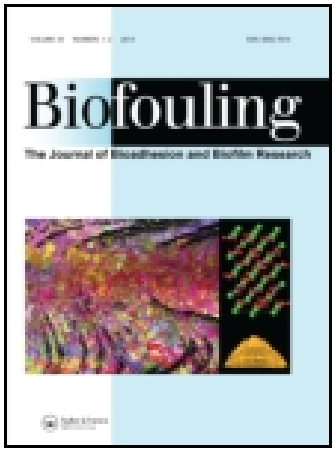

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Lorena I. Brugnoni[ab], María C. Tarifa[a], Jorge E. Lozano[a] & Diego Genovese[a]

[a] PLAPIQUI (UNS-CONICET), Bahía Blanca, Argentina

[b] Department of Biology, Biochemistry and Pharmacy, Universidad Nacional del Sur, Bahía Blanca, Argentina

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Lorena I. Brugnoni[a,b]*, María C. Tarifa[a], Jorge E. Lozano[a] and Diego Genovese[a]

[a]*PLAPIQUI (UNS-CONICET), Bahía Blanca, Argentina;* [b]*Department of Biology, Biochemistry and Pharmacy, Universidad Nacional del Sur, Bahía Blanca, Argentina*

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The aim of the present work was to investigate the *in situ* rheological behavior of yeast biofilms growing on stainless steel under static and turbulent flow. The species used (*Rhodototula mucilaginosa*, *Candida krusei*, *Candida kefyr* and *Candida tropicalis*) were isolated from a clarified apple juice industry. The flow conditions impacted biofilm composition over time, with a predominance of *C. krusei* under static and turbulent flow. Likewise, structural variations occurred, with a tighter appearance under dynamic flow. Under turbulent flow there was an increase of 112 µm in biofilm thickness at 11 weeks ($p < 0.001$) and cell morphology was governed by hyphal structures and rounded cells. Using the *in situ* growth method introduced here, yeast biofilms were determined to be viscoelastic materials with a predominantly solid-like behavior, and neither this nor the G'_0 values were significantly affected by the flow conditions or the growth time, and at large deformations their weak structure collapsed beyond a critical strain of about 1.5–5%. The present work could represent a starting point for developing *in situ* measurements of yeast rheology and contribute to a thin body of knowledge about fungal biofilm formation.

Keywords: yeast; biofilms; apple juice; rheology; flow conditions; stainless steel

Introduction

Microbial biofilms are the populations of microorganisms that concentrate at wetted solid surfaces in both industrial and natural environments. Biofilm cells are typically surrounded by a matrix of extracellular polymeric substances (EPS) consisting of cell clusters (aggregates of cells) surrounded by interstitial water channels.

The persistence of microorganisms in biofilms is a serious hygienic problem in the food industry, causing processing and post-processing cross-contamination leading to reduced product shelf life and effectiveness of sanitizing treatments as well as potentially affecting the consumer's health. Moreover, a buildup of biofilms in industrial pipelines can cause loss of hydrodynamic pressure leading to increased power consumption and decreased plant efficiency, as well as possible cooling failures (Characklis 1981).

In industrial processes, the geometrical design of machines, pipes and tanks is usually complex, and biofilms are thus grown under a wide distribution of local hydrodynamic strengths (Lelièvre et al. 2002; Blel et al. 2007). Depending on their location within the process equipment and the local wall shear stress, biofilms are consequently characterized by their thickness, architecture, and rheological properties.

The rheological properties of a biofilm determine its mechanical stability and its detachment processes (Stoodley et al. 1999). Biofilms are persistent in a variety of settings where knowledge of their mechanical properties would be helpful in the maintenance of clean surfaces as well as for preserving the efficiency and effectiveness of fouled components (Pavlovsky et al. 2013). Knowledge of biofilm rheology is crucial to fully interpret their behavior, particularly in the case of biofilms growing on surfaces exposed to flowing fluids, which may be subjected to shear stresses that vary widely in both magnitude and frequency. Such information may lead to a better understanding of how the interaction between a flowing liquid and the viscoelastic biofilm results in cell-detachment and the potential dissemination of microorganisms in the fluid bulk (Costerton et al. 1987; Marshall 1992). Stoodley et al. (1999) found that biofilm viscoelasticity allows the biofilm to structurally deform when exposed to varying shear stresses, which enables the biofilms to resist detachment due to increased fluid shear by deformation, while remaining attached to the surface.

However, there is limited information on the material properties of biofilms and how the environment influences their growth (Stoodley et al. 2002). There are two main reasons for this gap in knowledge: first, only a few groups have recognized the importance and implications of considering biofilms as materials, and second, biofilms are very difficult to test mechanically.

The studies of biofilm rheology reported in the literature usually involve removing the biofilm and testing the suspension (Ohashi & Harada 1994). Although this

*Corresponding author. Email: brugnoni@uns.edu.ar

approach can supply interesting rheological data it does not provide direct information regarding the *in situ* behavior of intact biofilms. To date, few studies have been conducted on the material properties of intact biofilms (Towler et al. 2003; Pavlovsky et al. 2013).

The development of yeast biofilms on food processing equipment will potentially adversely affect the quality and the shelf life of foods (Fleet 2007; O'Brien et al. 2007). In food processing lines, yeasts belonging to the genera *Saccharomyces*, *Candida*, and *Rhodotorula* have been isolated from biofilms on conveyor tracks, and on can and bottle warmers in the packaging department of a beverage industry (Salo & Wirtanen 2005).

