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# *Candida krusei* development on turbulent flow regimes: Biofilm formation and efficiency of cleaning and disinfection program

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ABSTRACT

In food processing lines or in complex equipment such as pumps or valves, microorganisms are exposed to varying hydrodynamic conditions caused by the flow of liquid food, and biofilms are thus grown under a wide distribution of local hydrodynamic strengths. Using an industrially relevant strain of *Candida krusei*, we demonstrated that biofilms formed on stainless steel for 4 days at Reynolds (Re) numbers ranging from 294,000 to $1.2 \times 10^6$ proceeds through three distinct developmental phases. These growth phases transform adherent blastospores to well-defined cellular communities encased in an extracellular matrix and biofilm formation increases when increasing Reynolds number and time. In all growth phases, the morphology of *C. krusei* biofilm revealed the influence of hydrodynamic drag. Indeed, we study the effect of cleaning and sanitation procedure in the control of turbulent flow-generated biofilm. This procedure involves alkali (NaOH 0.5%) and sodium hypochlorite (500 ppm). In terms of total biofilm mass, removal decreases with increasing biofilm age. The largest reduction post-treatment (between 57% and 62%) was observed, to all Reynolds numbers, on 24 and 48 h-old biofilms. Removal was between 39% and 46% on 72 h-old biofilms and was close to 30% for all Reynolds numbers on 96 h-old biofilm.

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## 1. Introduction

One of the most important factors affecting biofilm structure and behavior is the velocity field of the fluid in contact with the microbial layer (Vieira et al., 1993; Stoodley et al., 1999; Hall-Stoodley and Stoodley, 2002; Pereira et al., 2002; Tsai, 2005; Purevdorj-Gage et al., 2006). Hydrodynamic conditions will determine the rate of transport of cells and nutrients to the surface, as well as the magnitude of shear forces acting on a developing biofilm (Characklis, 1980; Characklis and Marshall, 1990; Stoodley et al., 1999). Thus, these conditions significantly influence many of the processes involved in biofilm development (Vieira et al., 1993).

In industrial processes, the geometry design of machines, pipes and tanks are usually complex, and biofilms are thus grown under a wide distribution of local hydrodynamic strengths (Lelièvre et al., 2002; Blel et al., 2007). Dead ends, corners, cracks, crevices, gaskets, valves and joints are vulnerable points for biofilm accumulation. Despite the above, little work has been done on the formation of biofilms at high Reynolds numbers.

In contrast to the extensive literature describing bacterial biofilms, little attention has been paid to fungal biofilms, although yeasts are usually contaminants that affect the quality and the shelf life of foods (Fleet, 1992) and yeast biofilm development may cause adverse effects on processing equipment (Salo and Wirtanen, 2005; O'Brien et al., 2007). 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 the beverage industry (Salo and Wirtanen, 2005).

On turbulent flow, previous studies of this type have focused on the pseudomonads (Pujo and Bott, 1991; Lewandowski and Stoodley, 1995; Melo and Vieira, 1999; Stoodley et al., 1998) and *Listeria innocua* (Perni et al., 2006), but no information has been found about yeast biofilm formation under hydrodynamic conditions relevant to food processing lines. In a previous work, (Brugnoni et al., 2011) we were able to demonstrate that a wild strain of *Candida krusei* was capable to colonize and to form biofilms in flow conditions at Reynolds numbers up to 130,000 and shear stress up to 90 N $m^{-2}$.

Since the hydrodynamic conditions under which the biofilms are formed play a significant role in their composition and structure, it is not surprising to find differences in the efficacy of cleaning and sanitation process when applied to biofilms formed under turbulent and laminar flow (Simões et al., 2005b). To understand

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the above it is important to develop a model that closely represents the industrial conditions in which cells colonize and biofilms form.

