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DISINFECTION EFFICACY OVER YEAST BIOFILMS OF JUICE PROCESING INDUSTRIES

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Highlights

- Sodium hypochlorite was more effective on SS than with PVDF membranes.

- Flux recovery was achieved with 200 mg $Cl \cdot L^{-1}$ with no effect over attached cells.

- Biofilm's stage of maturation should be considered in disinfection planning.

Abstract

Membrane separation systems represent a *hot – spot* for biofilm formation in juice industries. Sodium hypochlorite (NaOCl) has been traditionally the disinfectant of choice; however, its effectiveness over well-established biofilms is limited. In this work the study of biofilm formation on ultrafiltration membranes was proposed. The effectiveness of cleaning and disinfection procedures commonly used in juice industry was tested on the removal and killing of cells. The species used (*Rhodototula mucilaginosa*, *Candida krusei*, *Candida kefyr* and *Candida tropicalis*) were isolated from ultrafiltration modules of a clarified apple juice industry. Industrial concentrations of NaOCl (200 mg $CL \cdot L^{-1}$) showed to be effective against planktonic cultures with more than 4 log reductions, whereas their overall efficiency against adhered cells was smaller. Recovery of viable cell counts to initial numbers was evidenced regardless of the time of colonization. The topography of the surface showed to have an impact on the efficiency of the disinfectant, presenting membranes smaller log reductions than stainless steel (~1.09 -1.53 log CFU). At 200 mg $Cl \cdot L^{-1}$ only membrane's cross flow recovery was reached with no long-term effect over the attached cells. The overall results demonstrated the recalcitrance of these biofilms to typical cleaning and disinfection process which may confer them with a selective advantage.

Keywords: Ultrafiltration membrane, yeast biofilm, apple juice, sodium hypochlorite

1. Introduction

Membrane filtration processes are increasingly used in juices and beverage industry. Ultrafiltration, like reverse osmosis, is a cross-flow separation process where the liquid stream to be treated (feed) flows tangentially along the membrane surface, thereby producing two streams: 1) the permeate or stream of liquid that comes through the membrane and; 2) the rejection which consists of the remaining liquid that does not come through the membrane and that will contain all of the suspended solids, pectins, microorganisms, getting progressively concentrated in those species removed by the membrane (Wolf, Siverns, & Monti, 2005).

Ultrafiltration (UF) is the most used process for clarification and can be characterized according to the membranes material and the configuration they present (Constenla & Lozano, 1997; Wagner, 2001). UF membranes have a pore size of 0.001-0.05 μm and molecular weight cut-off (MWCO) between 1 and 300 kDa (Singh, 2015) and operate under a pressure of 0.2-0.5 MPa, retaining most of the suspended solids and microorganisms. Fruit juices are then concentrated to allow economy in storage, transportation, distribution and commercial operations, as well as conservation due to reduced water activity (Echavarría et al., 2012).

Unfortunately, membrane fouling caused by the deposition of inorganic, organic or biological material (biofouling*)* on the surface or in the pores of membranes limits its application in the food processing industry (Mohammad, Ng, Lim, & Ng, 2012). Biofilms have a negative impact as they can form on the product and on the food-contact surfaces and provoke contamination (Martínez-Vaz, Fink, Diez-Gonzalez, & Sadowsky, 2014). In food-processing lines, yeasts belonging to *Saccharomyces*, *Candida* and *Rhodotorula* have been isolated from biofilms of conveyor tracks and can and bottle warmers in packaging

departments of a beverage industry (Brugnoni, Cubitto, & Lozano, 2012; Salo & Wirtanen, 2005).

Membrane separation systems represent a *hot – spot* for biofilm formation in food processing industries, the topography provides shelter to the cells and thus they are less exposed to the disinfectant agents and drag forces of the liquid flow, being difficult to remove (Hijnen et al., 2012; Khan, Manes, Aubry, & Croué, 2013; Vrouwenvelder et al., 2008). In the context of UF, biofilms formed on these membranes cannot be completely removed by backwashes; this implies that chemical cleaning methods should be used to properly recover the operating parameters (Liikanen, Yli-Kuivila, & Laukkanen, 2002; Vrouwenvelder & Van der Kooij, 2001). The development of biofilms on UF membranes inevitably leads to operating difficulties with a sharp decline in permeate flux, extending along time the operating cycle length (He, Chen, Ji, & Li, 2009).

The regular application of cleaning and disinfection procedures is a common strategy employed to control microbial implantation on either industrial equipment or the products themselves (Jahid & Ha, 2012). However, such procedures are not fully effective on biofilm structures and can induce the selection of resistant phenotypes (Simões, Simões, & Vieira, 2010).

Although there are many biocides that are marketed as effective against fouling, only a few of them are suitable for large-scale applications (Xu, Jia, Li, & Gu, 2017), e.g. chlorine is indeed widely used in the food industry (Sagong et al., 2011; Van Haute, Sampers, Holvoet, & Uyttendaele, 2013). Nevertheless, one setback is that cells immersed in biofilms are very resilient against treatment efforts (Xu et al., 2017). Traditionally sodium hypochlorite has been used in food industry as a universal disinfectant (Møretrø, Heir, Nesse, Vestby, & Langsrud, 2012). Despite being corrosive at high concentrations, is one

of the most used disinfectants in food industry due to its low cost, facility to apply and wide spectrum of antimicrobial effectiveness (Ramos, Miller, Brandão, Teixeira, & Silva, 2013; Van Haute et al., 2013).

If the cleaning steps are insufficient, residues of food matrix may remain on the surface therefore affecting the effectiveness of the process. In industrial processes, the geometrical design of machines, pipes and tanks is usually complex and cleaning and disinfection procedures are thus complex. Moreover, cells as part of a biofilm are more resistant to conventional disinfection strategies compared to planktonic cultures and are more difficult to eradicate (Simões et al., 2010).

Being a multifactorial problem involving several variables that need to be taken in mind is that the following objectives were proposed 1) the study of biofilm formation on industrial surfaces in a simulated industrial environment, and 2) the study of the effectiveness of cleaning and disinfection procedures commonly used in juice industry on the removal and killing of cells. To do so, four wild yeast strains isolated of UF modules were used in their different growth forms: planktonic, sessile and forming biofilms over food contact surfaces (stainless steel and UF membranes) under variable flow conditions.

2. Materials and methods

2.1 Microorganisms and stock culture

The yeast strains used in this work were *Rhodototula mucilaginosa*, *Candida krusei*, *Candida kefyr* and *Candida tropicalis*, all of them isolated from the surfaces of polyvinylidene–fluoride (PVDF) UF membranes of a large-scale apple and pear juice processing industry located in Argentina (Tarifa, Brugnoni, & Lozano, 2013).

Stock culture of the strains were suspended in 20% ($v \cdot v^{-1}$) glycerol in Yeast Extract Glucose chloramphenicol (YGC) broth: 0.5% $w \cdot v^{-1}$ yeast extract (Merck KGaA, Darmstadt, Germany), 2% $w \cdot v^{-1}$ glucose (Merck KGaA, Darmstadt, Germany) and 0.01% $w \cdot v^{-1}$ chloramphenicol (Fluka Chemie AG, Buchs, Zwitzerland), and stored at -70°C until use.

2.2 Food soiling system (growth media) and inocula preparation

To better represent the ongoing reality of juice industries, the juice used was provided by a national producer/exporter company located in the Alto Valle de Río Negro y Neuquén (Argentina). The reconstituted juices used as growth media, according to each assay, were prepared from 72 °Brix concentrated apple juice and sterilized by microfiltration (pore size 0.45 µm) (Metricel®Grid, Gelman-Sciences, Ann Arbor, MI, USA). The approximate composition of clarified apple juice can be seen in Lozano (2006).

For the preparation of yeast suspensions, a loop of frozen cells of each strain used (section 2.1) were suspended in YGC broth and incubated at 25 ± 1 ºC for 48 h and harvested by centrifugation at 2,500 x *g* for 5 min (Labofuge 200, Kendro, Germany). Suspensions were made in 12 °Brix juice until reaching optical density (OD) at 550 nm of 0.125 (~ $5x10^6$ cells$\cdot mL^{-1}$) using a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA, USA).

