ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of Cultivation Conditions On Biofilm Formation of *Bacillus Subtilis.*

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ABSTRACT

Biofilms are the moving, constantly changing, heterogeneous bacterial communities. Microorganisms growing in a biofilm are highly resistant to both antimicrobial agents and immune mechanisms. Objective of this paper was to study the effect of extracellular and membrane-bound proteases *B. subtilis* on the formation of biofilms in liquid media. The strains *B. subtilis 168* (wild type) and *B. subtilis BRB14* (protease-deficit strain) were examined on the ability to form biofilms in liquid medium. The LB and 1% glucose LB media were used as rich culture medium, and the synthetic *E*-medium was chosen as poor culture medium. A decreased formation of biofilms by 20% was observed during growth in the glucose-containing LB medium as compared with the glucose-free LB medium. The level of biofilm formation in the synthetic E-medium was 7 times higher than the total level in both LB media. Under identical culture conditions, the ability of mutant strain *B. subtilis* to form biofilms was 10%-15% higher than the level of biofilm formation by a wild strain. By the 48th hour of growth, the level of biofilm content in the culture of both strains reached maximum and declined significantly by the 72nd hour of growth. pH-Optimum of biofilm formation by both strains *B. subtilis* was 7.4. The temperature optimum for the formation of biofilms for both strains *B. subtilis* also ranged from 22°C to 45°C. The results of the studies allow us to conclude that the extracellular and membrane-bound proteinases *B. subtilis*, deleted in the mutant strain, do not play a key role in the formation of biofilm, however, their effect is more significant during growth of bacteria in a nutrient-rich environment rather than in synthetic one.

Keywords: Bacillus subtilis, biofilms, proteinase-deficit strain, culture medium, pH optimum, temperature optimum.

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ISSN: 0975-8585

INTRODUCTION

Naturally, most of bacteria form biofilms [Henrici 1933; Costerton et al. 1999; Hall-Stoodley et al. 2004, 2009]. In general, the biofilm can be characterized as bacteria, embedded in a thick mucous layer consisting of sugars and proteins. This cellular barrier protects microorganisms from external influences [Iliina et al., 2004]. The formation of biofilms is one of the major virulence factors of pathogenic bacteria. Microorganisms growing in a biofilm are highly resistant to both antimicrobial agents and immune mechanisms. Biofilms are associated with a variety of human diseases, such as valve endocarditis, cystic fibrosis, periodontitis, otitis media, biliary tract infection, etc., they colonize medical equipment and implants, causing thereby the recurrent infections [Hancock et al., 2007]. In the laboratory environment, strains of bacteria become less able to form biofilms [Lemon et al., 2008]. Therefore, the natural strains suit more for studying the ways of regulation of biofilm formation. Exopolysaccharidic structure of biofilms is common to all species of biofilm-forming bacteria [Donlan et al., 2002]. This suggests common genetic mechanisms of regulation of the biofilm formation. Therefore, the identification of specific genes involved in the global process of formation of a biofilm structure is of particular interest. A non-pathogenic gram-positive spore-forming soil strain *B. subtilis* is a model system for the investigation of biofilms [Branda et al., 2001; Hamon & Lazazzera, 2006]. To find out the ways of biofilm formation by *B. subtilis*, we examined two strains: the natural isolate of *B. subtilis 168,* and the protease-deficit strain *B. subtilis BRB14* with the deleted eight genes of extracellular proteases (*nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA*) and two membrane-bound proteases (*htrA* and *htrB*).

Objective of this paper was to study the effect of extracellular and membrane-bound proteases *B. subtilis* on the formation of biofilms in liquid media.

MATERIALS AND METHODS

Objects of the study were a wild type strain *Bacillus subtilis 168* from the museum laboratory of the Department of Microbiology at Kazan Federal University, and a mutant strain *Bacillus subtilis BRB14* with deleted genes of extracellular proteases (neutral proteinase *nprB*, subtilisin E *aprE*, minor extracellular proteinases *epr* and *vpr*, bacillopeptidase *bpr*, bacillolysine *nprE*, metalloproteinase *mpr*, cell wall-associated proteinase *wprA*) and membrane-bound proteases (serine proteases *htrA* and *htrB*), kindly provided for by prof. R. Cranenburgh, “Cobra Biologics”, UK.

