feed and also have indirect effects on human health. However, bacteriocins do not induce resistance in pathogenic microorganism and also have no residual effect as public health concern (14).

Bacteriocins are a heterogenous group, characteristically selected for evaluation and use as specific antagonists against problematic bacteria; however, their effectiveness in foods can become limited for various reasons, and cost remains an issue impeding broader use of bacteriocins as food additives.

Because LAB and their metabolites have been consumed in high quantities by countless generations of people in cultured foods with no adverse effects, the LAB continue as the preferred source for food-use bacteriocins, either in the form of purified compounds or growth extracts.

Depending on the producer organism and classification criteria, bacteriocins can be classified into several groups (2,3) in which Class I modified bacteriocins, known as lantibiotics, Class II, heat stable minimally modified bacteriocins, class III, larger heat labile bacteriocins and Class IV, complex bacteriocins carrying lipid or carbohydrate moiety (4,12).

Biological control has received considerable attention over the last decades, as an alternative to the use of chemical bactericides and/or fungicides. Keeping in view the above mentioned facts, the study was designed to characterize the bacteriocin produced by *Lactococcus lactis* ssp. *lactis* which is a common dairy starter.

II. MATERIAL AND METHODS

A. Materials

The material required was bacterial sources (Raw milk and sour cream) and growth medium.
B. Methodology:

**Isolation and purification of bacterial strains**

Raw milk and sour cream were used as bacterial source. *Lactococcus lactis* ssp lactis was isolated and purified by repeatedly growing on selective media followed by morphological study. Purified strain was identified by sugar fermentation and biochemical tests and the purified culture was maintained in glycerol at -80°C.

**Bacteriocin Production**

1% of bacterial culture (*Lactococcus lactis* ssp. lactis) having 1X108 cfu/mL was dissolved in selective broth (M17) in a conical flask. The flask was put in shaker at 37°C and 120 rpm for 48 hours. The mixture was subjected to centrifugation at 10,000 rpm (4°C) for 20 minutes. The residue was discarded and cell free supernatant was concentrated up to 200 mL in a rotary evaporator at 25°C by evaporating the water and then stored in a flask at 4°C (5).

**Preparation of bacterial inoculum (test organism)**

Nutrient broth (oxide) was mixed at a concentration of 1.3g/100mL in distilled water and autoclaved at 121°C for 15 min. A loop full from pure culture of bacterial strain (*Staphylococcus aureus*) was mixed in the medium after cooling and the flask was placed in shaker at 37°C for 24 hours. Inocula for all the bacterial strains were prepared in this manner and stored at 4°C. The inoculum with 1×108 colony forming unit /ml is best for assay.

**Antibacterial assay by disc diffusion method**

Nutrient agar was prepared by adding 2.8 g in 100mL of distilled water and autoclaved at 121°C for 15 min. Before transferring this medium in sterilized petri plates, 10 µL inoculum was added to it while it was liquid and quite cool. Mixed them and then poured into Petri plates. After this, small filter paper (Whatman paper) discs were laid flat on growth medium and 100µL of extract was put on each disc. The petri plates were then incubated at 37°C for 48 hours, for growth of bacteria. The extracts having antimicrobial activity, inhibited the bacterial growth, and the clear zones were formed. The zones of inhibition were measured in millimeters using zone reader (6,7).

C. Characterization of Bacteriocin

**Sensitivity of bacteriocin to heat**

Sensitivity of bacteriocin to heat was checked by heating the culture supernatant for 15 minutes at 65°C, 100°C and 121°C. The disc diffusion assay was performed to detect activity against test organism (1).

**Sensitivity of bacteriocin to pH**

Sensitivity of bacteriocins to pH was checked by changing the pH of culture supernatant by 0.1N NaOH and 0.1N HCl at 3.8 and 6.8, while control was at 5.3 pH. The residual activity was checked by disc diffusion method (1).

