Original Research Article

BACTERIOCIN PRODUCTION BY *LACTOBACILLUS PLANTARUM* NRIC 0383, MEASURED IN TERMS OF ITS ANTAGONISTIC ACTIVITY AGAINST BACTERIA PATHOGENS OF SELECTED VEGETABLES

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Authors' contributions

This work was carried out in collaboration between all authors. Author TAI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors CCI and IOO managed the analyses of the study. Author GMG managed the literature searches. All authors read and approved the final manuscript.

Abstract

This study was carried out to measure the optimum condition for Bacteriocin production by *Lactobacillus plantarum*. Bacteriocin production with the candidate bacterium *Lactobacillus plantarum* NRIC 0383) was optimized by varying the incubation temperature and adjusting the pH of the production medium, whilst estimating bacteriocin production in terms of the antagonistic activity (zone of inhibition mm) against bacteria pathogens of selected vegetables using the well diffusion assay. Bacteriocin production was highest at temperatures of 30 and 35^{o}C and pH values of 6.0 and 6.5, further decrease or increase in temperature and pH values markedly decreased bacteriocin production. Increase in bacteriocin production as regards time was observed between the 24th -36th hours of incubation, contrariwise increased incubation time was accompanied by a decline in bacteriocin production. The present study provides detailed information on bacteriocin production (estimated in terms of the antagonistic activity) as it relates to specific isolates strains.

Key words: Bacteriocin; *Lactobacillus plantarum*; antagonistic activity,

1. INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of gram positive, catalase negative, oxidase negative, and micro-aerophilic organisms (17). They are generally recognized as safe bacteria (GRAS status) and play an important role in food and feed fermentation and preservation, either as the natural microflora or as starter cultures under controlled conditions (13). Bacteriocins are of interest in the food industry and their potential application in food bio preservation has prompted the need to isolate and identify best performing LAB species in terms of bacteriocin production and for their subsequent degree of antagonism to pathogenic and food spoilage micro-organisms (1). Many bacteriocins are heat-stable and retain their activity after several months of frozen or refrigerated storage and after drying. Bacteriocin production can be influenced by culture conditions, such as incubation atmosphere, pH, temperature and microbial growth phase (3; 13; 17; 18; 15). Several studies have shown that bacteriocin production is dependent on environmental factors like pH, temperature, composition and constitution of food (2). When investigating novel candidates, there are many considerations that will determine their usefulness in food systems. One of the most significant criteria is the ability to withstand thermal processing (6). Thermal processing is used extensively within the food manufacturing process and can have adverse effects on the bio-active capability of a bacteriocin, potentially rendering it less effective (5). The chemical and physical properties of a food, e.g. pH and fat content, can also have a significant role in the suitability of a particular bacteriocin (9). Antimicrobial effects of bacteriocins against sensitive microorganisms depends on environmental factors like pH, temperature, composition and constitution of food (6). Whereas bacteriocins are mostly synthesized by gram (+) bacteria, they are also produced by gram (-) bacteria (2). Due to their

nature, they are inactivated by proteases in the gastrointestinal tract. Most of the LAB bacteriocins identified so far are thermostable cationic molecules that have up to 60 amino acid residues and hydrophobic patches (4). When investigating novel candidates, there are many considerations that will determine their usefulness in food systems. One of the most significant criteria is the ability to withstand thermal processing (6). Thermal processing is used extensively within the food manufacturing process and can have adverse effects on the bio-active capability of a bacteriocin, potentially rendering it less effective (5). In this study bacteriocin production was estimated against different bacterial strains.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Bacterial strains (Sta*phylococcus aureus CIP* 9973, *Pectobacterium carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Proteus mirabilis* UPMSD3 and *Escherichia coli* 2013C-3342) used in this study were isolated from selected vegetable (Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Cabbage (*Brassica oleracea*), Eggplant (*Solanum melongena*), Green Beans (*Phaseolus vulgaris*) and Pumpkin (*Telfairia occidentalis*) samples and were referred to as the indicator (test) strain. Lactobacillus *plantarum* NRIC 0383 originally isolated from fermented cow milk in the Microbiology Laboratory of the Federal university Makurdi and characterized using 16S rRNA gene sequencing.

