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EFFECT OF ENZYMES, TEMPERATURE AND pH ON BACTERIOCIN ACTIVITY OF *Bacillus megaterium* AGAINST SOME FUNGAL PATHOGENS

Djadouni Fatima

Department of Biology, Faculty of Natural Sciences, Mascara University, Mascara, Algeria
e- mail: *Houarisalwa@yahoo.fr*

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ABSTRACT

The members of genus *Bacillus* are known to produce a wide range of antimicrobial substances, including peptide and lipopeptide antibiotics and bacteriocins that show wide antimicrobial spectra against food-spoilage microorganisms. The present study was aimed to evaluate the efficacy of bacteriocins from *Bacillus megaterium* against some selected spoilage fungi. *B. megaterium* was isolated from anise (*Pimpinella anisum*). The spoilage fungi were isolated from banana, strawberry, orange, lemon, apple, anion, cucumber, carrot, turnip and corn. Antifungal activity of extracted bactreriocin was determined at wide pH range (2-10), storage temperatures (-18, 4, 30 and 45°C for 15 days) and enzymes treatment (trypsin, pepsin, amylase and lipase) by agar well diffusion method against nine fungi. *Aspergillus niger*, *Penicillium digitatum*, *Penicillium italicum* and *Fusarium oxysporum* were found sensitive to bacteriocin and showed maximum zone of inhibition (12-16 mm). Partial characterization showed that the bactériocin was a glycolipoprotein, stable at 2-8 pH range, actives at all storage temperatures and was extracted in stationary phase of growth.

Key words: Anise, *Bacillus megaterium*, bacteriocin, characterization, spoilage fungi

INTRODUCTION

Bacillus megaterium is found in a wide range of habitats such as soil, dried food, seawater, sediments, fish, normal flora and even in bee honey (Djadouni and Madani, 2016). It is a Gram-positive rod-shaped bacterium, catalase positive, motile by peritrichous flagella, spore former and aerobic or facultatively anaerobic, having wide physiological diversity with respect to heat, pH and salinity. *B. megaterium* is completely apathogenic and produces a several biotechnologically relevant substances (Abriouel *et al.*, 2011) that prevent and destroy the contaminations caused by various species of *Cladosporium*, *Aureobasidium*, *Aspergillus*, *Penicillium*, *Phoma*, *Botrytis*, *Fusarium*, *Epicoccum*, *Geotrichum*, etc., occurring naturally on fruit and vegetable surfaces which induce premature decay and human illness (Léon *et al.*, 2009).

Bacteriocins are described as ribosomally synthesized small polypeptides that exert antimicrobial effects against closely or non-closely related bacteria (Djadouni, 2016). Some bacteriocins produced by *B. megaterium* are marketed under brand names like Megacin C (Donoghue, 1977), Megacin Cx (Brusilow and Nelson , 1981), Megacin 19 (Khalil *et al.*, 2009a) and Megacin 22 (Khalil *et al.*, 2009b). Recently, *B. megaterium* has been reported to produce thermostable lipase, penicillin G acylase and biopolymers during submerged fermentation (Anurag *et al.*, 2006; Malten *et al.*, 2006) as well as it has shown antifungal and antiviral effect including against HIV, hepatitis B

virus and herpes simplex corneal ulcers (Rodrıguez *et al.*, 2013). The bacterium has the ability to decolourize and biodegrade different azo dyes (Maulin, 2014). *B. megaterium* is also found in unusual and toxic environments where they use toxic compounds including the persistent insecticides as carbon source, so these organisms have potential for toxic waste cleanup. These features make *B. megaterium* well applicable in food and feed agricultural industry, pharmaceutical industry and medical field (Djadouni, 2016). The present study was aimed to evaluate the potential antifungal activity of *B. megaterium* against some fruit and vegetable spoilage fungi.

MATERIALS AND METHODS

Bacterial strain and culture conditions

A strain of *Bacillus megaterium* was isolated from a spice plant anise (*Pimpinella anisum*) that belongs to the family Apiaceae (anise spices). Anise seeds (1 g) in 50 mL water was heated for 10 min at 90°C to kill vegetative cells and the resultant spore suspension was plated on LB (Luria Bertani) agar and incubated at 30°C (Warth, 1978). The bacterial strain was identified on the basis of its culture-morphological, physiological and biochemical characteristics. Bacterial strain was maintained on LB broth (LB; Merck, Darmstadt, Germany), kept frozen in 20% (v/v) glycerol at -20°C until use and sub-cultured twice before use (Barrow and Feltham, 1993).

