Princípios de Química

Carmen Lúcia Voigt
(Organizadora)

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APRESENTAÇÃO

Química é a ciência que estuda a estrutura das substâncias, a composição e as propriedades das diferentes matérias, suas transformações e variações de energia. A Química conquistou um lugar central e essencial em todos os assuntos do conhecimento humano, estando interligada com outras ciências como a Biologia, Ciências Ambientais, Física, Medicina e Ciências da Saúde.

Pesquisas na área da Química continuam evoluindo cada dia, sendo benéficas devido maior conscientização de como usar os conhecimentos químicos em prol da qualidade de vida e do desenvolvimento da sociedade; prezando pelo meio ambiente, surgindo assim processos e novas tecnologias com menor agressão e impacto.

Muitas são as fontes degradadoras da natureza, porém os resíduos químicos são considerados os mais agressivos. Ao longo dos anos inúmeros tipos de contaminantes foram lançados no meio ambiente, causando contaminação e poluição em diversos tipos de compartimentos ambientais como solos, rios e mares. O avanço e crescimento industrial no mundo é uma das principais causas da poluição excessiva e liberação de resíduos químicos.

Devido estudos na área da Química é possível realizar remoção de poluentes por diversos processos e o desenvolvimento de técnicas e materiais é abordado neste volume, que trata de processos como adsorção para retirada de contaminantes da natureza. Além destes processos, este volume também trata de novos materiais para aplicação em substituição aos polímeros convencionais, como os biopolímeros, produzidos a partir de matérias-primas de fontes renováveis, ou seja, possuem um ciclo de vida mais curto comparado com fontes fósseis como o petróleo o qual leva milhares de anos para se formar.

Fatores ambientais e sócio-econômicos estão relacionados ao crescente interesse por novas estratégias que buscam alternativas aos produtos e processos convencionais. Neste enfoque, os trabalhos selecionados para este volume oportunizam reflexão e conhecimento na área da Química, abrangendo aspectos favoráveis para ciência, tecnologia, sociedade e meio ambiente.

Boa leitura.

Carmen Lúcia Voigt

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EFFECT OF BIOSSURFACTANTS IN PATHOGENIC BACTERIA ADHESION ON THE SURFACE OF FLEXIBLE FILMS

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RESUMO: Na indústria alimentícia, há uma grande preocupação com a contaminação de alimentos por bactérias, já que pequenas brechas podem se transformar em exposição e, consequentemente, em contaminação da produção. Uma vez que a bactéria tenha encontrado uma superfície adequada, ela pode se estabelecer e iniciar a formação de um biofilme bacteriano, que quando formado, sua remoção se torna difícil. Para evitar que isso aconteça, algumas substâncias antimicrobianas podem ser usadas, como Surfactina e Ramnolipídeo, que atuam destruindo a célula bacteriana. Estes foram testados contra vários microoganismos em diferentes polímeros utilizados na indústria de alimentos: *P. aeruginosa, Bacillus cereus, Sthaphylococcus aereus, Salmonella sp e E. coli*. Assim, foram realizados testes de adesão, testes físico-químicos de caracterização da superfície celular, adesão de microrganismos patogênicos em teste de plásticos e teste de adesão de microplacas de poliestireno. O teste de atividade microbiana mostrou que a Surfactina obteve melhores resultados que o Ramnolipídeo, exceto para o tratamento em *S. aureus* onde o ramnolipídio foi mais eficiente, nos tratamentos com *P. aeruginosa* não houve zona de inibição que comprovasse sua resistência tanto à surfactina quanto ao Ramnolipídeo. A adesão dos microorganismos patogênicos ao teste de plásticos mostrou alta eficiência contra todos os microrganismos, com exceção de *P. aeruginosa*, que apresentou redução de aderência microbiana inferior a 50%. No teste de adesão das microplacas de poliestireno, a surfactina e o ramnolipídeo foram eficientes, mas em experimentos de longo prazo a eficiência diminuiu, mostrando que em cenários longos não são muito eficientes.

KEYWORDS: Microorganismos, biofilme,

biosurfactantes, filmes flexíveis.

ABSTRACT: In the food industry, there is a large concern about food contamination by bacteria, since small breaches can turn into an exposure and consenquently into a production contamination. Once the bacteria have found an adequate surface, it can settle and start the formation of a bacterial biofilm. When the biofilm is formed, its removal becomes difficult. To prevent this from happening, some antimicrobial substances can be used such as surfactin and rhamnolipid, they act destroying the bacterial cell. Surfactin and rhamnolipid were tested against several microoganisms in different plastics used in the food industry: *P. aeruginosa, Bacillus cereus, Sthaphylococcus aereus, Salmonella sp* and *E. coli*. Thus, were performed adhesion tests, physical and chemical cell surface characterization tests, pathogenic microorganisms adhesion in plastics test, polystyrene microplates adhesion test and Scanning Electron Microscopy. The microbial activity test showed that surfactin obtained better results than rhamnolipid, except for the treatment in *S. aureus* where rhamnolipd were more efficient, in the *P. aeruginosa* treatments there were no inhibition zone that proves its resistance against both surfactin and rhamnolipid. The pathogenic microorganisms adhesion in plastics test showed high efficiency against all microorganism except *P.aeruginosa*, which had less than 50% microbial adhesion reduction. In the polystyrene microplates adhesion test, surfactin and ramnolipid were efficient, but in a long-term experimentation the efficiency dropped, showing that in long case scenarios they are not very efficient.
KEYWORDS: Microorganisms, biofilm, biosurfactants, flexible films.

