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Role of hydrophobicity in adhesion of wild yeast isolated from the ultrafiltration membranes of an apple juice processing plant

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The role of cell surface hydrophobicity in the adhesion to stainless steel (SS) of 11 wild yeast strains isolated from the ultrafiltration membranes of an apple juice processing plant was investigated. The isolated yeasts belonged to four species: *Candida krusei* (5 isolates), *Candida tropicalis* (2 isolates), *Kluyveromyces marxianus* (3 isolates) and *Rhodotorula mucilaginosa* (1 isolate). Surface hydrophobicity was measured by the microbial adhesion to solvents method. Yeast cells and surfaces were incubated in apple juice and temporal measurements of the numbers of adherent cells were made. Ten isolates showed moderate to high hydrophobicity and 1 strain was hydrophilic. The hydrophobicity expressed by the yeast surfaces correlated positively with the rate of adhesion of each strain. These results indicated that cell surface hydrophobicity governs the initial attachment of the studied yeast strains to SS surfaces common to apple juice processing plants.

Keywords: ultrafiltration membranes; yeast; apple juice; hydrophobicity; adhesion; stainless steel

Introduction

The use of ultrafiltration (UF) in the clarification of apple juice greatly simplifies the process operation and results in an increase in juice yield, improved product quality and avoidance of filtering aids that are costly and present disposal problems (Scott 1995).

A major operational problem in UF processes is caused by fouling, which is the undesirable formation of deposits on membranes, resulting in flux decline and/or an increase in pressure drop during filtration (Flemming 1997, 2002).

Several types of membrane fouling have been introduced including inorganic fouling or scaling, colloidal fouling, organic fouling and biofouling. Of these, the formation of biofilm on the membrane surfaces or membrane biofouling has been regarded as the most serious problem (Baker & Dudley 1998; Nagaoka et al. 1998; Pan et al. 2010). Membrane biofouling is initiated by irreversible adhesion of microorganisms to the membrane surface followed by growth and multiplication of the sessile cells at the expense of liquid food nutrients. Initial microbial cell deposition (called 'primary adhesion') is a critical early stage event in the overall process of biofouling (Characklis 1990). This initial attachment of microorganisms to surfaces is the initial part of adhesion, which makes the molecular or cellular phase of adhesion possible.

Interactions with the substratum involve Lifshitz–van der Waals, electrostatic and Lewis acid–base forces as well as hydrophobic forces (Carré & Mittal 2011). Several authors have demonstrated the importance of the physico-chemical characteristics of the microbial cell surface in the initial steps of adhesion to solid surfaces especially cell surface hydrophobicity and charge (Fletcher & Loeb 1979; Marshall 1991; Smith et al. 1998; Gottenbos et al. 2002; Carré & Mittal 2011).

A number of assays have been proposed to characterize cell surfaces based on the adhesion of cells to hydrocarbons (van der Mei et al. 1995), solvents (Bellon-Fontaine et al. 1996) and surfaces (Rosenberg 1984). It has been argued (van der Mei et al. 1998) that these methods essentially probe interplay of the physico-chemical and structural factors involved in microbial adhesion, rather than 1 single factor, eg the cell surface hydrophobicity.

Once deposited, cells can grow, multiply and produce extracellular polymers (EPS). Given adequate nutrients, time and a suitable temperature, the first attached cells can eventually form a confluent lawn of microorganisms on the membrane surface (Ridgway et al. 1999), which form a matrix that provides structure to the assemblage termed 'biofilm'.

Once formed, a biofilm is difficult to remove. Microorganisms growing as biofilms colonize membrane surfaces, blocking the membrane pores and resulting in

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reduced flux. In extreme cases, the membranes have to be replaced. Eradication of biofilms is more problematic than that of microorganisms in the planktonic mode of growth, since biofilms are more resistant to mechanical removal and cleaning and disinfection treatments (Costerton et al. 1999). Hence, a more promising strategy that has been proposed is to prevent biofilm formation through interference with the earliest steps of formation (Fux et al. 2005). Therefore, efforts to combat membrane biofouling by developing UF membrane materials, process optimization strategies and cleaning regimens must begin with an understanding of the fundamental mechanisms of microbial deposition.

Despite the substantial efforts towards understanding the fundamentals of membrane fouling, knowledge about fouling during fruit juice clarification is limited. Many apple juice processing plants are challenged by low-production yield and frequent membrane cleaning.

A proper understanding of the cause of fouling in UF membranes during fruit juice clarification is lacking, such as where fouling occurs, what type of fouling is dominant, what operating conditions and what types of membranes are more favourable regarding minimizing fouling in fruit juice clarification.

Argentina is the largest apple juice producing country in the Southern Hemisphere, and exports large amounts of concentrated apple juice. The 'Alto Valle de Río Negro y Neuquén' area of Argentina, located across 2 states of the Patagonia Argentina, is a region of apple growth where numerous processing plants are located. The juice processing industries have recently made important capital investments in new machinery and at present there is an increasing effort to improve the juice quality.

Apple juices are acidic beverages (*ca* pH 3–4) with a high sugar content (~12 °Bx). Under these conditions, lactic acid bacteria (LAB), moulds and yeasts comprise the typical microbiota. LAB are the primary spoilage bacteria in fruit beverages. However, their numbers are greatly reduced after pasteurization, concentration and refrigeration. Moulds and yeasts tolerate high-osmotic and low-pH conditions, and grow at refrigeration temperatures; therefore, they can cause spoilage in the processed product (Swanson 1989; Querol & Fleet 2006; Tournas et al. 2006).

The present authors believe that microorganisms colonizing membrane surfaces in apple juice processing plants can potentially contaminate the final product, slow membrane flux and reduce product yields. Determination of the physico-chemical surface properties of microbial strains collected from UF membranes provided from a concentrate apple juice processing plant would therefore provide a better understanding of their relative ability to attach to them.

The specific objectives were: (1) to isolate and identify the microorganisms attached to UF membranes used for clarify apple juice, (2) to study the physico-chemical surface characteristics of these microorganisms and (3) to correlate the cell surface physico-chemical properties and the kinetics of adhesion on stainless steel (SS) in the presence of apple juice.

