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GROWTH KINETICS AND THE EFFECT OF SURACTANTS ON BACTERIOCIN PRODUCTION BY LACTOBACILLUS PLANTARUM NRIC 0383

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ABSTRACT
Lactobacillus species are of major interest in the food industry because of their bio preservative properties. They produce various types of antimicrobial compounds, including bacteriocins. Lactobacillus isolates from locust bean were identified using biochemical and molecular means. Growth kinetics of bacteriocin producing Lactobacillus plantarum NRIC 0383 was determined by inoculating one ml inoculum (108CFU/ml) of Lactobacillus plantarum NRIC 0383 in 1 L of MRS broth medium and testing its activity against test bacteria (Escherichia coli 2013C-3342, Staphylococcus aureus CIP 9973, Pectobacterium carotovorum Pec 1, Enterobacter cloacae AS10, Klebsiella aerogenes OFM28 and Proteus mirabilis UPMSD3) from selected vegetable (Tomato (Solanum lycopersicum), Eggplant (Solanum melongena) and Pumpkin (Telfairia occidentalis) samples. Maximum OD (600 nm) of 0.6 ± 0.03 mm was observed at the 24th hour of growth of Lactobacillus plantarum NRIC 0383 while maximum antibacterial activity of bacteriocin produced during growth (shown as an inhibition zone) against the test bacteria occurred at the 24th and 30th hour of incubation. The effect of different surfactants (Sodium dodecyl sulfate (SDS), Tween 20, Tween 80, Urea, Triton 114 and Triton 100) on bacteriocin activity was also tested. Maximum activity of bacteriocin against the test bacteria in the presence of SDS ranged from 70.74 ± 3.22 to 91.46 ± 4.59% on the other hand activity of bacteriocin in the presence of tween 20 ranged from 45.82 ± 1.05 to 67.65 ±1.65 % while in the presence of tween 80 it ranged from 34.56 ± 1.77 to 59.17 ± 5.15 %.

Study of growth kinetics is an indispensable tool not only in the applied fields of industrial and environmental biotechnology, but also in fundamental areas such as microbial genetics. Activity of Lactobacillus plantarum NRIC 0383 bacteriocin was not hampered in the presence of surfactants.

Keywords: Growth, Kinetics, Effect, Suractants, Bacteriocin, Production.

INTRODUCTION

Bacteriocins are also generally regarded as safe (GRAS) substances which could be used as food additives or natural preservatives (Perez et al., 2014). In order to develop high performance fermentation process, a better understanding of the influencing factors that affect growth of bacteriocin-producing LAB and their ability to produce bacteriocin is essential. Microbial growth kinetics, i.e., the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s), is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology, or biotechnology, and therefore it is an important part of the basic teaching of microbiology (Mohammed et al., 2013a). Kinetics of growth refers to the rate at which the number of individual cells (or, more general, of active biomass) changes in a defined system (Hwanhlem et al. (2013). Study of growth kinetics is therefore an indispensable tool not only in the applied fields of industrial and environmental biotechnology, but also in fundamental areas such as microbial genetics, physiology, and ecology, including competition, selection, and evolution (Broomberg et al., 2014). Surfactant, also called surface-active agent, substance such as a detergent that, when added to a liquid, reduces its surface tension, thereby increasing its spreading and wetting properties. In the dyeing of textiles, surfactants help the dye penetrate the fabric evenly Parapouli et al. (2013). They are used to disperse aqueous suspensions of insoluble dyes and perfumes. Surfactants are thought to enhance the sensitivity of the indicator strain and form micelles with proteinaceous compounds thus stabilizing the bacteriocins (Pei et al. (2014). Bacteriocin are also used as biopreservatives in some dairy products where surfactants are also used

as emulisfying agents. It is important to study the stability of bacteriocins in the presence of these surfactants. According to Todorov and Dicks (2010) In some cases the addition of surfactants increased the concentration of bacteriocins produced as a consequence of cell growth acceleration. Surfactants may enhance the sensitivity of the indicator strain and form micelles with proteinaceous compounds thus stabilizing the bacteriocins (Corcionivoschi et al., 2010). Factors affecting bacteriocin production may be strain dependent and could vary with different types of bacteriocin hence its important to study their growth pattern as well as their stability in the presence of surfactants.

Methodology

Sample collection

Locust bean (500 g) measured using a weighing scale (EMC LVD C-Tick Rohs, China) was purchased from five different vendors at random locations in Modern Market, Benue state. All samples were kept at a temperature of 4oC in an ice pack before being transported to the Microbiology Laboratory for further analysis. Media used in the study are as listed below and were prepared according to the manufacturers' recommendations and guidelines.

Test bacterial strains

Bacterial species (Staphylococcus aureus CIP 9973, Enterobacter cloacae AS10, and Escherichia coli 2013C-3342) previously identified using 16S rRNA gene sequencing, were isolated from selected vegetable (Tomato (Solanum lycopersicum), Eggplant (Solanum melongena) and Pumpkin (Telfairia occidentalis) samples and were referred to as the indicator strain.

Processing of samples

Processing of locust bean

Locust bean sample (300 g) was weighed using a weighing scale, and crushed into tiny bits using a mortar and pestle pre sterilized using 70% alcohol. The different homogenates were collected in labeled sterile tubes

and stored in sterile containers at 4ºC in the refrigerator (Haier Thermocool, China) for further use.

Isolation of Lactobacillus species from locust beans samples

Processed locust beans sample were serially diluted, by transferring 1 g/1 ml of each sample separately into 9 ml of sterile water to make a stock mixture. Homogenized mixture (1 ml) was then taken into appropriately labelled test tube to make 10-1 of the mixture. The serial dilution was continued until 10-8 was obtained. An aliquot (0.1 ml) of the respective dilutions was spread over MRS (de Man, Rogosa, Sharpe) agar plates. The MRS agar plates were then incubated anaerobically in an anaerobic jar (Microbiology AnaerotestX Merck, Darmstadt, Germany) at 37 $\pm$ 2oC for 48 hours. Growth on MRS plates were then observed after 48 hours.