1 I INTRODUCTION

Biofilms are microbiological ecosystems adhered to several types of surfaces, produced by cells of microorganisms that interact with a surface and multiply, producing colonies that gather nutrients and a polymeric wrap shield.

In the food industries, gaps in the steps of sanitization or lack of them into equipments and/or packages are sources of contamination, by the accumulation of nutrientes and microbial cell avaliability, promoting the formation of biofilms.

Biofilms are resistant to removal and its presence can cause infections of high risk to human health. In this way, the food industries seek to maintain a rigid control over the adhesion of microorganisms to surfaces in the manufacturing processes, sometimes by the addition of antimicrobial agents in food, equipments and packagings. According to the US National Institutes of Heath, approximately 80% of the medical infections worldwide are associated with biofilms (NIH, 2007).

An alternative to reduce the adhesion of pathogenic microorganisms on surfaces are biosurfactants, amphipathic compounds produced by microorganisms, which generally have antibiotics, hydrophobic properties, high biodegradability and low toxicity. They are produced by fermentation processes using renewable substrates,

which reduces production costs, acting in a wide range of temperature and pH. Among the most studied biosurfactants are surfactin, produced by bacteria of the genus *Bacillus* and the rhamnolipids synthesized by *Pseudomonas*, due to properties as water surface tension reduction and high antimicrobial activity.

The bacterial adhesion in abiotic surfaces depends on the hydrophobicity and the load on the cell surface of the microorganism (primary colonizers). Such properties can be modified by Biosurfactants, which, together with the antibiotic characteristics, are the main responsible for the adhesion of pathogenic microorganisms on surfaces.

2 I MATERIAL AND METHODS

2.1 Inoculum Preparation

For the inoculant preparation, 100 mL of Luria Bertani broth (LB) (10 g L^{-1} NaCl, 10 g L^{-1} tryptone and 5 g L^{-1} yeast extract) were produced and sterilized by autoclaving at 121 ˚C per 30 minutes.

After cooling to room temperature, the microorganisms *Bacillus subtilis* (UFPEDA 86) and *Pseudomonas aeruginosa* (UFPEDA 741), from mother inoculants, in LB broth, were inoculated separately in the proportion of 10% (v/v), incubated in an incubator (Incubator TE -420, São Paulo, Brazil) at 37 ° C, under agitation at 150 rpm for 48 hours, where the first inoculum was obtained. At the time, an aliquot of 15 mL of this first inoculum was transferred to 150 mL of the enriched Luria Bertani broth (30g L^{-1} Glucose, 30g L^{-1} NaCl, 1g L^{-1} $(NH_4)_2SO_4$, 0.25 g L^{-1} of $MgSO_4$, 10 g L^{-1} yeast extract and 10 g L^{-1} salt solution [0.10 g L^{-1} of $ZnSO_4$, 3 g L^{-1} of $MnSO_4$, 0.10 g L^{-1} $FeSO_4$, 0.10 g L^{-1} $CaCl_2$, 0.10 g $CoCl_2$ L^{-1}, 0.01 g $CuSO_2$ L^{-1}, 0.01 g L^{-1} H_3BO_3 and 0.01 g L^{-1} of $KMnO_4$ in 0.2 mol L^{-1} phosphate buffer at pH 7.0]) and incubated in an incubator (Incubator TE -420, São Paulo, Brazil) at 37 ° C, kept on agitation at 150 rpm for 48 hours.

2.2 Fermentative Process for the Production of Biosurfactant

For the production of biosurfactants, it was used 200 mL of Mineral Saline culture supplemented with carbon sources (0.2 g L^{-1} of $MgSO_4$, 7.0 g L^{-1} of Na_2HPO_4, 1.0 g of L^{-1} of $(NH_4)_2SO_4$, 3.0 g L^{-1} of KH_2PO_4, 1.7 g L^{-1} of NH_4NO_3, 10 g of L-1 of motor oil, 10 g of L^{-1} of frying soybean oil and 10 g of L-1 of glycerol in 0.2 mol L^{-1} phosphate buffer at pH 7.0). In this culture medium, 10% (v/v) of the microorganisms from the enriched Luria Bertani broth were inoculated and incubated in an incubator (Incubator TE-420, São Paulo, Brazil) at 37 °C kept on agitation at 150 rpm for 120 hours.