The strains were cultured at pH 7.4 and temperature of 37ºC in LB medium and LB with glucose at final concentration of 1%, as well as in the synthetic *E*-medium, which composition is described in [Morikawa et al., 2006]. Culture was conducted on the vibration table (“B.Broun”, Germany) with vibration intensity of 200 rpm at 37ºC. Ratio of the medium volume to the volume of the flask was 1:7. Seed material was a 16-hour inoculum (1% v/v).Bacterial growth was monitored by the change in the optical density of the culture at 590 nm. Biomass amount was expressed in absorbance units. Spore formation was determined by counting cells and spores by Peshkov’s method in microscopy mode with the microscope Carl Zeiss Jena (Germany) at 1600x magnification in 4 visual fields. The amount of free spores was expressed as percentage of the total number of vegetative and sporulating cells.

The biofilm formation was identified by the method of incubation with crystal violet (CV) [O'Toole et al., 1999] with modifications [Merritt J. et al., 2005]. The overnight culture was diluted to OD_{600} 0.3, 1 µl was added to 99 µl of *E*-medium and placed in a 96-well round-bottom sterile plastic plates with a cover into an incubator at 37°C for 48 hours, without vibration. Then, the well content was carefully removed, flushed twice with distilled water, 150 µl of 1% CV was added to each well, and incubated for 25 minutes at room temperature. Then flushed twice again with distilled water. The biofilm-bound CV was solubilized with 150 µl of dimethylsulfoxide (DMSO) and the solution absorbance was measured at 570 nm. The measurement was conducted with a spectrophotometer BioRad xMark Microplate (USA).

Experimental data were analyzed with Microsoft Excel program. Characteristics were described and compared with the use of data of four independent experiments. he results were considered statistically significant at the mean standard deviation of $\sigma \leq 10\%$.

ISSN: 0975-8585

RESULTS AND DISCUSSIONS

We have studied the effects of different culture media on the intensity of biofilm formation by strains *Bacillus subtilis* (Fig. 1). The LB medium and LB medium with glucose at final concentration of 1% were used as rich culture media. Adding an additional source of readily available carbohydrates (glucose) to the culture medium could be a stimulating factor for the formation of biofilms. The results of the experiment showed a slight (<10%) reduction in biofilm growth in a medium supplemented with glucose as compared with the glucose-free LB (Fig. 1). Using a composite synthetic *E*-media for culture ensured increase in the level of biofilm formation by both strains approximately by 7 times as compared with both LB media.

The level of biofilm formation by a wild strain exceeded the same of the mutant strain by 30% -40% in LB media and by 10% in E-synthetic medium (Fig. 1).

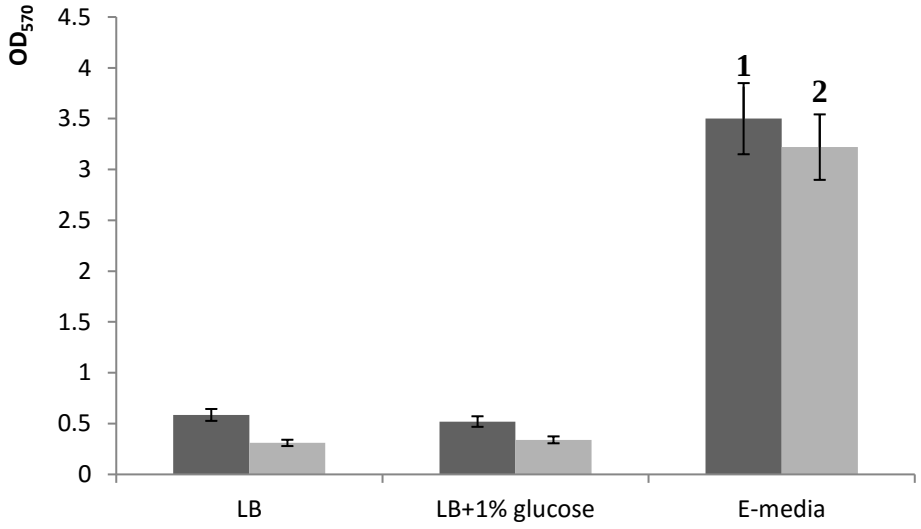

Fig. 1. Biofilm formation by a wild strain *B.subtilis 168* (1) and protease-deficit strain *B.subtilis BRB14* (2) in various media for 48 hours at 37°C and pH 7.4. σ≤10%.

