

Full Length Research Paper

Isolation and partial characterization of bacteriocin produced from *Lactobacillus plantarum*

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Bacteriocin producing *Lactobacillus plantarum* strain isolated from marine shrimp (*Penaeus monodon*) gut, showed broad range of antibacterial activity against some major food born pathogens. Maximum bacteriocin production was observed at 50°C, pH 4 and 0.9% sodium chloride solution. The bacteriocin has purified by ammonium sulphate precipitate and ion exchange (DEAE cellulose) chromatography. Biochemically it was pure protein moiety and the molecular weight was 2.5 KDa based on the plasmid curing experiment, suggesting that the bacteriocin was a plasmid encoded protein molecule. The study revealed the possibility of using bacteriocin as a food preservative and the *L. plantarum* strain as probiotic.

Key words: Antagonistic activity, bacteriocin, chromatography, *Lactobacillus plantarum*.

INTRODUCTION

The genus *Lactobacillus* is a diverse group of microorganisms consisting of a number of different species. They are non-spore formers, Gram-positive rods ranging from 0.5 - 1.2 × 1 - 10 µm in size and produce lactic acid as a fermented end product (Aasen et al., 2000). The genus comprises over 25 species and the first level of differentiation is based on end-product composition. Some are homofermentative where as others are heterofermentative in nature. Lactic acid bacteria (LAB) are useful in the food industry. They reduce the pH in food, low enough to inhibit the growth of most of other microorganisms including common human pathogens, thus increasing the self life of fermented food (Ivanova et al., 2000).

In the search for food biopreservatives, investigations on certain antibacterial proteins (bacteriocins) from lactic acid bacteria have been popular (Nisin) (Daeschel, 1990). Bacteriocins are proteinaceous compounds of bacterial origin and are lethal to bacteria other than the producing strain. Bacteriocin secreting microbes have selective advantage in a complex microbial niche. Gene-rally, the compounds are named according to the genus or species of the bacterial strain that produces them. For example, plantaricin is produced by *Lactobacillus plantarum* (Joerger and Hoover, 2000). Bacteriocins produced

by lactic acid bacteria have received considerable attention during recent years for their possible application as biopreservatives in food, with the hope that the use of chemical preservatives in foods will be reduced.

L. plantarum is one of the most important LAB used for the production of fermented meat, grass and vegetable products (Ritz Barba et al., 1991). Several bacteriocin-producing strains have been isolated from marine habitat. The antimicrobial peptides produced by one of these strains, later identified as *L. plantarum* (Van Reenen and Dicks, 1996), inhibits the growth of a number of food spoilage bacteria. This paper reports on the production, characteristics, isolation and spectrum of antibacterial activity of bacteriocin from *L. plantarum*.

MATERIALS AND METHODS

Isolation and identification of bacterial strains

L. plantarum was isolated from the guts of death shrimp (*Penaeus monodon*) using the method of (Todorov and Dicks, 2004). To prepare the guts of *P. monodon* for isolation of the bacteria, 1 g of the shrimp gut were homogenized in 99 ml of normal physiological saline and ground together to obtain a uniform homogenate and then used for the purpose of isolation of bacteria. To isolate the bacteria, 1 ml of the gut homogenate was mixed with 9 ml of sterile physiological saline and serially diluted up to 10⁻⁹. 1 ml of the 10⁻⁶ dilution was then inoculated into plates of de Man, Rogosa and Sharp (MRS) agar supplemented with 50 µg/liter of Natomycin. The culture was then incubated at 37°C for 72 - 96 h.

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After incubation, the isolates were identified by observing their morphological, physiological and biochemical characteristics as described by Michae (1981). Biochemical characteristics observed included gas production, sugar fermentation, resistance to biliary salts and pH determination. The test for gas production, the isolates were inoculated onto medium comprising of peptone 15 g/l, casein 5 g/l, glucose 1 g/l, potassium acetate 0.2 g/l, sodium thiosulphate 0.08 g/l, magnesium sulphate 0.05 g/l, agar 15 g/l and Tween 80 1 ml/l, which was incubated for 72 h at 37°C.