2.3 Crude Biosurfactant Extraction

The fermentation broth with the biosurfactant produced was acidified with HCl (6 Mol L^{-1}) to pH 2.0 and maintained at 4ºC for 24h to the biosurfactant precipitattion and according to Pollack (2000) to promote the death of microorganism. This precipitate

was separated by centrifugation (Excelsa 4, model 280-R, Fanem, São Paulo, Brazil) at 7000 rpm for 30 minutes, followed by three successive washes with distilled water.

2.4 Preparation of Pathogenic Microorganisms

Pseudomonas aeruginosa (UPEDA 416), *Staphylococcus aureus* (TSB 25923) and *Bacillus cereus* (isolated in the UTFPR Microbiology Laboratory), were inoculated in 100 mL of sterile nutrient broth and incubated at 37 ° C ± 1 °C until a bacterial suspension was obtained with 10^{10} CFU mL^{-1}. The cell growth control was verified by optical density (0.6 nm) in a UV spectrophotometer, with a length of 600 nm. This bacterial suspension was used to perform adhesion tests on flexible films and to evaluate the antimicrobial activity of biosurfactants.

2.5 Analysis of The Antimicrobial Activity of Biosurfactants

The antimicrobial activity of bissurfactants was assessed in triplicate by the method of diffusion in Agar (OSTROSKY et al., 2008), using cellulosic material films (6 mm in diameter and 2 mm in thickness) and impregnated with solutions of 0.1% (w/v) surfactin and 0.45% (w/v) ramnolipids.

The films were added on the surface of Petri plates containing 4 cm of selective culture medium, according to the pathogenic strain used for the test (strains prepared, as Salmonella-Shigella Agar (SS) for *Salmonella sp.*, Agar Methylene Blue Eosin (EMB) for *Escherichia coli*, Cetrimide Agar for *Pseudomonas aeruginosa*, Baird Parker Agar for *Staphylococcus aureus*). 0.1 mL of the respective microorganisms, previously stored in Nutrient Broth, were inoculated and incubated at 37 °C for 24 hours. The films with the respective biosurfactants were also placed on the surface of the Manitol Agar Yolk Egg Polymyxin (MYP) for the Bacillus cereus and incubated at 30 °C for 24 hours.

The selection criteria were the diameter of the zone of inhibition (Sensitive (S) ≥ 3 mm; Moderately sensitive (MS)> 2 mm and <3 mm; Resistant (R) ≤ 2 mm) of growth in relation to sensitivity and due to its importance as pathogen agent and food contaminant. Figure 1 exemplifies the formation of the zone of inhibition of antimicrobial activity for most microorganisms.

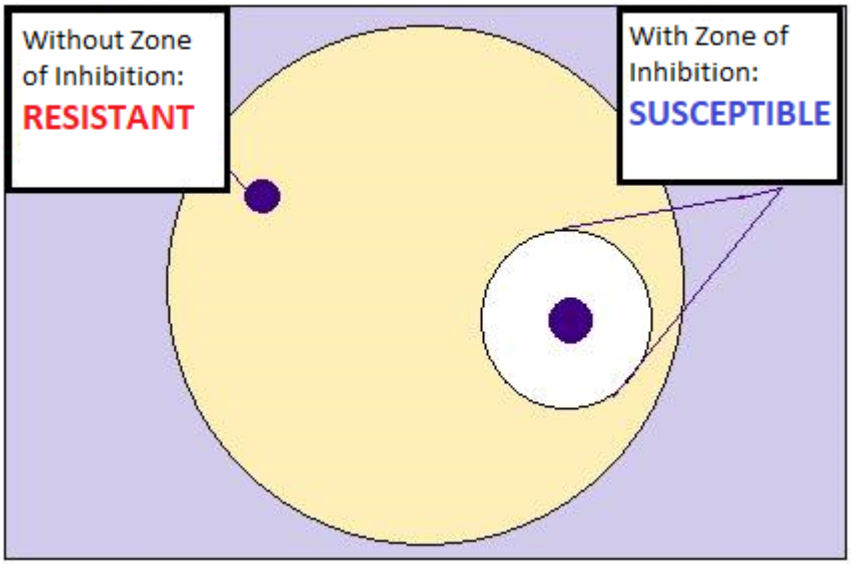

Figure 1. Example of zone of inhibition formation

Own source

2.6 Preparing the Surface of Flexible Films

The 3 cm^2 test specimens of flexible polypropylene (PP) films, high density polyethylene (HDPE), 0.05 mm thick low density polyethylene (LDPE) and 0.1 mm thick low polyethylene density (LDPE) were immersed in PA acetone (98%) and placed under ultrasonic bath for 10 minutes. Them, they were rinsed in sterile distilled water, immersed in 2% commercial neutral detergent solution for 15 minutes and a further ultrasonic bath for 10 minutes. After that period, the specimens were washed three times with sterile distilled water and dried at a temperature of 35 °C for 2 hours.

