Antimicrobial effect of cleaning formulations of industrial interest on free-living cells and biofilms

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Efecto antimicrobiano de formulaciones limpiadoras de interés industrial en células de suspensión y biopelículas

Efecte antimicrobià de formulacions netejadores de interès industrial en cèl·lules en suspensió i biopel·lícules

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RESUMEN

Las biopelículas son una fuente de contaminación de difícil eliminación debido a su resistencia a los procedimientos de limpieza y de higiene convencionales. En este trabajo se ha evaluado las propiedades antimicrobianas a temperatura ambiente de tensioactivos de interés industrial en la formulación de detergentes. En primer lugar se estudió la capacidad de adhesión de la levadura *Yarrowia lipolytica* y del moho *Aspergillus niger* a superficies de espuma de poliuretano y de vidrio, así como su habilidad para formar biopelículas bajo diferentes condiciones de cultivo. Las biopelículas más estables fueron tratadas a temperatura ambiente con una mezcla de un alquilpoliglucósido y un alcohol graso etoxilado, y con un ácido alquil éter carboxílico. Los resultados experimentales mostraron que células formando parte de biopelículas eran más resistentes al tratamiento desinfectante que células libres en suspensión del mismo microorganismo. Aunque las soluciones de tensioactivos ensayadas fueron poco eficaces contra biopelículas, mostraron un gran efecto inhibidor sobre células en disolución. Además inhiben la formación de nuevas biopelículas. Por lo tanto, los buenos efectos antimicrobianos de estos tensioactivos ecológicos a temperatura ambiente podrían proporcionarles un interés potencial en procedimientos de limpieza a baja temperatura.

Palabras clave: alcohol graso etoxilado, alquilpoliglucósido, biopelícula, éter carboxilato.

SUMMARY

Biofilms are a challenging source of contamination due to their resistance to conventional cleaning and hygiene procedures. In this work, we assessed the antimicrobial properties at room temperature of surfactants of industrial interest in detergent formulation. The adhesion abilities of the yeast *Yarrowia lipolytica* and the mould *Aspergillus niger* to polyurethane foam and glass surfaces and their ability to develop as biofilm was firstly studied under different culture conditions. The most stable biofilms were then treated at room temperature with a mixture of an alkylpolyglucoside and an ethoxylated fatty alcohol, and with an ether carboxylate. Experimental results illustrated that surface-attached cells were more resistant to disinfectant treatment than cells of the same microorganism grown in suspension. Although the assayed surfactant solutions hardly removed attached biofilms, they showed a great inhibitory effect on free-living cells. What is more, biofilms did not grow during treatments. Therefore, the good antimicrobial effects of these ecological surfactants at room temperature could provide them a potential interest in low-temperature cleaning in place procedures.

Keywords*:* Alkylpolyglucoside, biofilm, ether carboxylate, ethoxylated fatty alcohol.

RESUM

Les biopel·lícules son una font de contaminació de difícil eliminació degut a la seva resistència als procediments de neteja i higiene convencionals. En aquest treball s'han avaluat les propietats antimicrobianes a temperatura ambient de tensioactius d'interès industrial en la formulació de detergents. En primer lloc es va estudiar la capacitat d'adhesió del llevat *Yarrowia lipolytica* i de la floridura *Aspergillus niger* a superfícies d'espuma de poliuretà i de vidre, així com la seva habilitat per a formar biopel·lícules sota diferents condicions de cultiu. Les biopel•lícules més estables van ser tractades a temperatura ambient amb una barreja d'un alquilpoliglucósid i un alcohol gras etoxilat, i amb un àcid alquil èter carboxílic. Els resultats experimentals mostren que cèl·lules formant part de biopel·lícules eren mes resistents al tractament desinfectant que cèl·lules lliures en suspensió del mateix microorganisme, encara que les solucions de tensioactius assajades van ser poc eficaces contra biopel·lícules, mostrant un gran efecte inhibidor sobre cèl·lules en dissolució. A més inhibeixen la formació de noves biopel·lícules. Per tant, els bons efectes antimicrobians d'aquests tensioactius ecològics a temperatura ambient podrien proporcionar-lis un interès potencial en procediments de neteja a baixa temperatura.