Identification and Characterization of Lactobacillus Isolates from locust beans

Bacterial isolates were identified macroscopically, microscopically (based on colonial and cellular morphology), and by various biochemical means. Their identities were further confirmed using 16S rDNA gene identification.

Biochemical tests for the identification of isolates from locust beans

Biochemical tests (motility, spore reaction, catalase, nitrate and indole) for the identification of the different isolates were performed according to Bergey's manual of systematic bacteriology (Whitman et al., 2012).

Gram staining

Gram stain was carried out by placing a drop of water on a clean glass slide, part of a single colony taken aseptically from freshly grown culture was mixed with the water to make a smear. It was passed through Bunsen flame intermittently to fix the smear to the glass slide. The smear was flooded with crystal violet for one minute and rinsed under running tap water. It was further flooded with Gram iodine for 30 seconds and rinsed gently with tap water. The smear was then decolourized by adding 70% (v/v) ethanol to the cells and rinsed off with tap water immediately until no

colour effluent was seen. A counter stain, safranin was added to the smear for 1 minute, rinsed with water, drained and air dried. Under a compound light microscope (Bausch and Lomb Dynazoom, SMZ1500) using oil immersion objective lens (x 100) the smear was observed for cell colour and shape. The smear was also observed for the presence of more than one type of cell to check the purity. Gram positive cells showed a purple colouration while the red or pink coloured cells indicated Gram negative cells (Fawole and Oso, 2001).

Sugar fermentation test for all isolates

Sugar fermentation patterns were assessed according to the method of Whitman et al. (2012). Ten percent solution of each sugar to be used (Glucose, arabinose, mannitol, maltose, sucrose, galactose, raffinose and lactose) was added to peptone water using phenol red as the indicator and dispensed into test tubes containing inverted Durham's tubes. These were then autoclaved (Hydrolab, DWK Life Sciences, Duran) at 121 oC for 5 min and inoculated with each isolate. One test tube of each medium was left un-inoculated serving as control. The tubes were then incubated at 37 $\pm$ 2oC for 24 hours; a colour change indicated acid production in all sugars used. Airspace in the Durham's tube showed gas production.

Catalase test

A loop (full) of 24 hours old culture of each isolate was placed on a clean slide, a drop of 3% H2O2 was then added to it, effervescent bubble formation indicated a positive catalase test and the absence of bubble formation indicated the absence of catalase enzyme.

Preparation of cell-free supernatant (CFS) and neutralized cell free supernatant (NCFS)

Lactobacillus isolates from locust beans were incubated in 20 ml of MRS broth at 37 $\pm$ 2oC for 24 hours in an anaerobic jar (Microbiology Anaerotest X Merck, Darmstadt, Germany). Cell-free supernatant (CFS) was then obtained by centrifugation at 10,000 g for 10 minutes (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) to separate bacterial cells from 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 1 ml of 1N NaOH to exclude the antimicrobial effects of organic acids. Inhibitory activity due to hydrogen peroxide (H2O2) was eliminated by the addition of 1 ml of catalase. Samples were then heat to 100oC using a water bath for 10 minutes to inhibit enzyme activity. The CFS and NCFS were stored at 20oC in 3 ml of 0.05 M phosphate buffer until needed for screening the sensitivity of isolated test bacteria by the agar well diffusion assay (Noraphat et al., 2017).

Confirmation of bacteriocin-producing ability of LAB isolates

Vulnerability of the bacteriocin like substances (BLS) produced by Lactobacillus isolates to breakdown by different enzymes (proteinase K, α-chymotrypsin trypsin, catalase and amylase) were confirmed by checking for their inhibitory effects on the test bacteria as described by Noreddine et al. (2007). For this test 50 µl of CFS of Lactobacillus plantarum and L. acidophilus exhibiting antimicrobial potential (after acid neutralization and H2O2 elimination) were treated with 1 g/ml of the different enzymes proteinase k, α-chymotripsin, trypsin, pepsin, catalase and α-amylase. All enzymes used were products of Sigma Aldrich Corporation, USA. The assays were performed at a final concentration of 1 ml at pH 6.5 in phosphate buffer (0.1 M), except for pepsin which was dissolved in 0.02 N HCl (pH 2). Treated samples were incubated at 37 °C in a water bath for 2 hours. After the incubation process, samples were boiled using a water bath at 100 °C for 5 min to stop the reaction. Brain heart infusion soft agar was seeded separately with 1 ml (1.5 x108 CFU/ml) of the test bacteria, mixed and poured into sterile Petri dishes. After setting, agar wells, 8 mm in diameter were punched into the agar using a sterilized well cutter. Enzyme treated NCFS (50 µl) were then added separately in each well and further incubated for 24 hours at 37 $\pm$ 2oC. A control without enzyme treatment was included. Diameters of the transparent zones formed were measured in mm to determine the zones of inhibition. Isolates that produced bacteriocin like substance were further characterized.

Molecular identification of Lactobacillus specie producing bacteriocin

Selected specie of Lactobacillus producing bacteriocin like substance earlier identified by employing standard schemes of morphological, physiological and biochemical characteristics was further identified up to genetic level. Fresh culture of Lactobacillus on Man Ragosa Sharpe (MRS) plate was duplicated in sterile McCartney bottles containing 20 ml of MRS broth and incubated for 12 hours at 37 $\pm$ 2oC. Molecular identification was done using 16S rDNA gene sequencing. Deoxyribonucleic acid (DNA) extraction was carried out as earlier described. Polymerase chain reaction was also carried out using 16S rDNA gene universal primer 16S rDNA gene forward primer (50-AGAGTTTGATCCTGGCTCAG-30), reverse primer: 50 (GTGTGACGGGCGGTGTGTAC-30). Polymerase chain reaction was performed under the following conditions: denaturation: consisting of 30 cycles at 95 °C for 1 min, annealing: 55 °C for 1 min and extension:72 °C for 1.5 min, followed by a final extension step for 5 min at 72 °C. After cycling, the PCR products were detected by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under Ultraviolet (UV) light. Polymerase chain reaction amplified fragments were then sequenced using a Genetic Analyzer 3130 x l sequencer from applied bio systems using manufacturers' manual while the sequencing kit used was that of big dye terminator cycle sequencing kit. Bio- Edit software and MEGA 6 were used for the genetic analysis. The isolate were thus named: Lactobacillus plantarum NRIC 0383.