2.7 Application of Biosurfactants in Flexible Films

The 3 cm^2 specimens of 0.05 mm and 0.1 mm thick PP, HDPE, LDPE films were immersed in 0.1% (w/v) surfactin solution and 0.45% rhamnolipids (w/v) for 24 hours at room temperature. For control, the specimens were kept only in distilled water under the same time and temperature conditions, according to Zeraik and Nittsche (2010).

2.8 Adhesion Test of Pathogenic Microorganisms in Flexible Films

The adhesion test of pathogenic microorganisms in flexible films was adapted from Fernandes et al. (2007).

The specimens added to the biosurfactants (0.1% (w/v) surfactin and 0.45% (w/v)) rhamnolipids and control were washed with 5 mL of sterile distilled water and immersed in the suspension (10^{10} CFU mL^{-1}) for 1 minute without agitation and at room temperature. After this period, they were washed with 5 mL of sterile distilled water to remove non-adherent cells.

The test specimens were cleaned, but still with potential adherent cells, placed

in test tubes containing 10 mL of sterile 0.85% saline solution and maximum agitation on shakers test tubes (Vortex - Mixer, model VX-28 São Paulo, Brazil) for 2 minutes to remove adherent cells. As a result, the solution with liberated cells was diluted to 10^{-8} and 0.1 mL was inoculated into Petri plates containing selective medium, according to the strain that was used, as Salmonella-Shigella Agar (SS) for *Salmonella sp.*, Blue Eosin Agar Methylene (EMB) for *Escherichia coli*, Cetrimide Agar for *Pseudomonas aeruginosa*, Baird Parker Agar for *Staphylococcus aureus* incubated at 37 ° C for 24 hours, and Manitol Agar Yolk Polymyxin (MYP) for *Bacillus cereus* incubated at 30 ° C for 24 hours. Their analysis was performed in triplicate and the results expressed in Colony Forming Unit (CFU cm^{-2}), according to Equation 2.

$$CFU/cm^2 = (V_D/V_A).\ M.\ D/A \qquad \text{(Eq.2)}$$

Where: *CFU/cm²* - Colony-forming unit / square centimeters, *VD* - diluent rising volume (mL) (5 mL), *VA* - aliquot volume used on the surface plating (mL) (0,1 mL), *M* - Average counting obtained on plates (CFU), *D* - Dilution (10^{-10}) and *A* - Specimen area (cm^2) (2,9 cm^2).

2.9 Adhesion in Polystyrene Microplates

The pathogenic bacterial adhesion test was performed on sterile polystyrene microplates. They were filled with 200 **µ**L of each biosurfactant [0.1% (w/v) surfactin and 0.45% (w/v) rhamnolipids] for 24 hours. The microplate wells were washed with sterile distilled water and filled with 180 **µ**L of Nutrient broth. Then inoculated with 20 **µ**L of the bacterial suspension (10^8 CFU mL^{-1}) in each well. The incubation period occured at 35 °C for diferent time intervals (2, 5, 7, 10, 12, 25, 27 and 30 hours).

After those times, the wells were washed with sterile distilled water, fixed for 15 minutes with methanol and coloured for 20 minutes with 1% (w/v) crystal violet. After washing with sterile distilled water, the coloured wells were discolored twice with 200 µL of ethanol.

The optical density (550 nm) of the coloured solution obtained from the ethanol washing was used to measure the adhered cells. Wells not inoculated with the microorganisms were used as controls to determine the amount of microorganisms (CFU cm^{-2}) that adhered to the microplate over time, since the greater the amount of microorganisms present, the higher the absorbance of the solution.

The respective analysis was performed in triplicate and the adhesion kinetics results were expressed in optical density per hour (nm h^{-1}).

3 I RESULTS

3.1 Antimicrobial Activity of Biossurfactants

The 0.1% surfactin and 0.45% rhamnolipids showed antimicrobial activity (susceptible) for *Salmonella sp*., *E. coli* and *S. aureus*. However, both were resistant to *P aeruginosa*. *B. cereus* was sensitive to 0.1% surfactin and moderately sensitive to 0.45% ramnolipid (Table 1).