Materials and methods

Selection of samples

The UF membranes were obtained from JUGOS S.A. Villa Regina City, Rio Negro Province, a large-scale Argentinian apple juice processing industry. Figure 1 shows the flow chart of apple juice production. The tubular UF membrane used throughout the study was a product of aqueous-PCI Membrane Systems, Inc. (USA & UK) (Figure 2). FP100 tubular membrane is made of polyvinylidene–fluoride (PVDF) with the following characteristics: a working pH range of 1.5–12; a maximum pressure of 10 bar; a temperature up to 80 °C; an apparent retention character of 100,000 MW (100 kDa); and hydrophobic and high solvent resistance (PCI-Information).

All membranes had been in routine use in the manufacturing plant's processing cloudy apple juice to

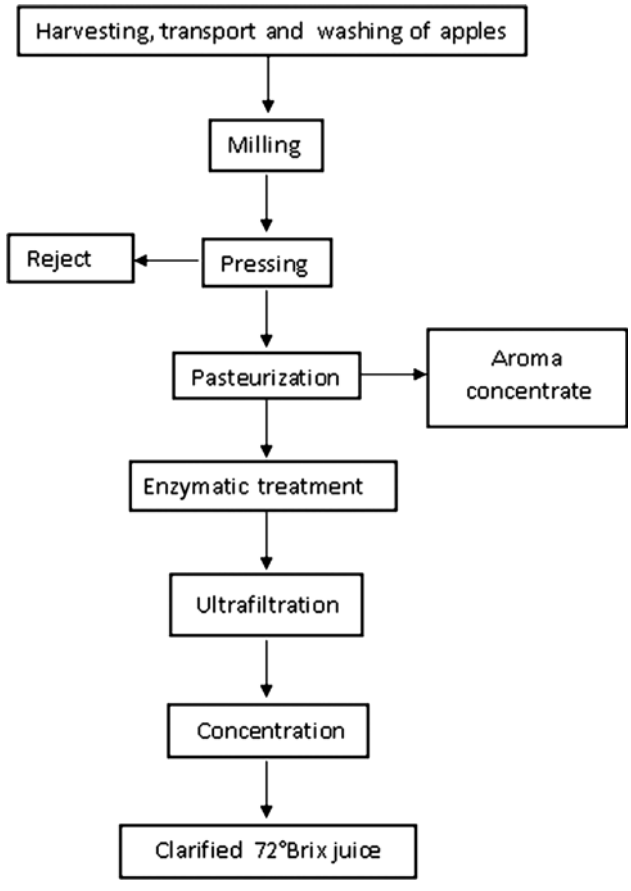

Figure 1. Flow chart of the process for the production of clarified-concentrate apple juice.

Figure 2. Cutaway of a tubular membrane module used for apple juice clarification.

obtain clarified apple juice. All operated at 15–20 °C under turbulent flow (lineal velocity = 2.5 m s^{-1} and flux = 20 m^3 h^{-1}) at pH 3.0–4.5.

Membranes had been cleaned, using the standard caustic based clean-in-place (CIP) system in the plant. CIP cleaning and sanitizing occurs on an intermittent basis (8–48 h) with dilute (1.5–2%) solutions of sodium hydroxide at temperatures ranging from 50 to 60 °C over a wide range of times, a sanitization step with 200 ppm sodium hypochlorite for 15 min and a final rinse in sterile double-distilled water. When the permeate flux is not recovered, enzymatic cleaning is used to hydrolyze the hemicelluloses which could block the pores of the membranes. Then, they were removed, sealed in plastic bags to retain moisture and sent by courier to the authors' research laboratory.

Isolation of microorganisms from membranes

To obtain microbial isolates from the surfaces of the PVDF UF multitube modules, the membrane tubes (1.25 cm diameter, 36 cm length) were cut into small pieces (1 cm length) using sterile scissors. Membrane surface structure and morphology were analysed by scanning electron microscopy (SEM). For SEM, samples were gold coated in a Pelco Model 3 Sputter Coater 91000 metal evaporator (Ted Pella Inc., Tustin, California) (Lozano 1990) and viewed with a Scanning Electronic Microscope (Zeiss Evo 40 VP, Cambridge, UK).

As the microflora on the membranes had survived cleaning, it was assumed that much of the population was firmly attached to the membrane and therefore difficult to remove. To isolate these firmly attached cells, membrane samples were shaken in 0.1% peptone water (Britania, Argentina) in the presence of glass beads (3 mm, Britania) for 15 min. Then, they were diluted in 9 ml of sterile 0.1% peptone water and successive decimal dilutions were prepared.

Enumeration of moulds and yeast was carried out on Yeast–Glucose–Chloramphenicol agar (YGC) (Merck, Germany) (see below) incubated at 25 °C for 5 days. A total heterotrophic bacteria count was carried out in Plate Count Agar (Britania, Argentina) and incubated at 25 °C for 72 h. A LAB count was carried out in MRS Agar (Biokar, France) and incubated at 25 °C for 72 h under reduced oxygen tension. Also, Enterobacteriaceae (EB) were investigated on Violet Red Bile Dextrose Agar (VRB dextrose-agar, Biokar, France) by placing either the permeate side or the retentate side directly onto the VRBA, and incubated aerobically at 35 °C for 24 h. Because no bacteria were isolated from membranes, the studies continued with yeast colonies grown on YGC.

The different yeast colonies were enumerated and identified by their morphological and biochemical characteristics (Kreger van Rij 1984). They were identified according to the following criteria: culture characteristics (colour, shape and texture), asexual structures (shape and size of cells, bipolar, fission, multipolar or unipolar 'budding', absence or presence of arthroconidia, ballistoconidia, blastoconidia, clamp connections, endoconidia, germ tubes, hyphae, pseudohyphae, or sporangia and sporgangiospores), physiological studies (assimilation, fermentation, nitrogen utilization, urea hydrolysis) and the identifications were compared with the results obtained with the Rapid ID Yeast Plus system (Remmel, USA). To confirm the identification results the isolated yeast strains were submitted to a reference laboratory (Instituto Dr Carlos G. Malbrán, Buenos Aires, Argentina). Selected strains were stored at −70 °C in YGC broth supplemented with 20% glycerol for further studies.