Determination of bacteriocin genes harbored in Lactobacillus plantarum NRIC 0383

Polymerase Chain Reaction (PCR) assay was used to determine the presence of bacteriocin genes (Plantaricin and Laf operon) harbored in Lactobacillus plantarum NRIC 0383 using the method of Macwana et al. (2012). Polymerase chain reaction primers and annealing temperatures used are shown in Table 3. The PCR reaction was performed in a total volume of 30 µl containing10 p mole/µl of each primer, 2x Taq pre-mix (SolGent 2x Taq PCR Pre Mix, SolGent Co., Ltd.), and 200ng genomic DNA. The reaction mixture was amplified in a GTC thermal cycler (Cleaver Scientific, UK). Initial denaturation was carried out at 95°C for 3 min and

the target DNA was amplified in 35 cycles. Subsequently, each cycle consisted of denaturation at 95°C for 30 sec, followed by annealing at 61°C for 40 sec. Elongation was carried out at 72°C and the extension time at 1 min. The final extension step was performed at 72°C for 5 min and the holding temperature was 10 sec. Amplified PCR products were checked for the expected size on 1% (w/v) agarose gel and visualized after staining with ethidium bromide under ultraviolet light. A DNA molecular weight marker (Gene aid/ Korea) was used to measure the weight of the fragments.

Determination of the growth kinetics of bacteriocin producing L. plantarum NRIC 0383

Growth kinetics of bacteriocin producing Lactobacillus plantarum NRIC 0383 was determined according to the method of Hwanhlem et al. (2013). In this method, one ml inoculum (108CFU/ml) of Lactobacillus plantarum NRIC 0383 was inoculated in 1 L of MRS broth medium and incubated at 37 $\pm$ 2oC for 48 hours. In every 6 hour, 1 ml of sample was taken and the optical density at 600 nm measured, the anti-bacterial activity of produced bacteriocin using the well diffusion assay was also observed with another 1 ml of NCFS (Neutralized Cell free supernatant) (pH 7.0) against the test bacteria (Escherichia coli 2013C-3342, Staphylococcus aureus CIP 9973, Pectobacterium carotovorum Pec 1, Enterobacter cloacae AS10, Klebsiella aerogenes OFM28 and Proteus mirabilis UPMSD3) at intervals of 6 hours for up to 48 hours. Eight mm diameter wells were made on BHI agar that had previously been seeded with 1ml (106 CFU/ml) of the various test bacteria. The plates were then examined for zones of clearance around the individual wells after an incubation period of 37 $\pm$ 2oC for 24 hours. The diameters of the transparent zones were measured in mm to determine the zones of inhibition according to the method of Ogunbanwo et al. (2003). The analysis was carried out in triplicates.

Production and extraction of bacteriocin of Lactobacillus plantarum NRIC 0383

Maximum production of bacteriocin by Lactobacillus plantarum NRIC 0383 was determined according to the method of Palanisamy et al. (2013).

Lactobacillus plantarum NRIC 0383 was propagated in MRS broth (1000 ml) seeded with 1 % (v/v) of overnight culture and incubated at 35°C ± 2 for 48 hours in an incubator (Swiss model NU-5700, UK). Cell-free supernatant was then obtained by centrifuging the culture broth at 12 000 g, for 15 min at 40 oC. After centrifugation the supernatant was collected in fresh sterile tubes and the pellets discarded. The CFS was adjusted to pH 6.5 using 1N NaOH and 5ml catalase (C-100 bovine liver, Sigma) was added to eliminate peroxides and acids effect before filter sterilization using Whatman® membrane nylon filter (0.22 µm) to eliminate any viable cells that could be present. The cell-free supernatant was tagged as bacteriocin crude extract (BCE).

Partial purification of crude extract produced by L. plantarum NRIC 0383

Bacteriocin produced by the candidate bacteria (Lactobacillus plantarum NRIC 0383) was purified using the scheme of Sharma et al. (2012). Ammonium sulfate (80% saturation, Sigma Aldrich) was added to the cell free supernatant of Lactobacillus plantarum NRIC 0383 in fermented MRS broth. The bacteriocin was then precipitated from the supernatant by centrifugation (14,000 rpm for 30 min at 4°C). The precipitate so obtained was pelleted by centrifugation and the pH adjusted to 6.5 with 1 M NaOH (Spectrum Labs, Rancho Dominguez, California). The pellet containing bacteriocin was suspended in 3 ml of 5 mM sodium phosphate buffer (pH 7.0) (Spectrum Labs, Rancho Dominguez, California) and dialyzed in the same buffer for 24 hours at 4 °C using a membrane with a 3.5 KDa cut off (Spectrum Medical Inc., Los Angeles, USA) as per the manufacturer's recommendation to remove salts and low-molecular weight impurities. The retenate was then tested for its antagonistic activity against the test bacteria (Escherichia coli 2013C-3342, Staphylococcus aureus CIP 9973, Pectobacterium carotovorum Pec1, Enterobacter cloacae AS10 Klebsiella aerogenes OFM28 and Proteus mirabilis UPMSD3) using the well diffusion assay to confirm the bioactivity of bacteriocin after purification. The dialyzed material was placed in vials and freeze dried in a freeze dryer (LYOVAC™ Pharma Freeze Dryer, Japan) at -20oC.

