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The effect of shear stress on the formation and removal of *Bacillus cereus* biofilms

*Madalena Lemos, Filipe Mergulhão, Luís Melo, Manuel Simões**

LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal

ABSTRACT

The influence of the shear stress (τ_w) under which biofilms were formed was assessed on their susceptibility to removal when exposed to chemical and mechanical stresses. A rotating cylinder reactor was used to form biofilms, allowing the simulation of τ_w conditions similar to those found in industrial settings, particularly in areas with low τ_w like elbows, corners, valves and dead zones. *Bacillus cereus* was used as a model bacterium for biofilm formation. Biofilms were formed on AISI316 stainless steel cylinders under different τ_w (estimated at 0.02, 0.12 and 0.17 Pa) for 7 days. Some phenotypic characteristics, including thickness, biomass production, cellular density and extracellular proteins and polysaccharides content were assessed. Biofilm density was found to increase significantly with τ_w while the thickness decreased. Also, biofilms formed at 0.02 Pa had lowest biomass content, cell density and extracellular polysaccharide content. Those characteristics were not statistically different for the biofilms formed under 0.12 and 0.17 Pa.

Ex situ tests were performed by treating the biofilms with the biocide benzyldimethyldodecyl ammonium chloride (BDMDAC), followed by exposure to increasing τ_w conditions, up to 1.84 Pa (whereas the maximum τ_w used during growth was 0.17 Pa). The biofilms formed under low τ_w were more resistant to removal caused by the BDMDAC action alone. Those formed under higher τ_w were more resistant to the mechanical and the combined chemical and mechanical treatments. The amount of biofilm remaining on the cylinders, after both treatments was statistically similar for biofilms formed under 0.12 and 0.17 Pa. The resistance of biofilms to removal by mechanical treatment (alone and combined with BDMDAC) was related to the amount of matrix polysaccharides. However, none of the methods investigated were able to remove all the biofilm from the cylinders.

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Keywords: *Bacillus cereus*; Benzyldimethyldodecyl ammonium chloride; Biofilm control; Cleaning; Hydrodynamic conditions; Rotating cylinder reactor; Shear stress

1. Introduction

There is a lack of efficient strategies to clean stagnant zones in industrial plants (Brooks and Flint, 2008). Crevices, corners, dead zones, valves or areas where the mixing rate is low are almost inevitable. Stagnation promotes bacterial accumulation, ultimately leading to biofouling (Manuel et al., 2010). Biofouling is a damaging problem, affecting the energetic efficiency of industrial processes, causing corrosion of the surfaces, decreasing product quality and eventually promoting the spread of pathogens and resistant infectious diseases (Costerton et al., 1999; Ludensky, 2003; Srey et al., 2013). In industrial settings, surface disinfection is usually focused on the use of biocides, aiming to inactivate the microorganisms (Cloete et al., 1998; Faille et al., 2013). Since biofilms are complex biological structures adhered to a surface, these strategies often fail, as the removal of biomass is neglected. Hence, cleaning the biomass from the surfaces is fundamental for controlling biofilm development (Flemming, 2011).

* Corresponding author. Tel.: +351 225081654; fax: +351 225081449.
E-mail address: mvs@fe.up.pt (M. Simões).
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Nomenclature

Latin

D_a	diameter of the cylinder (m)
f	Fanning friction factor (dimensionless)
N	rotation speed (s^{-1})
Re_A	Reynolds number of agitation (dimensionless)
V	velocity ($m\,s^{-1}$)

Greek

μ	fluid viscosity (Pa s)
ρ	fluid density ($kg\,m^{-3}$)
τ_w	wall shear stress (Pa)

In the biofilm formation process, the hydrodynamic conditions define the transport of the cells, oxygen and nutrients from the bulk fluid to the microbial film (Bryers and Characklis, 1981; Simões et al., 2007b; Stoodley et al., 1999). In fact, the overall flow conditions affect the biofilm structure, as they influence the bulk liquid velocity and shear stress exerted on the surface (Cao and Alaerts, 1995; Peyton, 1996; Vieira and Melo, 1999). The substrate loading rate also affects strongly the transport of the substrate to the cells, therefore influencing their metabolic growth, the production of extracellular polymeric substance (EPS) and ultimately the structure of the biofilm (Vieira et al., 1993).

