Bulgarian Journal of Agricultural Science, 30 (No 2) 2024, 263–269

Degradation of dual-species biofilms using hydrolytic enzymes produced by *Bacillus subtilis* 170 strains

Ivo Ganchev* **and Georgi Dzhelebov**

Trakia University, Faculty of Technics and Technologies of Yambol, 8602 Yambol, Bulgaria
**Corresponding author:* ivotg@abv.bg

Abstract

Ganchev, I. & Dzhelebov, G. (2024). Degradation of dual-species biofilms using hydrolytic enzymes produced by *Bacillus subtilis* 170 strains. *Bulg. J. Agric. Sci., 30*(2), 263–269

In this study, we evaluated the ability of enzymatic extracts produced by *Bacillus subtilis* 170 to remove multispecies biofilms, formed by the interaction of *Bacillus subtilis* and *Escherichia coli* strains. After culture in liquid medium, containing inorganic nitrogen sources, the bacterial hydrolytic extracts showed protease (250 U/mL) activity. Cell-free supernatants of *B. subtilis* 170 strains with proteolytic activity were the most effective, and promoted the complete removal of *Bacillus subtilis* and *Escherichia coli* dual-species biofilms. Of the treatments using cell-free supernatants of *B. subtilis* 170 with proteolytic enzyme activities with 250 U/mL, total biofilm degradation was observed for both dual-species biofilms in this study. The exoprotease secreted by *B. subtilis* 170 strain in the culture medium lead to a significant decrease in the biomass, average thickness, relative area of spread of biofilms formed with the participation of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains and strains is determined by their concentration. Thus, the hydrolases produced by *Bacillus subtilis* 170 strains evaluated here are highlighted as an interesting tool in the soil biofilm formation process and the potential ecological roles of soil biofilms.

Keywords: Rhizobacteria; protease; *Bacillus subtilis* 170; microbial biofilm

Introduction

Biofilms play an important role in colonization of surfaces – soil, roots, or shoots of plants and enable proliferation in the desired niche, besides enhancing soil fertility (Velmourougane et al., 2017; Blackledge et al., 2014; Balaban et al., 2003). Soil biofilms composed of single or multiple species were found to colonize various substrata and interfaces including mineral surfaces, pore spaces and plant roots, fungal hyphae or decomposing organic materials and secreted extracellular polysaccharides to form aggregates (Wang et al., 2004; Li, 2005; Chen et al., 2013; Brooks & Flint, 2008). In the soil, biofilms represent a hotspot where robust biogeochemical processes and intensive interactions take place (Flemmingand & Wuertz, 2019; Delaviz et al., 2015; Kaplan et al., 2003). In soil environments, extracellular polymeric substances (EPSm), most of them are negatively charged owing to the presence of anionic functional groups, such as carboxyl, phosphoryl, hydroxyl, and sulfhydryl groups, are produced during the growth and metabolism of heterotrophic bacteria. The main components of the matrix of soil biofilms are carbohydrates, proteins, humic substances, and nucleic acids, with carbohydrates and proteins accounting for 75%–90% of EPS (Longhi et al., 2008). Biofilm formation is a five-stage process: planktonic, attachment, microcolony formation, macrocolony, and dispersion stage (Velmourougane et al., 2017; Vishwakarma, 2019; Chen et al., 2013).

The soil biofilms, containing *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans*

and *Paenibacillus amylolyticus*, has shown how different interspecies interactions can drive synergistic community growth (Wang et al., 2004), that revealed a greater biofilm biomass production in the multispecies cultures than in biofilm formed by a single species culture (Røder et al., 2019; Li et al., 2005; Kaplan et al., 2003). Currently, the investigation of soil biofilms is only in an early stage, especially the composition, functions and genetic regulation of EPS, keep staying overly broad (Herschend et al., 2017).

