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ORIGINAL ARTICLE

Antimicrobial Activity of Biosurfactant Strain *Bacillus licheniformis 9/2 A*

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ABSTRACT
The antimicrobial activity of the lipopeptide biosurfactant of the Bacillus licheniformis 9/2A strain was studied against gram-positive (Bacillus subtilis, Bacillus cereus, Staphylococcus aureus), and gram-negative (Pseudomonas aeruginosa, Escherichia coli) bacteria, yeast Candida albicans, and some types of phytopathogenic fungi, including Aspergillus sp. 4/13, Aspergillus carbonarius UZB-1, Fusarium culmorum 1/6, Fusarium oxysporum 6/1, Cladosporium sp. 4/9, Alternaria tenuissima 4/7, Aspergillus sp. 2/5, Fusarium solani 1/5, Alternaria alternata 2b, Alternaria alternata 4, Penicillium sp., Fusarium sp., Alternaria sp. The maximum antimicrobial activity of BS strain B. licheniformis 9/2A was detected against test cultures of Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, yeast Candida albicans and phytopathogenic fungi Fusarium solani 1/5, Alternaria alternata and Aspergillus sp. Based on the results of studies of the antimicrobial effect of lipopeptide BS, it can be assumed that they are promising biomolecules for use in medicine and agriculture.
***Key words**: biosurfactant, Bacillus licheniformis 9/2A, antimicrobial activity, phytopathogens.*

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INTRODUCTION

Currently, there is growing interest in the study and practical use of biosurfactants (BS), surfactants of microbial origin, which are a wide range of surface-active organic compounds of various structures produced by many prokaryotic and eukaryotic microorganisms. Due to their amphiphilic structure, they are able to localize between liquids of different polarity, reducing surface and interfacial tension, they are characterized by low critical concentrations of micelles, they are non-toxic and biodegradable [1, 2], according to their chemical structure they are classified as glycolipids, lipopeptides, lipopolysaccharides, phospholipids, fatty acids and neutral lipids, and according to microbial origin, bacterial, yeast and fungal biosurfactants [3, 4]. One of the main advantages of BS of microbial origin is their biological activity (antimicrobial and antiviral). Presumably, the biological properties of BS are the result of their interaction with the membranes of target cells, the mechanism of action of which is associated with their adhesive activity, causing a change in the integrity of the cell membrane and disruption of the cell life cycle, as well as in their amphiphilic structure. By their structure, BS consist of a hydrophilic head, which usually consists of a peptide, amino acid, monosaccharide, disaccharide or polysaccharide, and a hydrophobic tail, which is a linear, branched, saturated, unsaturated or hydroxylated fatty acid. The insertion of BS fatty acid components into the cell membrane causes an increase in membrane size and significant ultrastructural changes in cells. Desai J. et al. in their studies described the possibility of inserting shorter acyl tails into the cell membrane, thereby causing a gap between the cytoskeletal elements and the plasma membrane, allowing the membrane to tear away from the cytoplasmic contents [5]. Carrillo C. et al suggest that lipopeptide BS increases membrane permeability due to interaction with phospholipids of the cell membrane, as well as due to their ability to form pores in biological membranes and destabilize the lipid structure [6].

The demonstrated antimicrobial and antiviral properties of BS of microbial origin allow their use in cosmetics; examples of commercially available products containing antimicrobial BS are Kanebo moisturizing cream (Kanebo Cosmetics, Japan), Sopholiance facial cleanser (Givaudan Active Beauty, France) and moisturizing cream for the body Relipidium (BASF, Germany) [7]. The bacterial strain *B. licheniformis SV1*, producing glycolipid BS, is used in the composition of a wound-healing ointment, promoting intensive tissue binding and a higher rate of re-epithelialization, enhancing the wound-healing effect [8-10]. Some of the microbial BS exhibit antimicrobial properties that are effective against a large number of plant pathogens. In addition, rhamnolipids and lipopeptides have been shown to stimulate the plant immune system, providing resistance to fungal and bacterial pathogens [11]. Research is underway on the use of microbial BS as part of antitumor drugs, as well as in the fight against the syndrome coronavirus-19 (SARS-CoV-19), the main treatment methods for which were related to the fight against the symptoms of the disease, but not the virus [12,13]. Since, for the ability of the virus to maintain its integrity and pass through the phospholipid bilayer of cells, initiating the infection mechanism, the lipid membrane plays a role along with the built-in spike proteins, then the main mechanism of action that allows interaction with the hydrophobic domain of the viral membrane is the amphiphilic nature of BS, which contributes to the destruction and disruption of the structure and, consequently, to the suppression of virus reproduction [14]. Currently, microbial BS are considered multifunctional biomolecules of the 21st century [2].

