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EFFECT OF DIFFERENT TEMPERATURES ON BIO PRESERVATIVE ACTIVITY OF BACTERIOCIN

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Abstract
The use of bacteriocin in the food industry has to a large extent replaced the use of chemical preservatives in enhancing the shelf-life and safety of foods. Consumer demand for higher quality and naturalness of foods has recently increased. 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. Stability of bacterioicn of *L. plantarum* NRIC 0383 at temperatures of 30, 50, 70,90 and 121^{o}C was determined. Antimicrobial activity of bacterioicn of *L. plantarum* NRIC 0383 against six bacteria isolates (*Staphylococcus aureus CIP* 9973, *Pectobacterium carotovorum* subsp. *carotovorum* Pec 1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Escherichia coli* 2013C-3342 and *Proteus mirabilis* UPMSD3) from selected vegetable samples remained stable at temperatures of 30, 50,70 and 90°C save at 121^{0}C when over 50% of its activity against the test bacteria was lost at exposure time of 15 minutes. This research has therefore established the ability of bacteriocin of *L. plantarum* NRIC 0383 at inhibiting food-borne pathogens while remaining stable and active at high temperatures thereby providing a safer and reliable alternative to the use of harmful chemical food preservation.

Key words: Food, Lactic acid Bacteria, Microbial, Preservation

Introduction
Bacteriocins are ribosomally synthesized, extracellular released low- molecular-mass peptides or proteins (usually 30–60 amino acids) which have a bactericidal or bacteriostatic effect on other bacteria (Bromberg *et al*., 2014), either in the same species (narrow spectrum) or across genera (broad spectrum) (Campion *et al*., 2013). Production of bacteriocins on an industrial scale usually involves enrichment of LAB growing media by different food grade by-products, for example whey, grape waste and

soybean residue (Perez *et al.*, 2014). The review of Garsa *et al.* (2014) emphasized the main points, such as bacteriocin media composition, fermentation conditions, recovery and purification, for improving bacteriocin production at small and large scale. Production conditions are unique to each strain (Ahire and Dicks, 2015). Bacteriocin production occurs throughout logarithmic growth of the producer organism, it proceeds to the stationary phase and then decreases immediately after the stationary phase (Ogunbanwo *et al*., 2003). The production of bacteriocins is dependent on the growth and physiological activity of the producing strains, culture conditions such as changes in pH, change in temperature and elimination or addition of nutrients to growth media may influence both the production and the amount of bacteriocin produced (Ahire *et al*., 2015).

Antimicrobial effects of bacteriocins against sensitive microorganisms depends on environmental factors like pH, temperature, composition and constitution of food (Mahdi *et al.,* 2011). Whereas bacteriocins are mostly synthesized by Gram (+) bacteria, they are also produced by Gram (-) bacteria (El-Batal *et al*., 2015). 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 (Hwanhlem *et al*., 2013). 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 (Kamrun *et al*., 2016). 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 (Jang and Gun, 2016). 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 (Agharkar *et al.*, 2014).

Methodology

Bacterial strains

The Lactic acid Bacteria (LAB), which is *Lactobacillu plantarum.* NRIC 0383, previously isolated from traditionally prepared locust beans and identified molecularly was used throughout this study. Bacterial isolates previously isolated from vegetables (tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), cabbage (*Brassica oleracea*), eggplant (*Solanum aethiopicum*), green beans (*Phaseolus vulgaris*), fluted pumpkin leaves (*Telfairia occidentalis*) were obtained from the Microbiology department of the Federal University of Technology Makurdi and identified as *Staphylococcus aureus CIP* 9973, *Pectobacterium carotovorum* subsp. *carotovorum* Pec 1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Escherichia coli* 2013C-3342 and *Proteus mirabilis* UPMSD3 were used as indicator microorganisms for the quantification of bacteriocin-like inhibitory substance (BLIS) produced by *Lactobacillus plantarum.* NRIC 0383. All strains were maintained in glycerol (20% v/v) at -80°C as stock culture. The cultures

were purified by streaking method on agar medium. Prior to use, the bacteria were subcultured twice in an appropriate media at 37°C for 24 hours.

