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Research Paper

STUDY ON THE INFLUENCE OF THE CULTURAL CONDITIONS AND THE COMPOSITION OF THE CULTURE MEDIUM ON THE ANTIMICROBIAL ACTIVITY OF *Bacillus methylotrophicus* BM47 AGAINST SOME FUNGAL PHYTOPATHOGENS

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Abstract

Members of genus *Bacillus*, in particular *Bacillus methylotrophicus* are known to produce a broad spectrum of substances, which possess different antibacterial and antifungal activities. The aim of the present study was to study the influence of cultural conditions and the composition of culture medium on the antimicrobial activity of *B. methylotrophicus* strain BM47 against the phytopathogenic fungi *Fusarium oxysporum* and *Aspergillus flavus*, by the agar-well diffusion assay. The fungi were preliminarily inoculated in the agar media, whereas the supernatant, the cell biomass and the culture liquid of *B. methylotrophicus* BM47 were pipetted into the wells. The influence of the cultural conditions on the inhibitory activity of *B. methylotrophicus* BM47 was determined by cultivation in agar media with pH ranging between 5.0 and 8.0, at temperature 25°C and 30°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. After 72 hours of incubation, the antimicrobial effect was determined by measuring the diameter of the zones of inhibition around the wells. The results we obtained demonstrated that *B. methylotrophicus* BM47 possess highest antifungal activity against *Fusarium oxysporum* at 30°C when glucose as a carbon source and tryptone as a nitrogen source (standard LBG-medium) were used, and at 30°C when a modified soy peptone medium was used. The highest inhibitory activity of *B. methylotrophicus* BM47 against the fungus *Aspergillus flavus* was observed at 30°C when fructose as a carbon source was used, and at 25°C and 30°C when a modified soy peptone medium was used.

Key words: *Bacillus methylotrophicus*, *Fusarium oxysporum*, *Aspergillus flavus*, antimicrobial activity, antifungal activity, bacteriocins, thermal springs.

INTRODUCTION

Antibacterial substances are widely produced by a large number of microorganisms. Currently microorganisms of the genera *Bacillus*, *Penicillium*, *Streptomyces*, *Cephalosporium* and *Micromonospora* are known to produce more than 5000 different antibiotics. *Bacillus* is the largest antibiotic producing genus, producing both antibacterial and antifungal, and also a wide range of other bioactive compounds. *Bacillus* sp. and its related genera have been identified as

potential biocontrol agents as they produce a wide range of cyclic lipopeptides active against various microorganisms. Most *Bacillus* antibiotics are active against Gram-positive bacteria, but small number have been found to have activity against Gram-negative bacteria, yeast and fungi [1].

The bacterial genus *Bacillus* includes aerobic or facultatively anaerobic Gram-positive or Gram-variable spore-forming rods. The vegetative cells range from 0.5 by 1.2 to 2.5 by 10 m in diameter and can grow at optimal temperatures ranging from 25 to 37ºC, although thermophilic and psychrophilic members are capable of growth at temperatures as high as 75ºC or as low as 3ºC. Some species can flourish at extremes of acidity and alkalinity, ranging from pH 2 to 10. The extreme heterogeneity of the genus is reflected in the wide variety of ecological niches that the many species occupy and in the debate over their taxonomic status. The guanine and cytosine (G+C) content of the DNA of species within the genus can vary between 32 and 69%, and many species may subsequently be reclassified into different taxonomic groupings. Most strains are catalase positive, possess peritrichous flagella and sporulate in air, which differentiates them from the clostridia [2, 3].

Methylotrophic strains of the genus *Bacillus* 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 Gram-negative bacteria remain the best-studied plant-interacting microbes, but representatives of high and low G+C Gram-positive bacteria also have excellent biocontrol, plant-growth-promoting and bioremediation activities. In addition, actinorhizal symbioses largely contribute to global biological nitrogen fixation and many Gram-positive bacteria promote other types of symbioses in tripartite interactions [4].