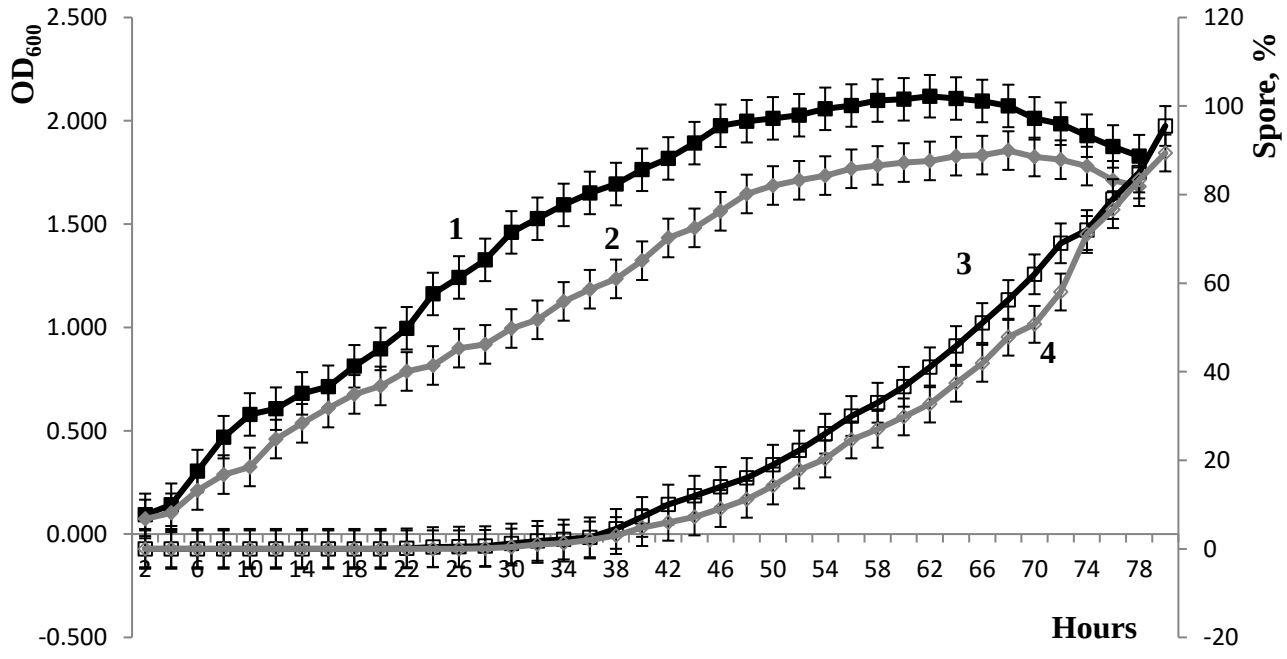

Fig. 2. – Dynamics of growth and sporulation of strains *B. subtilis.* σ≤ 10%.

1 - the growth of culture of the strain *B. subtilis 168*; 2 - the growth of culture of strain *B. subtilis BRB14*; 3 - sporulation dynamics of the strain *B. subtilis 168*; 4 - sporulation dynamics of the strain *B. subtilis BRB14*;

Obviously, a high content of energy-rich nutrients in the culture medium does not contribute to the formation of biofilms. Perhaps, the transition of the culture to life strategy of biofilm formation is caused by nutritional deficiencies. Thus, the extracellular proteinase deleted in the mutant strain *B. subtilis BRB14*, apparently, do not play a key role in the process of biofilm formation, since biofilms are formed by both strains. It is interesting that the difference in the level of biofilm formation by wild and mutant strains is higher (to 40%) in rich nutrient media and virtually unreliable (<10%) in a synthetic medium. All subsequent studies were carried out on a liquid synthetic *E*-medium at pH 7.4 and 37°C.

Comparison of the dynamics of growth of wild and protease-deficit strains *B. subtilis* showed that the growth of the wild strain exceeded on average by 15% -45% the level of growth of the mutant strain (Fig. 2). At the same time, we have shown that the accumulation of free spores in the culture medium occurred equally in both strains - free spores (10%) were found in the cultures of 44-46th hours of growth (Figure 2).

Further, we studied the dynamics of biofilm formation by both strains *B. subtilis* in the liquid medium at pH 7.4 and 37°C (Fig. 3).

