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RESEARCH ARTICLE

BACTERIAL BIOFILMS FORMATION AT AIR LIQUID INTERFACES

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Abstract
Bacterial biofilms have great significance for food industry as spoilage agents and for wastewater and contaminated air biological treatments. To date most attention has focused on biofilms formed from the colonization of solid-liquid or solid-air interfaces. The colonization of the interface between air and liquid, which can be selectively advantageous for aerobic or facultative aerobic bacteria, has been rarely studied. In this work the ability of the *Bacillus subtilis* ATCC 19659, *Bacillus cereus* ATCC 10876 and *Pseudomonas fluorescens* ATCC 13525 to form biofilms at the interface between air and liquid (pellicle) in medium with different composition were investigated. The ability of commercial strains of bacteria to generate biofilms under different experimental conditions for a total period of 9 days at 25°C and 37°C was studied. The results in this article indicate that tested bacterial strains can form monospecies or mixed biofilms and can behave physiologically different in biofilm formation.

Key words: biofilm, air-liquid interface, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas fluorescens*

Introduction

Biofilms can be defined as communities of microorganisms attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community (O'Toole, 2000).

Attachment is the first stage in the formation of a biofilm and subsequent growth often occurs concomitantly with the production of an extensive network of exocellular polymers (often exopolysaccharides) that facilitate firm adherence of bacteria to daughter cells and to the surface (Kuchma and O'Toole, 2000; Sutherland, 2001; Wilson, 2001). Biofilm architectures are highly variable, ranging from open structures containing channels and columns of bacteria, to structures with no obvious pores and densely packed regions of cells (Lawrence et al., 1991; Watnick and Kolter, 1999, 2000; Wimpenny et al., 2000). The close physical association of cells within these biofilms results in a structure with significant physical properties and changes in bacterial physiology compared with free-living cells (Costerton et al., 1995; Kuchma and O'Toole, 2000; Wimpenny et al., 2000; Sutherland, 2001).

In actual studies most attention has focused on biofilms formed at solid-liquid (S–L) interfaces, but also to biofilms formed on interface between air and liquid (A–L interface), which provides bacteria access to both the gaseous (e.g. oxygen) and liquid (e.g. nutrient) phases.

The purpose of this work was to investigate the *Bacillus subtilis* ATCC 19659, *Bacillus cereus* ATCC 10876 and *Pseudomonas fluorescens* ATCC 13525 abilities to form at air liquid interface biofilms, in media with different organic composition and to study morphological features of biofilms and their composition.

Experiments were achieved using several work protocols based on comparative analysis studied. The physical and biological parameters effects, strain of bacteria, substrate composition and temperature on biofilm formation and evolution were analysed.

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Materials and methods

Bacteria and conditions of cultivation for biofilm formation

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

Cells biomass obtained after reactivation of stock cultures, was separated by centrifugation at 10.000 rpm, for 15 min and re-suspended in a volume of 5 ml of distilled water.

For *Bacillus subtilis* and *Bacillus cereus* strains, obtained suspensions were pasteurised at 70-80˚C for 20 min and immediately cooled on ice, to inactivate vegetative cells. 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, MgSO4 2 g/l, CaCl20.05 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.

Monospecies 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, varying the following factors: culture media composition, time and temperature of incubation:

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

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	
Protocol IV	Basal medium	Incubation 37˚C, for 2, 3, 7, 9 days
Protocol V	Basal medium supplemented with 5g/l glycerol	
Protocol VI	Basal medium supplemented with 5g/l glucose	

The qualitative and quantitative evaluation of biofilms

For qualitative description of monospecies biofilms formed by *Bacillus subtilis, Bacillus cereus*, *Pseudomonas fluorescens* and mixed biofilm *(Pseudomonas fluorescens* and *Bacillus subtilis)* on the surface of the liquid media were assessed following characteristics: morphological characteristics, surface geometry, density and opacity.

