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Partial Characterization of Bacteriocins Produced by *Bacillus* Species Isolated from Common Food Products in South West Nigeria

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Abstract: Forty strains out of 246 of *Bacillus spp* isolates from common food products were found to produce proteinaceous substances which showed antimicrobial activity against strains of *Salmonella enteritidis* (3), *Micrococcus luteus* (1) and *Staphylococcus aureus* (2). The zones of inhibition ranged between 5.90±0.000 -27.00±0.000 mm. Three bacteriocin producers with the highest (12-23 mm) zones of inhibition were studied in more details. They were found to exhibit optimal antimicrobial activity at pH range 4.5-7.0. The antimicrobial property was totally lost after heat treatment at 100°C for fifteen mins. The isolation of this bacteriocin producing strains from a variety of food shows the ability of these strains to grow and produce antimicrobial compounds in various environmental conditions. These bacteriocins present potential for use in bio-preservation of foods to replace the synthetic chemical preservation.

Key words: Bacteriocins, *Bacillus* spp, traditional food products, antimicrobial activity

INTRODUCTION

In an attempt to control pathogenic bacteria in food, the production of antimicrobial peptides from bacteria "bacteriocins" has received much consideration. Bacteriocins are compounds produced by bacteria that have a biologically active protein moiety and bacteriocidal action (Line *et al*., 2008). Antimicrobial peptides from Gram positive organisms such as Lactic acid producing bacteria have attracted much attention and have been the subject of intensive investigation due to their extensive incorporation as bio-preservative ingredient into model foods, particularly in the diary industry (Diop *et al*., 2007) and also in human therapeutics (Martin-Visscher *et al*., 2008).

Consequently only limited data exist on bacteriocins from *Bacillus spp*, *Bacillus spp* therefore presents an interesting genus to investigate since it produces diverse array of antimicrobial peptides representing several different basic chemical structures (Bizani and Brandelli, 2002). The production of bacteriocins or bacteriocin-like substances has already been described for some bacilli such as *B. substilis*, *B. cereus*, *B. stearothemophilus* and other *Bacillus* species (Zheng *et al*., 1999; Cherif *et al*., 2001; Stein *et al*., 2002). Some strains produce bacteriocin with broad spectrum activity including important pathogens such as *Listeria monocytogenes* and *Streptococcus pyogenes* (Cherif *et al*., 2001), some have been well characterized such as; lichenin and megacin produced by *B. megaterium*, also bacteriocin have been isolated from *B. amyloloquefaciens* (Lisboa *et al*., 2006).

However, inspite of these approaches taken by researchers to gather data on Bacillus bacteriocins, their importance and the industrial value have been largely underestimated and thus, have attracted minimal attention. This study reveals the production of bacteriocin by some *Bacillus* species isolated from some traditional food products in South-western Nigeria.

MATERIALS AND METHODS

Bacterial strains: Six laboratory stock isolates: *Salmonella enteritidis* (3), *Micrococcus luteus* (1) and *Staphylococcus aureus* (2) implicated in food spoilage were used to test antimicrobial activity of the *Bacillus* spp.

Sampling of food sources: Twenty-five food samples namely: 'Wara' (cheese) (10), 'Fura' (fermented millet) (5), 'Elubo' (yam flour) (5), 'Kulikuli' (groundnut cake) (5) were sampled randomly from 5 markets in Ibadan city Nigeria. Samples were collected into sterile bijou bottles and kept on ice pack on transit to the laboratory to maintain the microbial population.

Preparation of samples: 5 grams of each sample collected was weighed aseptically and homogenized in 10 mls of sterile (0.1%) peptone water for all samples except 'elubo' which was homogenized in 20 mls of sterile (0.1%) peptone water. All samples were serially diluted to 10-fold.

Preparation of the media: A partial selective media was prepared for the isolation of the *Bacillus* species.

