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The Ability of *Bacillus licheniformis* Protease to Remove *Bacillus cereus* and *Pseudomonas aeruginosa* biofilm

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Abstract. Microbial biofilms are active communities of microorganisms attached to a surface and surrounded by a self-produced matrix composed of extracellular polymeric substances, acting as a shield against desiccation, cleaning procedures and antimicrobial substances. No matter which biomolecule is most responsible in the matrix formation, all these extracellular polymeric substances can nevertheless be broken down with different enzymes leading to a disruption of the biofilm matrix and the removal of the biofilm. Enzyme treatments can be used to break down extracellular polymeric substances as an alternative when standard cleaning agents do not give satisfactory results in removing biofilms. The aim of the study was to analyze the activity of a protease from *B. licheniformis* against the biofilm formed by *B. cereus* and *P. aeruginosa*. Fluorescent microscopy and confocal laser scanning microscopy was performed in order to assess the effect of the enzyme on the biofilm structure and to characterize the biofilm formed by both microorganisms. The results show that the enzyme used was more active against the *B. cereus* biofilm matrix compared to the activity on the *P. aeruginosa* biofilm matrix.

Keywords: *B. cereus* biofilm, enzymatic removal, matrix, protease, *P. aeruginosa* biofilm

INTRODUCTION

Microbial biofilms are multicellular, tridimensional structures, attached to a solid surface and embedded in a self-produced matrix. This matrix is made of extracellular polymeric substances (EPS), mostly polysaccharides, DNA and proteins (Ohtani *et al.*, 2002; Rasko *et al*., 2004) and act as a shield, protecting bacteria within the biofilm. Biofilms are therefore persistent structures, resisting to desiccation, cleaning procedures and antimicrobial substances (Schauder and Bassler, 2001), which makes them a challenge in human and animal health and industrial processes. EPS composition in biofilms varies greatly from one bacterial species to another and is strongly affected by growth conditions (Costerton *et al*., 1995; Kolter and Greenberg 2006). Studies showed that EPS influences biofilm shape and size, resistance to shear forces (Sharma *et al*., 2005; Stoodley *et al*., 2002), and resistance against antimicrobial compounds (Stewart and Costerton, 2001).

Because microorganisms in biofilms can become resistant to chemical and physical treatments applied during cleaning and sanitizing procedures in the food industry (Chmielewski and Frank, 2003) enzyme treatments can be used to break down EPS as an

alternative when standard cleaning agents do not give satisfactory results in removing biofilms. Studies on biofilm enzymatic removal have been made using different types of enzymes mainly polysaccharidases and proteolytic enzymes. Lequette *et al.* (2010) used seven proteases and polysaccharidases to remove biofilms of bacterial isolates of 16 species sampled in the food industry. The efficiency of the polysaccharidases and proteolytic enzymes against biofilms was studied, as well as enzyme effects on composition of extracellular polymeric substances. Proteolytic enzymes showed a higher reduction effect to a larger range of bacterial species than polysaccharidases. Chaignon *et al.* (2007) in another study on enzymatic detachment of biofilm showed that enzymatic removal is dependent on the nature of their constituent and varies between isolates. In this study, Chaignon *et al.* (2007) tried to remove *Staphylococcus epidermidis* biofilm from five clinical isolates with periodate, Pectinex Ultra SP, proteinase K, trypsin, pancreatin and dispersinB. DispersinB followed by proteases (proteinase K and trypsin) were capable of eliminating biofilms of a variety of strains on inert surfaces. Many other studies have showed that proteases in commercially available detergents are already used to clean, for example, ultrafiltration units, contact lenses, medical apparatus, and laundry (Lequette *et al*., 2010). In addition, amylases are widely used in the formulation of enzyme detergents, mainly to remove food residues of starch-based foods (Lequette *et al*., 2010).

The aim of the present study was to analyze the efficiency of the protease from *Bacillus licheniformis* in removing biofilms developed by *Bacillus cereus* and *Pseudomonas aeruginosa* on stainless steel surfaces.

MATERIALS AND METHODS

Bacterial strains. In this research, *Bacillus cereus* ATCC 10876 and *Pseudomonas aeruginosa* ATCC 27853 bacterial strains were used. Stock cultures were stored at -50˚C in Brain Heart Infusion (BHI) broth (Biokar Diagnostics, France) with 20% glycerol. The strains were streaked on BHI agar plates with incubation at 37˚C for 24 h prior to each experiment.

