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Combination of natural antimicrobials and sodium dodecyl sulfate for disruption of biofilms formed by contaminant bacteria isolated from sugarcane mills

Rachel Tereza Rigotti, Jessica Audrey Feijó Corrêa, Natalia Janaina Lago Maia, Giovanna Cesaro, Edvaldo Antônio Ribeiro Rosa, Renata Ernlund Freitas de Macedo, Fernando Bittencourt Luciano *

School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, 80215-901 Curitiba, Paraná, Brazil

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ABSTRACT

Bacterial contamination is found during ethanol production in the form of biofilms, which can decrease ethanol yield by 30%. Carvacrol, nisin, and hops extract, alone and in combination with SDS, were tested against planktonic and preformed biofilms of *Lactobacillus fermentum*, *Lactobacillus plantarum*, and/or *Leuconostoc mesenteroides*. MICs of carvacrol, nisin, hops extract and SDS were 250, 30, 5 and 37.5 mg/L, respectively, against all bacteria. Synergism between SDS and carvacrol ($FIC_{index} = 0.125$), and SDS and nisin ($FIC_{index} = 0.25$) was also found against all bacteria. Bacterial viability within biofilms and removal of biomass were indirectly measured using MTT and crystal violet methods, respectively. Metabolic activity of biofilms was inhibited up to 92% with nisin + SDS, while carvacrol + SDS could remove 82% of the biomass. Thus, combined use of these antimicrobials and SDS may provide an effective method for cleaning equipment of ethanol plants and prevent formation of biofilms.

1. Introduction

The Brazilian sugarcane harvest of 2014/2015 generated an estimated 36.36 million tons of sugar and 28.66 billion L of ethanol (CONAB, 2014), making Brazil the second largest ethanol producer in the world, only behind the USA. Common fermentation tanks contain 300,000 to 1 million L of sugarcane must which are usually fermented by *Saccharomyces cerevisiae*. Contaminating bacteria can easily reach levels of $<10^7$ cells/mL in these tanks, resulting in significant competition for the utilization of sugars as an energy source and forming undesirable substances, such as lactic and acetic acids, which affect the metabolism of the fermenting yeast. Moreover, some bacteria form polymers of dextran, which are responsible for clogging pipes, while other can adhere to the yeast, causing their flocculation. These processes can reduce the viability of *S. cerevisiae*, reducing the sugar-to-ethanol conversion rate by >15% and thus causing losses of 10,000–30,000 L of ethanol per day in a medium-sized industry (Skinner & Leathers, 2004; Amorim, Lopes, & Oliveira, 2011; Narendranath, Thomas, & Ingledew, 2001; Lucena et al., 2010; Rich, Leathers, Nunnally, & Bischoff, 2011).

Several gram-positive bacteria, including species of *Bifidobacterium*, *Clostridium*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Weissella* and, with much higher frequency, *Lactobacillus*, have been isolated from one wet mill and two dry grind corn-derived fuel ethanol facilities in the USA (Skinner & Leathers, 2004). This same research group also collected samples from commercial fermentation vats and tested the capacity of contaminant bacteria to form biofilms under laboratory conditions (Skinner-Nemec, Nichols, & Leathers, 2007). From 129 biofilm-forming isolates, most were lactic acid bacteria, mainly from the genus *Lactobacillus* (Skinner-Nemec et al., 2007). Studies have also shown that the genus *Lactobacillus* is the most prevalent in sugarcane mills throughout various regions of Brazil (Lucena et al., 2010; Costa et al., 2015). However, few studies have examined the main species that form biofilms on tanks, pipes, heat exchangers and valves of ethanol production plants. Moreover, obligatory heterofermentative bacteria, such as *Lactobacillus fermentum* and *Lactobacillus mucosae*, were found to greatly inhibit the conversion of glucose in ethanol by *S. cerevisiae* (Rich, Leathers, Bischoff, Anderson, & Nunnally, 2015). Gram-negative bacteria are not often present since they have a thin cell wall and their outer and inner membranes are solubilized in the alcohol present in the fermentation vats, which reduces the probability of contamination by these microorganisms (Messetti, Santos, Angelis, Chierice, & Neto, 2010).