In previous studies, Brugnoni et al. (2007) and Tarifa et al. (2013), reported on the adhesion and biofilm formation of yeasts isolated from apple juice processing equipment. One of these yeasts, a strain of *Candida krusei*, was capable of colonizing stainless steel and forming biofilms under laminar, transitional, and turbulent flow (Brugnoni et al. 2011a, 2011b; 2012). To the authors' knowledge, there are no published studies on the rheology of yeast biofilms and how it is influenced by the growth environment.

The objective of this work was to investigate *in situ* the rheological behavior of yeast biofilms growing on stainless steel under both static conditions and turbulent flow. By developing a model that closely represents industrial conditions, the goal was to gain a better understanding of the rheology of yeast biofilms in the fruit juice industry.

Materials and methods

Yeast strains

The yeast strains used in this work were isolated from the surfaces of polyvinylidene–fluoride ultrafiltration (UF) membranes, obtained from a large-scale apple juice processing industry located in Argentina (Tarifa et al. 2013). These yeasts had survived repeated cycles of production and membrane cleaning procedures. Hence, it was assumed that much of the community was firmly attached to the membrane and therefore constituted a biofilm.

Phenotypic identification was performed through the analysis of typical morphological features, along with biochemical and physiological characterization (fermentation of seven carbohydrates, assimilation of two nitrogen and 19 carbon sources, urea hydrolysis and growth at 37°C) (Kurtzman & Fell 1998). The genotypic identification was performed through the analysis of the single gene sequence of the domains D1/D2 region of 26S rDNA and from the internal transcribed space (ITS) region, commonly used to identify yeast species (Taverna et al. 2013). The D1/D2 variable domain was amplified by PCR using the primers NL1 (5′-GCATATCAA-TAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCTGTTT-CAAGACGG-3′) (White et al. 1990) whereas the ITS region was amplified using the ITS1 primer (5′-TCCGTAGGT- GAACCTGCGG-3′) and the ITS4 primer (5′-TCCTCCGCTTATTGATATGC-3′). The DNA extraction was performed using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA, USA) and preserved at −20 °C until use. For the purification of the PCR products a PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) was used. The PCR products were sequenced in the reference laboratory Instituto Nacional de Enfermedades Infecciosas 'Dr Carlos G. Malbrán' (Buenos Aires, Argentina). Once obtained, the sequences were edited using BioEdit 7.0.0 and the similarity was obtained using the BLASTN tool of the National Center for Biotechnology Information (Library of Medicine, Bethesda, MD, USA, www.ncbi.nlm.nih.gov/BLAST/).

Stock culture of the strains were suspended in 20% (v/v) glycerol in Yeast Extract Glucose chloramphenicol (YGC) broth: 0.5% w/v yeast extract (Merck KGaA, Darmstadt, Germany), 2% w/v glucose (Merck KGaA, Darmstadt, Germany) and 0.01% w/v chloramphenicol (Fluka Chemie AG, Buchs, Switzerland), and stored at −70 °C until use.

Culture conditions

The mixed culture inoculum was composed of four yeast species: *Rhodototula mucilaginosa*, *Candida krusei*, *Candida kefyr* and *Candida tropicalis*, all of which were isolated from apple juice UF membranes as described above. For the preparation of the mixed species suspension, a loop of frozen cells of each strain used was suspended in YGC broth at 25 ± 1 °C until they reached the stationary phase and harvested by centrifugation at 2,500 x *g* for 5 min (Labofuge 200, Kendro, Germany). Afterwards each strain was re-suspended in sterile clarified apple juice until the suspensions reached an OD_{550} of 0.125 (~$5x10^6$ cells ml^{-1}) using a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA, USA). Equal quantities of the adjusted suspensions of each yeast strain were mixed for biofilm formation.

Food soiling system (growth media)

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 (AVRNN) (JUGOS SA, Argentina).

The composition of apple juices depends on the variety, the origin, the growing conditions of the apples, the quality of the fruit, and the processing procedures and storage (Lee & Mattick 1989; Lea 1990). The AVRNN region concentrates its production on the apple varieties Red Delicious (65%), Gala (15%), with their respective

clones, Granny Smith (15%), and Pink Lady, Rome Beauty, Golden Delicious, Fuji and Braeburn (the remaining 5%) (Bruzone 2008). As a result of the production dynamics of the company, two different batches of concentrated apple juice were provided. The reconstituted-clarified apple juices used as growth media (~ 12°Brix) were called J1 and J2, respectively, and were sterilized by microfiltration (pore size 0.45 μm) (Metricel®Grid, Gelman Sciences, MI, USA) prior to use.

The major components of apple juice are carbohydrates, acids, nitrogen compounds, polyphenols, minerals and vitamins (Lee & Mattick 1989; Lea 1990). The approximate composition of the 12º Brix clarified apple juice can be seen in Lozano (2006).

Biofilm formation in static conditions

The system used for static biofilm formation consisted of beakers in which stainless steel (SS) disks (50 mm diameter, 0.5 mm thickness; AISI 304 2B, food grade) were placed. Previously, the disks and the beakers were soaked for 15 min with 2% v/v of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50°C, and rinsed with hot tap water, following sterilization for 15 min at 120°C.

To allow attachment, the beakers were filled with the mixed culture suspension (prepared as described above), with a working volume of 500 ml per beaker system. Yeasts cells were allowed to settle on the SS disk surfaces for 24 h at 20 ± 1°C. Afterwards, yeast suspensions were replaced every 72 h for up to 11 weeks with sterile apple juice in order to provide fresh nutrients for the cells. SS disks were removed after 3 (S-3) and 11 (S-11) weeks and analyzed by confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and rheology. Each analysis was carried out in duplicate.