**Sensitivity of bacteriocin to enzymes**

Sensitivity to proteolytic enzymes was checked when culture supernatant was treated with proteinase k, trypsin and chloroform. 1 mL of crude extract was treated with 1mg proteinase-k, 0.5mg trypsin and 1mL of chloroform separately, incubated at 37°C for 2 hours and then boiled at 100°C for 5 minutes. The residual activity was checked by disc diffusion method (1).

**Effect of NaCl on production of bacteriocin**

The effect of NaCl on bacteriocin production was checked by growing the bacteriocin producing strain in agar medium with 2%, 4% and 6% NaCl. Then indicator strain was mixed with fresh agar and spread onto the surface of agar containing the bacteriocin producing colonies followed by overnight incubation. Then bacteriocin activity was assayed by disc diffusion method (1).

III. RESULTS AND DISCUSSION

*Lactococcus lactis* ssp. *Lactis* was isolated from indigenous dairy sources and purified using different microbiological techniques. Identification and characterization was done by sugar fermentation, biochemical and enzyme activity tests. The pure culture was maintained in selective broth and used for bacteriocin production.

A. Production and characterization of bacteriocin

Lactic acid bacteria are important because they produce antimicrobial compounds (bacteriocins). These compounds have grown substantially due to their potential usefulness as natural substitute for chemical food preservatives to enhance shelf life of food. (Clavelend et al., 2001).

The isolated and purified bacteria were used to produce bacteriocin and then it was purified and characterized.

B. Characterization of bacteriocin

a) Effect of temperature

Bacteriocin is probably stimulated by environmental conditions like temperature and pH.

The rate of thermal inactivation of the bacteriocins was determined by heating crude samples of bacteriocin at various temperatures (37°C, 65°C, 100°C and 121°C) for 15 minutes. The bacteriocin was stable at 37°C and 65°C but partially stable at 100°C and retained its activity. But bacteriocin completely lost its activity when heated at 121°C.

In colony count, at 65°C less colonies of target organism (*Staph aureas*) appeared ranging from 30-35 indicating that bacteriocin is active at this temperature and inhibit the growth of target organism i.e Staph aureas. At 100°C, 240-270 colonies appeared indicating that bacteriocin is partially active at this temperature but it showed complete inactivation at temperature 121°C because colonies appeared were greater than 300 showing no inhibition against Staph aureas.

Crude sample of bacteriocin without any heat treatment showed maximum inhibition or activation revealed its proteinaceous nature that denatures at certain temperature.

This bacteriocin is markedly less thermostable than the Bacteriocides T1-I bacteriocin, as discussed by (1). According to their results, 3% fraction of B.fragilis bacteriocin is stable after heating at 121°C. As it was stable at low temperatures, the microorganism could act as a potential barrier to inhibit the growth of psychrotrophic or mesotrophic
spoilage and food born pathogens, such as Lactobacillus ssp., L. monocytogenes, S. aureus, B. cereus and C. perfringens, frequently found in foods stored under refrigeration.

b) Effect of pH
Activity of bacteriocin was determined at different pH levels; sterile cell-free supernatant was adjusted with 1 mol NaOH/HCl to different pH values (3.8, 5.3 and 6.8). When this pH treated sample sample assayd against Staph aureus it showed negative results in acidic pH i.e. 3.8 but showed maximum inhibition at pH 6.8. It was also active at pH 5.3 indicated that activity increased towards basic pH. In colony count fewer colonies appear at pH 6.8 (5-7) than at pH 5.3 (17-20). But maximum colonies were appeared at pH 3.8 showed maximum inactivation against target organism.

Significant results were found by (1) and (9). They described that the bacteriocin produced by strains of Lactococcus lactis isolated from fermented milk was active at higher level of pH. They also reported Lactococcus lactis isolated from fermented milk also grow at higher level of pH which may reflect the adaptations of these substances to the environmental conditions in which bacteriocin-producer bacteria develop (9).

c) Effect of enzymes
Antibacterial activity of crude extract of bacteriocin was determined by treating the sample with Proteinase k, Trypsin and chloroform. The samples treated with them showed no activity against Staph aureus when disc diffusion method was performed as shown in table. This indicated that bacteriocin is protein in nature and digested by these proteolytic enzymes.