2.2 Screening of bacteriocin-producing isolates

2.2.1 Preparation of Cell-free supernatant (CFS) and Neutralized Cell Free Supernatant (NCFS)

Lactobacillus plantarum isolate from Cow milk was incubated in 20ml of MRS broth for 24 hours. Cell-free supernatant (CFS) was then obtained by centrifugation at 10,000 rpm for 10 minutes (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) to separate

bacterial cells and supernatant. The supernatant was filtered through a sterile 0.22 µm syringe filter (Chromatographic Specialties Incorporated, Canada). Neutralized Cell Free Supernatant (NCFS) was prepared by adjusting the pH of CFS to 7.0 with 1ml of Na OH to exclude the antimicrobial effects of organic acids. Inhibitory activity due to hydrogen peroxide (H_2O_2) was eliminated by the addition of 1 mL of catalase. Samples were then heated to 100^{o}C for 10 minutes to inhibit enzyme activity (10).

2.2.2 Determination of the Influence of different growth conditions on bacteriocin production

Bacteriocin production with the candidate bacterium (*Lactobacillus plantarum* NRIC 0383) was carried out using the method of Ogunbanwo *et al*. (11). Bacteriocin production was optimized by varying the incubation temperature of the production medium individually at 25°C, 30°C, 35°C, 40°C, 45°C and 28°C and by adjusting the pH of the production medium to 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 (control) using 1N HCL (36.5 grams of hydrochloric acid (HCl) is a 1 N (one normal) solution of HCl) or 1N NaOH (prepared by dissolving 40.0 g of NaOH in 1 litre of water). A normal is one gram equivalent of a solute per liter of solution. All the Flasks containing 100 ml of MRS broth were then aseptically inoculated with 100 µL of overnight culture of *Lactobacillus plantarum* NRIC 0383 and incubated at 120 rpm in an orbital shaker (IKA™ 10316411) for 60 hours at temperature of 35^{o}C. Neutralized cell free supernatant (NCFS) was collected from each flask by centrifugation and membrane (0.22µm) filtration at every 12 hour interval (0, 12, 24, 48, 60), bacteriocin production in terms of antagonistic activity (mm) was then examined against the different vegetable pathogens using the well diffusion assay

2.2.3 Bacteriocin production in terms of antagonistic activity (mm)

Antimicrobial activity of NCFS of *Lactobacillus plantarum* was determined using the method of Noraphat *et al.* (10). This was done by inoculating One ml of each indicator strain (*Escherichia coli*, *Staphylococcus aureus*, *Pectobacterium carotovorum*, *Enterobacter cloacae*, *Klebsiella aerogenes* and *Proteus mirabilis*) previously adjusted to 1.5×10^8 CFU/mL into 20 ml of semisolid Brain heart infusion agar (BHI) medium maintained at 50°C and then poured into a petri dish. After solidification, two wells (8 mm diameter) were cut. Neutralized CFS (NCFS) of *L. plantarum* earlier treated with 1 ml of catalase (Sigma-Aldrich Corporation, USA) at 25°C for 30 mins to eliminate the possible inhibitory action of H_2O_2 and previously adjusted to pH 7.0 with 1 m of NaOH in order to rule out possible inhibition effects due to organic acids, was then placed in the second well. After an incubation period of 37°C for 24 hour, the incubated plates were examined for zone of clearance around the individual wells. If inhibitions zones were found in the second well, the isolates were considered to be able to produce bacteriocin like substances (BLS).

3. RESULTS AND DISCUSSION

3.1 Optimization of growth conditions for Bacteriocin production

Bacteriocin production can be significantly influenced by pH, temperature, incubation time, and other environmental factors (14). Bacteriocin production was estimated in terms of the antagonistic activity (zone of inhibition mm) against the indicator strains (*Enterobacter cloacae* AS10, *Escherichia coli* 2013C-3342, *Pectobacterium* Pec1, *Klebsiella aerogenes* OFM28, *Proteus mirabilis* UPMSD3 and *Staphylococcus aureus* CIP 9973).