For mixed cultures equal quantities of the adjusted suspensions of each yeast strain were mixed together in a ratio 1:1:1:1.

2.3 Disinfectant and microbicidal effect

The chemical agent used for disinfection was NaOCl as is the most widely used disinfectant in the food industry (Fukuzaki, 2006; Sagong et al., 2011). For all experiments, the sanitizing solutions were prepared in sterile distilled water at room temperature (21 ± 1 ºC). Ready to use solutions of NaOCl were prepared from a stock solution (60 $g \cdot L^{-1}$ active

chlorine, Clorox Argentine, S.R.L., Argentine) and used at 50 (pH 6.5), 150 (pH 8.0) and 200 mg $Cl \cdot L^{-1}$ (pH 9.0).

Results were expressed as Microbicidal Effect (ME) according to Ono, Yamashita, Murayama, and Sato (2012), using phosphate-buffered saline (PBS) (PBS: 0.15 $mol \cdot l^{-1}$ NaCl, 0.05 $mol \cdot l^{-1}$ KH_2PO_4, 0.05 $mol \cdot l^{-1}$ K_2HPO_4, pH 7.2) as control. The ME was calculated using the following equation (Salo & Wirtanen, 2005):

$$ME = Log\ N_0 - Log\ N_i$$

where N_0 is $CFU \cdot ml^{-1}$ (or cm^{-2} in case of biofilm assays) in the control and N_i is $CFU \cdot mL^{-1}$ (or cm^{-2} in case of biofilm assays) with disinfection treatment. In order to be considered effective, disinfectants must reduce the amount of yeasts counts by at least 4 log-units in the case of planktonic cells and by 3 log-units in the case of cells adhered to a surface (Mosteller & Bishop, 1993).

After each treatment time, the disinfectant effect was neutralized using PBS containing 0.2 % of sodium thiosulphate, homogenized for 15 s and then analyzed as described in the sections below (Stewart, Rayner, Roe, & Rees, 2001).

2.4 Disinfection of planktonic cells

Suspensions tests were carried out to investigate the effectiveness of the disinfectant against planktonic cultures of each strain and a mixture of all.

First each strain was assayed separately to see the resistance to increasing disinfectant concentrations mentioned in Section 2.3, and then a mix of the four strains was used to test possible influence of interspecies interactions on resistance.

Yeast suspended cultures were made as described in Section 2.2. The disinfection tests were carried out as proposed by Brugnoni, Lozano, and Cubitto (2012), two mixing milliliters of each strain adjusted suspension in 12 ºBrix apple juice were mixed, separately, with 18 mL of each disinfectant solution (50, 150 and 200 mg $Cl \cdot L^{-1}$) under low stirring (50 rpm) on an orbital shaker at 21 ± 1°C. In the case of the mixed suspension, aliquots of each of the four cultures with similar OD were mixed in equal proportions and vortexed until full mixture. In this case one higher additional concentration of NaOCl was used (1000 mg $Cl \cdot L^{-1}$, pH 10).

After 5, 10, 15 and 30 min exposure, a sample (1 ml) was neutralized with sodium thiosulphate as described in 2.3 and then serially diluted for counting as described in section 2.6.

The results were expressed as ME based on the survival relative to appropriate controls in PBS.

2.5 Abiotic substrata used for biofilm development

After analyzing the performance of disinfections assays over planktonic cultures, the tests were made against attached cells.

Stainless steel (SS) coupons (25 x 15 x 1 mm, type AISI-304) were used as substrate for biofilm formation, as one of the most universal materials used for the manufacture of food-processing equipment (Wijman, de Leeuw, Moezelaar, Zwietering, & Abee, 2007). Before the experiments, the coupons were properly cleaned and disinfected as in Brugnoni, Lozano, and Cubitto (2007), and autoclaved for 15 min at 120°C.

Also, PVDF flat membranes of 14 x 19 cm were used (Synder Filtration, Vacaville, CA, USA) with an effective filtration area of 140 cm^2 and a MWCO of 100 kDa, as part of the clarification step of juice industries. Wet flat sheet membranes came sealed packaged in a

solution of 2% sodium metabisulphate. After removing the flat membrane from the storage bag, they were carefully rinsed with sterile distilled water to remove any preservatives or unwanted material from the surface.

Scanning electron microscopy (SEM) was used to see the topography, structure and morphology.

2.5.1 Biofilm test – static condition

For each surface, adjusted suspensions in apple juice were done as stated in Section 2.2. For yeast colonization assays a mixed suspension with the four yeast species was poured into a modified homemade Petri dish with divisions, previously described in Brugnoni et al. (2007). Each division contained the surfaces (SS coupons or PVDF membrane), and was filled until full coverage (6 mL) followed by incubation at 21 ± 1°C for 2, 8 and 16 h, under static conditions. After each time, the surfaces were carefully removed from the division using sterile forceps and thereafter rinsed by immersing it for 2 min in 5 ml of PBS with shaking, to remove the loosely attached cells.

For the disinfection trial, a ready-to-use concentration of NaOCl 200 $mg \cdot L^{-1}$ Cl was used, following the daily use concentrations in juice industries as stated in the protocol of Section 2.3 ("*Desinfection and microbiocidal effect*"); at the same time another set of surfaces were put in contact with PBS as control. After each time (5, 10 and 30 min contact) SS coupons and PVDF membranes were later used for epifluorescence microscopy (EM) and for counts. All results were expressed as $CFU \cdot cm^{-2}$, whereas treatment was expressed as ME.

2.5.2 Biofilm test - UF membranes under flow conditions

For this, a cross-flow (CF) cell was used for biofilm evaluation (Sepa CF System by Osmonics, Minetonka, MN, USA), simulating the UF of the clarification process. It has been recommended for simulating the flow dynamics of larger commercially spiral-wound

membrane elements (Mosqueda-Jimenez, Narbaitz, & Matsuura, 2004). Moreover the efficiency of NaOCl on both attached cells and membrane performance (e.g. cross-flow recovery) was done.

The feed source used was a mixed suspension of *R. mucilaginosa, C. krusei, C. tropicalis* and *C. kefyr* prepared in 12 ºBrix apple juice (~ 10^4 cells·mL^{-1}). The duration of the filtration was of 2 h at 21 ± 1 ºC. The permeation rate was maintained by a peristaltic pump (Cole Parmer Instrument Co, Illinois, USA.) and runs were performed by total retentate recycling. Fouling development was monitored by measuring the pressure drop over the membrane, using a pressure gauge at the rejection outlet. The permeate flux was measured during the filtration experiment, in order to see changes along the filtration run.

Figure 1 shows the Cleaning and Disinfection Protocol followed. The adjusted suspension in apple juice simulated the microbial load of fresh juice that could reach the clarification point (Brugnoni et al., 2007). Afterwards a washing step was done with sterile distilled water followed by the disinfection step; separately two solutions of NaOCl (200 and 500 mg Cl·L^{-1}) were applied for 15 min, in independent trials. At this point samples were taken for counting as described in Section 2.6.

Moreover, to simulate what would happen if disinfection steps are inefficient and a contribution of nutrient remains in the system, membrane pieces were removed and placed in sterile modified Petri dishes with apple juice of different concentrations (3, 6 and 12 ° Brix) for 2, 8 and 16 h. At the end of each time the corresponding counts were performed on YGC and Chromagar *Candida*.

2.6 Quantification of cells

For counts, surfaces were placed into a test tube with enough glass beads (diameter ~ 3 – 4 mm) to cover the surface and vortexed to full speed for 3 min (to remove the adherent

microorganisms) (Lindsay & Von Holy, 1997). In the case of membranes, pieces of 2 cm^2 were cut small using sterile scissors.

