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Biofilm formation by the yeast *Rhodotorula mucilaginosa*: process, repeatability and cell attachment in a continuous biofilm reactor

Jasmin Gattlen[a], Manfred Zinn[a]*, Sébastien Guimond[b], Enrico Körner[b], Caroline Amberg[c] and Laurie Mauclaire[a]

[a]*Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Biomaterials, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland;* [b]*Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Advanced Fibers, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland;* [c]*Empa Testmaterials AG, Moevenstrasse 12, CH-9015 St. Gallen, Switzerland*

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Yeast biofilms contribute to quality impairment of industrial processes and also play an important role in clinical infections. Little is known about biofilm formation and their treatment. The aim of this study was to establish a multi-layer yeast biofilm model using a modified 3.7 l bench-top bioreactor operated in continuous mode ($D = 0.12\ h^{-1}$). The repeatability of biofilm formation was tested by comparing five bioprocesses with *Rhodotorula mucilaginosa*, a strain isolated from washing machines. The amount of biofilm formed after 6 days post inoculation was 83 $\mu g\ cm^{-2}$ protein, 197 $\mu g\ cm^{-2}$ polysaccharide and 6.9×10^6 CFU cm^{-2} on smooth polypropylene surfaces. Roughening the surface doubled the amount of biofilm but also increased its spatial variability. Plasma modification of polypropylene significantly reduced the hydrophobicity but did not enhance cell attachment. The biofilm formed on polypropylene coupons could be used for sanitation studies.

Keywords: yeast; *Rhodotorula mucilaginosa*; model biofilm; test system; bioprocess

Introduction

The wealth of information on the development, structure and impact of bacterial biofilms in different fields such as medicine, biotechnology or ecology is enormous. In comparison, knowledge about fungal biofilms (both yeast and filamentous fungi) is still in its infancy.

Yeast biofilm development is similar to that of bacteria (Harding et al. 2009). The phases of biofilm formation such as adherence, microcolony formation, maturation, biofilm maintenance and dispersal occur (Harding et al. 2009). The main difference in biofilm development is the morphological transition of some yeast cells during maturation. Dimorphic yeasts, like *Candida albicans* and *Saccharomyces cerevisiae*, attach to the surface and build a monolayer as spherical cells and pseudohyphae during maturation (Vopalenska et al. 2010). *Rhodotorula* sp. also has the ability to form pseudohyphae but their formation has not been reported during biofilm formation. For the examination of cell attachment, biofilm growth and production reactors such as perfusion chambers (Palmer 1999), modified Robbins devices (Kharazmi et al. 1999) or rotating disk reactors (Hentzer et al. 2001) were used. These systems focused on bacterial biofilm, whereas few studies with yeast cells have been performed (Busscher et al. 1994). From a practical point of view yeasts are more complicated to cultivate than bacteria because of a rather slow growth and a higher susceptibility to contamination by bacteria (Saithong et al. 2009) or other yeasts (Kronlof and Haikara 1991). Yeast can grow directly on plastics (Reynolds and Fink 2001) or stainless steel (Brugnoni et al. 2007) but also on bacterial (Jenkinson and Douglas 2002) or fungal biofilms (Webb et al. 2000) as a secondary colonizer.

Biofilm comprised of yeast occurs not only on implants (Douglas 2002) but also on industrially relevant devices such as photo-processing tanks (Elvers et al. 1998) and food processing plants (Brugnoni et al. 2007) where yeast biofilms influence the quality and taste of the product. They are also found in domestic environments such as kitchen sponges, dish towels (Rayner et al. 2004) or household washing machines (Gattlen et al. 2010). Biofilms in household washing machines produce malodor and impair hygienic performance due to increased use of low-temperature and bleach-free washing (Munk et al. 2001). In industry, biofilms lead to costs of several billion dollars every year due to eg product losses (Kumar and Anand 1998), reduced heat transfer (Shi and Zhu 2009), increased fuel consumption (Chambers et al. 2006) and

*Corresponding author. Email: manfred.zinn@empa.ch

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the use of chemical agents for control and removal of biofilms (Lyon et al. 2008). Besides chemicals (eg antimicrobial agents), several mechanical strategies are available to remove biofilms (eg ultrasound) (Muller et al. 2007). However, treatment efficiency (especially of antimicrobial agents) for biofilms remains unclear because there are still very few test systems available to evaluate biofilm removal (Hamilton 2002; Pitts et al. 2003; Bloss and Kampf 2004). One reason why biofilm removal cannot be efficiently quantified is the lack of reference biofilms. Such reference material should represent the system of interest and be produced in a repeatable manner. Short-term studies of antimicrobial and biofilm removal tests can be performed in 96-well plates for bacteria (Pitts et al. 2003) and yeasts (Rambali et al. 2001). Chandra et al. (2001) produced a 24 h-old *C. albicans* model biofilm on prosthesis material cultivated in 12-well tissue culture plates for testing antifungal agents. Ramage et al. (2001) developed a high throughput 96-well plate system to produce and study *C. albicans* biofilms. However, all these biofilms were cultivated in well plates. The cultivation of biofilms in well plates is limited to young biofilms (24–48 h old) because continuous nutrient supply is not possible. Therefore, they are not representative of thicker and/or older (initially mature) (Harding et al. 2009) or mature biofilms as they are typically found in industrial plants or home appliances. An alternative to study initially mature biofilms is the use of bioreactors. This was successfully done for bacterial biofilms using either rotating disk reactors (Pitts et al. 2001) or a reactor developed by the Centers for Disease Control (CDC reactor) (Goeres et al. 2005; Hadi et al. 2010).

Available yeast models that are not used for testing antimicrobial susceptibility focus more on the developmental characteristics of biofilm formation (Ramage et al. 2001). A laminar flow or biofilm bioreactor system is more convenient for the study of initially mature biofilms because flow systems can be adjusted to represent particular physiological conditions (eg nutrient limitations and different shear stress) better than well plates.

A further aspect for the development of a model biofilm is that the resulting biofilm is repeatable and reproducible (eg amount of cells, total protein). Repeatable biofilm formation has already been successfully achieved with bacteria grown in rotating disk reactors (Pitts et al. 2001), rotating annular reactors (Chen and Stewart 2000) and in the CDC reactor to evaluate the effects of chemical agents (Goeres et al. 2005; Hadi et al. 2010). First attempts to grow *C. albicans* in a CDC reactor were described by Honraet et al. (2005), where the main goal was to test different quantification techniques rather than producing a model biofilm.

