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Air-Liquid Interface Biofilms of *Bacillus cereus*, *Bacillus subtilis* and *Pseudomonas fluorescens*

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Abstract

Biofilms can be defined as communities of microorganisms attached to a surface. The purpose of this work was to investigate the roles of strain diversity and environmental conditions in biofilm formation by Bacillus subtilis, Bacillus cereus and Pseudomonas fluorescens using various assays and culture conditions and a range of strains.

Experiments were achieved using several protocols based on comparative analysis studies. The physical and biological parameters effects, strain of bacteria, substrate composition and temperature on biofilm formation and evolution were analyzed. The results in this article indicate that tested bacterial strains can form monospecies or mixed biofilms and can behave physiologically different in biofilm formation.

Keywords: biofilm, Bacillus subtilis, Bacillus cereus, Pseudomonas fluorescens

1. Introduction

A biofilm is a multicellular complex, formed of microorganisms that are attached to a surface and embedded in a matrix, consisting of Extracellular Polymeric Substances (EPS). Cells within a biofilm are surrounded by the EPS matrix and cells in the outer layers of the biofilm, protecting them from harsh influences from the environment, thereby making them more resistant to cleaning agents and other antimicrobial substances [6].

Biofilm formation consists of initial attachment, microcolony and EPS (extracellular polymeric substances) production, followed by maturation [3]. It has been observed that attachment of bacterial cells is affected by several factors, including the medium in which they are grown, motility, growth phase of the cells, type and properties of the inert material, presence of organic material ("conditioning film"), temperature, pH, length of contact time, production of extracellular polysaccharides, and cell-to-cell communication [1-4].

The purpose of this work was to investigate the *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas fluorescens* abilities to form biofilms at the air liquid interface, in media with different organic composition and to study morphological features of biofilms and their composition.

2. Materials and methods

The bacterial strains used, were purchased from the American Type Culture Collection: *Pseudomonas fluorescens* ATCC 13525, *Bacillus subtilis* ATCC 19659 and *Bacillus cereus* ATCC 10876. Cell biomass obtained after reactivation of stock cultures, was separated by centrifugation at 10.000 rpm, for 15 minutes and re-suspended in a volume of 5 ml of distilled water. For *Bacillus subtilis* and *Bacillus cereus* strains, obtained suspensions were pasteurized at 70-80˚C for 20 minutes and immediately cooled on ice, to inactivate vegetative cells.

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Spores have been preserved as a dense suspension in sterile distilled water at 4˚C, until use.

Pseudomonas fluorescens cells were cultivated on a basal medium (peptone 7 g/l, $MgSO_4$ 2 g/l, $CaCl_2$ 0.05 g/l) containing agar, at 25˚C, for 24h. The cells obtained were preserved in sterile distilled water as concentrated suspension at a temperature of 4˚C, until use.

The media for biofilms formations were liquid, basal medium, simple or supplemented with carbon sources, glucose 5 g/l glucose or 5 g/l glycerol.

Mono species or mixed biofilms were formed by cultivation in tubes, containing 4.5 ml liquid medium, in the stationary conditions. Each medium was inoculated with 0.5 ml suspension of cells containing about 10^4 CFU/ml of bacterial cells. Different conditions for biofilm formation were used:

Protocol I	Basal medium	Incubation at 25˚C, for 2, 3, 7, 9 days
Protocol II	Basal medium supplemented with 5g/l glycerol	
Protocol III	Basal medium supplemented with 5g/l glucose	

The following factors were varied: culture media composition and time of incubation. Culture media used for inoculation was a basal medium with 3% agar.

Biofilm mass was determined by weighing the pellicle of biofilm before and after lyophilization. Freeze drying regime used was: primary drying reached -42˚C temperature and a pressure 0.90 atm, time 2 h and final drying reached a temperature of -36˚C and a pressure of 0.80 atm, time 10min.

