**SAT-23-P-BFT(R)-07**

# ANTIMICROBIAL ACTIVITY OF *BACILLUS METHYLOTROPHICUS* 47 AGAINST PHYTOPATHOGENIC FUNGI

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***Antimicrobial activity of Bacillus methylotrophicus 47 against phytopathogenic fungi:*** *A Gram-positive, spore-forming, rod-shaped bacteria strain was isolated from healing spring waters in the village of Mineralni Bani, Haskovo region. It was identified as Bacillus methylotrophicus by the application of modern molecular-genetic methods – ARDRA-analysis and sequencing of 16S rRNA gene. The strain was designated as Bacillus methylotrophicus 47. The strain was cultured under aerobic or anaerobic conditions at 30°C or 37°C at different pH values of the medium (LBG-broth or MPB). Its antimicrobial activity on the growth of phytopathogenic fungi of Aspergillus sp. was examined. Bacillus methylotrophicus 47 showed high inhibitory activity against fungi of Aspergillus sp. after culturing under aerobic or anaerobic conditions at pH values in the range from pH = 5.0 to pH = 8.0. It was determined that the inhibitory activity was due to the synthesis of an antibiotic substance of a peptide nature with Rf value of 0.65.*

***Key words:*** *Bacillus methylotrophicus, antifungal, phytopathogenic, antimicrobial*, *Aspergillus.*

## INTRODUCTION

Representatives of the genus *Bacillus* produce substances with antimicrobial activity of different chemical nature. The antimicrobial agents include peptides, amino sugars, phospholipids, etc. [4].

According to Leifert et al., 1995; Yazgan et al., 2001; Montesinos, 2007 [1, 2, 5] the antimicrobial compounds produced by strains of the genus *Bacillus* exhibit antifungal activity against phytopathogenic fungi.

In recent years aflatoxins in food and the protection of the population from their carcinogenic effect are the subject of growing social interest. The development of preparations with high antifungal activity against *Aspergillus flavus* would allow to process both soil and plant individuals. This in turn would enable the production of safe food.

The purpose of the present work was to study the antifungal activity of a *Bacillus methylotrophicus* strain against pathogenic fungi of the genus *Aspergillus*.

## MATERIALS AND METHODS

***Microorganisms.*** *Bacillus methylotrophicus* 47 isolated from spring waters in the village of Yabalkovo, Haskovo region, Bulgaria and identified to species level by sequencing the 16S rDNA

was used in the present study. As test microorganisms were used the following plant isolates: *Aspergillus niger, Aspergillus awamori, Aspergillus flavus.*

***Media***

a. MPB. Composition (g/dm$^3$): peptone - 10; NaCl - 5; meat extract - 3; glucose - 10. Sterilization - 20 min at 121 °C.

b. LBG-broth. Composition (g/dm$^3$): tryptone - 10; yeast extract - 5; NaCl - 10; glucose - 10 Sterilization - 20 min at 121 °C.

c. LBG-agar. Composition (g/dm$^3$): tryptone - 10; yeast extract - 5; NaCl - 10; glucose - 10 agar - 15. Sterilization - 20 min at 121 °C.

d. MPA. Composition (g/dm$^3$): tryptone - 10; yeast extract - 5; NaCl - 10; glucose - 10 Sterilization - 20 min at 121 °C.

***Methods of analysis***

***Culturing of Bacillus methylotrophicus* 47**

The culturing of *Bacillus methylotrophicus* 47 was carried out in 500 cm$^3$ Erlenmeyer flasks that contained 10 cm$^3$ of culture medium (LBG-broth or MPB) under aerobic conditions obtained by a rotary shaker (220 min$^{-1}$) and under anaerobic conditions (static culturing in a thermostat) at a temperature of 37 °C for 24-48h. The inoculum was 1% (v/v) 18-hour vegetative inoculum. The culturing was conducted at various pH values of the broth media (LBG-broth or MPB) ranging from pH=4.5 to pH=8.0.

***Preparation of a sterile filtrate of the culture suspension***

After the culturing the culture suspension was centrifuged at 3500 min$^{-1}$ for 15 min to separate the biomass. The supernatant was filtered through a membrane filter (pore size 0,45 μm). The biomass was washed twice with saline solution and was adjusted to the initial sample volume.