Biofilm formation. The biofilm was formed on 316 (AISI 2B finish) stainless steel coupons (10x30x1 mm). The bacterial cultures on BHI agar (Biokar Diagnostics, France) were used to make an inoculum that matches 1.0 McFarland standard. This solution was then diluted 1:30 in TSB (Tryptone Soya Broth) growth medium. A quantity of 250 µL of TSB containing the bacterial strains were spread on each stainless steel coupons surface and incubated at 37°C for 72h in an incubator with 100% RH. After 72h incubation the stainless steel coupons were divided for enzymatic treatment and control for both bacterial strains used.

Enzymatic treatment. A *Bacillus licheniformis* protease (Sigma Aldrich - P4860 activity ≥2.4 U/g) was used in order to remove the biofilm formed by *B.cereus* and *P.aeruginosa*. The enzyme solution was prepared using sterile distillate water. After the incubation time, the coupons were washed twice in saline solution (0.9%), and then were divided for enzymatic treatment and control. Each coupon was placed in separate tubs containing 5 mL enzyme solution or sterile distillate water for control and incubated for 60 min at 37°C.

After the incubation time one set of coupons (enzymatic treatment and control for both bacterial strains) were rinsed twice in saline solution then placed in separate tubes containing 5 mL peptone saline solution. The tubes containing the coupons were sonicated for 5 min and vortexed for 2 min then spot plating was performed on BHI agar.

The second set of coupons (enzymatic treatment and control for both bacterial strains) after enzymatic treatment was washed twice in saline solution then staining with acridin

orange (AO) for 2 min, rinsed with distillate water and air dried at room temperature. Fluorescent microscopy and confocal laser scanning microscopy (CLSM - *Leica* DM 2500) was performed to compare the effect of the enzyme on the biofilm structure and to characterize the biofilm formed by both microorganisms. CLSM images were analyzed using special computer software programs (ImageJ and COMSTAT).

RESULTS AND DISCUSSION

B. cereus is a Gram-positive, spore-forming bacterium. *B. cereus* is frequently identified as the causative agent of food-borne diseases. This ubiquitous organism can easily contaminate food processing systems (Kotiranta *et al*., 2000) and forms biofilms that are highly resistant to cleaning procedures (Peng *et al*., 2002). *Pseudomonas spp.* are Gram-negative bacteria that are ubiquitous in nature and are among the most notorious spoilage organisms in food systems (Jay *et al*., 2005; Liao, 2006).

The results obtained from the CLSM images analysed by COMSTAT (Heydorn *et al*., 2000a, 2000b) show that *P. aeruginosa* compared to *B. cereus*, is able to form denser biofilm and is more efficient in occupying the surface aria even if they have the same thickness. Heydorn *et al.* (2000a) in a study on 4 *Pseudomonas* species biofilms found that *P. aeruginosa* colonized the entire substratum very rapidly (substratum coverage of 98-100%) and formed a uniform biofilm compared to the other three strains. The CLSM images are presented in Fig. 1. The surface to volume ratio reflects what fraction of the biofilm is in fact exposed to the nutrient flow, and thus may indicate how the biofilm adapts to the environment (Heydorn *et al*., 2000a). In this study the surface to volume ratio results suggest that *P. aeruginosa* biofilm is more adapt to the environmental conditions compared to *B. cereus* biofilm. It is known that *Pseudomonas* spp. are among the most well-studied microorganisms capable of biofilm formation in clinical and agricultural settings (O'Toole and Kolter, 1998; Parsek and Greenberg, 1999). They are considered as primary colonizers of surfaces and can provide the biofilm backbone to which other bacteria could adhere and become part of the biofilm in natural and processing environments (Luo, 2005).

