Development of a Microtiter Plate Method for Biofilm Formation

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Abstract

Biofilms have a dynamic and complex architecture that is now being recognized as having significance in many environments. The formation of biofilms depends on the substrate properties, nutrient availability and flow hydrodynamics. The aim of this article is to investigate the cells attachment of *Bacillus subtilis*, Bacillus cereus and Pseudomonas fluorescens to polystyrene and bacteria capacity to form biofilms.

Keywords: biofilm, microbial adhesion, Microtiter plate

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1. Introduction

Biofilms play an important role in food processing, sanitation, and food safety. Microorganisms capable to form biofilm, which may include harmful food-borne pathogens, secrete extra-cellular polymeric substances – EPS that allows them to adhere to various surfaces. Such surfaces include stainless steel and plastic that are used in food industry plants. Biofilms protect the cells that secrete them and they tend to make the cells more resistant to cleaning agents and disinfectants, thereby making removal of pathogens more difficult. With the cells embedded in a polysaccharide matrix, biofilms are highly resistant to disinfectants than planktonic cells. Therefore, the control of harmful biofilms is an important issue in food industry.

Attachment to surfaces by microorganisms enhances their survival in diverse environments (Bryers, 2000). A microorganism can undergo many different physiological responses in environmental niches which are influenced by the substrata upon which it attaches and the environmental changes that it encounters (Fletcher, 1991). Microbes can live in the environment as individual free floating organisms (planktonic) or attached to surfaces (sessile) as single cells or as a network structure in a biofilm (Frank, 2001;

Costerton et al., 1999; Fletcher, 1991). A biofilm is generally defined as a structured community of bacterial cells enclosed in a self-producing matrix and adherent to an inert or living surface (Costerton et al., 1999). Development of biofilms in the food environment increases resistance of cells to environmental stresses and protect cells from cleaning and sanitation procedures (Reisner et al., 2006; Ryu and Beuchat, 2005; Costerton et al., 1999). The ability of bacteria to attach to and form biofilms on surfaces may influence their persistence during manufacturing and retail, as well as their ability to cause disease (Kumar and Anand, 1998).

2. Materials and Method

2.1. Bacterial strains, culture conditions

Microorganisms used for experimental tests were *Bacillus subtilis* ATCC 19659, *Bacillus cereus* ATCC 10876 and *Pseudomonas fluorescens* ATCC 13525, purchased from American Type Culture Collection (Microbiologics). These reference strains are marketed as commercial kits Kwik-StikTM. The selection of these strains was motivated by their ability to form biofilms as shown in literature.

In brief, cell suspensions consisting of 106 cfu ml^{-1} were prepared using cells from medium (0.5%

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peptone, 0.25% yeast extract and 0.1% glucose). The initial cell count for each cell suspension was determined by serial dilution onto (0.5% peptone, 0.25% yeast extract, 0.1% glucose, 1.5% agar).

2.2. Biofilm assay using a microtiter plate assay

The microtiter plate assay was performed as outlined by Djordjevic et al. (2002). The Microtiter Plate test involved the analysis of bacterial adhesion and biofilm formation on sterile polystyrene wells by measuring the optical density at different time intervals: 24 h, 48 h and 72 h. Microorganisms were inoculated in a volume of 5 ml culture medium (0.5% peptone, 0.25% yeast extract, 0.1% glucose) and incubated for 18 h at 37˚C for Bacillus subtilis and Bacillus cereus, respectively at a temperature of 25˚C for Pseudomonas fluorescens. After this incubation period, a 20μl volume of cultures were transferred into 5 ml medium and incubated again for 18 h at 20˚C. A new passage was made later: 125μl in a volume of 5 ml medium.

Final cultures thus obtained were shaken for 1 min and a volume of 100 ml of each sample was transferred to sterile polystyrene wells. Well support was covered and then incubated at 20˚C for 24, 48 and 72 h. For each sample was made a corresponding control sample. After the incubation, cell densities were determined by measuring the turbidity at the wavelength of 580 nm.