Biofilm formation in turbulent flow

The Rotating Disk System (RDS) used in the present work was described in Brugnoni et al. (2011b) (Figure 1). A 0.2 m diameter, 5 mm thickness acrylic disk was rotated in a SS container (30 cm in diameter and 19 cm in height). The drive motor was a Boeco Model OSD-20 stirrer (Boeckel Co., Hamburg, Germany) with a speed controller. SS disks (50 mm diameter, 0.5 mm thickness; AISI 304 2B, food grade) used as the substratum were fixed to the acrylic disk with a contact adhesive (POXIPOL™, Akapol, Argentina). Previously, the disks and the SS container were soaked for 15 min in 2% v/v of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50°C, rinsed with hot tap water and autoclaved for 15 min at 120°C.

The hydrodynamics of the liquid flow along the surface of a RDS are described by Levich (1962). Fluid flow was characterized by the Reynolds number (Re),

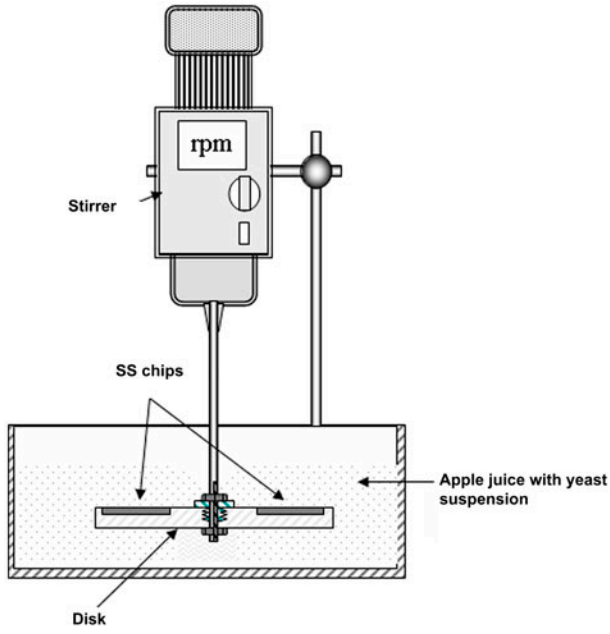

Figure 1. A schematic representation of the rotating disk system (RDS) used in the present work (Brugnoni et al. 2011b).

defined as $\rho\omega r^2\mu^{-1}$, where ρ is the fluid density (apple juice density: 1,020 kg m^{-3}), ω is the angular velocity (rad s^{-1}), r is the radius of the disk (m), and μ is the fluid viscosity (apple juice viscosity: 1.24 x 10^{-3} Pa s). In practical terms this parameter represents the ratio of inertial to viscous flow. For turbulent flow (Re > 200,000), the shear stress (τ_{turb}) is given by Visser (1973): $\tau_{turb} = 0.534\rho r^{8/5}\mu^{1/5}\omega^{9/5}$. The rotation speed was adjusted to obtain Re numbers equal to 210,000 (shear stress: 204.5 N m^{-2}).

To allow attachment, the SS container was filled with the yeast suspension (prepared as described above) and yeasts cells were allowed to settle on the SS disk surfaces for 24 h at 20 ± 1°C. In order to provide continuous conditions when the disk was in place and rotating, the working volume was 1,500 ml of apple juice (the difference in the starting volume between the turbulent and static assays relates to the influence of the vortex on the liquid volume in the system). During the experiment the acrylic disk was rotated for 8 h day^{-1} with the sterile apple juice being replaced every 72 h for up to 11 weeks. SS disks were removed after 3 (T-3) or 11 (T-11) weeks and samples were analyzed, at least in duplicate, through CLSM, SEM, and rheology.

The time and temperature (15–25°C) used in the present study were selected based on the average time and temperature of the first stage of concentrated apple juice production, in the Southwest of Argentina.

To determine the species prevalence in the trial, the biofilms grown on the surfaces were removed into a test tube with YGC broth and homogenized. Samples were

serially diluted with PBS and plated onto CHROMagar *Candida* (CHROMagar Microbiology, Paris, France) medium in triplicate, and incubated at 25°C for 48 h. 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 et al. 2003), culture identification was also performed with an identification system (Rapid ID Yeast Plus system, Remmel, USA).

Microscopic observations

CLSM

To observe the adhering yeasts on the SS surfaces, samples were stained with fluorescein diacetate (FDA). This fluorescent probe is widely used as an indicator of cell viability; the principle behind using FDA is that only living cells convert FDA to fluorescein. FDA stains cells possessing esterase activity and intact cell membranes (Ki-Bong & Hideaki 2002).

A standard stock solution of 2 mg ml^{-1} (0.2%) FDA ($C_{24}H_{16}0_7$, Sigma-Aldrich Chemical Co., St Louis, MO, USA) was prepared in acetone (Dorwil, Industria Argentina) and stored at -18°C. For the observation of yeast cells on SS the disks from each experimental condition were stained with sterile FDA acetonic solution in 0.1 mol l^{-1} phosphate buffer (0.04%), pH 7.5. After 90 min shaking (50 rpm) at 25 ± 1°C in darkness, the disks were rinsed twice with sterile distilled water. Stacks of images in all samples were obtained with a CLSM (Leica TCS SP2 acoustic optical beam splitter (AOBS), Leica Microsystems, Richmond Hill, ON, Canada) through a 20 × water immersion objective. Image capture and two-dimensional (2D) projections of z-stacks were performed using Leica Confocal Software (LCS, Leica Microsystems).