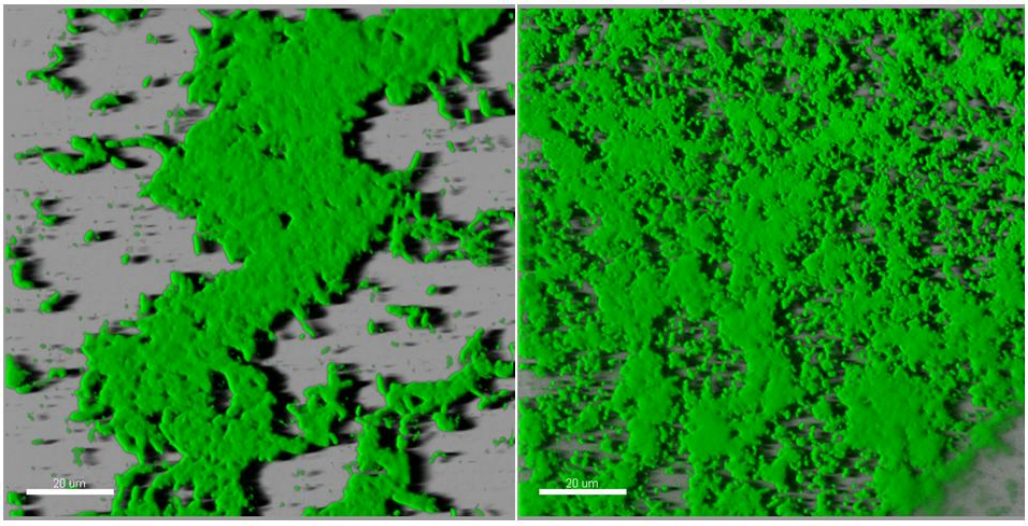

Fig. 1. CLSM images of the biofilm formed by B.cereus (left) and P.aeruginosa (right) and some of the characteristics calculated by COMSTAT (scale bar 20µm)

The results showing the ability of protease from *Bacillus licheniformis* to remove the biofilm formed by *B. cereus* and *P. aeruginosa* are presented in Fig. 2. The protease was more active on *B. cereus* biofilm matrix showing 89.37% reduction (from log 5.93 to 4.94). A lower activity of the protease was observed on *P. aeruginosa*, removing just 56.25% of the biofilm (from log 6.94 to 6.58). One of the explanations could be the composition of the matrix or the specificity of the enzyme. Lequette *et al.* (2010) in a study upon the enzymatic removal of the biofilm formed by 25 bacterial species including *B. cereus* and *P. fluorescens*, found that the efficacy of enzymes depends on the bacterial species: proteases were more efficient than polysaccharidases in removing *Bacillus* biofilms, while polysaccharide-degrading enzymes were more efficient in removing *P. fluorescens* biofilms. Johansen *et al.* (1997) have used a multicomponent enzyme preparation containing protease activity and a wide range of carbohydrases, including pectinase, arabinase, cellulase, hemicellulase, b-glucanase, and xylanase activities (Pectinex Ultra) for removing *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *P. fluorescens* biofilm. In general, *S. aureus* and *S. epidermidis* biofilms were more sensitive to enzymatic removal by Pectinex Ultra than *P. aeruginosa* and *P. fluorescens* biofilms. *S. aureus* biofilm was most sensitive to Pectinex Ultra, as 1.8 Pectinex Ultra SP units (PSU) of Pectinex Ultra per ml decreased the cell number on the substrate more than 1 log unit. *P. fluorescens* was the most resistant biofilm, as 1800 PSU of Pectinex Ultra per ml was needed to decrease the number of biofilm cells by 1 log unit. This difference may be due to the thinner biofilm obtained with *Staphylococcus* spp. or variations in the composition of the extracellular polymers in the biofilm.

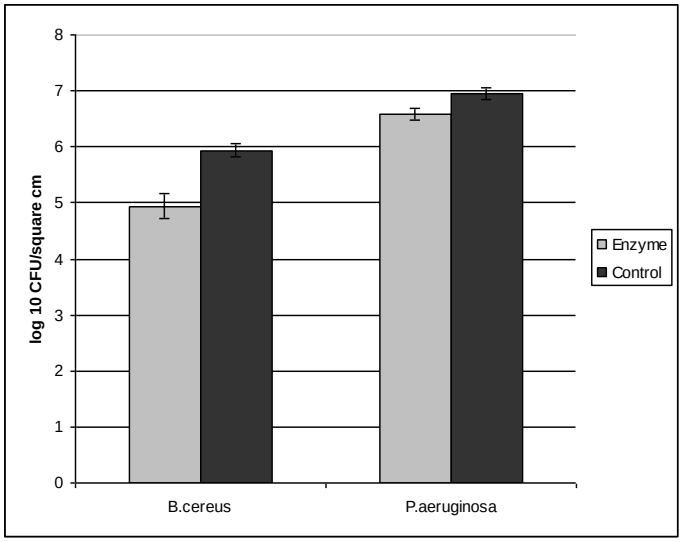

Fig. 2. Remaining *B. cereus* and *P. aeruginosa* biofilm (log CFU/cm^2) after 60min enzymatic treatment on stainless steel coupons.