In food industry, cleaning generally involves the use of sodium hydroxide, chemical originally selected for its ability to remove organic (proteins and fats) fouling layers. Following cleaning, in some instances, a disinfectant is also applied. Traditionally chlorine (sodium hypochlorite) has been used, however, a wide variety of disinfectants are currently in use or being evaluated for use in food industry (Alasri et al., 1992, 1993; Rossoni and Gaylarde, 2000; Joseph et al., 2001; Parkar et al., 2004).

Previous work in our laboratory (Brugnoni et al., 2012) has focused on cleaning and disinfection process of yeast biofilm formed under laminar flow. In this work, the combined action of 0.5% NaOH and 500 ppm sodium hypochlorite produced a reduction of viable cells greater than 70% for *C. krusei*.

This work had a dual purpose: first, to describe the effect of turbulent flow on biofilm formation of *C. krusei* in a simulated industrial environment, and second, to study the effectiveness of cleaning and disinfection on biofilm removal.

In order to investigate the effect of hydrodynamic forces (turbulent flow) on yeasts biofilm formation and control, we use a rotating disk system (RDS) designed in our laboratory. This reactor allows the simultaneous generation of different shear rates on the same inoculating population and the rotating speeds of the surface are proportional to their radius. The RDS can achieve the high flow rates that are common in industrial processes. The major advantage of the rotating disk is the ability to produce a linear range of shear stress at the surface of a sample in a single experiment with uniform chemical conditions over the whole sample surface (García et al., 1997; Detry et al., 2009). The rotating disk allows the generation of well controlled mass transfer conditions to study fouling and cleaning.

## 2. Materials and methods

### 2.1. Yeast strain and culture conditions

*C. krusei* was isolated in a large-scale apple juice processing industry located in Argentina from samples obtained at different processing steps (Brugnoni et al., 2007). It was isolated after being subjected to cleaning and disinfection processes similar to those used in our experiments.

Stock culture of *C. krusei* was 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, Zwitzerland) and stored at −70 °C. For experiments, a loop of frozen yeast cells was sub-cultured in sterile 12 °Brix clarified apple juice at 22 ± 1 °C and 100 rpm on an orbital shaker (Vicking M23, Vicking s.r.l., Argentina) until reaching the stationary phase (48 h), harvested by centrifugation at 1200*g* for 5 min (Labofuge 200, Kendro, Germany) and subsequently resuspended in sterile clarified apple juice diluted to half to a final concentration of $5.0 \times 10^4$ cells/mL.

### 2.2. Experimental apparatus

The rotating disk system (RDS) used in the present work was described in Brugnoni et al. (2011). A 0.2 m disk with 5 mm thickness was rotated in a stainless steel container (30 cm in diameter and 19 cm in height). The disk was made from a transparent acrylic sheet. Drive motor was a Boeco Model OSD-20 stirrer (Boeckel Co., Hamburg, Germany) with digital speed display. The stainless steel (SS) coupons (AISI 304 2B – 12 × 15 × 0.1 mm; food grade), used as a substrate onto which yeast cells were deposited, were fixed to disk with a contact adhesive (POXIPOL™, Akapol, Argentina) to 5, 7.5 and 10 cm to the center of the disk. This distance (radius) determines the values of hydrodynamic forces acting on the cells. The hydrodynamic of liquid flow along the surface of a RDS is described by Levich (1962).

Fluid flow was characterized by the Reynolds number (Re), defined as $\rho\omega r^2/\mu$, where $\rho$ is fluid density (apple juice density: 1020 kg m$^{-3}$), $\omega$ is angular velocity (rad/s), $r$ is radius of disk (m) and $\mu$ is fluid viscosity (apple juice viscosity: $1.24 \times 10^{-3}$ Pa s). In practical terms this parameter represents the ratio of inertial to viscous flow.

For turbulent flow (Re > 200,000), the shear stress $\tau_{turb}$ is given by Visser (1973):

$$\tau_{turb} = 0.534\rho r^{8/5}\mu^{1/5}\omega^{9/5}$$

To establish the relationship between the angular velocity and the average flow velocity in a straight pipe (linear velocity), we calculate linear velocity ($v$) as: $v$ (m s$^{-1}$) = $\omega r$.