Microbial adhesion to solvents assay

Each yeast strain isolated and identified as described above was grown in YGC broth: 0.5% yeast extract (Merck KGaA, Darmstadt, Germany), 2% glucose (Merck KGaA, Darmstadt, Germany) and 0.01% chloramphenicol (Fluka Chemie AG, Buchs, Zwitzerland) at 25 °C for 48 h. After culture, cells were harvested by centrifugation at 2000 × g for 5 min (Labofuge 200, Kendro, Germany), then washed twice and resuspended in 0.023 mol l^{-1} NaCl at pH 4.0. The pH of each suspending liquid was adjusted by adding 0.1 mol l^{-1} of NaOH or HCl. Standardized cell suspensions were

prepared by adjusting the optical density (OD) at 600 nm to 0.8 ($\sim 10^7$ cells ml^{-1}) using a visible spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA, USA).

The microbial adhesion to solvents (MATS) test is based on comparing microbial cell affinity to a polar solvent and microbial cell affinity to a non-polar solvent. The polar solvent can be an electron acceptor or an electron donor, but both solvents must have similar van der Waals surface tension components. The following pairs of solvents, as described by Bellon-Fontaine et al. (1996), were used: chloroform (Dorwil, Industria Argentina), an electron acceptor solvent and hexadecane (Mallinckrodt Baker, Inc., NJ, USA), a non-polar solvent; and ethyl acetate (Dorwil, Industria Argentina), a strong electron donor solvent and octane (Carlo Erba, Divisione Chimica Industriale, Milano, Italy), a non-polar solvent. Due to the surface tension properties of these solvents, differences between the results obtained with chloroform and hexadecane and the results obtained with ethyl acetate and octane indicated that there were electron donor–electron acceptor interactions at the microbial cell surface and revealed hydrophobic and hydrophilic properties.

In MATS test, the loss in absorbance of the aqueous phase relative to the initial absorbance value is taken to represent the numbers of cells adhering to each solvent and this is a reflection of the affinity of the cell surface.

The percentage of cells present in each solvent was subsequently calculated by using the equation: % Affinity $= 100 \times [1 - (A/A_0)]$, where A_0 is the OD_{600} of the cell suspension before mixing and A is the absorbance after mixing. Each test was performed in triplicate and the results expressed as means and standard deviations (SD).

Adhesion tests

To determine the adhesion capacity of the yeast strains to SS in the presence of apple juice, the yeasts were cultured in YGC broth at 25 °C for 48 h. Then, the cells were harvested by centrifugation at $2000 \times g$ for 5 min and subsequently washed and resuspended in sterile 12 °Bx clarified apple juice (mean composition: fructose: 70 g l^{-1}, glucose: 35 g l^{-1}, sucrose: 16 g l^{-1}, malic acid: 0.4–3.4 g l^{-1}, citric acid: <1 g l^{-1}, ascorbic acid: <40 mg l^{-1}, potassium: 1 g l^{-1}, calcium: 0.05–0.4 g l^{-1}, phosphorus 70–100 mg l^{-1}, sodium: 20 mg l^{-1}, free aminoacids: 1–5 g l^{-1} and pH: 3.2 (0.2, ionic strength 0.023 mol l^{-1}) (Lozano 2006). The OD_{600} was adjusted to 0.8 ($\sim 10^7$ cells ml^{-1}). The clarified apple juice was prepared from 72 °Bx concentrated juice and was sterilized by microfiltration (pore size 0.45 μm) (Zierdt 1979) (Metricel®Grid, GelmanSciences, MI, USA).

The surface used for adhesion experiments was AISI 304 L SS (mean roughness: 0.064), cut into rectangular chips (15 × 25 mm). Before the experiments, the chips were soaked for 15 min in 2% of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50 °C and rinsed 5 times for 5 min each with hot tap water followed by five rinses with distilled water. Finally, the chips were autoclaved for 15 min at 120 °C. The experiments were carried out in sterile glass Petri dishes divided into 6 sections by glass pieces (Brugnoni et al. 2007). The divisions were made by fusing the Petri dish base and the glass division to avoid overlapping of the chips during the experiment and cross contamination. A chip was put into each Petri dish section and 2 sterile dishes were used for each study strain. In one Petri dish, 6 coupons were examined and in other Petri dish, the remaining 2.

Six milliliters of the yeast suspension in apple juice were poured into each Petri dish. This volume was enough to cover the chips. The plates were incubated at 20 ± 1 °C with slow stirring (50 rpm). After incubation for 5, 10, 15, 20, 30, 60, 90 and 120 min, 1 Petri dish was taken out of the experiment. Each chip was washed twice for 1 min by immersion in sterile distilled water with agitation at 50 rpm to remove the poorly adherent cells. To determine the number of adhering yeasts on the SS surfaces, 3 chips from each Petri dish were stained with fluorescein diacetate (FDA). The principle behind a test using FDA is 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%) FDA, ($C_{24}H_{16}0_7$, Sigma–Aldrich Chemical Co., St Louis, MO, USA) was prepared in acetone (Dorwil, Industria Argentina) and stored to −18 °C. To quantify yeast cells on SS, the coupons 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 shaking for 90 min at 25 ± 1 °C in darkness, the coupons were rinsed twice with sterile distilled water. Chips were then allowed to air-dry and observed with an epifluorescence microscope (Olympus BX 51, NY, USA) using a 100× oil-immersion objective, and blue excitation U-MWB2. Twenty fields were examined per chip.

The other 3 chips were fixed with glutaraldehyde (2.5%) in phosphate buffer (0.1 mol l^{-1}, pH 7.2); washed 3 times with the same buffer, and dehydrated in increasing concentrations of acetone (25–100%). Samples were vacuum dried at 40 °C, gold coated in a Pelco Model 3 Sputter Coater 91000 metal evaporator (Ted Pella Inc., Tustin, California) (Lozano 1990) and viewed with a scanning electronic microscope (JEOL Model 35CF, Tokyo, Japan).

Adhesion tests were carried out on SS because it is the most widely used material in industry. Faille et al.