Determination of the effect of surfactants on the stability and activity of bacteriocin of Lactobacillus plantarum NRIC 0383

The effect of different surfactants (Sodium dodecyl sulfate (SDS), Tween 20, Tween 80, Urea, Triton 114 and Triton 100) on bacteriocin activity was tested using the method of Perumal et al. (2016). One ml of the various surfactants was added to 1 ml of purified bacteriocin in separate test tubes containing 4.5 ml of nutrient broth. The tubes were then incubated at 37 $\pm$ 2oC for 5 hours. Anti-bacterial activity of the surfactants against the test bacteria was determined by the agar well diffusion method of using the above preparations against the test bacteria. Diameters of the transparent zones if any were measured and recorded in millimeters (mm). In all determinations, untreated bacteriocins was considered as control and its activity taken as 100% (Perumal et al., 2016). Bacteriocin activity (%) was thus calculated as the:

Inhibition zone of treated sample x 100
Inhibition zone of untreated sample (control)

RESULTS

Lactobacillus isolates from locust bean were found to be catalase and oxidase negative and were able to reduce nitrate and ferment different carbohydrates (Glucose, arabinose, mannitol, maltose, sucrose, galactose, Raffinose and Lactose) to varying levels indicating that they are able to grow in a variety of habitats while utilizing different types of carbohydrates. When checked for gas production, all of them gave negative result (Table 1)

Table 1: Biochemical Characteristics of Lactobacillus isolates from locust

Isolates codes	Morphology	Gram reaction	Motility	Spore	Oxidase	Catalase	Indole	H2S	Glucose	Arabinose	Mannitol	Maltose	Sucrose	Galactose	Nitrate	Raffinose	Lactose	Gas	Probable Organism
IS 1	Rod	+	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	Lactobacillus acidophilus

IS 2	Rod	+	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+	Lactobacillus plantarum

Confirmation of bacteriocin producing ability of Neutralized cell free supernatant (NCFS) of L. plantarum and L. acidophilus against the test bacteria when treated with the different proteolytic enzymes (Trypsin, Proteinase K, Pepsin and Chymotrypsin) using the agar well diffusion assay, showed that the effect of trypsin on the activity of NCFS of L. plantarum and L. acidophilus against the test bacteria ranged from 1.20 ± 0.12 mm to 2.35 ± 0.17 mm and 1.64 ± 0.14 mm to 2.14 ± 0.18 mm respectively. A look at the effect of Chymotrypsin and Proteinase K on the antibacterial activity of NCFS of L. plantarum and L. acidophilus showed reduction in activity against the test bacteria ranging from 2.10 ± 0.15 mm to 4.00 ± 0.15 mm and 1.5 ± 0.21mm to 2.2 ± 0.14 mm for chymotrypsin and 1.00 ± 0.14 mm to 2.00 ± 0.15 mm and 1.5 ± 0.21 to 2.10 ± 0.14 mm for Proteinase K. Over all mean activity of NCFS of L. acidophilus and L. plantarum when treated with pepsin showed inhibitory values of 1.85 ± 0.71 mm and 1.85 ± 0.715 mm respectively. Activities of NCFS of both Lactobacillus isolates were retained to a large extent after being treated with α-amylase and catalase which excluded inhibition by hydrogen peroxide. The LAB isolates (L. plantarum and L. acidophilus) were found to be sensitive in the presence of the proteolytic enzymes as shown by the accompanying reduction in growth observed against the test bacteria after treatment with the proteolytic enzymes (Table 2 and 3). Lactobacillus plantarum was adopted for further study as its growth was reduced to a greater extent when compared to that of L. acidophilus. Polymerase chain reaction amplification of bacteriocin genes in the Lactobacillus isolates (L. plantarum NRIC 0383) showed that Plantaricin S and Laf operon genes were present in L. plantarum NRIC 0383 (Figure 1).

Table 2: Confirmation of Bacteriocin Producing ability of Lactobacillus acidophilus (NCFS)

Diameter of inhibition zone (mm) for test bacteria							
Enzymes	E. cloacae	E.coli	K. aerogenes	P. carotov	P. mirabilis	S. aureus	Overall mean
Trypsin	2.00 ±0.72d	1.93 ± 1.30d	2.00 ± 0.75e	1.15 ± 0.14e	2.35 ± 0.17d	1.20 ±0.12f	1.77 ± .74d
Proteinase k	2.20 ± 0.84d	1.40 ± 0.12e	1.70 ± 0.69f	1.10 ± 0.14e	2.00 ± 0.15d	1.30 ±0.15f	1.62 ± 0.56e
Chymotrypsin	3.31 ± 0.75c	2.10 ± 0.15c	2.60 ± 0.84d	2.50 ±0.15c	3.40 ± 0.69c	4.00 ±0.15d	2.98 ± 0.8c
Pepsin	1.30 ± 0.75e	2.10 ± 0.14c	2.50 ±.75d	1.42 ± 0.84d	1.80 ±.72e	2.00 ±0.69e	1.85±0.715d
Amylase	17.50 ± 1.0b	20.00 ± 1.0b	21.00 ± 3.1b	20.00 ± 2.00b	18.50 ±2.46b	22.40 ±0.7c	19.90±2.31b
Catalase	18.40±1.10b	23.00 ± 0.15a	15.00 ±.85c	20.20±2.46b	17.80 ±0.69b	25.00±3.21b	19.90 ± 3.73b
Control	22.70±1.10a	26.40 ±2.42a	28.00 ±0.51a	25.00 ± 3.21a	24.20 ±0.81a	28.38 ±2.28a	25.78 ± 2.67a

Values are means of three replicates ± standard deviation. Means within a vertical column with same superscript are not significantly different according to Tukey HSD post-hoc test at 5% level of significance; Over all mean: Over all mean activity of LAB across test bacteria; LAB = Lactic acid bacteria

Table 3: Confirmation of Bacteriocin Producing ability of Lactobacillus plantarum (NCFS)

Diameter of inhibition zone (mm) for test bacteria							
Enzymes	E. cloacae	E. coli	K.aerogenes	P. carotov	P. mirabilis	S. aureus	Overall mean
Trypsin	1.64 ±0.14d	1.93 ± 0.21c	2.1 ± 0.53c	2.11 ± 0.18c	2.3 ± 0.44c	2.3 ± 0.81d	1.77± 0.74d
Proteinase k	1.8 ± 0.15c	2.3 ± 0.44b	1.94 ± 0.61d	2.44 ± 0.22c	2.1 ± 0.19c	1.73 ± 0.46e	1.62 ± 0.56e
Chymotrypsin	1.84 ± 0.19c	1.5 ± 0.21d	2 ± 0.21c	2.2 ± 0.14c	1.61 ± 0.33d	2.16 ± 0.28d	2.99 ± 0.81c