In each incubation period, samples were serially diluted with PBS and viable counts were determined by plating 0.2 mL on Chromagar *Candida* (CHROMagar Microbiology, Paris, France) and 1 mL on YGC agar by pour plate technique, incubated at 25 ± 1 °C for 2 and 5 days. Chromagar *Candida* was used to have an estimate of the species prevalence. In the cases where the visual appearance and color of the colonies varied, the strains were identified using established morphological and physiological tests. Even though CHROMagar *Candida* medium proved to be suitable for the differentiation of a number of nonclinical yeasts (Tornai-Lehoczki, Péter, & Dlauchy, 2003), culture identification was also performed with an identification system (Rapid ID Yeast Plus system, Remmel, USA). YGC was used to determine the ME.

2.7 Microscopy: *SEM and EM*

SEM was used to characterize the morphology of UF membranes, analyzing the cross section and the surface to determine the adhesion patterns. The membranes were fixed with glutaraldehyde (2.5%) in phosphate buffer (0.1 $mol \cdot L^{-1}$, pH 7.2); washed three times with the same buffer and dehydrated by critical point drying (E3000, Polaron Instruments, Hatfield, PA, USA). Samples were gold coated (300 Å) in a Pelco Model 3 Sputter Coater 91000 metal evaporator (Lozano, 1990) and viewed with a Scanning Electronic Microscope (LEO EVO 40, Cambridge, UK) at 7.0 kV acceleration voltage.

For EM, the surfaces were stained with fluorescein diacetate (FDA). The principle behind a test using FDA is that only live cells will convert FDA to fluorescein. FDA specifically stains cells possessing esterase activities and intact cell membranes. This fluorescent probe is widely used as an indicator of cell viability (Ki-Bong & Hideaki, 2002). A standard stock

solution of 2 mg·mL^{-1} (0.2% w·v^{-1}) FDA, ($C_{24}H_{16}0_7$, Sigma–Aldrich Chemical Co., St. Louis, MO) was prepared in acetone (Dorwil, Industria Argentina) and stored to -18 °C. The surfaces from each experimental condition were stained with sterile FDA acetonic solution in 0.1 mol·L $^{-1}$ phosphate buffer (0.04% v·v^{-1}), pH 7.5. After 90 min shaking at 21 ± 1°C in darkness, the surfaces were rinsed twice with sterile distilled water. Surfaces were then allowed to air-dry and observed with an epifluorescence microscope (Olympus BX 51, NY, USA) using a 100 x oil-immersion objective, blue excitation U-MWB2.

2.7 Statistical analysis

Counts were converted to decimal logarithmic values (log CFU·cm^{-2}) to nearly match the assumption of a normal distribution. In all analyses, triplicate tests were performed under identical conditions in two independent trials and the results expressed as mean and standard deviation (mean ± SD). When appropriate, Student's t-test was used for comparison of means. Confidence level equal or higher than 95% was considered statistically significant.

3. Results

3.1 Disinfection of planktonic cells

As it can be seen in Table 1 after 5 min contact with increasing concentrations of NaOCl, only *R. mucilaginosa* exposed to a concentration of 50 mg Cl·L^{-1} showed a ME < 4 (below the limit to be considered effective), with a reduction of 3.68 log-units. The remaining three species presented ME > 4 for all the NaOCl concentrations, showing to be 100% effective against planktonic cells.

Similar results were found when the mix culture of the four species was faced to different concentrations of NaOCl (Table 2), only *R. mucilaginosa* showed to have an ME < 4 (3.66 log-units) at 5 min contact to the lowest concentration of NaOCl, being sensitive to the

remaining higher concentrations (200 and 1000 mg $Cl \cdot L^{-1}$). Sodium hypochlorite was effective against *Candida krusei*, *C. tropicalis* and *C. kefyr* in all tested concentrations and at exposure times.

3.2 Disinfection test of adhered cells

3.2.1 Biofilm test – static condition

The number of attach cells on PVDF membranes and SS coupons is shown in Figure 2, before (time 0) and after (time 5, 10 and 30 min) being exposed to the NaOCl solution. The results were expressed as log $CFU \cdot cm^{-2}$. As shown in the figure, the tendency observed was higher counts for SS surfaces than for PVDF membranes. After 2 h, counts ranged from 6.09 ± 0.39 to 5.57 ± 0.004 log $CFU \cdot cm^{-2}$ for SS and PVDF membranes, respectively ($p < 0.05$); whereas after 16 h values were of 7.16 ± 0.01 (SS) and 6.6 ± 0.17 log $CFU \cdot cm^{-2}$ (PVDF membranes) ($p < 0.05$). On the other hand for 8 h biofilm's no significant differences were observed ($p > 0.05$). After 5 min exposure to NaOCl, cells attached to the membrane for 2 h presented an ME < 3 whereas in the case of SS coupons the decrease was of more than 3 log-units. In the remaining exposure times (10 and 30 min) PVDF membranes presented smaller log reductions than SS coupons, but in both cases were > 3 log-units, being 3.40 and 4.91 log units at 10 and 30 min exposure for PVDF membranes, and 4.93 and 6.00 log units for SS coupons.

As colonization times increased to 8 and 16 h the log reductions decreased, lowering the effectiveness of the disinfectant requiring greater contact times. For PVDF membranes reductions were of 0.39 (16 h colonization and 5 min exposure) and 1.41 (10 min exposure), whereas SS coupons presented greater reductions, 2.1 and 3.79 for 5 and 10 min, respectively for 16 h colonization. When surfaces were face to a longer exposure time like 30 min, greater reductions were seen. For 2 h colonization of SS coupons NaOCl presented

to be 100% effective removing the yeast cells attached (< 10 CFU mL^{-1} counts); while membrane topography rendered in shelter for yeast cells having a remaining community of 0.66 ± 0.09 log CFU·cm^{-2}. At 8 and 16 h colonization reductions ranged 5 log-units, but part of the community survived with counts of 0.94 ± 0.05 and 1.45 ± 0.03 log CFU·cm^{-2}, respectively for SS; while membranes presented counts of 2.25 ± 0.02 and 1.93 ± 0.02 log CFU·cm^{-2} at 8 and 16 h. After 16 h adhesion and 30 min exposure to NaOCl samples of SS coupons were stained with FDA (Figure 3) and it was observed that the remaining cells were found to be metabolically active, similar results were seen for PVDF membranes (not shown).

3.2.2 Biofilm test – UF membranes under flow conditions

Figure 4 A shows a SEM image of a transversal cut of 100 kDa PVDF membrane section, whereas in Figure 4 B it can be seen the same membrane after 2 h run in which a mixed suspension of yeasts in 12 ºBrix apple juice was filtered, simulating the scenario where the contaminated with yeast cells juice reaches the clarification point. As can be seen in Figure 4 B yeast cells adhere to both the active part of the membrane (in charge of the filtration process), and to the fibers acting as support. Those cells that cross through the membrane not only stay attach to the fibers but also begin to multiply.

Following filtration, membranes were subjected to a washing step and then disinfection with NaOCl of 200 and 500 mg Cl·L^{-1} for 15 minutes, time at which counts were made.

In order to see the potential recovery of stressed cells after the chemical treatment membrane samples were left in juice of different concentrations (3, 6 and 12 ° Brix) for 2, 8 and 16 hours (Figure 4 C to E). In Figure 4 B to E it can be seen how remaining cells on top and inside the membrane structure present different morphologies, including blastospores and pseudohyphae. In all cases the resulting community consisted of a predominance of

Candida krusei and *Candida kefyr*, followed by *Candida tropicalis* and *Rhodotorula mucilaginosa*, with percentages ranging from 30-40%, 10-20% and 1-10% respectively.

As it can be seen in Figure 5 after 2 h filtration total counts were of 4.62 ± 0.17 log CFU·cm^{-2}, represented by *R. mucilaginosa* (8%), *C. krusei* (39%), *C. tropicalis* (12%) y *C. kefyr* (42%). After disinfection protocol with 200 mg Cl·L^{-1} the ME was of 2.07 log CFU·cm^{-2} (Figure 5A) meanwhile for solutions of NaOCl 500 mg Cl·L^{-1} no surviving cells were found (< 10 CFU mL^{-1} counts) (Figure 5 B).

