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Rhamnolipid and surfactin: Anti-adhesion/antibiofilm and antimicrobial effects

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ABSTRACT

Biofilm growth in food industrial processing environment increases the probability of product's microbial contamination by spoilage and pathogenic microorganisms. Biosurfactants represent a great strategy to reduce biofilms due to their environmental-friendly nature when compared to synthetic surfactants. In order to evaluate biosurfactants potential on controlling biofilms, surfactin and rhamnolipid were tested against *Listeria monocytogenes* and *Pseudomonas fluorescens*. Adhesion/biofilm formation on polystyrene and stainless steel 304 surfaces, and physicochemical parameters were used to analyze and discuss findings. Adhesion was reduced up to 79% and 54% on polystyrene, while biofilm formation was reduced up to 83% and 73% on stainless steel surfaces (rhamnolipids and surfactin, respectively). Surfactants altered surfaces physicochemical characteristics. When added to the culture medium, microbial adhesion was inhibited up to 100%, depending on the product. Biosurfactants showed antimicrobial activity. Results demonstrate good potential of tested biomolecules to decrease adhesion and biofilm formation on food-contact surfaces.

1. Introduction

Surfactants are amphiphilic molecules showing a wide range of applications in food, cosmetic and pharmaceutical industries as emulsifiers, cleaning and wetting agents, lubricants and stabilizers (Mukherjee, Das, & Sen, 2006). Many microorganisms such as bacteria, fungi, and yeasts, are able to synthesize different surface-active agents, known as biosurfactants (Banat, Makkar, & Cameotra, 2000). Biosurfactants (BS) show high biodegradability, low toxicity, low critical micellar concentration (CMC), high surface activity, stability to extreme pH, temperature, salt concentrations and biological activity (Bognolo, 1999; Desai & Banat, 1997). Different from synthetic surfactants, BS are not produced from petroleum feedstock but can be obtained from renewable substrates; and their chemical structure and physical properties can be modified by genetic, biological or biochemical manipulation to develop products for more specific requirements (Nitschke & Pastore, 2002). Therefore, BS can replace conventional synthetic surfactants with great advantages and additionally, their bio-based origin confers the status of natural (green) additives fulfilling the market needs (Freire, Araujo, Kronemberger, & Nitschke, 2009; Nitschke & Costa, 2007).

Biofilms are an important concern among food processing industries once their establishment can result in obstruction of pipelines, corrosion of equipment, reducing efficiency of temperature transfer systems and in addition, to spoilage and contamination of final products with pathogens (Kumar & Anand, 1998). Biofilm affects not only the economics of the process but also consumers health. Even with cleaning and sanitization procedures consistent with good manufacturing practices, microorganisms could remain on equipment surfaces and survive for prolonged periods (Cloete, Thantsha, Maluleke, & Kirkpatrick, 2009), so it is

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imperative to search for new biofilm controlling strategies.

Studies have shown that surface conditioning with biosurfactants can substantially decrease microbial contamination of materials and inhibit subsequent biofilm formation (Meylheuc, Renault, & Bellon-Fontaine, 2006). The prior adsorption of surfactin on polypropylene and stainless steel surfaces reduced the adhesion of the food pathogens *Listeria monocytogenes*, *Salmonella* Enteritidis and *Cronobacter sakazakii*. The number of adhered cells of *L. monocytogenes* on stainless steel was reduced by two log units (Nitschke et al. 2009). The anti-adhesive potential of rhamnolipid and surfactin on polystyrene surfaces was evaluated against *L. monocytogenes* strains and the results showed that both BS were efficient on retarding the surface colonization (Araujo et al. 2011).

Within the context of the effect on adhesion and biofilm formation when surfaces are conditioned with BS, it is important to investigate the relationship between physicochemical properties of surfaces and BS and the inhibition of adhesion and biofilm formation. The objective of this work was to relate physicochemical modifications on surfaces to anti-adhesive/antibiofilm and antimicrobial properties of rhamnolipids and surfactin biosurfactants using different materials. To perform this study two important biofilm forming bacteria were selected: the pathogen *L. monocytogenes* (a Gram-positive microorganism related to a foodborne disease), and the opportunistic (Gershman et al. 2008) *Pseudomonas fluorescens* (a Gram-negative microorganism commonly found at industrial water systems and related to food spoilage).

2. Material and methods

2.1. Microorganisms

Biosurfactants were obtained from *P aeruginosa* PA1 (Petrobras and INCQS P4046, Brazil) and *Bacillus subtilis* ATCC 21332 (INCQS, Brazil). The microorganisms chosen to evaluate biofilm formation were *L. monocytogenes*, ATCC 7644 and ATCC 19112 (INCQS, Brazil), previously described as strong biofilm formers (Araujo et al. 2011) and *P. fluorescens* ATCC 13525 (INCQS, Brazil). Microorganisms were stored in LB broth with 20% (v/v) glycerol at −80° C until use.