Pepsin	1.66 ± 0.15d	1.4 ± 0.18d	1.55 ± 0.19e	2 ± 0.15cd	2.3 ± 0.22c	1.5 ± 0.19e	1.85 ± 0.71d
Amylase	19.57± 0.39b	26 ± 0.81a	22 ± 0.44b	14.1 ± 0.51b	18.43±0.76b	20.6 ± 0.72b	19.9 ± 2.31b
Catalase	20.5 ± 0.53b	22.1 ± 0.46a	24 ± 0.81a	22.2 ± 0.34a	19.2 ± 0.72a	18.4 ± 0.43c	19.9 ± 3.73b
Control	28 ± 0.28a	28.03 ± 0.2a	26.2 ± 0.22a	23.98 ± 0.26a	21 ± 0.72a	25.6 ± 0.51a	25.78 ± 2.6a

Over all mean: Over all mean activity of LAB across test bacteria; LAB = Lactic acid bacteria

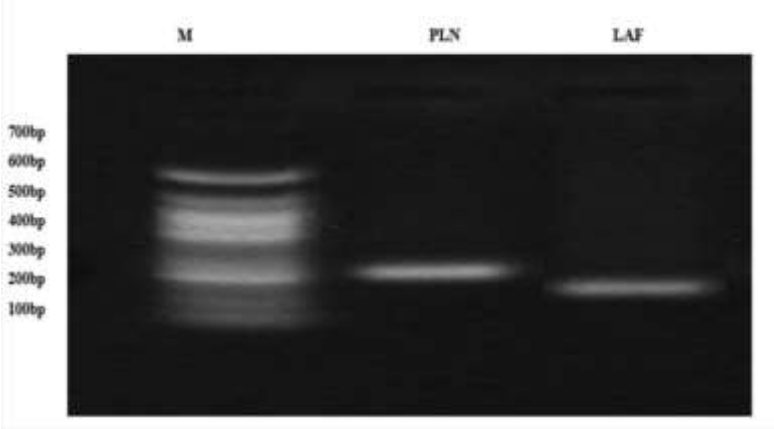

Figure 1: Amplified PCR product of L. plantarum NRIC 0383 with Bacteriocin Primers.

M: 100 bp DNA ladder; Lane 1: Plantaracin gene; Lane 2: Laferon gene

Growth of L. plantarum NRIC 0383 measured at optical density (600 nm), alongside the inhibitory activity of produced bacteriocin against the test bacteria (Escherichia coli 2013C-3342, Staphylococcus aureus CIP 9973, Pectobacterium carotovorum Pec1, Enterobacter cloacae AS10 Klebsiella aerogenes OFM28 and Proteus mirabilis UPMSD3) for 48 hours of incubation time, showed increase in growth of L. plantarum NRIC 0383 between the hours of 6 to 24, followed by a decline after the 24th hour .This trend was maintained up till the 48th hour. Maximum OD (600 nm) of 0.6 ± 0.03 mm was observed at the 24th hour of growth. Inhibitory activity of bacteriocin increased rapidly between the hours of 12 and 18. Maximum antibacterial activity of bacteriocin produced during growth (shown as an inhibition zone) as observed for the test bacteria occurred at the 24th and

30th hour of incubation and it declined slowly after the 36th hour of incubation until the end of the experiment (48 hours) (Figure 2). Antibacterial activity of bacteriocin produced during growth of L. plantarum NRIC 0383 showed higher antagonistic activity against Pectobacterium carotovorum (15.67 ± 0.03 mm and 15.74 ± 0.02 mm) and P. mirabalis (12.00 ± 0.01 mm and 11.90 ± 0.04 mm) between the hours of 24 and 30 respectively as compared to other tested bacteria. Maximum antibacterial activity of bacteriocin against S. aureus was observed between the hours of 30 and 36. Growth of L. plantarum beyond the 30th hour of incubation resulted in decreased bacteriocin production with accompanying reduction in inhibitory activity against the test bacteria (Figure 2).

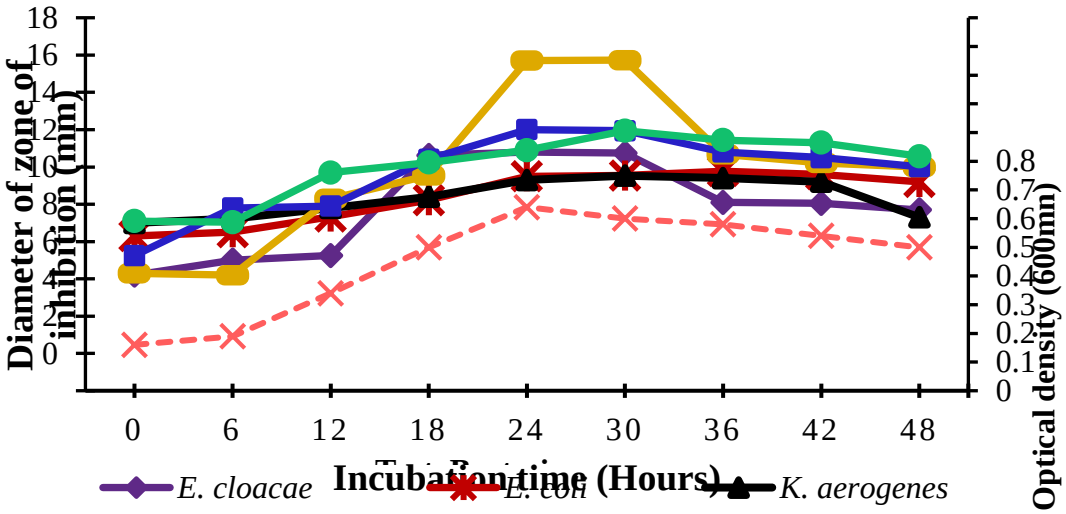

Figure 2: Kinetics of Bacteriocin Production by Lactobacillus plantarum NRIC 0383

E. cloacae = Enterobacter cloacae AS10; E. coli = Escherichia coli 2013C-3342; K. aerogenes =Klebsiella aerogenes OFM28; P. carotovorum =Pectobacterium carotovorum Pec1; P. mirabilis =Proteus mirabilis UPMSD3; S. aureus= Staphylococcus aureus CIP 9973; L. plantarum =Lactobacillus plantarum NRIC 0383

Antimicrobial activity of partially purified bacteriocin using ammonium sulphate and dialysis showed that Lactobacillus plantarum NRIC 0383 bacteriocin retained varying inhibitory activities against the test bacteria. The inhibition zones ranged from 15-20 mm against Staphylococcus aureus CIP 9973, Enterobacter cloacae AS10 and Proteus mirabilis

UPMSD3 while that against Pectobacterium carotovorum subsp. Carotovorum pec 1, Klebsiella aerogenes (OFM28) and Escherichia coli 2013C-3342 were from 10-15 mm (Table 4).

