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Chemically Modified Natural Substances Screening for Biofilms Inhibition and Biofouling Control

Maria da Glória C. Silva[a,b,c], Darne G. Almeida[b,c], Rita de Cássia F. Soares da Silva[b,c], Hugo M. Meira[b,c], Fabíola G. Almeida[b,c], Múcio L. B. Fernandes[c], Andrea K. P. Silva[c], Valdemir A. Santos [b,c], Leonie A. Sarubbo*[a,b,c]

[a] Northeast Biotechnology Network, Federal Rural University of Pernambuco – RENORBIO/UFRPE, Rua Dom Manoel de Medeiros, s/n, Dois Irmãos. CEP: 52171-900, Recife – Pernambuco, Brazil
[b] Centre of Science and Technology, Catholic University of Pernambuco, Rua do Príncipe, n. 526, Boa Vista, Cep: 50050-900, Recife-Pernambuco, Brazil
[c] Advanced Institute of Technology and Innovation (IATI), Rua Joaquim de Brito, n. 216, Boa Vista, CEP: 50070-280, Recife, Pernambuco, Brazil
leonie@unicap.br

Biofilms can be characterized as a microorganisms community attached to a surface and enveloped by an extra polymeric substances complex array in a moist environment. It is known that the growth and formation way of the biofilm structure induces resistance to several microbial agents, making it impossible to disintegrate and eliminate these structures. These biofilms can settle in the vessels surface, pipes, thermoelectric cooling systems and so forth causing serious economic problems in the most diverse strategic sectors of a country such as nautical, industrial, energy.
Search for substances capable of disturbing this formation is desired, since the formation of the biofilm is the crucial stage for the emergence of new organisms that will develop until the climax of the ecological succession with the arrival of macrofouling. These substances when isolated can be ideal inputs for the development of antifouling products and be incorporated into paints and coatings. Thus, the present study aim was synthesize antibacterial substances through chemical modification of natural substances, obtained from vegetable oils, specifically soybean oil (*Glycine max* (L.)). Selected substances and their variants for the synthesis were chosen based on/supported by studies that demonstrated the antifouling potential of some types of fatty acids and the efficacy of monoglycerides in combating the formation of biofilms. The following substances were synthesized: 9,10-dihydroxy octadecanoic acid; 9,10-dihydroxy octadecanoate; 9-octadecenoate 2,3-dihydroxypropanoyl; 2,3-dihydroxypropanoyl dodecanoate and 9,10-dihydroxy octadecanoate 2,3-dihydroxypropanoyl. For the synthesis of the substances, neutralization reactions using a catalyst base as well as esterification reactions with a polyalcohol (glycerol) and/or hydroxylation reactions by epoxidation were performed.
To assess the antimicrobial activity, a rapid antibiogram assay was developed to observe the bacterium *Pseudomonas aeruginosa* UCP0992 growth obtained from the Cultures Collection of Catholic University of Pernambuco – UNICAP, Brazil. The microorganism *Pseudomonas aeruginosa* used in this study is reported as a pioneer in the formation of resistant biofilms. *Pseudomonas aeruginosa* UCP0992 was seeded in Petri dishes containing medium LB then, antibiogram disks (Ø = 6 mm) each containing the synthesized substances to be tested were transferred to the plates and arranged in equidistant positions. Plates were incubated in an oven at 30 °C and the presence or absence of halos around each disc was verified after 24h, indicating whether or not the inhibition occurred. Any halo produced, regardless of its diameter, was considered as inhibition. All synthesized substances showed inhibition halo indicating potential use for application in the microorganisms inhibition and therefore the biofilm removal.