Among the tested pH maximum bacteriocin production in terms of antagonistic activity was recorded at pH of 6.5 against *E. cloacae* AS10 and valued at 4.80 ± 0.03 mm to17.80 ± 0.05 mm at incubation time of 12 to 60 hours. Highest inhibitory activity (17.35 ± 0.04 mm) of bacteriocin of *L. plantarum* NRIC 0383 against *E. cloacae* AS10 was observed at a temperature of 35°C (figure 1).

Highest Bacteriocin production measured as inhibitory activity against *E.coli* 2013C-3342 ranged from 5.51 ± 0.05 mm to 16.55 ± 0.02 mm at a temperature of 35°C with peak production occurring at the 36th hour while at pH of 6.5 peak bacteriocin production of 15.00 ± 0.03 mm was observed (figure 2).

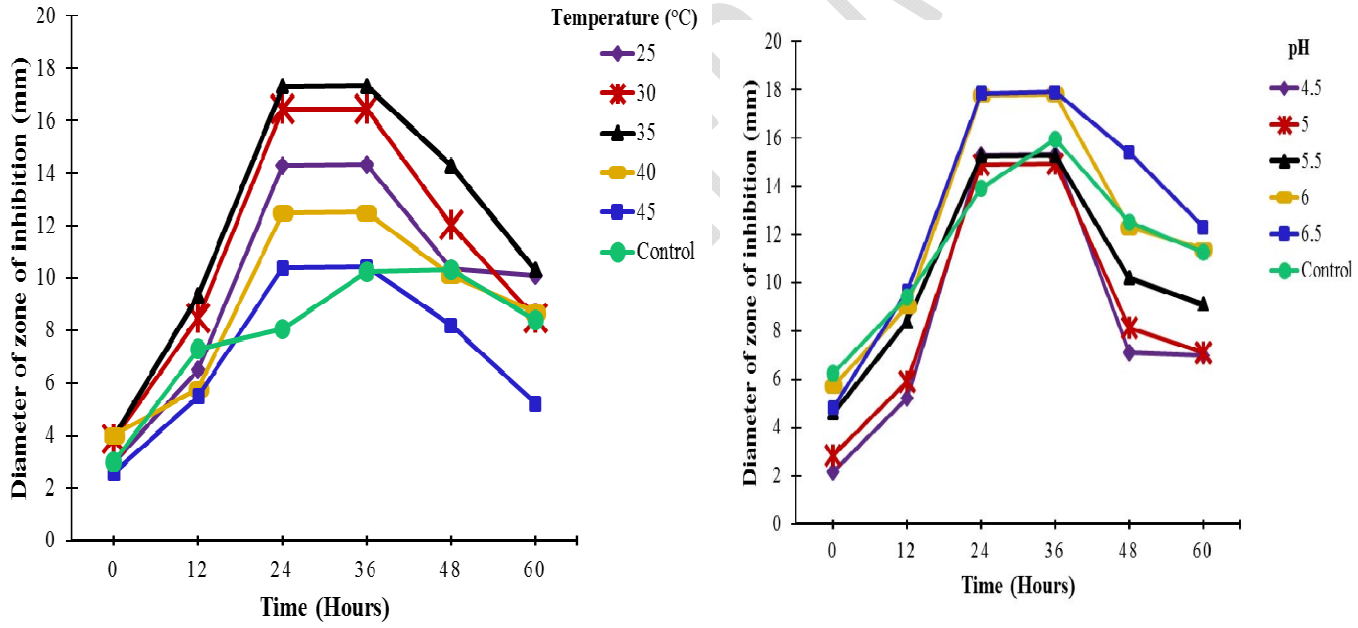
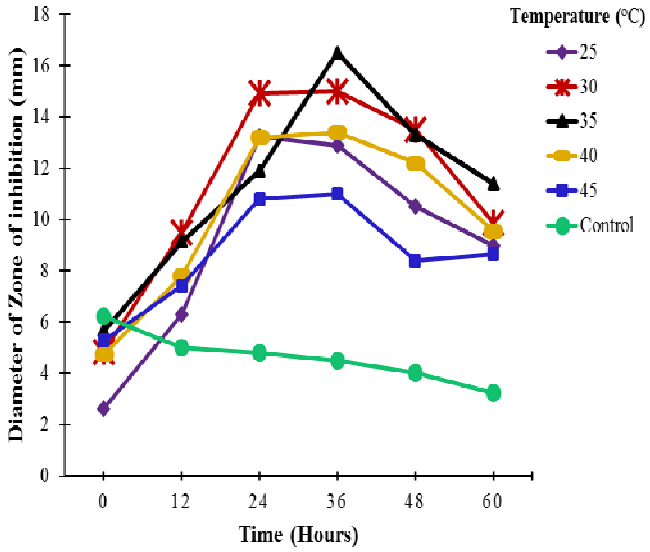