Table 4: Antimicrobial Spectrum of Partially Purified Lactobacillus plantarum NRIC 0383 Bacteriocin

Test bacteria	Antimicrobial activity of Purified bacteriocin
Staphylococcus aureus CIP 9973	+++
Pectobacterium carotovorum subsp. Carotovorum Pec1	++
Enterobacter cloacae AS10	+++
Klebsiella aerogenes OFM28	++
Proteus mirabilis UPMSD3	+++
Escherichia coli 2013C-3342	++
MRS broth	-

. -; No activity; +: Inhibitory zone diameter within 5-10 mm; ++: Inhibitory zone diameter within 10-15mm; +++: Inhibitory zone diameter within 15-20 mm.

Activity of bacteriocin against the test bacteria (Enterobacter cloacae AS10, Escherichia coli 2013C-3342, Klebsiella aerogenes OFM28, Pectobacterium carotovorum Pec1, Proteus mirabilis UPMSD3, and Staphylococcus aureus CIP 9973) was found to increase with the addition of sodium dodecyl sulphate (SDS), tween-20 and tween-80. Maximum activity of bacteriocin against the test bacteria in the presence of SDS ranged from 70.74 $\pm$ 3.22 to 91.46 $\pm$ 4.59% with highest activity against E. coli and K. aerogenes. On the other hand activity of bacteriocin in the presence of tween 20 ranged from 45.82 $\pm$ 1.05 to 67.65 $\pm$1.65 % while in the presence of tween 80 it ranged from 34.56 $\pm$ 1.77 to 59.17 $\pm$ 5.15 %. There was a reduction in activity of bacteriocin in the presence of triton x 100, triton x 114 and urea. Reduction in activity of bacteriocin against the test bacteria in the presence of triton x 100 was recorded at 20.90 $\pm$ 2.65%, 24.48 $\pm$ 1.9 %, 20.12 $\pm$ 1.27%, 21.3 $\pm$ 1.62 %, 25.68 $\pm$ 1.12 % and 29.74 $\pm$ 1.21 % respectively for E. cloacae, E.coli, K. aerogenes, P. carotovorum, P. mirabilis and S. aureus (Table 5). Over 50% of bacteriocin activity against

the test bacteria was lost upon exposure to triton x-114 with activity ranging from 28.94 ± 2.49 to 36.93 ± 3.19 %. Activity of bacteriocin in the presence of urea was 31.35 ± 1.53 to 35.96 ± 1.29 % with minimal activity observed against S. aureus. Statistical analysis indicated that inhibitory activities associated with triton x-100 and urea were not significantly different for Escherichia coli ($p = 0.094$) and Staphylococcus aureus ($p = 0.942$) while that for triton x-100, triton x-114 and urea were not significantly different for Staphylococcus aureus (Table 5).

Table 5: Effect of Surfactants on the Stability and Activity of Lactobacillus plantarum NRIC 0383 Bacteriocin

Bacteriocin activity (%) against test bacteria							
Surfactants	E. cloacae	E. coli	K.aerogenes	P. carotov	P. mirabilis	S. aureus	
SDS	87.37±3.41b	91.7±4.59b	91.46±1.86b	88.62±1.45b	88.18±2.66b	70.74±3.22b	
Triton X-100	20.9±2.65e	24.48±1.9f	20.12±1.27e	21.3±1.62e	25.68±1.12e	29.74±1.21e	
Triton X-114	38.23±3.08d	36.93±3.19e	36.77±3.47d	34.8±4.5d	37.67±5.4d	28.94±2.49e	
Tween 20	58.79±5.38c	67.65±1.65c	58.33±1.1c	59.42±3.4c	60.79±1.46c	45.82±1.05c	
Tween 80	55.03±2.22c	53.63±1.47d	59.17±5.15c	59.85±3.06c	58.91±1.91c	34.56±1.77d	
Urea	34.13±1.88d	32.35±2.68e	34.66±1.86d	33.96±3.4d	35.96±1.29d	31.35±1.53d	
*Control	100±0a	100±0a	100±0a	100±0a	100±0a	100±0a	

Values are means of three replicates ± standard deviation. Means within a vertical column with same superscript are not significantly different according to Tukey HSD post-hoc test at 5% level of significance.
Bacteriocin activity (%) = inhibition zone of treated / inhibition zone of control (untreated) sample *Control was processed at room temperature (28oC) and its activity considered as 100%

DISCUSSION

The presence of lactic acid bacteria (LAB) in locust been used in this study was an evidence of LAB as a normal microflora of fermented foods. It also shows that traditional fermented food products can potentially be good sources of probiotic organisms, producing antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins which are suspected to be associated with the preservation of many fermented food condiments in Nigeria. Bacteriocins produced by different strains of L.

plantarum isolated from food products have also been described by Ogunbawo et al. (2003). Tajabadi et al. (2011); Ravi et al. (2012); Zhao et al. (2016) and Balogun et al. (2017) also isolated LAB from yogurt, cheese, fermented milk and dough. These indicated that fermented foods represent an abundant resource of such potentially useful bacteria. The result showed that the molecule, produced by L. plantarum and L. acidophilus, is peptidic since the antibacterial activity of the molecule was reduced to minimal after digestion with proteolytic enzymes. Van et al. (2013) and Van and Gilmore (2014) opined that the loss of bacteriocin activity upon treatment with pepsin, trypsin or α-chymotrypsin qualifies the antimicrobial substance to be classified as bacteriocin or a bacteriocin like substance. This further confirmed that the antimicrobial substance produced by L. plantarum and L. acidophilus was bacteriocin or a bacteriocin like substance.