Scanning electron microscopy (SEM)

The disks were fixed with glutaraldehyde (2.5%) in phosphate buffer (0.1 mol l^{-1}, pH 7.2), washed three times with the same buffer, and dehydrated by critical point drying (E3000, Polaron). Samples were gold coated (300 Å) in a Pelco Model 3 Sputter Coater 91,000 metal evaporator (Ted Pella, Inc., Irvine, CA, USA) and viewed with a Scanning Electronic Microscope (LEO EVO 40, Cambridge, UK) at 7.0 kV acceleration voltage (Lozano 1990).

Rheometry

The viscoelastic properties of the yeast biofilms were determined by small deformation dynamic oscillatory measurements performed in a Paar Physica rheometer model MCR301 (Anton Paar GmbH, Graz, Austria), using a geometry of parallel plates (50 mm diameter). The temperature of the lower plate was set at 25°C. For each measurement the disk with the biofilm was attached to the rheometer's lower plate by means of a special ring designed *ad hoc* (Figure 2). The upper plate was lowered to the sample until the normal force was approximately 1 N. The samples were allowed to rest for at least 5 min to attain thermal equilibrium and stress relaxation. Then, a frequency (ω) sweep test was performed from 0.1 to 100 rad s^{-1} at 0.05% strain. Data obtained were the elastic modulus (G'), the viscous modulus (G''), and the loss tangent ($tan\ \delta = G''/G'$). The frequency sweep test is valid if it is performed within the linear viscoelastic range (LVR), where material functions (such as G' and G") do not depend on the magnitude of the deforming strain. One way of determining the LVR is to perform a strain amplitude sweep, and see the range of strains where G' and G" are approximately constant. However, this test destroys the structure of the sample and it is convenient to perform it last. The strain value used for the frequency sweep tests (0.05%) was chosen from preliminary studies which showed that it was typically within the LVR of yeast biofilms. Finally, the strain amplitude (γ) sweep (from 0.01 to 100%, at 10 rad s^{-1}) was performed to verify that the frequency sweep was within the LVR, and also to obtain data in the non-linear region. This type of test is known as large amplitude oscillatory shear (LAOS). As previously mentioned, each biofilm sample (S-3, S-11, T-3 and T-11) was replicated

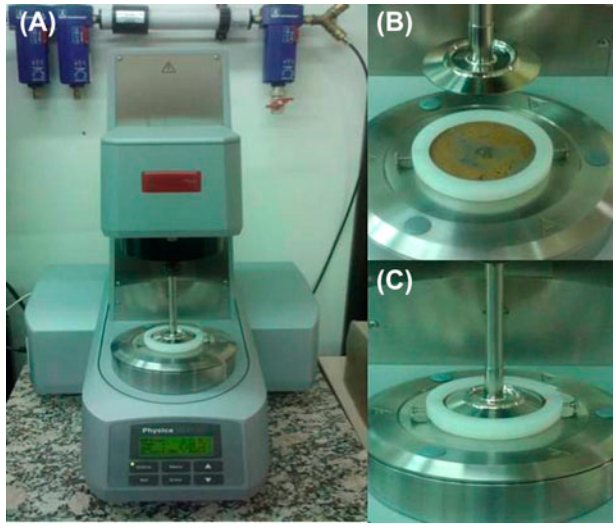

Figure 2. (A) Paar Physica rheometer model MCR301 using a geometry of parallel plates with a biofilm sample attached to the lower plate of the rheometer by means of a special ring designed *ad hoc*. (B) Overhead view of the special ring designed *ad hoc* and an SS disk sample used in a previous measurement. (C) Close-up view of the adjustment ring.

twice (two runs, J1 and J2, per sample). Each run was measured at least in duplicate, using a fresh disk+biofilm system each time.

Experimental design and statistical analysis

A two factor experimental design was employed, with two levels per factor (Table 1), and two replicates per level, one factor being the type of flow (static or turbulent) and the other factor the growth time (3 or 11 weeks). Then, a two factor ANOVA was performed, at a significance level of 5%. In the case of rheological data, the effect of using two different batches of juice in each replicate was eliminated by considering both juices as blocks, J1 and J2 (Table 2).

Results and discussion

Biofilm characterization

As described in a previous study (Tarifa et al. 2013) the resident community on biofouled UF membranes used in the apple juice processing industry was composed of four yeast species. In order to gain an insight into their behavior, a mixture of yeast species was examined under different hydrodynamic forces and exposure times.

After 11 weeks differences were observed in the composition and richness of the biofilms formed on the SS disks in comparison with the initial inocula. Using CHROM agar *Candida* as a differentiation medium it was observed that under static conditions *Candida krusei* dominated the biofilm community, with a viable population of 8.00 ± 0.134 Log cfu cm^{-2}, whereas under turbulent flow the community was represented by *Candida krusei* and *Candida tropicalis* with counts of 8.05 ± 0.009 Log cfu cm^{-2} and 5.80 ± 0.106 Log cfu cm^{-2}, respectively. These results show that *C. krusei* predominated in biofilms formed on SS disks in both hydrodynamic conditions. In the study by Tarifa et al. (2013) *C. krusei* represented the majority of the isolates from UF membrane samples, showing the adaptive advantage of this species. Brugnoni et al. (2007) reported similar results where *C. krusei*, isolated from a large-scale apple juice processing plant, presented the highest covered surface on SS compared with other yeasts. Kundu et al. (2013) found that after operation of a hybrid anaerobic reactor for 7 days, high shear intensity led to substantial changes in the richness or diversity of an archaeal community. However, in the control reactor no notable differences were observed, suggesting a stable community structure under fixed conditions. Brugnoni et al. (2011b) proposed that high shear environments would select for populations that produce stronger biofilms.