3. Results and Discussion

The growth was monitored by determining the CFU/g biofilm in the intervals: 2, 3, 6, 7 and 9 days (Fig. 1, 2 and 3). Protocol I (basal medium, 25˚C) showed a similar development between cell counts of biofilm formed by *P. fluorescens* (7.75, 8.46 and 7.79 log CFU) and cell counts of mixed biofilm *P. fluorescens* and *B. cereus* (7.67, 8.2 and 7.83 log CFU), in the time interval 2 to 6 days.

Since 7th day, each biofilm was characterized by a different development, highlighted by the cell counts, which was increasing by 1.5 log CFU for mixed biofilms and only 0.52 log CFU for strain *P. fluorescens* (Fig. 1). After 9 days of development, biofilms formed by *B. subtilis*, *P. fluorescens*, and mixed biofilms had an almost similar level of development of 8.66 log CFU, 8.82 and 9.13 log CFU. Only *B. cereus* preferentially formed biofilms on the surface liquid media. The biofilm *B. cereus* growth was rapid, by 9.56 log CFU after 3 days, followed by a sudden reduction of the cells number after 7 days and after 9 days of growth return at a rate of 10.15 log CFU.

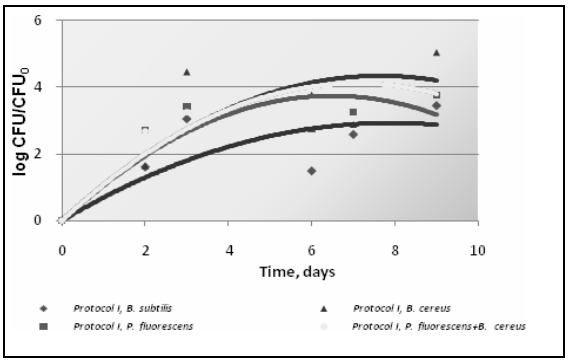

Figure 1. Dynamics of biofilm formation after 9 days of cultivation of bacteria in different environmental conditions – Protocol I

Biofilm mass for protocol I (basal medium, 25˚C) had a slight variation for all the species used. A similar behavior was observed between *B. cereus* biofilm mass (0.1241 g/g biofilm) and *P. fluorescens*/*B. cereus* biofilm mass (0.1445 g/g biofilm). Production of larger quantities of EPS is characteristic of mixed biofilm.

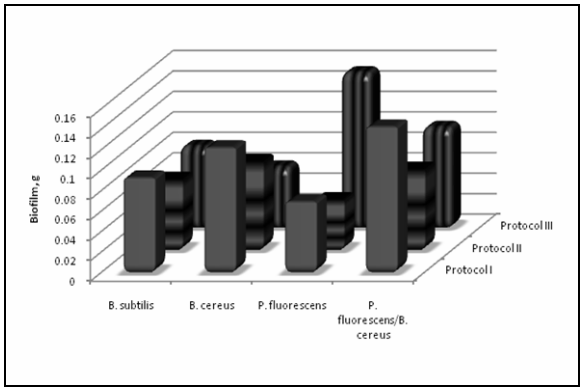

Figure 2. Mass variation on the biofilms formation

P. fluorescens biofilm exhibited a rapid increase (Fig. 3) for the protocol II (basal medium supplemented with 5g/l glycerol, 25˚C).

The degree of cell multiplication in mixed biofilm *P. fluorescens* and *B. cereus* is almost similar to the *P. fluorescens* biofilm for 2 to 7 days of cultivation, with a reduction of 0.81 log CFU in the 9th day of biofilm maturation. The *B. cereus* biofilm growth was rapid, by 9.06 log CFU after 6 days, to 10.71 log CFU after 9 days. In 9th day *B. subtilis* biofilm reached values similar to *B. cereus* biofilm (10.8 log CFU).

Biofilm masses obtained for protocol II (basal medium supplemented with 5g/l glycerol, 25˚C) have values lower than those from protocol I. *B. cereus* biofilm registered a maximum biomass accumulation (Fig. 2) for protocol II.