Growth metabolism and synthesis of bacteriocin by Lactobacillus plantarum NRIC 0383 depicted by the growth curve in this study showed the relationship of cell density to bacteriocin production by the producer strain thus: the higher the growth, the greater the synthesis of antagonistic compound produced. In this research, bacteriocin production was detected early in the logarithmic growth phase. Optimal bacteriocin production obtained at 24 hours of incubation, marked the beginning of the stationary growth phase of L. plantarum followed by decrease in production during the rest of the stationary phase, thus demonstrating that incubation time has a significant role in bacteriocin production. A decline in inhibitory activity during the late stationary growth phase as observed may be due to proteolytic degradation, adsorption to cells or bacteriocin aggregation. Similar observation was reported by Mohammed et al. (2013a) and Hwanhlem et al. (2013) in bacteriocin production from P. acidilactici strain where it displayed primary metabolite kinetics with the rate of production parallel to the growth rate. A similar trend in bacteriocin production have been reported in previous studies for nisin and other bacteriocins (Cabo et al., 2001; Broomberg et al., 2014). Relationship between bacteriocin production and growth could also have depended upon the strain used (Tajabadi et al., 2011).

Purification of L. plantarum NRIC 0383 bacteriocin, using ammonium sulfate precipitation and dialysis did not seem to affect the inhibitory activity of bacteriocin against the test bacteria thus indicating, that the purification methods applied were effective. Increased biological activity was reported by Wang et al. (2016) after purification of other bacteriocins in the pediocin family. Increase in activity of bacteriocin against the test bacteria (Enterobacter cloacae AS10, Escherichia coli 2013C-3342, Klebsiella aerogenes OFM28, Pectobacterium carotovorum Pec1, Proteus mirabilis UPMSD3, and Staphylococcus aureus CIP 9973) with the addition of sodium dodecyl sulphate (SDS), Tween-20 and Tween-80 could be attributed to the fact that Tween 80 stimulates the secretion of peptides through its influence on membrane fluidity. Tween 80 product is a non-ionic detergent and a water-soluble ester of oleic acid in which growth of microorganisms is enhanced with their presence (Balogun et al. (2017). Oleic acid has been known to be an essential growth factor for several microorganisms while non-ionic detergents containing oleic acid, free oleic acid and cis-vaccenic acid can be used to replace the requirement for biotin by Lactobacilli (Sahar et al., 2017). The presence of Tween 80 in the culture helped to incorporate oleic acid into the cell membrane and oleic acid is then converted into cyclopropane fatty acids. According to Saelim et al. (2012) it is believed that the role of cyclopropane fatty acids is to increase fluidity of LAB membranes as in the case of polyunsaturated fatty acids and to protect LAB from different environmental conditions viz. low pH, deleterious effects of oxygen, and extreme temperatures. Several reports (Sahar et al., 2017; Pei et al., 2014; Balogun et al., 2017) claimed that Tween 80 improved the production of bacteriocin by preventing the aggregation of their molecules. Addition of SDS in culture medium resulted in enhanced bioactivity which could be due to the increased permeability of the cell membrane of indicator organism or due to the breakup of bacteriocin complex into active subunits with enhanced lethal effect. Increase in bacteriocin production in the presence of surfactant Tween 20 can be attributed to its effect on membrane fluidity. In a similar study carried out by Corcionivoschi et al. (2010) they found out that the addition of surfactants such as hexadecyl trimethylammonium bromide, ethylene diaminetetraacetic (EDTA), and sodium dodecyl sulphate (SDS) to crude

bacteriocin from Lactobacillus acidophilus NCIM5426 increased the antibacterial activity against food-borne (L. monocytogenes and S. aureus) and human pathogens (E. coli and S. typhi). Hence, supplementation of surfactant in basal media such as TSB and Nutrient broth (NB) significantly increased the yield of LAB cells and subsequently raised the bacteriocin activity. According to Simova et al. (2009) surfactants have unique chemical structures that can strongly affect the stability of colloid systems, interact with all the main components of flour (starch, gluten and lipids), act as lubricants, emulsify oil or fat in butters, build structure, aerate, improve certain qualities of the final product, extend shelf life, modify crystallization, prevent sticking, and retain moisture. Study of the growth kinetics of beneficial organisms will go a long way in maximizing the benefits accrued from the use of their metabolites.

Conclusion

The study of the growth kinetics of Lactobacillus plantarum NRIC 0383 could be enhance to monitor bacterioicn production whilst also improving the quality and quantity of bacteriocin produced. Increased stability and inhibitory activity of bacteriocin in the presence of the surfactants helps to broaden its use as a bio preservative as its activity was not hindered in the presence of the surfactants.

REFERENCES

Balogun, T. V., John, J. and Abdulsalam, A. (2017). Cultivation, Isolation and Characterization of Bacteriocin from Fresh Cow Milk and Meat Samples obtained from Lapai Market in Niger State Nigeria. Journal of Applied Science and Environmental Management, 1 (3): 413-418.

Broomberg, R., Barnby, S.A. and George, F. M. (2014). Isolation of Bacteriocin-producing Lactic acid bacteria from meat products and its spectrum of inhibitory activity. Brazilian Journal of Microbiology, 35: 21-27.

Cabo, M. L., Murado, M. A., Gonza´lez, M. P. and Pastoriza, L. (2001). Effects of aeration and pH gradient on nisin production: A mathematical model. Enzyme Microbiology Technology, 29 (4):264–273.

Corcionivoschi, N., Drinceanu, D. L., Stef, I., Luca, C., Julean, B. and Mingyart, O. (2010). Probiotics-identification and ways of action. Romanian Food Biotechnology, 6: 1-11.

Fawole, M. O. and Oso, B. A. (2001). Laboratory Manual of Microbiology. Spectrum Books Limited Ibadan, Nigeria. 127 pp.

Hwanhlem, N., Biscola, V., El-Ghaish, S., Jaffr-es, E., Dousset, X. and Haertl, V.S. (2013). Bacteriocin-producing lactic acid bacteria isolated from mangrove forests in southern Thailand as potential bio-control agents: Purification and characterization of bacteriocin produced by Lactococcus lactis subsp. Lactis KT2W2L. Probiotics and Antimicrobial Proteins, 5(4): 264-278.