1. Introduction

In the marine environment, microorganisms associated with local organisms produce secondary metabolites that may increase the chance of survival of colonized under unfavourable conditions, as well as attract or eliminate the process of fixing and developing larvae of fouling organisms (Satheesh et al., 2016). For the establishment of biofouling, it is necessary the presence of biofilms and other organisms that secrete molecules to the environment with attractive signals, initiating the fixation and the larval metamorphosis (Dobretsov, 2010; Maruzzo et al., 2012). The basic structural unit of the biofilm is the micro colony. The proximity of cells within the micro colony (or between micro-colonies) provides an ideal environment for the creation of nutrient gradients, gene exchange, and quorum detection (Donlan, 2002). Industrial water pipes are highly favorable sites for the development and proliferation of microorganisms. Consequently, the formation of biofilms in equipment and channels is a nearly impossible process to prevent (Cristiani, 2005).
It was observed that a mixture of palmitic, stearic, oleic and linoleic acids was able to inhibit AI-2 activity, disturbing the microbial quorum sensing, thus indicating that fatty acids inhibit biofilm formation and, in turn, inhibit the larval establishment (Soni et al 2008).
In general, experiments that evaluate the inhibition of biofilms first employ disk diffusion tests with active tests (Flemming, 2009). The anti-fouling activity of fatty acids was first reported more than 20 years ago, however its anti-fouling potential was shelved until very recently (Goto et al., 2009). Research reports that there is still no certainty as to how specific fatty acids produce antibacterial effect. The ideas of a destabilization of the bacterial membrane due to its surfactant properties, formation of hydroperoxides of fatty acids that cause oxidative stress, decoupling of the synthesis of ATP and increase of the fluidity of the membrane due to the incorporation of unsaturated fatty acids in phospholipids are some of the effects reported in the literature when bacterial microorganisms are in the presence of some fatty acids (Parsons et al., 2012).
Given the fact that microorganisms are essential for the development of the biofouling process, it is imperative to explore solutions that retard this development. Thus, the objective of this experiment was to synthesize substances with antibacterial activity using the fatty acid obtained from soybean oil (*Glycine max* (L.)) as the main molecule.

2. Materials and Methods

2.1 Materials

The substances evaluated were synthesized by the techniques mentioned below or by the combination thereof. They were: 9,10-dihydroxy octadecanoic acid (hydroxylated oleic acid), sodium 9,10-dihydroxy octadecanoate (sodium salt of hydroxylated oleic acid), 2,3-dihydroxypropanoyl 9-octadecenoate (oleate of glyceryl), 2,3-dihydroxypropanoyl dodecanoate (glyceryl laurate) and 2,3-dihydroxypropanoyl 9,10-dihydroxy octadecanoate (hydroxylated glyceryl oleate).

2.2 Growth medium

Bacteria evaluated in the present study were cultured in LB medium containing the following composition (1L): 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g Agar dissolved in mL deionized water.

2.3 Obtaining monoglycerides

The monoglyceride was produced by the direct esterification reaction through acid catalysis. A ratio of 1: 6 moles of fatty acid:glycerol and 3% of sulfuric acid was used as catalyst, relative to the mass of the respective fatty acid (oleic, lauric and hydroxylated oleic). Glycerol was added in a beaker followed by the addition of sulfuric acid under constant stirring. Thereafter, the fatty acid was added to the mixture. After complete compounds addition, the mixture was heated at 150 °C under constant stirring for 48 hours. At the end of the reaction, the mixture was transferred to a separatory funnel and the lower phase containing excess glycerol was discarded. The upper phase containing the respective monoglyceride (2,3-dihydroxypropanoyl 9-octadecenoate, 2,3-dihydroxypropanoyl dodecanoate and 2,3-dihydroxypropanoyl 9,10-dihydroxy octadecanoate) was diluted with acetate of ethyl acetate, washed three times with a saturated sodium chloride solution in an amount equal to one half of the volume present in the organic phase. At the end of the washes, the organic phase containing the monoglyceride was dried with anhydrous sodium sulfate, mixed with activated charcoal, filtered and put to evaporate, to remove the solvent (Nascimento et al., 2011; Solomons and Fryhle, 2012).

2.4 Oleic acid hydroxylation

Hydroxylated oleic acid was produced by the epoxidation reaction, followed by the opening of the epoxide ring in aqueous acidic medium. In a glass flask equipped with reflux condenser, a mixture containing 2: 1: 1 mol of

hydrogen peroxide, glacial acetic acid and oleic acid, respectively, was added. The mixture was kept under constant stirring at a temperature of 90 °C for a period of 24 hours. Afterwards, the mixture was transferred to a beaker and allowed to stand, allowing complete separation of the layers and solidification of the upper layer. The lower phase was discarded and the hydroxylated oleic acid was melted and washed three times with distilled water. At the end of the washes, the hydroxylated oleic acid was recrystallized with a solution of ethanol and water (Abraham and Benenati, 1972; Al-Qasiri, 2010; Vogel, 1971).