2.2. Surfactants

2.2.1. Crude and purified rhamnolipids

Crude rhamnolipids were obtained from *P aeruginosa* PA1 (Santa Anna et al. 2002; Santa Anna, Freire, Borges, Kronemberger & Perreira, 2004) according to the methodology described by Santos et al. (2002). After production, cells were removed by centrifugation, and the supernatant was sterilized, filtered (0.22 μm) and stored at 4 °C until use. The cell-free broth containing rhamnolipids was purified (Costa, Nitschke, Haddad, Eberlin, & Contiero, 2006). After purification, the biosurfactant was re-dissolved in water at different concentrations. The aqueous solutions were filtered through a 0.22 μm filter and stored at 4 °C until use. Rhamnose quantification was carried out using the method of Pham, Webb, and Rehm (2004). Rhamnose values were adjusted to rhamnolipids content according to Santa Anna (2005).

2.2.2. Rhamnolipids homologue composition

Homologue composition of the produced rhamnolipids and their percentage were determined according to Déziel et al. (1999).

2.2.3. Surfactin

Surfactin was produced and purified according to Nitschke and Pastore (2006). Aqueous solutions at different concentrations were filtered through a 0.22 μm pore size filter and stored at 4 °C until use.

2.3. Physicochemical properties

The following parameters were analyzed in order to compare the respective tensoactive properties of the products: surface tension (ST), interfacial tension (IT, against *n*-hexadecane) and critical micellar concentration (CMC). Those parameters were determined using the pendant drop technique (DSA 100S Goniometer, Model: OF 3210) according to Song and Springer (1996). The results were expressed as the mean value of at least ten pendant drops at 23 °C and 55% relative humidity. Biosurfactant serial dilutions in water were performed and analyzed also using the pendant drop technique to determine the critical micellar concentration (CMC) according to Sheppard and Mulligan (1987).

The emulsification index (E_{24}) using *n*-hexadecane was evaluated according to Cooper (1986). The height of the emulsified layer was monitored during 30 days to check for emulsion stability.

2.4. Solid surfaces preparation and surface conditioning

The surface samples were 2 cm^2 coupons and also microtiterplate wells of stainless steel AISI 304 and polystyrene. All of them were cleaned and conditioned according to Nitschke et al. (2009). The surfaces were conditioned with aqueous solutions of biosurfactants in four different concentrations (5000, 2500, 1000, 500 μg/mL). Water was used as control. The samples were then washed with sterile water and left to dry at room temperature until further analysis.

2.5. Solid surfaces and microbial physicochemical properties

The sessile drop technique was used to measure the contact angle between surfaces and different liquids such as water, formamide and ethylene glycol (Krüss DSA 100S goniometer, model: OF 3210). Angles were measured following the approach described by Van Oss (1995). Results are expressed as the mean value of at least ten drops (10 μl) at 23 °C and 55% relative humidity.

Surface free energy was calculated from the surface tension components of each known liquid obtained from the contact angle using Eq. (1) (Van Oss, Chaudhury, & Good, 1988):

$$(1+\cos\theta)\gamma_i^{TOT} = 2\left[\left(\sqrt{\gamma_S^{LW}\gamma_i^{LW}}\right) + \left(\sqrt{\gamma_S^{+}\gamma_i^{-}}\right) + \left(\sqrt{\gamma_S^{-}\gamma_i^{+}}\right)\right] \tag{1}$$

where: θ is the contact angle between the liquid and the surface; γ^{TOT} is the total surface free energy; $\gamma^{\mathrm{LW}}$is the Lifshitz–van der Waals component; γ^{AB} is the Lewis acid–base property; γ^{+} and γ^{-} are the electron acceptor and donor components, respectively; $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$ *and* $\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}}$

The surface hydrophobicity was determined by contact angle measurements and by the approach of Van Oss et al. (1988) and Van Oss (1995), which states that the degree of hydrophobicity of a material (*i*) is expressed as the free energy of the interaction between two entities of that material when immersed in water (w), ΔGiwi. If the interaction between the two entities is stronger than the interaction of each entity with water, the material is considered hydrophobic (ΔGiwi<0). Hydrophilic materials have a ΔGiw*i* > 0. The results were calculated according to Eq. (2)(2):

$$\Delta G_{iWi} = -2\left(\sqrt{\gamma_l^{LW} - \gamma_W^{LW}}\right) - 4\left(\sqrt{\gamma_l^{+}\gamma_W^{-}} + \sqrt{\gamma_l^{-}\gamma_W^{+}} - \sqrt{\gamma_l^{+}\gamma_l^{-}} - \sqrt{\gamma_W^{+}\gamma_W^{-}}\right) \tag{2}$$