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Nutrient agar was measured out and weighed using an automatic weighing balance. 5.6 grams of the nutrient agar was measured into 200 mls of distilled water. Maltose sugar was incorporated at 1% concentration. The mixture was brought to boil using a water bath before autoclaving at 121°C for 15 min. At about 39-40°C 125 mg of neomycin was added to the viscous media and mixed. This was then dispensed into sterile petri dishes for isolation of *Bacillus* species.

Enumeration of *Bacillus* spp: 0.1 mls of 10- fold diluted sample was inoculated on the semi-selective nutrient media prepared above and incubated at 37°C for 24-48 h. Bacterial identification was done on pure isolates by cultural, morphological, physiological and biochemical characteristics according to Barrow and Feltham (1993).

Detection of antimicrobial activity in *Bacillus* specie: Isolates were examined for inhibitory activity to strains of *Salmonella enteritidis* (3), *Micrococcus luteus* (1) and *Staphylococcus aureus* (2) using the surface diffusion method and Agar Well Diffusion (AWD) assay (Lasta *et al*., 2008).

Screening for bacteriocin-producing *Bacillus* specie by Agar Well Diffusion (AWD) assay: Eight strains showing zones of inhibitions of 6-27 mm diameter were tested further for bacteriocin production. Overnight broth culture (10 ml) was centrifuged at 3500 g for 15-20 min and neutralized with 1 mol/L NaOH to a final pH of 7.0. Antimicrobial activity was then tested with five organisms (*Micrococcus luteus*, *Salmonella enteritidis*, *Staphylococcus aureus* and 2 yeast isolates from yoghurt using the Agar Well Diffusion method (AWD). Nutrient agar (35-39°C) was seeded with indicator organism and poured into sterile petri dishes. Wells of 5 mm diameter were cut into the agar and filled with 50 µL of crude bacteriocin prepared from *Bacillus* species; Plates were pre-incubated at 4°C for few minutes to allow diffusion of any inhibitory metabolites into the surrounding agar and then incubated at 37°C for 24 h. The plates were afterwards examined for clear zones in the agar surrounding the wells. The experiment was done in two replicates.

Sensitivity of bacteriocin to heat treatment: Three of these bacteriocin producing strains (Ok2a, Oe2a and In5a) were studied in more details. The neutralized sterile bacteriocins of these strains as prepared for the AWD assay were subjected to heat treatment at 45, 60, 75, 90 and 100°C for 15 min and then cooled. Their antimicrobial activity against the indicator microorganisms were then tested for, using the AWD assay (Naclerio *et al*., 1993; Mota-Meira *et al*., 2005).

Activity of bacteriocin at different pH values: Ten mls supernatants of test strains (*Bacillus* spp: Ok2a, Oe2a and In5a) prepared as described above were adjusted to pH 4.5, 5.5, 6.5, 7.0 and 7.5 (Ogunbawo and Sanni, 2003a,b) with 1 mol/L NaOH and concentrated acid (glacial acetic acid). Nutrient broth adjusted to the same pH values served as control. After 5 h incubation at 30-37°C, the supernatants were neutralized to pH 7.0 and the activity of the bacteriocin in the supernatant was detected using AWD assay (Bogovic-Matijasic *et al*., 1998). The experiment was done in two replicates.

RESULTS

Detection of antimicrobial activity: Forty isolates showed antimicrobial activity against the six indicator microorganisms using surface diffusion method. The average diameter of the inhibition zones ranged from 5.9-27 mm in size (data not shown). Among the isolates, three isolates Ok2a, Oe2a and In5a isolated from kulikuli, Elubo and Nono respectively were subjected to other treatments.

Screening and identification of bacteriocinogenic strains: Results of the AWD method showed that eight out of the 40 *Bacillus spp* isolates produced antimicrobial metabolites with wide spectrum of activity that inhibited the growth of representatives of at least three indicator organisms and two yeast strains (Table 1). Inhibition zones greater than 5.9 mm were observed (Fig. 1).