2.5 Obtaining sodium salt of hydroxylated oleic acid

Hydroxylated sodium oleate was produced by direct equimolar neutralization between the respective fatty acid and sodium hydroxide. In a beaker, the hydroxylated oleic acid was added, followed by the addition of the sodium hydroxide under constant stirring. The generated salt was washed with acetone to remove any excess of the reactants and then vacuum filtered. Finally, the salt was placed in a greenhouse to remove the solvent and water (Solomons and Fryhle, 2012; Vogel, 1971).

2.6 Nuclear magnetic resonance spectroscopy

The selected chemically modified substance was re-dissolved in deuterated chloroform (CDCl3) and the respective ^{1}H and ^{13}C NMR spectra were recorded at 25 °C using an Agilent 300Mz spectrometer operating at 300.13 MHz. Chemical shifts (δ) are given on the ppm scale relative to tetramethylsilane (TMS).

2.7 Bacterial growth inhibition tests using synthesized substances

The synthesized substances were evaluated using the Bauer method (Bauer et al., 1966). To this end, a strain of the bacterium *Pseudomonas aeruginosa* UCP0992 obtained from the Collection of Cultures of Catholic University of Pernambuco - UNICAP was seeded at the 10^{7} CFU/ml concentration in Petri dishes (Ø = 12 cm) with the aid of swabs, containing LB medium for microbial growth. Then, antibiogram disks (Ø = 6 mm) containing each synthesized substances to be tested, at the concentration of 10% (w/v), were transferred to the plates and arranged in equidistant positions. The plates were incubated in an oven at 30 °C and the presence or absence of halos around each disc was verified after 24 h, indicating whether or not the inhibition occurred. Any halo produced, regardless of its diameter, was considered as inhibition. Penta-acetate glucose was used as negative control.

2.8 Statistical analysis

All determinations were performed at least three times and the data were analyzed using the Statistica® program, version 10.0 (Statsoft Inc, USA).

3. Results and Discussion

3.1 Synthesized substances and bacterial growth inhibition tests using synthesized substances

Figure 1 shows the results for the tests for inhibition of microbial growth. It was found that all chemically modified substances caused some kind of inhibition of microbial growth, highlighting the sodium salt of hydroxylated oleic acid and glyceryl laurate, which presented the highest inhibition halos (Table 1). Penta-glucose acetate (negative control) did not inhibit bacterial growth. There was no significant statistical difference ($p < 0.05$) between the inhibition halos produced by glyceryl oleate and hydroxylated oleic acid. The Gram-negative bacterium *Pseudomonas aeruginosa* UCP0992 used in this preliminary test was chosen because of its great capacity to form resistant biofilms (Jagani et al, 2009). It is known that biofilms are the first structures formed in the ecological succession that precedes the fouling-causing fouling process (Doghri et al., 2011). In addition, microorganisms that grow in a biofilm are much more resistant to inhibitory agents than planktonic cells, requiring 1000 times more inhibitors to kill biofilm cells than planktonic cells (Jagani et al, 2009).

Table 1: Results of the microbial inhibition and halos produced by chemically modified substances

Chemically modified substances	Inhibition zone diameter(cm)
Glyceryl oleate	2,86
Hydroxylated oleic acid	2,88
Glyceryl laurate	3,38
sodium salt of hydroxylated oleic acid	4,84
Hydroxylated glyceryl oleate	2,11
Penta-Acetate Glucose (Negative Control)	0,20