Bacteria of the genus *Bacillus* are producers of several types of lipopeptide BS (iturins, fengycins and surfactins). Lipopeptide BS can be either cyclic or linear, and consist of 7–11 amino acid residues associated with β-amino- or β-hydroxy fatty acids [15]. Iturins, especially mycosubtilin, exhibit high antifungal activity [16].

The aim of this study is to study the antimicrobial effect of BS, strain *Bacillus licheniformis 9/2A.*

MATERIAL AND METHODS

Nutrient medium and cultivation conditions for the strain *Bacillus licheniformis 9/2A*.

To prepare the inoculum, the bacterial strain was grown in 5 ml of TSB (tryptone soy broth, Himedia) for 14-18 hours at 30°C and 150 rpm. Then, in an amount of 2% (containing $0.8x10^7$ CFU/ml), with an initial OD_{600} of (0.7-0.8), MSM was added to the liquid nutrient medium of the following composition (g/l): Na_2HPO_4 - 2,2; KH_2PO_4 - 1,4; $MgSO_4$ x$7H_2O$ - 0,6; $FeSO_4$ - $7H_2O$-0,01; NaCl - 0,3; $CaCl_2$ - 0,02 and 0,1% solution of microelements containing (g/l); $ZnSO_4$ x$7H_2O$ - 2,32; $MnSO_4$ x $4H_2O$ - 1,78; H_3BO_3 - 0,56; $CuSO_4x5H_2O$ - 1,0; $NH_4MoO_4x2H_2O$ - 0,39 and KI - 0,66. pH 7,0 – 7,5. 4% glycerol was used as a carbon source, and 2 g/l urea was used as a nitrogen source. The strain was grown in 1000 ml flasks, with a nutrient medium volume of 500 ml, with an initial pH value of 7.0, at a temperature of 30°C, at 150 rpm for 72 hours.

Determination of the optical density (OD) of the cell suspension was carried out on a TP-X8 UV/VS Scanning Spectrophotometer using a 1.0 cm cuvette. Bacterial growth was assessed by the optical density of the cell suspension at λ=600 nm, taking into account dilution, as well as by measuring absolutely dry biomass obtained by centrifugation of the culture liquid for 10 min at 10,000 rpm.

Surface tension measurement. The surface tension of the coolant liquid of the supernatant was measured using an automatic tensiometer (Model GD8541A, China). Measurements were carried out at 20°C. Surface tension was calculated using the formula: $\sigma = mg/2l$, where m – the plate separation mass, g = 9.8 m/s^2 – the acceleration of free fall; l = 15.7 mm – length of the tear line (width of the plate). The decrease in surface tension ($\Delta\sigma$) was calculated as the difference between the surface tension values of the sterile medium (control) and the test culture sample.

Isolation of BS was carried out using acid precipitation according to Vater J. et al. [17]. Cell-free supernatant was obtained by centrifugation at 10,000 rpm at 4°C for 20 minutes. The supernatant was acidified with 6N HCl to ph 2, after which it was kept for 14–18 hours at 4°C to enhance the precipitation of BS. The precipitate was separated by centrifugation at 10,000 rpm and then dissolved in 10 mM phosphate buffer (pH 8.0) to redissolve. The solution was concentrated by lyophilization to obtain a dried mass of BS, which was subsequently used to calculate the yield of BS (weight of BS per dry mass of cells).

Thin layer chromatography (TLC). The chemical nature of BS was determined using TLC plates detected with 0.25% ninhydrin in 96% alcohol (followed by heating to 110°C for 5 minutes) to detect peptides, and with 10% copper sulfate in phosphoric acid to detect lipids. BS obtained after lyophilization of BS was dissolved in phosphate buffer, then extracted with chloroform: methanol in a ratio of 2:1 and 20 µl of the fraction (1 mg/ml) was applied to silica gel plates for preparative TLC (Merck, 0.2 mm, 60 F25). TLC plates were eluted with the solvent system acetone: acetic acid: water (70:30:10) for the detection of amino acids and chloroform: methanol: water (60:35:8) for the detection of lipids.