Presently, little is known about the antimicrobial activity of *Bacillus methylotrophicus* and the influence of different conditions on this important property. Therefore, the aim of present study is to inquiry the influence of the cultural conditions (pH and temperature) and the composition of the culture medium (by changing of the carbon and nitrogen sources) on the antimicrobial activity of *B. methylotrophicus* strain BM47 against two of the most common phytopathogenic fungi - *Fusarium oxysporum* and *Aspergillus flavus*, by the classical agar-well diffusion assay.

2. Materials and methods

The following microorganisms from the collection of Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria, were used:

2.1. *Bacillus methylotrophicus* strain BM47

The strain was isolated from a natural thermal spring (with water temperature 57°C), located in Haskovo mineral spa, Haskovo district, Southern Bulgaria. The comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 99% pairwise similarity of *Bacillus methylotrophicus* strain BM47 to the reference strain *Bacillus methylotrophicus* PY5.

2.2. Test-microorganisms

Filamentous fungi from the genera: *Aspergillus (Aspergillus flavus*) and *Fusarium* (*Fusarium oxysporum*).

2.3. Culture media

2.3.1. LBG-broth medium.

Bacillus methylotrophicus BM47 was cultured in standard LBG-medium, containing 10g casein trypsic peptone (tryptone), 5g yeast extract, 10g NaCl and 10g glucose dissolved in 1L of deionized water. The final pH was adjusted to 7.5 and medium was autoclaved for 20 min at 121°C.

2.3.2. LBG-agar medium.

This agar medium was used for the implementation of the agar-well diffusion assay. For this purpose we prepared LBG-agar media with pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (by the prescription above) and added 15g/L agar before autoclaving.

2.3.3. Modified agar media.

To investigate the influence of the composition of the culture medium on the antimicrobial activity of *B. methylotrophicus* BM47, we changed the carbon source (glucose) in the standard

LBG-medium with fructose and the nitrogen source (tryptone) with another one of organic origin - soy peptone. They were added to the LBG-agar medium in the same quantities as above.

2.3.4. Malt extract agar (MEA).

This medium was used for the cultivation of the test fungi. Ingredients (per 1L of deionized water): 20g malt extract, 20g dextrose, 6g peptone and 15g agar. The final pH was corrected to 5.5 and the medium was autoclaved for 10 - 15 min at 121°C.

2.4. Agar-well diffusion assay

The antimicrobial activity of *B. methylotrophicus* BM47 was determined by the classical agar-well diffusion assay [5].

2.4.1. Cultivation of test-microorganisms and preparation of spore suspensions.

The fungi were grown on MEA at 30ºC for 7 days or until sporulation. The spore suspensions were prepared by adding sterile 0.5% NaCl into the tubes and vigorous shaking. After this, the suspensions were filtered and collected. The concentration of spores in the spore suspensions was determined by using a Thoma's haemocytometer. The final concentration of spores in the suspensions for inoculation was adjusted to $1.0x10^5$cfu/ml. Then the spore suspensions were inoculated in a preliminarily melted and tempered to 45 - 48ºC LBG-agar media. The inoculated LBG-agar media were transferred in quantity of 20ml in sterilized Petri dishes (d=10 cm) and allowed to solidify. After this, six wells (d=6 mm) per dish were cut [6].

2.4.2. 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 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.

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 hours of incubation at 25ºC (room temperature) and 30ºC, the antimicrobial activity was determined by measuring the diameter of the zones of inhibition around the wells.

Microorganisms with inhibition zones of 18 mm or more were considered as sensitive; moderately sensitive were those in which the zones were from 12 to 18 mm; resistant were those microorganisms where the inhibition zones were up to 12 mm or completely missing [6].

3. Results and discussion

As seen from the results below, *Bacillus methylotrophicus* strain BM47 possesses high antagonistic activity against both phytopathogenic fungi used as test-microorganisms.