Figure 1: Influence of different levels of temperature and pH on bacteriocin production estimated in terms of antagonistic activity of bacteriocin against *Enterobacter cloacae* AS10

Legend:
Control = Room temperature at 28°C; pH: 7.0
Time = Incubation time per Hour



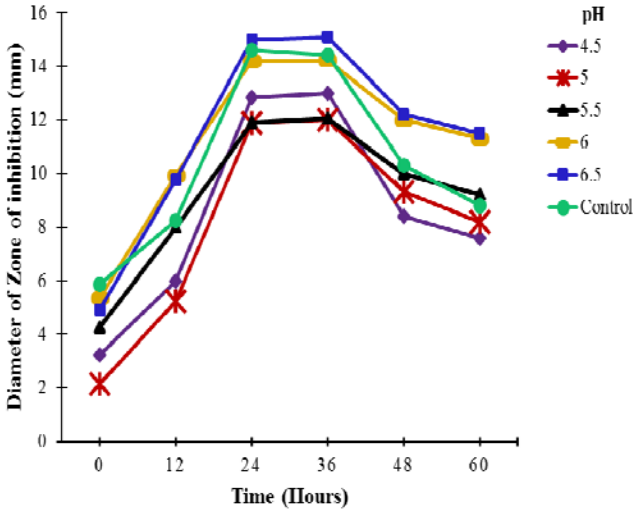


Figure 2: Influence of different temperature and pH values on bacteriocin production estimated in terms of the antagonistic activity of bacteriocin against *E. coli* 2013C-3342

Legend:

Control = Room temperature at 28^{o}C, pH: 7.0
Time = Incubation time per Hour

Maximum bacteriocin production determined from its inhibitory activity against *K. aerogenes* OFM28 occurred between the 12th and 36th hour at all tested temperatures and pH, with maximum production value of 3.90 ± 0.04 mm to 14.27 ± 0.01 mm occurring at 35^{o}C and 5.44 ± 0.10 mm to 15.40 ± 0.04 mm at pH 6.5 respectively (figure 3).

Increased bacteriocin production expressed as antibacterial activity (12.42 ± 0.01 mm and 14.33 ± 0.15 mm to) against *P.carotovorum* Pec 1 was observed at the 36th hour at temperature of 35^{o}C while at pH of 6.5 maximum Bacteriocin production of 15.30 ± 0.02 mm and 15.31 ± 0.05 mm was recorded at the 24 and 36th hours of incubation (figure 4).

Increased Bacteriocin production against *P. mirabalis* UPMSD3 (10.60 ± 0.13 mm to 15.28 ± 0.03 mm) occurred between the hours of 12 and 48, with peak production occurring at temperature of 35^{o}Cwhile at pH of 6.5, Maximum bacteriocin production expressed as antimicrobial activity against *P. mirabalis* UPMSD3 was measured at 12.49 ± 0.04 mm at the 36th hour as shown in figure 5. Maximum bacteriocin production of 14.61 ± 0.02 mm expressed as inhibitory activity against P. *mirabalis* at pH of 7.0 was observed at the zero hour of incubation.

Bacteriocin production measured in terms of inhibitory activity against *S. aureus* CIP 9973 was observed to be 12.51 ± 0.04 mm and 13.07 ± 0.02 mm at temperature of 35^{o}C and pH 6.5 (Figure 6) respectively. At pH of 5.0 maximum inhibitory activity of 12.13 ± 0.12 mm was observed at the 36th hour.