For determination of sugar fermentation, the isolates were inoculated onto a medium comprising containing peptone 10 g/l, NaCl 5 g/l, K₂PO₄ 0.3 g/l and bromothymol blue (pH 6.5), while for bile salt resistance determination, the isolates were inoculated onto medium comprising tomato juice 10 ml, peptone 1.5 g/l, glucose 2 g/l, NaCl 0.5 g/l, yeast extract 0.6 g/l, soluble starch 0.05 g/l, tauroglycocholate 2 g/l and Tween 80 0.1 ml/l. To determine the pH of isolates, the organisms were grown in MRS broth adjusted to various pH ranges of 3, 3.5, 4.5, 5.0, 5.5 and 6.0.

Screening of isolates for antibacterial activity

The *L. plantarum* isolates were screened for antibacterial activity using the agar spot method as described by (Eamanu et al., 2005). Briefly, 10 µl of the 18 - 24 h culture suspension of indicator strains (*L. bulgaricus*) were mixed with 10 ml of sterilized Brain Heart Infusion soft agar (BHIA-soft agar) earlier cooled to 47°C and then poured onto BHI firm agar (10 ml) plates and allowed to solidify. An aliquot of 10 µl cell free culture fluid of bioassay strains (*L. plantarum*) was spotted onto agar plates seeded with indicator organism. After incubation (37°C for 18 - 24 h) the plates were examined for the presence of 2 mm clear zone around the spot (Van Reenen and Dicks, 1996).

The antimicrobial activity of isolates was tested against the ten pathogenic organisms as method described by (Todorov and Dicks, 2004). *L. plantarum* was inoculated into MRS broth and incubated at 37°C for 12 - 14 h. Aliquot of 5 l cell-free culture supernatant was spotted on the surface of agar plate seeded with actively growing cells of the test organism. Plates were incubated at the optimal growth temperature (37°) of the test organism. The test organisms are *Latobacillus bulgaricus*, *Salmonella typhimurium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella paratyphi 'B'*, *Escherichia coli*, *Klebsiella sps*, *Serratia marsacence*, *Pseudomonas aeroginosa*, *Vibrio cholerae*.

Bacteriocin production and biomass in different media

The growth and bacteriocin production were measured as described by (Eamanu et al., 2005). The test organism was inoculated into the MRS broth (Peptone 10.0 g/l, yeast extract 5.0 g/l, meat extract 5.0 g/l, glucose 20.0 g/l, dipotassium phosphate 2.0 g/l, dibasic ammonium citrate 2.0 g/l, sodium acetate 5.0 g/l, maganous sulphate 0.050 g/l, magnesium sulphate 0.010 g/l, tween 80 1 ml/l (pH 6.5) and GP broth, glucose 2%, peptone 1%, CaCO₃ 1% (pH 6.5) . OD was measured at 600 nm at regular interval intervals (1 h) and respectively evaluates the bacteriocin titer.

The titer of bacteriocin was quantified as described by (Todorov and Dicks, 2004). Cell free extracts of *L. plantarum* was two fold diluted using physiological saline solution. Aliquots of 0.1 µ l from each dilution were spotted in plates seeded with the 18 - 24 h indicator strain. The plates were incubated at 37°C for 18 - 24 h and examined for the presence of 2 mm wider clear zone of inhibition around the spot. The antimicrobial activity of bacteriocin was expressed as arbitrary unit (AU), defined as the reciprocal of the highest dilution showed the inhibitory activity (Todorov and Dicks, 2004).

Effect of pH, temperature and NaCl concentration on bacteriocin production

To determine the effect of pH on bacteriocin production, six batches 100 ml MRS broth was prepared and adjusted to pH 1, 2, 3, 4, 5 and 6, respectively with 6 M HCl or 6 M NaOH, and then autoclaved. Flask were inoculated with 2% v/v of 18 h old culture of *L. plantarum* and incubated at 30°C for 20 h without aeration. The pH of the supernatants was adjusted to 6.0 with sterile NaOH, from which bacteriocin titer was assessed.