Mots clau*:* alcohol gras etoxilat, alquilpoliglucósid, biopellícula, èter carboxilat.

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INTRODUCTION

The formation of stable biofilms on surfaces protects colonies from cleaning and sanitation. A biofilm is a group of microorganisms that stick to each other on a surface, which can be composed of single species or mixtures of them, including bacteria, moulds, yeasts, protozoa, and other microorganisms. Biofilms can be found in any environment (natural, clinical or industrial) in which microorganisms live as they just need a hydrated medium and a minimum amount of nutrients in order to develop. Microorganisms attached to surfaces are more resistant to sanitization than free-living cells[1]. Generally, the extracellular matrix made of proteins and polysaccharides is responsible for the adherence of biofilms. This matrix protects the cells within it and facilitates communication among them through biochemical signals.

A particularly challenging problem in many areas is the presence of these biofilms as a result of adhesion of microbial species to equipment surfaces. Therefore, a cleaning operation is required to avoid hygiene problems and damage in equipment. Generally, control of microbial strains in closed processing lines is carried out by cleaning in place (CIP) procedures, which involve the circulation of cleaning solutions[2]. The CIP process is designed for automatic cleaning without disassembly and assembly operations. However, due to the increased resistance of food-borne bacteria and biofilms, compared with free-living bacteria, sterilization in place (SIP) process is also required[3]. The use of a combined detergent–disinfectant can reduce time and costs.

Commonly, industry uses aqueous washing systems that work at high temperatures to improve the effectiveness of the cleaning solutions. However, heating, insulation and ventilation requirements increase operating costs. Low temperature cleaning can overcome this problem without resorting to costly heating systems, thus leading to a more energy-efficient cleaning system.

Widespread use of chemical surfactants as cleaning agents is discouraged due to their inherent toxicity[4]. Nowadays, detergent formulations of non-ionic surfactants such as alkylpolyglucosides and ethoxylated fatty alcohols are steadily increasing because they are not only readily biodegradable and highly efficient on fatty soils[5,6], especially in the presence of an α-amylase[7], but also come from renewable sources. On the other hand, ether carboxylates derived from ethoxylated alcohols are mild crytoanionic surfactants of growing importance in shampoos and liquid hand cleaners[8]. As for sanitizers, new disinfecting agents are needed due to the increasing resistance of pathogenic microbes to traditional sanitizers such as sodium hypochlorous acid and benzalkonium chloride[9]. As the above-mentioned surfactants have proved to be efficient detergents, the next step is to assess their antimicrobial properties at room temperature to include them in low-temperature CIP or SIP processes.

In this work, we assessed the antimicrobial effect at room temperature of a mixture of alkylpolyglucosides and ethoxylated fatty alcohols, a solution of an ether carboxylate in the presence of acetic acid, and of an α-amylase solution. To this end, we first studied the characteristics and stability of biofilms formed by the yeast *Yarrowia lipolytica* and the filamentous fungus *Aspergillus niger* on glass coupons and polyurethane foam. Subsequently, the most stable biofilms were used to analyse the antimicrobial effect of the cleaning formulations. As it was established that microorganisms attached to surfaces are more resistant than free-living cells, we also studied the cell growth of the yeast in culture medium containing the detergent solutions to assess their performance against it, using as control the traditional sanitizer benzalkonium chloride.

EXPERIMENTAL

Microorganisms and maintenance medium

Yarrowia lipolyica, an acid-producing yeast, and the strain *Aspergillus niger* NB2, a citric acid-producing filamentous fungus, used throughout this study, were maintained on potato dextrose agar slants at 4ºC.