A biofilm is a structured community of microorganisms bound to a biotic or abiotic surface and incorporated into a matrix of extracellular polymeric substances (EPSs) (Abee, Kovács, Kuipers, & van der Veen,

* Corresponding author at: School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição, 1155, 80215-901 Curitiba, PR, Brazil.
E-mail address: fernando.luciano@pucpr.br (F.B. Luciano).

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2011). This extracellular mass is formed by polysaccharides, proteins, nucleic acids, and lipids, providing a three-dimensional support structure that immobilizes and links cells; this provides mechanical stability and allows adhesion of the biofilm to the surface. An estimated 90% of biofilm mass is formed by extracellular material, and only 10% is formed by actual microorganisms (Flemming & Wingender, 2010). Biofilms are difficult to remove and require the manufacturing facilities be shut down for periodic cleaning, resulting in economic losses (Skinner-Nemec et al., 2007). More importantly, when embedded in a biofilm, microorganisms become up to 1000 times more resistant to antibiotics compared with planktonic cells (Aslam, Trautner, Ramanathan, & Darouiche, 2007; Murphree, Heist, & Moe, 2014).

Despite the common use of antibiotics to combat contaminant bacteria populations in Brazilian sugarcane mills, there are many market-based factors pressuring ethanol manufacturers to reduce the use of these substances. For example, yeast biomass, which is a by-product of the ethanol industry, has high added value as supplement for animal feed. However, European Union countries, who are major importers of this yeast, have banned the use of antibiotics as additives in animal feed. For this reason, Brazil has already suffered embargoes in yeast extract export to Europe due to the presence of monensin (Feed Info News Service, 2008). Therefore, natural alternatives have been used by mills to reduce the bacterial population in the fermentation process; of these alternatives, hops extract is the main product adopted by industry for this purpose (Leite, 2011; Muthaiyan, Limayem, & Ricke, 2011). Additionally, the bacteriocin nisin has demonstrated efficacy against *Lactobacillus* strains isolated from ethanol plants in the USA (Limayem et al., 2011). However, commercial use of nisin is difficult due to the high cost of the compound. Essential oils are potent antimicrobial agents that have gained attracted interest as alternatives to synthetic preservatives used in food. For example, carvacrol is the major component of the essential oil obtained from oregano (*Origanum vulgare*) and exhibits activity against several biofilm-forming microorganisms (Nostro et al., 2012; Davidson, Critzer, & Taylor, 2013), which could support its use in the ethanol production industry.

Most antimicrobial agents are able to kill microorganisms within a biofilm when used at high concentrations but are unable to remove the formed biomass from equipment surfaces and piping (Simões, Simões, Machado, Pereira, & Vieira, 2006). Hence, it is necessary to use a surfactant agent to aid in the removal of the biomass. Sodium dodecyl sulfate (SDS) is an anionic detergent containing a 12-carbon chain bound to a sulfate group. In the industry, SDS is used as an antimicrobial agent and surfactant to prevent the formation and growth of biofilms (Liu et al., 2012; Li, Molin, Yang, & Ndoni, 2013).

Based on these data, we aimed to determine the activity of the natural antimicrobials carvacrol, nisin Z, and hops extract combined with the surfactant SDS against biofilms formed by gram-positive bacteria isolated from Brazilian ethanol plants (*L. fermentum*, *Lactobacillus plantarum*, and *Leuconostoc mesenteroides*).

2. Materials and methods

2.1. Antimicrobials and microorganisms

Hops extract Betabio 45 was provided by Hopsteiner SS (Mainburg, Germany), and nisin Z 2.5% (w/w) was provided by Handary SA (Lendelede, Belgium). Carvacrol was purchased from Sigma-Aldrich Brasil Ltda. (Sao Paulo, Brazil), and SDS was purchased from Neon Reagentes Analíticos (Sao Paulo, Brazil). *L. mesenteroides* (LM) CCT 0848 and *L. fermentum* (LF) CCT 1629 were acquired from the Tropical Culture Collection of Foundation André Tosello (Campinas, Brazil) and had been isolated from Brazilian ethanol production plants. *L. plantarum* PUCPR 44 (LP) was acquired from the Culture Collection of Laboratório de Tecnologia de Produtos Agroalimentares of Pontifical Catholic University of Paraná (Sao José dos Pinhais, Brazil).