However, yeast model biofilms have not been produced in a comparable reactor system which would allow the reproducible production of biofouled test coupons. Such standardized biofilms could be of use for testing the removal efficiency of cleaning and sanitation (either mechanical or with antimicrobial and chemical agents). Further, they could be applied for testing tolerance towards detergents or antimicrobial agents as well as dosage effects. The potential field of applications could be medical devices but also water pipes (either cooling or water distribution systems) and manufacturing procedures (eg plate heat exchanger of pasteurizers for diary processing).

The goal of this study was to establish a model yeast biofilm to be used as reference for testing removal efficiency of household washing machines (Gattlen et al. 2010). The yeast *Rhodotorula mucilaginosa* that was originally isolated from household washing machines was chosen as model organism. *R. mucilaginosa* was grown in a modified bench-top reactor to firstly determine the cultivation conditions in minimal medium for the optimal production of a multi-layered yeast biofilm and secondly, to assess repeatable biofilm formation on polypropylene coupons with different surface characteristics (smooth and rough, as well as plasma treated).

Material and methods

Bioreactor set-up

For the experiment a modified 3.71 bench-top bioreactor (KLF2000, Bioengineering AG, Wald, Switzerland) was used (Figure 1). A stainless steel cylinder (height: 20 cm, maximal diameter: 6 cm) designed to hold six removable coupon holders (stainless steel) with space for 20 test coupons was mounted on the stirrer axis replacing the stirrer blades. The coupons were immersed in 70% ethanol (EtOH) and sonified in a water bath for at least 10 min before mounting into the metal holders for chemically cleaning the coupon surfaces. In order not to modify the surface, ethanol was chosen for cleaning. Autoclaving sterilizes the surface but is not able to remove grease. The test coupons were used only once. A pH probe (RedCap 405–60-T-S7/120/9848, Mettler Toledo, Greifensee, Switzerland) was calibrated with two reference solutions with pH = 4 and pH = 7 (BioChemika). An external aeration loop was connected to the reactor to avoid bubble formation that could result in additional shear force and remove biofilm from test coupons. A trap column for liquids was connected between the 50 l medium bag and the reactor to prevent back contamination of the medium bag.

The biofilm reactor was filled with 2.5 l 30% Sabouraud dextrose broth (SDB, pH = 5.6) to control

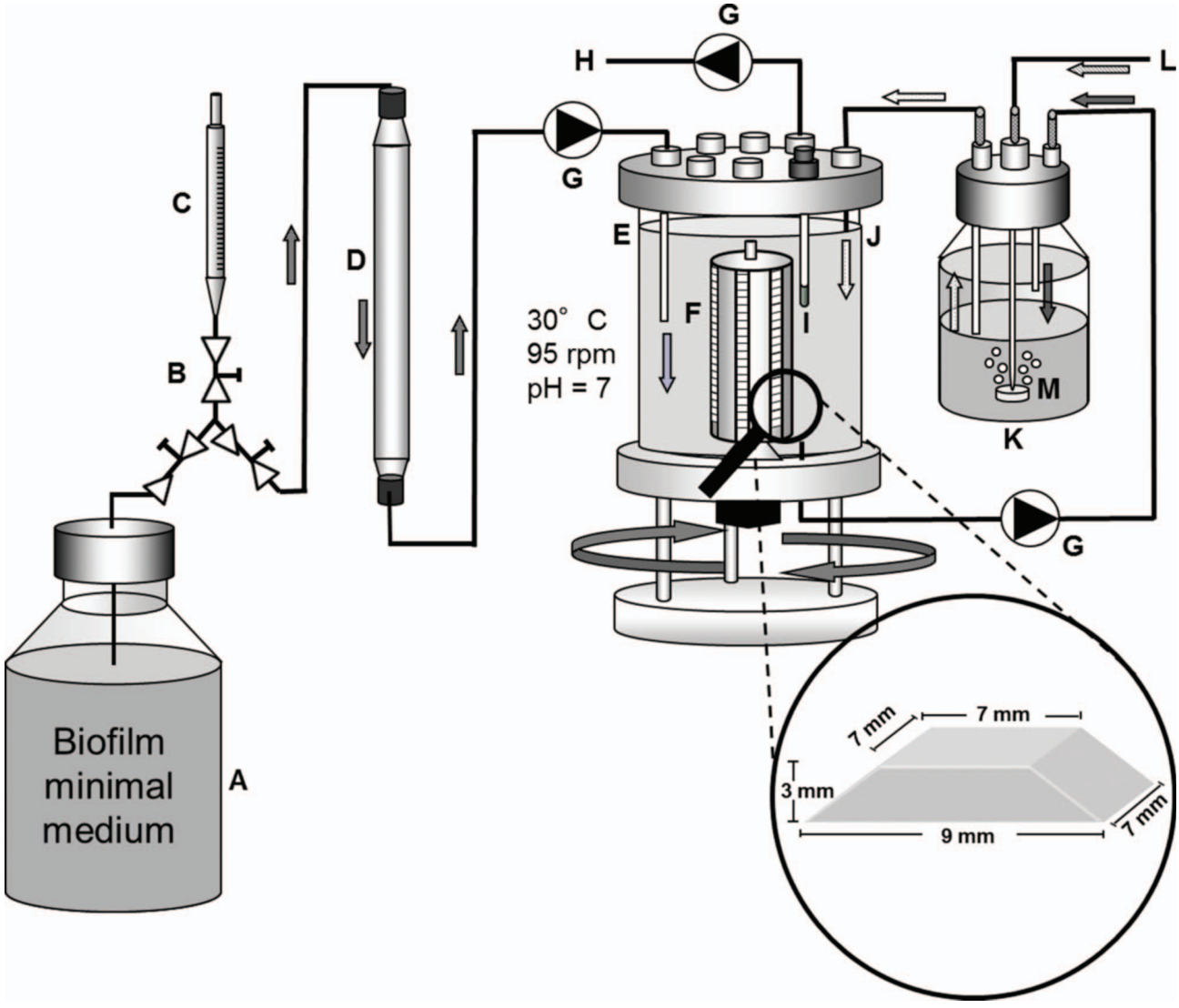

Figure 1. Set up of reactor system for biofilm formation. A: medium reservoir (50 l) with biofilm minimal medium, B: valve, C: glass burette for flow measurements, D: glass column for prevention of back contamination, E: submerged inlet tube for biofilm minimal medium feed, F: rotating cylinder with coupons, G: peristaltic pumps, H: outlet waste, I: pH meter, J: inlet of aerated medium, K: aeration bottle, L: inlet for pressurized air, M: ventilation frit, magnification of the trapezoid PP coupons. Biofilm formation of coupon occurred only on the top surface (7 × 7 mm).

and slow down cell growth as well as to adjust the cells to a poor nutritional environment as will follow during continuous cultivation. The medium was sterilized (30 min at 121°C). The external aeration bottle filled with *ca* 500 ml of 30% SDB (Figure 1K), the whole tubing system as well as the glass columns (see Figure1C,D) were autoclaved separately. The aeration of the biofilm reactor *via* external aeration loop (Figure 1K) was initiated *ca* 12–15 h prior to inoculation to stabilize the system and ensure sufficient dissolved oxygen tension during inoculation. The cyclic flow in the aeration loop was maintained by a peristaltic pump (Periplex, Bioengineering AG) (Figure 1G) running at maximum speed and an overpressure triggered by the aeration of filtered air *via* a ventilation frit (Figure 1M).