The liquid growth medium was composed of: glucose, 60 g/l; NH_4Cl, 3.0 g/l; KH_2PO_4, 0.4 g/l; $MgSO_4.7H_2O$, 0.5 g/l; yeast extract, 0.5 g/l. Cultivation (preculture) was performed in 250 ml Erlenmeyer flasks on shaker at 150 rpm for 40 h at 30°C, and the pH was stabilized between 4.5 and 5.5 with 7 g/l of $CaCO_3$.

Biofilms formation

Several combinations coupon-microorganism were assayed. Polyurethane foam (PUF) R60 (Espe Inc., Finland) (Fig. 1), free of isocyanate-reactive moieties, and glass coupons (2.5 x 3.5 x 0.15 cm) were used as surfaces for biofilm growth.

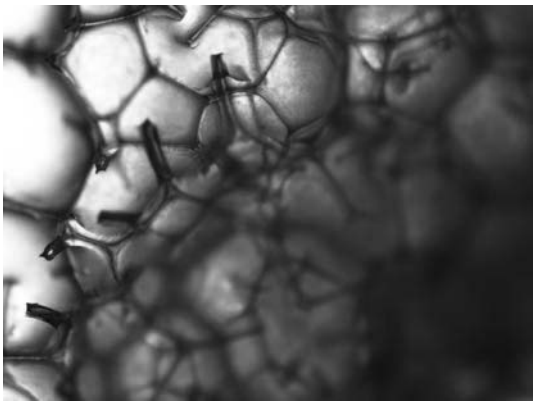


Figure 1. *PUF coupons*

Three schemes were assayed for *Y. lipolytica* and *A. niger* biofilms formation: biofilms formation on PUF coupons in a continuous 400-ml bioreactor; biofilms growth on PUF surface in submerged culture in 250-ml conical flasks; and biofilms formation on the surface of glass coupons in Petri dishes (90 mm diameter) in stationary phase.

The cultivation of the microorganisms before biofilm formation was performed in 250-ml Erlenmeyer flasks containing 100 ml of production medium (glucose, 80 g/l; $MgSO_4.7H_2O$, 0.7 g/l; yeast extract 0.1 g/l), as described elsewhere[10,11].

Continuous biofilm formation from *Y. lipolytica* was performed under the following conditions: temperature, 28ºC; residence time, 90 min; cell concentration, 1.3×10^6 cell/ml, treatment time, 14 h. The **s**ubmerged cultivation for the formation of biofilms from both *Y. lipolytica* and *A. niger* on PUF was carried out in batch mode in 250-ml Erlenmeyer flasks under the following conditions: temperature, 28ºC; stirring, 220 rpm; cell concentration, 1.3×10^6 cell/ml;

treatment time, 14 h. Finally, for cell adherence and biofilm formation in Petri dishes from both microorganisms, glass coupons were covered with 15 ml of cell suspension (cells in growth stationary phase) for 24 h at room temperature. Afterwards, glass coupons were placed in a laminar flow cabinet for 12 h at room temperature.

Cleaning solutions

The first cleaning solution assayed was composed of 1 g/l of an ethoxylated fatty alcohol supplied by Kao-Chemical Europe, and an alkylpolyglucoside supplied by Henkel-Cognis in a ratio 4:1 in a dry basis. This ratio was selected because it was previously illustrated that the wetting power of this mixture of surfactants increases when the ratio of etholylated fatty alcohol: alkylpolyglucoside is between 1:1 and 4:1[12].

The second cleaning solution was composed of 1 g/l of capryleth-6 carboxylic acid supplied by Kao-Chemical Europe, whose low environmental impact was previously investigated[13], and 0.3 g/l of acetic acid. The trade names, main properties and chemical structures of the above-mentioned surfactants are shown in Table I.