Pathogenic microorganisms	Inhibition halo (mm)/Biosurfactants		Antimicrobian activity
	Surfactin 0,1%	Rhamnolipid 0,45%	
Salmonella sp.	6,5	3,5	Susceptible
E.coli	6,5	3,5	Susceptible
P. aeruginosa	0,0	0,0	Resistant
S. aureus	4,5	6,0	Susceptible
B.cereus	5,5	2,0	Susceptible - Surfactin 0,1% Moderately susceptible - Rhamnolipid 0,45%

Table 1. Antimicrobial activity of biosurfactants on pathogenic microorganisms

3.2 Adhesion of Pathogenic Microorganisms in Flexible Films Impregnated With Surfactin 0.1%

When the control films were compared with those added with 0.1% surfactin, it was verified that in PP there was a reduction of one (01) logarithmic cycle in the adhesion of *Salmonella sp* and *E. coli*. However, no efficiency was observed in the decrease of colonies of *P. aeruginosa*, *S. aureus* and *B. cereus*. In HDPE, one (01) logarithmic cycle was observed for *E coli, P. aeruginosa* and *B. cereus*, and eight (08) cycles for *Salmonella sp*, but with no effect on *S. aureus*. In 0.05 mm LDPE films, a reduction of one (01) logarithmic cycle was observed for E coli and *B. cereus* and, for eight (08) cycles for *Salmonella sp*, but without efficiency on *P. areruginosa* and *S. aureus*. On the films of 0.1mm LDPE, only one logarithmic reduction was observed for Salmonella sp and had no effect on the growth of *E. coli, P. aeruginosa, S. aureus* and *B. cereus* (Table 2).

Flexible films	Adhesion of pathogenic microorganisms/ UFC cm² in flexible films with 0,1% surfactin				
	Salmonella sp.	*E.coli*	*P. aerugi-nosa*	*S. aureus*	*B.cereus*
PP	$3,8 \times 10^7$	$7,5 \times 10^7$	$1,4 \times 10^9$	$2,8 \times 10^8$	$4,0 \times 10^8$
PP control	$1,7 \times 10^8$	$6,2 \times 10^8$	$1,5 \times 10^9$	$5,1 \times 10^8$	$6,8 \times 10^8$
HDPE	< 10	$3,8 \times 10^7$	$9,4 \times 10^7$	$3,0 \times 10^8$	$9,5 \times 10^7$
HDPE control	$3,0 \times 10^8$	$7,9 \times 10^8$	$3,6 \times 10^8$	$5,7 \times 10^8$	$7,9 \times 10^8$

LDPE 0,05 mm	< 10	$5,5 \times 10^8$	$3,8 \times 10^8$	$1,9 \times 10^8$	$6,6 \times 10^8$
LDPE 0,05 mm Control	$3,0 \times 10^8$	$3,0 \times 10^9$	$4,9 \times 10^8$	$9,2 \times 10^8$	$1,6 \times 10^9$
LDPE 0,1 mm	$3,8 \times 10^7$	$2,3 \times 10^8$	$1,7 \times 10^8$	$3,8 \times 10^8$	$4,5 \times 10^8$
LDPE 0,1 mm Control	$1,3 \times 10^8$	$9,1 \times 10^8$	$2,6 \times 10^8$	$4,9 \times 10^8$	$9,1 \times 10^8$

Table 2. Adhesion of pathogenic microorganisms in flexible films with 0.1% surfactin

Note: The values presented are the average of triplicates of the zone of inhibition.

Among the films of LDPE, it was verified that the lower thickness of the plastic (0.05mm) provided greater efficiency in the reduction of cell adhesion. This aspect may be related to the biosurfactant fixing and / or absorption capability in the plastic.

Surfactin has negative charge due to its anionic group, but when applied on the surface of the films (negative charge), it may promote a low hydrophobic characteristic, in other words, an increase of positive charges on the surface of the material. According to Gang; Liu; Mu (2010), surfactin in situations of reduced translational and rotational mobility can adjust the polar group (negatively charged) to stay in contact with the aqueous medium, and the non-polar group with the hydrophobic surface, which would lead to a reduction of hydrophobicity on the surface of the material. However, this alteration was not sufficient to prevent E. coli (positive charge) adhesion in 0.05 mm and 0.1 mm LDPE films (Table 2), despite having been verified the antimicrobial ability of 0.1% surfactin on this microorganism (Table 1).

In relation to the other microorganisms analyzed, all of them had a negatively charged cell surface. In this aspect, the low hydrophobicity of 0.1% surfactin (positive charge increase) was not sufficient to maintain cell adhesion, which suggests the occurrence of an anti-adhesive effect between the surface of the film impregnated with the biosurfactant (negative charge) and micro-organisms tested (negative charge). The effect may be related to electrostatic repulsion, reduction of hydrophobic interactions and/or interference of some factors such as the production of extracellular polymeric matrix (EPM) by some microorganisms. The EPM are mostly anionic and can change the surface charge promoting electrostatic repulsion (SIMÕES; SIMÕES; VIEIRA, 2010).

Another aspect should be considered in the reduction of cellular adhesion in films such as the antimicrobial action of surfactin, being observed in all microorganisms, except for *P. aeruginosa* (Table 2).