Fungal isolation and media

Nine (09) fungal species [*Aspergillus niger* (isolated from strawberry), *Aspergillus* spp. (isolated from corn), *Colletotricum musae* (isolated from banana), *Fusarium oxysporum* (isolated from cucumber), *Penicillium digitatum*, (isolated from orange) *P. italicum* (isolated from lemon), *P. expansum* (two isolates from apple and onion), *P. viridicatum* (isolated from carrot) and *Penicillium* spp. (isolated from turnip)], were procured from the Collection of Laboratory of Applied Microbiology of the Biology Institute, Oran University, Algeria. The fungal species were cultured on PDA (potato dextrose agar) {PDA; Merck, Darmstadt, Germany) and incubated at 25$\pm$1°C for 5-8 days. The isolated fungi were maintained at 4°C (Smaoui *et al.*, 2010).

Bacteriocin preparation

B. megaterium cells were grown as 2% in LB broth at 30°C, collected after 18 h at the stationary phase by centrifugation at 10,000 x g for 20 min at 4°C. The cell free supernatant (CFS) was passed through membrane filters (Renner GMBH D-67125, Germany) with a pore diameter of 0.22 µm and stored in a refrigerator for a maximum period of two weeks and periodically tested for the bacteriocin titer before renewal (Smaoui *et al.*, 2010).

Fungicidal effect of B. megaterium

The antifungal activity was detected by using agar well diffusion method. The culture plates were incubated at 25$\pm$1°C for 8 days and observed for inhibition zones diameter (Ogunbanwo *et al.*, 2014).

Detection of bacteriocin activity during growth (growth kinetics)

LB broth was inoculated with 2% (v/v) of an overnight preculture of test strain and incubated at 30°C. The changes in optical density were recorded after every 2 h at 600 nm by UV-VIS spectrophotometer (UV mini-1240, Shimadzu). The growth kinetics experiment was employed as per Periago *et al.* (2006) with slight modification. The indicator strain *Escherichia coli* (10^7 cfu mL^{-1}) was grown at 30°C in presence of CFS of bacteriocinogenic strain. The bacteriocin activity was expressed in terms of growth reduction of indicator strain and determined from the ratio between the optical densities of treated cultures and untreated ones (the indicator strains without the CFS). This optical density method was employed in all the antimicrobial assays (Khalil *et al.*, 2009b).

Influence of enzymes, heat and pH on bacteriocin activity

The proteinaceous nature of the bacteriocin was confirmed by testing their sensitivity to proteolytic enzymes. The cell-free supernatant of test strain was treated with enzymes including pepsin, trypsin, lipase and amylase (100 µg mL^{-1}, Oxford Laboratory Reagents) and incubated for 2 h at 30°C. The antimicrobial activity was monitored by growth reduction of the indicator strain *E. coli* using optical density method. Similarly, the effect of temperature and pH on the activity of bacteriocin was assessed by incubation at four temperatures (-18, +4, +30 and +45°C) for 15 days as well as tested to four pH levels (2, 4, 6, 8 and 10) and finally assayed for antimicrobial activity (Albano *et al.*, 2007).

Statistical analysis

The data were expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance on the replicates, where a p-value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

Bacillus is an interesting bacterial genus to investigate as it produces diverse antimicrobial peptides representing several different basic chemical structures. The production of bacteriocins or bacteriocin-like substances has been already described for some bacilli such as *B. subtilis*, *B. cereus*, *B. stearothermophilus* and other *Bacillus* species (Martirani *et al.*, 2002). The best-characterized ones are subtilin of *B. subtilis*, megacin of *B. megaterium*, bacteriocins of *B. cereus* and bacteriocins of *B. thuringiensis*. However, in spite of these approaches adopted by researchers to gather information on *Bacillus* bacteriocins, their importance and industrial value have largely been underestimated so have attracted minimal attention. This study describes the fungicidal effect of *B. megaterium* on nine fungi, isolated from different fruits and vegetables, which include *Colletotricum musae*, *Aspergillus niger*, *Aspergillus* spp., *Penicillium digitatum*, *P. italicum*, *P. expansum* (two isolate), *viridicatum*, *Penicillium* spp. and *Fusarium oxysporum*.