Table 1. Biofilm thickness as a function of growth time and flow conditions.

Flow	Time (weeks)	Sample	Thickness (μm)
Static	3	S-3	41.0 ± 12.6
	11	S-11	50.0 ± 0.0
Turbulent	3	T-3	21.0 ± 4.5
	11	T-11	133.0 ± 4.2

Table 2. Viscoelastic properties of biofilms obtained by fitting experimental data with Equation 1.

	Time (weeks)	Sample	Juice	G'_0 (Pa)	n (-)
Static	3	S-3	J1	37,478	0.0318
			J2	10,762	0.0366
	11	S-11	J1	61,974	0.0571
			J2	33,830	0.0330
Turbulent	3	T-3	J1	48,506	0.0431
			J2	20,511	0.0331
	11	T-11	J1	42,388	0.0360
			J2	10,172	0.0350

Note: The pre-exponential coefficient G'_0 represents the predicted elastic modulus at a frequency of 1 rad s^{-1}, and the exponential coefficient n represents the slope of the curve.

Unlike *C. krusei*, *C. tropicalis* is capable of producing multicellular, filamentous forms of growth, pseudohyphae and pseudomycelium. It has been suggested that the presence of pseudohyphae may be important in the structural integrity of multilayered biofilms (Baillie & Douglas 1999; Ramage et al. 2002; Paramonova et al. 2009). The pseudohyphal content was found to be a determining parameter for the strength of fungal biofilms (Paramonova et al. 2009). Strength is one of the parameters used to describe the mechanical properties of biofilms. It is defined as the ability of a material to resist applied forces, such as shear originating from liquid food flow or cleaning protocols. Pseudohyphal cells contain at least three times as much chitin as yeast cells (Braun & Calderone 1978; Chaffin et al. 1998). Chitin is a hydrophobic material and has been shown to increase the mechanical and flexural strength of bone substitutes (Chen et al. 2005). A higher amount of chitin, due to the presence of more pseudohyphal cells in biofilms, may be responsible for the increased compressive strength of the biofilm.

The typical hydrodynamic condition prevailing in the membranes of clarified apple juice manufacturing plants is turbulent flow (Yazdanshenas et al. 2005; Tarifa et al. 2013). In order to adhere to surfaces and subsequently to form biofilms, yeast cells grown in high-velocity flowing systems must overcome shear stress at the fluid–surface interface. It may be assumed that those cells are able to form a dense network of pseudohyphae on inert surfaces (eg SS and plastics) and adhere in an easier way, forming biofilms resistant to the removal effect of the liquid flowing through the system. In this environment, the ability of *C. tropicalis* to adhere strongly to SS surfaces

may provide it with a competitive advantage over other yeasts.

Diversity in microbial communities is shaped by a variety of complex relationships involving inter- and intraspecies interactions (Berry et al. 2006; Elenter et al. 2007; Hansen et al. 2007). Kreth et al. (2005) postulated that when two species grow simultaneously, both proliferate. However, prior establishment of one of the species prevents the others from occupying the same niche, and also the rapid multiplication of yeast cells can speed up starvation and increase stress (Palková & Váchová 2006).

CLSM was used to compare the three-dimensional architecture and thickness. As already mentioned, in industrial processes the geometrical design is varied and complex so biofilms are grown under a wide range of local hydrodynamic strengths (Lelièvre et al. 2002; Blel et al. 2007). As biofilms became thicker, the multiple layers of cells formed over time impeded the counts by the FDA method or by plate count agar, so thickness was the measure selected to observe the profile reaction to the stress imposed.

After exposure for 3 weeks, the biofilm thickness showed no significant differences ($p > 0.05$) between the turbulent and static culture conditions (see Table 1). However, significant differences ($p < 0.001$) were observed after 11 weeks, where biofilms grown under turbulent flow were 83 μm thicker than those grown under static conditions (see Table 1).

How liquid flow affects the thickness of the biofilm can be explained in the light of the factors that influence the structure of biofilms growing in aqueous environments: flow velocity and nutrient status (Bott 1995). Flow velocity determines the hydrodynamic shear and the mass transfer characteristics of a system (Stoodley et al. 1999). Mass transfer can be described as the efficiency of delivery of suspended solids (nutrient) from the bulk phase (flowing solution) to the attached phase (biofilm). During the initial stages of biofilm development (3 weeks in the present experiment), internal mass transfer limitations were probably not significant due to the reduced thickness, and so no significant differences were observed between biofilms formed under static and turbulent flow conditions ($p > 0.05$).

As the biofilm thickness increased, mass transfer limitations became increasingly important (Stewart & Franklin 2008). In this case increasing the flow velocity increases the substrate supply and mixing, leading to faster and more stable growth.

In potable water pipe systems, Ohl et al. (2004) showed that increasing the flow velocity in the bulk phase leads to higher biofilm density and higher maximum substrate flux. Santos et al. (1991) found that in filtered tap water *Pseudomonas fluorescens* biofilms were thicker when grown at 2.5 m s^{-1} than at 0.5 m s^{-1}. In this case increasing the flow velocity increases substrate supply and mixing, leading to faster and more stable growth.