3.3 Adhesion of Pathogenic Microorganisms in Flexible Films Impregnated With Rhamnolipid 0.45%

Considering the control films with the films impregnated with 0.45% ramonolipids,

it was observed that in PP there was a reduction of the adhesion of one (01) logarithmic cycle of *P. aeruginosa* and eight (08) cycles for *Salmonella sp*, but with no effect on *E. coli, S. aureus* and *B.cereus*. In HDPE with 0.45% ramnolipid there was a reduction of one (01) logarithmic cycle in *Salmonella sp*. and *E coli*. However, there was no influence on *P. aeruginosa, S. aureus* and *B. cereus*. The influence of 0.45% ramnolipid was higher in LDPE films with a lower thickness (0.05 mm). A logarithmic reduction of B. cereus and eight (08) cycles for *Salmonella sp*. In the films of 0.1 mm LDPE, only one logarithmic reduction was observed for *Salmonella sp*., having no effect on *E. coli, P. aeruginosa, S. aureus* and *B. cereus* (Table 3).

Flexible films	Adhesion of pathogenic microorganisms/ UFC cm² in flexible films with 0,45% rhamnolipids				
	Salmonella sp.	*E.coli*	*P. aerugi-nosa*	*S. aureus*	*B.cereus*
PP	<10	$1,5 \times 10^8$	$1,5 \times 10^8$	$3,2 \times 10^8$	$3,2 \times 10^8$
PP control	$1,7 \times 10^8$	$6,2 \times 10^8$	$1,5 \times 10^9$	$5,1 \times 10^8$	$6,8 \times 10^8$
HDPE	$5,6 \times 10^7$	$9,5 \times 10^7$	$2,8 \times 10^8$	$3,2 \times 10^8$	$4,0 \times 10^8$
HDPE control	$3,0 \times 10^8$	$7,9 \times 10^8$	$3,6 \times 10^8$	$5,7 \times 10^8$	$7,9 \times 10^8$
LDPE 0,05 mm	< 10	$1,9 \times 10^9$	$2,4 \times 10^8$	$6,6 \times 10^8$	$2,6 \times 10^8$
LDPE 0,05 mm Control	$3,0 \times 10^8$	$3,0 \times 10^9$	$4,9 \times 10^8$	$9,2 \times 10^8$	$1,6 \times 10^9$
LDPE 0,1 mm	$1,9 \times 10^7$	$2,6 \times 10^8$	$2,2 \times 10^8$	$3,6 \times 10^8$	$1,3 \times 10^8$
LDPE 0,1 mm Control	$1,3 \times 10^8$	$9,1 \times 10^8$	$2,6 \times 10^8$	$4,9 \times 10^8$	$9,1 \times 10^8$

Table 3. Adhesion of pathogenic microorganisms in flexible films with 0.45% rhamnolipds

Note: The values presented are the average of triplicates of the zone of inhibition

3.4 Comparison Between the Adhesion of Pathogenic Microorganisms in Flexible Films Impregnated With 0.1% Surfactin and 0.45% Rhamnolipids

The PP covered with 0.45% ramnolipid showed a greater ability to reduce cell adhesion, but only for *Salmonella sp*. However, 0.1% surfactin impregnated in HDPE and 0.05 mm LDPE was efficient for most tested microorganisms. The 0.1 mm LDPE with both biosurfactants had the same efficiency, but only for *Salmonella sp*.

In general, both biosurfactants showed efficiency in the antimicrobial activity when the films were impregnated with *Salmonella sp., E. coli, P. areruginosa* and *B. cereus*, having no effect on *S. aureus*.

Among the films, PP, HDPE and 0.05 mm LDPE showed a higher performance in the reduction of microbial adhesion when impregnated with 0.1% surfactin and 0.45% ramnolipid.

Using the values of pathogenic microorganisms adhesion (CFU cm-2) of Tables

2 and 3, were calculated the averages in the reduction of bacterial adhesion presented in Table 4.

Microrganismo	Polymer	Surfactin	Surfactin rate	Rhamnolipid	Rhamnolipid rate
B. cereus	HDPE	88%	59%	50%	67%
	PP	42%		53%	
	LDPE (0,05 mm)	58%		83%	
	LDPE (0,1 mm)	50%		85%	
S. aureus	HDPE	47%	48%	43%	34%
	PP	44%		37%	
	LDPE (0,05 mm)	80%		29%	
	LDPE (0,1 mm)	23%		27%	
Salmonella sp.	HDPE	100%	87%	81%	91%
	PP	78%		100%	
	LDPE (0,05 mm)	100%		100%	
	LDPE (0,1 mm)	71%		86%	
P. aeruginosa	HDPE	74%	35%	21%	43%
	PP	10%		90%	
	LDPE (0,05 mm)	23%		50%	
	LDPE (0,1 mm)	36%		14%	

Table 4. Average percentage reduction of bacterial adhesion in flexible films

Considering the biosurfactants activity and their effects on microorganisms, it was observed that the average adhesion of *B. cereus* to the flexible films had a reduction of 59% when treated with 0.1% surfactin and 67% when using 0,45% rhamnolipid. For *S. aureus* the average reduction was 48% in films with 0.1% surfactin and 34% in films with 0.45% rhamnolipids. For *Salmonella sp*. the use of 0.1% surfactin in films showed an average reduction of 87% and 91% when using 0.45% rhamnolipids. The reduction for *P. aeruginosa* was 35% on films with 0.1% surfactin and 43% for 0.45% ramnolipids.