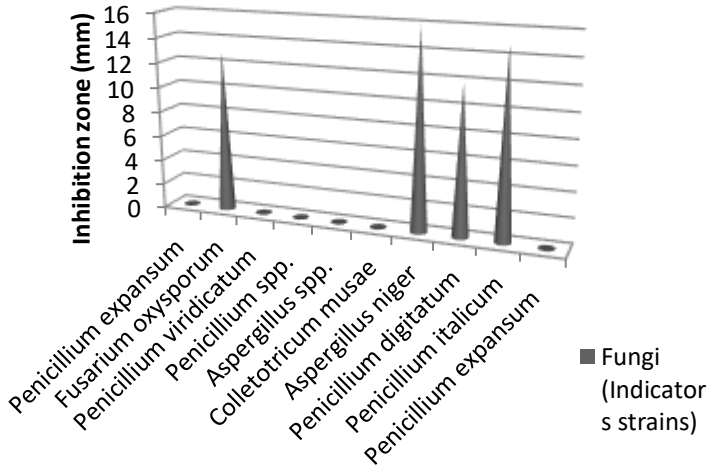

Fig. 1: Bacteriocin activity of *B. megaterium* on fungi isolated from fruits and vegetables using agar-well diffusion method

Bacteriocin of *B. megaterium* exhibited high inhibitory activity against *A. niger*, *P. digitatum* and *P. italicum* that affect acidic fruits such as strawberry, orange and lemon with inhibition zone ranging in between 12 and 16 mm. In contrast, bacteriocin was less effective to inactivate other fungi that altered fruits and vegetables, except *F. oxysporum* with inhibition of 13 mm (Fig. 1). The preliminary screening of *B. megaterium* for antifungal activity depicted the ability of this bacterium to produce antimicrobial substances that elicit antifungal property

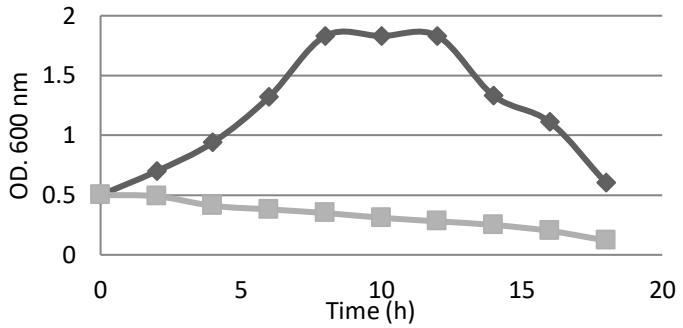

Fig. 2: *B. megaterium* growth kinetics ( ). The strain was grown aerobically in LB broth at optimal conditions of temperature (30°C), for 18 h. Activity of bacteriocin preparations against *E. coli* was expressed as reduction of growth ( )

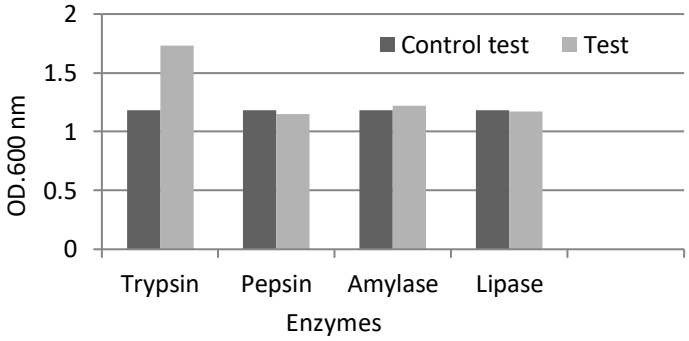

Fig. 3: Effect of enzymes (100 ug mL^{-1}) on bacteriocin activity against *E. coli* as indicator stain; control test was the growth of *E. coli* in BL broth and the test was the growth of *E. coli* in presence of bacteriocin

