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ANTIMICROBIAL ACTIVITY OF *BACILLUS METHYLOTROPHICUS* BM47 AGAINST *BOTRYTIS CINEREA* IN DIFFERENT CONDITIONS OF CULTIVATION

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Summary

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Members of the bacterial genus *Bacillus*, in particular *Bacillus methylotrophicus* are known to produce a broad spectrum of biologically active substances, with various antimicrobial activities. The aim of the present study was to evaluate the antimicrobial activity of *Bacillus methylotrophicus* strain BM47 against the plant pathogenic fungus *Botrytis cinerea*, in different conditions of cultivation (temperature, pH and composition of the culture medium) by the standard agar-well diffusion method. The influence of the conditions of cultivation on the inhibitory activity of *B. methylotrophicus* BM47 was determined by using agar media with pH ranging between 5.0 and 8.0, and temperatures 25 ^{o}C and 30 ^{o}C. The influence of the composition of culture medium on the inhibitory activity of *B. methylotrophicus* BM47 was determined by changing the carbon and nitrogen sources. The antimicrobial effect was determined by measuring the diameter of the inhibition zones around the wells at 48th and 72nd hour of incubation. The results demonstrated that *B. methylotrophicus* BM47 exhibited the highest inhibitory activity against *B. cinerea* at temperature of cultivation 30 °C when the standard LBG-agar medium was used. The change of the carbon and nitrogen sources in the composition of the culture medium did not increase the antimicrobial activity of the studied *B. methylotrophicus* BM47 strain.

Key words: antimicrobial activity, *Bacillus methylotrophicus*, *Botrytis cinerea*

INTRODUCTION

The bacterial genus *Bacillus* is the largest group of microorganisms that produces a wide arsenal of biologically active substances with antibacterial and antifungal activities. This group of antibiotic compounds includes peptides, lipopeptides and bacteriocins (Abriouel *et al.*, 2010). Due to their antimicrobial properties against various pathogenic microorganisms, the antibiotics synthesised from *Bacillus* sp. and its related genera are identified as potential biocontrol agents. Most *Bacillus* antibiotics are active against Gram-positive bacteria, but a small number has been found to have activity against Gram-negative bacteria, yeast and fungi (Dev Sharma *et al.*, 2013).

Members of the genus *Bacillus* are aerobic or facultatively anaerobic Gram-positive spore-forming rods. Methylotro-

phic *Bacillus* strains display a strong resistance to high methanol concentrations and the molar growth yields on methanol at the optimum growth temperatures in methanol-limited chemostats are among the highest reported for any methylotrophic bacteria. Currently, the Gram-negative bacteria remain the best studied plant-interacting microorganisms, but some representatives of Gram-positive bacteria with high guanine and low cytosine (G+C) content of the DNA also have excellent biocontrol, plant-growth-promoting and bioremediation activities (Madhaiyan *et al.*, 2010; Gerbore *et al.*, 2016). Biocontrol with beneficial microorganisms such as *B. methylotrophicus* has been shown to be an environmentally sound option to increase crop yields and prevent outbreaks of pathogenic bacteria (Ge *et al.*, 2016).

The fungus *Botrytis cinerea* is a causal agent of a disease called grey mould. *B. cinerea* causes significant economic losses throughout the world as a destructive pathogen of many soft fruits, vegetables, grapes and flowers, particularly after their harvest (Bezier *et al.*, 2002).

At present, little is known about the antimicrobial properties of *B. methylotrophicus* and its capacity for application against some major pathogens. Therefore, the aim of current study was to investigate the antimicrobial activity of *B. methylotrophicus* strain BM47 against one of the most important plant pathogenic fungi *B. cinerea* in different conditions of cultivation.