The last solution assayed contained 10 μl of α-amylase type XII-A from *Bacillus licheniformis* (Termamyl 300L type DX) supplied by Sigma in 1 l of pH 7 phosphate buffer solution. To compare effectiveness of cleaning solutions, replica of the experiments were made with distilled water.

Finally, a surfactant largely used as cleaner and sanitizer in the industry, such as benzalkonium chloride, supplied by Sigma-Aldrich, was used in concentrations 0.10 %, 0.05%, and 0.01% (w/w) to compare the effectiveness of the above-mentioned cleaning solutions as biocides.

Biofilm treatment

Biofilm (from *Y. lipolytica*) glass coupons were introduced in Petri dishes (90 mm diameter) containing 20 ml of cleaning solution and placed in an orbital shaker at room temperature. The variables studied were time (1 h, 2 h, 12 h and 24 h) and stirring (0 and 50 rpm). Experiments were carried out in duplicate.

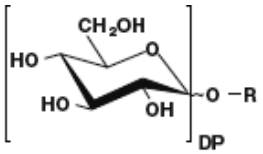
Culture medium treatment

1 ml of culture medium from *Y. lipolytica* was added to 9 ml of cleaning solution for 10, 25 and 40 min at room temperature and then cultivated in agar. 3 replicas of each experiment were made.

Analytical methods

Fig. 2 shows a scheme of the method used to assess the effect of the cleaning solutions assayed on biofilms formed on glass coupons. Biofilms were non-destructively observed by light microscopy. After treatments, biofilm on coupons were stained[15] to differentiate living cells by microscopy using oil immersion. Biofilms of replicas of the same experiments were recovered using 20 ml of physiological solution (0.9% NaCl) for 7 h at 50 rpm at room temperature. The optical density of the resulting solutions was measured at 660 nm and then samples were cultivated on solid culture medium (39 g of glucose agar in 1 l for distilled water) in Petri dishes in dilutions up to $1:10^{-7}$. Sample incubation was performed as described elsewhere[16] and CFU were determined in an IUL Countermat Automatic Colony Counter. The cleaning solutions were also cultivated in the same solid culture medium and their biomass concentration was estimated by both optical density at 600 nm and by gravimetric measures (6 ml of cleaning solution centrifuged at 8,000 rpm for 10 minutes).

Table I. *Main properties of the surfactants assayed.*



Akylpolyglucoside
where R is the carbon chain length and DP is the degree of polymerization

Trade name	DP	R	Humidity (%)[12]	HLB
Glucopone 650EC	1.3	C_8-C_{14}	50	11.9

(HLB value supplied by Sigma)

Ethoxylated fatty alcohol $R\text{-}(OCH_2CH_2)_nOH$
where R is the alkyl chain length and n the average number of ethylene oxide units per molecule

Trade name	Name	n	R	Humidity (%)[14]	HLB
Findet 1214/N23	Polyoxyethylene (11) alkyl (C_{12} 70% and C_{14} 30%) ether	11	$C_{12.6}$	0.3	14.3

(HLB value supplied by Kao-Chemical Europe)

Ether carboxylate $R\text{-}O\text{-}(CH_2CH_2O)_nCH_2COOH$
where R is the alkyl chain length and n the average number of ethylene oxide units per molecule

Trade name	Name	n	R	Humidity (%)	HLB
Akypo LF1	Capryleth-6 carboxylic acid	5	C_8	11.5	13

(Humidity and HLB values supplied by Kao-Chemical Europe)