Control samples (control) were studied to highlight the absence of cell growth. After removal of cultures and control samples check, wells were rinsed 3 times with a volume of 150 ml sterile distilled water to remove unattached cells and air dried at temperature of 30˚ C for 30 min. Then was added 150 μl aqueous solution of 1% (w/v) crystal violet in each well and incubated at 20˚C for 45 min. After staining, wells were rinsed with very gently running distilled water until no stain was visible (Fig. 3) and dry air flow at a temperature of 30˚C for 30 min.

The quantitative analysis of the biofilm production was performed by adding 150 μl of 95% ethanol to destain the wells and the absorbance (CV-OD$_{580}$) of the crystal violet present in the destaining solution was measured at a wavelength of 580 nm.

The average absorbance from the control wells (OD$_{580}$) was subtracted from the optical density at wavelength of 580 nm of all test wells.

Samples were made in three parallel samples for each test microorganism were calculated and mean values and standard deviation. For statistical analysis of data obtained was used SPSS 11.0 Windows Student Version. Student t test was used in order to determine the difference between the averages of two determinations dependent (pairs).

Strains were classified as follows: CV-OD$_{580}$≤OD$_{580}$=no biofilm producer, OD$_{580}$<CV-OD$_{580}$≤ (2×OD$_{580}$) =weak biofilm producer, (2×OD$_{580}$) <CV-OD$_{580}$≤ (4×OD$_{580}$) =moderate biofilm producer and (4× OD$_{580}$) < CV-OD$_{580}$=strong biofilm producer (Stepanovic et al., 2004).

3. Results and Discussion

The test was adapted to evaluate the biofilm formation after incubation for 24 h, 48 h (Fig. 4) and 72 h at 20 ˚ C, and the protocol results are presented in Fig. 1 and 2.
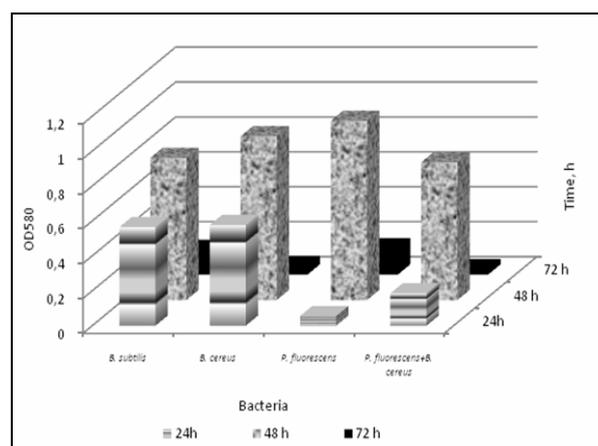

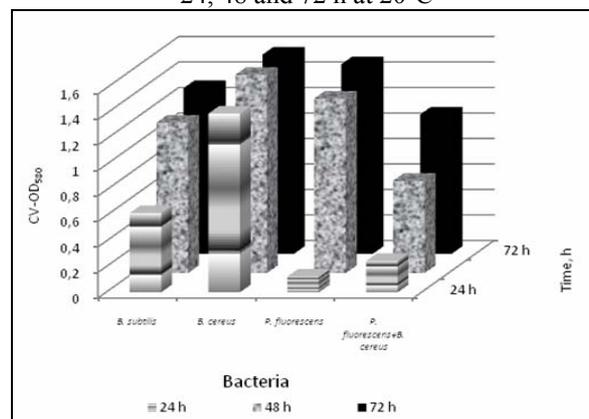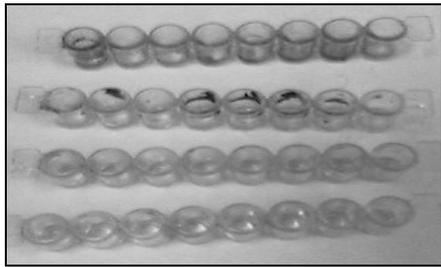**Figure 1**. Evolution of planktonic bacteria suspension at 24, 48 and 72 h at 20˚C

**Figure 2.** Mean absorbance (CV-OD$_{580}$) on microtiter plates after 24, 48 and 72 h at 20˚C

Figure 3. Appearance of biofilms stained with crystal violet 1, after 48h of development

For planktonic and sessile cultures, a significant difference ($p < 0.05$) was found for biofilm production between bacteria at both 24 and 48 h.