MATERIAL AND METHODS

The following microorganisms from the collection of Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria, were used: 1) *Bacillus methylotrophicus* strain BM47, isolated from a natural thermal spring (with water temperature 57 ^{o}C) in Haskovo mineral spa, Bulgaria. The comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 99% pairwise similarity of *B. methylotrophicus* strain BM47 to the reference strain *B. methylotrophicus* PY5; 2). *Test-microorganism* – phytopathogenic fungus *Botrytis cinerea* (a plant isolate).

Culture media

LBG-broth. This medium was used for cultivation of *B. methylotrophicus* BM47. LBG-broth was prepared by the following prescription: 10 g tryptone, 5 g yeast extract, 10 g NaCl and 10 g glucose dissolved in 1 L of deionised water. The final pH was adjusted to 7.5 and the medium was autoclaved for 20 min at 121 °C.

LBG-agar media. LBG-broth media with pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were prepared by the prescription above and 15 g/L agar before autoclaving was added.

Modified agar media. To investigate the influence of the composition of culture medium on the antimicrobial activity of *B. methylotrophicus* BM47, the carbon source (glucose) in the standard LBG-agar media was changed with fructose (10 g/L) and the nitrogen source (tryptone) was changed with soy peptone (10 g/L).

Malt extract agar (MEA). This medium was used for cultivation of fungus *B. cinerea*. Ingredients (per 1 L of deionised water): 20 g malt extract, 20 g dextrose, 6 g peptone and 15 g agar. The final pH was corrected to 5.5 and the medium was autoclaved for 15 min at 121 °C.

Antimicrobial assay

The antimicrobial activity of *B. methylotrophicus* BM47 was determined by the conventional agar-well diffusion method

in standard and modified LBG-agar media (Tumbarski *et al.*, 2016). The antimicrobial assay was implemented in two steps:

Cultivation of B. cinerea and preparation of spore suspension. The fungus was grown on MEA at 30ºC for 7 days or until sporulation. The spore suspension was prepared by addition of 5 mL of sterile 0.5% NaCl into the tube and vigorous shaking. Then suspension was filtered and replaced in another tube before use. The concentration of fungal spores in the suspension was determined by Thoma's haemocytometer. The final spore concentration was adjusted to $1.0x10^5$ cfu/mL and then inoculated in a preliminarily melted and tempered to 45–48ºC LBG-agar media. The inoculated LBG-agar media were transferred in quantity of 20 mL in sterile Petri dishes (d=10 cm) and allowed to solidify. Then six wells (d=6 mm) per dish were cut.

Cultivation of B. methylotrophicus BM47 and experimental procedure. B. methylotrophicus BM47 was propagated in two tubes containing LBG-broth medium for 24 hours at 30ºC. Then the culture liquid in the first tube was stored at 4ºC (until use) and the other one was centrifuged at 3000 rpm for 10 min and the supernatant was collected. The supernatant was filtered through a bacterial filter with diameter of the pores 0.20 μm. The cell biomass was washed and resuspended in sterile 0.5% NaCl. The cell-free supernatant and the cell biomass were also stored at 4ºC until use. *B. methylotrophicus* BM47 samples (supernatant, cell biomass and culture liquid) were pipetted in quantity of 60 μL into the agar wells in two replicates. After 72 h of incubation at 25 ºC and 30 ºC, the antimicrobial activity was determined by measuring and recording the diameter of inhibition zones around the wells.

Microorganisms with inhibition zones of 18 mm or more were considered as sensitive (strong inhibitory effect); moderately sensitive were those in which the zones were from 12 to 18 mm (moderate inhibitory effect); resistant were those where the inhibition zones were up to 12 mm or completely missing (insignificant or no inhibitory effect).

RESULTS

As seen from the results, *B. methylotrophicus* BM47 demonstrated different antimicrobial activity against the fungus *B. cinerea*, depending on the conditions of cultivation (Table 1).