Before the experiments, the coupons and the stainless steel container were soaked for 15 min with 2% w/v of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50 °C and rinsed five times for 5 min each with hot tap water followed by five rinses with distilled water. Finally, they were autoclaved for 15 min at 120 °C.

The rotational speed was adjusted to obtain Re numbers varying between 294,000 at 5 cm (shear stress = 1455.16 N m$^{-2}$, lineal velocity equal to 5.76 m s$^{-1}$), 661,000 at 7.5 cm (shear stress = 2783.93 N m$^{-2}$, lineal velocity equal to 8.63 m s$^{-1}$) and $1.2 \times 10^6$ at 10 cm (shear stress = 4411.24 N m$^{-2}$, lineal velocity equal to 11.51 m s$^{-1}$). The three flow regimes resulted in a twofold difference in Reynolds number. These Reynolds numbers were indicative of fully turbulent flow.

### 2.3. Food soiling system

Food soiling system (or food matrix) was prepared from 72 °Brix concentrated apple juice and sterilized by microfiltration (pore size 0.45 μm) (Metricel®Grid, GelmanSciences, MI, USA). Subsequently, it was diluted to half with sterile distilled water because surfaces in food industry premises provide low nutrient concentrations (Leriche et al., 2003).

### 2.4. Biofilm development

To allow adhesion, the disk was rotated in the vessel filled with the yeast suspension (prepared as described in Section 2.1) during 5 h at 17 ± 1 °C. Then, yeast suspension was replaced with sterile food matrix in order to provide fresh nutrients for the cells. The time and temperature used in the experience was selected considering the temperature and the average time-consuming in the first stage of concentrated apple juice production. In industries located in the Southwest of Argentina, this production is performed at room temperature, in the range of 15–23 °C approximately.

Non adhering yeasts were removed by rinsing with sterile distilled water. Four days biofilms were developed with the same incubation conditions but new food matrix was replaced daily. At 24, 48, 72 and 96 h, SS coupons (control coupons) were removed and analyzed in duplicate by epifluorescence microscopy and biofilm mass determination.

### 2.5. Cleaning and disinfection protocol

Biofilms formed as described in Section 2.4 were subjected every 24 h to a cleaning and disinfection procedure involving alkali

and sodium hypochlorite. Previous to the daily treatment, the coupons used as control coupons (untreated) were carefully removed from the RDS. The remaining SS coupons (treated coupons) were exposed to the following cleaning and disinfection regime: NaOH solution 0.5% (10 min at 60 °C, pH 12.0), followed by five water rinses with sterile distilled water (for 2 min each) at 20 °C. Then, a sodium hypochlorite solution (500 ppm active chlorine, pH 9.0) was circulated for 5 min at room temperature (20 °C) followed by rinse with sterile distilled water. Treated coupons were removed and analyzed in duplicate by epifluorescence microscopy and biofilm mass determination.

The remaining control coupons were fixed again to the disk together with the treated coupons and the experience continued for 96 h as was described in Section 2.4, repeating cleaning and disinfection protocol described above every 24 h.

This cleaning and disinfection procedure is the traditional system used in pipes, tanks and other accessories not subjected to high temperatures in fruits and vegetables processing plants.

The removal of the chips was carefully carried out to produce the least distortion of the biofilms. Furthermore, the experiments were repeated in three different occasions in order to reduce possible experimental artifacts due to processing.

Before placing control coupons, the stainless steel container was cleaned for 15 min with 2% w/v of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50 °C and rinsed five times for 5 min each with hot tap water followed by five rinses with distilled water. Then, it was disinfected with diluted bleach. Water with bleach was totally removed and the system was rinsed with sterile water in aseptic conditions.