***Determination of the antimicrobial activity – well-diffusion method***

To determine the antimicrobial activity of *Bacillus methylotrophicus* 47 against phytopathogenic fungi were prepared three variants of each sample: culture suspension (CS), biomass in saline solution (BSS) and acellular supernatant (ASN), obtained after 24-48 hours of culturing of *Bacillus methylotrophicus* 47. The antimicrobial activity was investigated against *Aspergillus niger, Aspergillus awamori, Aspergillus flavus*. For the preparation of the fungal spore suspensions the respective test fungal microorganism was grown in an incubator at 30 °C on LBG-agar for 3 to 7 days. Spore suspensions of each of the test microorganisms ($10^6$-$10^7$ cfu/cm$^3$) were prepared and they were used to inoculate Petri dishes (pour plating) with LBG-agar, the final spore concentration in the inoculated LBG-agar medium was $10^5$cfu/cm$^3$. After the solidification of medium wells (d=6mm) were prepared. 0,06 cm$^3$ of the CS, BSS and ASN were pipetted in the wells and the Petri dishes were incubated at 30 °C or 37 °C for 24h to 48 h. The experiments were performed in quadruplicate. The results as average inhibition zones in mm were reported on the 24$^{th}$ or 48$^{th}$ hour of incubation at 30 °C or 37 °C.

**Thin layer chromatography (TLC)**

TLC for the separation of the produced antibiotic substances in the culture suspension (CS), biomass in saline solution (BSS) and the acellular supernatant (ASN) was performed. The system used was butanol: acetic acid: water (3: 1: 1 v/v/v). Chromatographic silica gel plates $COF_{254}$ 20x20 cm (Merck, Germany) were used. The plates were placed in a chromatography chamber in a layer with height of 0,5 cm. At the start of the chromatographic plate with a micropipette were placed 50μl of the culture suspension (CS), the acellular supernatant (ASN) or the biomass in saline solution (BSS). The chromatograms were run in duplicate. After the thin layer chromatography, the chromatograms were dried at 60 °C for about 15min and were sprayed with ninhydrin solution (to prove the presence of peptide antibiotics according to the method of Niederwieser, 1972 [3]). After

the spraying of the chromatograms pink spots appeared. The Rf - values of the active substances were determined.

## RESULTS AND DISCUSSION

The most appropriate temperature range for the growth of *Bacillus methylotrophicus* 47 is in the temperature range of 30-37°C. Thus, its antimicrobial action was determined at two temperatures - 30 °C and 37 °C, after static or dynamic culturing in two broths – LBG-broth and MPB. The culturing was conducted at various pH values of the broth media ranging from pH=4.5 to pH=8.0. The inhibitory activity was determined by the well-diffusion method. The results as inhibition zanes in mm were reported on the $24^{th}$ or $48^{th}$ hour of incubation at 30 °C or 37 °C.

The test results for the antimicrobial activity of *Bacillus methylotrophicus* 47 cultured under anaerobic conditions at 30 °C are presented in Table 1.

Table 1.

Antimicrobial activity of *Bacillus methylotrophicus* 47 cultured under anaerobic conditions for 48 hours against phytopathogenic fungi at incubation temperature of 30 °C

| Media | | Inhibition zones, mm | | |
|---|---|---|---|---|
| | | Test-microorganism | | |
| | | *Aspergillus niger,* $3x10^5 cfu/cm^3$ | *Aspergillus awamori,* $1,2x10^5 cfu/cm^3$ | *Aspergillus flavus,* $1,12x10^5 cfu/cm^3$ |
| LBG pH = 4,5 | ASN | 17 | 25 | 27 |
| | CS | 18 | 25 | 35 |
| | BSS | 20 | 25 | 34 |
| LBG pH = 5,0 | ASN | 30 | 25 | 28 |
| | CS | 37 | 35 | 37 |
| | BSS | 37 | 37 | 35 |
| LBG pH = 6,0 | ASN | 30 | 30 | 28 |
| | CS | 39 | 37 | 32 |
| | BSS | 39 | 39 | 28 |
| LBG pH = 7,0 | ASN | 32 | 30 | 35 |
| | CS | 36 | 35 | 40 |
| | BSS | 27 | 35 | 33 |
| LBG pH = 8,0 | ASN | 28 | 25 | 30 |
| | CS | 37 | 27 | 37 |
| | BSS | 34 | 32 | 27 |
| MPB pH = 4,5 | ASN | 20 | 25 | 23 |
| | CS | 28 | 32 | 27 |
| | BSS | 27 | 28 | 25 |
| MPB pH = 5,0 | ASN | 27 | 30 | 23 |
| | CS | 33 | 32 | 28 |
| | BSS | 33 | 30 | 27 |
| MPB pH = 6,0 | ASN | 27 | 20 | 22 |
| | CS | 30 | 24 | 23 |
| | BSS | 27 | 27 | 24 |
| MPB pH = 7,0 | ASN | 30 | 32 | 30 |
| | CS | 34 | 37 | 35 |
| | BSS | 34 | 34 | 30 |
| MPB pH = 8,0 | ASN | 30 | 30 | 27 |
| | CS | 30 | 35 | 35 |
| | BSS | 27 | 32 | 40 |

*B. methylotrophicus* 47 exhibited strong antifungal activity, the largest inhibition zones being reported at samples obtained by culturing at pH=7.0 in LBG-broth and MPB under anaerobic conditions for 48 h (Table 1). The inhibition zones were very clear which is a proof of the fungicidal action of *B. methylotrophicus* 47.