Many bacteria of soil biofilms have an ability to secrete biofilm formation-inhibiting molecules, such as biosurfactants, polysaccharides, and molecules that interfere with bacterial quorum sensing, and these secreted molecules enable to exclude unfavorable competitors from biofilms. *B. Subtilis* produces a wide array of antibiotics, that are non-ribosomally synthesized peptide compounds, such as surfactin, bacillaene, fengycin, iturin, and bacilysin (Kaplan et al., 2003), and many extracellular degradative enzymes, such as proteases, levansucrase, α-amylase, β-glucanases, and xylanase (Heydorn et al., 2000), that play an important role in competition within biofilms or in bacterial competitions and nutrient acquisition in nature. The production of extracellular proteases are a part of late stages of biofilm formation, but a functional role for proteases in breaking down the protein components of the biofilm matrix has not yet been established, such activity is supported by the knowledge that the protease nattokinase of *B. subtilis*in their association with other species has amyloid-degrading capabilities. Extracellular proteases could have a role in disassembly of the TasA amyloid-like fibres in the matrix (Delaviz et al., 2015). The dispersion coincides with the expression of genes encoding matrix-degrading enzymes, and requires several matrix-degradative enzymes that work together to degrade multiple matrix components, including extracellular DNA, polysaccharides and adhesins (Flemming & Wuertz, 2019). Although most attention has been focused on animal and human pathogens, the ability of soil drawing bacteria in their consortia to form and detach from biofilms may equally have considerable implications for the completion of their life cycle and its role in the soil processes.

The main objective of the present work is to study the effects of cell-free supernatants of *Bacillus subtilis* 170 strains with proteolytic activity on the biofilm development and architecture of biofilms of the bacterium (*B. subtilis*) during their interactions with *Escherichia coli* K-12 1655 strain. To further evaluate the effects of biofilms on soil biogeochemical processes, microbial metabolic activities of soil with biofilms/free-living cells were compared and the fraction of active microbes was estimated by using of confocal laser scanning microscopy. This study provides the role of secretory microbial metabolites on the soil biofilm formation process and the potential ecological roles of soil biofilms, which are critical to understanding soil biogeochemical processes.

Material and Methods

Bacterial strains and culture media

In this research, *Bacillus subtilis* and *Escherichia coli*, both acquired from the BCCM/LMG bacteria collection of NBIMCC, NCIPD and the "Stephan Angeloff"Institute of Microbiology in Sofia, were used. Stock-cultures were stored at −80°C in Luria Bertani Broth (LB, NCIPD, Sofia), which were both supplemented with 20 (v/v) % glycerol (NCIPD, Sofia). For every experiment, a purity plate was prepared by spreading a loopful of stock-culture onto a LB agar plate [Plate Count Agar (NCIPD, Sofia). The purity plates for *Bacillus subtilis* and *Escherichia coli* were incubated for 24 h at 37°C. Starting from the purity plates, pre-cultures were prepared by transferring one colony into an Erlenmeyer flask containing 20 mL of LB medium (LB, NCIPD, Sofia). *Bacillus subtilis* and *Escherichia coli* pre-cultures were incubated for 24 h at 37°C. Following this incubation period, stationary phase cultures with a cell density of ~10^9 CFU/mL were obtained.

Biofilm development conditions

The stationary phase pre-cultures were used to develop a 100-fold diluted inoculum with a cell density of ~107 CFU/mL. The investigated pre-culture ratios (*Bacillus subtilis* and *Escherichia coli*) were 1:1 and the growth media was Luria Bertani Broth (NCIPD, Sofia), which proved to be the optimal media for single-species and multispecies biofilm development by *Bacillus subtilis* and *Escherichia coli*, respectively. To develop the biofilms, 1.2 mL of the inoculum was transferred to a small Petri dish made out of polystyrene (50 mm diameter, 9 mm height, Simport, Canada). After inoculation, Petri dishes were closed and gently shaken to make sure the inoculum covered the entire surface. Dependent on the applied (incubation) conditions, Petri dishes were incubated for 24 h at 20°C, which were the optimal temperatures for *Bacillus subtilis* and *Escherichia coli* single-species and multispecies biofilm formation, respectively.

Crystal violet assay

Biofilms were stained during 15 min with 200 μL of Crystal Violet at 0.01% (w/v) and rinsed thrice with NaCl (36 $g.l^{-1}$) and dried for 10 min. The quantification of biofilm was evaluated by releasing the stain from the biofilm with absolute ethanol for 10 min at 20°C, at 120 rpm and measuring the absorbance of the Crystal Violet solution at 595 nm.