We used a RDS to monitor the fouling and cleaning of biofilms under conditions which simulated a pre-heat section of apple juice processing plant in terms of flow velocity, temperature, contact surface and contaminating micro-organisms. This system was inexpensive to construct, easy to run, and permitted testing of multiple SS chips in a single run which in turn allowed the effectiveness of the complete cleaning and disinfection system, as well as its individual components, to be tested. Throughout this study apple juice was obtained from a large-scale apple juice processing industry located in Argentina in order to maintain similarities between the plant conditions and the laboratory system. Further, to reduce any differences between trials and to help produce a consistent and reproducible biofilms on the surface of the SS coupons all the apple juice came from a single batch.

### 2.6. Microscopy of biofilms: fluorescence microscopy

Biofilm development on treatment and control coupons was evaluated by epifluorescence microscopy with fluorescein diacetate (FDA). The principle behind a test using FDA is only live cells will convert FDA to fluorescein and the amount of fluorescence is directly related to the number of live cells.

A standard stock solution of 2 mg/mL (0.2% w/v) 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 characterize the yeast biofilm on stainless steel, the coupons from each experimental condition were stained with sterile FDA acetonic solution in 0.1 M phosphate buffer (0.04% w/v), pH 7.5. After 90 min shaking at 25 ± 1 °C in the darkness, the coupons were rinsed twice with sterile distilled water. FDA specifically stains cells possessing esterase activities and intact cell membranes. This fluorescent probe is widely used as an indicator of cell viability. The stained surfaces were examined under an Olympus BX 51 epifluorescence microscope (Olympus, Buenos Aires, Argentina) with a suitable filter combination. At least twenty fields (area: 0.038 $mm^2$) were examined per coupon.

### 2.7. Biofilm dry weight determination

The dry mass of the biofilm accumulated on the stainless steel slides before and after the cleaning and disinfection process was determined. Briefly, treated and control coupons with biofilms were removed from the RDS. Afterwards, the slides were dried until constant weight at 55 °C and weighed. The SS coupons were then carefully washed, dried again and weighed (empty control). Biofilm dry weight was calculated from the difference between these measurements.

The biofilm mass accumulated was expressed in mg of biofilm per $cm^2$ of surface area (mg biofilm/$cm^2$). In each experiment, the percentage of the biofilm removal was determined through the following equation: Biofilm removal (%) = $[(W - W_1)W^{-1}] \times 100$, where $W$ is the biofilm mass in control coupons and $W_1$ is the biofilm mass in treatment coupons.

### 2.8. Statistical analysis

The experiments were repeated in three different occasions by performing three independent biofilm formation experiments. The mean and standard deviation within samples were calculated for all cases. Statistical comparisons of biofilm removal were analyzed by Student's *t*-test. Statistical calculations were based on confidence level equal or higher than 95% ($P$<0.05) was considered statistically significant.

## 3. Results and discussion

### 3.1. Influence of flow on biofilm development and morphology

Fig. 1 shows that *C. krusei* biofilm formation on stainless steel progresses in three distinct developmental phases: early, intermediate and maturation.

Early (24 h-old biofilm), the majority of *C. krusei* cells were present as blastospores (yeast forms) adhering on the surface of stainless steel coupons. The cells are aligned in the main flow direction (Fig. 1aI, bI and cI). Statistical analysis showed that, in control coupons to all Reynolds numbers, 24 h-old biofilms had similar biofilm mass and no significant differences ($p > 0.05$) were observed (Fig. 2).

An intermediate developmental phase was characterized, at 48 h-old biofilms, by microcolonies showing alignment with the direction of flow and elongated in the downstream direction (Fig. 1aII, bII and cII). No significant differences ($p > 0.05$) in biofilm mass were observed to all Reynolds numbers (Fig. 2).