For determination of the effect of temperature on bacteriocin production, 100 ml each of sterilized MRS broth in six different sets of flasks were inoculated with 2% v/v 18 h old culture of *L. plantarum* and incubated without aeration at various temperatures of 10, 20, 30, 40, 50 and 60°C for 20 h. After incubation the bacteriocin titer in the medium was determined.

The effect of NaCl on bacteriocin production was determined by means of preparing ten sets of 100 ml MRS broth, their salt concentrations were differ from 0.1 - 1.0% respectively. The flasks were inoculated with 2% v/v 18 h old culture of *L. plantarum* and incubated without aeration at 37°C for 20 h. After incubation the bacteriocin titer in the medium was determined as described by (Todorov and Dicks, 2004).

Purification and characterization of bacteriocin

Two methods were used for this purpose; Ammonium salt precipitation and ion-exchange chromatography (Yang et al., 1992). For Ammonium salt precipitation, various concentrations of Ammonium Sulphate (10, 20, 30, 40, 50 and 60%) were added to 10 ml of crude bacteriocin in different sets of test tubes, precipitate for 24 h. The mixture was then centrifuged at 5.000 rpm for 10 min and the precipitate were resuspended in 25 ml of 0.05 M Potassium Phosphate buffer. The mixture was stirred for 24 h at 4°C, after which the suspension was dialyzed in a tubular cellulose membrane (1000 cut off) against 2 l distilled water for 24 h. After dialysis, the bacteriocin titer was then determined as earlier described.

Ion-exchange chromatography

The dialyzate was used for purification by cation exchange column (DEAE cellulose column) and elution was performed by using a linear gradient from citrate phosphate buffer ranging from pH 2.6 to 7.0 (Macher et al., 1980). Protein content was determined by Bradford method (Sadasivam and Manickam, 1996). The bacteriocin titer was assessed (Todorov and Dicks, 2004).

Effect of enzyme inhibitors on bacteriocin activity

The effect of the enzymes; amylase, protease and catalase at various concentrations (0.1 and 1.0 mg/ml) were added to the purified bacteriocin and incubate for 1 h at 37°C. After incubation the bacteriocin activity was determined as described by (Todorov and Dicks, 2004).

Determination of molecular size of bacteriocin

The molecular size of the purified bacteriocin was determined using SDS PAGE gel as described by (Sambrook et al., 2006). Briefly, sterile glass plates were assembled, 20 ml of 15% resolving gel were dispensed, 2 ml of butanol was then overlaid onto the gels, allowed to polymerize, after which the overlay was poured off and then the gel surface rinsed with deionized water. To the gel, 8 ml of 5% stacking gel was overlaid and fixed in an electrophoresis apparatus. To the electrophoresis wells, equal volumes 20 µl of 1 x SDS and

Table 1. Physiological and biochemical characteristic.

| Physiological and biochemical characteristic | Result |
|--|---|
| Colony morphology | Cream, beige, little sticks and smooth round colonies |
| Gram staining | gram positive rod |
| Growth in MRS broth | uniform turbidity |
| Type of fermentation | Homofermentative |
| Galactose, glucose, fructose, mannitol, lactose, sucrose and maltose | Fermentation positive |
| Rhamnose. | Fermentation negative |
| Catalase and amylase production | Negative |
| Growth in Biliary salt | Resistant |
| H ₂ S production | positive |

Table 2. Inhibitory activity of bacteriocin against ten food spoilage bacteria. Inhibitory zone <2(positive for inhibitory activity). Inhibitory zone >2(negative for inhibitory activity) (Todorov et al., 2004).

| Test organism | Inhibitory zone (mm) |
|---------------------------------|----------------------|
| <i>Bacillus subtilis</i> | 3.00 |
| <i>Staphylococcus aureus</i> | 2.30 |
| <i>Lactobacillus bulgaricus</i> | 4.00 |
| <i>Salmonella typhimurium</i> | 3.75 |
| <i>Salmonella paratyphi 'B'</i> | 2.90 |
| <i>Escherichia coli</i> | 2.50 |
| <i>Kelbsiella sps</i> | 2.10 |
| <i>Serratia marcescens</i> | 3.10 |
| <i>Pseudomonas aeruginosa</i> | 2.00 |
| <i>Vibrio cholerae</i> | 0.90 |

test sample preheated at 100°C in a test tube for 30 min and marker (2,500 - 40,000 KDa) respectively were loaded in the gel. The gel was then run 100 V for 5 h at 4°C, after which it was stained with Coomassie brilliant blue.

