NATURAL ZEOLITE AS A TOOL FOR MODELLING BACTERIAL BIOFILM DEVELOPMENT IN STATIC CONDITIONS

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ABSTRACT

In the environment bacteria mainly exist adhered to solid surfaces in communities known as biofilms. Bacterial biofilm is a sessile community whose cells are attached to the substrate and to each other, engulfed in the matrix of extracellular polymeric substances that they have created. Currently, a five stage model for biofilm development is widely accepted for every biofilm growth, in spite the fact that it was modelled based on biofilm growth in continuous flow conditions. We hypothesize that in static conditions, which are nowadays mostly used for biofilm investigations, the five stage model is perhaps not applicable. So, we used particles of natural zeolite as a unique tool that allows simultaneous visualization and statistically satisfactory quantification of biofilm growth during 24 h. The results from testing two morphologically distinguishable bacterial strains suggested that, when grown in static conditions, five-stage model could be applicable for non-motile bacteria *Acinetobacter junii*, but not for motile bacteria *Bacillus cereus*.

Key words: motility; five-stage model; zeolite; biofilm.

INTRODUCTION

Biofilm is commonly defined as a community of microorganisms adhering to a surface engulfed in a complex matrix of self-produced extracellular polymeric substances (EPS). It can be best envisaged as a city of microbes covered by a dome. Same as a dome, the EPS protects the cell from harsh environmental conditions: antibiotics, disinfectants, or desiccation [1,2]. Same as in a city, the cells communicate with various mechanisms and even exchange genetic material enhancing the overall community fitness [3].

Recognizing the abundancy and importance of bacterial biofilms in the environment, industrial settings and medicinal surroundings, has led to vast scientific researches being conducted to investigate many phenomena linked, and arising from the biofilm. The model of bacterial biofilm growth has been one of the first items concluded from such research [4], and five-stage model is now universally accepted. However, the five-stage model has been developed from observations obtained on biofilm growth in flow systems, where biofilm is attached on solid surface and nutrient media is continuously replenished for a period of time usually counted in days [4]. On the contrary, majority of experiments on biofilm nowadays are being done in static systems that offer simplified experimental setup compared to flow systems and much faster results since the biofilms are usually grown for 24-48 h [5,6]. In addition, static systems do not replenish the nutrient media, hence the bacterial populations follow classic lag/log/static phase growth curve. Despite the obvious significant differences in experimental setup, it has become widely accepted that five-stage biofilm growth model is applicable to static conditions as well.

The goal of here presented research was to investigate whether the five-stage biofilm growth model is valid and applicable when experiments were done in static conditions. We have used the particles of natural zeolite – clinoptilolite, as a solid substrate for biofilm growth. The experimental system with zeolite particles enabled simultaneous visualization and qualitative along with quantitative analysis of bacterial biofilms, in a relatively simple and completely reliable and reproducible experimental setup.

EXPERIMENTAL

Biofilm growth of gram positive, motile bacteria with flagellar apparatus *Bacillus cereus* (4080 LBK) and gram-negative, non-motile bacteria with Type IV pili *Acinetobacter junii* (DSM 1532) was monitored in static and pseudo-flow conditions. In static conditions, a 0.5 g of natural zeolitized tuff (NZ) obtained from quarries in Donje Jesenje (Croatia) of 0.122-0.263 mm particle size was added to series of laboratory bottles already containing 50 mL of Luria Bertani liquid medium (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1000 ml distilled water, pH 7.0±0.1) and bacteria at starting concentration of ~106 CFU mL^{-1} (Colony Forming Units). The bottles were set for incubation with mixing (100 rpm, Orbital Shaker, Biosan OS-10, Latvia) at 35°C. Every three hours, from one of the bottles, the media was poured out, the NZ particles were gently washed with sterile physiological solution and aseptically transferred to plastic vials containing 9 mL of fresh physiological solution. The vials were vortexed (50 Hz, Kartell, Italy) for 3 min to allow the bacterial cells to detach from the NZ. The bacteria remained as planktonic cells in the suspension. The suspension was serially diluted and inoculated onto LB agar plates, incubated for 24 h at 35°C after which the grown colonies were counted, the NZ particles were weighted and bacterial number was expressed as logCFU g^{-1} of dry NZ.