At 72 h, *C. krusei* communities appear as thick tracks of fungal growth, due to cell growth and aggregation along the surface (Fig. 1aIII, bIII, cIII and aIV). As Reynolds number increase, the biofilm evolves into partial and completely covered fields with metabolically active cells. The overall orientation of these initial structures was parallel to the main flow direction. At 72 h-old biofilms, significant differences ($p < 0.05$) in terms of biofilm mass were observed on biofilms formed in control coupons at Reynolds equal to 294,000 compared to those formed at 661,000 and 1200,000 Reynolds (Fig. 2).

During the maturation phase shows the presence of extracellular material (Fig. 1bIV and cIV) which appeared as a haze-like film. In this phase, *C. krusei* communities were completely encased within this material (Fig. 1cIV). At this stage it was difficult to focus on the basal blastospore communities covered by the extracellular material. Interestingly, this biofilm behavior was observed to the greatest Reynolds number (Re = 1.2 × 106, shear stress = 4411.24 N $m^{-2}$, lineal velocity equal to 11.51 m $s^{-1}$).

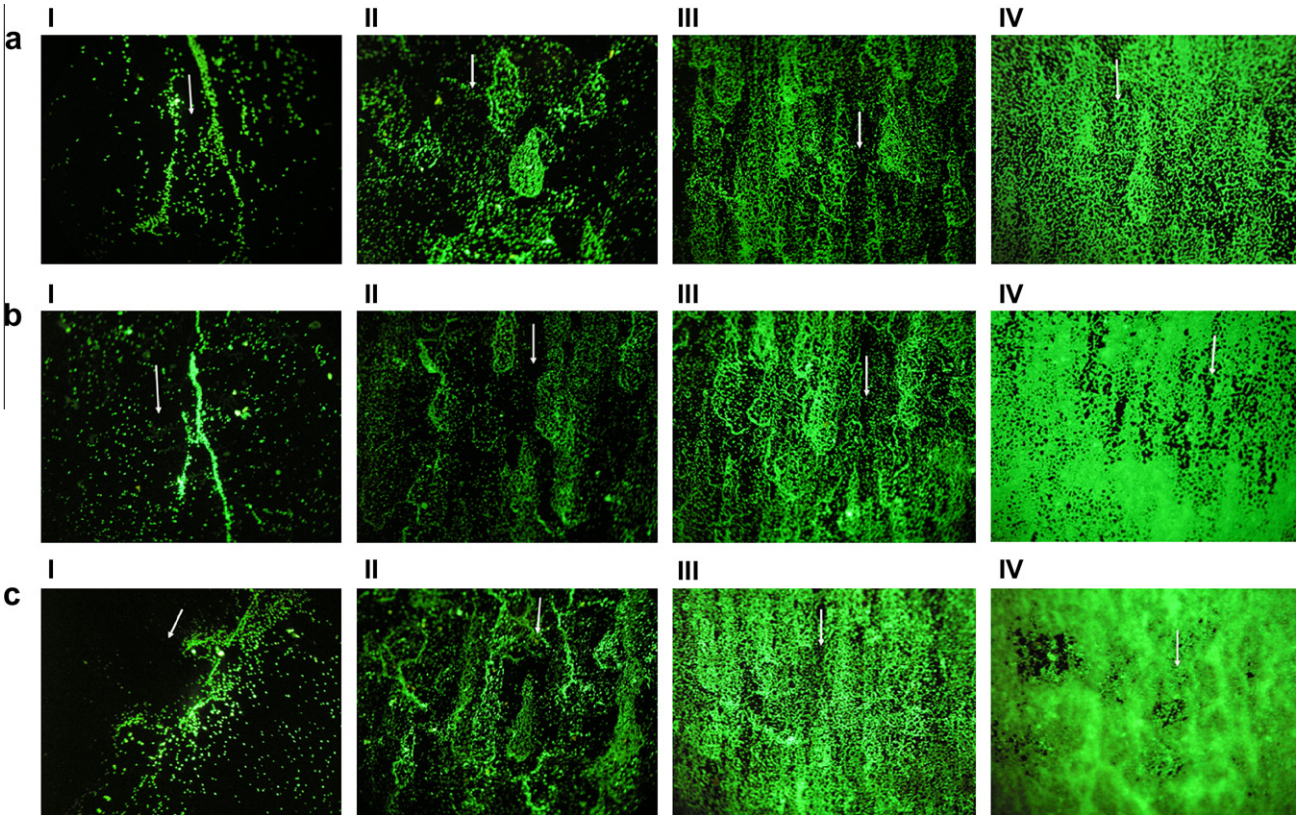


**Fig. 1.** Epifluorescense microscopy characteristics of *Candida krusei* biofilms formed on stainless steel at Reynolds number equal to: (a) 294,000, (b) 661,000 and (c) 1200,000 after 24 (I), 48 (II), 72 (III) and 96 (IV) h. 200× Magnification. Arrows indicates main flow direction.

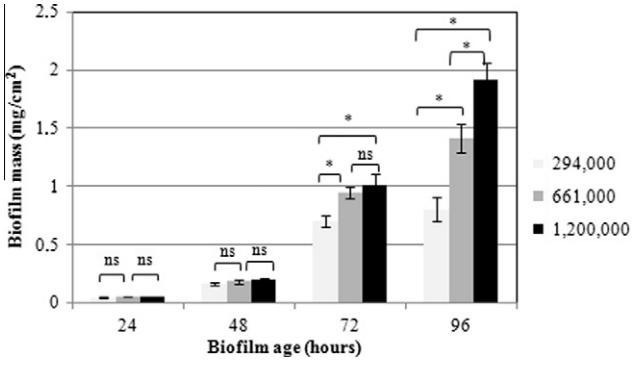


**Fig. 2.** Biofilm mass of *Candida krusei* biofilms formed at Reynolds number equal to 294,000, 661,000 and 1200,000 after 24, 48, 72 and 96 h. Means ± SD. *Significant differences ($p < 0.05$), ns, no significant differences.

On 96 h-old biofilms, significant differences ($p < 0.05$) in biofilm mass were observed comparing biofilms formed at all Reynolds numbers in control coupons (Fig. 2).

The results obtained here cast doubt on the "rule of thumb" widely quoted in industry that velocities of 1.00 m $s^{-1}$ are sufficient to prevent biofilm formation (Pujo and Bott, 1991).