Isolation of DNA

Plasmid profiling was carryout by using the method described by (Burger et al., 1994). The DNA was separated on an agarose gel, according to the method described by (Sambrook et al., 2006).

Isolation of bacteriocin-deficient mutants

Curing experiments were conducted as described by (Ruiz Barba et al., 1991). *L. plantarum* was incubated with various concentrations of novobiocin (0.125 - 8 µgml⁻¹) and incubated at 37°C for 72 h. Cul-tures that grow at the highest concentration of Novobiocin (8 µg/ml) were assess for loss of bacteriocin production by agar spot test method (Todorov and Dicks, 2004) and the plasmid profiling were perormed (Burger et al., 1994).

RESULTS

The viable cell count of LAB was around 4.0×10^6 cfu / g of *P. monodon* gut. The isolated *Lactobacillus* strains

show antagonistic activity against *L. bulgaricus*. The strains which showed the largest zone of growth inhibition was selected for further identification.

The selected strain was identified as *L. plantarum* based on its physiological and biochemical characteristic (Table 1). Antagonistic activity was tested against ten major pathogens. Among the ten pathogens tested, all the human pathogens were found to be susceptible to bacteriocin except *V. cholerae* (Table 2).

Measurement of biomass and screening for bacteriocin production

Measurement biomass and screening for bactericin production are shown in Figures 1 and 2. Results showed that *L. plantarum* produced bacteriocin in both MRS and GP broths. The bacteriocin production was started after 14 h incubation, 3400 AU/ml bacteriocin produced was recorded in MRS broth and 800 AU/ml in GP broth.

Effect of pH, temperature and salt concentration on bacteriocin activity

The bacteriocin activity was tested with different temperatures (10, 20, 30, 40, 50 and 60°C) and the activity was found to vary from 1600 to 12800 AU/ml, the maximum arbitrary unit was measured as 12800 AU/ml at 40°C (Figure 3). Regarding pH the maximum inhibitory activity was found to vary from 400 AU/ml to 12800 AU/ml, the maximum arbitrary unit was measured as 12800 AU/ml at pH 5.0 (Figure 4). Regarding various salinity (NaCl %) tested from 0.1 to 1.0% NaCl, the bacteriocin production activity was vary from 3800 AU/ml to 12800 AU/ml, 0.9% was found to be suitable for the bacteriocin production (Figure 5).

Purification and characterization of bacteriocin

The maximum inhibitory activity was measured at 50% saturation. In ion exchange chromatography (DEAE cellulose) the active fraction was eluted with pH 5.0-citrate phosphate buffer. The molecular weight of the bacteriocin

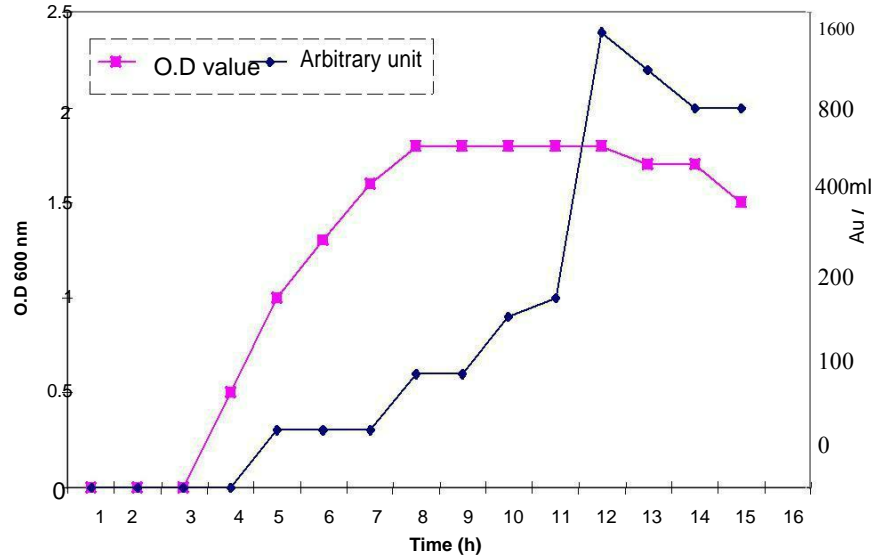


Figure 1. Bacteriocin production in MRS broth.