3.1. Influence of the carbon source on the antimicrobial activity of *B. methylotrophicus* BM47

The antifungal effect of *B. methylotrophicus* BM47 on *Fusarium oxysporum* was low only when it was cultivated in standard LBG-medium at room temperature (25°C) and in this case no activity of the supernatant was detected. The change of the carbon source (glucose) with fructose in the composition of the standard culture medium, led to a significant increase in the inhibitory effect on *Fusarium oxysporum* at 25°C; a slight decrease at pH-range between 5.0 and 6.5, and almost an insignificant increase between pH 7.0 and 8.0 at temperature of cultivation 30°C (Table 1).

The change of the carbon source in the standard LBG-medium, led to decrease in the inhibitory effect on *Aspergillus flavus* at 25°C, and appearance of inhibition zones at pH 5.0. In the presence of fructose in the culture medium and cultivation at 30°C, activity of the supernatant at all pH-values was detected, and an increase of the fungicidal effect of the cell biomass and the culture fluid between pH 6.0 and 7.5 was established (Table 2).

Table 1. Antimicrobial activity of *B. methylotrophicus* BM47 against the fungus *Fusarium oxysporum.*

pH	*B. methylo-trophicus* BM47	Inhibition zones, mm											
		Fusarium oxysporum ($1.0x10^5$ cfu/cm^3)											
		Standard LBG-medium				Modified medium with fructose				Modified medium with soy peptone			
		25°C		30°C		25°C		30°C		25°C		30°C	
		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	-	-	14.0	14.0	12.0	12.0	15.0	12.0	-	-	11.0	12.0
	Cell biomass	19.0	19.0	19.0	20.0	22.0	22.0	16.0	19.5	16.5	25.0	17.0	18.5
	Culture liquid	18.0	18.0	20.0	20.0	25.0	25.0	18.0	16.0	20.0	25.0	25.0	25.0
5.5	Supernatant	-	-	20.0	20.0	15.0	15.0	13.5	17.5	-	-	13.0	13.0
	Cell biomass	19.0	17.0	25.5	25.5	24.0	27.5	19.0	19.0	16.5	27.5	34.0	35.0
	Culture liquid	17.5	17.0	32.0	32.0	29.0	29.0	18.5	19.0	19.0	37.5	37.5	37.5
6.0	Supernatant	-	-	16.0	16.0	13.5	25.0	15.0	15.0	-	-	13.0	13.0
	Cell biomass	10.0	10.0	32.0	32.0	31.0	34.0	27.5	27.5	19.0	32.0	38.0	38.0
	Culture liquid	8.0	8.0	34.5	33.0	34.0	35.5	27.0	23.5	18.5	39.0	37.0	37.0
6.5	Supernatant	-	-	19.5	19.0	14.0	15.0	13.5	13.0	10.0	10.0	13.0	13.0
	Cell biomass	10.0	10.0	37.0	37.0	22.0	26.5	32.0	32.0	18.0	30.0	32.0	33.0
	Culture liquid	10.0	10.0	33.0	31.5	24.0	29.0	31.5	32.0	19.0	31.5	35.0	36.0
7.0	Supernatant	-	-	24.0	24.0	21.0	23.0	26.0	26.5	10.0	10.0	14.0	14.0
	Cell biomass	8.0	10.0	33.5	33.5	30.0	33.0	32.5	32.5	17.0	21.0	32.0	32.0
	Culture liquid	8.0	8.0	30.0	30.0	31.0	36.0	33.5	34.5	19.0	25.0	35.0	35.0
7.5	Supernatant	-	-	23.5	23.5	12.0	13.0	30.0	30.0	12.0	12.0	14.0	14.0
	Cell biomass	11.0	11.0	33.0	33.0	33.0	35.0	33.0	33.0	17.0	24.0	36.0	38.0
	Culture liquid	10.0	10.0	34.5	34.5	29.0	31.5	33.0	33.0	18.0	27.0	36.5	39.0
8.0	Supernatant	-	-	18.5	18.5	18.0	19.0	28.5	28.5	10.0	10.0	15.0	15.0
	Cell biomass	11.0	11.0	30.0	30.0	30.0	33.0	27.0	27.0	12.0	30.0	26.0	32.5
	Culture liquid	11.0	11.0	34.0	33.0	37.5	37.5	27.0	27.0	16.0	35.0	32.5	36.0
Average value (Σ/21)		**8.1**	**8.1**	**26.8**	**26.7**	**24.1**	**26.5**	**24.6**	**24.7**	**13.7**	**21.5**	**26.0**	**27.0**

Legend: d_{well} = 6 mm; „-“ – no inhibition.