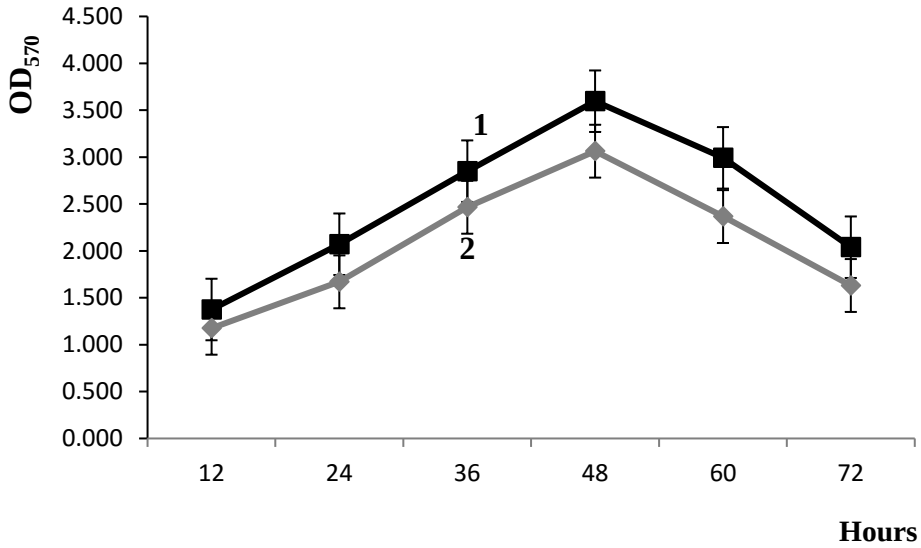

Fig. 3. Biofilm formation dynamics by strains *B. subtilis* in the liquid medium at pH 7.4 and 37°C. σ≤ 10%.
1 - *B. subtilis 168;* 2 - *B. subtilis BRB14*

Investigation of the dynamics showed that the strains are similar in formation of biofilms with a maximum at 48th hour of growth. At this hour, the level of biofilm formation by the strain *B. subtilis 168* exceeded the same by the strain *B. subtilis BRB14* by 15% (Fig. 3). This indicates that the proteases deleted in the mutant strain have a positive effect on the formation of biofilm, however, do not play a key role. During the rest hours, the difference in the level of biofilm formation by both strains was less than 10%, and therefore was not significant.

To determine the optimal culture conditions for the formation of biofilms by strains *B. subtilis* we examined the effect of factors such as pH and temperature of the medium on the process. Measurements were conducted on 48th hour of culture growth at maximum content of biofilms structures in the medium of both strains *B. subtilis* (Fig. 4).

It was found that pH 7.4 is the best for biofilm formation by the strains *B. subtilis* (Fig. 4). This is pH of slightly alkaline soil - a typical habitat of *B. subtilis.* Increase in pH in both strains up to 8.0 resulted in the reduced biofilm formation on average by 6%, the further increase up to pH 8.5-9.0 resulted in a sharp decrease in the biofilm content in the environment. Obviously, more acidic pH is optimal for biofilm formation. The

ISSN: 0975-8585

difference in the level of biofilm formation by both strains was less than 10%, and therefore was not significant.