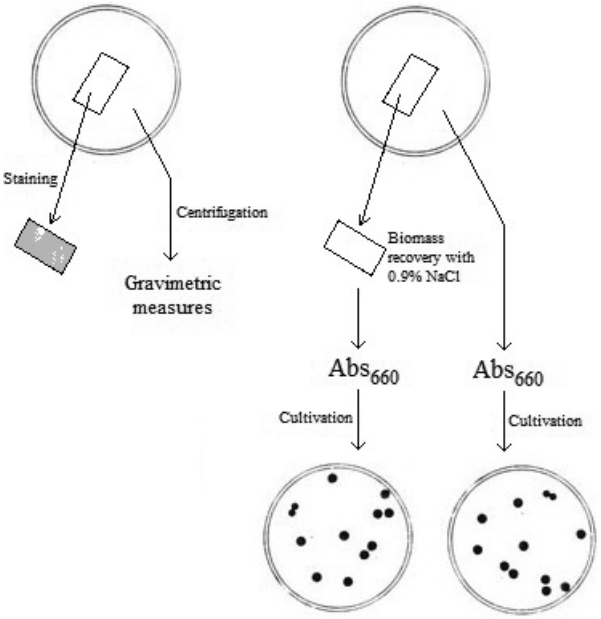


Figure 2. *Scheme of the analytical procedure for biofilm removal assessment.*

RESULTS AND DISCUSSION

Yarrowia lipolytica biofilms formation

Y. lipolytica biofilms were developed in both PUF and glass coupons. When working with the continuous bioreactor, biofilms on PUF grew on the coupons surface closer to the inlet pipe of the cell flow to the bioreactor, biofilms forming on the external surface of PUF. By contrast, biofilms were also formed inside the PUF coupons for the submerged cultures. Regarding glass coupons, biofilms from cells in stationary phase uniformly grew on the coupons surface. Some areas showed biofilms more developed than others, where only small biofilm colony units were observed (Fig. 3).

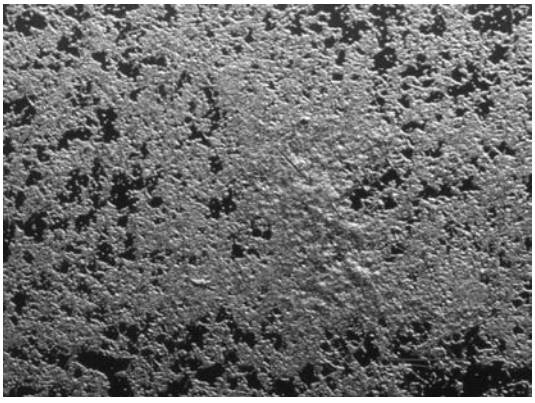


Figure 3. *Immobilized Y. lipolytica on glass coupons.*

The strong interaction between the microorganism used and the characteristic of each coupon was demonstrated. Of note was the instability of the biofilms of *Y. lipolytica* cells formed on PUF and the good cell-coupon compatibility with regards to the formation of immobilized biomass on glass coupons. For these reasons, glass coupons were selected for *Y. lipolytica* biofilms formation.

Aspergillus niger biofilms formation

A. niger biofilms were also obtained on PUF and glass coupons. Fig. 4 shows the abundant biofilm formed on glass coupon; a mycelial network with densely packed hyphal ramifications and spores formation were observed. Fig. 5 shows the immobilized *A. niger* culture on PUF coupons. The coupon surface was completely covered by fungus structures. PUF characteristics did not allow the fungus to grow in the same way than in the glass coupons: dense mycelial structures were formed within PUF pores.

Figure 4. *Immobilized A. niger on glass coupons.*

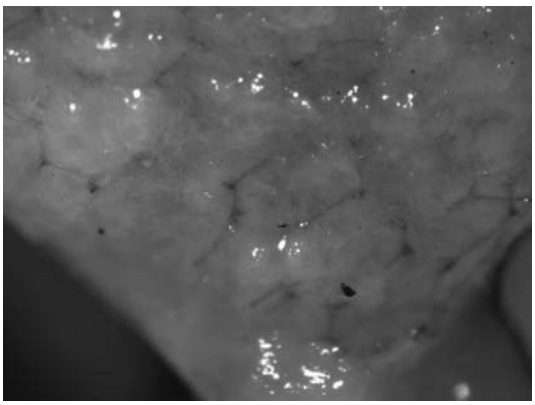


Figure 5. *Immobilized A. niger on PUF.*

Therefore, different cell-coupon relationships were found in the assays of biofilm formation with the fungus *A. niger*: a well-developed but unstable biofilm was formed. When working with PUF, hyphal ramifications and fungus colonies grew inside the PUF structure throughout the pores. As a result, the fungus *A. niger* was discarded for biofilm formation in the subsequent trials of biofilms removal.