3.2. Influence of the nitrogen source on the antimicrobial activity of *B. methylotrophicus* BM47

The change of the nitrogen source (tryptone) with soy peptone in the composition of the standard culture medium, led to a significant increase in the antimicrobial activity of the cell biomass and the culture liquid of *B. methylotrophicus* BM47 against *Fusarium oxysporum* at 25°C and 30°C. In contrast, changing the nitrogen source decreased the activity of the supernatant in the pH-range between 5.0 and 8.0 (Table 1.).

The change of the nitrogen source in the standard LBG-medium led to an increase in the inhibitory effect on *Aspergillus flavus* at 25°C, and decrease in the effect at pH values between pH 5.0 and 6.0 at 30°C. The presence of soy peptone in the culture medium at pH-values greater than 6.5 led to pronounced increase in the antagonistic activity of *B. methylotrophicus* BM47 at both temperatures of cultivation (Table 2).

Table 2. Antimicrobial activity of *B. methylotrophicus* BM47 against the fungus *Aspergillus flavus.*

pH	*B. methylo-trophicus* BM47	Inhibition zones, mm											
		Aspergillus flavus ($1.0x10^5$ cfu/cm^3)											
		Standard LBG-medium				Modified medium with fructose				Modified medium with soy peptone			
		25°C		30°C		25°C		30°C		25°C		30°C	
		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	-	-	-	-	-	-	15.5	15.0	-	-	-	-
	Cell biomass	-	-	19.5	19.5	13.0	12.0	18.0	15.5	20.0	20.0	18.0	18.0
	Culture liquid	-	-	35.0	36.0	35.0	35.0	40.0	40.0	25.0	13.0	22.0	22.0
5.5	Supernatant	11.0	10.0	-	-	-	-	16.0	22.0	16.0	-	13.5	-
	Cell biomass	14.0	14.0	30.0	30.0	19.0	19.0	29.0	28.5	23.0	17.0	16.5	22.0
	Culture liquid	21.0	21.0	33.5	38.0	22.5	21.0	34.0	30.0	29.0	26.5	26.0	28.0
6.0	Supernatant	17.0	17.0	-	-	13.0	15.0	14.0	16.0	23.5	18.0	15.0	21.0
	Cell biomass	24.5	24.5	31.5	31.0	15.0	22.5	37.0	37.5	25.0	24.5	22.0	23.0
	Culture liquid	40.0	40.0	32.0	31.0	19.0	21.0	40.0	39.0	35.0	37.5	36.0	35.0
6.5	Supernatant	30.5	30.5	16.5	16.5	13.5	20.0	21.5	21.5	25.0	25.0	25.0	29.0
	Cell biomass	35.5	35.5	19.5	19.5	21.0	24.0	33.5	33.5	37.5	37.5	37.5	39.0
	Culture liquid	40.0	40.0	18.5	18.5	20.5	25.5	38.5	38.5	40.0	40.0	40.0	40.0
7.0	Supernatant	30.5	25.5	30.0	30.0	14.0	16.0	21.5	23.5	27.5	27.5	32.5	32.5
	Cell biomass	35.5	35.5	33.0	33.0	33.0	36.0	33.5	39.0	31.0	31.0	37.5	35.0
	Culture liquid	40.0	40.0	35.0	35.0	30.0	32.5	38.5	39.0	40.0	40.0	40.0	40.0
7.5	Supernatant	25.0	25.0	27.0	27.0	18.0	19.0	15.5	18.0	30.0	27.5	27.5	27.5
	Cell biomass	34.0	34.0	31.0	31.0	36.0	38.5	24.5	21.0	40.0	37.5	39.0	40.0
	Culture liquid	40.0	40.0	35.5	35.5	34.0	34.0	37.5	37.5	40.0	40.0	40.0	40.0
8.0	Supernatant	27.0	26.0	27.5	27.5	19.5	19.0	18.0	19.5	29.0	30.0	25.0	25.0
	Cell biomass	37.0	37.0	31.0	31.0	36.5	33.0	24.5	26.5	30.0	30.0	40.0	32.5
	Culture liquid	40.0	40.0	33.0	33.0	32.0	30.5	32.5	35.5	37.5	37.5	40.0	40.0
Average value (Σ/21)		**20.8**	**25.5**	**24.7**	**24.9**	**21.2**	**22.5**	**27.8**	**28.4**	**28.8**	**26.7**	**28.2**	**28.1**