(2001) found contact angles for SS of 75°, confirming its hydrophobic characteristics. It is believed that the results of adhesion to this material would be comparable to those that could be obtained on PVDF membranes, which are also hydrophobic in nature.

Statistical analysis

In all analyses, triplicate tests were done under identical conditions and the results expressed as means and SD. When appropriate, the Student's *t*-test was used for comparison of means. Confidence levels equal to or higher than 95% ($p < 0.05$) were considered statistically significant. Cell surface hydrophobicity was correlated with the attachment of the yeast strains to SS in the presence of apple juice using a logarithmic regression model.

Results and discussion

One of the most important processes used in beverage clarification is cross-flow UF. However, the process is limited by permeate flux decline due to severe membrane fouling. To avoid membrane fouling, it is extremely important to identify the foulants and the mechanisms that govern the process. This work focused only on the microbiota present in UF membranes used in apple juice clarification, assuming that the presence of microorganisms on cleaned membrane surfaces is a good indication of their adhesion ability.

A limited group of yeasts, moulds and other microorganisms are able to survive at the low pH range of fruit juices. The acidic condition of juices might be favourable for the growing of these microorganisms by limiting competition from other groups. In addition, apple juice has been traditionally pasteurized by thermal means using continuous pasteurization, which may be carried out by passage through plate heat exchangers, and by tunnel pasteurizers. Currently, high-temperature short-time (HTST) pasteurization is the mode commonly used for heat treatment of apple juice previous to the UF process. In HTST pasteurization, the temperature used is 76.6–87.7 °C for a holding time between 25 and 30 s (Moyer & Aitken 1980). This treatment usually inactivates a large proportion of the microorganisms responsible for spoilage during refrigerated storage, along with the pathogenic microorganisms (Qin et al. 1995).

In the experimental conditions used in this study, bacteria could not be isolated from membrane samples. These results do not necessarily imply the absence of LAB and EB in membrane samples. These microbial groups typically comprise the natural microbial load of apple juice; however, their numbers can be greatly reduced after pasteurization, concentration and refrigeration (Arias et al. 2002; Suárez-Jacobo et al. 2010). In a previous study (Brugnoni et al. 2007), counts of 10^7 colony forming units (CFU) ml^{-1} of yeast were reported in apple juice samples collected from the post-UF process from a concentrate apple juice processing plant. The authors believe that the predominance of yeast in apple juice processing equipment is due to their resilience in this environment and their potential to form biofilms.

As explained previously, only yeasts were isolated from UF membranes. The mean yeast total counts obtained from membrane samples were in the range 7×10^5–1×10^6 CFU g^{-1}. Eleven isolates of yeast were obtained from membrane samples. Five isolates corresponded to *Candida krusei* (L2, L6, L8, L9 and L10), 3 isolates belonging to *Kluyveromyces marxianus* (L3, L7 and L11), 2 isolates to *Candida tropicalis* (L4 and L5) and 1 isolate to *Rhodotorula mucilaginosa* (L1).

These results show that yeasts are the microorganisms which predominate in membrane samples and are the principal cause of microbial fouling in UF membranes during fruit juice clarification. The diversity and high numbers of yeasts found in this study indicates that the most likely source of contamination may be a general plant hygiene problem. These results must be taken into account in developing effective control strategies in the apple juice industry.

This is, as far as the authors know, the first report of yeast strains being isolated from the UF membranes used for clarify apple juice. Tang et al. (2009) reported 1 yeast strain (*Blastoschizomyces capitatus*) isolate from a dairy reverse osmosis membrane plant. In food processing lines, yeasts belonging to *Saccharomyces*, *Candida,* and *Rhodotorula* have been isolated from biofilms on conveyor tracks, and can and bottle warmers in packaging departments of a beverage industry (Salo & Wirtanen 2005). Yeasts are also found in domestic environments such as kitchen sponges, dish towels (Rayner et al. 2004) or household washing machines (Gattlen et al. 2010). It is evident that there is a lack of information on yeasts that forms biofilms on food processing equipment and home appliances.

Moreover, large amounts of solid material were visible by SEM (Figure 3a–c). This clearly shows enclosure of the membrane pores. After filtration of apple juice, some of the materials in the feed are adsorbed on the membrane surface. Membrane fouling may be caused by pectin, tannins, proteins, starch, hemicelluloses and cellulose (Santón et al. 2008). The apple juice clarification process is mainly limited by the accumulation of matter on the filter, including concentration polarization and membrane fouling (formation of a gel layer or a deposit) (Carvalho & Bento da Silva 2010).

In this trial, it was presumed that the presence of yeasts on cleaned membrane surfaces is a good indication of their adhesion ability and survival of process conditions.

According to van Oss (2003), in biological systems, hydrophobic attraction is the strongest of all long-range