Characterization of test coupon material

PP composition and surface roughness

The supporting material for biofilm formation was white polypropylene (PP) reinforced with glass fibers (Lot Nr.: PP Miele Granulate Hostacom EKG W92535, Germany). The PP plates were cut to trapezoid coupons (top surface 7 mm × 7 mm, bottom surface: 7 mm × 9 mm, thickness: 3 mm) (Figure 1) and either used unchanged or with a roughened top surface with a sandpaper/abrasive paper (150 grains

cm^{-2}), perpendicular to the direction of rotation in the reactor. Alternatively, a set of dye-casted PP (dcPP) with a defined roughness (roughness 24, defined according to VDI 3400; Treff AG, Degersheim, Switzerland) was used to examine the influence of the coupon material on biofilm formation. The composition of the dcPP was similar to the PP provided by Miele, however, without glass-fibres and zinc oxide (white color) as additives.

Plasma treatment of coupons

To reduce the hydrophobic nature of the dcPP coupons, they were plasma activated or plasma coated using the following gases and gas mixtures: Ar/O_2, N_2, NH_3/C_2H_4 (ratios 1:1 and 2:1) and CO_2/C_2H_4 (ratios 2:1 and 6:1). The C_2H_4 based gas mixtures led to the deposition of plasma polymer thin films where either N- or O-containing functional groups were embedded. The functional groups based on N_2 and Ar/O_2 were directly grafted onto the PP surface. The exact procedure was carried out as described elsewhere (Hegemann et al. 2007; Hossain et al. 2007; Koerner et al. 2009). Prior to reactor experiments the coatings were tested for heat stability (121°C, in the presence of culture broth). The composition of the coupon surface was characterized by X-ray photoelectron spectroscopy (XPS; PHI 5600 spectrometer, USA, $n = 1$) and static contact angle measurements using a droplet of distilled water (~ 5 μl) ($n = 3$). A set of 18 coated coupons (three coupons for each plasma coating condition) was autoclaved in the presence of 30% SDB to simulate the conditions within the reactor during medium sterilization and to check the influence of the culture medium on the plasma-coating. The samples were air-dried under laminar flow in the sterile bench for 2.5 h and contact angles were measured (Krüss G10 apparatus, Hamburg, Germany). In order to evaluate the stability of the functionalization after 2 days, the samples were dipped for 30 s in nanopure water, dried for 2.5 h and water contact angles were measured again. Freshly coated coupons were used for cultivation experiments.

Cultivation of the yeast R. mucilaginosa

R. mucilaginosa, a pigmented yeast typically living in terrestrial and aqueous habitats, was isolated from a household washing machine (Gattlen et al. 2010) and was used throughout all experiments.

Preparation of frozen stocks

Since frozen stocks are a potential source of variability, the preparation of the stocks was performed with special care. A colony of *R. mucilaginosa* grown on Sabouraud 4% glucose agar (SDA) was transferred into 15 ml of SDB and incubated for *ca* 18 h (30°C, 150 rpm). The culture was used to inoculate a shake flask containing 100 ml of SDB. The cells were grown (150 rpm, 30°C) until an OD_{600} of about 0.5–1.0 was reached. The culture broth was mixed 1:1 (v v^{-1}) with 30% glycerol and 2 ml aliquots were prepared. Cells were frozen at −20°C overnight and stored at −80°C until usage.

Preparation of pre-cultures

For the preparation of the bioreactor inoculum one vial with frozen yeast cells (2 ml) was transferred into a baffled shake flask containing 150 ml of SDB supplemented with anhydrous ampicillin (final concentration: 50 μg ml^{-1}) and chloramphenicol (final concentration: 500 μg ml^{-1}). Antibiotics were used to prevent contamination with bacteria. Cells were incubated at 30°C, 150 rpm for *ca* 24 h until reaching an optical density of 2.6 ± 0.1.

Inoculation of the bioreactor

Cells reaching the late exponential phase were inoculated into the biofilm reactor at 30°C and a cylinder rotation of 95 rpm. Cell growth was followed by measurements of OD_{600}. When the maximal growth rate μ_{max} (0.23–0.27 h^{-1}) was reached the washout of cells in suspension was initiated. In order to wash out the non-adhering cells, continuous cultivation was started with an initial dilution rate of *ca* 0.52 h^{-1} with biofilm minimal medium. The biofilm minimal medium (pH = 7) for continuous cultivation consisted of 1 g l^{-1} 3-(N-morpholino)propanesulfonic acid (MOPS), 1.1 g l^{-1} $(NH_4)_2SO_4$, 0.15 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4 \times 7\ H_2O$, 0.1 g l^{-1} $FeSO_4 \times 7\ H_2O$, 0.2 g L^{-1} ethylenediaminetetraacetic acid disodium salt and 1 ml of filter-sterilized (0.22 μm, Milex, Milipore AG, Zug, Switzerland) trace element stock solution (1.5 g l^{-1} $CaCl_2 \times 2H_2O$, 3.96 g l^{-1} $MnCl_2 \times 4H_2O$, 5.62 g l^{-1} $CoSO_4 \times 7H_2O$, 0.34 g l^{-1} $CuCl_2 \times 2\ H_2O$, 1 g l^{-1} $ZnSO_4 \times 7H_2O$, 1 g l^{-1} $MoO_4Na_2 \times 2H_2O$, pH = 1) with 4 g l^{-1} glycerol as carbon source. All chemicals (Sigma-Aldrich, Buchs, Switzerland) except for the micronutrient solution were autoclaved prior to filter-sterilization (0.45 μm + 0.2 μm; Sartorius) to minimize risk of contamination. In previous experiments it has been observed that filter-sterilization was not sufficient to remove contaminants from the chemicals. The dilution rate was set twice as high as the μ_{max} to wash out non-adhering cells for 13 ± 1 h. After washout the dilution rate was reduced to 0.12 h^{-1} for further cultivation.