Diverse studies have demonstrated the influence of hydrodynamic conditions on biofilm behaviour. Douterelo et al. (2013) examined biofilms from drinking water distribution systems and found that the hydraulic regime influenced the bacterial composition and community structure. They also observed that flushing (sudden flow of fluid at high shear stresses inside the system) did not succeed in total biofilm removal but altered the biofilm microbial community. Paul et al. (2012) studied the influence of the substrate and hydrodynamic conditions on biofilm formation and erosion, measuring biofilm thickness and density. Their results showed that increasing the shear stress experienced during growth resulted in biofilms with lower thickness and mass, and higher volumetric density, compared with low shear stress conditions. The authors also found that the biofilms presented stratified cohesion: exposure to shear stress $\leq$2 Pa caused detachment while shear stress >2 Pa caused compression of the biofilm. The effect of environmental conditions on biofilm formation, their structure, composition and physical properties have been reported by Cloete et al. (2003), Derlon et al. (2008) and Rochex et al. (2008).

The aim of this study was to investigate the influence of the shear stress under which biofilms were formed on their resistance to removal by chemical and mechanical treatments. A rotating cylinder reactor (Simões et al., 2005) was used to form biofilms on stainless steel cylinders at low shear stresses, mimicking conditions found in engineered systems. The low shear stresses simulated with this reactor are often found in elbows, valves, dead zones, corners and in sudden pipe expansions (Jensen and Friis, 2005; Lelièvre et al., 2002). Also, typical shear stress values found in drinking water distribution systems are in the range of those used in this study (Gomes et al., 2014).

The combination of mechanical action and chemical treatment was used to challenge biofilms formed by *Bacillus cereus*. *B. cereus* is an industrial contaminant and a public health hazard widespread in nature and frequently isolated from dairy products and equipment (Blel et al., 2008; Faille et al., 2014; Lee et al., 2010; Nam et al., 2014; Peng et al., 2002).

2. Materials and methods

2.1. Bacteria and culture conditions

Biofilms were formed by a *B. cereus* strain, previously isolated from a disinfectant solution and identified by 16S rRNA gene sequencing (Simões et al., 2007a). The bacterial growth conditions were 27 ± 1 °C, pH 7 and glucose as the carbon source (Simões et al., 2005).

The bacterium planktonic culture grew in a sterile concentrated nutrient medium (CNM) consisting of 5 g L^{-1} of glucose, 2.5 g L^{-1} of peptone and 1.25 g L^{-1} of yeast extract, in 0.2 M phosphate buffer (KH_2PO_4; Na_2HPO_4) at pH 7. For biofilm formation, a sterile diluted nutrient medium (DNM), which is a 1:100 dilution of the CNM in the same phosphate buffer (PB) was used.

2.2. Antimicrobial chemical

The antimicrobial compound used to challenge the biofilms was benzyldimethyldodecyl ammonium chloride (BDMDAC) (Sigma-Aldrich, Portugal), at a concentration of 300 μg mL^{-1}. This concentration was selected based on previous experiments with chemically related products (Simões et al., 2005).

2.3. Biofilm formation

Biofilms were grown on cylinders of AISI316 stainless steel (SS) with a surface area of 34.6 cm^2 (diameter = 2.2 cm, length = 5.0 cm), using a 5 L rotating cylinder reactor (RCR) (Fig. 1). The main reactor contained three cylinders immersed in a bacterial suspension. The three cylinders were driven at the same rotation speed by an overhead stirring engine via a synchronizing belt.

A planktonic culture of *B. cereus* grew in a 0.5 L chemostat fed with the CNM described above, at a flow rate of 10 mL h^{-1}. This chemostat was agitated by a magnetic stirrer and fed the RCR at a steady flow rate, set by gravity, at approximately 0.8 L h^{-1}. The period for biofilm formation and growth was 7 days, in order to obtain steady-state biofilms (Simões et al., 2008). Sterile aeration by filtration was provided to the RCR and to the chemostat.

The RCR was assumed to be an agitated vessel and therefore the Reynolds number of agitation (Re_A) was calculated according to (Geankoplis, 1993):

$$Re_A = \frac{D_a^2 N \rho}{\mu} \quad (1)$$

where D_a is the diameter of the cylinder; N is the rotation speed, ρ is the fluid density and μ is the fluid viscosity.

The Fanning friction factor establishes the relation between the τ_w and the velocity head $\rho V^2/2$, and is defined by (Perry and Green, 1999):

$$f = \frac{2t_w}{\rho V^2} \quad (2)$$

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