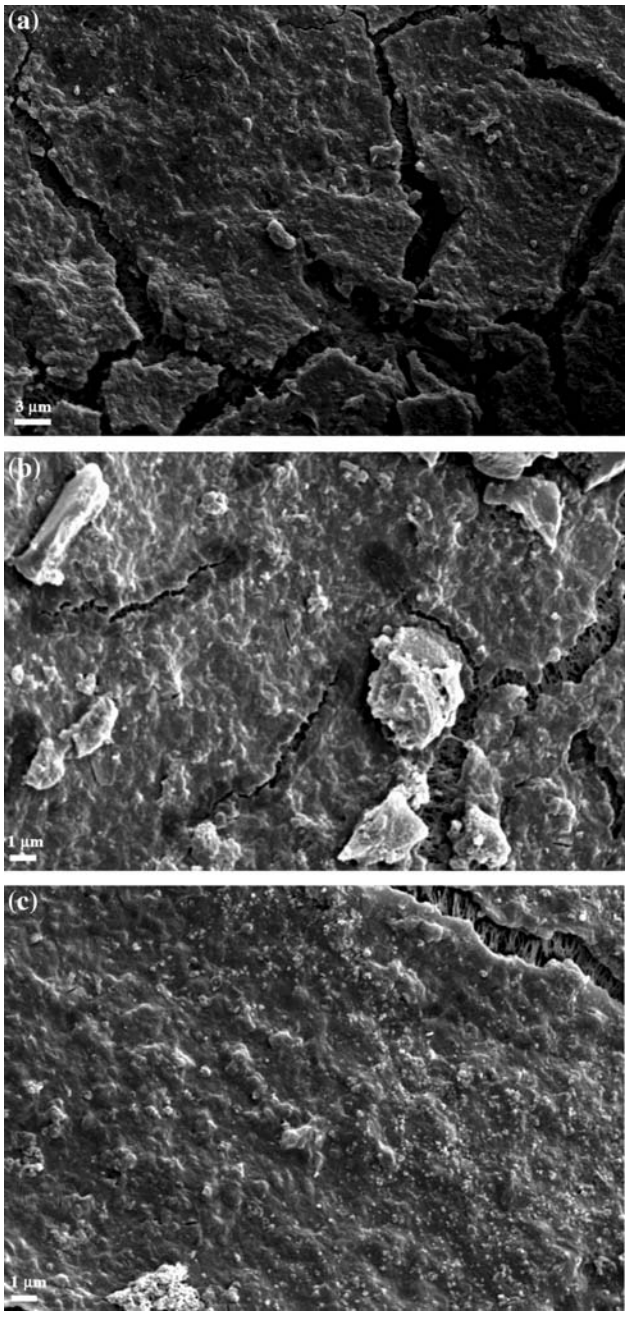

Figure 3. SEM images of the fouling layer.

non-covalent, non-electrostatic binding forces occurring between particles immersed in water. This force is based on electron donor–electron acceptor (Lewis acid–base) interactions in polar media. In aqueous media, Lewis acid–base interactions can be attractive (hydrophobic attraction) or repulsive (hydrophilic repulsion), depending upon the degree of surface hydrophobicity or hydrophilicity of the entities involved. Quantitatively, the strongest Lewis acid–base interaction in water is the hydrophobic effect, which is always attractive.

Figure 4 shows the cell surface affinity percentages for polar and non-polar solvents measured by the MATS method. Due to their surface tension properties, differences in the results between chloroform/hexadecane and ethyl acetate/octane enabled the authors to indicate the presence of electron donor–acceptor interactions at the yeast cell surface as well as hydrophobic/hydrophilic properties.

After growth at 25 °C, the highest affinity for chloroform (the acidic solvent) was always significantly higher ($p < 0.05$) than to hexadecane, except for *C. krusei* strains L6 and L8 and *C. tropicalis* strain L5. These values of affinity were compared because both solvents possess the same van der Waals properties (Bellon-Fontaine et al. 1996). The important difference observed was due to the implication of Lewis acid–base interactions resulting from the electron donor and basic character of yeast strains. The quantitatively important existence of chemical groups such as $–COO^-$ and $–HSO_3$ at the surface of microorganisms could explain their strong electron donor character (Pelletier et al. 1997). These data therefore demonstrate the capacity of these 8 yeasts to establish some interactions with a support other than those of van der Waals, for example. Also, the 11 yeast strains tended to be electron donors (ie basic) rather than electron acceptors (ie acidic).

Based on the values of percentage adhesion of the yeast strains to *n*-hexadecane obtained at pH and ionic strength that simulate 12 °Bx apple juice (Figure 4), and in accord with the classification suggested by Li and McLandsborough (1999), 5 strains (*R. mucilaginosa* L1,

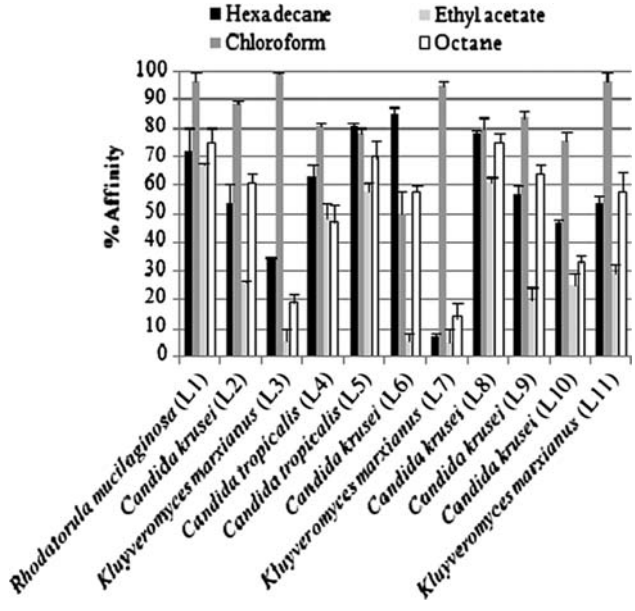

Figure 4. Affinities of yeast strain cells for the four solvents used in the MATS analysis after growth in YGC broth medium at 25 °C and resuspension in 0.023 mol l^{-1} NaCl at pH 4.0. Affinity values are means ± SDs ($n = 3$).

C. tropicalis L4 and L5 and *C. krusei* L6 and L8) were strongly hydrophobic (>55%); 5 strains (*C. krusei* L2, L9 and L10 and *K. marxianus* L3 and L11) were moderately hydrophobic and 1 strain (*K. marxianus* L7) was hydrophilic (<10%). The origin of hydrophobicity of the cell surface is 'hydrophobic' cellular materials such as glycans and mannoproteins, and the hydrophilic group may originate from amino acids and phosphates (Lipke & Ovalle 1998).

Except for strain L6, which showed a moderate basic character and strong hydrophobicity, and strain L7, which was strongly hydrophilic, the remaining yeast strains expressed a strongly basic character and a moderate or high affinity for hexadecane. From these results, it can be concluded that, in experimental conditions which reproduce the pH and ionic strength of apple juice, these strains not only expose chemical groups of basic character (hydrophilic groups) but hydrophobic groups. In consequence, they could adhere, in theory, to both, hydrophilic and hydrophobic surfaces.

Cell surface charges and substratum surface charges affect hydrophobic interactions, which are favoured by low electrostatic repulsion (van der Mei et al. 1995). Therefore, it is important to consider that SS presents its isoelectric point around pH 4.0 (Boulangé-Petermann et al. 1995). It would imply that this substratum has a low surface charge in 12 °Bx apple juice possibly minimizing electrostatic repulsion with the cell surface, and therefore, favouring cell attachment.