Quantification of colonies

Each specimen was individually placed in a centrifuge tube containing 4.5 mL of sterile physiological solution, and these tubes were vortexed for 1 min to detach the biofilms from the acrylic samples. After this, aliquots of 25 µL of serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) were seeded in duplicate on Plate Count Agar (NCIPD, Sofia) and MacConkey Agar (NCIPD, Sofia) for the identification of *Bacillus subtilis* and *Escherichia coli*, respectively. Red and pink colonies grown on MacConkey Agar (NCIPD, Sofia) were presumptively identified as *Escherichia coli*. After incubation at 37°C for 24 h, the colony-forming unit per milliliter (CFU/ml) was determined and log-transformed (log10).

Effect of enzymatic solutions on dual-species biofilms

For protease production, bacterial strains were cultivated in medium prepared with $MgSO_4.7H_2O$ – 0.5 %; KH_2PO_4 – 0.5 %; $FeSO_4.7H_2O$ – 0.01%, glucose – 0.5 %; peptone – 0.75 %; at pH 5.0, 37°C, for 48 h and 150 rpm. The protease activity was determined based on the reaction of 150 µL of azocasein (0.5%) with an equal volume of the enzymatic extract for 40 min at 55°C. After reaching room temperature, 500 µL of 10% trichloroacetic acid (TCA) was added. The mixture was centrifuged at 4400 rpm for 15 min; then, 700 µL of supernatant was collected and the same amount of NaOH 1 M was added. The absorbance reading was performed on a spectrophotometer at 440 nm. A standard curve was prepared with BSA (bovine serum albumin).

The biofilm removal action of cell-free supernatants of *B. subtilis* 170 and *B.subtilis* 168 strains with proteolytic activity in the range from 50 µg/cm^3 to 250 µg/cm^3 was evaluated against 24 h biofilms. Three coupons were washed as before and then placed in a clean well. Negative controls were run in parallel by adding the corresponding buffer solution without enzyme. After the end of the fermentation process, the culture medium was subjected to centrifugation at 10 000 rpm at 4°C to separate the cells. Samples of the culture medium were filtered through a filter with a porosity of 0.2 µm. Determination of remaining adhered cells and visualisation of coupons was performed as described above. Results were expressed as the reduction in log CFU/cm^2, calculated as the mean of each replica difference in log CFU/cm^2 before enzymatic and after enzymatic treatment. After this, the two most effective enzymes were used in the following experiments.

Multispecies biofilm formation

For the dual-species biofilm, bacteria in post-exponential growth phase were suspended in ASW and inoculated in 24 well plates (Corning Incorporated Costar®, New York, NY, United States) to a final OD600 nm of 0.3 (0.15 per strain). For the biofilms involving two bacterial strains, the final OD600 nm was 0.3 or 0.4 (0.1 per strain). Controls included single species biofilms formed in the same concentrations and conditions than the multispecies biofilm. For the purposes of the task, 18-hour cultures of *B. subtilis* 170, *B. subtilis* 168 and *E. coli* K-12 1655 strains were prepared in advance in a medium broth (Laboratory "BulBio" – Sofia). 50 µl of the liquid cultures were inoculated into 5 cm^3 of M63 liquid medium (0.02 M KH_2PO_4, 0.04 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$ and 0.04 M glucose) to which cell-free supernatants of *Bacillus subtilis* 170 strains with proteolytic activityof 250 U/ml was added at concentration of 50 µg/cm^3, 150 µg/cm^3 and 250 µg/cm^{3}respectively. The control sample was cultured only in M63 medium without the addition of cell-free supernatants of *Bacillus subtilis* 170 strains with proteolytic activity.

Matrix components staining

For the matrix staining, a static biofilm of 48 h was performed. Each biofilm was stained with DAPI at 5 µg/mL (Sigma-Aldrich, Darmstadt, Germany) and one of the following matrix dyes. Exopolysaccharides were stained with the Wheat Germ Agglutinin (WGA) associated with the Alexa FluorTM 555 conjugate (Thermo Fisher Scientific, Waltham, MA, United States) at 100 µg/mL to label N-acetyl-glucosamine. After 30 min of incubation of each probe, each coverslip was washed 3 times in PBS 1×. Finally, the coverslips were mounted with a drop of ProlongTM Diamond Antifade before observation with confocal laser scanning microscopy **Leica TCS SPE** at wavelength of 540 nm.

Data Extraction From Images and Statistics

The biovolume, the average thickness and the evaluation of the maximum coverage in the CLSM pictures was determined with the COMSTAT software developed in MATLAB R2015a (MathWorks, Natick, MA, United States) as previously performed.