In general the present authors' observations seem to be in agreement with those displayed by *C. albicans* biofilms formed under conditions of flow, as reported in other studies where *C. albicans* produced higher amounts of extracellular matrix and had a high cell density compared with biofilms grown under static conditions (Hawser et al. 1998; Al-Fattani & Douglas 2006; Ramage et al. 2008).

The samples that supported turbulent flow showed a significant increase ($p < 0.001$) in thickness (112 μm) between 3 and 11 weeks exposure (see Table 1). In contrast samples held under static conditions did not show a significant change in biofilm thickness between 3 and 11 weeks ($p > 0.05$).

CLSM images were also used to determine the three-dimensional architecture of the biofilms formed. The observations proved the ability of the species to form robust mature biofilms within the times sampled (Figure 3 A to D). Biofilms are complex biological structures the thickness of which comprises the distance from the substratum to the peaks of the highest cell clusters in contact with the liquid flow. Consequently, the channels that surround the cell clusters are an integral part of the biofilm. When comparing biofilms grown under static and turbulent flow at 3 and 11 weeks, a higher proportion of channels were observed in the static conditions (Figure 3 A–B) compared with turbulent flow (Figure 3 C–D). Liquid channels facilitate nutrient transport within the biofilm (Stoodley & Lewandowski 1994). Once mature colonies are established, their metabolic activity can lead to modifications of the surface/solution interface, inducing changes in the type and concentration of ions, pH, oxygen levels, flow velocity and the buffering capacity of the liquid microenvironment or the interface (Videla & Characklis 1992). In this way as hydrodynamic flow decreases, biofilms must establish a way to dispose of this matter. As already mentioned, high shears are involved in mass transfer, which results in a better distribution of nutrients and acts as a disposal medium for cellular detritus.

Moreover, SEM analyses showed differences in the tightness and cell morphology of the species involved. Yeasts cells grown under static condition (Figure 4 A–B) generally consisted of elongated ovoid cells, arranged in a loosely isotropic pattern on the surface, covering the surface abundantly at 3 weeks (Figure 4 A) and reaching a mature biofilm at 11 weeks (Figure 4 B). Although the morphology of the biofilm may have been altered by the dehydration carried out on it, the SEM observations give some useful information that is confirmed by CLSM, which was performed without any drying of the biofilms.

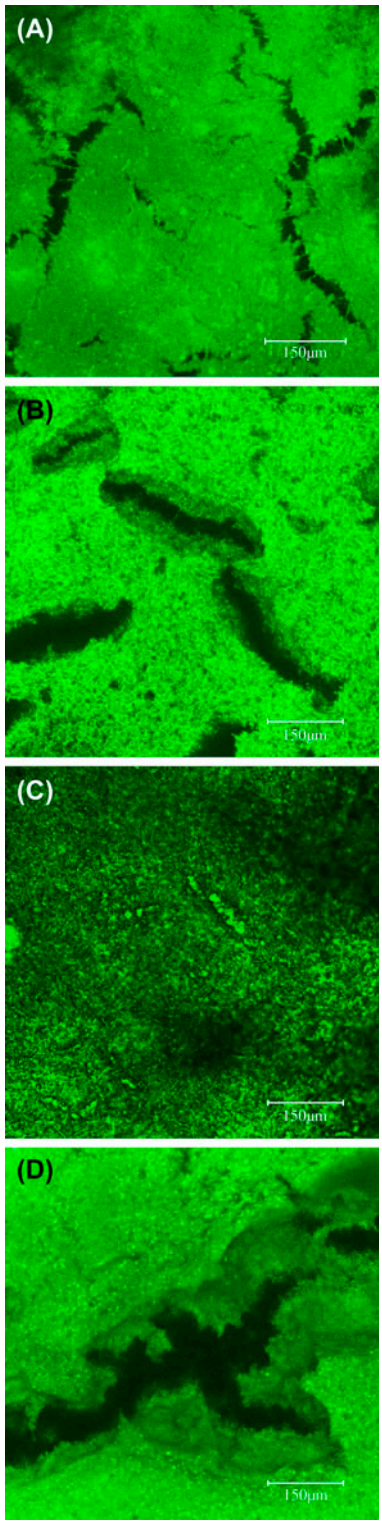

Figure 3. CLSM images (using a 20 × water immersion objective) of mixed yeasts biofilm stained with FDA grown as follows: (A) 3 weeks – static flow; (B) 11 weeks – static flow; (C) 3 weeks – turbulent flow; (D) 11 weeks – turbulent flow.

The biofilm microcolonies grown under turbulent flow (Figure 4 C–F), however, showed a tight appearance with more rounded cells and major development of hyphae structures, while maintaining a porous network structure. The formation of filamentous forms at high shear certainly imply some type of structural alignment (Jahnke et al. 2001).

Dynamic oscillatory rheological measurements

In flowing systems the processes of biofilm deformation and detachment depend on the hydrodynamics (the stresses applied by the moving fluid) and the mechanical properties of the biofilm. Previous studies have shown that biofilms are viscoelastic in nature (Stoodley et al. 2002; Towler et al. 2003; Houari et al. 2008; Pavlovsky et al. 2013).