Inoculum preparation and standardization

Inoculum was prepared by aseptically inoculating a colony picked from each of the culture streaked plates into sterile 9 ml De Mann Rogosa Sharpe Agar (MRS) and NB broth in sterile capped bottles using sterile inoculating loop. The broth was then incubated with shaking at 10,000 rpm using an orbital shaker (IKA™ 10316411) at 37°C until the visible turbidity was equal to or greater than that of 0.5 McFarland standard (NCCLS, 1999).

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 °C. 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) and used for bacteriocin antibacterial assays.

Determination of the effect of different growth conditions on the stability and activity of purified bacteriocin

Purified bacteriocin of *Lactobacillus plantarum* NRIC 0383 was tested for its stability and 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) at different temperatures.

Determination of the effect of temperature on the stability and activity of bacteriocin

Effect of temperature on the stability and activity of *Lactobacillus plantarum* NRIC 0383 bacteriocin was determined by the method of Ravi *et al*. (2012). Purified bacteriocin (0.5 ml) was added to 4.5 ml of nutrient broth in test tubes. Each of the test tube was then overlaid with paraffin oil to prevent evaporation and the water bath was set according to the required temperatures (30, 50, 70, and 90 °C) using a thermometer at a time range of 15 to 60 minutes respectively. Temperature of 28 ± 2°C was taken as the control. The preparations containing nutrient broth (4.5 ml) and bacteriocin (0.5 ml) in test tubes were plugged with non- absorbent cotton and covered with aluminum foil and

autoclaved at 121 °C for 15 minutes to check its activity at very high temperature. The heat treated bacteriocin was cooled to room temperature and the activity of the residual bacteriocin was then assayed 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 agar well diffusion method as earlier described by Ogunbanwo *et al*. (2003).

Determination of the Antibacterial activity of Purified Bacteriocin on the Test Bacteria

Antibacterial activity of bacteriocin of *L. plantarum* NRIC 0383 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) was determined using the well diffusion assay as described by Ivanova *et al*. (2004). A 100 µl suspension of each test bacteria containing 1.5×10^8 CFU/ml were first grown on Muller-Hinton agar (Hi Media, India) at 37 °C for 8 hours. Aliquots of purified bacteriocin (pH 6.5) at 50, 75 and 100 µl were then added in 8 mm wells on the different agar plates. The plates were incubated in an incubator (Swiss *model* NU-5700, UK) for 24 hours at 37 ± 2°C. After incubation, the plates were examined for zone of clearance around the individual wells. The diameter of the zones of clearance, was calculated as a measure of bacteriocin activity against the test bacteria (Bhaskar *et al*., 2007). Cell free supernatant treated with 1 ml of catalase (Sigma-Aldrich Corporation, USA) to eliminate possible inhibitory action of H_2O_2 and adjusted to pH 7.0 with 1 m of NaOH to rule out possible inhibition effects of organic acids was used as control. The test was carried out in triplicates.

Statistical Analysis

A one-way analysis of variance (ANOVA) with Tukey's test was used to test the significance differences among treatment means.

Results

Results from this study showed that bacteriocin retained more than 80% of its stability at temperatures of 30 to 70°C against *E. cloacae* AS10. Maximum activity of 99 ± 0.87 % to 99. 84 ± 0.16% against *E. cloacae* AS10 was observed when bacteriocin was held at temperature of 30°C at 15 to 60 minutes exposure time. The activity peak was 99.84 ± 0.16% at 30°C but at 50°C and 70°C ranged from 90. 19 ± 0.32% to 91.65 ± 0.16% and 81.18 ± 0.16% to 88.08 ± 5.78% respectively. Results from ANOVA and TukeyHSD tests showed that the effect of temperature levels on bacteriocin production varied significantly ($p<0.001$) and affected bacteriocin activity against *E. cloacae* AS10 (Table 1).