Ivanova, I., P., Kabadjova, A., Pantev, S. and Danova, T. (2004). Detection, purification and partial characterization of a novel bacteriocin substance produced by Lactococcus lactis subsp. lactis b14 isolated from Boza-Bulgarian traditional cereal beverage. Journal of Biocatalysis, 41(6): 47-53.

Macwana, S. J. and Muriana, P. M. A. (2012). 'Bacteriocin PCR array' for identification of bacteriocin-related structural genes in lactic acid bacteria. Journal of Microbiological Methods, 88:197–204.

Mohammed, S. S., Damisa, D., Oyeleke, S.B. and Jigam, A. A. (2013a). Bio preservation Studies of White Cheese (Wara) using Acidophilin produced by Lactobacillus acidophilus FCF2 isolated from Fermented Corn Flour. Journal of Engineering and Applied Science, 2:1-9.

Noraphat, H., Ivanova, T., Thomasm, H., Emmanuel, J. and Xavier, D. (2017). Inhibition of food-spoilage and foodborne pathogenic bacteria by a nisin Z-producing Lactococcus lactis subsp. lactis KT2W2L. Food Science and Technology, 82:170-175.

Noreddine, B., Yasmine, G. and Hakim, G. (2007). Screening for Bacteriocin-producing Lactic Acid Bacteria from Various Moroccan Food Products and Partial Characterization of Putative Bacteriocins. Journal of Biotechnology, 6: 481-488.

Ogunbanwo, S. T., Sanni, A. I. and Onilude, A. A. (2003), Characterization of bacteriocin produced by Lactobacillus plantarum FI and Lactobacillus brevis OFI. African Journal Biotechnology, 2: 219-227.

Palanisamy, I., Thirumalai, M., Ramasamy, R.., Santhiyagu, P., Chandrasekaran, K., Grasian, I. and Arunachalam, P. (2013). Optimization of bacteriocin production by Lactobacillus sp. MSU3IR against shrimp bacterial pathogens Journal of Aquatic Biosystems, 67:119-121.

Parapouli, M., Delbès-Paus, C., Kakouric, A., Koukkou, A. I., Montel, M. C. and Samelisc, J. (2013). Characterization of a wild, novel nisin A producing Lactococcus strain with an L. lactis subsp. cremoris genotype and an L. lactis subsp. lactis phenotype, isolated from Greek raw milk. Applied and Environmental Microbiology, 79:76–84

Pei, J. J., Yuan, Y. H. and Tue, T. L. (2014). Control of Alicyclobacillus acidoterrestris in fruit juices by a newly discovered bacteriocin. World Journal of Microbiology and Biotechnology, 30: 855–863.

Perez, R. H., Zendo, T. and Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): Various structures and applications. Microbiology Cell Fact, 13:24-27.

Perumal, V., Repally, A., Dasari, A. and Venkatesan, A. (2016). Partial purification and characterization of bacteriocin produced by Enterococcus faecalis DU10 and its probiotic attributes. Preparative Biochemistry and Biotechnology, 34:168–176.

Ravi, S.V., Deepthi Priyanka, P., Srinivas Reddy, P., Rajanikanth, V., Kiran, K. and Indira, M. (2012). Optimization of Bacterioicn production. International Journal of Microbiological Research, 3 (2): 133-137.

Saelim, K., Sohsomboon, N., Kaewsuwan, S. and Maneerat, S. (2012). Probiotic properties of Enterococcus faecium CE5-1 producing a bacteriocin-like substance and its antagonistic effect against antibiotic-resistant enterococci in vitro. Czech Journal of Animal Science, 57(11): 529–539.

Sahar, A.M., Joo, S.T., Tengku, A. T., Ibrahim, F.B., Nagasundara, R.R., Shuhaimi, M. and Arbakariya, B. A. (2017). Fermentation factors influencing the production of bacteriocins by lactic acid bacteria: Royal Society of Chemistry, 5(7): 29395-29420.

Sharma, T. K., Mahak, S., Aradhana, C., Rekha, Y.A., Supriya, D. P., Ravinder, K. M., Ranjana, P. and Naveen, K. N. (2012). Interaction of Bacteriocin-Capped Silver Nanoparticles with Food Pathogens and their Antibacterial Effect. International Journal of Green Nanotechnology, 2:93-110.

Simova, E. D., Beshkova, D. B. and Dimitrov, Z. P. (2009). Characterization and antimicrobial spectrum of bacteriocins produced by lactic acid bacteria isolated from traditional Bulgarian dairy products. Journal of Applied Microbiology, 106:692–701.

Tajabadi, E. M, Ouwehand, A. C, Hejazi, A. M, Jafari, P. (2011). Traditional Iranian dairy products: A source of potential probiotic lactobacilli. African Journal of Microbiology Research, 5: 20-27.

Todorov, S.D. and Dicks, L.M.T. (2010). Characterization of bacteriocins produced by two strains of Lactobacillus plantarum isolated from Beloura and Chouriço, traditional pork products from Portugal. Journal of Meat Science, 84: 334–343.

Van, T.D. and Gilmore, M. S. (2014). Friend turned foe: evolution of enterococcal virulence and antibiotic resistance. Annual Review of Microbiology, 68:337–356.

Van, T.D., Martin, M. J. and Gilmore, M. S. (2013). Structure, function, and biology of the Enterococcus faecalis cytolysin. Toxins, 5(5): 895–911.

Wang, W., Liu, Q.J. and Cui, H. (2016). "Rapid desalting and protein recovery with phenol after ammonium sulfate fractionation". Journal of Electrophoresis, 28 (14): 58–68.

Whitman, W.B., Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M.E., Ludwig, W. and Suzuki, K.I. (2012). Bergey's Manual of Systematic Bacteriology, Second edition, Springer-Verlag press, New York, 49 pp.

Zhao, S, Han, J., Bie, X., Lu, Z., Zhang, C. and Luv, F. (2016). Purification and characterization of plantaricin JLA-9: a novel bacteriocin against Bacillus spp.produced by Lactobacillus plantarum JLA-9 from Suan-Tsai, a traditional Chinese fermented cabbage. Journal of Agriculture and Food Chemistry, 64:2754–2764.