3.5 Analysis of Adhesion in Polystyrene Microplates

During the time of adhesion of the pathogenic microorganisms on polystyrene microplates, it was generally observed that the adhesion of *Salmonella sp., E. coli*, *P. aeruginosa* and *S aureus* were lower when covered with 0.1 surfactin %, than with 0.45% ramnolipid. Only for *B. cereus* the number of adhered cells were very close when submitted to both biosurfactants analyzed (Figure 2: *a,b,c,d* and *e*).

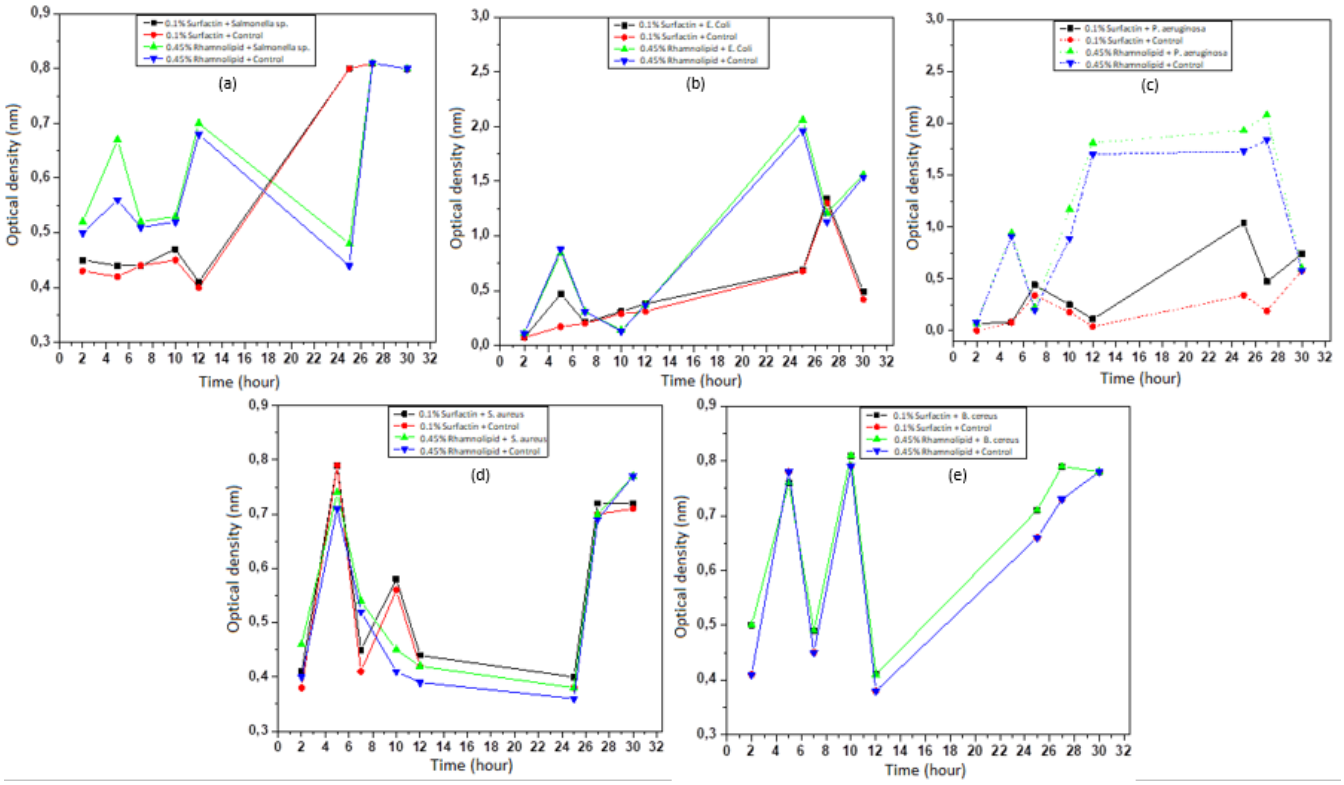

Figure 2. (a) Adhesion kinetics of Salmonella sp. in plates of polystirene coated with surfactina 0.1% and ramnolipídeo 0.45%; (b) Adhesion kinetics of E. coli in plates of polystirene coated with surfactina 0.1% and ramnolipídeo 0.45%; (c) Adhesion kinetics of P. aeruginosa in plates of polystirene coated with surfactina 0.1% and ramnolipídeo 0.45%; (d) Adhesion kinetics of S. aureus in plates of polystirene coated with surfactina 0.1% and ramnolipídeo 0.45%; (e) Adhesion kinetics of B. cereus in plates of polystirene coated with surfactina 0.1% and ramnolipídeo 0.45%

The lower adhesion of the investigated microorganisms had a greater efficiency in 2 hours (0.5 nm) for both biosurfactants, for *B cereus* and *P. aeruginosa*. *E coli* showed low cell adhesion in 2h (0.07nm) and *Salmonella sp*. (0.44nm) in 5h when applied to 0.1% surfactin. *S. aureus* showed reduced cell adhesion in 25h (0.38nm) when using 0.45% ramnolipid.