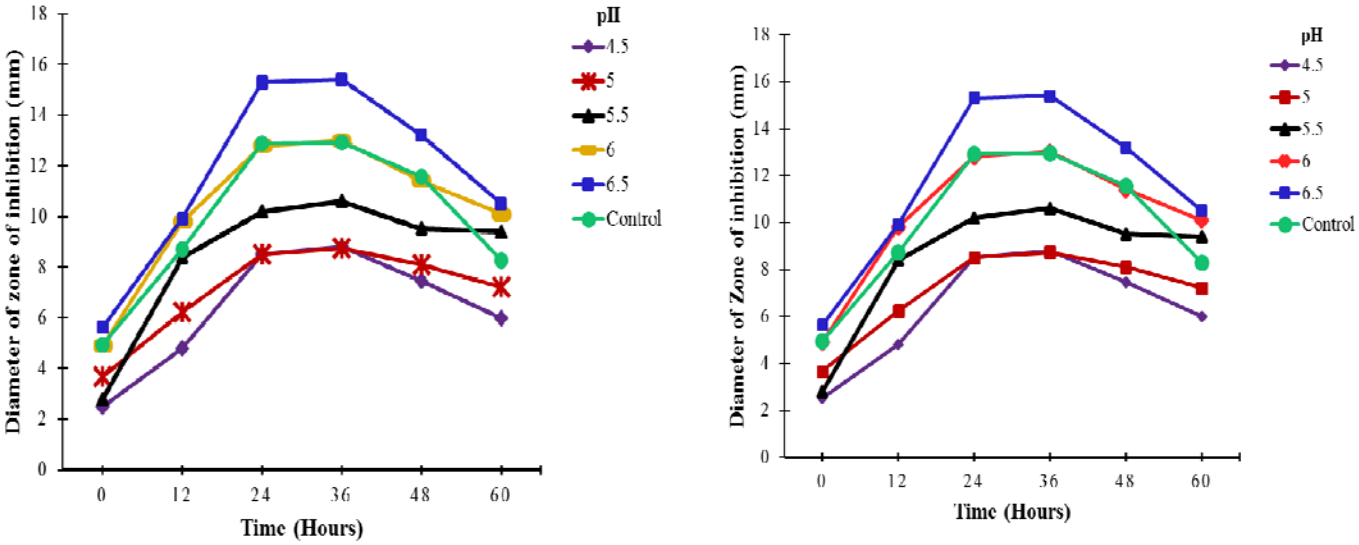


Figure 3: Influence of different levels of pH and Temperature on bacteriocin production estimated in terms of the antagonistic activity of bacteriocin against *K. aerogenes* OFM28

Legend:

Control= Room temperature at 28^{o}C, pH: 7.0

Time = Incubation time per Hour

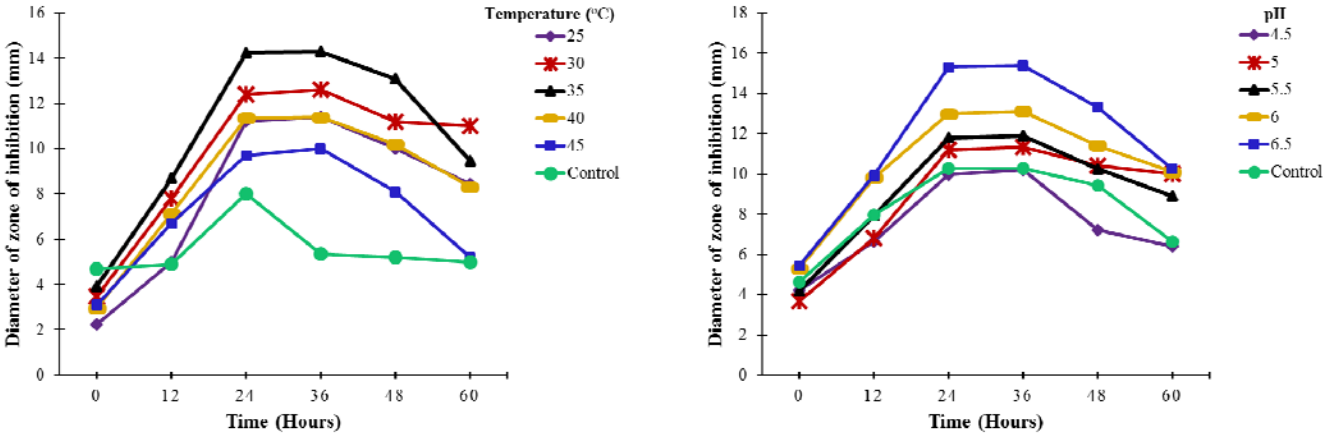


Figure 4: Influence of different levels of pH and Temperature on bacteriocin production estimated in terms of the antagonistic activity of bacteriocin against *P. carotovorum* Pec1

Legend:

Control = Room temperature at 28^{o}C, pH: 7.0
Time = Incubation time per Hour

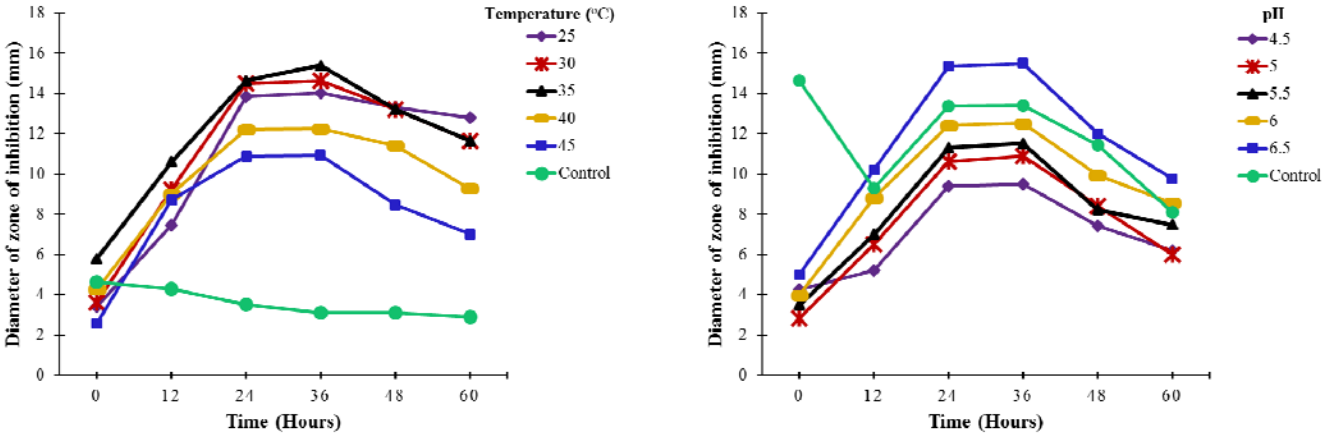


Figure 5: Influence of different levels of pH and Temperature on bacteriocin production estimated in terms of the antagonistic activity of bacteriocin against *P. mirabilis* UPMSD3

Legend:

Control = Room temperature at 28°C, pH: 7.0
Time = Incubation time per Hour

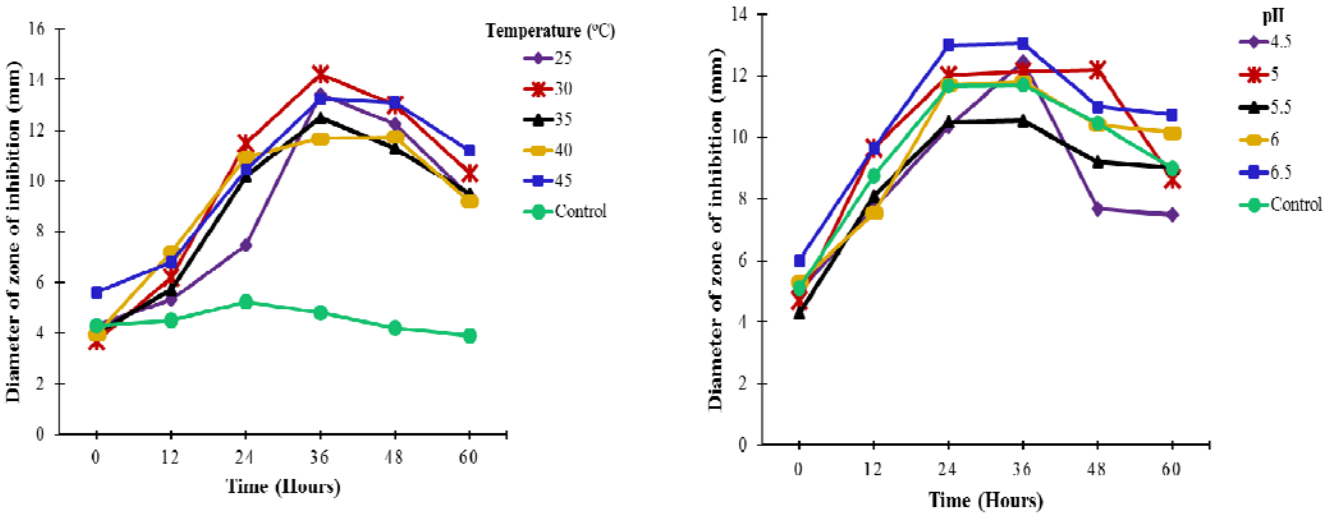


Figure 6: Influence of different pH values on bacteriocin production estimated in terms of the antagonistic activity of bacteriocin against *S. aureus* CIP 9973

Legend:

Control = Room temperature at 28°C, pH: 7.0
Time = Incubation time per Hour

The results obtained in this study revealed that the optimal production of bacteriocin from *Lactobacillus plantarum* NRIC 0383 occurred at pH of 6, comparably the optimum pH for certain bacteriocin production was reported to be less than 5.0 as reported by Noraphat *et al.* (10). According to Yi *et al.* (16), pH has the greatest influence on bacteriocin production and the more the bacteriocins being pH tolerant the better are the chances for its selection in the food system. Results from the study showed that Neutralized cell free supernatant remained active, against sensitive target strain (*Staphylococcus aureus* CIP 9973, *Pectobacterium carotovorum subsp. carotovorum* Pec1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Proteus mirabilis* UPMSD3 and *Escherichia coli* 2013C-3342) even when the pH was adjusted to pH 7. Maximum bacteriocin production in terms of antagonistic activity was recorded at pH 6.0, however, further increase in pH was found to mitigate bacteriocin production as expressed in the inhibitory activities. The result of Saranya and Hemashenpagam (12) report is in consonance with the result obtained in this study who found out that the optimum pH for bacteriocin production was usually 5.5 to 6.0. Bacteriocin production in alkaline conditions are now gaining more attention in many food industries because the pH of several food products vary from natural to alkaline (7).

Bacteriocin production was highest at temperatures of 35°C, further decrease or increase in temperature markedly decreased bacteriocin production. Highest activity of bacteriocin of *L. plantarum* NRIC 033 (15.31 ± 0.05mm) was recorded against *P. carotovorum* Pec1 at 35 °C while the activities of bacteriocin decreased with increasing temperature. This is not in consonance with the results of Moonchai *et al*. (8) who reported that bacteriocin production by *L. lactis* was optimum at 30°C. Control samples at temperatures of 28°C and pH of 7.0 showed varying levels of bacteriocin production as seen in the inhibitory activities. Optimal bacteriocin production by *L. plantarum* NRIC 0383 was best at pH of 6.5 and temperature of 35°C.

Conclusion

This research has shown that bacteriocin production by *L. plantarum* NRIC 0383 was highest at temperatures of 30 and 35°C and pH values of 6.0 and 6.5, further decrease or increase in temperature and pH values markedly decreased bacteriocin production. Increase in bacteriocin production as regards time was also observed between the 24th -36th hours of incubation irrespective of the target strain, contrariwise increased incubation time was accompanied by a decline in bacteriocin production. The study of the optimal conditions for bacteriocin production is important as it fosters maximal bacteriocin production, inhibitory activity as a measure of bacteriocin production further authenticates the potency of bacteriocin being produced for use as antimicrobial agents against food spoilage pathogens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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