When membrane samples were put in contact with juice of different concentrations (3, 6 and 12 ºBrix) to see the potential recovery of the cells, no recovery was found for samples exposed to 500 mg Cl·L^{-1}, while at 200 mg Cl·L^{-1} recovery was evidenced regardless of the time of colonization ($p > 0.05$). In the case of membranes in contact with juice of 6 and 12 ° Brix, a maximum recovery of 75 and 100%, was reached respectively after 6 h of exposure.

Throughout the experiments the permeate flux was measured, with a reestablishment using both solutions of NaOCl although only the higher concentration (500 mg Cl·L^{-1}) presented a dual effect of 100% flux recovery and no viable cells. At 200 mg Cl·L^{-1} only the hydraulic properties of the membrane were reached with no long-term effect over the attached cells.

4. Discussion

High dosages of biocides are typically required in field applications to eradicate sessile cells because of the various defense mechanisms employed by biofilms (Li, Jia, Al-Mahamedh, Xu, & Gu, 2016). As expected, the results indicate that NaOCl at industrial concentrations of 200 mg Cl·L^{-1} is effective when applied against planktonic cells of the four species tested, with a ME of more than 4 log reductions, taking 10 minutes to reach its effectiveness, both separately and in mix cultures. This indicates a good potential to

eradicate planktonic cultures. Nevertheless, the overall response over adhered cells on either SS or PVDF membranes was a greater resistance towards NaOCl. Once cells completely colonized the surfaces they survived up to 30 minutes of exposure to industrial NaOCl concentration (200 mg $Cl \cdot L^{-1}$) (Figure 2). Communities of 8 and 16 h required 30 minutes to reach log reductions greater than 3, increasing resistance over time (16 h biofilms) ($p < 0.05$). As mentioned by Bridier, Briandet, Thomas, and Dubois-Brissonnet (2011) transport limitations may be a mechanism that contributes to the resistance of biofilms to disinfectants. In fact, biofilm insusceptibility is sometimes considered to be a tolerance rather than a real 'resistance' being mainly induced by a physiological adaptation to the biofilm mode of life (sessile growth, nutrient stresses, contact with repeated sub-lethal concentrations of disinfectant). This can be lost or markedly reduced when biofilm cells revert to the planktonic state (Russell, 1999).

Surfaces of food processing premises are exposed to regular cleaning and disinfection regimes, using biocides that are highly effective against planktonic cells. However, the ones growing in surface associated communities (biofilms) are typically more tolerant towards these procedures than their individual free cells counterparts (Fagerlund, Møretrø, Heir, Briandet, & Langsrud, 2017). In the context of UF and nanofiltration (NF), biofilms cannot be completely removed mechanically by backwashing. This implies that chemical cleaning methods should also be used, which usually recover the hydraulic properties of the membranes with little impact on the composition and organization of the deposited material (Di Martino et al., 2007), as evidenced in this work in which full recovery of the flux was reached with little impact on viable cells at NaOCl solutions normally used (200 mg $Cl \cdot L^{-1}$). In fact, as seen in Figure 3, coupons after 16 h colonization and treated for 30 min with NaOCl, present a resulting community of green cells when stained with FDA, indicating its

viability, with counts ranging 1.45 log $CFU \cdot cm^{-2}$. Cells that survive the disinfection process have the potential to act as a source of future contamination, with a greater resistance to environmental factors. The four strains used were previously cited as biofilm formers with potential to harbor pathogenic bacteria (Brugnoni et al., 2007; Brugnoni, Tarifa, Lozano, & Genovese, 2014; Tarifa et al., 2013; Tarifa, Lozano, & Brugnoni, 2015, 2017).

Even though test times were short enough to speak of a mature biofilm, it could be assumed that as time passes the attached microorganisms increase their number, even after cleaning and disinfection of the surfaces. As was seen in Figure 5A after apple juice was filtered through a 100 kDa PVDF membrane, and disinfected with 200 mg $Cl \cdot L^{-1}$ sections were allowed to be in contact with increasing concentrations of nutrient represented by apple juice of 3, 6 and 12 ºBrix. Results show how viable cells can recover from the stress imposed by the chemical treatment, taking 6 hours to fully recover to initial values. At higher concentrations of NaOCl cells were not able to recover but membrane properties could be modified, affecting the filtration process. The influence of nutrients on the overall process of biofouling is evident and crucial. The presence of different concentrations of juice as variations in the nutritional input allows the recovery of cells that had survived the disinfection process. As reported by several authors (Brugnoni, Lozano, et al., 2012; Dionisio-Ruiz et al., 2014), biofilms can develop even after the application of chlorination, which is a routinely process. In the case of Rojas-Serrano, Marín, Pérez, and Gómez (2015) when performing autopsies of UF membranes with a pre-treatment of coagulation, they observed that the affluent with organic matter acted as a protective barrier of biofilms.

In general, surface roughness can create conditions for the favorable initial adhesion of a single cell, possibly in a topological feature and this in turn forms the seed for the subsequent growth of a micro-colonies (Semião et al., 2013).

As shown in SEM images, membranes present a topography that provides shelter to the cells which is not the case of SS surfaces in which they are most exposed to disinfectant agents. Evidence of this is shown in the ME of PVDF membranes *vs* ME of SS coupons, 0.65 to 2 log reductions, respectively for 16 h biofilms exposed to 30 minutes of solutions of NaOCl - 200 mg $Cl \cdot L^{-1}$ (concentration of use recommended disinfection of surfaces in contact with food) (Figure 2). Even though hypochlorite is among the most corrosive chemicals used, having detrimental corrosive consequences on SS (Craig & Andersen, 1994), Neville, Hodgkiess, and Destriau (1998) concluded that the corrosive effect on stainless steel could be controlled with concentrations up to 500 mg $Cl\ L^{-1}$.

It has been shown that the concentration polarization also maintains the presence of biofilms by concentrating nutrients at the membrane surface from the bulk environment (Chong, Wong, & Fane, 2008; Vrouwenvelder, Von Der Schulenburg, Kruithof, Johns, & Van Loosdrecht, 2009). This is important as the attached cells multiply at the expense of local nutrient (Chong et al., 2008). For example, in the case of water, its pre-treatment helps to reduce nutrient loading and limits the extent of biofilm growth in membranes (Huang, Schwab, & Jacangelo, 2009; Huck et al., 2011; Peldszus, Benecke, Jekel, & Huck, 2012) comprising a form of intervention.

The development of biofilms in filtration systems has also been associated with decreases in membrane wettability and permeability (Houari et al., 2013), fact that was observed in the present filtration tests where a drop in permeate flux was observed along the 2 h experience. This leads to progressive increases in filter pressures leading to the development of increasingly compact biofilms in high shear systems. Houari, Seyer, Kecili, Heim, and Martino (2013) observed that these biofilms presented a higher proportion of polysaccharides than those developed under lower shear. An increase in the proportion of

polysaccharides could lead to better cohesion of the cells in the biofilms by protecting them and increasing their resistance to flow.

To sum up, biofilms represent a major problem for juice industries as major sources of contamination, causing operational, economic and potentially sanitary problems. Hence the mechanism involved in biofilm formation and maintenance of its structural integrity has become one of the most important concerns to develop efficient criteria for controlling biofouling in food processing facilities.

In this study it was demonstrated that yeast species native from juice production facilities can survive cleaning and disinfection at concentrations used in the industrial environment. How microorganisms interact for biofilm formation with the different surfaces used in the productions lines should be taken in mind to properly design control strategies.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Figure captions:

Figure 1. Cleaning and Disinfection Protocol followed against biofilms formed on ultrafiltration membranes under variable flow conditions.

Figure 2. Microbicidal Effect (ME) of a ready to use solution of NaOCl 200 mg $Cl \cdot L^{-1}$ over yeast biofilms formed under static conditions, along 5, 10 and 30 min exposure. The black line marks out the limit for a disinfectant to be considered effective. Results are expressed as log $CFU \cdot cm^{-2} \pm$ SD. M: Membranes, PVDF-UF 100 kDa; SS: Stainless Steel AISI 304.