B. subtilis was found to produce moderate biofilm as defined by Stepanovic et al. (2004), after 24 h of development.

In the case of biofilm mixed and thus formed by *P. fluorescens* the optical densities were very low, showing a reduced adhesion to the surface of the PVC wells, being weak biofilm producer.
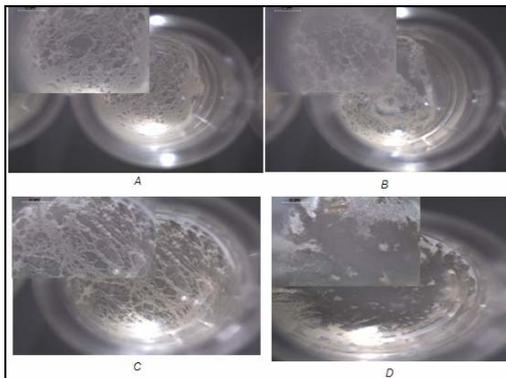

Figure 4 Biofilm formations by *B. cereus* (A), *B. subtilis* (B), P. fluorescens (C) and *P. fluorescens and B. cereus* (D) after 48 h

A maximum adhesion can be observed if the species *B. cereus*. Sommer et al (1999) showed that cell wall hydrophobicity may promote adherence to either inert hydrophobic or hydrophilic supports. For hydrophobic surfaces like PVC surfaces (wells), hydrophobic interactions are considered as the main forces involved in adhesion which can compete to hydrophobicity of cell walls of species studied.

After a period of 48 h of development, optical density values reached levels higher by 1.9, 1.1, 11.1 and 2.95 times compared with the first 24 h of adhesion.

Optical density values, after 72 h of development, have supported a few changes compared to 48 h, probably due to nutrient depletion contribution.
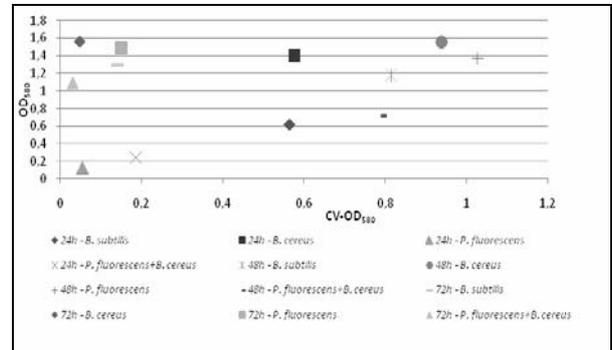
Figure 5. Evolution of optical density of planktonic bacteria suspension (OD_{580}) versus optical density (CV-OD_{580}) obtained after staining with crystal violet

Correlation shown in Figure 8, the optical densities of planktonic bacteria (OD_{580}) versus optical density (CV-OD_{580}) obtained after staining with CV after 24, 48 and 72 h, showed a difference between the amounts of cells attached to surfaces test (biofilm) and planktonic cells in culture medium.

The results indicate that *Microtiter Plate* test has the advantage of analysis in as short a time adherence of bacteria, but can not provide information on the biofilm formed. Adsorption of drawing crystal violet is used as an indicator of the amount of biofilm formed.

Correlation shown in Figure 5, the optical densities of planktonic bacteria (OD_{580}) versus optical density (CV-OD_{580}) obtained after staining with CV after 24, 48 and 72 h, showed a difference between the amounts of cells attached to surfaces test (biofilm) and planktonic cells in culture medium.

4. Conclusion

Experimental data obtained indicate differences in biofilm formation correlated with species as being possible to say that no one species can not be a model of behavior for the type of part.

The microtiter plate has the advantage of enabling researchers to rapidly analyze adhesion of multiple bacterial strains or growth conditions within each experiment. The major disadvantage is that the microtiter plate method is an indirect indication of the level of biofilm produced; the adsorption of the crystal violet in the destaining solution was used as an indication of the amount of biofilm.

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