Removal of biofilms formed on glass coupon

Biofilm growth was not observed during treatments, which was a major finding. The assayed solutions limited *Y. lipolytica* growth. On the contrary, treatments with distilled water, a mixture of an ethoxylated fatty alcohol and an alkylpolyglucoside, a mixture of capryleth-6 carboxylic acid and acetic acid, and an α-amylase in a pH 7 phosphate buffer solution were ineffective against biofilms at room temperature, as no biofilm detachment was observed at the maximum treatment time assayed (24 h). Petri dishes were let stand in orbital shaker for up to 1 week. A little cell removal was observed from 24 h on and probably could be

not due to the cleaning solutions action, since it is known that, at some point after the formation of the biofilm, cells and sometime spores are released, or pieces of the biofilm are sloughed off[17].

Concentrations of biomass recovered by centrifugation in cleaning solutions are shown in Table II. Stirring did not exert a defined effect on biomass recovery. As it can be observed, the concentration of α-amylase assayed was totally ineffective against biofilms. Data from Table II suggests a gradual, ongoing removal of biomass by the mixture of an ethoxylated fatty alcohol and an alkylpolyglucoside. The ether carboxylate solution led to the highest biomass recovery. After a rapid initial release, the cell concentration decreased when increasing the treatment time, which could indicate that this solution not only had a positive effect in removing biofilms, but also contributed to the cell elimination within the medium. On the contrary, the cleaning solution could not exert any further effect on biofilm after the initial removal. Finally, α-amylase and ethoxylated fatty alcohol solutions presented turbidity after treatment while the ether carboxylate formulation was clean.

Table II. *Concentration (g/l) of biomass in cleaning solutions after biofilm treatments with 10 μl/l of α-amylase, a mixture of an ethoxylated fatty alcohol and an alkylpolyglucoside (solution 1), and mixture of capryleth-6 carboxylic acid and acetic acid (solution 2) at different times.*

Time (h)	Stirring (rpm)	Biomass concentration (g/l)		
		α-amylase	Solution 1	Solution 2
2	0	0.00 ± 0.00	0.13 ± 0.01	1.02 ± 0.07
2	50	0.00 ± 0.00	0.11 ± 0.02	0.93± 0.03
12	0	0.00 ± 0.00	0.28 ± 0.01	0.70 ± 0.02
12	50	0.00 ± 0.00	0.22 ± 0.03	0.70 ± 0.04
24	0	0.00 ± 0.00	0.45 ± 0.03	0.46 ± 0.04
24	50	0.00 ± 0.00	0.34 ± 0.01	0.50 ± 0.01

There were no differences between staining of the biofilms coupons before and after treatments, and resulting staining could indicate that non-living-cell remained within the biofilm wall after biofilm formation. What is more, cell suspensions recovered from biofilms using the physiological solution and afterwards plated on agar did not grow. By contrast, cleaning solutions plated on agar after treatment grew in all the condition tested, which means that living cells were detached from biofilms.

Compared to distilled water as cleaning solution, the surfactant solutions assayed led to higher optical density at 660 nm after treatment, which could indicate a slight improvement in biofilm detachment (Table III). What is more, absorbances in physiological solutions used to recover remaining biofilms on coupons after treatments with surfactants solutions were slightly lower than that for untreated biofilm, which indicated that a small part of biofilm was removed during treatment. However, Table III also suggests that this effect on biofilms found for surfactant solutions is not much higher than the effect exerted by distilled water. Finally, as it was above mentioned, stirring did not exert a marked effect on biomass recovery.