Legend: d_{well} = 6 mm; „-“ – no inhibition.

The results demonstrated that *B. methylotrophicus* BM47 possesses highest antifungal activity against *Fusarium oxysporum* at 30°C when glucose as carbon source and tryptone as nitrogen source (standard LBG-medium) were used, and at 30°C when a modified soy peptone medium was used. The highest inhibitory activity of *B. methylotrophicus* BM47 against the fungus *Aspergillus flavus* was observed at 30°C when fructose as carbon source was used, and at 25°C and 30°C when a modified soy peptone medium was used. This can be taken into account in the optimization of the culture media for cultivation of *B. methylotrophicus* strains for obtaining bacteriocins.

B. methylotrophicus BM47 and the compounds with antimicrobial activity it synthesizes, can find wide application in agriculture for biocontrol of some plant pathogens. It is well known that *Fusarium oxysporum* is among the major pathogens, which cause severe losses on most vegetables and flowers, several field crops such as cotton and tobacco, plantation crops such as banana, plantain, coffee and sugarcane, and a few shade trees [7]. Other fungal species as *Aspergillus flavus* are known as producers of extremely toxic metabolites, which can cause poisonings and even liver cancer in humans and animals when contaminated food is consumed [8].

Moussa and Abdel Azeiz (2013) reported for other applications of *B. methylotrophicus* strains such as production of biosurfactants. Biosurfactants are chemical compounds produced by microorganisms and comprise both hydrophilic and hydrophopic moieties in one molecule (surface-active molecules). As alternative surfactants, biosurfactants have outstanding 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, coating sand plastics [9].

The synthesis of silver nanoparticles (AgNPs) with antimicrobial activity by microorganisms is an area attracting growing interest in nanobiotechnology, due to the applications of these nanoparticles in various products including cosmetics and biosensors, and in the biomedical, clinical, and bioimaging fields as well. Various microorganisms as *Bacillus methylotrophicus* strain DC3, isolated from the soil of Korean ginseng (a traditionally known oriental medicinal plant in Korea) have been found to be able to synthesize AgNPs when silver salts are supplied in the reaction system [10].

Based on the results we obtained, we can conclude that *B. methylotrophicus* BM47 possess strong inhibitory effect on both of the tested fungi - *Aspergillus flavus* and *Fusarium oxysporum*, regardless of the cultural conditions and the composition of the medium. This makes the examined *Bacillus* strain very perspective as producer of bacteriocins for application as plant-protecting agents against these fungal phytopathogens, which cause diseases of economic importance.

CONCLUSION

Using environment-friendly and food-hygienically-safe methods with agents of biological origin is of great significance in the intensive agricultural production systems. The increasingly proven harm from chemical pesticides resulted in a growing interest in considering the bacteriocins produced by *Bacillus* strains as alternative plant-protecting agents (antimicrobials) against a broad spectrum of microorganisms. Bacteriocin-producing *Bacillus* strains, in particular *B. methylotrophicus* could be used for different applications against diverse microorganisms in agriculture, medicine and food industry. Based on the results obtained, *B. methylotrophicus* BM47 could be successfully used as bacteriocin-producing strain and its examination has to continue in future.

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