*C. krusei* biofilm mass increased when increasing Reynolds number, and time. Several studies (Vieira et al., 1993; Pereira et al., 2002; Boyle and Lappin-Scott, 2006, 2007; Simões et al., 2007) reported that progressively increasing the flow rate had an escalating effect on the attachment of cells, biofilm mass, metabolic activity and total protein content.

How liquid flow affects the mass of the biofilm can be explained considering the factors that influence the structure of biofilms growing in aqueous environments: flow velocity and nutrient status (Bott, 1995). The 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 suspended solids (nutrient) delivery from the bulk phase (flowing water) to the attached phase (biofilm). During the initial stages of biofilm development, internal mass transfer limitations were probably not significant due to the reduced thickness. As the biofilm thickness increased, mass transfer limitations become increasingly more important (Stewart and Franklin, 2008).

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. Howsam (1995) reported that at velocities of 0.4–4 m $s^{-1}$, the worst biofouling occurs at the fastest flow rates. It would appear that biofilms are able to compress under pressure and exhibit a high resistance to shear. Santos et al. (1991) observed less stable *Pseudomonas fluorescens* biofilms formed at low velocities of 0.5 m $s^{-1}$, and suggested that the biofilm formed was less than the biofilm formed at 2.5 m $s^{-1}$. Moreover, Santos et al. (1991) found that when filtered tap water was used to grow *P. fluorescens* biofilms at 2.5 m $s^{-1}$, it was much thicker than one grown 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 all stages, the morphology of *C. krusei* biofilms in turbulent flow revealed the influence of hydrodynamic drag. Similar cell disposition has been previously reported and has been implicated with increased frictional energy losses in water pipelines (Picologlou et al., 1980; Bryers and Characklis, 1981; McCoy et al., 1981; Stoodley et al., 1998; Santos et al., 1991). These results are in agreement with those obtained for us under transitional flow (Brugnoni et al., 2011). Bott (1995) explained this behavior