Generally, it has been believed that a hydrophobic membrane exhibits higher biofouling potentials than hydrophilic membranes (Leslie et al. 1993; Knoell et al. 1999; Pasmore et al. 2001). Other studies also confirmed that the increase in the hydrophobicity both of cells and membranes results in the increase in adhesion rate due to higher interaction energies between cells and membranes (van Oss 1995; Ghayeni et al. 1998; Knoell et al. 1999; Ong et al. 1999; Pasmore et al. 2001). However, it can be the very opposite when particles or cells have a hydrophobic surface. Brant and Childress (2002) showed that hydrophobic polystyrene colloids adhered more weakly to three hydrophilic membranes compared to hydrophilic silica colloids.

In this study, MATS analysis confirmed that the majority of cell surfaces were mainly hydrophobic, and cells were expected to adhere more easily onto hydrophobic membranes than hydrophilic membranes by the hydrophobic–hydrophobic interactions between cells and membrane surfaces. But the interaction between hydrophilic cell surface groups with hydrophilic membrane also should be taken into account because cell surfaces had an electron-donating nature proven by MATS.

In order to get more insights into the adhesion process of yeasts the adhesion ability of the strains isolated from UF membranes to SS, the most common surface used in food processing plants, has been evaluated. Figure 5a–d shows the number of attached yeast cells on AISI 304L SS surfaces in the presence of 12 °Bx clarified apple juice.

Only the first 30 min of *C. tropicalis* cell adhesion (Figure 5c) are reported. As more mature *C. tropicalis* biofilms consisted of a dense network of yeast cells and filamentous forms (Figure 6), these are not easily counted by direct method.

The adhesion rate (cells cm^{-2} min^{-1}) was determined by linear regression in the first 30 min of cell attachment and R^2 values > 0.95 were found. The percentage of hydrophobicity expressed by the yeast surfaces at pH 4.0, correlated positively with the rate of adhesion (Figure 7) of each strain on SS in the presence of 12 °Bx apple juice, especially in the first 30 min of contact until saturation was reached ($R^2 = 0.91$). In this study, considering that within the same species there may be different strains, the authors analysed separately the 11 isolates because different strains could have different behaviour. It was evident that all yeast strains adhered to SS, although differences were observed according to species and strains. Strain variation was particularly evident for *C. krusei* (Figure 5a). *C. krusei* L6 and L8 adhered at equivalent levels to SS at 5 and 10 min of contact time ($p > 0.05$). This was significantly higher ($p < 0.05$) than for *C. krusei* L2, L9 and L10. This could be associated with the highest hydrophobicity of *C. krusei* L6 and L8 (strongly hydrophobic) compared with the lowest (and similar) hydrophobicity of *C. krusei* L2, L9 and L10 (moderately hydrophobic) (Figure 4).

C. tropicalis strains had a similar adhesion ability with no significant differences ($p > 0.05$) except for the first adhesion time (5 min) (Figure 5c). This significant difference ($p < 0.05$) could be associated with the lowest cell surface hydrophobicity expressed for L4. *K. marxianus* (L7) exhibited the lowest capacity of adhesion to SS along with the lowest percentage of hydrophobicity at pH 4.0. The combination of these 2 antagonistic parameters, a hydrophilic cell envelope and a hydrophobic surface, leads to a significant decrease ($p < 0.05$) in the adhesion capacity of this strain in the first 30 min of cell attachment compared to *K. marxianus* strains L3 and L11. These results indicate that the hydrophobicity of the cell surface would govern the initial attachment of the studied yeast strains to SS.

The present results clearly show differences in the adhesion ability among the different isolates. Different intra-species adherence ability was also reported by other authors for *Candida* species (Panagoda et al. 2001; Henriques et al. 2007; Okawa et al. 2008) and *Yarrowia lipolytica* strains (Amaral et al. 2006). The differences observed between the behaviour of the yeast strains may reflect the fact that the early events in yeast attachment involve differences in the composition of the cell wall