The antimicrobial activity of BS was studied against various gram-positive and gram-negative bacteria, yeasts and phytopogenic fungi. Test culture of bacteria, yeast and phytopathogens *Aspergillus sp. 4/13, Fusarium culmorum 1/6, Fusarium oxysporum 6/1, Cladosporium sp. 4/9, Alternaria tenuissima 4/7, Aspergillus sp. 2/5, Fusarium solani 1/5, Alternaria alternata 2b, Alternaria alternata 4, Aspergillus carbonarius UZB-1, Penicillium sp., Fusarium sp.* and *Alternaria sp*., obtained from the collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. Strains of phytopathogenic fungi *Aspergillus carbonarius UZB-1, Aspergillus sp. 5, Penicillium sp. 24.1, Fusarium sp. – 32, Alternaria sp.* and *Axt sp31* were kindly provided by the authors of [18].

To prepare the inoculum, 24-hour broth cultures of bacterial strains *E. coli, P. aeruginosa, S. aureus* grown in soybean-casein broth, 48-hour culture of *C. albicans, S. cerevisiae* grown in Sabouraud liquid nutrient medium were diluted 0.9 % sterile NaCl solution to a concentration of 10^5 CFU/ml. A suspension of *B. cereus* spores was also diluted to a concentration of 10^5 per ml. 0.1 ml of a suspension of each test microorganism was added to previously prepared sterile Petri dishes kept for 48 hours with nutrient agar medium (Himedia) for bacteria (E*. coli, P. aeruginosa, S. aureus, B. cereus*), and on the medium Sabouraud for *C. albicans, S. cerevisiae* cell suspensions of test microorganisms were evenly distributed throughout the dish, wells were made with a diameter of 0.8 cm, and 100 µl of BS solution was dropped into the wells. To prepare the inoculum of phytopathogenic fungi, the strains were grown at 28°C for 4 to 6 days on potato agar medium with the addition of glucose. To wash off fungal conidia from the surface of the agar slant, a sterile 0.9% sodium chloride solution was used and the titer was adjusted to 10^8 using the McFarlane turbidity standard. The titer of conidia test of fungal strains brought to 10^8 was diluted with physiological solution to 10^6. Then, molten potato agar with the addition of 20 g/l glucose, 25 ml each, was poured into Petri dishes placed on tables with a strictly horizontal surface. The cups were dried in a thermostat. The inoculum of fungal spores was inoculated onto the agar in an amount of 100 µl and evenly distributed using a spatula over the entire surface of the agar. A sterile metal cylinder with a diameter of 0.8 cm was used to punch holes in the agar. Equal volumes of 100 µl of the test sample were added to the wells of each dish. The dishes were kept in the refrigerator for 3-4 hours. Then the dishes were incubated at a temperature of 28 ^{o}C for 72 hours. After the end of the incubation period, zones of suppression of the growth of phytopathogenic fungi were measured.

RESULTS AND DISCUSSION

Currently, the potential commercial applications of BS are becoming increasingly in demand due to the fact that they are environmentally friendly and non-toxic biomolecules. Lipopeptide BS, the main producers of which are bacteria of the genus Bacillus, are one of the promising groups of BS [1-4]. Previously, a bacterial strain belonging to the genus B. licheniformis 9/2A, a BS producer, was isolated from a contaminated sample of an oil and gas well in the Kashkadarya region. For biosynthesis of BS, the strain was cultivated on a selected optimal nutrient medium MSM, where glycerol at a concentration of 4% was used as a carbon source, and urea at a concentration of 2 g/l was used as a nitrogen source. BS of the CS supernatant reduced surface tension from 81.7 to 39.4 mN/m; when determining the emulsification index (E24) of the CS supernatant, E24 was more than 60% and was stable for 5 days. Analysis of oil displacement (with a density of 0.9 g/cm^3) when adding 10 µl of sample, the oil displacement zone varied in the range of 20-25 mm. [19].

After isolation, freeze drying, and extraction of BS with organic solvents, the yield of BS varied from 3.0 to 3.5 g/l. To study the nature of BS, chloroform: methanol extracts were used using TLC. It was found that when eluting with a solvent system of acetone: acetic acid: water (70:30:10) and spraying with ninhydrin to identify amino acids, a dark pink color was observed (Rf = 0.372), which characterized the presence of a peptide fragment of BS (Figure 1), while after elution in the system chloroform: methanol: water (60:35:8) and development with α-naphthol to detect carbohydrates, the absence of spots was observed, indicating the absence of carbohydrates in the sample.