For each strain analyzed, biofilms profiles were shaped having regard to biofilms development. For quantitative evaluation of each biofilms formed by each protocol used was given a score from 0 to 4 as follows:

0 - Not visible;
1 – Film appearance on the surface medium;
2 – Very thin pellicle on the surface medium;
3 – Film on walls and the surface medium;
4 – Pleated pellicle on the surface medium;

Results and discussion

The morphological characteristics of biofilms after two and nine days of cultivation of bacteria in mentioned conditions are presented in Tables 1 and 2.

In second day, all of bacteria form a biofilm, like a thin pellicle, particularly at a temperature of 25˚C (protocols I, II and III) (Table 1).

Table 1 Morphological characteristics of biofilms formed by bacteria after 2 days of growth on liquid media, in stationary conditions

Strain of bacteria	Protocols of cultivation conditions	Morphological characteristics of biofilm	The biofilm formation level
B. subtilis	I	visible traces of biofilm, brittle pellicle on tube walls	1
	II	fragments of biofilm	1
	III	visible traces of biofilm, light pellicle on tube walls	1
	IV	visible traces of biofilm, light veil on the tube walls	1
	V	not visible signs of biofilm	0
	VI	visible traces of biofilm, light veil on the tube walls	1
B. cereus	I	light pellicle on tube walls and at medium surface	1
	II	light pellicle on tube walls and at medium surface	2
	III	light pellicle on tube walls and at medium surface	1

	IV	pleated veil on the walls and the surface medium	3
	V	light pellicle on medium surface and on tube wall	2
	VI	light pellicle on medium surface and on tube wall	2
P. fluorescens	I	light visible pellicle	1
	II	light pellicle	1
	III	light pellicle on medium surface and on tube wall	2
	IV	visible traces of biofilm, light pellicle on wall tube	1
	V	no visible pellicle	0
	VI	light pellicle on medium surface and on tube wall	2
P. fluorescens and B. cereus	I	very light pellicle on medium surface and on tube wall, fragmented	2
	II	pellicle on medium surface and tube wall	2
	III	light pellicle on medium surface and on tube wall	2
	IV	pleated pellicle on the walls and on medium surface, non-fragmented	3
	V	pellicle on medium surface and tube wall	3
	VI	light pellicle on medium surface and on tube wall	2

Biofilms development grades: 0 no biofilm, 1 - low development, 2- medium development, 3 - intense development

Table 2 Morphological characteristics of biofilms formed by bacteria after 9 days of growth on liquid media, in stationary conditions

Strain of bacteria	**Protocols of cultivation conditions**	**Morphological characteristics of biofilm**	**The biofilm formation level**
B. subtilis	I	slight pellicle on tube walls, sediment	2
	II	pellicle on medium surfaces, sediment	3
	III	pellicle on wall	2
	IV	sediment in the medium	2
	V	sediment in medium	1
	VI	traces of pellicle, the slight pellicle on the walls of the tube, sediment in medium	1
B. cereus	I	very thin pellicle	2
	II	very thin pellicle, sediment in the medium	3
	III	thin pellicle on medium surface, sediment, non-fragmented	2
	IV	thin pellicle on medium surface, sediment	3
	V	pellicle on medium surface, very thin sediment	3
	VI	pellicle on surface and tube wall, sediment in the medium	3
P. fluorescens	I	traces of biofilm on walls, slight veil, sediment	2
	II	thin pellicle, sediment, yellow green fluorescent	3
	III	stable pellicle on medium surfaces, greenish yellow fluorescent medium	1
	IV	traces of biofilm on walls, slight veil, sediment	2
	V	traces of biofilm, sediment	3
	VI	thin pellicle, sediment	2
P. fluorescens and B. cereus	I	pellicle on walls and medium surface, , sediment in the medium	2
	II	pellicle on walls and medium surface, greenish yellow fluorescent	3
	III	pellicle on walls and medium surface, greenish yellow fluorescent	2
	IV	pellicle on the walls, sediment in the medium	3
	V	pellicle on the surface and fragmented medium, sediment in the medium	3
	VI	thin pellicle	2

Biofilms development grades: 0 - no biofilm, 1 - low development, 2- medium development, 3 - intense development.

In protocol IV, *B. cereus* biofilm formed basal medium have grew rapidly, being characterized by a homogeneous pellicle. In protocol IV mixed biofilms *(Bacillus cereus* and *Pseudomonas fluorescens)* had a similar development with the *B. cereus* biofilm formed for the same conditions of cultivation.