Significant results were also discussed by (10). They described that bacteriocin produced by L. lactis subsp. lactis showed sensitivity to proteases similar to that of nisin. However, several factors can have an effect on antimicrobial activity including the interaction between bacteriocin and constituents from the cell or the growth medium, purity and concentration of enzyme and the technique used to test for enzyme sensitivity. The inactivation of antimicrobial activity by proteases suggested that the substances evaluated in this study could be antimicrobial peptides or bacteriocin.

They described that loss of the antimicrobial activity after treatment with enzymes indicated that sensitivity of the active compounds secreted by Lactococcus lactis strains. All bacteriocins including niacin were fully or partially inactivated by proteinase-K, trypsin and chloroform.

d) Effect of NaCl
The research led to environmental conditions that maximized bacteriocin activity, which can be expressed as polynomial function of NaCl. The culture isolated was treated with different concentrations of (0%, 2%, 4% and 6%) NaCl to check the bacteriocin production. The culture showed maximum production in the absence of NaCl but stable at 2% and 4% NaCl. The culture showed complete inactivation when 6% NaCl was added. (13) showed that the production of lacticin was higher in the presence of NaCl than in its absence.

IV. CONCLUSION
It can was concluded that Lactococcus lactis ssp. lactis can produce bacteriocin that is usable as antimicrobial in different foods and also it can retain its activity on normal processing conditions.

REFERENCES

### TABLE I
**EFFECT OF TEMPERATURE ON BACTERIOCIN ACTIVITY**

<table>
<thead>
<tr>
<th>Temperature treatments (15 minutes each)</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C (Control)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>65°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100°C</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>121°C</td>
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</table>

### TABLE II
**COLONY COUNT VALUES FOR BACTERIOCIN ACTIVITY INFLUENCED BY TEMP.**

<table>
<thead>
<tr>
<th>Temperature treatments (15 minutes each)</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C (Control)</td>
<td>15</td>
<td>18</td>
<td>20</td>
<td>17.67</td>
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<tr>
<td>65°C</td>
<td>30</td>
<td>35</td>
<td>32</td>
<td>32.33</td>
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<tr>
<td>100°C</td>
<td>245</td>
<td>260</td>
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<td>257.0</td>
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<tr>
<td>121°C</td>
<td>&gt;300</td>
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### TABLE III
**EFFECT OF PH ON BACTERIOCIN ACTIVITY**

<table>
<thead>
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<th>pH values</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>5.3 (Control)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6.8</td>
<td>+</td>
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### TABLE IV
**COLONY COUNT VALUES FOR BACTERIOCIN ACTIVITY INFLUENCED BY PH**

<table>
<thead>
<tr>
<th>pH values</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Means</th>
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</thead>
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<tr>
<td>3.8</td>
<td>180</td>
<td>165</td>
<td>168</td>
<td>171.0</td>
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<tr>
<td>5.3 (Control)</td>
<td>18</td>
<td>20</td>
<td>17</td>
<td>18.33</td>
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<td>6.8</td>
<td>5</td>
<td>7</td>
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### TABLE V
**EFFECT OF ENZYMES ON BACTERIOCIN ACTIVITY**

<table>
<thead>
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<th>Enzyme treatment</th>
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<th>Sample 3</th>
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<tbody>
<tr>
<td>No enzyme (Control)</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Proteinase-K</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Trypsin</td>
<td>-</td>
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### TABLE VI
**EFFECT OF NaCl CONCENTRATION ON BACTERIOCIN PRODUCTION**

<table>
<thead>
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<th>NaCl Concentration</th>
<th>Sample 1</th>
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<th>Sample 3</th>
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<td>0%</td>
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<td>+</td>
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</tr>
<tr>
<td>2%</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4%</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6%</td>
<td>-</td>
<td>-</td>
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