The substances obtained in this work and its variants (Figure 2) were based on studies with marine organisms in which the antifouling potential of some types of fatty acids and the efficacy of monoglycerides in the fight against biofilm formation were discovered (Fusetani, 2011; Schlievert et al., 1992). Among the main fatty acids isolated from marine organisms with potential for application in antifouling coatings described in the literature are 2-hydroxy-tetradecanoic acid (hydroxylated myristic acid), 9-octadecenoic acid (oleic acid), 12-methyl-tetradecanoic acid (methylated myristic acid), 3,5-dihydroxy-decanoic acid (hydroxylated capric acid). The antimicrobial and hence antifouling activities of such substances arise from the surface interactions with the plasma membrane of the organisms' cells. For such interactions to occur, it is necessary for the molecule to exhibit amphipathic characteristics, such as those obtained in the present study (Projan et al., 1994; Schlievert et al., 1992; Vetter and Schlievert, 2005). Figure 3 illustrates the ^{1}H and ^{13}C NMR spectrums of sodium salt of hydroxylated oleic acid, which was the substance that showed the highest degree of inhibition. The signals between δ 0.5 and 2.5 ppm suggests the presence of hydrogen bonded to the aliphatic group and that between δ 3.5 and 4.5 ppm indicate the presence of hydrogen bonded to the hydroxyl groups (Figure 3A). Already the peaks shown between 60 and 80 ppm (Figure 3B) are the carbon bound to the hydroxyl groups.

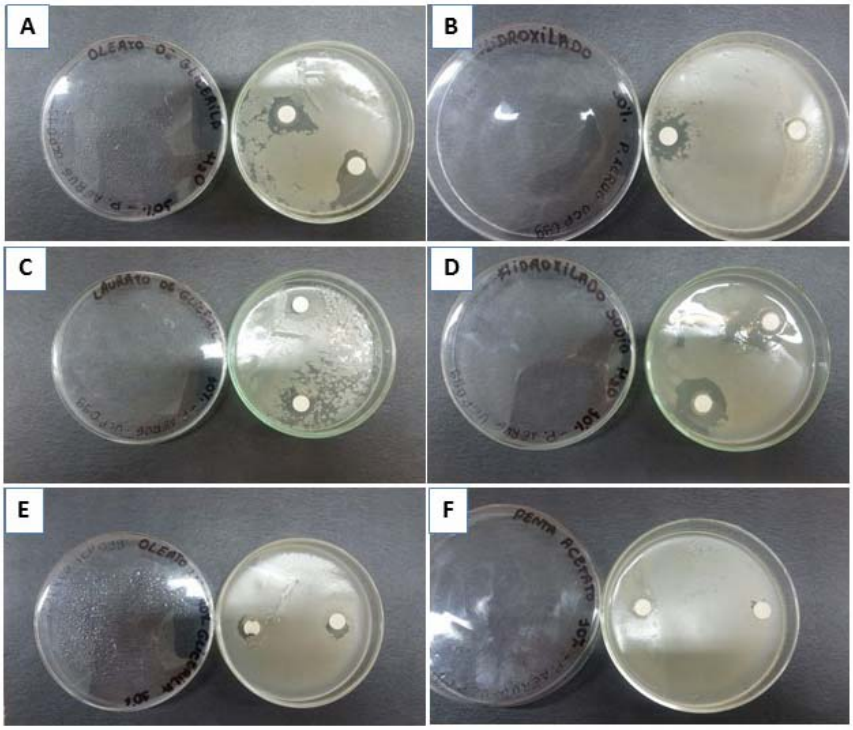

Figure 1: Results of microbial inhibition of the chemically modified substances. (A) Glyceryl oleate. (B) Hydroxylated oleic acid. (C) Glyceryl laurate. (D) sodium salt of hydroxylated oleic acid. (E) Hydroxylated glyceryl oleate. (F) Penta-Acetate Glucose (Negative Control)