Sampling and quantification of biofilms

Biofilm formation was assessed on PP coupons mounted in holders on a rotating cylinder (Figure 1). For the temporal development of biofilm formation, one holder harbouring 14 test coupons (7 rough and 7 smooth) was harvested after 1, 3, 6, 9, and 13 days post inoculation. One smooth and one rough coupon were prepared for microscopic observation by confocal laser scanning microscopy (CLSM).

For the repeatability test, all six holders of a bioreactor numbering 16–23 smooth and 18 rough coupons were sampled for biofilm quantification on day 6. The remaining coupons were only used to fill the other positions.

The plasma treated coupons were harvested after 1, 3 and 6 days post inoculation and biofilm was quantified ($n = 4$) as follows.

Sampling of coupons

After removal from the holder unit, each coupon was briefly submerged in sterile 0.9% NaCl solution to remove loosely attached cells. Each coupon was then transferred into 5 ml of 0.9% NaCl and treated with ultrasound (sonifier tip, Branson sonifier) at 0°C (10% amplitude, 30 s with alternating 1 s pulse on and 1 s pulse off) to detach the cells from the coupon. The suspensions were stored at 4°C for a maximum of 4 h due to the large number of samples that needed to be sonified. The samples were vortexed before subsampling for further analysis.

Optical density

One ml of cell suspension derived from sonified biofilms was measured with a spectrophotometer (Spectronic® Genesys™ 6, UV-visible spectrophotometer, Thermo Electron Schweiz AG, Allschwil, Switzerland) at 600 nm.

Polysaccharide quantification

This was based on Dubois et al. (1956). Because the main constituents of the EPS matrix are polysaccharides the total amount of polysaccharide was quantified (Sutherland 2001; Flemming and Wingender 2010). One ml of cell suspension was taken, 25 μl of 80% (v v^{-1}) phenol dissolved in distilled water were added and the sample was vortexed. Subsequently, 2.5 ml of 98% sulphuric acid (Merck, Zug, Switzerland) were added within 20–30 s in the center of the solution to ensure perfect mixing, followed by vortexing for 1 min, cooling at room temperature for 10 min, vortexing again and finally incubating in the water bath at 26 ± 1°C for 20 min. Before reading the light absorption at 485 nm, the samples were vortexed again. A standard curve was prepared with D(+)-glucose dissolved in distilled water (0–35 μg ml^{-1}). Samples containing sugar concentrations >35 μg ml^{-1} glucose equivalents were diluted with 0.9% NaCl and re-analysed.

Protein quantification

Proteins are a large component of the microbial cell and also found in the matrix consisting of exopolymeric substances (EPS) (Sutherland 2001), therefore the total amount of protein was analysed. For the quantification of the total protein, the micro BCA protein assay kit (Thermo Scientific, Rockford Illinois, USA), based on the biuret reaction, was applied according to manufacturer's instructions. A standard curve was prepared with BSA (0–40 μg ml^{-1}).

Viable cell counts

Viable cell counts by colony forming units (CFU) were performed by serial dilutions of the suspension and plating on SDA plates. The plates were incubated for *ca* 2 days at 30°C before counting.

Confocal laser scanning microscopy

Sampled coupons were placed on wet paper and kept under humid atmosphere at 4°C for no longer than 5 h before staining. The coupons were stained for 30 min in the dark with 100 μl of a mixture of 0.1 M Tris buffer (pH = 7.5) and Syto BC (Molecular Probes, Invitrogen, Lucerne, Switzerland, final concentration: 0.5 μM) for staining cell DNA. ConcanavalinAlexa-633 (Molecular Probes, Invitrogen, final concentration: 0.1 mg ml^{-1}) was used for staining lectins of the EPS sugar residues of the biofilm matrix.

The cells were examined with a confocal laser scanning microscope (Axioplan 2 Imaging LSM 510, Zeiss) at wavelength of 488 and 632 nm for Syto BC and ConcanavalinAlexa-633, respectively. The micrographs were recorded and analyzed with the LSM Image examiner (Zeiss, version 4.0.0.2).

Statistical analysis of samples

In general, the mean values and standard deviations (SDs) for the OD_{600}, polysaccharide, protein and viable cell counts were determined for each sampling day. An exception was the first experiment of the repeatability tests where neither cell number nor polysaccharide quantification was done.

Determination of the repeatability of the bioprocess

Spatial variability within the bioreactor

Data sets for each parameter of all five experiments with smooth and rough coupons were checked for normal distribution using Kolmogorov–Smirnov ($\alpha = 0.05$). It is essential for ANOVA-2 analysis that the sample size for each reactor experiment is the same. In case a coupon was lost during harvesting and consequently no data could be obtained, the mean of the samples with the same position on other holders in the reactor was taken as a value. However, this procedure had to be done only in the case of three coupons, two smooth and one rough.

To determine the homogeneity of the growth conditions along the vertical axis, one holder of each reactor experiment was analysed from top to bottom (position 4 to 18) for smooth and rough coupons ($n = 3$–7 and 3, respectively). The presence of a vertical gradient was tested using the linear regression model ($\alpha = 0.05$). For regression analysis outliers were determined applying the Grubbs test.

Homogeneity of growth conditions within bioreactors was analyzed for five bioprocesses using two-way analysis of variance without repetition (ANOVA-2, $\alpha = 0.05$). Eventually, $\log_{10}$ transformation was applied to achieve normal distribution of the parameters. The total variability was split into the three parameters: vertical positions, horizontal positions and residual error that includes undefined parameters such as handling or cultivation. For the analysis of the vertical position and for the horizontal position three times six coupons were evaluated.

Repeatability of biofilm formation

To test repeatability of biofilm formation five independent reactor experiments with *R. mucilaginosa* were conducted. The biofilm samples were analyzed as previously described. ANOVA-2 with repetition was performed in order to determine the source of variation ($\alpha = 0.05$, $n = 18$). The total variability was split into 'position' and 'repeatability', 'interaction' and residual error. Missing values ($n = 2$ per analysis) were replaced by the mean of the values measured at the same position on the five remaining holders. The Levene test was used to test the homoscedasticity of each single reactor experiment. In the case of variance equality, one-way ANOVA (ANOVA-1) was used to test average equality of each repetition. Under unequal variance conditions the results of the Brown–Forsythe test was considered.