B. subtilis and *P. fluorescens* biofilms were not visible after 48 h of growth for protocol V.

On the third day of cultivation all bacterial strains used formed biofilm as a pellicle attached particularly to the tubes walls (protocols I-VI).

A maximum development was observed for *B. cereus* biofilm (protocol IV) on basal medium. In this case was obtained a rapid growth, constant, maintaining the characteristics of the second day of growth and characterized by the formation of a non-fragmented pellicle.

In protocol IV mixed biofilms had a rapid evolution, similar to B. cereus biofilm formation in the same conditions of cultivation. For *B. subtilis* and *P. fluorescens* strains in protocol V (with addition of 5g/l glycerol) formation of very thin pellicle was noted (Figure 1).

A slight development of *P. fluorescens* biofilm was observed in protocol III by increasing the quantity to the pellicle at air liquid interface and on tube wall.

In the seventh days of biofilm development, all strains formed biofilm as homogeneous surface pellicles (protocols I-VI). A maximum growth was observed for all strains in protocol II, using basal medium supplemented with 5g/l glycerol (Figure 2). A maximum development can be observed in protocols V (addition of 5g/l glycerol) and VI (5g/l added glucose) for all strains used, particularly for *Bacillus cereus* and mixed biofilms, observing the formation of pleated veil and the sediment presence into the medium.

In the nine day of biofilm development, all strains used formed biofilm as a homogeneous pellicle on the medium surface (protocols I - VI). A maximum growth was seen for protocols II and V for all strains used. At basal medium surface, supplemented with glycerol, glucose respectively, an exponential growth was observed from day 9 of biofilm development (protocol II), characterized by the formation of a homogeneous pellicle and the sediment in the medium (Table 2).

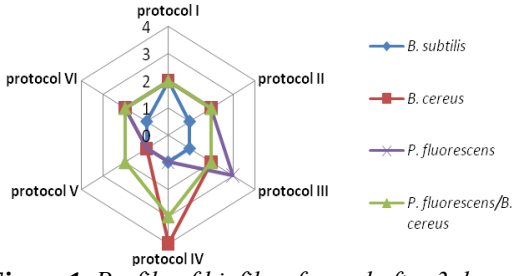

Figure 1. *Profile of biofilms formed after 3 days of development of bacteria on liquid media, in stationary conditions*

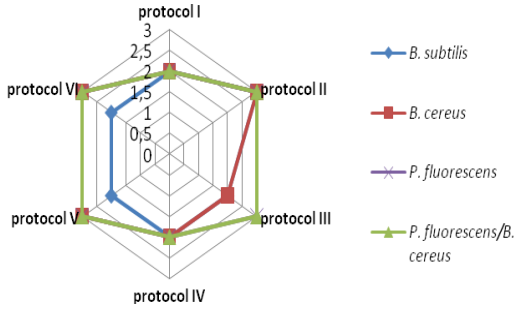

Figure 2. *Profile of biofilms formed after 7 days of development of bacteria on liquid media, in stationary conditions*

Protocol V is characterized by the presence of a fragmented pellicle for mixed biofilm and by a reduced quantity of pellicle, especially for *B. subtilis* biofilm.

Conclusions

The results presented in this paper allowed the following conclusions:

- Species of bacteria: *B. subtilis, B. cereus* and *P. fluorescens* can grow and can form biofilms on the surface of liquid media, in stationary conditions of cultivation.
- The tested bacterial strains *Bacillus subtilis* ATCC 19659, *Bacillus cereus* ATCC 10876 and *Pseudomonas fluorescens* ATCC 13525 behave physiologically different in biofilm formation.
- The combined effect of temperature - nutrients on the biofilm formation can influence the rate of multiplication and aggregation of cells of bacteria in biofilm.
- At temperature of 25˚C, the level of development of biofilm was higher compared to the 37˚C, correlated with the nutritional composition of the culture medium.
- By stationary cultivation of bacteria in liquid media supplemented with glycerol or glucose

biofilm pellicles are firm, uniform, often valued with 4, according scoring method used.

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