Figure 3. EM images (200 x magnification) of stainless steel coupons colonized for 16 h by yeasts (*C. krusei, C. tropicalis, C. kefyr* and *R. mucilaginosa*) and treated with 200 mg $Cl \cdot L^{-1}$ for 30 min. Samples were stained with FDA showing in Green metabolic active cells.

Figure 4. SEM images of 100 kDa PVDF-UF membranes. (A) Cross section of a virgin membrane; (B) Section after 2 h filtration of a mixed suspension of *R. mucilaginosa, C. krusei, C. tropicalis, C. kefyr* in 12 ºBrix apple juice; (C to E) Membrane subjected for 16 h to a recovery step in 3ºBrix (C), 6ºBrix (D) and 12ºBrix (E).

Figure 5. PVDF-UF membranes of 100 kDa MWCO after 2 h filtration of a mix yeast suspension, a disinfection step and lately a recovery step where membranes were incubated in 3, 6, and 12 ºBrix juice. Two NaOCl solutions were used: A) 200 mg $Cl \cdot L^{-1}$ and B) 500 mg $Cl \cdot L^{-1}$. Results are expressed as log $CFU \cdot cm^{-2} \pm$ SD.

Table 1. Microbicidal Effect (ME) of a ready to use solutions of sodium hypochlorite (NaOCl) over *Rhodotorula mucilaginosa, Candida krusei, C. tropicalis* and *C. krusei.* The disinfection efficiency was evaluated in suspensions for 5 min at 21 ± 1 ºC.

Table 2. Microbicidal Effect (ME) of a ready to use solutions of sodium hypochlorite (NaOCl) over mixed cultures of *Rhodotorula mucilaginosa, Candida krusei, C. tropicalis*

and *C. krusei*. The disinfection efficiency was evaluated in suspensions for 5 min at 21 ± 1 ºC.

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Table 1

Time (minutes)	NaOCl (mg Cl L^{-1})			
	50	150	200	500
	EM			
5	***R.mucilaginosa*: 3.68**	>4	>4	>4
10	>4	>4	>4	>4
15	>4	>4	>4	>4
30	>4	>4	>4	>4

Table 2

Species	NaOCl (mg Cl L^{-1})			
	50	150	200	1000
	EM			
Candida krusei	>4	>4	>4	>4
Candida tropicalis	4	>4	>4	>4
Candida kefyr	>4	>4	>4	>4
Rhodotorula mucilaginosa	**3.66**	>4	>4	>4