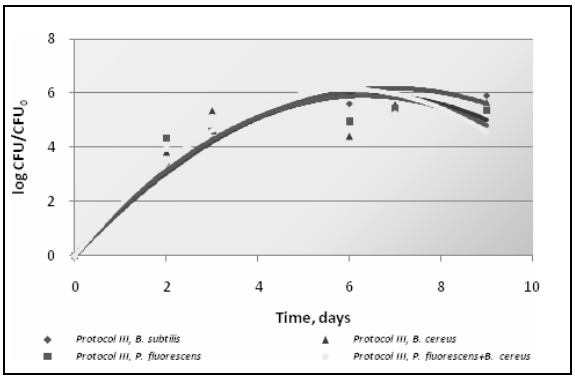

Figure 3. Dynamics of biofilm formation after 9 days of cultivation of bacteria in different environmental conditions – Protocol II

B. subtilis biofilm had an exponential increase on 9th day (2.52 log CFU) for the protocol III (basal medium supplemented with 5g/l glucose, 25˚C), probably due to the presence of glucose, compared with the number of cells after 2 days of growth (Fig.4). Cell counts of mixed biofilm *P. fluorescens* and *B. cereus* decreased between 6 to 9 days, with a reduction of 1.12 log CFU on 9th day by comparison with the degree of multiplication after two days of growth. *B. cereus* biofilm after 6 days of development, reached a decrease in the number of cells, followed by an increase after 7 days, obtaining a value of 10.77 log CFU, similar to that of *B. subtilis* biofilm. According to the centralization shown in Fig. 4, protocol III is considered the best choice for biofilm formation and development, especially for *B. subtilis*. In this variant, the medium is supplemented with glucose, at a temperature of 25˚C.

In protocol III (basal medium supplemented with 5g/l glucose, 25˚C) there was an increase of mass for *P. fluorescens* biofilm, compared with all versions of the protocols.

The increase may be linked to a high amount of EPS (extracellular polymeric substances). Such a correlation can be established also for mixed biofilm.

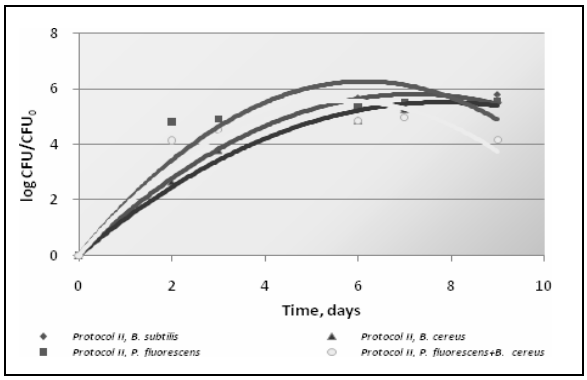

Figure 4. Dynamics of biofilm formation after 9 days of cultivation of bacteria in different environmental conditions – Protocol III

One of the factors that stimulate the biofilm formation on the liquid media surface is oxygen [5] Limiting access to oxygen for *P. fluorescens*, adversely affects the pellicle development of air-liquid interface biofilm [7].

4. Conclusion

Experiment results allowed formulating the following conclusions:

The bacteria species used in this study: *B. subtilis, B. cereus* and *P. fluorescens*, can grow and constitute cell communities (biofilms) on the surface of liquid nutrient media;

The study showed the combined effect of factors: temperature - nutrients on the biofilm formation influenced the rate of multiplication of bacteria in biofilm. Nutrient concentrations influenced biofilm morphology; biofilms on basal media supplemented with 5g/l glycerol or 0.05% glucose, are firm and uniform;

B. cereus, B. subtilis and *P. fluorescens* biofilms behave physiologically different. *B. cereus* and *B. subtilis* biofilms were mainly formed by cell associations, whereas the mixed (*B. cereus* and *P. fluorescens*) and *P. fluorescens* biofilms contained relatively high amounts of EPS.

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