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Use of phenyl isothiocyanate for biofilm prevention and control

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ABSTRACT

The purpose of the present study was to assess the antibacterial activity of phenyl isothiocyanate (PITC), a synthetic isothiocyanate, on biofilms of *Escherichia coli* and *Staphylococcus aureus*. The effects of PITC on bacterial free energy of adhesion and motility were also investigated. Biofilm formation in 96-well polystyrene microtiter plates was quantified by crystal violet staining and the metabolic activity of those biofilms was assessed with alamar blue. The viability and culturability of the biofilm bacteria after exposure to PITC were determined. The highest removal and metabolic activity reduction of biofilms with PITC was around 90% for both bacteria. Treatment with PITC enabled 4.5 and 4.0 $\log_{10}$ reductions of the number of viable cells for *E. coli* and *S. aureus*, respectively; and no colony forming units (CFUs) were detected. PITC also affected the adhesion process and motility of bacteria, greatly preventing biofilm formation. In conclusion, PITC enabled both biofilm prevention and control, promoting high biofilm removal and inactivation activities, suggesting that this compound is a promising disinfectant.

1. Introduction

In the last decades, an increasing incidence of food poisoning cases has been reported due to the contamination of food with pathogens and spoilage organisms. This has prompted the need for hygiene and sanitary actions and regulatory practices regarding food control, food safety and food trade processes (Kühn et al., 2003). The emphasis on safer foods and longer shelf-life has led to higher frequency of disinfection of food-contact surfaces, equipment, utensils, etc. (Langsrud et al., 2003). The recurrent application of disinfectants at sub-optimal conditions (e.g. concentration, temperature and exposure time) has resulted in the increased resistance of microorganisms (Zottola and Sasahara, 1994). Resistant microorganisms have been responsible for a decreased efficiency of disinfection programs and, consequently, for severe contaminations in industrial, environmental and biomedical settings (Talon, 1999; Chorianopoulos et al., 2011). The ability of bacteria to adhere to surfaces is one of the most important causes for the failure of disinfection programs (Gilbert et al., 2002a, 2002b). Bacterial adhesion is implicated in the contamination of medical devices, industrial cooling water systems and food processing equipment (Glinel et al., 2008). These attached microorganisms can form biofilms, where they enjoy a number of advantages over their planktonic cells, particularly an increased tolerance to antimicrobials (Busetti et al., 2010; Simões et al., 2010a). Bacteria in biofilms can be 10 to up 1000 times more resistant to the effects of antimicrobial agents than their planktonic counterparts (Amorena et al., 1999; Mah and O'Toole, 2001; Smith, 2005; Zahin et al., 2010). Biofilm resistance is not completely understood and the persistence of biofilms, even after aggressive antimicrobial treatment, continues to motivate the search for new control strategies (Xu et al., 2000; Inoue et al., 2008).

New disinfection compounds and processes are necessary in order to ensure high levels of sanitation (Grönholm et al., 1999; Wutzler et al., 2005). Substantial resources have been invested in the research of new antimicrobial compounds (Cowan, 1999; Simões et al., 2008; Zahin et al., 2010). These compounds must be efficient in the inactivation of pathogens while maintaining the organoleptic properties of the product (Bermúdez-Aguirre and Barbosa-Cánovas, 2012). Plants are an interesting source of such compounds as they produce an enormous array of phytochemicals with antimicrobial activity, most of them related to defense/stress mechanisms against microorganisms, insects, nematodes and even other plants (Dangl and Jones, 2001; Dixon, 2001). Glucosinolates are sulphur-containing glucosides found in several plants, including the *Cruciferae* family (which comprises well-known plants such as broccoli, cabbage, cauliflower, watercress), and are hydrolyzed as a defense mechanism of the plant, releasing several compounds with

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antimicrobial activity (glucosinolate hydrolysis products – GHP) (Gomes de Saravia and Gaylarde, 1998; Fahey et al., 2001). Amongst these products, isothiocyanates (ITCs) are the most potent inhibitors of microbial activity (Saavedra et al., 2010). ITCs have an electrophilic character, due to the presence of a –N=C=S group, which is responsible for their strong reaction with amines and cellular thiols. Thus, ITCs are capable to interaction with diverse biomolecules (Saavedra et al., 2010).

In the present study, a synthetic ITC – phenyl isothiocyanate (PITC) (Fig. 1) was tested in the prevention and control of biofilms formed by *E. coli* and *S. aureus* in 96-well polystyrene microtiter plates. *S. aureus* is an important foodborne pathogen and a major cause of *staphylococcal* food poisoning cases (Chorianopoulos et al., 2011). The presence of *E. coli* in foods such as meat, fish and milk is an indicator of fecal contamination, causing outbreaks of diarrhea, gastroenteritis and hemolytic uremic syndrome (Mauriello et al., 2005). *E. coli* has also shown the ability to attach strongly to leafy structures (Bermúdez-Aguirre and Barbosa-Cánovas, 2012). To our knowledge, there are no studies on the activity of PITC against biofilms. Since biofilm formation is a multistep process, starting with transient adhesion to a surface, the effects of the application of PITC were also evaluated on bacterial adhesion and motility. Different approaches were tested in order to characterize the activity of PITC in the prevention of biofilm formation and in the removal and inactivation of 24 h-aged biofilms. Also, the effects of PITC were tested in pre-stressed biofilms, whose cells were previously exposed to the chemical.