The samples obtained after the aerobic culturing of the strain demonstrate large inhibition zones (Table 2). High antifungal activity against *Asp. niger* and *Asp. awamori* was reported in the samples obtained after culturing in LBG-broth and MPB with pH = 5.0. The high antimicrobial activity of the acellular supernatant (ASN) and the biomass in saline solution (BSS) indicated that the antimicrobial components were associated with the cell surfaces as well as secreted into the medium.

Table 2.

Antimicrobial activity of *Bacillus methylotrophicus* 47 cultured under aerobic conditions for 48 hours against phytopathogenic fungi at incubation temperature of 30 °C

| Media | | Inhibition zones, mm | | |
|---|---|---|---|---|
| | | Test-microorganism | | |
| | | *Aspergillus niger,* $3x10^5 cfu/cm^3$ | *Aspergillus awamori,* $1,2x10^5 cfu/cm^3$ | *Aspergillus flavus,* $1,12x10^5 cfu/cm^3$ |
| LBG pH = 4,5 | ASN | 20 | 30 | 35 |
| | CS | 26 | 32 | 34 |
| | BSS | 18 | 23 | 37 |
| LBG pH = 5,0 | ASN | 32 | 33 | 37 |
| | CS | 43 | 42 | 37 |
| | BSS | 40 | 42 | 40 |
| LBG pH = 6,0 | ASN | 27 | 35 | 30 |
| | CS | 36 | 40 | 37 |
| | BSS | 31 | 40 | 32 |
| LBG pH = 7,0 | ASN | 24 | 37 | 35 |
| | CS | 32 | 43 | 43 |
| | BSS | 34 | 35 | 37 |
| LBG pH = 8,0 | ASN | 28 | 23 | 42 |
| | CS | 32 | 32 | 42 |
| | BSS | 32 | 34 | 40 |
| MPB pH = 4,5 | ASN | 29 | 39 | 26 |
| | CS | 30 | 37 | 30 |
| | BSS | 33 | 35 | 34 |
| MPB pH = 5,0 | ASN | 28 | 30 | 35 |
| | CS | 37 | 37 | 37 |
| | BSS | 30 | 33 | 43 |
| MPB pH = 6,0 | ASN | 25 | 22 | 25 |
| | CS | 31 | 27 | 32 |
| | BSS | 30 | 27 | 31 |
| MPB pH = 7,0 | ASN | 33 | 30 | 34 |
| | CS | 36 | 35 | 34 |
| | BSS | 36 | 32 | 34 |
| MPB pH = 8,0 | ASN | 35 | 32 | 23 |
| | CS | 30 | 35 | 32 |
| | BSS | 37 | 33 | 35 |

When the temperature of culturing was raised to 37°C the inhibitory activity of the strain was also amended. From the samples obtained after the 48-hour anaerobic culturing of *B. methylotrophicus* 47 the highest inhibitory activity was established in the samples with culturing in LBG-broth medium with pH=7.0 and in MPB with pH=6.0 (Table 3).

Table 3.

Antimicrobial activity of *Bacillus methylotrophicus* 47 cultured under anaerobic conditions for 48 hours against phytopathogenic fungi at incubation temperature of 37 °C

| Media | | Inhibition zones, mm | | |
|---|---|---|---|---|
| | | Test-microorganism | | |
| | | *Aspergillus niger*, $3x10^5 cfu/cm^3$ | *Aspergillus awamori*, $1,2x10^5 cfu/cm^3$ | *Aspergillus flavus*, $1,12x10^5 cfu/cm^3$ |
| LBG pH = 4,5 | ASN | 12 | 22 | 17 |
| | CS | 12 | 23 | 17 |
| | BSS | 13 | 20 | 20 |
| LBG pH = 5,0 | ASN | 25 | 24 | 15 |
| | CS | 27 | 27 | 17 |
| | BSS | 34 | 27 | 20 |
| LBG pH = 6,0 | ASN | 25 | 30 | 34 |
| | CS | 30 | 32 | 36 |
| | BSS | 27 | 36 | 39 |
| LBG pH = 7,0 | ASN | 30 | 27 | 33 |
| | CS | 25 | 32 | 33 |
| | BSS | 34 | 34 | 35 |
| LBG pH = 8,0 | ASN | 36 | 30 | 27 |
| | CS | 30 | 27 | 25 |
| | BSS | 35 | 32 | 32 |
| MPB pH = 4,5 | ASN | 26 | 30 | 29 |
| | CS | 26 | 28 | 30 |
| | BSS | 32 | 30 | 30 |
| MPB pH = 5,0 | ASN | 28 | 25 | 28 |
| | CS | 34 | 35 | 33 |
| | BSS | 29 | 32 | 33 |
| MPB pH = 6,0 | ASN | 30 | 32 | 30 |
| | CS | 30 | 32 | 32 |
| | BSS | 36 | 35 | 36 |
| MPB pH = 7,0 | ASN | 30 | 26 | 22 |
| | CS | 28 | 30 | 22 |
| | BSS | 45 | 25 | 23 |
| MPB pH = 8,0 | ASN | 30 | 27 | 25 |
| | CS | 30 | 32 | 30 |
| | BSS | 33 | 27 | 30 |