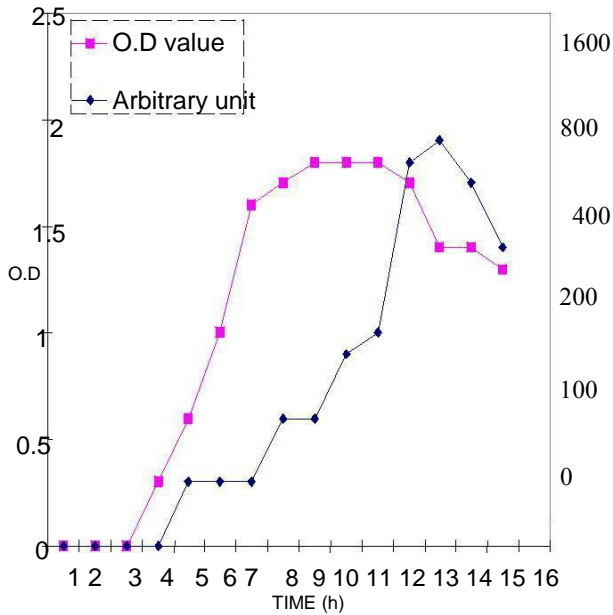


Figure 2. Bacteriocin productions in GP broth.

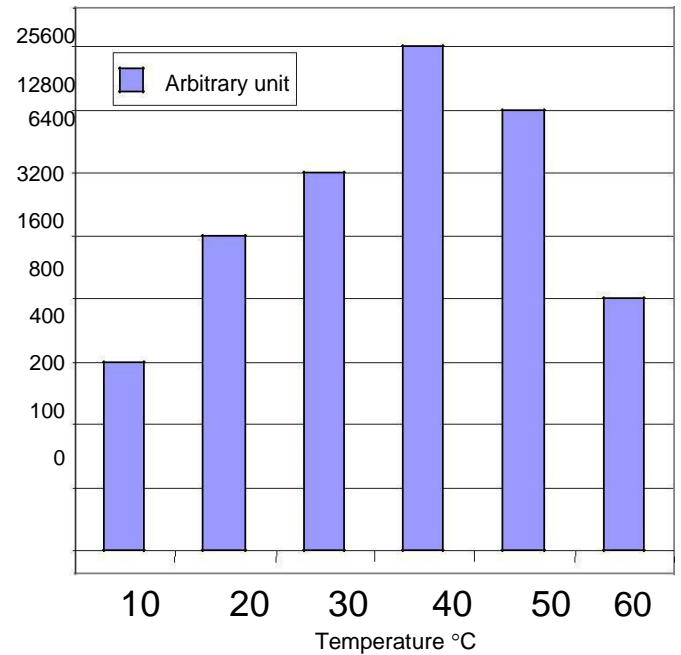


Figure 3. Bacteriocin production in different temperatures.

was determined as 2.5 KDa (Figure 6).

Effect of enzyme inhibitors on bacteriocin activity

Effect of enzyme inhibitors on bacteriocin activity is shown in (Tables 3 - 5). Results showed that catalase and amylase had no effect on bacteriocin activity, but protease completely inhibited the activity of the compound.

Isolation of bacteriocin-deficient mutants

Curing with novobiocin yield one mutant of *L. plantarum*, designated as A, which lost a plasmid of approximately 5.5 kbp (Figure 7) and the ability to produce bacteriocin.

