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Fungicidal effect of bacteriocins harvested from *Bacillus* spp.

Victoria Olusola Adetunji* and Opeyemi Oyinda Olaoye

Veterinary Public Health Unit, Department of Veterinary, Public Health and Preventive Medicine, University of Ibadan, Ibadan Oyo State, Nigeria.
Email: vadetunji@gmail.com; vo.adetunji@mail.ui.edu.ng

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ABSTRACT

Aims: This study investigated the ability of bacteriocins isolated from *Bacillus* spp. (*Bacillus* species) to inhibit four different yeast isolates obtained from common food products (nono, yoghurt, ogi and cheese) commonly consumed by Nigerians with minimal heat treatment.
Methodology and results: Forty-five *Bacillus* spp. was isolated and identified from common food products using cultural, morphological, physiological and biochemical characteristics. These isolates were tested for antimicrobial activity against *Salmonella enteritidis* (3), *Micrococcus luteus* (1) and *Staphylococcus aureus* (2). Eight bacteriocin producing strains were identified from an over- night broth culture centrifugated at 3500 revolutions for five minutes. Fungicidal effects of these bacteriocins were tested against four yeast strains using the Agar Well Diffusion method. The bacteriocins produced wide zones of inhibition ranging from 5.9±0.000 to 24.00±0.000 mm against the 4 yeast strains tested. There was a significant difference (at $p<0.05$) between the yeast organisms and the bacteriocins from the *Bacillus* spp.
Conclusion, significance and impact of study: The study reveals the antifungal property of bacteriocins from *Bacillus* spp. and serves therefore as a base for further studies in its use in the control of diseases and extension of shelf-life of products prone to fungi contamination.

Keywords: *Bacillus* spp., bacteriocins, yeasts, fungi, products

INTRODUCTION

Due to increased interest in food bio-preservation, there has been tremendous development of new and natural anti-microbial compounds possessing ability to suppress the survival of several food spoilage microorganisms, of which bacteriocins present a great potential for use. The problem of fungal spoilage of food is of great concern in the food industry and many researchers have been done to minimize this (Soomro *et al*., 2002). However, there is an urgent need to prevent fungal contamination of these foods, because of accompanying economic and/or health hazards due to loss in nutritional and organoleptic properties and/or production of mycotoxins that could result (Pitt and Hocking, 1999).

Bacteriocins from Lactic Acid Bacteria (LAB) have attracted much attention and have been the subject of intensive investigation, especially their antibacterial activity (Seuk-Hyun and Cheol, 2000; Mataragas *et al*., 2002; Adebayo and Aderiye, 2007) and more recently their antifungal property (Adebayo and Aderiye, 2010). 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 *Bacillus* spp. such as *B. substilis*, *B. cereus*, *B. stearothemophilus* and other *Bacillus* spp. (Zheng, 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, in spite 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.

Many *Bacillus* spp. including *B. cereus*, *B. subtilis*, *B. mycoides* are known to suppress several fungal pathogens growth such as *Rhizoctonia*, *Sclerotinia*, *Fusarium*, *Gaeummanomyces*, *Nectria*, *Pythium* and *Phytophthora* (McKnight, 1993; Fiddaman and Rossall, 1994). The bacterial antagonists assume their antagonistic effects mainly by the production of antifungal antibiotics (Katz and Demain, 1977; Korzybski *et al*., 1978), which seems to play a major role, in the biological

*Corresponding author

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control of plant pathogens (Phae *et al*., 1990; Leifert *et al*., 1995) and post-harvest spoilage fungi (Klich *et al*., 1994). Antifungal peptides produced by *Bacillus* spp., includes mycobacillins (SenGupta *et al*., 1971), iturins (Isogai *et al*., 1982), bacillomycins (Besson *et al*., 1977; Peypoux *et al*., 1981), surfactins (Kluge *et al*., 1988), mycosubtilins (Besson and Michel, 1990), fungistatins (Korzybski *et al*., 1978), and subsporins (Ebata *et al*., 1969). Most of these antibiotics are cyclic peptides composed entirely of amino acids, but some may contain other residues.

Many strains of *B. subtilis* have been shown to be potential bio-control agent against fungal pathogens. It was reported that the principal mechanism of this antifungal involves the production of antibiotics (Fravel, 1988). In addition, *B. subtilis* strains produce volatiles that antagonise a range of soil-borne plant pathogen including *R. solani* and *Pythium ultimum* (Fiddaman and Rossal, 1993). *B. cereus* 65 strain (producing a chitobiosidase) is effective against *R. solani* (Pleban *et al.*, 1997). *B. cereus* 28-9 excreted two chitinases which had inhibitory activity against *Botritis elipitica* (Huang *et al*., 2005). All these portray great potentials of the *Bacillus* spp. to inhibit fungal organisms.