All the SS surfaces analyzed under differing time and flow conditions showed a complete coverage progression. The viscoelastic properties of the biofilms were determined by small deformation, dynamic oscillatory measurements. The curves of elastic (G') and viscous (G") moduli obtained as a function of frequency (mechanical spectra) are shown in Figure 5. As previously mentioned, each biofilm sample (S-3, S-11, T-3, and T-11) was obtained and measured for two runs (J1 and J2). For the sake of clarity, only the average curve of the two runs was plotted. In all cases, G' was higher than G" at all frequencies studied, confirming that yeast biofilms were viscoelastic materials with a predominant solid-like behavior at these small deformations. Also G' (but not G") exhibited a slight power law increase with angular frequency. This behavior is characteristic of gel-type structures. Similar results were found in previous studies on other biofilm systems (Houari et al. 2008; Pavlovsky et al. 2013). This behavior has been attributed to the EPS matrix surrounding the microcolonies of the biofilm, having the rheological properties of a gel.

In order to compare the samples, elastic modulus *vs* angular frequency, data were fitted with a power law model:

$$G' = G'_0.\omega^n \quad (1)$$

where G'_0 predicts the value of G' at $\omega = 1$ rad s^{-1}, and n indicates how G' changes with ω (slope of the curve). The values of G'_0 and n obtained by fitting experimental data with Equation 1 are listed in Table 2. The values of n ranged from 0.0318 to 0.0571. Statistical analysis indicated that there were no significant differences between the n values of all the samples. Values of G'_0 ranged from 10,172 to 61,974 Pa. For all samples, there was a significant decrease in G'_0 values when the growth medium was changed from J1 to J2. This difference may be

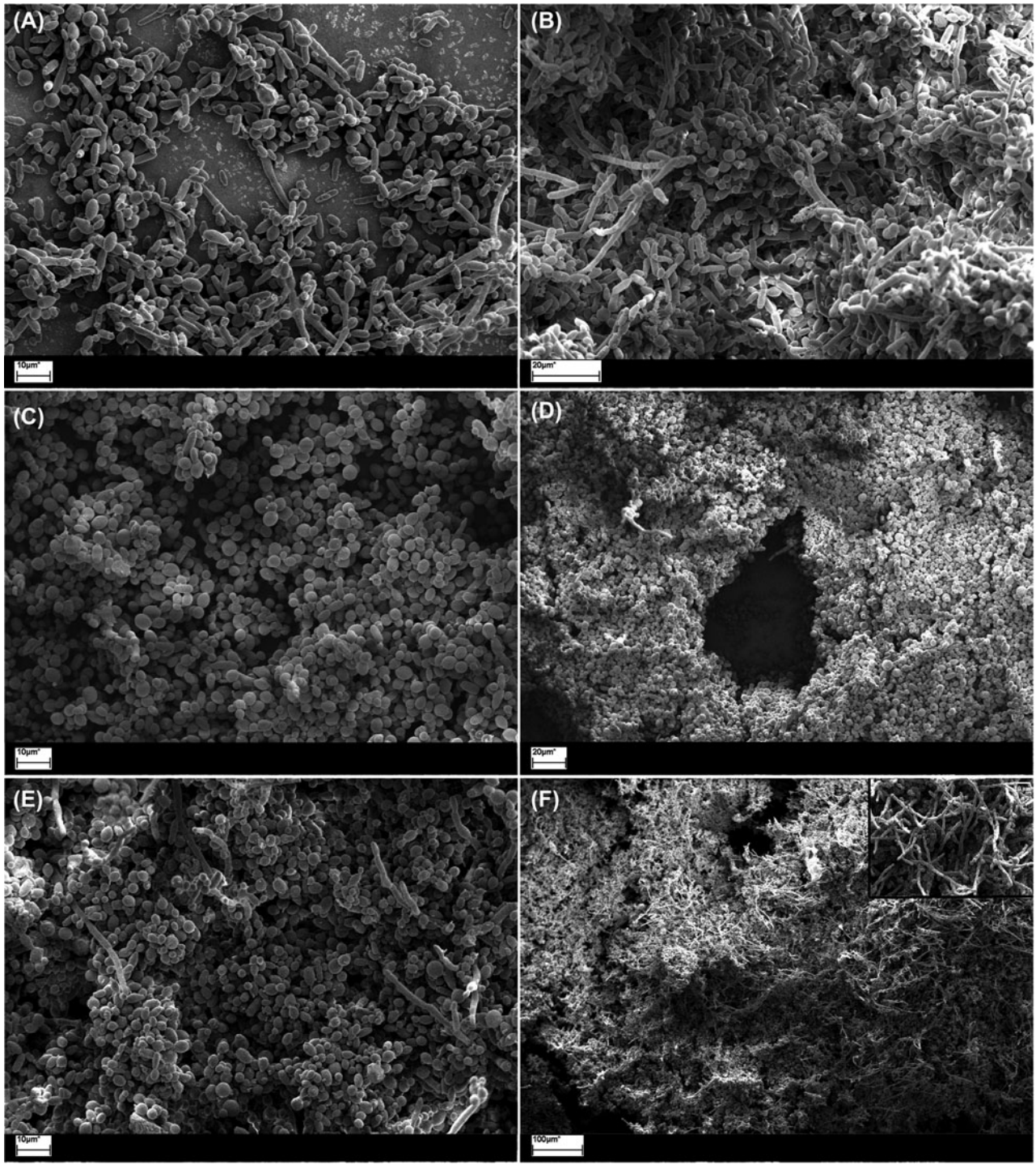

Figure 4. SEM images of mixed yeasts biofilm grown on SS AISI 304. Static growing conditions: (A) 3 weeks; (B) 11 weeks. Turbulent flow: (C–D) 3 weeks; (E–F) 11 weeks. In (F), upper right, the hyphal structures are shown.

attributed to the fact that juices J1 and J2 were prepared from concentrates of different batches, implicating that J1 and J2 probably had different compositions, which may have affected biofilm development. Excluding this effect as a block, the ANOVA results indicated that neither the flow conditions nor the growth time had a significant effect on G'_0 values.