DISCUSSION

The present investigation highlights the isolation, charac-

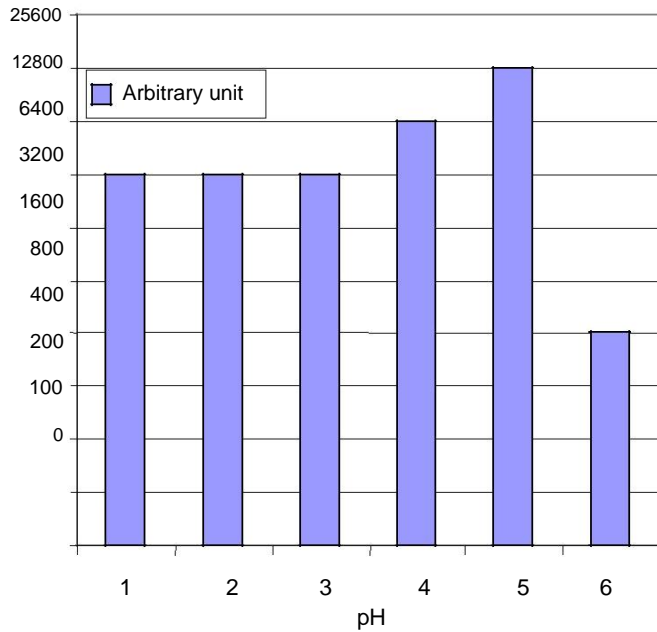


Figure 4. Bacteriocin production in different pH.

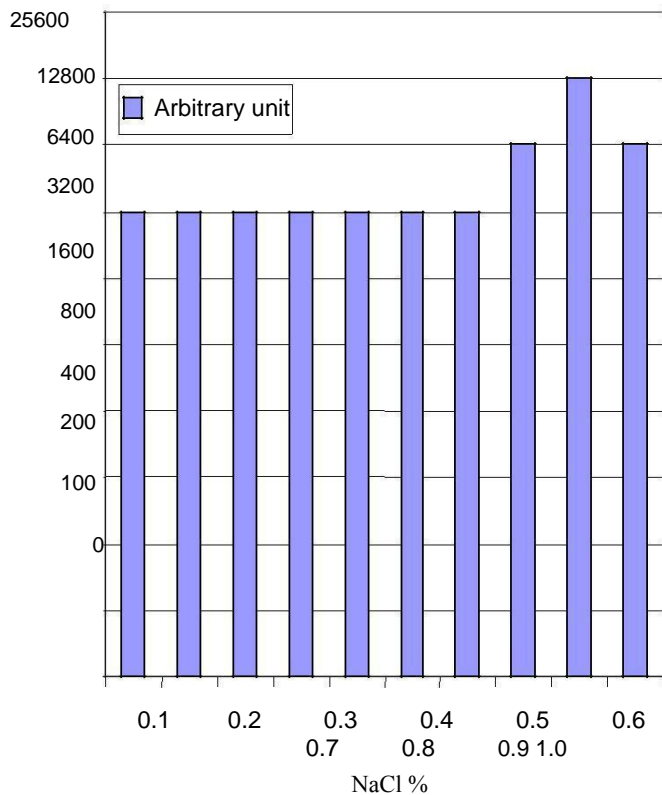


Figure 5. Bacteriocin production in different salinity.

terization and activity of bacteriocin produced by *L. plantarum*. Prawn gut seems to be a good source of LAB.

Present study revealed that *P. monodan* harbored LAB at the level of 4.0×10^5 CFU/g in its gut, among strains

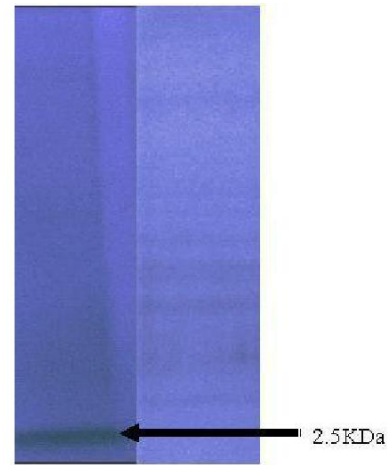


Figure 6. SDS Gel: Purified Protein (Bacteriocin) was about 2.5 KDa.



