Biofilm Formation on Ultrafiltration Membranes of Yeast Strains Isolated from Apple Juice Processing Industries

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Abstract. The aim of the present research was to study monospecies adhesion and biofilm formation on ultrafiltration membrane of four yeast strains isolated from an apple juice industry (*Candida tropicalis, C. krusei, C. kefyr* and *Rodhotorula mucilaginosa*). Cell adhesions of each strain were estimated by plate count at different contact times, 2, 6, 24, 48, 72 h and one week in apple juice as food matrix and incubated at 25 ºC. All assays were carried out by triplicate. The highest counts recorded after one week were for *C. tropicalis* and *C. krusei*, reaching 9.02 ± 0.30 and 9.30 ± 0.29 log colony-forming units (CFU) cm^{-2}, respectively, following *C. kefyr* with 8.63 log CFU cm^{-2} and *R. mucilaginosa* with the lowest adhesion, 7.68 log CFU cm^{-2}. The present study supports the importance to examine the interactions of members of resident microbiota on surfaces commonly used in juice industries for clarification processes and should alert about adopting and improving the best cleaning and disinfecting practices as they can create a persistent source of contamination that generates a serious hygienic-sanitary problem.

Keywords: Biofilms, Yeast, *Candida* spp., Ultrafiltration membranes, Apple juice.

1 Introduction

Microorganisms can grow and settle on different abiotic and biotic surfaces on which they firmly adhere and produce an extracellular polymeric matrix (EPS) in which they are embedded in forming biofilms. In food industry, they contaminate different stages in production lines, interfering with different processes and damaging equipment [1-2]. Membrane technology is applied in many branches of production, including the fruit juice industry. A high permeate flux is necessary for filtration to be practical and economic, and product quality should at least meet those obtained by other clarifica-

tion processes. Ultrafiltration (UF) is the most used process for clarification of juices and can be characterized according to the membranes material. They have a pore size of 0.001–0.05 μm and molecular weight cut-off (MWCO) between 1 and 300 kDa and are constantly in contact with liquid food [3]. One of the major obstacles that limit the potential of this technology is membrane fouling, which significantly reduces the filtration flux, shortens the lifespan of the membrane, and increases energy consumption and overall costs with the consequent losses of yield and product. Therefore is essential to evaluate conditions and to establish appropriate strategies for the reduction and elimination of biofilm development on membranes. The predominantly spoilage microorganisms isolated from acid food products like juices are yeasts; they are usually contaminants that affect the quality and shelf life, and their development into biofilms on food processing equipment can cause adverse effects to the products being processed [4]. Taking into account that fruit juice producing plants are compromised by the colonization of yeasts, the present study describes the dynamics of adhesion and biofilm formation of *C. krusei*, *C. tropicalis*, *C. kefyr* and *R. mucilaginosa* on UF membranes under conditions of industrial relevance.

2 Materials and methods

2.1 Culture conditions.

C. tropicalis, C. krusei, C. kefyr and *R. mucilaginosa* were previously isolated from the surfaces of polyvinylidene fluoride (PVDF) UF membranes, of an apple juice processing industry located in Alto Valle de Río Negro, Argentina [5]. Stock cultures of yeasts were stored at -70°C until use in yeast extract glucose chloramphenicol (YGC; Biokar diagnostics, Beauvais, France) broth supplemented with 20% v/v glycerol. A loop of frozen cells of the strains was suspended in YGC broth at 25 ± 1°C until reaching the stationary phase (48 h). Cultures were then harvested by centrifugation at 1200 x *g* for 5 min (Labofuge 200, Kendro, Germany) and washed twice with phosphate-buffered saline. Pellets were subsequently re-suspended in sterile clarified 12°Brix apple juice and the suspensions were adjusted by optical density at 550 nm to 0.125 (~ 10^6 cells ml^{-1}) using a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, Waltham, MA, USA).

2.2 Food soiling system (culture media) and biofilm formation

The 12° Brix clarified apple juice used in the successive assays was prepared from 72° Brix concentrated apple juice obtained from a large-scale apple juice processing industry. This juice was provided by a national producer/exporter company located in the Alto Valle de Rio Negro y Neuquén (Argentina) and their principal components and characteristics of apple juice are described in Lozano [6]. Assays were carried out on PVDF UF membranes (Synder Filtration, Vacaville, CA, USA), with a MWCO of 100 kDa, and operation characteristic resembling the ones seen in juice processing industries [36]. For this experiment sections of 1 x 2 cm of PVDF membrane were cut with sterile scissor. According to industry recommendations, the membrane was im-

mersed for 15 min in sterile distilled water to eliminate possible antibiotic residues that it contains. The sections were placed into each well of a sterile 6-well plate (Jet Biofil, Argentina). To completely cover the surfaces, 4 ml of each adjusted suspension was put in contacted with it for 2, 6, 24, 48, 72 hours and 1 week at 25 ± 1 °C. Initially, yeast cells were allowed to deposit on the membrane for 2 h at 25 ± 1°C. Afterwards, the food matrix was replaced every 48 h with sterile juice in order to provide fresh nutrients for the cells.