Table 1: Inhibition zones of bacteriocin producing *Bacillus spp* against foodborne pathogens in mm diameter

Bacillus strains	Indicator organism				
	Micrococcus luteus	*Salmonela enteritidis*	*Staphylococcus aureus*	Yeast 1	Yeast 2
Bf4b	6.00±0.000	8.95±4.313	5.90±0.000	6.00±0.000	5.90±0.000
Bk4b	6.00±0.000	5.90±0.000	5.90±0.000	6.00±0.000	5.90±0.000
In5a	6.00±0.000	20.00±0.000	5.90±0.000	24.00±0.000	20.00±0.000
In5c	6.00±0.000	5.90±0.000	5.90±0.000	5.90±0.000	5.90±0.000
Mk1a	6.00±0.000	20.00±0.000	5.90±0.000	5.90±0.000	5.90±0.000
Oe2a	6.00±0.000	25.00±0.000	5.90±0.000	20.00±0.000	5.90±0.000
Ok2a	6.00±0.000	17.00±0.000	5.90±0.000	27.00±0.000	5.90±0.000
Sw3b1	6.00±0.000	5.90±0.000	5.90±0.000	5.90±0.000	6.00±0.000

Values represents:- mean ± standard error of mean in mm

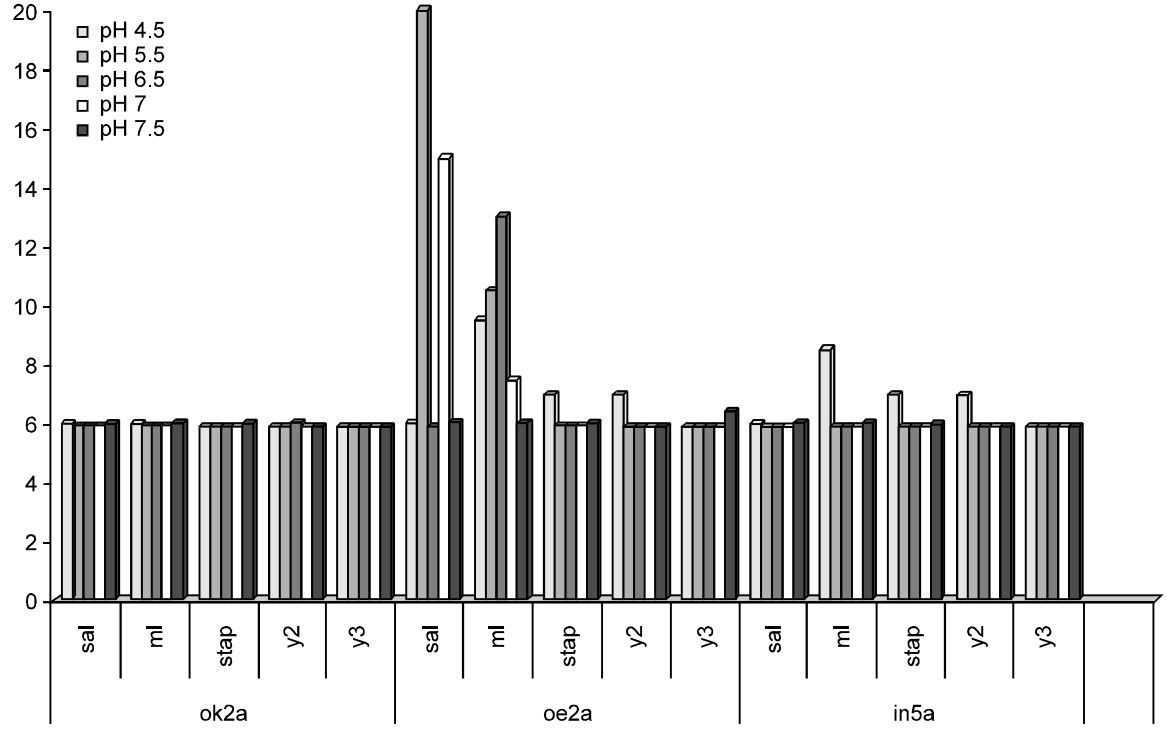

Fig. 1: Effect of pH variations on the antimicrobial activity of bacteriocin

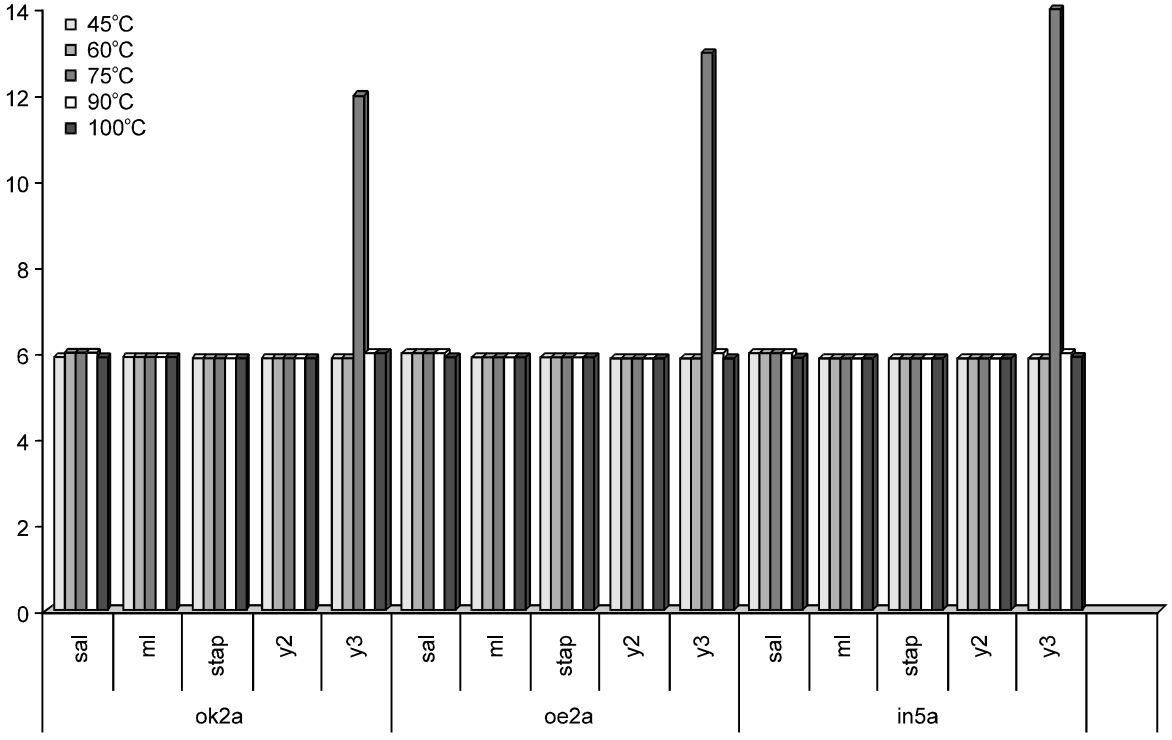

Fig. 2: Effect of heat treatment on the antimicrobial activity of bacteriocins

The antimicrobials in the supernatants of the three tested bacilli strains showed zones of inhibition ranging from (5.90±0.000 - 9.50±0.000)mm at low acidic pH of 4.5, sensitivity decreases as the pH increases for

bacteriocins of strains Ok2a and In5a, except for strain Oe2a; such that at pH = 7.5 zones of inhibition observed were not prominent (5.90±0.000). Hence stability was observed at pH range 4.5-7.0, with total loss of antimicrobial activity of bacteriocins produced by Ok2a and In5a at pH 7.5 after 5 h of incubation at 7°C.

The bacteriocins were not heat stable, after heating for 15 min at 45°C the activity of the tested bacteriocins on the microbial floral of treated soft cheese was not significantly different from the untreated cheese. The bacteriocins were still potent after 15 min heat treatment at 75 and 90°C. A complete loss of antimicrobial activity was obtained after 15 min heat treatment at 100°C.