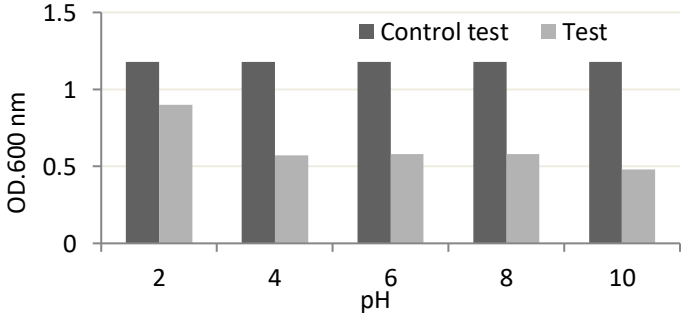

Fig. 4: Effect of pH on bacteriocin activity against *E. coli* as indicator stain; control test was the growth of *E. coli* in BL broth and the test was the growth of *E. coli* in presence of bacteriocin

against three moulds *viz.*, *Aspergillus*, *Fusarium* and *Penicillium*. This activity spectrum clearly showed that megacin is not active principally against species phylogenetically related to the producer strain.

Detection of bacteriocin activity and production during 18 h of growth at 30°C in LB medium (Fig. 2) showed that the activity occurred during late logarithmic phase (OD, 0.4 growth reduction of indicator strain *E. coli*) and reached its maxima during stationary phase (OD, 0.2). The results suggest that antimicrobial peptide is a secondary metabolite (Lisboa *et al.*, 2006).

The proteinaceous nature of bacteriocin was confirmed by testing their sensitivity against proteolytic enzymes. The bacteriocin of *B. megaterium* was completely inactivated by the tested enzymes which displayed a higher growth reduction (range OD 1.2 and 1.8) potential against *E. coli* (Fig. 3). These data clearly showed that the antimicrobial substance was a complex protein and possibly the bacteriocin may also be bound to other molecules like a carbohydrate-lipid moiety because it was completely digested by trypsin, pepsin, lipase, and amylase. These results are vital for use of *B. megaterium* bacteriocins in biopreservation of vegetables, biological control of plant pathogens (microbial antagonists) and can used as biofertilizers in agriculture to improve soil fertility and productivity as well as increase vegetative growth and nitrogen fixation (Tournas, 2005; Radhakrishnan *et al.*, 2017).

The bacteriocin activity remained extremely stable in the tested temperature range and the activity was unaltered by freezing at -18°C and 45°C after 15 days (Fig. 4). The pH stability was studied in the range of pH 2–10 and this bacteriocin was found active throughout this range (Fig. 5). The results suggested that this bacteriocin from *B. megaterium* can be added to acidic and basic foods and feeds to eliminate spoilage microbes like fungi, yeast and bacteria (Khalil *et al.*, 2009a and b,

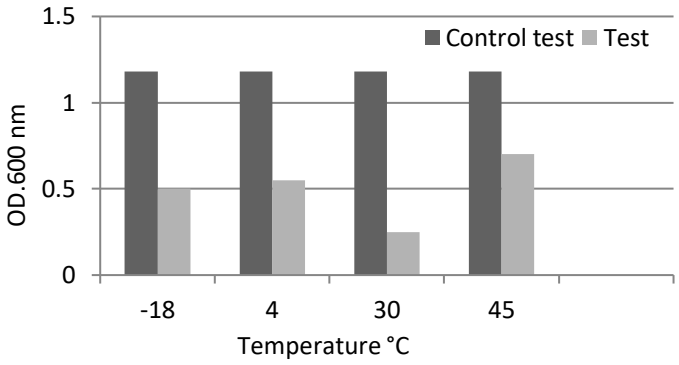

Fig. 5: Effect of temperature on bacteriocin activity against *E. coli* as indicator stain; control test was the growth of *E. coli* in BL broth and the test was the growth of *E. coli* in presence of bacteriocin

Tournas, 2005; Radhakrishnan *et al.*, 2017).

Conclusion: The bacteriocins secreted by *Bacillus megaterium* can be applied as biopreservatives during the storage of vegetables under unfavorable conditions to eliminate fungal growth that produce mycotoxins and other related fungal metabolites which might be hazardous to human health. It can also prevent the growth of pathogens as well as reduce the occurrence of diseases.

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