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METABOLITES OF *Bacillus subtilis* SKB 256, GROWTH INHIBITORS OF PHYTOPATHOGENIC FUNGI

U. R. Radzhabov and K. Davranov*

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Use of bacterial antagonists or preparations prepared from them is an effective biological method for battling phytopathogenic microorganisms. In this respect, spore-forming bacteria of the genus *Bacillus* are promising and widely used [1–5]. Several compounds with antibiotic activity were isolated from different *Bacillus* species and characterized [5, 6]. The metabolites of *B. subtilis* contain low-molecular-weight peptides [5, 6]. We showed earlier [7, 8] that *B. subtilis* SKB 256 is an active antagonist of *Fusarium oxysporum* and *Pseudomonas syringiae*, which is consistent with the broad spectrum of antagonistic compounds from this strain.

Our goal was to study and isolate exometabolites with antifungal activity from culture liquid (CL) of strain *B. subtilis* SKB 256.

The total antifungal activity of exocellular metabolites of *B. subtilis* SKB 256 after ultrafiltration through a membrane with pore diameter ≤ 20 kDa was about 50% (5–6 U/mL) of the activity of starting CL (9–10 U/mL). Such a significant reduction of the initial activity could be explained by partial inactivation as a result of the ultrafiltration. On the other hand, *B. subtilis* SKB 256 could have included fungicides with MW ≤ 20 kDa. In fact, use of denser membranes, i.e., with pore diameter ≤ 10 kDa, increased the yield of antifungal activity by 17–20%. Thus, the overall yield of antifungal activity was about 70–72%. Therefore, we used a membrane with pore diameter ≤ 10 kDa for further work.

The action of extracellular metabolites of *B. subtilis* SKB 256 on *F. oxysporum* under nutrient medium conditions was characterized by destruction of the morphological development of fungal mycelium accompanied by limited growth of hyphae and partial lysis of spores.

A study of the physicochemical properties of the low-molecular-weight fraction of *B. subtilis* SKB 256 showed that its active components were stable at pH 6.0–8.0 and 40–60°C (Fig. 1). The preparation activity was practically unaffected at 40–50°C and retained over 80% of the initial activity for 1 h at 60°C.

TLC and PC found six components of differing mobility in fractions after membrane purification of extracellular metabolites of *B. subtilis* SKB 256. The PC data showed that three components with antifungal activity against *F. oxysporum* were present.

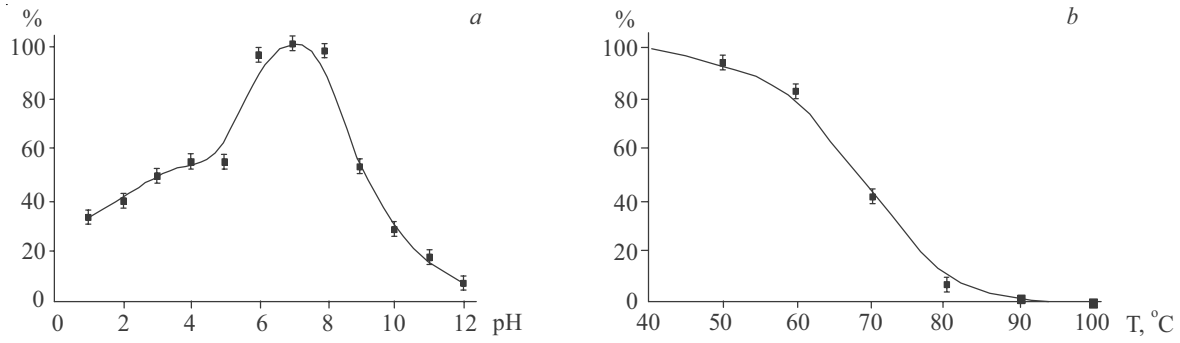

Fig. 1. Stability of fractions (≤ 20 kDa) of *Bacillus subtilis* SKB 256 metabolites with antifungal activity. Along the ordinate, antifungal activity (%); pH stability (*a*); thermal stability (°C) (*b*).

Mirzo Ulugbek National University of Uzbekistan, Tashkent, e-mail: K_davranov@mail.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 135–136, January–February, 2010. Original article submitted June 15, 2009.

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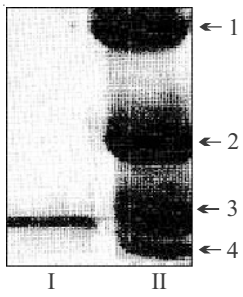

Fig. 2. Electrophoregram of protein component with MW 14 kDa (I) and antifungal activity in PAAG (12.5%) with Na-DDS (1%). Markers (II): carboanhydrase, 30 kDa (1); soy trypsin inhibitor, 21 kDa (2); cytochrome *c*, 12.4 kDa (3); insulin, 6.2 kDa (4).