To test for statistically significant differences ($P < 0.05$) between two conditions a t-test was performed and between different time points, a two-way analysis of variance including the Bonferroni post-test were performed using SPSS 13.0 (IBM, Armonk, NY, United States).

Results and Discussion

The formation of biofilms is accompanied by the synthesis of extracellular matrix, which ensures the implementation of intercellular contacts in the structure of biofilms and its attachment to surfaces. One of the ways for the dispersion of this structure is the decomposition of the matrix. Among the

microbiota of biofilms, species stand out, characterized by the ability to form enzymes that specifically catalyze the hydrolysis of the main components that make up the structure of the matrix. Hydrolytic enzymes have an ability to break down proteins, nucleic acids and carbohydrates of extracellular matrix into their simplest units, produced by microorganisms, and to catalyze hydrolysis reactions. They belong to the special class of transferases, with water serving as the acceptor of the transferred group in the lysis of biopolymers (Matias et al., 2021; Balaban et al., 2003; Mikhailova et al., 2007; Blackledge et al., 2014).

The content of cell-free supernatants of *B. subtilis* 170 and *B. subtilis* 168 strains with proteolytic activity in the range from 50 μg/cm^3 to 250 μg/cm^3 leads to a statistically significant reduction in optical density reduction at 540 nm after staining with 0.1% solution of crystal violet in the process of cultivation of mixed biofilms ($p < 0.05$), as its value is established in a narrow range from 0.046 to 0.035 in the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains ($p > 0.05$) and from 0.036 to 0.031 in the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains ($p > 0.05$), which is significantly lower than the control samples (in the absence of protease activity) ($p < 0.05$). The values of the optical density after the staining with 0.1% crystal violet solution of the biofilms formed by the interaction of the two pairs of strains do not differ significantly ($p > 0.05$), which is a result of the similar mechanism of action of proteinases, secreted by *B. subtilis* 170 and *B. subtilis* 168 strains (Figure 1).

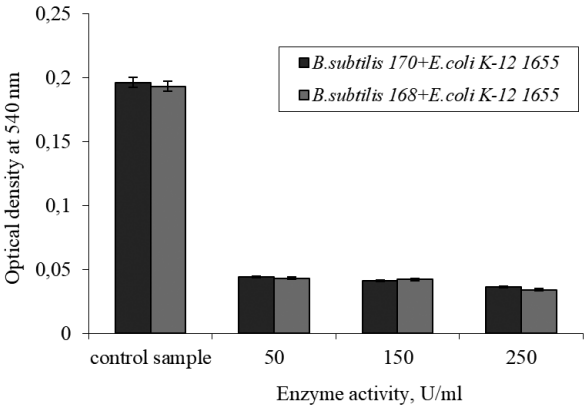

Fig. 1. The Influence of content of cell-free supernatants of *Bacillus subtilis* 170 strain with protease activity of 250 U/ml in the cultural medium on the dual-species biofilm growth

- $y = -0.0059.x+0.0523$, $r^2 = 0.9927$, $p < 0.05$ – *B. subtilis* 170 and *E. coli* K-12 1655
- $y = -0.0021.x+0.0379$, $r^2 = 0.9720$, $p < 0.05$ – *B. subtilis* 168 and *E. coli* K-12 1655

Subtilisin protease and glutamyl endopeptidase secreted by *Bacillus subtilis* JB 20-36 strain in the study of Mitrofanova et al. (2017) inhibit the formation of biofilms from strain *S. marcescens* SR 41-8000, resulting in a loss of biomass of 60–70% and is close to the results in the present study related to the influence of secreted proteinases from *B. subtilis* 170 and *B. subtilis* 168 strains on the formation of mixed biofilms involving *E. coli* K-12 1655 strain ($p > 0.05$). Proteases isolated from *Staphylococcus epidermidis* strains have a negative effect on the formation of biofilms of *Staphylococcus aureus* strains and their colonization on the surface layer of tissues and organs in the human body (Chen et al., 2013; Sugimoto et al., 2013). The effect of metalloproteases on *Serratia marcescens* strains has been associated with inhibition of the biofilm formation of strains of *Pseudomonas aeruginosa*, *S. epidermidis* (Kaplan et al., 2003) and *Listeria monocytogenes* (Longhi et al., 2008).