The production of bacteriocins or bacteriocin-like substances (BLS) has been described for many *Bacillus* spp. such as *B. subtilis* (Zheng, 1999) and *B. cereus* (Bizani and Brandelli, 2002). *Bacillus* spp. are considered safe biological agents (Kim *et al*., 2003), different antagonists studies with *Bacillus subtilis*, *B. megaterium*, *B. cereus*, *B. pumilus*, and *B. polymyxa* have been done (Silo-suh *et al*., 1994; Kim *et al*., 2003).

Among *Bacillus* spp., *B. cereus* is known to produce antibacterial antibiotics (Naclerio *et al*., 1993; Oscariz *et al*., 1999; Oscariz and Pisabarro, 2000). This species have been reported to produce zwittermicyne A, an aminopolyol antibiotic very effective to suppress damping-off of alfalfa caused by *Phytophthora medicaginis* (Stabb, *et al*., 1994). This study is a preliminary investigation on the anti -fungal property of bacteriocin from *Bacillus* spp. isolated from some traditional food products in South-Western Nigeria.

MATERIALS AND METHODS

Bacillus strains, isolation and identification

The isolation and identification of the *Bacillus* spp. used in this study have been previously described by Adetunji and Olaoye (2011). A total of 45 *Bacillus* spp. were isolated from common food samples namely; 'Fura' (fermented millet), 'Kulikuli' (groundnut cake), 'Elubo' (yam flour) and 'Wara' (cheese) and identified as *Bacillus* spp. by cultural , morphological, physiological and biochemical characteristics according to Barrow and Feltham (1993).

The yeast organisms used were laboratory stock isolates from fermented food products like nono, ogi, yoghurt and cheese. These fungal agents were cultured and stored on Potato Dextrose agar (PDA) (Oxoid, London) before use.

Detection and screening of antimicrobial activity of *Bacillus* strains

A preliminary study was undertaken to test the 45 *Bacillus* spp. 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).

Harvesting of the bacteriocins

Eight strains of the 45 *Bacillus* spp. showing zones of inhibitions of 6-27 mm in diameter were further screened for bacteriocin production. Test for bacteriocin production was done according to Adetunji and Adegoke (2007) with slight modification. An overnight 10 mL broth cultures in modified Mueller-Hinton broth grown at 30-37 °C for each of the 8 strains was centrifuged at 3500 rpm for 15-20 min. The supernatant was decanted into sterile test tubes, adjusted to pH 6.5 - 7.0 with 1 mol/L NaOH (Karmen and Bogovi, 2003) to remove organic acid effect. H_2O_2 was neutralized by addition of catalase from bovine liver at 200 µ/mL. The mixture of the decanted supernatant, NaOH and catalase was filtered and sterilized with a 0.2 µm Millipore filter membrane. There after the inhibitory effect of free bacteriocin on test bacteria was then determined by agar well diffusion method using the filtrate stored at 4 °C. An uninoclulated modified Mueller-Hinton broth was used as a control.

Test for the antimicrobial activity of the harvested bacteriocin

Antimicrobial activity was then tested again with five indicator organisms (*Micrococcus luteus*, *Salmonella enteritidis*, *Staphylococcus aureus* and 2 yeast isolates) 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 4 replicates.

Fungicidal effect of bacteriocins harvested

The bacteriocins harvested were examined for their anti-fungal activity against four yeast strains from fermented food using the Agar Well Diffusion method. Sterilized PDA was prepared and poured into Petri dishes, each plate was seeded with the yeast organism grown in an overnight broth culture and left to solidify. After which wells of 5 mm diameter were bored into the agar plate

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using a sterile cork borer, each filled with 50 µL of neutralized cell-free culture supernatant fluid of each *Bacillus* spp. The plates were pre-incubated at at 4 °C for few minutes, to aid the diffusion of the CFNS (cell-free culture filtrate) before incubating at 37 °C for 24 h. The anti-fungal activity was determined by measuring clear zones of inhibition with sharp edges around the wells. The zones of inhibitions were recorded for 4 replicates.

RESULTS

Eight of the 45 *Bacillus* spp. strains showing maximum zones of inhibition with average diameter of the inhibition zones measured (5.90±0.000-30.00±0.000) mm (data not shown) were further screened. Bacteriocin harvested from the eight strains selected examined for inhibition against the five indicator organisms used, showed that the bacteriocins posses a wide range of antimicrobial spectrum against the indicator organisms. This was observed by zones of inhibition measuring about (5.90±0.000-25.00±0.000) mm (data not shown). Inhibition zones ranging from 5.90±0.000-24.00±0.000 were observed with strain Sw3b1 producing the highest inhibition against Yeast 3 after 24 h incubation, no fungal growth was observed after 24 h (Table 1). Yeast 2 produced the highest zones of inhibition for all the bacteriocins tested (Table 1). There were some significant differences between the inhibition zones of 4 yeast strains and the bacteriocins from the 8 *Bacillus* spp. (Table 1).