When studying the antimicrobial activity of the resulting lipopeptide BS, it was determined (Figure 2) that all tested bacteria showed sensitivity; the strains *S. aureus* and *B. subtilis* showed the greatest sensitivity to BS. The results obtained in this study are consistent with the results of studies on the BS activity of the strain *B. licheniformis 09IDYM23*, which showed antimicrobial activity against *S. aureus, P. aeruginosa, E. coli, B. cereus* and *A. niger* fungi [20].

A study conducted by Zammuto V. et al. showed the ability of BS of the thermotolerant strain *B.licheniformis B3-15* against adhesion and biofilm formation by strains *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 to polystyrene and human cells, based on the results of studies of BS of this strain *B. licheniformis B3-15* also showed maximum antibacterial activity against *S. aureus* [21]. As known, the treatment of infections caused by *S. aureus* bacteria is currently a serious threat, since the resistance of

these bacteria to beta-lactam antibiotics is widespread; data obtained on the antimicrobial effect of BS synthesized by the *B. licheniformis 9/2A* strain in relation to *S. aureus* will allow the resulting BS to be used in medicine.



A	B	C

Figure 1. TLC chromatograms of BS strain *B. licheniformis 9/2A.* (A) - manifested by iodine vapor; (B) - spraying with ninhydrin (system - acetone: acetic acid: water (70:30:10); (C) - development of α - naphthol (system - chloroform: methanol: water (60:35:8). (A), (B) - 1-BS *B. licheniformis 9/2A*, 2-surfactin standard, 3-phospholipid, 4-glycolipid. (C) - 1-surfactin, 2-phospholipid, 3-BS *B. licheniformis 9/2A,* glycolipid

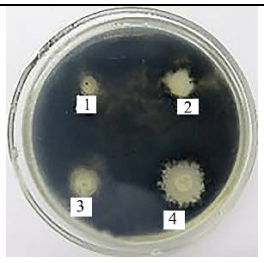
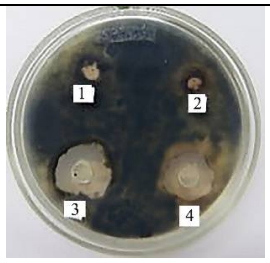
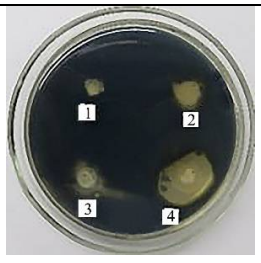




E. coli	*St. aureus*	*Ps. aeruginosa*	*B. cereus*	*B.subtilis*
Growth inhibition zone (mm)				
14-16	18-20	10-12	14-16	16-18

Figure 2. Antibacterial effect of chloroform:methanol fractions of BS of strain *Bacillus licheniformis 9/2A* (c-control, 1, 2 - BS concentration - 3 mg/ml, 3-6 BS concentration - 5 mg/ml.

When studying the phytopathogenic effect of chloroform: methanol extracts of BS of the strain *Bacillus licheniformis 9/2A*, it was found that BS showed activity against three species of the genus *Alternaria: Alternaria alternata 4, Alternaria alternata 6, Alternaria alternata 2b*, the growth inhibition zone of which varied from 14 to 18 mm, in relation to two phytopathogens of the genus *Aspergillus sp. 4/13, Aspergillus sp. 2/5 and one Fusarium solani 1/5*, the growth suppression zone of which varied from 12-16 mm (Figure 3). Based on the results of research conducted by a group of scientists, the potential of the strain *Bacillus subsp. Spizizenii MC6B-22* was studied as a biocontrol agent in agriculture. The lipopeptide BS of the strain showed activity against phytopathogens of tropical crops, with a fungicidal mechanism of action, the minimum inhibitory concentration of which ranged from 25 to 400 μg/ml [22]. According to the results of studies conducted by Sarwar A. et al., [23] it was shown that lipopeptide BC of bacteria of the genus *Bacillus* exhibit antifungal activity against *Fusarium moniliforme, Fusarium oxysporum, Fusarium solani* and *Trichoderma atroviride*. In addition, lipopeptide BS extracts showed biotechnological potential as biocontrol agents against the phytopathogenic fungi *Fusarium* and *Trichoderma*. BS strain *B. licheniformis 9/2A* can be used to create biological products with antifungal action against the fungi *Alternaria, Aspergillus sp.* and *Fusarium*.