Table III. *Absorbance at 660 nm in cleaning and physiological solutions after biofilm treatment for 1 h with distilled water, a mixture of an ethoxylated fatty alcohol and an alkylpolyglucoside (solution 1), and mixture of capryleth-6 carboxylic acid and acetic acid (solution 2)*.

Sample	Stirring (rpm)	Abs_{660} (cleaning solution)	Abs_{660} (physiological solution)
Untreated biofilm	-	-	0.0564
Distilled water	0	0.0594	0.0496
Distilled wáter	50	0.0772	0.0514
Solution 1	0	0.1892	0.0489
Solution 1	50	0.1902	0.0483
Solution 2	0	0.1102	0.0474
Solution 2	50	0.1208	0.0465

Elimination of living cells

In the experiments in which inoculums were treated with mixtures of surfactants and then plated, the whole cultures grew. Compared to the treatment of the inoculums with distilled water, Table IV shows that cultures treated with surfactant solutions at room temperature were growth-inhibited, especially the one which contained an ether carboxylate and acetic acid, which is consistent with the results obtained from biofilm removal. Nevertheless, it remained unclear whether the antimicrobial effect was due to the surfactant itself or to the acetic acid. The cultivation measurement also showed that 40- min treatment was not enough to kill all the living cells.

Table IV. *Plate counting of cultures from distilled water, mixture of an ethoxylated fatty alcohol and an alkylpolyglucoside (solution 1), mixture of capryleth-6 carboxylic acid and acetic acid (solution 2) and different concentrations of benzalkonium chloride (ADBAC) with the treatment time.*

t (min)	Plate counting × 10^4 (CFU/ml)					
	Distilled water	Solution 1	Solution 2	0.10% ADBAC	0.05% ADBAC	0.01% ADBAC
10	98 ± 7	10 ± 1	5 ± 1	0 ± 0.0	0 ± 0.0	0 ± 0.0
25	82 ± 3	9 ± 1	5 ± 1	0 ± 0.0	0 ± 0.0	0 ± 0.0
40	70 ± 4	7 ± 2	4 ± 1	0 ± 0.0	0 ± 0.0	0 ± 0.0

The antimicrobial effect of both cleaning solutions was lower than that found with benzalkonium chloride, which was able to eliminate all the biomass within the media in all the concentrations and treatment times assayed. Of note are the concentrations of benzalkonium chloride assayed, much lower than 10% (w/w). Above this concentration, benzalkonium chloride solutions are toxic to humans, causing irritation to the skin and mucosa, and death if taken internally[18]. By contrast, the advantages of the detergents assayed, such as the low toxicity, biodegradability, and high detergent power[12,13], and the fact that experiments were carried at room temperature illustrate the potential use of these surfactants in low-temperature CIP processes.

CONCLUSIONS

Yarrowia lipolytica, under the conditions assayed, provided well-developed, stable biofilms from cells in stationary phase that uniformly grew on glass coupons surface.

The aqueous solutions of mixtures of surfactants assayed have inhibitory effect on free yeast cells and limit their adhesion to surfaces, but are unable to remove attached

biofilms from the microorganism assayed, at least at room temperature. These surfactants could be included in the formulation of soap and detergents for domestic and industrial purposes as they have good detergent properties and low toxicity. As for low-temperature CIP procedures, the good antimicrobial effect showed at room temperature makes these surfactants an interesting alternative for CIP processes. The combination of the mechanical forces due to high flow rates used in industrial cleaning processes and the cell inhibitory effect of the assayed surfactants could enhance the removal of adherent bacterial spores and biofilms avoiding the use of high temperatures (operational costs reduction) and toxic cleaning agents and sanitizers. Nevertheless, the effect of the studied surfactants on hard-surface cleaning where well-established attached biofilms from numerous microorganisms are present remains to be demonstrated. Further research at different conditions is required.

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