At 30 to 90°C *L. plantarum* NRIC 0383 bacteriocin remained stable and recorded more than 50% activity against *Escherichia coli* 2013C-3342 at exposure time of 15 to 45

minutes. A decline in activity was observed at 60 minutes exposure time and temperature of 90°C. Variations in activity against *E. coli* 2013C-3342 due to temperature was statistically significant ($P < 0.001$). However, its activity at 30°C was not significantly different from that of the control at 60 minutes incubation period (Table 2).

When held at 90°C and exposure time of 15 to 60 minutes a decline in activity of bacteriocin against *K. aerogenes* OFM28 and *P. carotovorum* Pec1 was observed. Minimum bacteriocin activity of 37.95 ± 0.34%, 36.73 ± 0.17%, 36.25 ± 0.18% and 36.23 ± 0.17 % were recorded against *K. aerogenes* OFM28 (Table 3), and 27.22 ± 0.17%, 29.69 ± 0. 17%, 29.44 ± 0.27%, 28.42 ± 0.37%, 28.69 ±1.04% against *P. carotovorum* Pec1 at 90°C (Table 4).

Table 1: Stability of *Lactobacillus plantarum* NRIC 0383 Bacteriocin Activity against *Enterobacter cloacae* AS10 at Different Temperatures

Bacteriocin activity (%) in minutes				
Temperature (°C)	15 min	30 min	45 min	60 min
30	99.84 ± 0.32^{a}	99.68 ± 0.16^{a}	99.52 ± 0.24^{a}	99 ± 0.87^{b}
50	91.65 ± 0.24^{b}	91.56 ± 0.16^{b}	90.95 ± 0.32^{b}	90.19 ± 0.24^{c}
70	88.33 ± 0.24^{c}	82.08 ± 0.16^{c}	81.33 ± 0.16^{c}	81.18 ± 5.78^{d}
90	49.84 ± 0.32^{d}	49.37 ± 0.24^{d}	48.42 ± 0.24^{d}	48.08 ± 0.24^{e}
121	27.05 ± 0.32^{e}			
***Control**	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

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 (28± 2°C) and its activity considered as 100%

Table 2: Stability of *Lactobacillus plantarum* NRIC 0383 Bacteriocin Activity against *Escherichia coli* 2013C-3342 at Different Temperatures

Bacteriocin activity (%) in minutes				
Temperature (°C)	15 min	30 min	45 min	60 min
30	99.20 ± 0.33^{a}	98.88 ± 0.25^{b}	99.19 ± 0.25^{a}	99.51 ± 0.16^{a}
50	90.84 ± 0.25^{b}	90.68 ± 0.24^{c}	90.91 ± 0.16^{b}	91.34 ± 0.25^{b}
70	60.77 ± 0.16^{c}	59.52 ± 0.24^{d}	59.42 ± 0.33^{c}	58.09 ± 0.17^{c}
90	51.05 ± 0.33^{d}	50.28 ± 0.16^{e}	50.41 ± 0.25^{d}	50 ± 0.25^{d}

121	28.78 ± 0.16^{e}			
***Control**	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

Table 3: **Stability of *Lactobacillus plantarum* NRIC 0383 Bacteriocin Activity against *Klebsiella aerogenes* OFM28 at Different Temperatures**

Bacteriocin activity (%) in minutes				
Temperature (°C)	15 min	30 min	45 min	60 min
30	98.98 ± 0.17^{a}	99.66 ± 0.26^{a}	99.54 ± 0.26^{b}	99.44 ± 0.17^{b}
50	92.70 ± 0.17^{b}	90.37 ± 0.17^{b}	90.31 ± 0.35^{c}	88.71 ± 0.18^{c}
70	65.20 ± 0.26^{c}	64.38 ± 0.26^{c}	61.42 ± 0.26^{d}	60.65 ± 0.39^{d}
90	37.95 ± 0.34^{d}	36.73 ± 0.17^{d}	36.25 ± 0.18^{e}	36.23 ± 0.17^{e}
121	22.41 ± 0.26^{e}			
***Control**	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

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 (28 ± 2°C) and its activity considered as 100%

Table 4: Stability of *Lactobacillus plantarum* NRIC 0383 Bacteriocin Activity against *Pectobacterium carotovorum* subsp. *carotovorum* Pec1 at Different Temperatures