Results and discussion

Temporal development of biofilm formation and influence of roughness

Temporal development of the yeast biofilms on smooth and rough PP surfaces was determined over a period of 2 weeks. In general as observed in CLSM micrographs (Figure 2) *R. mucilaginosa* colonized the rougher test coupons significantly better than the smooth ones, which is in accordance with previous studies that showed that microbial cells prefer rough surfaces for attachment (Quirynen et al. 1991; Muller et al. 2007) because cracks provide a protection from the shear forces (Zottola and Sasahara 1994; Palmer et al. 2007). The microscopic observations were confirmed by biofilm quantification (Figure 3). The number of living cells was significantly lower on smooth coupons compared to rough ones (6×10^6 *vs* 4×10^7 CFU cm^{-2}, respectively) and did not change significantly over the cultivation period (Figure 3a). The decrease in cell number and protein on the rough coupons observed on day 3 was due to sampling errors (ie additional immersion of the coupons in the cultivation medium as the coupon holder was blocked during sampling). The protein content on the smooth coupons remained stable over the entire cultivation period, whereas the median increased regularly on the rough coupons together with the variability (Figure 3b). The total amount of polysaccharides increased regularly together with the variability between samples. This increase was more marked for the rough coupons (Figure 3c). It was also observed that after day 6, when the rotation of the bioreactor was stopped for harvesting the coupons, parts of the biofilm detached from the rough coupons on the different holders.

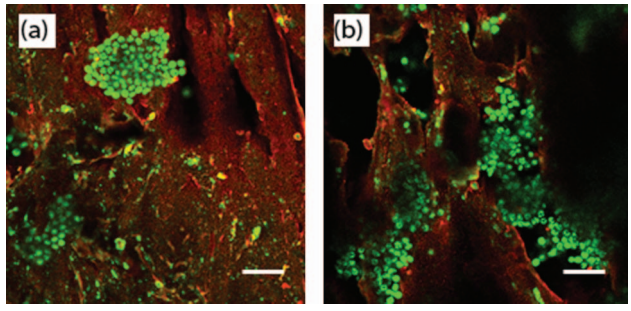

Figure 2. CLS micrographs of 1-day old biofilms on (a) smooth and (b) rough coupons. Two types of staining were used for EPS (ConcanavalinA, red) and cellular DNA (Syto BC, green). The dark red signal originated from polypropylene. Most of the cells were gathered around small scratches that increased the overall surface area for attachment and also protected the cells from shear forces. Scale bar = 20 μm.

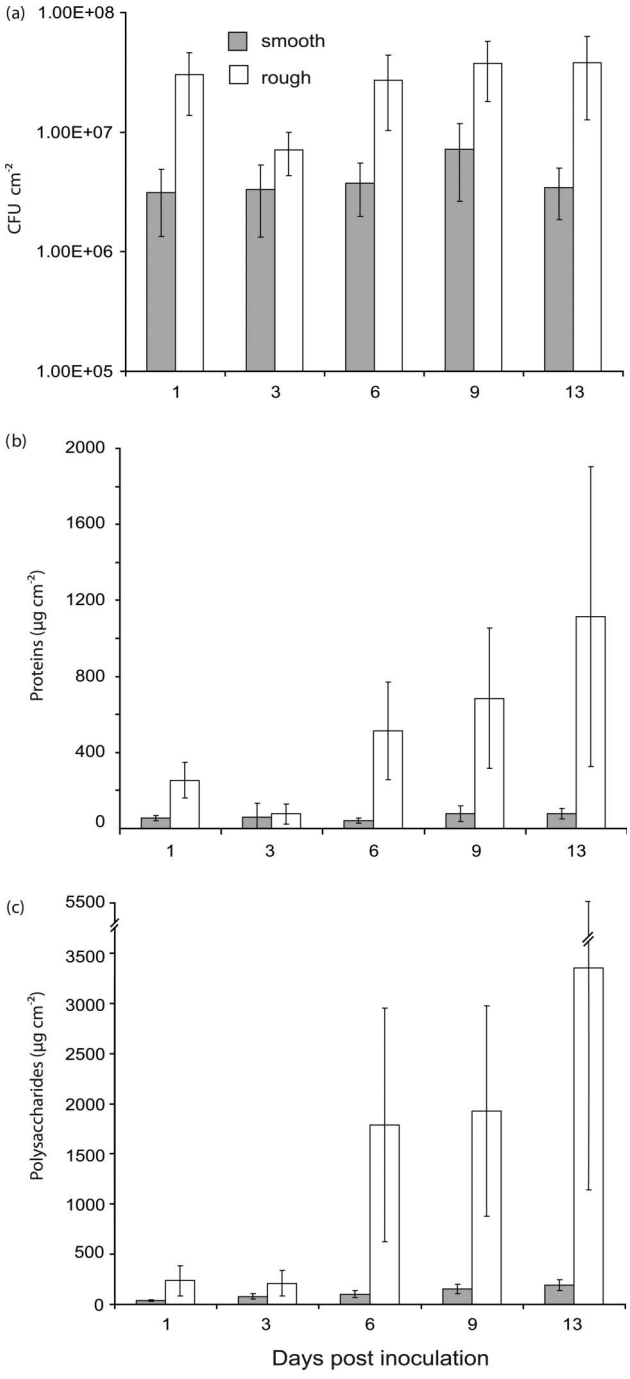

Figure 3. Time course experiment with *R. mucilaginosa* over 13 days post inoculation. Mean values and SD of (a) the colony forming units (CFU) cm^{-2}, (b) amount of proteins cm^{-2}, and (c) amount of polysaccharides cm^{-2}. ■: smooth coupons, □: rough coupons.

In order to achieve reproducible biofilm coverage on coupons, random events like erosion or sloughing should be limited. In the present experiments, sloughing was observed when the biofilm thickness increased and led to a higher flow resistance. This is a random event, which creates heterogeneity within the biofilm and does not lead to a reproducible biofilm (Lewandowski et al. 2004). Therefore, it was important to define the optimal surface and time period to harvest the produced biofilm before it started to detach. Figure 3 shows that reproducible biofilm coverage was achieved after 3 days and 6 days for the rough and smooth coupons, respectively. Further experiments were performed with the smooth coupons because the process of roughening increased the variability of the surface material. Prolongation of the bioprocess up to 6 days on the smooth coupons also offered the possibility of studying the different phases of biofilm development. Thus, in subsequent experiments biofilms were grown on smooth coupons for a period of 6 days.