2.2. Preparation of sugarcane juice supplemented with nutrients (SSN)

In order to mimic Brazilian ethanol plant conditions, SSN was used for the experiments performed in this study. The broth preparation, adapted from Nobre, Horii, and Alcarde (2007), consisted of a combination of 0.3% yeast extract and 0.6% peptone in clarified sugarcane juice. The clarified sugarcane juice was obtained from the addition of two whipped egg whites per liter of sugarcane juice. This mixture was autoclaved until the temperature reached 121 °C. The liquid was then cooled to room temperature, filtered with gauze and cotton for removal of existing soils in the broth, and adjusted to a soluble solids concentration of 10°Brix by adding sterile deionized water. After supplementation with yeast extract and peptone, the SSN broth was autoclaved again for final sterilization. This medium was used for all experiments performed in the present work.

2.3. Determination of the minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FIC_{Index})

First, we determined the MICs of carvacrol, nisin Z 2.5%, hops extract, and SDS against *L. fermentum*, *L. plantarum*, and *L. mesenteroides*. The microdilution method was applied using 96-well microtiter plates (CLSI, 2012). Each well was filled with 297 μL of broth plus the experimental treatment agents and 3 μL of bacterial inoculum containing about 10^8 CFU/mL. The antimicrobials were added at different concentrations for each row of the plate. The solvent dimethyl sulfoxide (DMSO) was used for solubilization of carvacrol and hops extract, with a maximum concentration of 2% per well, at which no antimicrobial activity was observed. Nisin Z 2.5% and SDS were directly dissolved in the broth. No antimicrobials were used in the control group. The plates were incubated for 24 h at 37 °C under continuous agitation at 110 rpm. The MIC was determined as the lowest dose at which the well showed no turbidity by microbial growth.

After evaluation of the MICs of compounds alone, the compounds were tested in combination with SDS to determine the FIC_{Index}. The compounds were used at their MICs, and antimicrobials were also tested at 1/2, 1/4, 1/8, 1/16, and 1/32 of their MICs. The FIC_{Index} value of each combination was thus determined using the following the formulas:

$$\text{FIC} = \frac{\text{Concentration of the compound used in the combination MIC}}{\text{MIC of the compound alone.}}$$

$$\text{FIC}_{\text{Index}} = \text{FIC of the natural antimicrobial} + \text{FIC of the surfactant}$$

FIC_{Index} values of >0.5 and <4.0 indicated insignificant interactions between the compounds, whereas FIC_{Index} values of 0.5 or less or 4.0 or more indicated synergism and antagonism, respectively (Palaniappan & Holley, 2010).

2.4. Quantification of biofilm mass by crystal violet staining

Antimicrobial-dependent biofilm mass removal was quantified using the methods described by Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000). Each well of a polystyrene 96-well microplate was filled with 200 μL of bacterial suspension in a 0.5 McFarland scale in SSN (~10^8 CFU/mL). For the bacterial pool, 66.7 μL of each bacterial suspension at 0.5 McFarland was added to the wells (total population of ~10^8 CFU/mL or ~3.33×10^7 CFU/mL of each bacterial strain). Plates were then incubated for 24 h at 37 °C with shaking at 110 rpm. The broth was then refreshed, and the plates were incubated for more 24 h at 37 °C with shaking at 110 rpm. After incubation, the medium was replaced with 200 μL of treatment solution (containing antimicrobials at varying concentrations up to 128 × MIC), and the plates were incubated again at 37 °C with shaking at 110 rpm for 1 h. No antimicrobials were added to the control wells. The test was performed for each of the bacteria separately and for the combined bacterial pool.