Components of the low-molecular-weight fraction (after membrane filtration) were partially separated also by size-exclusion chromatography over a column of Toyopearl HW-40 equilibrated with Tris-HCl buffer (pH 7.5, $5 \cdot 10^{-3}$ M). The separated compounds also included three components with antifungal activity. Gel electrophoresis in PAAG (12.5%) under denaturing conditions found a component that gave a single protein band of ~14 kDa (Fig. 2). Two other components had MW <6 kDa.

Further studies confirmed the antifungal activity of the protein component with MW 14 kDa that was isolated from the low-molecular-weight fraction of *B. subtilis* SKB 256. The method of dilutions found that its minimum inhibiting concentration for *F. oxysporum* was 0.10–0.15 mg/mL.

Thus, it was shown that *B. subtilis* SKB 256 had high antifungal activity against *F. oxysporum*, a cause of wheat root rot.

It was found that a fraction of *B. subtilis* SKB 256 had antifungal activity and consisted of three components of MW 14 kDa and <6 kDa.

We used strain *B. subtilis* deposited in the microorganism collection of the Institute of Microbiology, Academy of Sciences, Republic of Uzbekistan, No. SKB 256.

Culture was grown in liquid nutrient medium containing (g/L) cotton meal extract (10.0), molasses (30.0), K_2HPO_4 (7.0), $MgSO_4 \cdot 7H_2O$ (0.1), NH_4NO_3 (1.0), $Na_3C_6H_5O_7 \cdot 3H_2O$ (0.5), corn extract (2.5), and tapwater (1 L). Cultivation was carried out for 2 d in Ehrlenmeyer flasks on a rocker at 200 rpm and 36–38°C.

Antagonist activity of *B. subtilis* SKB 256 metabolites was determined by the method of dilutions. The test sample was phytopathogenic fungus *F. oxysporum*. The unit of activity was the amount of CL inhibiting growth of fungus with a 1:100 ratio of CL volume to total nutrient medium volume. Growth inhibition of fungus was estimated visually.

Isolation of Total Fraction of Low-Molecular-Weight Metabolites of *Bacillus subtilis* B-1. CL was centrifuged for 10 min at 2000 g. Supernatant was ultrafiltered on UVA-PS-20-1040 fiber modules (Russia) with pore diameter ~20 kDa. The filtrate was lyophilized on an Inei-26 apparatus (Russia). An aqueous solution of the preparation was desalted over a column (1 × 25 cm) of Sephadex G-10 (Pharmacia, Sweden).

The number of components in the isolated fraction (≤ 20 kDa) was determined by ascending TLC on cellulose plates (Merck, Germany) and paper (Whatman, GB). The mobile phase as a mixture of *n*-BuOH:H_3CCO_2H:H_2O (12:4:6). Chromatograms were detected by ninhydrin solution (0.25%) in acetone. Pure active components were found by placing uncolored paper chromatograms into sterile Petri dishes and covering with a thin (1–2 mm) layer of Chapek agar. The surface of the medium was innoculated with a suspension of *F. oxysporum* spores. The dishes were incubated for 7 d at 28°C. Antifungal activity of the separated compounds was estimated by the formation at their sites of sterile zones in the fungus layer.

Gel chromatography of the fraction ≤ 20 kDa was carried out over a column (1.5 × 40 cm) of Toyopearl HW-40 (Toyosoda, Japan). The equilibrating and eluting buffer was $5 \cdot 10^{-3}$ M Tris-HCl buffer at elution rate 0.5 mL/min. Fractions of 3 mL were collected. Active components of the fractions were obtained by gel chromatography and lyophilization.

Electrophoresis of components was performed in PAAG (12.5%) with Na-DDS (0.1) according to Lemmly. Gel was colored with coomassie G-250 solution (0.02%, Serva, Germany) in $HClO_4$ (3.5%). The molecular-weight markers were (kDa) carboanhydrase (29), soy trypsin inhibitor (21), cytochrome C (12.4), and insulin (6.2).

Thermal stability of the total fraction of low-molecular-weight metabolites of *B. subtilis* SKB 256 was estimated as follows. Tubes with preparation (1–3 mL) were held for 1 h at 50–100°C; cooled to room temperature; and treated with a suspension of test-fungus spores (1 mL), six-fold concentrated Chapek medium (1 mL), and distilled water to a total volume of 6 ml. The innoculated samples were left at 28°C for 7 d. Fungicidal activity of preparation in a control sample that was not heat treated was taken as 100%.

pH stability of preparation was determined by incubating it for 12 h at room temperature at pH values 1.0–12.0. Then samples were neutralized to pH 7.0, treated with Chapek medium, and innoculated with a suspension of fungus spores.

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