Spatial variability within the reactor

Biofilms produced in five independent reactor experiments were harvested, sampled, and quantified on day 6. For all tested parameters in all experiments the largest source of variability was the vertical position (ie variability between the coupons located on the same sample holder) which was 24–83% within the reactor compared to the horizontal position (ie variability among the 6 sample holders) and 1–34% in a reactor (Table 1). However, the residual error contributed significantly to the overall variability (10–74%).

The extent of the vertical gradient present in the bioreactor was illustrated for the protein content on the smooth (Figure 4) and rough coupons (see Figure 4, Supplementary data [Supplementary material is available *via* a multimedia link on the online article webpage]). Analysis of the biofilms on the smooth coupons with linear regression revealed that the vertical gradient was significant ($p < 0.05$) only for the reactor experiment No. 2 for protein (Figure 4), optical density and viable cell counts. For the rough coupons (see Figure 3, Supplementary data [Supplementary material is available *via* a multimedia link on the online article webpage]) no significant vertical gradients were detected. For the remaining experiments and for the rough coupons the vertical gradients were not significant (Figure 4).

The presence of horizontal and vertical gradients with respect to the thickness of the biofilm was reported for the rotating annular reactor (RAB reactor) (Gjaltema et al. 1994; Neu and Lawrence 1997). By contrast, CDC reactors appear to depict no significant spatial gradient (Goeres et al. 2005). One possible explanation is that the distance between the three coupons of the CDC reactor was relatively small (*ca* 5 cm), whereas in the present system the whole

Table 1. Summary of results for biofilm accumulation at day 6 post inoculation for smooth coupons and distribution of the source of variability within a reactor experiment.

Parameter	Components of total variability	Exp. 1	Exp. 2	Exp.3	Exp. 4	Exp. 5
OD_{600}	Average value	0.07	0.05	0.06	0.04	0.06
	SD	0.02	0.03	0.03	0.02	0.02
Components of total variability	Vertical (%)	42.7	30.0	82.6	27.1	25.1
	Horizontal (%)	15.9	4.7	7.9	1.3	1.1
	Residual error (%)	41.4	65.4	9.5	71.6	73.9
Proteins	Average value (μg cm^{-2})	85.0	87.1	77.7	57.0	82.6
	SD (μg cm^{-2})	23.1	43.2	36.9	38.1	24.7
Components of total variability	Vertical (%)	35.5	25.1	73.7	38.9	24.1
	Horizontal (%)	10.0	3.7	5.3	2.4	29.4
	Residual error (%)	54.5	71.2	21.1	58.7	46.5
Polysaccharides	Average value (μg cm^{-2})	n. d.	253.9	189.9	127.4	166.1
	SD (μg cm^{-2})	n. d.	175.5	99.1	38.7	69.2
Components of total variability	Vertical (%)	n. d.	25.6	43.3	27.1	30.2
	Horizontal (%)	n. d.	4.0	8.0	6.3	30.4
	Residual error (%)	n. d.	70.4	48.7	66.6	30.4
CFU	Average value (CFU cm^{-2})	n. d.	1.0E+07	5.1E+06	4.8E+06	6.1E+06
	SD (CFU cm^{-2})	n. d.	9.8E+06	3.3E+06	2.7E+06	4.2E+06
Components of total variability	Vertical (%)	n. d.	39.1	25.7	28.7	38.6
	Horizontal (%)	n. d.	5.4	33.9	11.8	2.2
	Residual error (%)	n. d.	55.5	40.4	59.5	59.2

Notes: The variance components are shown as percentages of the total variability; n. d. not determined.

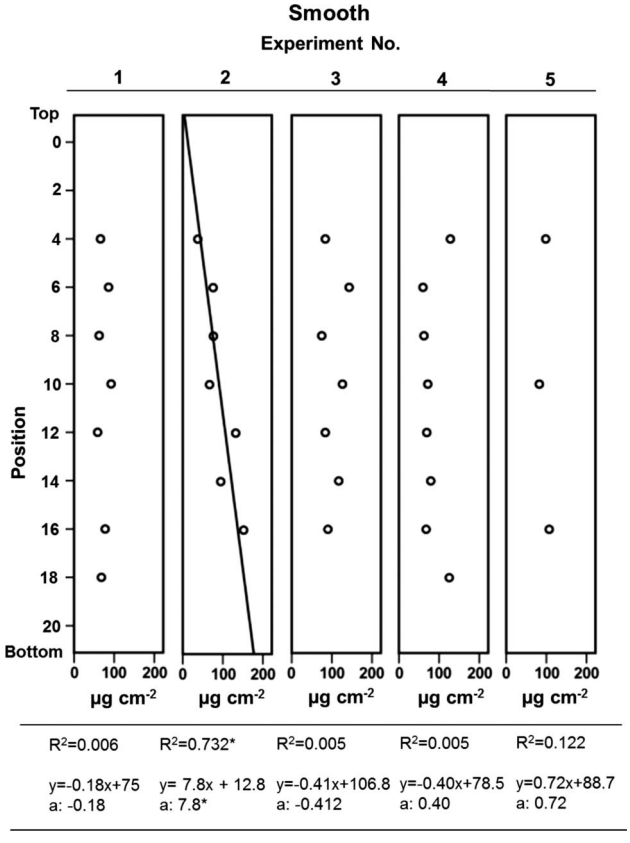

Figure 4. Vertical distribution from top to bottom of the protein amount for the five independent experiments on smooth coupons. Linear regression models with significance of R^2 and slope are indicated below ($\alpha = 0.05$), (*): significant.

length of coupons in a row was 14 cm and in the RAB 10–15 cm (Lawrence et al. 2000; Milferstedt et al. 2006). A possibility to prevent sedimentation and the formation of thick biofilm at the bottom of the reactor would be to increase the shear flow or turbulence. In the present system the mixing was performed by the rotation of the cylinder holding the test coupons and by the flow caused by the external aeration loop.

Repeatability of the bioprocess

The amount of biofilm on the smooth coupons of each reactor experiment was quantified and displayed in Figure 5. The biofilm characterized by quantification of the amount of protein and polysaccharides, viable cell count, and optical density was similar from one experiment to another for both the smooth and rough coupons. The medians of the tested parameters (OD_{600}, protein and polysaccharide amount and viable cell count) varied between reactor experiments, but the values for the $n = 4$–5 experiments always overlapped, none being completely different from another experiment. Also the ranges (minimal to maximal) of the values for the single reactor experiments were similar except for reactor No. 3 which also showed the largest variability between the coupons.