2. Materials and methods

2.1. Bacteria

E. coli CECT 434 and *S. aureus* CECT 976 were used in this study. These bacteria were already used as model microorganisms for antimicrobial tests with phytochemical products (Simões et al., 2008; Saavedra et al., 2010; Borges et al., 2013). Bacteria were picked from overnight cultures in 250 mL flasks with 100 mL of Mueller-Hinton broth (MHB, Merck, Germany) incubated at 30 °C and under 150 rpm of agitation (CERTOMAT® BS-1, Sartorius AG, Germany).

2.2. Minimum inhibitory concentration

PITC (Sigma, Portugal) was prepared in dimethyl sulfoxide (DMSO; Sigma). To determine whether the presence of PITC had effects on bacterial growth in liquid culture, the minimal inhibitory concentration (MIC) was determined using the standard broth microdilution method (CLSI, 2007). MIC was defined as the lowest concentration of the antimicrobial product that inhibited bacterial growth. Three independent experiments were performed for each bacterium and condition.

2.3. Free energy of adhesion

The free energy of adhesion between the bacterial cells and polystyrene (PS) surfaces was assessed according to the procedure described by Simões et al. (2010a) and calculated through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory expressed by the Dupré equation. When studying the interaction between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w), the total interaction energy, ΔG_{bws}^{TOT}, can be expressed by the interfacial tensions components as follows:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (1)$$

For instance, the interfacial tension for one system of interaction (bacteria/substratum – γ_{bs}) can be defined by the thermodynamic theory according to the following equations:

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (2)$$

$$\gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2 \times \sqrt{\gamma_{b}^{LW} \times \gamma_{s}^{LW}} \quad (3)$$

$$\gamma_{bs}^{AB} = 2 \times \left(\sqrt{\gamma_{b}^{+} \times \gamma_{b}^{-}} + \sqrt{\gamma_{s}^{+} \times \gamma_{s}^{-}} - \sqrt{\gamma_{b}^{+} \times \gamma_{s}^{-}} - \sqrt{\gamma_{b}^{-} \times \gamma_{s}^{+}} \right) \quad (4)$$

The other interfacial tension components, γ_{bw} (bacteria/water) and γ_{sw} (substratum/water), were calculated in the same way, which allowed the assessment of thermodynamic energy of adhesion. Thermodynamically, if $\Delta G_{bws}^{TOT} < 0$ mJ m^{-2}, the bacterial adhesion to the substratum is favorable. If $\Delta G_{bws}^{TOT} > 0$ mJ m^{-2}, adhesion is not expected to occur (Simões et al., 2010a). The hydrophobicity of PS was obtained from Simões et al. (2010a):$\Delta G_{sws}^{TOT} = -44$ mJ m^{-2}.

2.4. Motility

Overnight cultures grown on Luria–Bertani broth (LBB; Merck, Germany) were used to characterize bacterial motility. A volume of 15 μL of these cultures were applied in the center of plates containing 1% tryptone, 0.25% NaCl, and 0.3% or 0.7% (w/v) agar (Merck, Germany) for swimming/sliding and swarming motilities, respectively (Butler et al., 2010; Stickland et al., 2010). PITC at MIC and 5 × MIC were incorporated in the growth medium (tempered at 45 °C). Plates were incubated at 30 °C and the diameter (mm) of the bacterial motility halos were measured at 24, 48 and 72 h. Three plates were used to evaluate the motility of each bacterium. The control was performed with DMSO.

2.5. Biofilm formation in sterile 96-well polystyrene microtiter plates

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. (2000). For each bacterium, at least 16 wells of a sterile 96-well PS microtiter plate (Orange Scientific, USA) were filled with 200 μL of overnight batch cultures in MHB (with a density of 1 × 10^8 cells mL^{-1}) and incubated overnight at 30 °C and 150 rpm. The PS microtiter plates are commonly used as the standard bioreactor system for adhesion and biofilm formation of bacteria isolated from many different environments (Simões et al., 2010a; Moreira et al., 2013). Negative control wells contained MHB without bacterial cells. The plates were incubated for 24 h at 30 °C and agitated at 150 rpm. The prevention and control activities of PITC were tested as well as the cell adaptation to this product. Table 1 provides a schematic explanation of the biofilm tests.

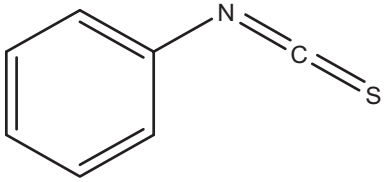

Fig. 1. Chemical structure of PITC.