A moderate to strong antifungal effect of *B. methylotrophicus* BM47 on *B. cinerea* was observed when the strain was cultivated in standard LBG-agar medium at room temperature (25 ^oC) and in this case no activity of the cell-free supernatant was detected. The maximal inhibitory activity was demonstrated at pH 7.5. The cultivation of *B. methylotrophicus* BM47 in standard LBG-agar medium at higher temperature (30 ^oC) led to a significant increase in the inhibitory effect of *B. methylotrophicus* BM47 on *B. cinerea* at almost all pH values. In this case activity of the cell-free supernatant at pH 5.0, 7.5 and 8.0 was not detected.

The change of the carbon source (glucose) with fructose in the composition of the standard LBG-agar medium, led to a pronounced decrease of the inhibitory effect of *B. methylotrophicus* BM47 on the test microorganism *B. cinerea* at both temperatures of cultivation and even loss of antimicrobial activity at pH 5.0 and 5.5 and temperature 30 ^oC.

The change of the nitrogen source (tryptone) with soy peptone in the composition of the standard LBG-agar medium

Table 1. Antimicrobial activity of *Bacillus methylotrophicus* strain BM47 against *Botrytis cinerea* in different conditions of cultivation

pH	*B. methylo-trophicus* BM47	Inhibition zones, mm											
		Botrytis cinerea (1.0×10^5 cfu/mL)											
		Standard LBG-agar medium				Modified agar medium with fructose				Modified agar medium with soy peptone			
		25 ºC		30 ºC		25 ºC		30 ºC		25 ºC		30 ºC	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
5.0	Supernatant	–	–	–	–	–	–	–	–	–	–	–	–
	Cell biomass	20.0	20.0	16.5	14.0	–*	12.5	–	–	–	–	–	–
	Culture liquid	13.5	13.5	18.0	16.0	–*	10.0	–	–	–*	15.0	–	–
5.5	Supernatant	–	–	19.0	17.0	–*	13.0	–	–	–	–	–	–
	Cell biomass	16.0	20.0	30.0	24.0	12.0	13.0	–	–	–*	11.0	–	–
	Culture liquid	15.0	20.0	30.0	20.0	14.0	13.0	–	–	–*	12.0	–	–
6.0	Supernatant	–	–	17.0	15.0	–*	20.0	12.0	12.0	–	–	24.0	–
	Cell biomass	16.0	20.0	32.0	23.0	12.0	13.0	14.0	16.0	–*	10.0	14.0	–
	Culture liquid	15.0	18.0	32.0	20.0	13.0	13.0	15.0	22.0	–*	10.0	30.0	–
6.5	Supernatant	–	–	12.0	12.0	10.0	12.0	10.0	10.0	–	–	24.0	–
	Cell biomass	14.0	20.0	27.5	22.0	13.0	13.0	12.0	12.0	12.0	10.0	14.0	–
	Culture liquid	13.0	20.0	26.0	20.0	13.0	13.0	14.0	13.0	12.0	10.0	30.0	–
7.0	Supernatant	–	–	15.0	20.0	10.0	10.0	11.0	10.0	–*	10.0	24.0	–
	Cell biomass	15.0	18.0	24.5	12.0	12.0	12.0	13.0	13.0	12.0	12.0	14.0	–
	Culture liquid	15.5	23.0	28.0	25.0	13.0	13.0	13.0	13.0	12.0	12.0	30.0	–
7.5	Supernatant	–	–	–	–	12.0	11.0	11.0	10.0	–*	10.0	18.0	–
	Cell biomass	30.0	29.0	19.0	19.0	14.0	12.0	12.0	10.0	12.0	12.0	22.0	–
	Culture liquid	23.0	12.0	20.5	25.0	15.0	12.0	12.0	12.0	12.0	12.0	31.0	–
8.0	Supernatant	–	–	–	–	11.0	11.0	10.0	10.0	–*	10.0	18.0	–
	Cell biomass	18.0	18.0	23.0	21.0	13.0	13.0	13.0	13.0	12.0	14.0	22.0	–
	Culture liquid	13.5	12.0	19.5	18.0	13.0	13.0	12.0	13.0	12.0	12.0	31.0	–