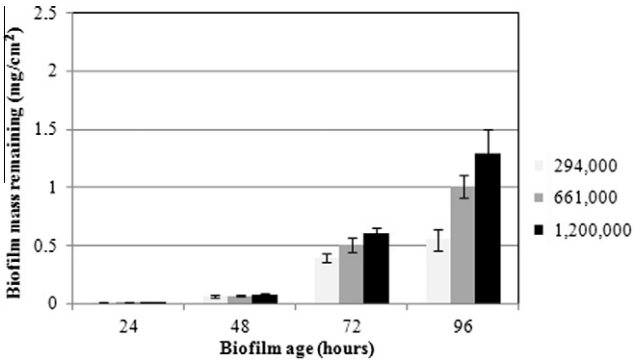


**Fig. 3.** Biofilm mass of *Candida krusei* remaining on stainless steel chips after cleaning and disinfection process. Means ± SD.

considering that cells in contact with flowing become orientated so that each cell offers the least resistance to flow thereby reducing the tendency to slough. It is not clear whether this is "deliberate" attempt by the cells to remain on the surface, or whether it is due to the natural elimination of cells that are susceptible to shear forces due to their position in relation to flow.

It is important to note that biofilm morphological patterns were produced in our system under a variety of environmental parameters (temperature, hydrodynamic forces and growth media) that simulate the industrial conditions prevailing in food processing plants. To our knowledge there are no references on biofilm formation by spoilage yeast on stainless steel at temperatures below 20 °C and under fully turbulent flow conditions.

### 3.2. Effectiveness of cleaning and disinfection program

Fig. 3 shows biofilm mass (containing microbial cells and the extracellular matrix they produce) remaining on stainless steel chips after cleaning and sanitation process.

In terms of total biofilm mass, the largest reduction post-treatment (between 57% and 62%) was observed, to all Reynolds numbers, on 24 and 48 h-old biofilms. Removal was between 39% and 46% on 72 h-old biofilms and was close to 30% for all Reynolds numbers on 96 h-old biofilm. Removal decreases with increasing biofilm age. This is not surprising considering that older biofilms showed and increased resistance to removal (Lelièvre et al., 2002; Bremer et al., 2006). There is some debate in the literature about the time required to develop consistent, relevant biofilms (Bremer et al., 2006). For example, it has been reported that a minimum of 48 h is required to develop a "true" biofilm on SS using a meat soup test medium (Wirtanen and Mattila-Sandholm, 1992). Since yeasts are eukaryotic organisms with generation times greater than the bacteria, we can consider that a biofilm of 72 h or more corresponds to a "true" and relevant biofilm.

No differences in biofilm removal were observed between biofilms formed at different Reynolds. This could be due to the fact that the hydrodynamic forces used in the cleaning and disinfection process were the same which acted in biofilm formation. According to Stoodley et al. (1999) and Körstgens et al. (2001), the biofilm matrix develops an inherent internal tension, which is in equilibrium with the shear stress under which the biofilm is formed, and the removal of a well-established biofilm requires overcoming the forces which maintain the integrity of the biofilm.

The experiment showed that on biofilms subjected to 0.5% NaOH cleaning solution the removal was low, eliminating between 30% and 60% of the total mass of the biofilm. This remaining biofilm can act as an additional source of nutrients and/or as a suitable surface to further growth of cells. Residual biofilm acts as a microbial attachment site that allows rapid reestablishment of the biofilm (Neu, 1992) and the regrowth of the injured microorganisms can also be stimulated in these biofilms (Simões et al., 2005a). Furthermore, biofilm accumulation changes the hydrodynamics of flowing systems (Picologlou et al., 1980) and is essentially an autocatalytic process in that it increases surface roughness (Bouwer, 1987) which, in turn, provides shelter from shear forces and increases both the surface area and convective mass transport near the surface.