2.3 Quantification of cells and statistical analysis

After each incubation period membranes were carefully removed using sterile forceps and rinsed by immersing in sterile water for 1 min. To remove the adherent microorganisms, membranes samples were transferred to tube with sterile water and sonicated for 2 min at 20°C (Digital Ultrasonic Cleaner, PS-10A) and vortexed at full speed. Then, samples were serially diluted and counts determined by spread plate technique on YGC agar. Samples were incubated for 24– 48 h. In all analyses, triplicate tests were performed under identical conditions and the results expressed as means and standard deviations (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 (*P < 0.05, **P < 0.01, ***P < 0.001).

3 Results and discussion

The number of attach cells and biofilm formation on PVDF-membrane surfaces evaluated at different times (2, 6, 24, 48, 72 h and one week) is shown in **Table 1.** The results were expressed as log CFU cm^{-2}. The yeasts strains with the highest numbers of adhered cells were *C. krusei* and *C. tropicalis.* Comparing the start point (2 h) with final point (1 week) it was observed that *C. krusei* increased 2.09 log units, from 7.21 ± 0.22 to 9.30 ± 0.29 log CFU cm^{-2} , and *C. tropicalis* increased 2.08 log units with counts ranging from 6.94 ± 0.18 to 9.02 ±0.3 log CFU cm^{-2}. Similar results were reported for *C. krusei*, presented the highest covered surface on stainless steel compared with other yeasts and, also *C. krusei* represented the majority of the isolates from UF membrane samples, showing the adaptive advantage of this species [5]. Unlike *C. krusei, C. tropicalis* is capable of producing multicellular, filamentous forms of growth, pseudohyphae and pseudomycelium. It was suggested that the condition to switch their morphology modifies the ability to invade on different biotic and abiotic surfaces to form biofilms [1-2, 7]. On the other hand, *C. kefyr* reached 8.63 ± 0.32 log CFU cm^{-2} and cell counts increase 1.44 log units at the end of the trial. The lowest cell counts were for *R. mucilaginosa.* It presented significant differences (P < 0.001) only after one week of adhesion (**Table 1.** It may be assumed that those cells are able to form a dense network on inert surfaces and adhere in an easier way, forming biofilms resistant to the removal effect of the liquid flowing through the system. In this work, the four yeasts strains evaluated were able to strongly adhere and form mono-species biofilm on the PVDF membrane surface and increased their viable cell counts.

Table 1. Cells numbers of *R. mucilaginosa, C. tropicalis, C. krusei and C. kefyr* expressed as mean log CFU cm^{-2} ± SD in mono-species biofilms on PVDF-membranes surfaces at 25º C.

Time	2	6	24	48	72	One week
R. mucilaginosa	6.79 ± 0.14	6.84 ± 0.17 ns	7.27 ± 0.28 ns	7.35 ± 0.18 *	6.71 ± 0.08 Ns	7.68 ± 0.35 ***
C. tropicalis	6.94 ± 0.18	7.58 ± 0.24 **	8.33 ± 0.16 ***	8.31 ± 0.08 ***	8.50 ± 0.26 ***	9.02 ± 0.30 ***
C. krusei	7.21 ± 0.22	7.77 ± 0.22 *	8.16 ± 0.23 ***	8.41 ± 0.20 ***	8.90 ± 0.12 ***	9.30 ± 0.29 ***
C. kefyr	7.19 ± 0.18	6.26 ± 0.00 ***	7.52 ± 0.22 ns	7.91 ± 0.23 **	8.22 ± 0.18 ***	8.63 ± 0.32 ***

Significant differences reference are expressed through the following nomenclature ns, not significant, *p <0.05, **p < 0.01 ***p < 0.001, all are in reference at 2 h of own specie.

4 Conclusion

Research on biofilms has focused on understanding the mechanisms and factors governing biofilm formation in mono- or dual-species biofilm models and in the design of strategies to control them at lab scale. The results of this study should alert to the presence of complex communities that could affect the effectiveness of sanitation procedures and the microbiological stability of juice-producing plants and will provide a basis for the development of better strategies for biofilm control in food industry.

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