Bacteriocin activity (%) in minutes				
Temperature (°C)	15 min	30 min	45 min	60 min
30	99.41 ± 0.17^{b}	99.23 ± 0.26^{b}	99.04 ± 0.18^{b}	99.02 ± 0.26^{b}
50	92.22 ± 0.26^{c}	91.47 ± 0.34^{c}	94.74 ± 0.18^{c}	92.22 ± 0.18^{c}
70	58.24 ± 0.17^{d}	57.34 ± 0.26^{d}	57.54 ± 0.27^{d}	55.59 ± 0.27^{d}
90	27.22 ± 0.17^{e}	29.69 ± 0.17^{e}	29.44 ± 0.27^{e}	28.42 ± 0.37^{e}
121	24.26 ± 0.34^{f}			
***Control**	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

Bacteriocin activity (%) = inhibition zone of treated / inhibition zone of control (untreated) sample
*****Control was processed at room temperature (28°C) and its activity considered as 100%
Increase in exposure time of 45 and 60 minutes at 90°C decreased the activity of the bacteriocin against *P. mirabalis* UPMSD3 but at temperatures of 30°C to70°C and

exposure time of 15 to 60 minutes it remained stable with maximum activity of 99.16 ± 0.19% to 99.72 ± 0.28% and 93.85 ± 0.28% to 95.45 ± 0.28% observed at temperatures of 30°C and 50°C respectively. Statistical analysis further indicated that variation in bacteriocin activity against *P. mirabalis* UPMSD3 due to temperature was statistically significant (Table 5). Nevertheless, bacteriocin activity at 30°C was not significantly different from that of the control at incubation period of 15 minutes ($p = 0.583$).

More than 50% of the activity (73.45%, 67.59% and 56.91%) of *L. plantarum* bacteriocin against *S. aureus* CIP 9973 was retained at 90°C and exposure time of 15, 30 and 45 minutes respectively. Results from statistical analysis indicated that bacteriocin activity against *S. aureus* CIP 9973 differed significantly ($p < 0.001$) with different temperatures. However, bacteriocin activity at temperatures of 30°C and 50°C were not significantly different at incubation periods of 15 ($p = 0.998$), 45 ($p = 0.398$) and 60 minutes ($p \approx 1$) minutes (Table 6). Over 50% of bacteriocin activity against the test bacteria was lost at 121°C at exposure time of 15 minutes with values of 27.05 ± 0.32%, 28.78 ± 0.16%, 24.26 ± 0.34%, 22.41 ± 0.26%, 39.09 ± 1% and 31.10 ± 0.18 for *E. cloacae* AS10, *E. coli* 2013C-3342, *P. carotovorum* subsp. *carotovorum* Pec1, *K. aerogenes* OFM28, *S. aureus* CIP 9973 and *P. mirabilis* UPMSD3 respectively. Generally, it was observed that *L. plantarum* NRIC 0383 bacteriocin was more stable at temperatures of 30 and 50°C. In general the Tukey HSD tests indicated that at each period of time considered, bacteriocin activities varied significantly from each other due to temperature differences.

Table 5: Stability of *Lactobacillus plantarum* NRIC 0383 Bacteriocin Activity against *Proteus mirabilis* UPMSD3 at Different Temperatures

Bacteriocin activity (%) in minutes				
Temperature (°C)	15 min	30 min	45 min	60 min
30	99.72 ± 0.28^{a}	98.9 ± 0.28^{b}	98.79 ± 0.37^{a}	99.16 ± 0.19^{a}
50	94.59 ± 0.19^{b}	93.85 ± 0.28^{c}	95.45 ± 0.28^{b}	95.32 ± 0.38^{b}
70	73.39 ± 0.19^{c}	67.49 ± 0.37^{d}	67.41 ± 0.28^{c}	65.54 ± 0.19^{c}
90	45.87 ± 0.28^{d}	40.04 ± 0.28^{e}	37.14 ± 0.37^{d}	37.08 ± 0.38^{d}
121	31.10 ± 0.18^{e}			
***Control**	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