Taking all reactor experiments ($n = 5$) for the smooth coupons into account, the mean of the biofilm of each reactor experiment was statistically different from one run to another (eg ANOVA-1 for polysaccharide amount p value: 0.008) except for the

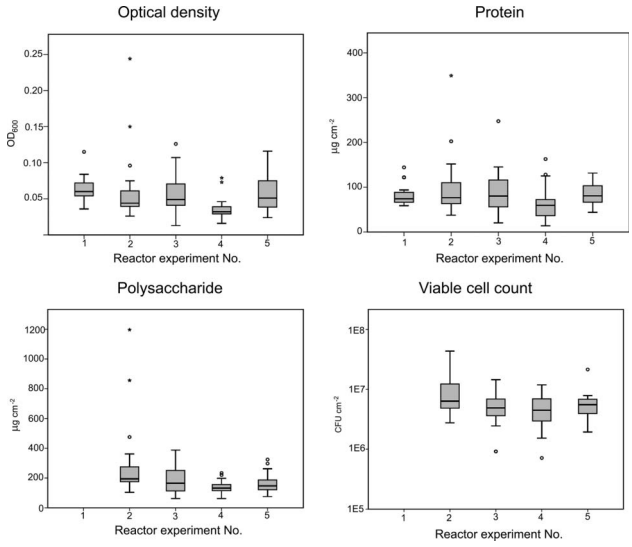

Figure 5. Box plot analysis of biofilm formed on smooth coupons. For 4–5 independent experiments OD_{600}, protein, polysaccharide, and viable cell counts were quantified. Whiskers: maximal and minimal values, bold line: median, ○: outlier, *: extreme values.

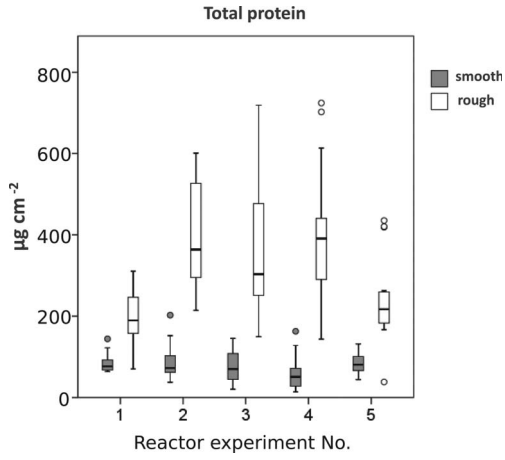

Figure 6. Box plot of total protein content of biofilm. Smooth (■) and rough PP (□) coupons (μg cm^{-2}) were used for five independent experiments with (n = 18–23) coupons per reactor. Whiskers: maximal and minimal values, bold line: median, ○: outlier, *: extreme values.

protein amount (p value: 0.066). The difference from the minimal mean to the maximal mean on the smooth coupons of the five independent reactor experiments was 45% for OD, 38% for protein, 52% for polysaccharide and 50% for CFU. The reactor experiment which showed the largest difference was reactor experiment No. 4 (Figure 5). For protein and OD the differences in the remaining reactor experiments did not exceed 20%, while for polysaccharide and CFU the minimal difference was still around 35% and 42%, respectively. The larger differences for polysaccharide and CFU could be mainly the result of the several handling and dilution steps of the analytical method.

Interestingly, the distribution of the medians and the single values were larger for the rough coupons than for the smooth coupons, indicating heterogeneity of biofilm formation probably due to sloughing (Figure 6). It can also be assumed that due to the increased surface area (Katsikogianni and Missirlis 2004; Palmer et al. 2007) and attachment possibilities the cells could establish biofilm faster on the rough coupons. This could consequently lead to earlier sloughing events. To compare the mean of the smooth and the rough coupons a T-test was conducted. It revealed that the biofilm was statistically different ($p < 0.000$) for the smooth and rough coupons.

The variability was analyzed using ANOVA-2 with 'repeatability' (between experiments) and 'position' (in-between experiments) as sources of variability (Table 2). A main source of variability was 'repeatability' ranging from 13–21% (smooth) and 12–34% (rough) of the total variability. The parameter 'position' had less influence on the total variability, ranging from 3–4% (smooth) and 2–7% (rough) of the total variability. However, the main source of variability was a 'residual error' that could be due to other undefined parameters such as harvesting, handling or the method of measurement that represented 71–75% and 54–74% for the smooth and rough coupons, respectively.

Table 2. ANOVA-2 of results for biofilm accumulation at day 6 post inoculation for smooth and rough coupons. All parameters were $\log_{10}$ transformed.

	OD_{600}	Proteins	Polysaccharides	CFU
Smooth coupons				
Repeatability (%)	21.2	17.9	18.9	13.2
Position (%)	2.6	4.4	4.4	9.2
Interaction (%)	3.7	2.2	5.9	2.9
Residual error (%) (handling, etc.)	72.5	75.5	70.9	74.6
Rough coupons				
Repeatability (%)	12.1	34.3	18.8	21.6
Position (%)	2.2	2.9	6.7	4.1
Interaction (%)	12.2	8.4	13.3	2.4
Residual error (%) (handling, etc.)	73.6	54.4	61.2	71.9

Note: The variance components are shown as percentages of the total variability.

Pitts et al. (2001) reported that the among-experiment variability contributed the least to the total variability. In experiments without chlorine treatment the within-reactor variability was about 60 and 73%, respectively, while the among-reactor variability was 40 and 27%. In our study most of the variation from

one to another reactor experiment was observed to come from the remaining variability (residual error)/parameters (eg handling). Therefore finding the most appropriate method for biofilm quantification resulting in smaller residual errors is crucial for testing repeatability.