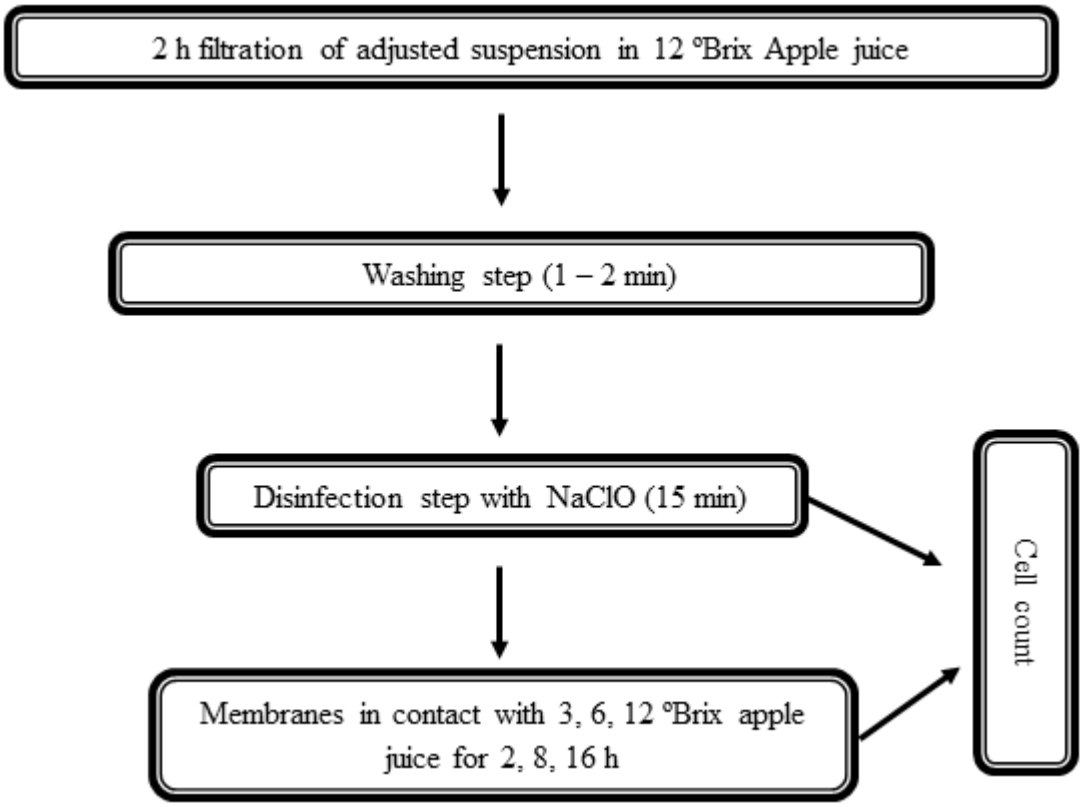


Fig. 1

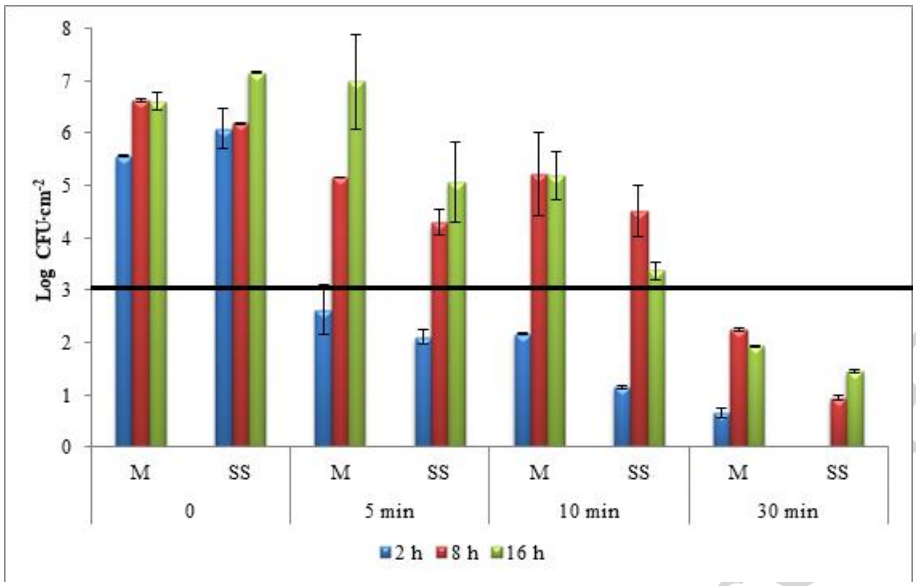


Fig. 2

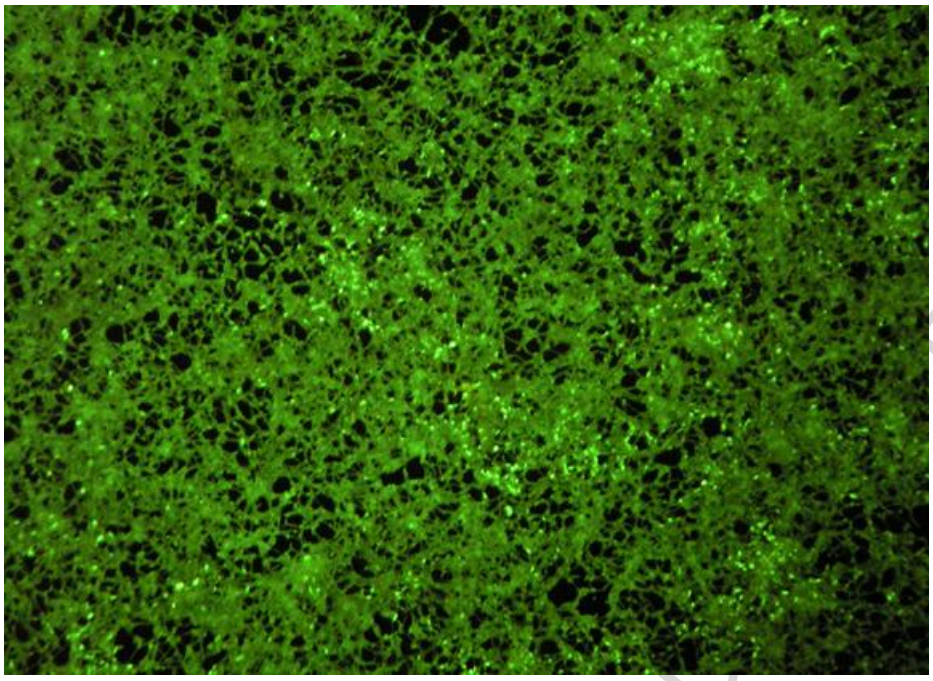


Fig. 3

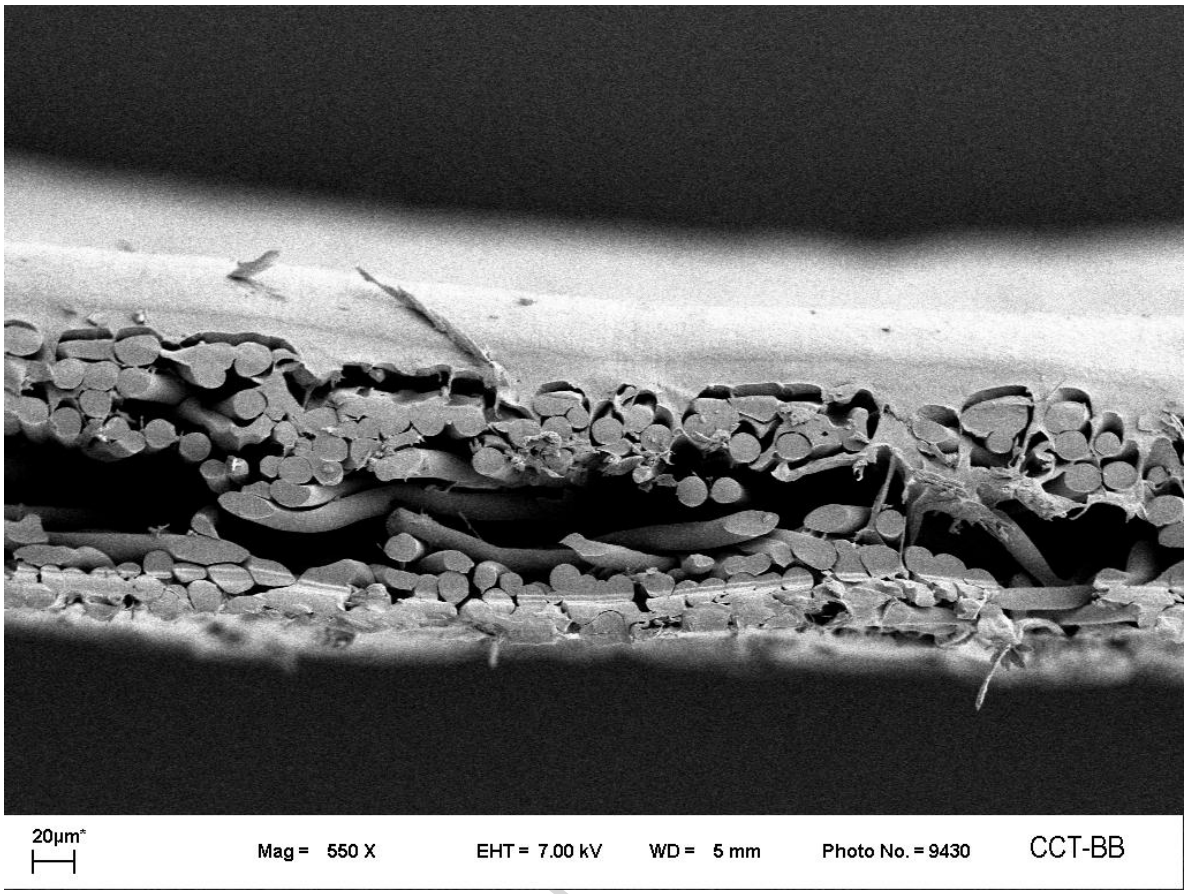
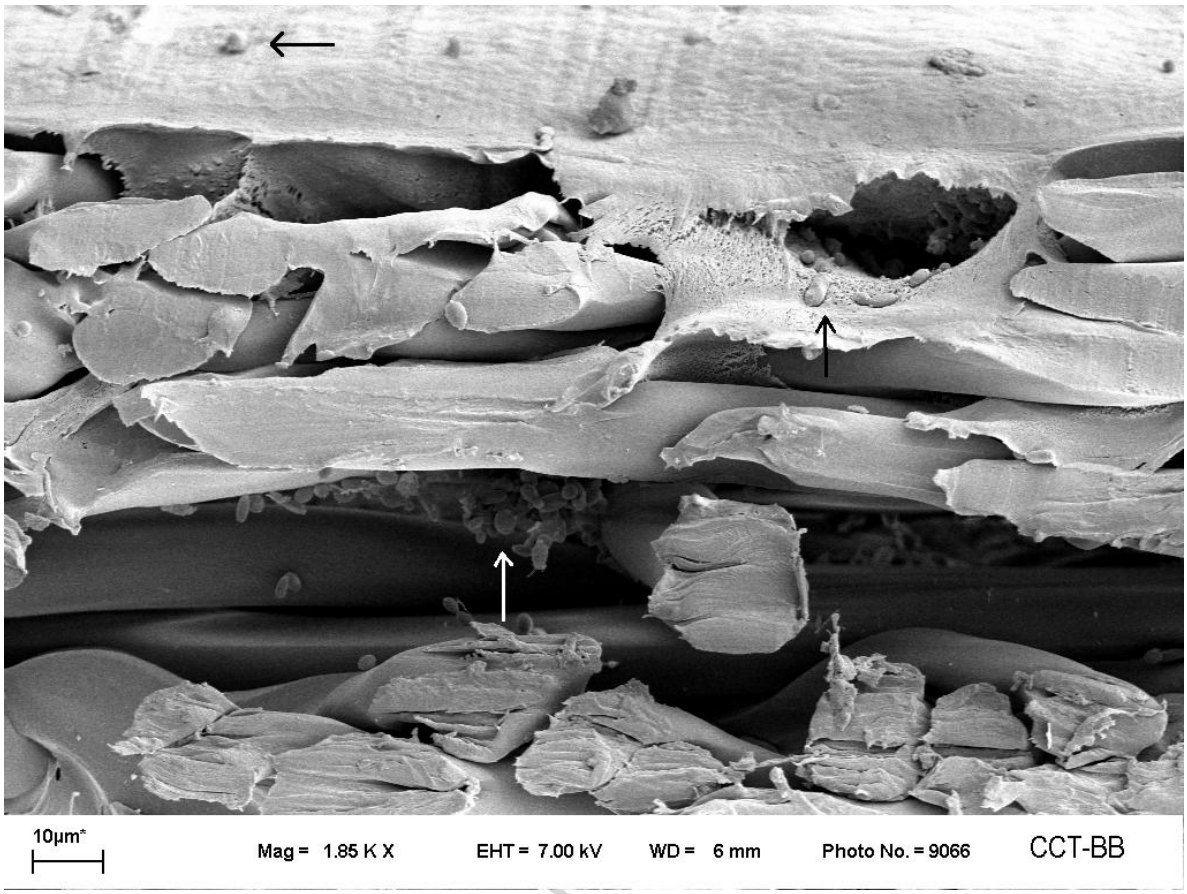