DISCUSSION

There is a paucity of information on bacteriocins produced by *Bacillus* species. Bacteriocin producing *Bacillus spp* were identified in traditional foods and characterized in this study. The isolation of *Bacillus spp* from both dairy and non dairy traditional foods in this study is consistent with previous reports associating the pathogen with milk (Karmen and Bojana, 2003) and agro-industrial wastes (Rowaida *et al*., 2009). Several food samples (dried condiments, cereals, spices, meat, eggs, milk and milk products, cooked and inappropriately kept food) have also been reported to have been contaminated with high levels of *B. cereus*. (Kramer and Gilbert, 1989; Becker *et al*., 1994).

The antimicrobial activities of the *Bacillus* strains in this study were detected against six bacteria indicator organisms (*Micrococcus luteus* (3), *Salmonella spp* (2), *Staphylococcus spp* (1)) and 2 yeast strains. Similar report was made by Karmen and Bojana (2003) where *B. cereus* strains isolated inhibited the growth of *E. coli* ATCC 11229, *S. aureus* ATCC 25923, *S. aureus SA* etc. The indicator organisms used are common food spoilage organisms of public health importance, therefore, the antimicrobial activity of the *B*acillus strain will be of importance in preserving food from spoilage.

Bacteriocins produced by the three strains (Ok2a, Oe2a and In5a) chosen for detailed study revealed antimicrobial spectrum that inhibited the growth of representative of at least three of the indicator organism used (*Salmonella spp* and two yeast strains used), with inhibition zones ranging from 5.9-30 mm. This result can be compared to that obtained by Rowaida *et al*. (2009) where the Bacillus strain isolated showed a zone of inhibition which ranged from 0.5-5 mm diameter in size, with bacteriogenic activity against *S. typhinurium* and *S. aureus*.

As regards the effect of pH, the results obtained are in consonance with that reported by Karaoglu *et al*. (2003), where the bacteriocin characterized showed antimicrobial activity in the acidic pH more that the basic pH. Zottola *et al*. (1994) also reported an enhanced inhibition of *Listeria monocytogenes* Scott A by *B. substilis* in an acidic media.

The antimicrobials in the supernatant of the three strains tested (Ok2a, Oe2a, In5a) showed stability at pH values ranging from (4.5-7.0) at 37°C with inhibition against at least three indicator organisms (*Salmonella spp*, *Staphylococcus spp* and *Micrococcus leteus*). This is in contrast with nisin which is unstable at neutral and alkaline pH values (Mota-Meira *et al*., 2005). Total loss of antimicrobial activity was observed for bacteriocins from strains Ok2a and In5a after 5 h of incubation at pH 7.5, similar to reports by Rowaida *et al*. (2009) in which the activity of the bacteriocin (from *B. megaterium*) was significantly ($p<0.05$) reduced after 12 h of incubation at pH 7.5. However, bacteriocin from strain Oe2a showed inhibition against the yeast strains at pH 7.5. This findings suggests that the antimicrobial activity of a bacteriocin is dependent on; the strain of the bacteria specie from which it was harvested, pH of the growth media and the incubation time.

In comparison with some of the bacteriocin produced by LAB, the bacteriocin from bacilli strains tested were not extremely heat stable. For example bacteriocin produced by *L. acidophilus* LF221 or Lactin NK24 form *Lactococcus lactis* could partially preserve their activity even after heating at 100°C for 30 min. In this study, total loss of antimicrobial activity was observed after heating the bacteriocin at 100°C for 15 min. Hence the bacteriocin will not be of much use in food preservation involving high thermal treatment.

Conclusion: On the basis of the results obtained *Bacillus spp* are present in food products and produce bacteriocins. More detailed research will be important to; characterize the bacteriocin on their synthesis system, the secretion and regulatory process including their purification and amino-acid sequencing.

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