Alternaria alternata 4	*Alternaria alternata 6*	*Alternaria alternata 2b*	*Uncimle sp.2*
Growth inhibition zone (mm)			
14-18	16-18	15-16	0

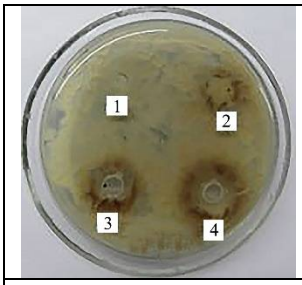
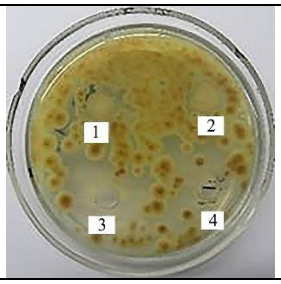
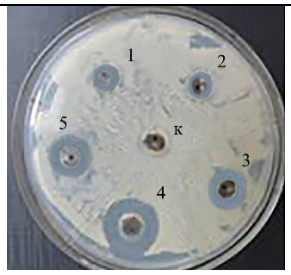
			
Aspergillus sp 4/13	*Aspergillus sp 2/5*	*Fusarium solani 1/5*	*C. albicans*
Growth inhibition zone (mm)			
12-14	14-16	14-16	18-20

Figure 3. Antifungal effect of chloroform:methanol fractions of BS of strain *B. licheniformis 9/2A* against some phytopathogenic fungi (1, 2 - BS concentration 3 mg/ml, 3, 4, 5 - BS concentration 5 mg/ml.

When studying the antifungal effect of BS strain *B. licheniformis 9/2A* against the yeast culture *C. albicans* (Figure 2), it was found that chloroform-methanol fractions of BS exhibited antifungal activity against *C. albicans* at a concentration of 5 mg/ml, growth inhibition zone strain was 18-20 mm.

As immunocompromised patients who have undergone transplantation, as well as patients with medical implants, are known to be susceptible to fungal infections caused by the yeast *C. albicans*, the ability of sophorolipid BS *Starmerella bombicola MTCC 1910* has been shown to inhibit the growth of *C. albicans* and biofilm formation, as well as reduce viability preformed biofilms. When using the obtained sophorolipid BS in combination with amphotericin B (AmB) or fluconazole, a synergistic effect was found both against the formation of biofilms and inhibition of the growth of *C. albicans* [24].

CONCLUSION

Based on the results of the studies, it was found that lipopeptide BS of the strain *Bacillus licheniformis 9/2A* showed maximum antimicrobial activity against test cultures of *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus*, yeast *Candida albicans*, as well as phytopathogenic fungi *Fusarium solani 1/5, Alternaria alternata* and *Aspergillus sp*. Obtained data on the antimicrobial effect of BS strain *B. licheniformis 9/2A*, which exhibits an antibacterial effect against *S. aureus*, and the yeast culture *C. albicans* have biotechnological potential for use in medicine. The demonstrated antifungal activity of BS against the phytopathogenic fungi *Alternaria alternata 4, Alternaria alternata 6, Alternaria alternata 2b, Aspergillus sp. 4/13, Aspergillus sp. 2/5* and *one Fusarium solani 1/5* will serve as biopotential as biocontrol agents in agriculture.

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Contributions of the authors. All authors discussed the results and contributed to the final version of the manuscript. Makhsumkhanov A.A. - supervised the results of this work, Alimova B.Kh. - participated in the development, interpretation of the results, working on the manuscript of the article, Pulatova O.M. - participated in the development and conduct of the study, in the analysis of the results, Abdukhalilova N. S - participated in the development and conduct of the study, Sayliev M. - conducted the experiments, Ishonkhodzhaev T.M. - conducted the experiments, Saidova I.M. - conducted the experiments, Amirsaidova D.A. - conducted the experiments, Khidirova M. A. - conducted the experiments,

Declaration of Conflicting Interests. The authors declare that they have no known conflicting financial interests or personal relationships that could have appeared to influence the work presented in this article.

Declaration of interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work presented in this article.

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