Fig. 4A



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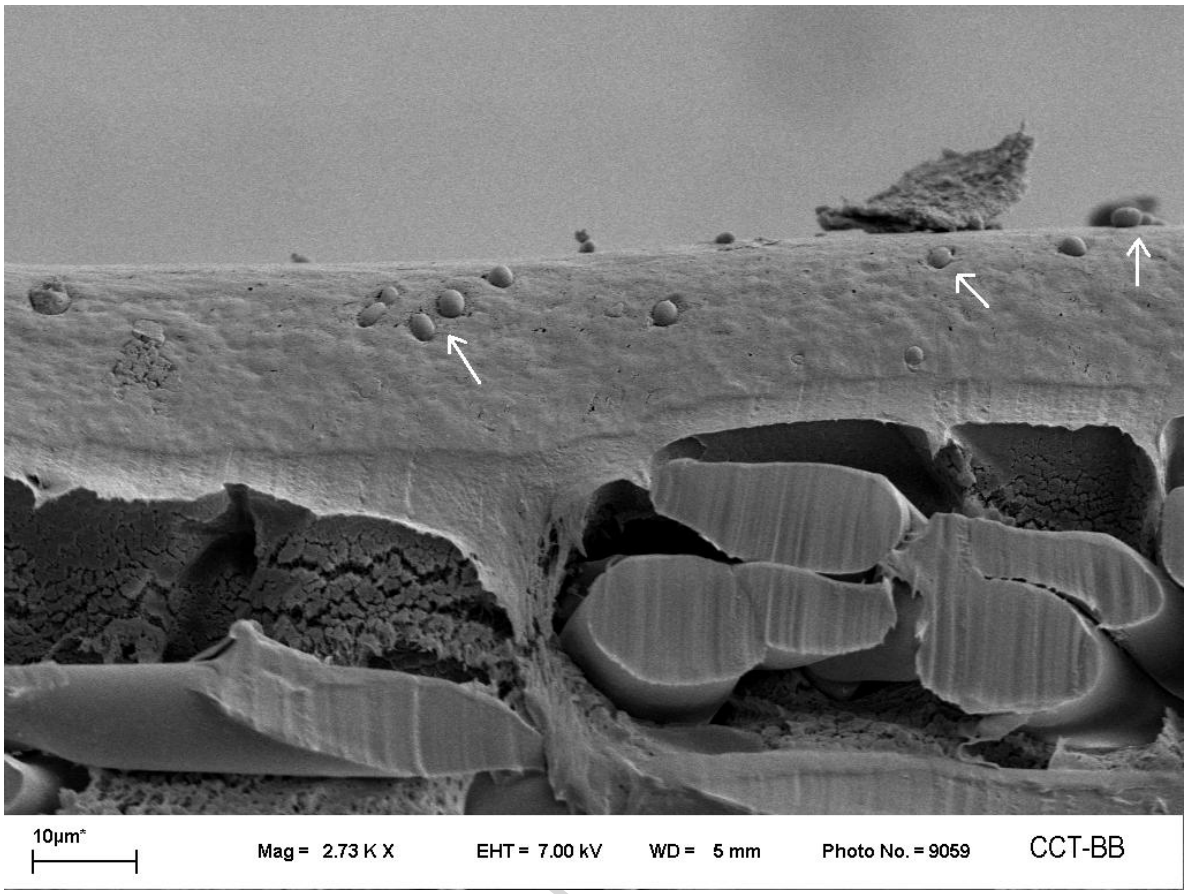

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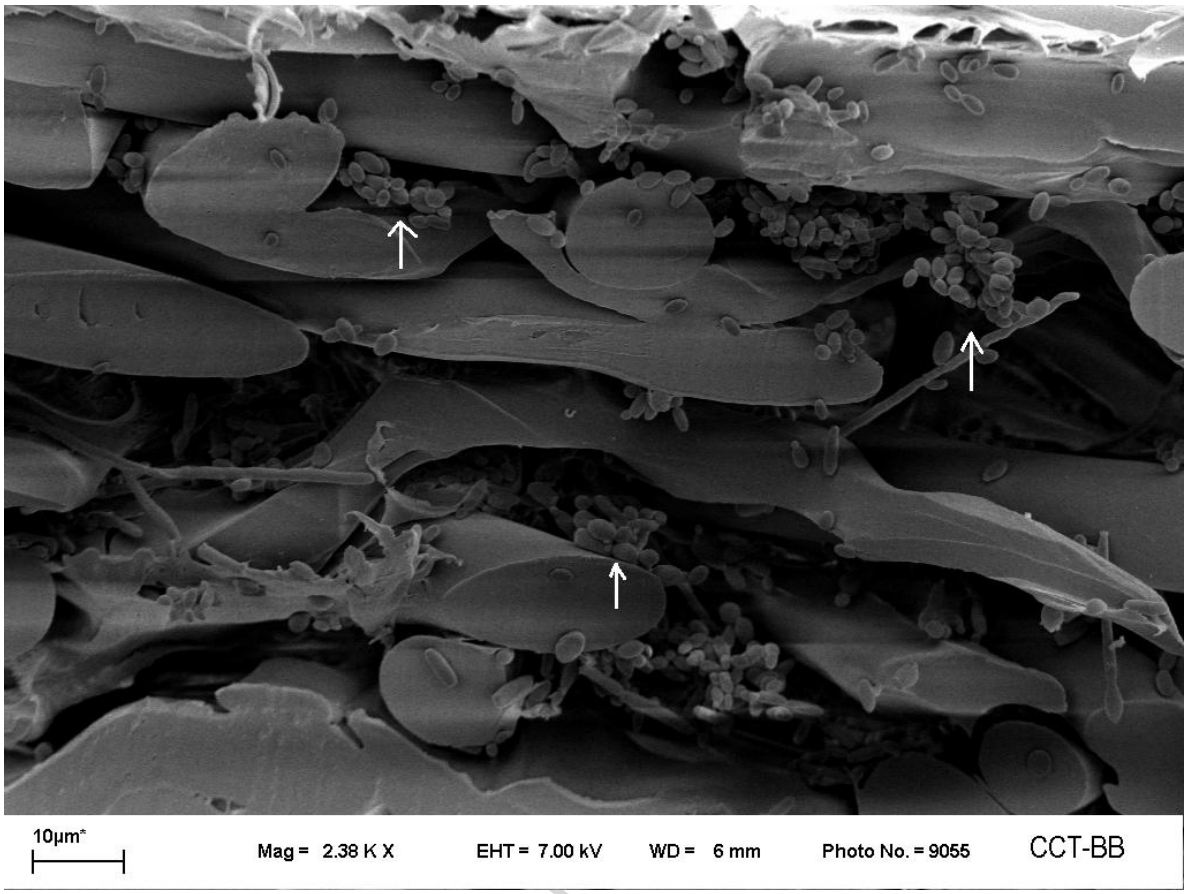