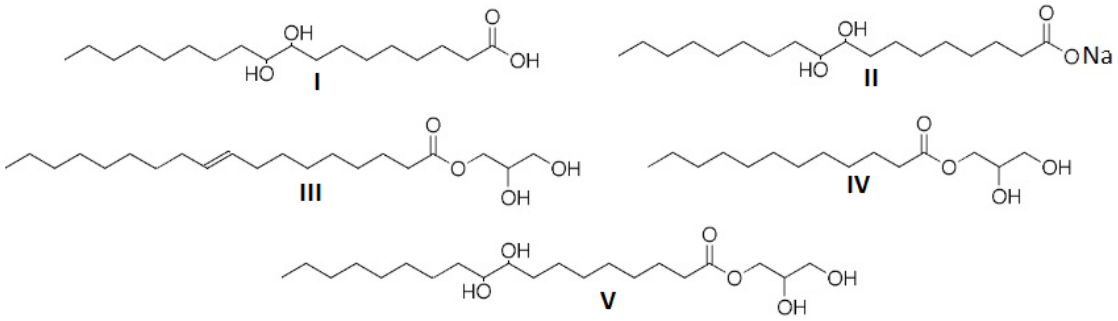

Figure 2: Substances synthesized and their variants: (I) 9,10-dihydroxy octadecanoic acid (hydroxylated oleic acid), (II) sodium 9,10-dihydroxy octadecanoate (sodium salt of hydroxylated oleic acid), (III) 2,3-dihydroxy-propanoate (glyceryl oleate), (IV) 2,3-dihydroxypropanoyl dodecanoate (glyceryl laurate) and (V) 2,3-dihydroxypropanoyl 9,10-dihydroxy octadecanoate (hydroxylated oleate of glyceryl)

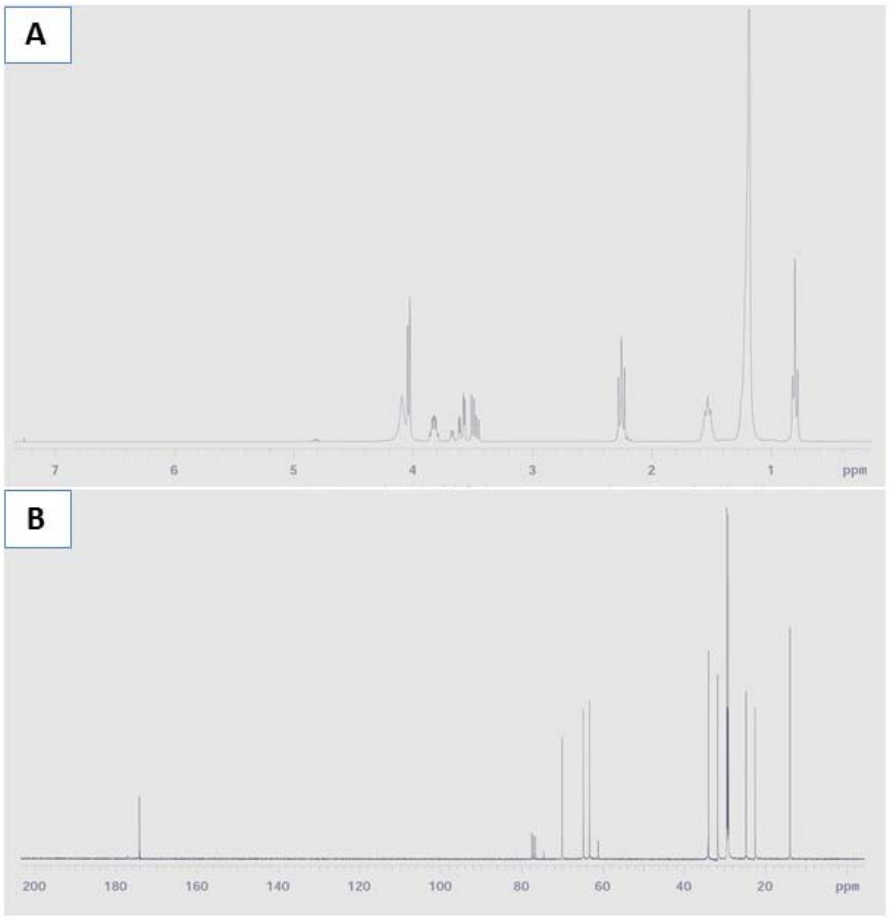

Figure 3: ^{1} H NMR (A) and ^{13}C NMR (B) spectra of the sodium 9,10-dihydroxy octadecanoate (sodium salt of hydroxylated oleic acid)

4. Conclusions

The results obtained in the present work demonstrate that it is possible to obtain chemically modified natural substances with antimicrobial and antimicrobial activity without having to extract directly from marine organisms, thus avoiding the degradation of marine fauna and flora. Besides that, these substances presented activity against bacterial growth, demonstrating potential for application in the inhibition of biofilm and consequently the biofouling.

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