The effect of the protease secreted by strains of *B. subtilis* 170 and *B. subtilis* 168 after their cultivation in medium containing nitrogen as a nitrogen source and glucose as a carbon source affects the population structure of mixed biofilms, with the highest sensitivity of *E. coli* K-12 1655 strains, whose number decreases significantly from (14.2 ± 0.10) .10^{6}cfu/cm^3 to (0.12 ± 0.00) .10^{4}cfu /cm^3 in its association with *B. subtilis* 170 strain and up to (0.02 ± 0.00).10^{4}cfu/cm^3 in their interaction with *B .subtilis* 168 strain due to the increase of protease activity to a value of 250 U/cm^3 (Tables 1 and 2). The degree of reduction in the population of *B. subtilis* 170 and *B. subtilis* 168 strains is significantly smaller when reaching the enzymatic activity of 150 U/cm^3, which is closely dependent on the number of spores in the structure of mixed biofilms. Their number is (0.6 ± 0.00). 10^{2}cfu/cm^3 in the structure of the mixed biofilms of *B. subtilis* 170 and *E. coli* K-12 1655 strains and represents 0.32% of the total number of microbial population in the formed structure at the enzyme activity of 150 U/cm^3. The number of spores reached a value of (4.2 ± 0.30) .10^{2}cfu/cm^3 in the biofilms of the the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains under the same culture conditions during the study. The increase of the enzymatic activity of the culture medium to 250 U/cm^3 in the present study prevents the formation of spores in the structure of the mixed biofilms (Tables 1 and 2).

The development of biofilms on slides is accompanied by the appearance of single structures whose thickness varies in the range from 5.96 ± 0.19 μm to 4.74 ± 0.29 μm with an unevenness coefficient of 0.05 ± 0.017 to 0.03 ± 0.0032 with increasing content of cell-free supernatants with protease activity in the culture medium from 50 to 250 μg/cm^3 in biofilms formed by the mixed microbial population of *B. subtilis* 170 and *E. coli* K-12 1655 strains, as well as in the range from

Table 1. The impact of different concentrations of cell-free supernatants of *Bacillus subtilis* 170 strain with protease activity of 250 U/ml in the cultural medium on colony forming units and spore forming units of *B. subtilis* 170 and *E. coli* 1655 strains in the structures of dual-species biofilms

№	Cell-free supernatants of *Bacillus subtilis* 170 strain, μg/cm^3	Colony forming units in dual-species biofilms, cfu/cm^3		Spores, cfu/cm^3
		B. subtilis 170	*E. coli* K-12 1655	
1	0	(15.3±0.16).10^6	(14.2±0.10).10^6	(5.8±0.2). 10^2
2	50	(2.17±0.02).10^4	(0.72±0.03). 10^4	(0.8±0.00). 10^2
3	150	(1.57±0.01).10^4	(0.27±0.03). 10^4	(0.6±0.00). 10^2
4	250	(0.52±0.02).10^4	(0.12±0.00). 10^4	0

Table 2. The impact of different concentrations of cell-free supernatants of *Bacillus subtilis* 170 strain with protease activity of 250 U/ml in the cultural medium on colony forming units and spore forming units of *B. subtilis* 168 and *E. coli* 1655 strains in the structures of dual-species biofilms

№	Cell-free supernatants of *Bacillus subtilis* 170 strain, μg/cm^3	Colony forming units in dual-species biofilms, cfu/cm^3		Spores, cfu/cm^3
		B. subtilis 168	*E. coli* K-12 1655	
1	0	(10.7±0.25).10^6	(10.36±0.32).10^6	(15.4±0.6). 10^2
2	50	(2.38±0.05).10^4	(0.42±0.02). 10^4	(4.6±0.4). 10^2
3	150	(1.66±0.02).10^4	(0.06±0.00). 10^4	(4.2±0.30). 10^2
4	250	(0.6±0.03).10^4	(0.02±0.00). 10^4	0

Table 3. Morphometric features of dual-species biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655, *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains, depending on the concentration of cell-free supernatants of *Bacillus subtilis* 170 strain with protease activity of 250 U/ml in medium