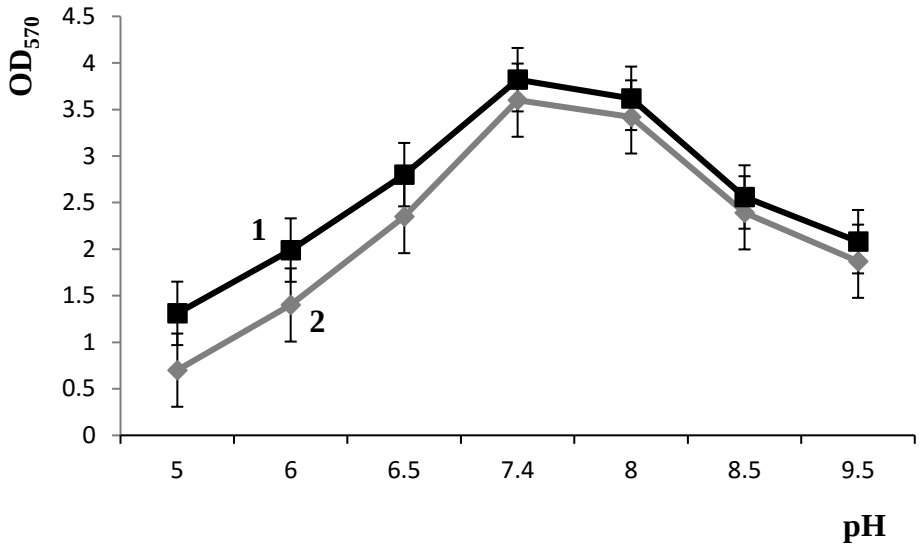

Fig. 4. – pH optimum for biofilm formations by strains *B. subtilis.* σ≤ 10%.
1 - *B. subtilis 168;* 2 - *B. subtilis BRB14*

We have studied the effect of temperature on the intensity of biofilm formation by strains *B. subtilis.* Our experiments have shown that the temperature range from 22°C to 45°C is optimal for film formation (Fig. 5). The difference in the level of biofilm formation by both strains was less than 10%, and therefore was not significant.

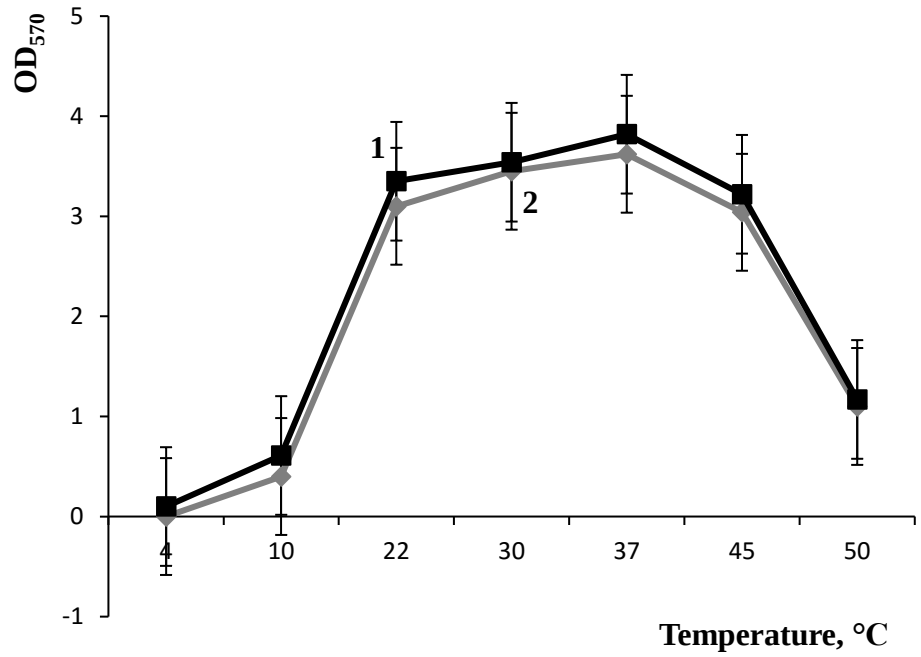

Fig. 5. – Temperature optimum for biofilm formations by strains *B. subtilis.* σ≤ 10%. 1 - *B. subtilis 168;* 2 - *B. subtilis BRB14*

SUMMARY

As a result of the study of the influence of culture medium factors on the intensity of the biofilm formations by strains *B. subtilis 168* and *B. subtilis BRB14,* we have shown that both strains demonstrate similar dynamic curves of biofilm formation with its maximum on hour 48. Both strains significantly better (700%) form biofilms in the *E*-synthetic medium than in LB rich culture media. Both strains also have an optimum pH for the formation of biofilms equal to 7.4 and a temperature optimum in the range from 22°C to 45°C. All experiments showed the level of biofilm formation by a wild strain higher than that of the mutant strain by not more than 15%, which is close to a significant level of difference (10%).

CONCLUSION

Slight differences in biofilm formation indicate that the extracellular and membrane-bound proteinases deleted in the mutant strain *B. subtilis BRB14* affect the process of biofilm formation, and possibly are managed by the same signaling systems which play a key role in the biosynthesis of proteases formed from bacilli secret.

ACKNOWLEDGEMENTS

This work is performed in the framework of the state support of Kazan (Volga) Federal University among the world's leading research and education centers, partially at the expense of subsidies allocated to Kazan Federal University to perform government tasks in the field of scientific research.

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