In the aerobic culturing of *B. methylotrophicus* 47 at a temperature of 37 °C high antimicrobial activity was determined in LBG-broth with pH=6.0 and in MPB with pH=7.0, the diameter of the inhibition zone exceeding 40 mm (Table 4) in the culture suspension (CS) and the biomass in saline solution (BSS), which reaffirmed the fact that the antimicrobial compounds were associated with the microbial cells. This showed that *B. methylotrophicus* 47 could grow under anaerobic conditions and in both media at pH=6-7 the samples demonstrated high antifungal activity. Higher antimicrobial activity was determined in the samples obtained after the aerobic cultivation at 37 °C in both media at pH=6.0 (Table 4).

Table 4.
Antimicrobial activity of *Bacillus methylotrophicus* 47 cultured under aerobic conditions for 24 hours against phytopathogenic fungi at incubation temperature of 37 °C

| Media | | Inhibition zones, mm | | |
|---|---|---|---|---|
| | | Test-microorganism | | |
| | | *Aspergillus niger,* $3x10^5 cfu/cm^3$ | *Aspergillus awamori,* $1,2x10^5 cfu/cm^3$ | *Aspergillus flavus,* $1,12x10^5 cfu/cm^3$ |
| LBG pH = 4,5 | ASN | 8 | 22 | 16 |
| | CS | 10 | 17 | 15 |
| | BSS | 11 | 20 | 13 |
| LBG pH = 5,0 | ASN | 30 | 35 | 35 |
| | CS | 22 | 25 | 28 |
| | BSS | 27 | 27 | 30 |
| LBG pH = 6,0 | ASN | 35 | 25 | 25 |
| | CS | 30 | 37 | 32 |
| | BSS | 25 | 32 | 35 |
| LBG pH = 7,0 | ASN | 32 | 24 | 25 |
| | CS | 27 | 30 | 32 |
| | BSS | 30 | 32 | 30 |
| LBG pH = 8,0 | ASN | 34 | 22 | 23 |
| | CS | 34 | 32 | 25 |
| | BSS | 25 | 29 | 25 |
| MPB pH = 4,5 | ASN | 32 | 22 | 24 |
| | CS | 34 | 28 | 30 |
| | BSS | 32 | 25 | 30 |
| MPB pH = 5,0 | ASN | 32 | 30 | 32 |
| | CS | 32 | 40 | 40 |
| | BSS | 32 | 37 | 37 |
| MPB pH = 6,0 | ASN | 28 | 32 | 25 |
| | CS | 37 | 44 | 37 |
| | BSS | 37 | 34 | 35 |
| MPB pH = 7,0 | ASN | 35 | 32 | 33 |
| | CS | 45 | 40 | 47 |
| | BSS | 43 | 40 | 45 |
| MPB pH = 8,0 | ASN | 26 | 25 | 23 |
| | CS | 28 | 37 | 30 |
| | BSS | 35 | 30 | 22 |

The strain manifested strong antagonism against phytopathogenic fungi after culturing in all media. The spectrum of its antimicrobial action in different culture media was similar, but not identical. This suggests that the antibiotic substances produced by *B. methylotrophicus* 47 are probably synthesized in different amounts and may be similar in nature, but not the same. This strain is a promising biocontrol agent against phytopathogenic fungi.

The Rf - values of the antibiotic substances produced by *B. methylotrophicus* 47 were determined by thin layer chromatography. *B. methylotrophicus* 47 synthesized antibiotic substances, which determined its antifungal activity. In the thin layer chromatogram a spot with Rf value of 0.65, ninhydrin-positive was established so the synthesized antibiotic substances were peptides since the ninhydrin solution proved the presence of a protein ingredient (the stain was with the characteristic pink-violet color).

## CONCLUSION

*B. methylotrophicus* 47 possesses high antifungal activity against *Aspergillus niger, Aspergillus awamori* and *Aspergillus flavus* after culturing in MPB or LBG-broth with different pH values under aerobic and anaerobic conditions. It was due to the synthesis of an antibiotic substance of a peptide nature with Rf value of 0.65.

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