Legend: d_{well} = 6 mm; (–) no inhibition; (–*) no growth at 48th hour.

at temperature of cultivation 25 °C, led to a significant decrease of the antimicrobial activity of *B. methylotrophicus* BM47 against *B. cinerea*. In addition, a loss of antifungal activity of the cell-free supernatant at 48th hour and pH ranging between 5.0 and 6.5 was observed. In contrast, the cultivation at higher temperature (30 °C) led to an increase of the inhibitory effect of *B. methylotrophicus* BM47 on *B. cinerea* at pH from 6.0 to 8.0, which was clearly pronounced in the cell-free supernatant and culture liquid. No inhibitory activity of *B. methylotrophicus* BM47 at 72nd hour of the cultivation at 30 °C was detected.

DISCUSSION

The results demonstrated that *B. methylotrophicus* BM47 exhibited the highest inhibitory activity against plant pathogenic fungus *B. cinerea* at temperature of cultivation 30 °C when the standard LBG-agar medium was used. The change of the carbon and nitrogen sources in the composition of the culture medium did not lead to an increase of the antimicrobial activity of the studied strain *B. methylotrophicus* BM47. The use of modified agar medium with soy peptone and temperature of cultivation 30°C showed an increase of the antimicrobial activity of cell-free supernatant and culture liquid (pH range 6.0 ÷ 8.0) but the antagonistic effect continued only up to the 48-th hour of cultivation. Based on the obtained results, we can conclude that the standard LBG-agar medium and temperature 30 °C are most suitable for cultivation of *B. methylotrophicus* BM47 for obtaining biologically active compounds for plant protection against *B. cinerea*.

The antimicrobial potential of *B. methylotrophicus* BM47 can find a wide and successful application in the field of agriculture for biocontrol of major plant pathogens. The high antimicrobial activity of *B. methylotrophicus* BM47 against some other fungal phytopathogens was proven in our previous study. The results showed that *B. methylotrophicus* BM47 possesses strong inhibitory effect on the fungi *Aspergillus flavus* and *Fusarium oxysporum*, regardless of the cultural conditions and the composition of the culture medium. This makes the examined strain very perspective as a producer of bacteriocins for application as natural protecting agents against these fungal pathogens, which cause plant diseases of economic importance (Tumbarski *et al.*, 2015). Moreover, some recent studies proved that *B. methylotrophicus* possesses not only antifungal, but also antibacterial properties. Dev Sharma *et al.* (2013) have examined the crude metabolite extracted from *B. methylotrophicus*-SCS2012, which exhibited strong antibacterial activity against *Streptococcus agalactae*, *Bacillus cereus*, *Escherichia coli*, *Shigella sonnei* and *Shigella dysenteriae*.

Some authors reported for other applications of *B. methylotrophicus* strains such as production of biosurfactants. Biosurfactants are surface-active compounds produced by microorganisms and comprise both hydrophilic and hydrophobic moieties in one molecule. As alternative surfactants, biosurfactants have a number of advantages, such as high biodegradability, low toxicity, environmental compatibility, high selectivity, and specific activity at extreme temperatures, pH and salinity. Biosurfactants have been widely used in different industries, such as cosmetics, special chemicals, pharmaceutics, microbial enhanced oil recovery (MEOR), cleaning oil sludge from oil storage facilities, anti-static agents, as emulsifiers in the food industry and for

production of dyes, coatings and plastics (Moussa & Abdel Azeiz, 2013).

CONCLUSION

The present study exhibited the great antimicrobial potential of *Bacillus methylotrophicus* strain BM47 against one of the most important phytopathogens *Botrytis cinerea*. The antifungal properties of *B. methylotrophicus* BM47 could find successful application in agriculture as a means for plant protection and biocontrol. The use of environment-friendly and safe products of biological origin as alternatives of the chemical fungicides is of great importance in the intensive agricultural and food production systems.

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