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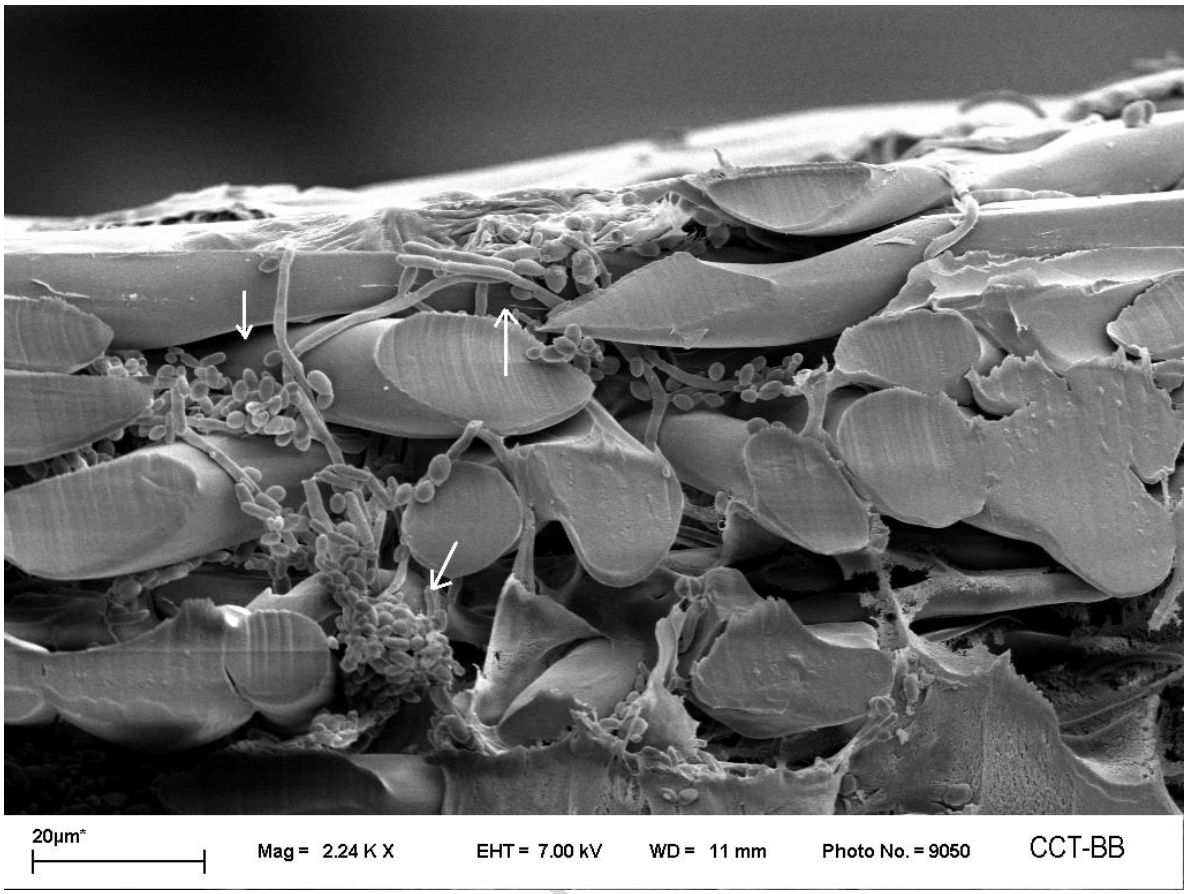

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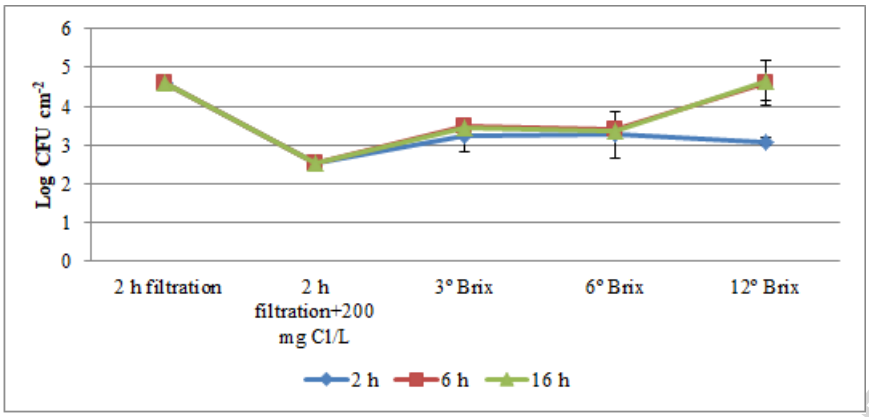


Fig. 5A

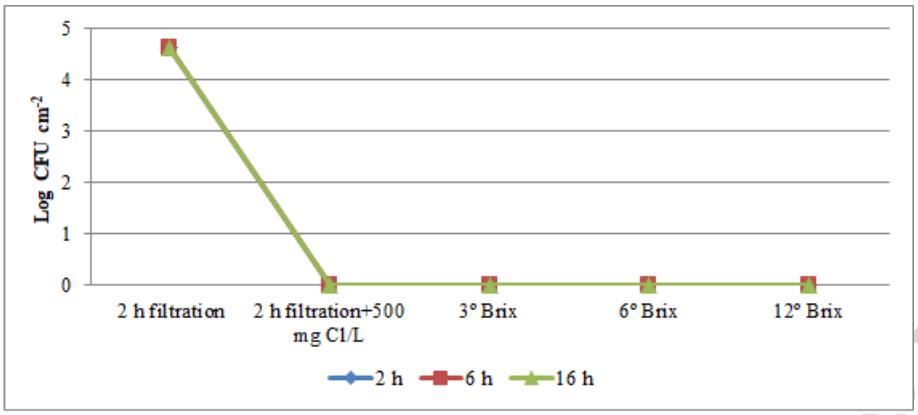
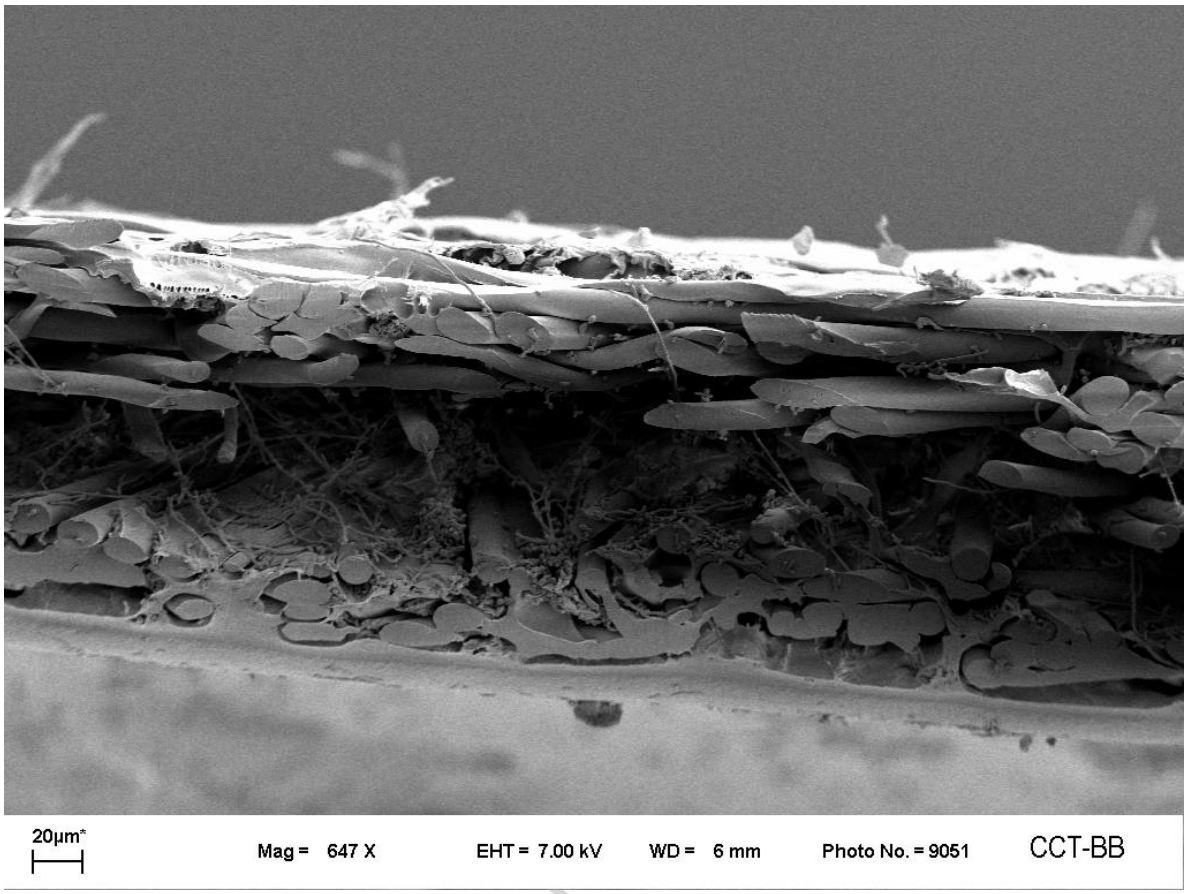


Fig. 5B



Graphical Abstract