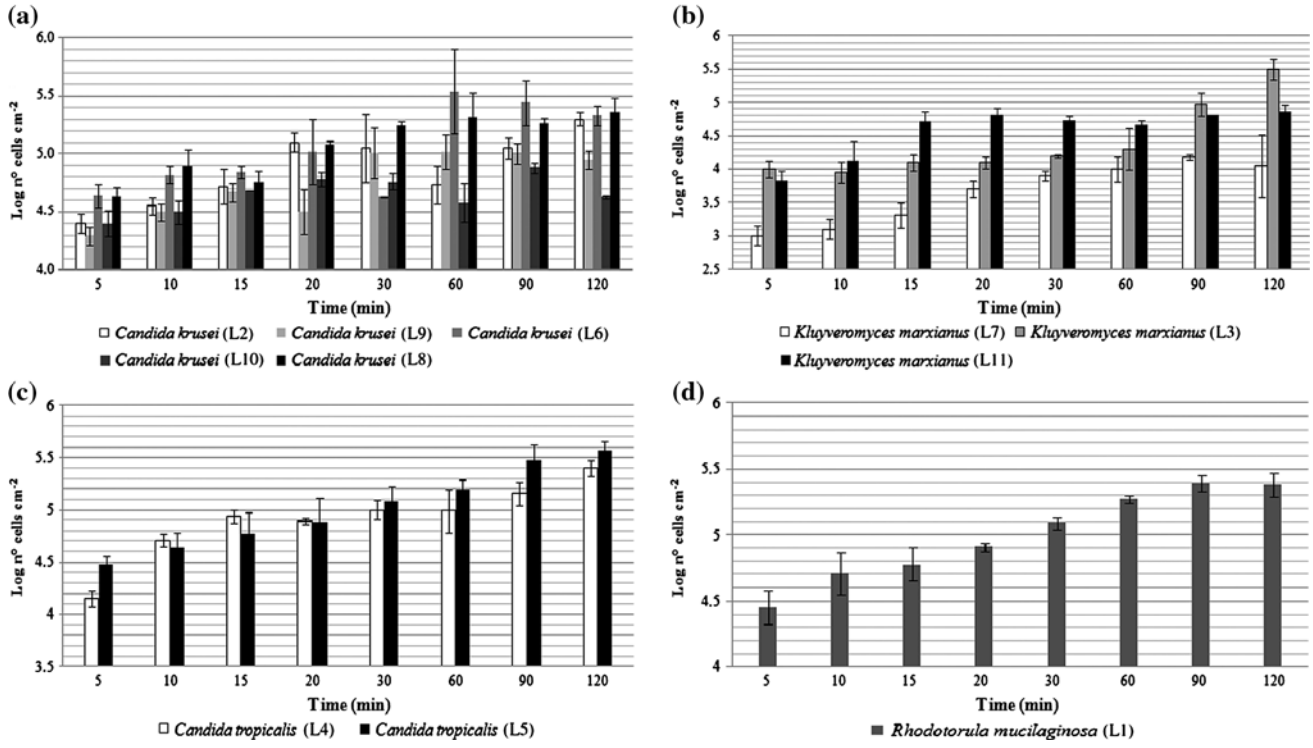

Figure 5. Number of yeast cells per cm^2 (Log nº of cells cm^{-2}) (means ± SD) adhered to SS measured by FDA staining: (a) *C. krusei* strains L2, L6, L8, L9 and L10; (b) *K. marxianus* strains L3, L7 and L11; (c) *C. tropicalis* strains L4 and L5; (d) *R. mucilaginosa* strain L1. Results expressed as means and SDs, which were from triplicates.

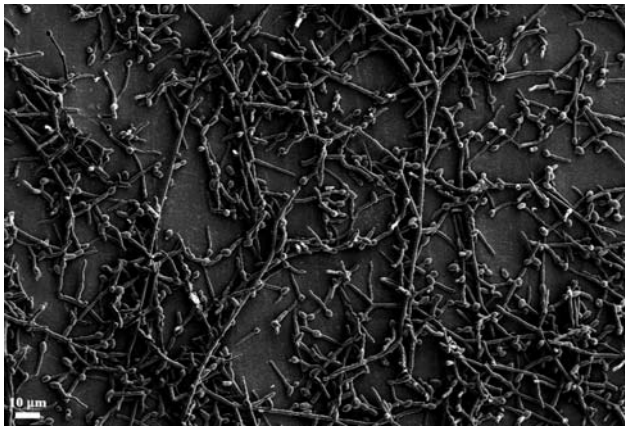

Figure 6. SEM observations of *Candida tropicalis* adhesion on SS incubated in 12 °Bx apple juice for 2 h.

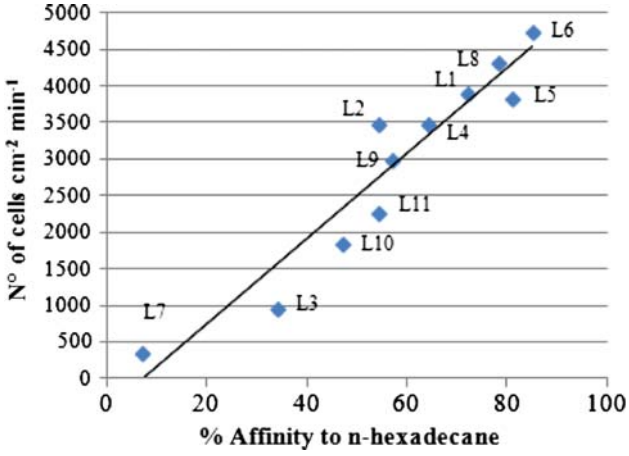

Figure 7. Adhesion rates (cells cm^{-2} min^{-1}) of the different strains of yeast cells as a function of affinity to *n*-hexadecane determined by linear regression in the first 30 min of cell attachment.

(Mercier-Bonin et al. 2004; Aguedo et al. 2005; Amaral et al. 2006).

The study of the relationship between cell surface properties, adhesion and biofilm formation has been extensively studied. Evidence that hydrophobicity is a strong predictor of cell attachment to surfaces varies from group to group, with van Lossdrecht et al. (1987), Gilbert et al. (1991), Peng et al. (2001), Iwabuchi et al. (2003) and Liu et al. (2004) suggesting a strong correlation between hydrophobicity and cell attachment to surfaces. Van Lossdrecht et al. (1987) went so far as to suggest that surface hydrophobicity is the key factor in determining bacterial attachment to solid surfaces and that surface charge can only become important when surface hydrophobicity is minimal. However, it must be noted that van Lossdrecht et al. (1987) used polystyrene

discs, which are very hydrophobic, to measure cell adhesion, thus possibly favouring hydrophobic interactions. On the other hand, Sorongon et al. (1991), Parment et al. (1992), Flint et al. (1997) and Parker et al. (2001) concluded that hydrophobicity had little to no relationship in determining bacterial cell attachment. The present results suggest that the hydrophobicity expressed by the surface of the yeast cells isolated in this study and determined at the pH corresponding to the food matrix, could be used as an index of the initial attachment ability of these yeasts to SS accessories in an apple juice processing plant. However, general predictions for the degree of biofilm formation on a particular material cannot be made because biofilm formation depends on the microorganisms, environmental factors and surface properties.

Several authors have postulated that in *Candida* spp. cell-surface hydrophobicity plays a critical role in the initial events leading to adhesion to biotic and abiotic surfaces. Samaranayake and Samanarayake (1994) concluded that cell surface hydrophobicity together with adherence may have clinical implications in fungal infections related to plastic devices such as implants and catheters (polymeric, hydrophobic inert surfaces). Miyake et al. (1986) reported a significant positive correlation between the adherence of *Candida* spp. to acrylic surfaces and their cell surface hydrophobicity. Hawser and Douglas (1994) observed that of all *Candida* spp. examined in their study, *C. krusei* produced the most extensive biofilm on the surfaces of poly(vinyl chloride) (PVC) disks irrespective of the growth medium, which either suppressed or promoted extracellular polysaccharide formation. One reason for this may be the dual attributes of very high cell surface hydrophobicity and adherence of *C. krusei* to inert plastic surfaces compared with other species which may have facilitated biofilm development.