According to Nitschke (2009) polystyrene is considered hydrophobic. Rhamnoliids when in contact with a hydrophobic surface can be organized in micelles, depending on their concentration and the pH of the medium. This aspect reduces the contact angle of the surface with water, making the surface hydrophilic, which leads to reduced adhesion of (hydrophilic) microorganisms.

However, there was no efficiency in adhesion reduction of polystyrene compared to the control, for both biosurfactants (Figure 2: *a,b,c,d* and *e*). In this way, the concentration of the biosurfactants tested is not appropriate for the anti-adhesive treatment of these pathogens for this type of plastic.

According to Rodrigues et al. (2006) the inefficiency of these biosurfactants might be related with the loss of adsorbed molecules on the surface after washing, since the surface adsorption occurs by weak interactions as Van der Waals forces and not with the inefficiency of the biosurfactant itself on the pathogens.

4 I DISCUSSION

Fernandes et al. (2007), also confirms the antimicrobial activity of surfactin in microorganisms such as *Salmonella sp., E. coli, Staphylococcus aureus* and other 26 bacteria.

Dong et al. (2005) and González et al. (2006) showed the antimicrobial activity of rhamnolipid for 18 different microorganisms, including *Salmonella sp.*, *E. coli, Staphylococcus aureus* and *B. cereus*, as it was observed in this research. The same authors emphasized that some of the differences found in the literature can be explained by the percentage of growth of microorganisms when the biosurfactant used is in crude or pure form, since other components may be present (virulence factors and signs of quorum sensing) in the rough.

According to Raza et al. (2010), due to the presence of the carboxyl and the ramnosil groups, the rhamnolipids are anionic (negative charge). When the rhamnolipids are exposed to a hydrophobic surface (positive charge), they organize into micelles, lamellae or vesicles depending on the pH of the medium, directing its polar part (negative) to the external environment (aqueous medium). The apolar part (positive charge) in contact with the surface, make the surface hydrophilic, facilitating the repulsion of microorganisms with hydrophilic cell surface. However, in general, the action of rhamnolipid had no antiadhesive effect on the majority of the interactions between microorganisms and flexible films, except in the interaction of *Salmonella sp.* and the flexible films investigated (Table 3).

In the research developed by Zezzi, (2011), surfactin has proved to be more effective in adhesion control of *Salmonella sp.*, as well as presented in this research (Figure *2a*), when compared to the rhamnolipid. Surfactin had no effect on *Staphylococcus aureus* adhesion. However, the author points out that the rhamnolipid promoted adhesion inhibition of *Staphylococcus aureus*, which was not observed in this study (Figure *2d*).

Rodrigues et al. (2006) pointed out that polystirene surfaces treated with rhamnolipid obtained inhibitions of 33.8% on the adhesion of *Staphylococcus aureus*, but when treated with 0.1% surfactin, the percentage of inhibition of adhesion rose to 61.2%, corroborating the results presented in this study (Figure *2d*).

Araujo et al. (2011) pointed out that the surfactin was more efficient than the rhamnolipid, when polystirene surfaces were treated against the adhesion of *E. coli* and *P. aeruginosa*. However, it is emphasized that the efficiency of rhamnolipid grows according to its purity, and when purified, it was more efficient than the surfactin in reducing the adhesion of *E. coli* on surfaces of expanded polystirene. In this study, 0.1 % surfactin was more efficient on the *E. coli* than 0.45% rhamnolipid; however, both biosurfactantes were not very effective on the *P. aeruginosa* adhesion in polystirene.

With this study we can conclude that 0.1% surfactin and 0.45% ramnolipids showed antimicrobial effect on *Salmonella sp*. inoculated in the flexible films of PP, LDPE 0.05mm and 0.1mm and HDPE.

The 0.1% surfactin reduced the bacterial development of *E coli* impregnated in PP, HDPE and 0.05mm LDPE, *P aeruginosa* in HDPE and *Bacillus cereus* in HDPE and 0.05mm LDPE. 0.45% ramnolipids inhibited bacterial growth of E coli impregnated in HDPE, *P aeruginosa* in PP and *Bacillus cereus* in 0.05mm LDPE.

There was no significant efficiency in the reduction of polystyrene adhesion compared to the control, for both biosurfactants, demonstrating that the concentrations of the biosurfactants tested were not adequate for the antiadhesive treatment of these pathogens.

Therefore, there was an effect of the biosurfactants tested on the adhesion of pathogenic bacteria on the surface of the flexible films tested.

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