After cleaning process, we observed fields covered with metabolically active cells (Fig. 4b). We present only the images of day 4 (control and treatment coupons, 96 h-old biofilm, Fig. 4a and b) for being the best evidence of the consequences of an ineffective cleaning process.

If the cleaning process is not efficient, disinfectant action cannot be evaluated. The aim of disinfection is to reduce the surface population of viable cells after cleaning and prevent microbial growth on surfaces before restart of production. Disinfectants do not penetrate the biofilm matrix left on a surface after an ineffective cleaning procedure, and thus do not destroy all the living cells in biofilms (Holah, 1992; Carpentier and Cerf, 1993). Note that there was no input of yeast cells between one CIP process and another. We may therefore conclude that the phenomenon of re-growth between treatments is virtually inevitable when cleaning and disinfection process is inadequate, because all it takes for yeast to get established is a single microbial input (in this case, the microbial input was $5 \times 10^4$ cells/mL). Clearly, the cleaning and disinfection protocols commonly used by juice industries and cited as effective by others authors in dairy industries (Gilbert, 1994; Bird and Barlett, 1995; Lelièvre et al., 2002) are not sufficient to ensure the removal of biofilm from surfaces. Hotter temperatures, increased alkali concentration, and inclusion of chelators (EDTA) as part of a cleaning protocol will adequately remove biofilm. Indeed,

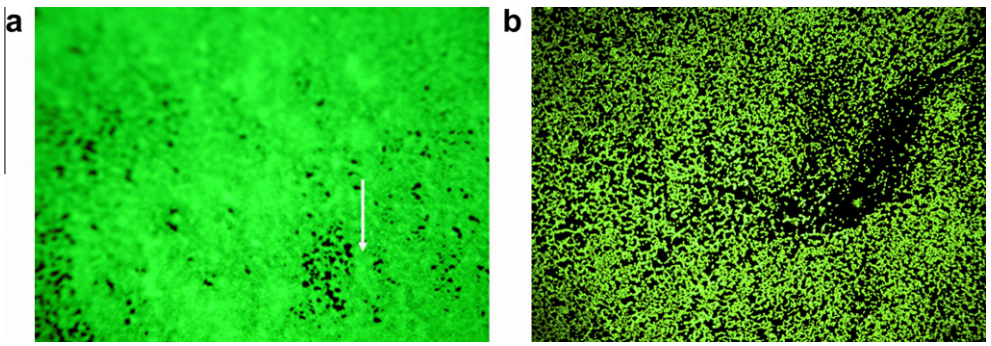


**Fig. 4.** Epifluorescense microscopy characteristics of 96 h-old *Candida krusei* biofilms formed on stainless steel at Reynolds number equal to 1200,000. (a) Before treatment and (b) after treatment. 200× Magnification. Arrow indicates main flow direction.

in food processing equipment, because of the presence of water on the surfaces, or because of the inaccessibility of some places, the real concentration in contact with the surfaces may be lower than expected.

## 4. Conclusions

The studies reported here are the first, to our knowledge, to demonstrate biofilm formation by spoilage yeast species under fully turbulent flow. *C. krusei* biofilms can metabolically respond to these flow conditions and those metabolic changes in turn should favor the formation of a strong and stable microbial community against the stressful hydrodynamic situation.

The recalcitrance of these biofilms to typical cleaning and disinfection process may confer a selective advantage for this species when making biofilms on surfaces with high hydraulic flow and cleaning and disinfection conditions, such as, e.g. in juice processing plants. Improper cleaning and disinfection of food contact may lead to accumulation of *C. krusei* cells on the surfaces and consequently increase the difficulty of its entire removal. This can lead to problems in food quality and safety with significant financial losses to the food industry.

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