The NZ sample used in the research consisted mostly of clinoptilolite (50–55%) with major constituents being celadonite, plagioclase feldspars and opal-CT (10–15% each), whilst analcime (another zeolite group mineral) and quartz were present in traces [7].

The procedure was identical for pseudo-flow conditions, with difference that after every three hours one of the bottles was analyzed for number of bacteria attached on the NZ, and the remaining bottles were emptied of current nutrient media, the NZ particles were gently washed with sterile saline, and refilled with 50 mL of fresh sterile LB broth.

From each bottle, a few grains of NZ were placed onto microscope slides, dyed by Alcian-blue method and visually examined under the microscope (Olympus CX-21, 1000× magnification). The photos were taken using mobile phone.

All the experiments were done in duplicate. The results were statistically analyzed using Statistica software version 13.3. We used ANOVA with Duncan's post-hoc analysis and Student's *t*-test to compare independent variables. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

The idea and main hypothesis of the experiment was the following; the five stage model implies clonal growth, meaning that a single cell (or aggregate of few cells) adsorbs to a solid surface and continues to grow attached to the surface, producing the EPS, and eventually forming a biofilm [8]. If this was applicable to static systems than in both growth conditions of our experiment, the static without changing media and pseudo-flow with regular removal of spent media and replenishment with fresh sterile media, the number of bacteria in the biofilm on NZ particles should be the same. The bacteria attach to surface of NZ and form biofilm regardless of the surrounding media change.

Visual observations of microscopic slides confirmed that in the case of *A. junii* the amount of bacteria attached on the NZ particles after 1 and 24 h in the experiments without (Figure 1 a and b) and with media change (Figure 1 c and d) were comparable: few cells attached on NZ after 1 h and many cells on and surrounding the NZ particle after 24 h, regardless of static or pseudo-flow conditions.

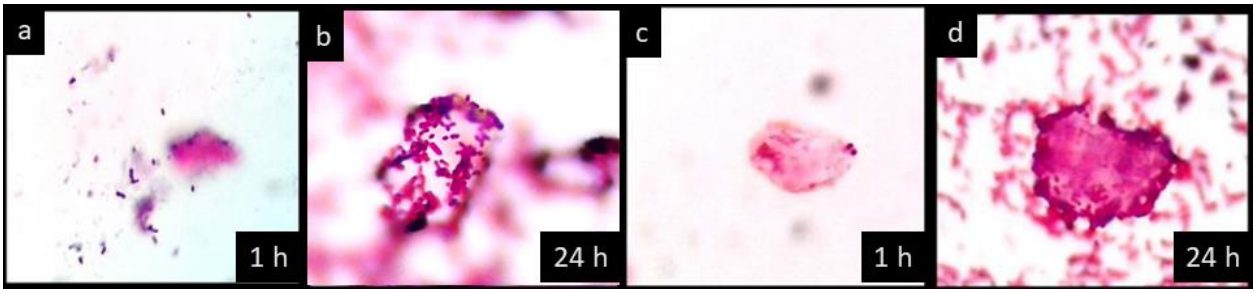

Figure 1. Cells of *A. junii* and NZ particles imaged after 1 and 24 h of experiment in static conditions without nutrient media change (a and b) and in pseudo-flow conditions with regular media change (c and d).

However, in the case of *B. cereus* the observations differed: few cells attached after 1 h in both experimental conditions (Figure 1a and c), many cells on and surrounding NZ particle after 24 h in static condition (Figure 2b) but much fewer cells on, and almost none surrounding the NZ particle in pseudo-flow condition (Figure 2d).

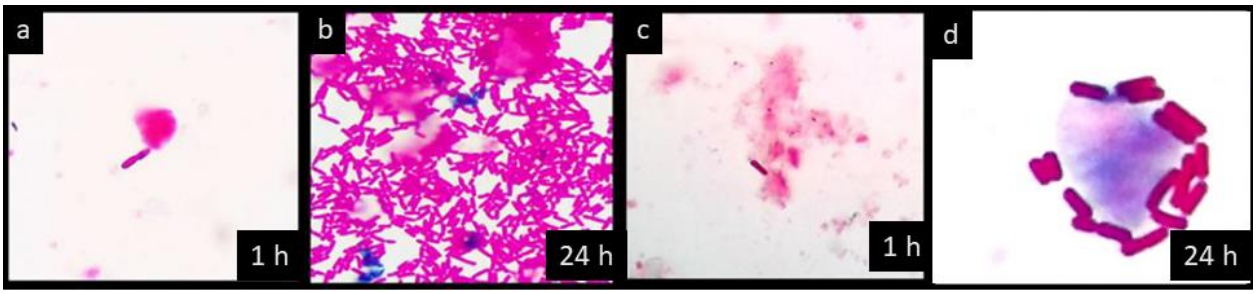

Figure 2. Cells of *B. cereus* and NZ particles imaged after 1 and 24 h of experiment in static conditions without nutrient media change (a and b) and in pseudo-flow conditions with regular media change (c and d).