The microscopic image analyses also confirmed the plate counting results. Aspects of the biofilm before and after the treatment can be seen in Fig. 3. The microscopic studies of the enzyme effect on *B. cereus* and *P. aeruginosa* biofilm revealed a reduction in biofilm cells and substantial degradation of the microcolonies attached to stainless steel surface. The reduction was more obvious for the *B. cereus* biofilm.

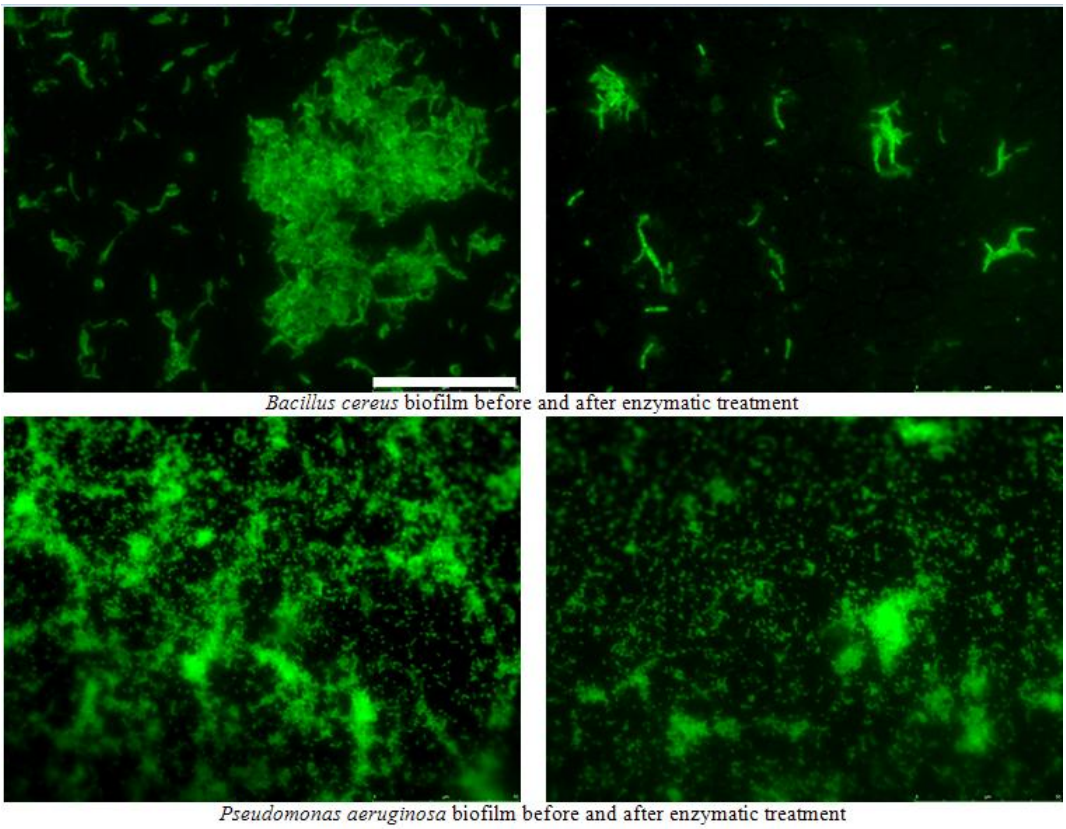

Fig. 3. Aspects of *B. cereus* and *P. aeruginosa* biofilm, before and after the enzymatic treatment (scale bar 50 µm).

CONCLUSIONS

- *Pseudomonas aeruginosa* is able to form a denser biofilm and is more efficient in occupying the surface aria, compared to the biofilm formed by *Bacillus cereus*.
- The protease from *Bacillus licheniformis* is more active on the *Bacillus cereus* biofilm matrix compared to the activity on the *Pseudomonas aeruginosa* biofilm matrix.

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