The strain sweep tests confirmed that the frequency sweeps were performed within the linear viscoelastic region. Strain sweep tests also generate a non-linear

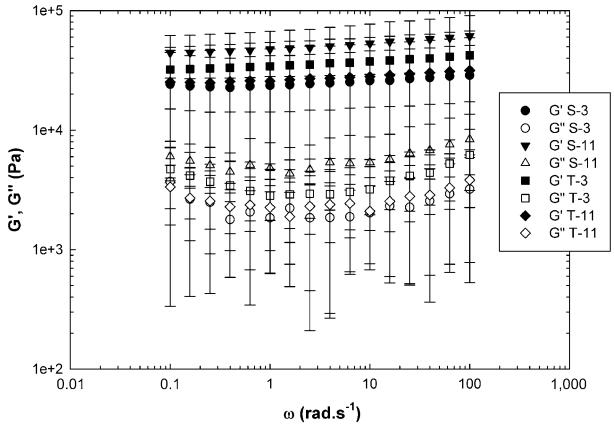

Figure 5. Elastic modulus (G', black symbols) and viscous modulus (G", white symbols) *vs* angular frequency (ω) of biofilms grown under static flow conditions for 3 and 11 weeks (S-3 and S-11, respectively), and under turbulent flow conditions for 3 and 11 weeks (T-3 and T-11, respectively).

experiment known as the LAOS, which can provide additional information on complex fluids such as biopolymers (Hyun et al. 2002). The average curves obtained for the yeast biofilms are shown in Figure 6. All curves followed the same behavior: when the strain amplitude exceeded the linear region, G' decreased. On the other hand, G" first increased (surpassing G'), and then decreased like an overshoot phenomenon. It can also be observed that the G'–G" crossover point was coincident with the G" maximum. This behavior was previously observed in a 4% solution of xanthan gum, where it was classified as 'weak strain overshoot' (Houari et al. 2008). The similarities between the two

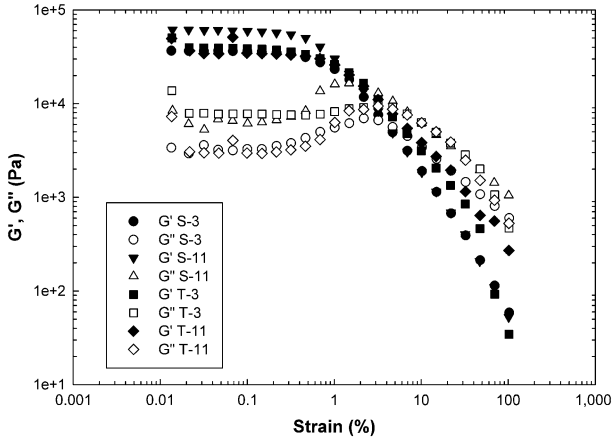

Figure 6. Elastic modulus (G', black symbols) and viscous modulus (G", white symbols) *vs* strain amplitude (γ) of biofilms grown under static flow conditions for 3 and 11 weeks (S-3 and S-11, respectively), and under turbulent flow conditions for 3 and 11 weeks (T-3 and T-11, respectively).

systems may be attributed to the fact that both xanthan gum and the EPS matrix in the biofilms are biopolymers. The critical strain in biofilms lay between 1.5% and 5% (Figure 6), while in xanthan gum solutions it was much higher (Hyun et al. 2002). It was explained that when an external strain is imposed on a weakly structured material, the complex structure resists deformation (G" increases) up to a certain strain. Then, the complex structure is destroyed by large deformation over the critical strain, after which the polymer chains align with the flow field, and G" decreases. As detachment is influenced by flow velocity, a weakly structured biofilm will be more susceptible to detachment as pressure conditions increase, representing an effective but serious dissemination mechanism. Finally, no significant differences were observed between the curves of the different biofilms.

Conclusions

The *in situ* rheology of yeast biofilms is an important step in understanding the communal lifecycles of food industry environments, as it measures how they bind together and dissociate. By attaching a special ring (designed *ad hoc*) to the rheometer, the need to remove the biofilm from the growth surface was eliminated, gaining the ability to perform *in situ* rheology. This allowed the study of small and large amplitude oscillatory shear on biofilms formed by a spoilage yeast isolated from apple juice processing equipment. Using this method, yeast biofilms were determined to be viscoelastic materials with a predominant solid-like behavior, and it was shown that neither the flow conditions nor the growth time had a significant effect on n and G'_0 values, and that at large deformations their weak structure was destroyed above a critical strain.

Regarding the composition of the biofilm, *C. krusei* predominated in both the hydrodynamic conditions tested, whereas *C. tropicalis* adhered strongly to SS only in turbulent flow conditions. In addition, it was observed that flow velocity does affect the thickness of yeast biofilms. Accordingly, the presence of these yeasts in the environment of fruit juice processing plants may lead to accumulation of biofilms on the surfaces and consequently worse cleaning problems.

Knowledge of biofilm structure and rheological properties allows a better understanding of how biofilm development is influenced by the surrounding environment, and leads to a better interpretation of biofilm processes, both of which are important for more effective control strategies.

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