The visual observations were indicative but purely qualitative and insufficient to make significant conclusions. Therefore, an analysis of bacterial numbers attached to solid particles was needed. The NZ was previously reported to be an excellent material enabling reliable and statistically reproducible counting of bacterial cells forming the biofilm on particle surfaces [7,9]. Experimental system using NZ particles as substrate for biofilm formation and quantitative analysis enabled investigation of influence of harsh environmental conditions on bacterial biofilm [10] or dynamics of biofilm formation in consortium of few bacterial species [7].

The quantification of biofilm bacteria revealed that there was no significant difference in the static and pseudo-flow experimental system with *A. junii* (Figure 3). However, significant difference occurs when static and pseudo-flow conditions were compared in the system with *B. cereus* (Figure 3). The results confirmed visual observations and showed much higher number of *B. cereus* cells in static conditions after 9 h until 24 h of growth when mature biofilm formation was expected. Most reasonable explanation would be that both bacterial species initially assume clonal growth after attachment to NZ particles, but in pseudo-flow conditions, with regular media change, motile *B. cereus* cells begin to detach from the biofilm in much higher proportion than non-motile *A. junii* cells, probably taking advantage of fresh nutrient media. In static conditions the *B. cereus* cells attach on the NZ particles not just as a result of clonal growth but also migrating from the surrounding media which after few hours becomes saturated bacterial suspension.

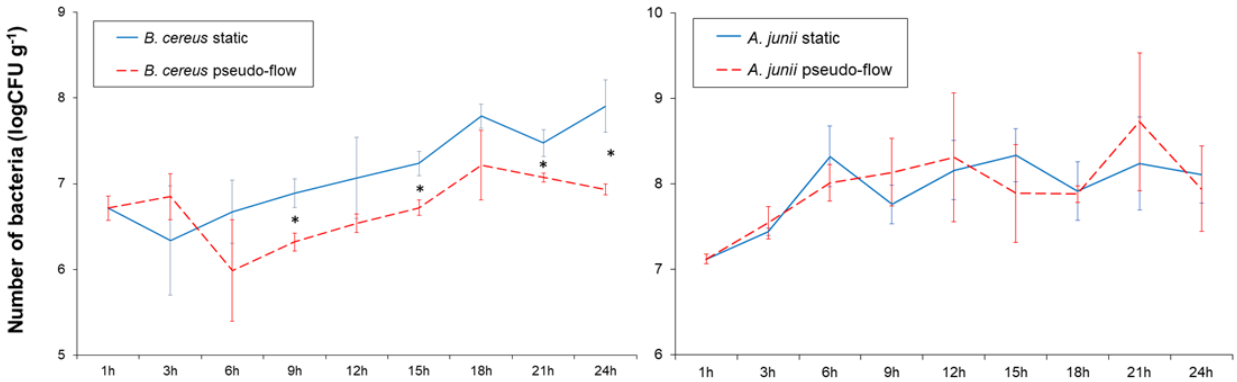

Figure 3. Number of *A. junii* and *B. cereus* attached to NZ particles after different period of incubation in static or pseudo-flow experimental conditions. * marks statistically significant difference between measurements (p<0.05).

CONCLUSION

The particles of NZ have shown to be an excellent tool for investigation of biofilm enabling not just qualitative but reliable and statistically reproducible quantitative analysis. Enumeration of bacterial numbers attached to the surface of NZ indicated that if bacterial biofilm was grown in static conditions, the five-stage biofilm model was applicable to *A. junii* but not to *B. cereus*. The difference is probably due to active motility by flagellar apparatus of *B. cereus*. Additional experiments with a battery of bacterial species or motile/non-motile mutants of the same bacterial species would give definite answer whether five-stage model is relevant for static biofilms.

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