Cell-free supernatants of *Bacillus subtilis* 170 strain, μg/cm^3	Mean thickness, μm*	Coefficient of unevenness	Relative area, μm^{2**}	Relationship area/volume, μm^2.μm^{-3***}
Dual-species biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655 strains				
0	10.35±0.41	0.02±0.03	0.99±0.12	0.090±0.003
50	6.28±0.11	0.01±0.02	0.33±0.02	0.159±0.003
150	5.96±0.19	0.03±0.03	0.27±0.02	0.167±0.005
200	4.74±0.29	0.05±0.07	0.16±0.01	0.211±0.013
Dual-species biofilms of *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains				
0	10.78±0.37	0.03±0.03	0.97±0.21	0.090±0.002
50	5.83±0.48	0.06±0.04	0.30±0.02	0.172±0.014
150	5.44±0.29	0.04±0.02	0.28±0.01	0.183±0.009
200	3.52±0.28	0.06±0.03	0.13±0.01	0.284±0.023

* $y = -3.913.\ln(x)+9.941$, $r^2 = 0.931$, $p < 0.05$ – *B. subtilis* 170-*E. coli* K-12 1655; $y = -4.998.\ln(x) + 10.36$, $r^2 = 0.942$, $p < 0.05$ – *B. subtilis* 168-*E. coli* K-12 1655

** $y = -0.593.\ln(x)+0.909$, $r^2 = 0.905$, $p < 0.05$ – *B. subtilis* 170-*E. coli* K-12 1655; $y = -1.473.e^{0,61.x}$, $r^2 = 0.901$, $p < 0.05$ – *B.subtilis* 168-*E. coli* K-12 1655

*** $y = -0.0812.\ln(x)+0.092$, $r^2 = 0.952$, $p < 0.05$ – *B. subtilis* 170-*E. coli* K-12 1655; $y = -0.07.e^{0,351.x}$, $r^2 = 0.915$, $p < 0.05$ – *B. subtilis* 168-*E. coli* K-12 1655

5.83 ± 0.48 μm to 3.52 ± 0.29 μm for biofilms obtained as a result of co-cultivation of of *B. subtilis* 168 and *E. coli* K-12 1655 strains. The average thickness of the formed structures changed significantly relative to its value in the control sample ($p < 0.05$). The ratio of the area of distributionof the formed structures to their volume changes exponentially in the studied pair of strains under the influence of cell-free supernatants with protease activity in the range from 50 to 250 μg/cm^3. It reaches a maximum value of 0.211 ± 0.013 μm^2.μm^{-3} for the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains and 0.284 ± 0.023 μm^2.μm^{-3} for the co-culture of *B. subtilis* 168 and *E. coli* K -12 1655 strains at a content of cell-free supernatants of 250 μg/cm^3, which significantly exceeds the value of the control sample, characterized by the absence of enzymatic activity in the study ($p < 0.05$).

The area of distribution of biofilms formed by mixed microbial communities is equal to 0.34 ± 0.002 μm^2 for the pair of *B. subtilis* 170 and *E. coli* K-12 1655 strains and 0.30 ± 0.002 μm^2 for the pair of *B. subtilis* 168 and *E. coli* K-12 1655 strains at a cell-free supernatant content of 50 μg/cm^3

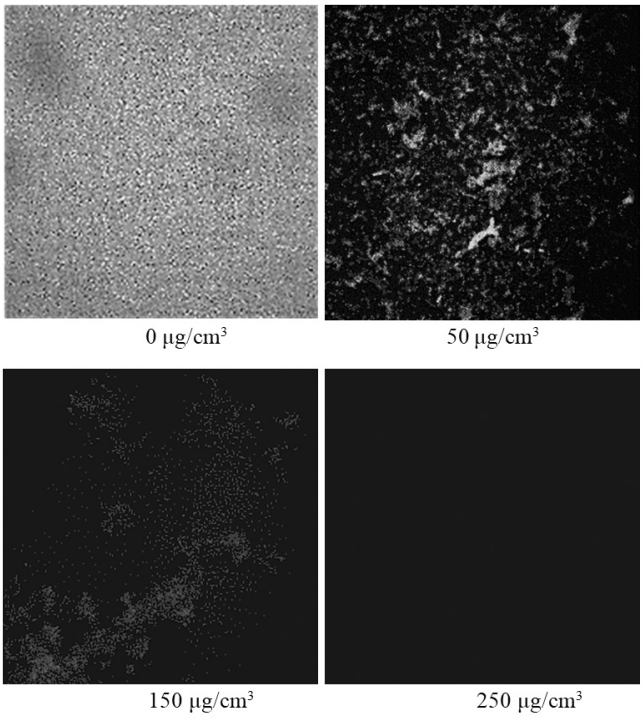

Fig. 2. Microscope study of biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655 strains at different concentrations of cell-free supernatants of *Bacillus subtilis* 170 strain with protease activity of 250 U/ml in the cultural medium