Table1: Mean zones of inhibition of bacteriocin harvested against four different yeast strains.

Indicator organism *Bacillus* strains	Yeast 1	Yeast 2	Yeast 3	Yeast 4
Bf4b	7.45±2.192$_{abA}$	16.00±8.485$_{cB}$	7.95±2.891$_{abA}$	12.00±0.000$_{bAB}$
Bk4b	9.95±3.727$_{abA}$	12.00±4.243$_{cAB}$	7.95±2.891$_{abA}$	5.90±0.000$_{aA}$
In5a	5.90±0.000$_{aA}$	12.45.00±9.263$_{cAB}$	5.90±0.000$_{aA}$	9.45±5.020$_{abA}$
In5c	5.90±0.000$_{aA}$	18.50±9.192$_{cdBC}$	7.95±2.891$_{abA}$	15.45±13.50$_{bcB}$
Mk1a	5.90±0.000$_{aA}$	5.90±0.000$_{aA}$	5.90±0.000$_{aA}$	5.90±0.000$_{Aa}$
Oe2a	5.90±0.000$_{aA}$	7.95±2.899$_{abA}$	5.90±0.000$_{aA}$	5.90±0.000$_{aA}$
Ok2a	5.90±0.000$_{aA}$	12.50±3.53$_{cAB}$	5.90±0.000$_{aA}$	5.90±0.000$_{aA}$
Sw3b1	5.90±0.000$_{aA}$	5.90±0.000$_{aA}$	24.00±0.000$_{cC}$	5.90±0.000$_{aA}$

Values represents:- Zones of inhibition mean ± standard error of mean (mm) of test strains against yeast organism.
similar lower case letters in the same column are not significantly different at p<0.05
different lower case letters in the same column are significantly different at p<0.05
similar upper case letters in the same column are not significantly different at p<0.05
different upper case letters in the same column are significantly different at p<0.05

DISCUSSION

Screening and identification of bacteriocin producing strains

The isolation of *Bacillus* spp. from 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). 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.

Anti-fungal activity of the *Bacillus strains*

The eight bacteriocin producing *Bacillus* spp. possess antifungal property by inhibition zones produced against the four (yeast) fungal organisms used in this study. This preliminary screening of *Bacillus* spp. for anti-fungal activity demonstrated that, they are able to produce antimicrobial substances that elicit anti-fungal property. This is significant as bacteriocins are generally believed to inhibit bacteria growth (Nes *et al.*, 1996). Hence, from our study, we can deduce that *Bacillus* spp. like the LAB strains have interesting potential for anti-fungal activity against a wide variety of food borne fungi, since the fungal organism used in this study were isolated from common fermented food products prone to contamination by fungal organisms. This indicates their prospective usage in the reduction of fungal mass in food systems as against common chemicals like propionic acid and its salt, which have been observed to act by a fungistatic mechanism (Lacey *et al.*, 1991) that causes only temporary inhibition of microbial growth.

The antagonistic action was observed to be produced by neutralized, cell-free culture filtrate (CFNS) which indicates that the antifungal activity was not due to the action of organic acids or hydrogen- peroxide produced by the strains. Hence the bacteriocins were responsible for the inhibitions observed, and this is in agreement with findings from LAB strains by Adebayo and Aderiye, (2010). However Vanne *et al.*, (2000) had earlier reported

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inhibition of test fungi (*Penicillum* spp.*)* after 48 h, of incubation which is slightly different from findings in this present study where an inhibition was observed within 24 h of incubation. This suggests a high potency of these antifungal metabolites.

It is also important to note that the *Bacillus* spp. did not exhibit the same level of inhibition to the various yeast strains used. This depicts variations in the expression of antifungal activity by *Bacillus* spp., in addition it accentuate the fact that these yeast organisms are of different species. Similar report of strain dependent antifungal activity was made by Adebayo and Aderiye, (2010) in lactic acid bacteria.

CONCLUSION

Bacteriocins harvested from *Bacillus* spp. in this study have antifungal properties with some variations in potency. Therefore, they could be of use in the control of diseases and extension of shelf-life of food products prone to fungi contamination. However, further studies on safety and factors contributing to these variations in the antifungal activities of the bacteriocins are needed in other to fully maximize antimicrobial potentials possessed by this class of bacteriocin producing bacteria species.

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