Figure 7. Plasmid profile (agarose gel electrophoresis).

tested; the most potential strain was selected and used for further study. The physicochemical characterization of the strain revealed that it was *L. plantarum*.

To state that the isolate *L. plantarum* was tested for antibacterial activity against *L. bulgaricus*, *S. typhimurium*, *E. coli*, *B. subtilis*, *S. aureus*, *S. paratyphi 'B'*, *Klebsiella spp*, *S. marcescens*, *P. aeruginosa* and *V. cholerae* associated with food borne illnesses. The highest inhibitory activity was demonstrated against *L. bulgaricus* (4 mm inhibitory zone) while the least activity was demonstrated against *V. cholerae*. The inhibitory effect demonstrated by *L. plantarum* against these bacteria is an indication of possession of antibacterial activity.

Results also revealed the presence of the compound

Table 3. Effect of protease on bacteriocin activity (+; inhibitory activity positive, inhibitory activity negative).

| Protease mg/ml/dilution Factor | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | 1:25600 | 1:51200 |
|--------------------------------|-------|-------|-------|-------|--------|--------|--------|---------|---------|---------|
| 0.1 | + | + | + | + | - | - | - | - | - | - |
| 1 | + | - | - | - | - | - | - | - | - | - |

Table 4. Effect of α amylase on bacteriocin activity (+; inhibitory activity positive, inhibitory activity negative).

| α amylase mg/ml/dilution Factor | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | 1:25600 | 1:51200 |
|--|-------|-------|-------|-------|--------|--------|--------|---------|---------|---------|
| 0.1 | + | + | + | + | + | + | + | + | + | + |
| 1 | + | + | + | + | + | + | + | + | + | + |

Table 5. Effect of catalase on bacteriocin activity (+; inhibitory activity positive, inhibitory activity negative).

| Catalase mg/ml/dilution Factor | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | 1:25600 | 1:51200 |
|--------------------------------|-------|-------|-------|-------|--------|--------|--------|---------|---------|---------|
| 0.1 | + | + | + | + | + | + | + | + | + | + |
| 1 | + | + | + | + | + | + | + | + | + | + |

bacteriocin in the test organisms. Bacteriocins have been reported to be inhibitory against several other bacteria (Klaenhammer, 1983; Flythe et al., 2004). Possession of bacteriocin by *L. plantarum* is an indication that the bacteria can be used as probiotic and as biopreservative.

Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity was noted at pH 5, temperature 40°C and 0.9% NaCl. From the results proved that it can be used in fermented acidic foods like pickle or yogurt. MRS seemed to be more suitable medium compared to GP broth for the bacteriocin production and the bacteriocin secretion was started at 14 h culture, it might be a secondary metabolites.

Bacteriocin is a bacterial substance, biological protein moiety and a bactericidal mode of action against the homologous species. Chemical analysis indicated that some bacteriocin, Example; plantaricin, are quite complex molecules, lipid and carbohydrate components in addition to protein or simple proteins. To state that the purified bacteriocin was purely protein moiety, because enzyme inhibitor study proved that the bacteriocin was only inhibited by protease and their molecular weight of the protein was determined about 2.5 KDa proteins.

Based on the result obtained with the curing experiments, bacteriocin is encoded by a plasmid of approximately 5.5 kbp in size. Pediocin like bacteriocins may be either plasmid encoded (Gonzalez and Kunka, 1987) or genomically encoded (Holck et al., 1994). It might even be that only certain of the genes encoding bacteriocin are located on the 6 kbp plasmid. The structural gene of plantaricin A was shown to be located on the chromosome

(Diep et al., 1994). The 5.5 kbp plasmid of *L. acidophilus* is currently being sequenced to determine the exact location of the structural gene of bacteriocin (Diep et al., 1994).

Nisin is the first used as a food biopreservative in 1931 and first received approval by Food and Drug Administration (FDA) to be used in pasteurized processed cheese in 1988 (Rosland et al., 2005). Like nisin the bacteriocin produced by *L. plantarum* in the present study also has the potential to develop probiotics and biopreservative.

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