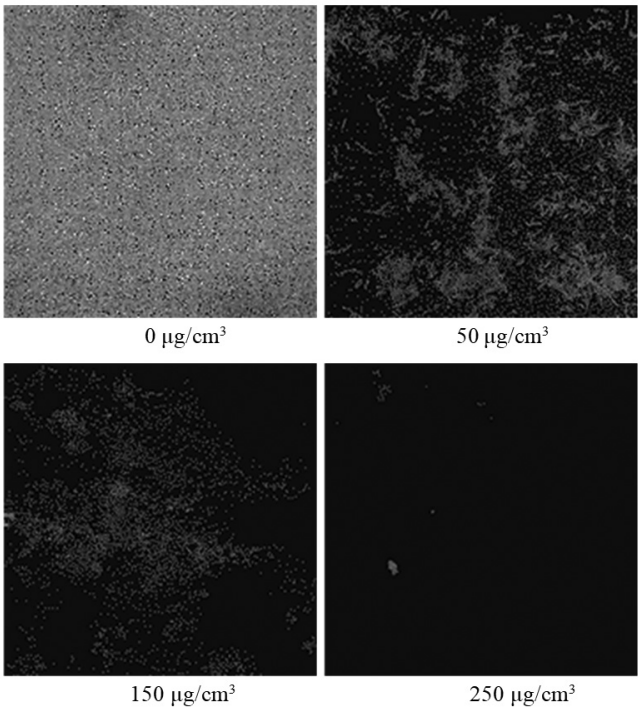

Fig. 3. Microscope study of biofilms of *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains at different concentrations of cell-free supernatants of *Bacillus subtilis* 170 strain with protease activity of 250 U/ml in the cultural medium

and constituted approximately 30% of the control sample in the absence of an inhibitory agent (Table 3, Figures 2 and 3). Their values decrease proportional to the increase in the activity of proteinase isolated from *B. subtilis* 170 and *B. subtilis* 168 strains, reaching 0.16 ± 0.01 μm² and 0.13 ± 0.01 μm², respectively, with increasing content of cell-free supernatants of 250 μg/cm³, which is significantly lower than their values in the cultivation of mixed biofilms in a medium containing glucose as a carbohydrate source and characterized by the absence of enzyme activity ($p < 0.05$).

Exposure to subtilin protease and glutamyl endopeptidase leads to disruption of biofilm structure, causing inhibition of cell adhesion to the surface layer of the substrate (Blackledge et al., 2014). At the heart of this mechanism is the hydrolysis of the protein components that make up the matrix of biofilms (Blackledge et al., 2014). Serine prosthesis and glutamylendopeptidase in relatively low concentrations cause destruction of the overall structure of biofilms. In the study of Mitrofanova et al. (2017) treatment with a protease isolated from *Bacillus subtilis* strain is accompanied by a significant reduction in protein content in the matrix of the biofilm of of *S. marcescens* strain. Significant reduction of protein content in the matrix of biofilms of *B. licheniformis* strain occurs after treatment of DNA-ase and proteinase K. About 64% of amyloid fibers of the structures undergo hydrolysis by exogenous enzymes (Kaplan et al., 2003). These observations and the results of the present study determine the ability of proteases to disperse biofilms formed by strains of one or more bacterial species and testify to the role of proteins in ensuring the structural stability of formed biofilms.

Conclusions

The exoprotease secreted by *B. subtilis* 170 strain in the culture medium lead to a significant decrease in the biomass, average thickness, relative area of spread of biofilms formed with the participation of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains. The negative effect of exoprotease, on the formation of biofilms of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains is determined by their concentration, and its decrease is accompanied by a proportional increase in

their optical density, average thickness, relative distribution area and decrease in the ratio of area to volume.It is important to further investigate these enzymes in regard to their purification and application on biofilms from other microbial species, in addition to other possible applications.

The findings described herein reinforce the relevance of research with soil-drawing bacteria of *Bacillus subtilis* strains. As far as we believe, this is the first study using a combination of hydrolytic enzymes, produced by members of microbiota of the rhizosphere, in the soil biofilm formation process and the potential ecological roles of soil biofilms.

References

Brooks, J. D. & Flint, S. (2008). Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science & Technology*, *43*(12), 2163-2176.