Table 6: Stability of *Lactobacillus plantarum* NRIC 0383 Bacteriocin Activity against *Staphylococcus aureus* CIP 9973 at Different Temperatures

Bacteriocin activity (%) in minutes				
Temperature (°C)	15 min	30 min	45 min	60 min
30	97.45 ± 2.82^{b}	99.07 ± 0.93^{a}	98.41 ± 0.19^{b}	97.23 ± 0.48^{b}
50	96.91 ± 1.19^{b}	97.5 ± 0.37^{b}	97.23 ± 1.05^{b}	96.95 ± 1.42^{b}
	78.18 ± 0.37^{c}	72.41 ± 0.19^{c}	69.16 ± 1.22^{c}	69.05 ± 2.38^{c}

70				
90	75.45 ± 0.2^{c}	67.96 ± 0.37^{d}	58.04 ± 1.13^{d}	47.62 ± 0.38^{d}
121	39.09 ± 1^{f}			
***Control**	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

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 (28^{o}C) and its activity considered as 100%

Discussion

When considering a new bacteriocin, one of the vital criteria to be evaluated is the ability to withstand thermal treatment. This characteristic determines its usefulness in food system especially in food manufacturing processes (Campion *et al*., 2013). If the bacteriocin is heat labile, it can have adverse effects on the bioactive capability of a bacteriocin, potentially making it less effective (Balogun *et al*., 2107). The activity of bacteriocin produced from *Lactobacillus plantarum* NRIC 0383 in this study was fairly stable when exposed at 121°C for 15 minutes as evident in the inhibitory activities 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. These characteristics are similar to bacteriocin-like peptides produced from *B*. licheniformis ZJU12 that was fairly stable at 121°C for 15 minutes (Dhanasekaran *et al*., 2010). With respect to temperature, the examined bacteriocin exhibited strong heat stability, which meant that it could be placed within the heat stable low molecular weight group of bacteriocins. According to Kamrun *et al*. (2016) an important factor in selecting a suitable bacteriocin for use as a preservative is its stable nature. Although a partial loss in the activity was observed with continuous increase in temperature, thermo stability of bacteriocin at high temperature makes it possible to sterilize the food products even at room temperature, thus avoiding their storage at low temperature. Similar results were reported for bacteriocin produced by *Lactobacillus* CA44 and also thuricin 7 from *B. thuringiensis* BMG 1.7 (Yang *et al*., 2012). The phenomenon of heat stability of bacteriocin for different LAB at temperatures of 121^{o}C for 15 min has been reported earlier in Lactocin RN 78 by Tabanelli *et al*. (2014). Ogunbawo *et al*. (2003) showed that the activity of bacteriocin of *L. acidophilus* DSM20079 and *L. acidophilus* OGI after heat treatment up to 80^{o}C for 30 min was stable and its activity measured in arbitrary unit was 6400 AU/ml, but declined after heating at temperature of 100^{o}C for 30 min (3200 AU/ml). However, this differed from the report of Zhao *et al*. (2016) who noted that bacteriocin C8 obtained from *Lactobacillus plantarum* isolated from Chinese pickle was heat-stable and retained 94.6% of its activity after 20 min at 121°C, towing the same trend as was observed by Hernandez *et al*. (2005)

who reported the stability of bacterocin at high temperatures of 121°C for 30 minutes. On the other hand, some plantaricins were heat sensitive, e.g. bacteriocin produced by *L. plantarum* ATCC 8014 (Bradley *et al*. 2005) and Plantaricin UG1 (Enan *et al*. 1996). Temperature stability is important if bacteriocins are to be used as a food preservative, because many procedures of food preparation involve different heating temperatures.

Conclusion

Bacterioicn of *Lactobacillus plantarum* NRIC 0383 can be considered as heat stable after exposure to high temperatures. The results also shows that bacterioicn of *Lactobacillus plantarum* NRIC 0383 can be applied as a bio preservative in food samples that require high thermal processing as its inhibitory activity against the indicator strains at the different temperatures tested was also maintained.

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