More recently, Borghi (2011), working with 37 clinical isolates of *C. tropicalis,* found a positive correlation between biofilm formation and cell surface hydrophobicity, and suggested that hydrophobicity plays a major role in biofilm formation in *C. tropicalis*. In a previous study, Brugnoni et al. (2007) evaluated the cell surface hydrophobicity expressed by 4 yeasts isolated from the SS surfaces of an apple juice processing plant and this parameter correlated positively with the rate of adhesion (number of cells min^{-1}) of each strain. On the other hand, to develop repeatable biofilm formation on polypropylene coupons, Gattlen et al. (2010) chose as a model organism a strain of *R. mucilaginosa* isolated from a household washing machine. They concluded that the modification of polypropylene to reduce its hydrophobicity did not enhance cell attachment compared with non-modified coupons. In accordance with Gattlen et al. (2010), Brugnoni (2008) reported that 1 strain of *R. mucilaginosa* isolated from fresh apple juice expressed a strongly basic character and a moderate affinity for hexadecane. In consequence, the author concluded that this strain could adhere, in theory, to both, hydrophilic and hydrophobic surfaces.

Membrane biofouling is a very complicated process that is affected by many factors, including some characteristics of the microorganism itself and the membrane surface. The surface topology of UF membranes plays an important role in determining their fouling propensity (Park et al. 2005). As mentioned by Carman et al. (2006), bioadhesion could also be influenced by the nanotopography of the material in relation to the formation of focal contacts. They chose as model system the motile zoospores of the marine alga *Ulva* (syn. *Enteromorpha*) and concluded that topography roughly equivalent to the diameter of the pear-shaped swimming spore at its widest point and the diameter of the settled spore was the most favourable for settlement.

Wong et al. (2009) characterized PVDF-FP100 membranes and they described hills measuring about 0.2 μm in lateral extent and large circular depressions (1.5–2.5 μm). This topography could favour the settlement of yeast cells due to their different sizes at the different growth stages (mother cells, daughter cells and pseudohyphae). Also, even though PVDF-FP100 membranes have a pore size distribution between 0.15 and 0.22 μm (Wang et al. 2008), pressure driven forces could enlarge the pores, with time, and permit the passage of yeast cells.

Once yeast attachment has occurred, the formation of a biofilm begins. Surface adhesion, defined as the binding of planktonic microorganisms to a surface, is the first step for biofilm formation (Gristina 1987; Donlan 2001). The next step is surface colonization, defined as the spread of adherent microorganisms across a surface through division (Anderson et al. 2007). Yeast biofilms are generally characterized in the following stepwise developmental process that proceeds through 3 stages: (1) an 'early' phase characterized by adhesion of blastospores (yeast cells) to the surface, (2) an 'intermediate' phase where the yeast cells have proliferated to cover a large surface area and have begun to produce EPS and (3) a 'maturation' phase.

C. tropicalis biofilms formed over 2 h consisted of a dense network of pseudohyphae, some yeast cells and compact cell clusters covering the entire length of the SS surface (Figure 6). Silva et al. (2009) showed this biofilm structure on polystyrene inoculated for 48 h with a clinical isolate of *C. tropicalis* in Sabouraud dextrose broth, and they considered it a 'mature' biofilm. These results have obvious implications of for the apple juice processing industry. The organisms used in the experiments were isolated directly from UF membranes from a concentrate apple juice processing plant, adhesion tests

were carried out on SS and cell suspensions were prepared in apple juice. To the authors' knowledge, this is the first report of biofilm formation on surfaces commonly used in food industry by wild strains of *C. tropicalis* isolated from food process equipment.

Interestingly, these strains do not have the highest index of hydrophobicity compared to, for example, *C. krusei* L6, confirming that biofilm formation is a multifactorial process. It is noteworthy that the presence of similar initial adherent populations does not imply the same kinetics of colonization with time, which suggests that the adhesion and biofilm formation phases could require different molecular adaptations (Chavant et al. 2002). This result is consistent with other studies (Bizerra et al. 2008; Silva et al. 2009), reporting differences between adhesion and biofilm formation abilities on polysterene and PVC surfaces under static conditions.

It has been suggested (Baillie & Douglas 1999; Ramage et al. 2002; Paramonova et al. 2009) that the presence of pseudohyphae may have importance in the structural integrity of multilayered biofilms. 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 increased compressive strength of the biofilm.

The typical hydrodynamic prevailing condition in the UF process is turbulent flow. In order to adhere to surfaces and subsequently to form biofilms, yeast cells submerged in high-velocity flowing systems must overcome shear stress at the fluid–surface interface. It may be assumed that those cells that are able to form a dense network of pseudohyphae on inert surfaces (eg SS and plastics) more easily adhere and form 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. The recalcitrance of these biofilms to typical cleaning and disinfection processes may confer a selective advantage for these species when making biofilms on surfaces with high hydraulic flow and cleaning and disinfection conditions, such as, for example, in juice processing plants.

The predominance of highly hydrophobic cells from apple juice processing plants could suggest that either these cells are most likely to form biofilms or that the conditions in an apple juice processing plant select for the most hydrophobic strains. It is possible that the cleaning systems used in these plants are more likely to remove the more hydrophilic cells, leaving the more hydrophobic cells to form biofilms and contaminate the product stream.

Conclusions

A better understanding of the factors involved in the adhesion process will help in designing methods to control biofilms through the prevention of adhesion or by enhancing the removal of attached bacteria. Studies on the initial adhesion of microbial cells to surfaces are important in any programme aimed at eliminating biofilms.

The results of this study suggest that the high affinity for SS demonstrated by all isolates could act, amongst others, as survival and selection factors that would explain the predominance of these yeasts in the analysed UF membranes. These results have obvious implications for the apple juice processing industry.

Further studies will investigate biofilm formation on membranes in a flowing system and the factors affecting removal of attached cells and mature biofilm.

This study contributes to a thin body of knowledge of fungal biofilm formation and helps to gain knowledge of which parameters are important for fungal adhesion in concentrated apple juice processing equipment.

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