Balaban, N., Sharipova, M., Gabdrakhmanova, L., Sokolova, E., Garusov, A., Milgotina, E. & Leshchinskaya, G. I. (2003). Isolation and characterization of glutamylendopeptidase 2 from *Bacillus intermedicus*. *Biochemistry*, *68*(11), 1217-24.

Blackledge, M. S., Worthington, R. J. & Melander, C. (2014). Biologically inspired strategies for combating bacterial biofilms. *Current Opinion in Pharmacology*, *13*(5), 699–706.

Chen, C., Krishnan, V., Macon, K., Manne, K., Narayana, S. & Schneewind, O. (2013). Secreted Proteases Control Autolysin-mediated Biofilm Growth of *Staphylococcus aureus*.*Journal of Biological Chemistry*, *288*(41), 29440 –29452.

Delaviz, Y., Santerre, J. P. & Cvitkovitch, D. G. (2015). Biomaterials and Medical Device – Associated Infections. Elsevier, 223-254.

Flemming, H.-C. & Wuertz, S. (2019). The biofilm matrix. *Nat. Rev. Microbiol*., *17,* 1-15.

Herschend, J., Damholt, Z. B. V., Marquard, A. M., Svensson, B., Sørensen, S. J., Hagglund, P. & Burmølle, M. (2017). A meta-proteomics approach to study the interspecies interactions affecting microbial biofilm development in a model community. *Sci. Rep*., 7. H.

Kaplan, J., Ragunath, C., Ramasubbu, N. & Fine, D. (2003). Detachment of Actinobacillus actinomycetemcomitans Biofilm Cells by an Endogenous -Hexosaminidase Activity. *Journal of Bacteriology*, *185*(16), 4693–4698.

Longhi, C., Scoarughi, G. L. & Poggiali, F. (2008). Protease treatmentaffects both invasion ability and biofilm formation in *Listeriamonocytogenes. Microbial Pathogenesis*, *45*(1), 45–52.

Li, B. (2005). Microbial Dynamics of Biofilms. In: Water Encyclopedia, *Wiley Science.*

Matias, R., Sepúlveda, A., Batista, B., Mesquita, J., de Lucena, V. & Albuquerque, P. (2021). Degradation of *Staphylococcus aureus* Biofilm Using Hydrolytic Enzymes Produced by Amazonian Endophytic Fungi. *Applied Biochemistry and Biotechnology*, *193,* 2145–2161.

Mikhailova, E. O., Mardanova, A. M., Balaban, N. P., Rudenskaya, G. N. & Sharipova, M. R. (2007). Isolation and characterization of a subtilisin-like proteinase of *Bacillus intermedius* secreted by the *Bacillus subtilis* recombinant strain AJ73 at different growth stages. *Biochemistry*, *72*(2), 192–198.

Mitrofanova, O., Mardanova, A., Evtugyn, V., Bogomolnaya, L. & Sharipova, M. (2017). Effects of *Bacillus* Serine Proteases on the Bacterial Biofilms. *BioMed Research International,* 1-10.

Røder, H., Liu, W. S., Sørensen, J. & Burmølle, M. (2019). Interspecies interactions reduce selection for a biofilm-optimized variant in a four-species biofilm model. *Environmental Microbiology Reports*, *11*(6), 835-839.

Sugimoto, S., Iwamoto, T., Takada, K., Okuda, K., Tajima, A., Iwase, T. & Mizunoe, Y. (2013). *Staphylococcus epidermidis* Esp Degrades Specific Proteins Associated with *Staphylococcus aureus* Biofilm Formation and Host-Pathogen Interaction. *Journal of Bacteriology*, *195*(8), 1645–1655.

Wang, W., Li, X., Wang, J., Xu, H. &Wu, J. (2004). Influence of biofilms growth on corrosion potential of metals immersed in seawater. *Materials and Corrosion*, *55*(1), 30-35.

Vishwakarma, V. (2019). Impact of environmental biofilms: industrial components and its remediation. *Journal of Basic Microbiology*, *60*(3), 198-206.

Velmourougane, K., Prasanna, R. & Saxena, A. (2017). Agriculturally important microbial biofilms: Present status and future prospects. *Journal of Basic Microbiology*, *57*(7), 548-573.

Received: **June, 08, 2023**; *Approved*: **August, 10, 2023;** *Published:* **April, 2024**