Influence of the supporting material

Wettability and surface composition

Plasma treatments were used to investigate the influence of wettability and surface composition on fouling. Non-treated PP coupons (rough, smooth and dcPP) had water contact angles of *ca* 90°. Plasma treatments led to a similarly significant increase in hydrophilicity that remained after both autoclaving and rinsing the SDB films (Table 3). The coupons were exposed to 30% SDB to simulate the conditions within the reactor as previously described in the *in situ* sterilization protocol. All surfaces contained an elevated number of N and O atoms. The surface composition remained stable after the sterilization process except for a few changes in the amount of O and N atoms (Table 4). To evaluate the attachment of cells and further biofilm growth on the plasma treated surfaces, the coupons were harvested after 1, 3 and 6 days. The initial cell attachment as well as the early fouling (day 3) were similar for all plasma treated surfaces (Figure 7). After 6 days some differences could be observed, eg Ar/O_2 treated coupons were significantly less fouled than CO_2/C_2H_4 plasma treated surfaces (Figure 7). NH_3/C_2H_4 based polymers have already been described to enhance cell adhesion due to the high content of amino groups (Truica-Marasescu and Wertheimer 2008). For example, the attachment of mouse fibroblasts was significantly increased on NH_3/C_2H_4 treated poly(L-lactide) material compared to untreated material (Wan et al. 2003). Similarly, oxygen-based functional groups have been reported to enhance cell attachment (Wei et al. 2007) under static or low shear conditions. In the present experiments, these two types of plasma-based surface modifications did not enhance the attachment of cells although the surfaces were highly hydrophilic.

The influence of shear forces can be excluded because the smooth and roughened coupons showed significant biofilm formation under identical growth conditions. It seems that cell adhesion is enhanced only in the case of mammalian cells, which agrees with other reports where surfaces were modified using plasma polymerization techniques. For bacterial cells, the plasma-coating technique was applied to incorporate antimicrobials and other toxic compounds to prevent microbial attachment (Jansen and Kohnen 1995; Sen et al. 2009).

Relevance of the study

Standardized biofilms are essential to develop test systems to assess the efficacy of the methods of biofilm removal. Currently, limited data are available

Table 3. Static water contact angle measurement of plasma treated coupons before and after autoclaving ($n = 3$).

Treatment	Ratio	Before autoclaving	Autoclaved in SDB	Autoclaved in SDB and rinsed
None	–	~90°	n. d.	n. d.
CO_2/C_2H_4	2:1	55° ± 1°	3° ± 3°	41° ± 4°
CO_2/C_2H_4	6:1	54° ± 3°	Flat film	37° ± 3°
NH_3/C_2H_4	1:1	53° ± 2°	Flat film	36° ± 9°
NH_3/C_2H_4	2:1	54° ± 2°	Flat film	31° ± 5°
Ar/O_2	–	61° ± 2°	31° ± 4°	51° ± 1°
N_2	–	53° ± 2°	16° ± 9°	43° ± 4°

Note: n. d.: Not determined.

Table 4. X-ray photoelectron spectroscopy measurements of differently treated dcPP coupons before and after autoclaving.

	Before autoclaving			After autoclaving[a]		
	Relative atomic composition (%)			Relative atomic composition (%)		
Treatment	[C]	[O]	[N]	[C]	[O]	[N]
None	98	2	0	n. d.	n. d.	n. d.
CO_2/C_2H_4 (2:1)	79	20	1	75	19	6
CO_2/C_2H_4 (6:1)	78	22	0	76	19	5
NH_3/C_2H_4 (1:1)	70	14	16	70	17	12
NH_3/C_2H_4 (2:1)	72	13	15	71	16	13
Ar/O_2	82	15	3	83	12	5
N_2	75	11	14	80	12	8

Notes: [a]In presence of 30% Sabouraud dextrose broth and rinsing with nanopure water; n. d.: not determined.

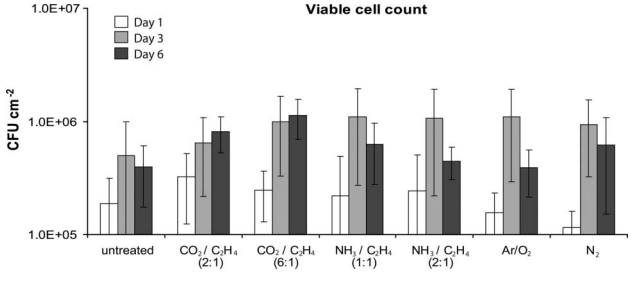

Figure 7. Cell attachment and biofilm formation by *R. mucilaginosa* on dye-cast rough PP coupons with differently treated plasma-based modifications. Sampling occurred after 1, 3, and 6 days post inoculation with $n = 4$ coupons per treatment.

regarding yeast biofilms and these studies have focussed on *C. albicans*. A 'new', up-coming group of opportunistic pathogens are *Rhodotorula* species that have caused fungemia in neonates and other immunocompromised individuals (Duggal et al. 2011).

In this study a reactor system was presented that allowed growth, sampling and quantification of *R. mucilaginosa* biofilms at different stages of development. This bioprocess made use of a commercially available laboratory fermenter with a custom-made rotating cylinder harbouring holders for test coupons. The bioreactor combined features of the CDC biofilm reactor (Donlan et al. 2002; Goeres et al. 2005) and the RAB reactor (Lawrence et al. 2000) and presented distinct advantages over other systems such as continuous exchange of growth medium or the possibility to conduct *in situ* sterilization, ie the cultivation medium together with the test coupons can be sterilized within the reactor. *In situ* sterilization of the medium reduced the risk of contamination. Due to integrated temperature and pH controls, the overall handling of the system is facilitated and repeatability of the bioprocess increased. In contrast to the CDC biofilm reactor and the RAB, this design allows a relatively large sample number of up to 120 coupons per bioprocess. This leaves more possibilities for designing of experiments, eg testing cell attachment onto different types of materials (silicone to mimic medical catheters or stainless steel for food industrial purposes) or surface treatments during a single experiment. A further advantage of this system is the ability to follow the development of biofilm formation by sampling and analysis of the biofilms at different time points.

The development of a standardized model biofilm enables the determination of cleaning, removal, and killing efficiency of mechanical procedures and chemical agents (Gattlen et al. 2010). With the possibility of sampling the biofilms at any phase of their development, antimicrobial studies can be performed with young, initially mature or mature biofilms. The antimicrobial action or efficacy of other chemical agents can be determined and their concentration-dosage effect can be adjusted against yeast or specifically against *R. mucilaginosa*.

Treatment with antimicrobial agents has to result in an at least a 4-log reduction of cell number for a fungicidal activity (DIN EN 1275; European Committee for Standardisation 1997), while >5-log reduction of bacterial CFU is required (DIN EN 1040; German Institute for Standardization 1997). Similarly, a 4-log reduction in cell numbers may be adequate for biofilm removal tests even though no requirements for these tests are currently available in international standards. With the test system described herein, biofilms of up to 10^7 cells cm^{-2} could be grown after 6 days. Considering that the detection limit for CFU is about 10 cells cm^{-2}, the biofilm produced should consist of $> 10^6$ yeast cells cm^{-2} in order to enable the determination of up to 5-log a removal efficiency.

Conclusions

The aim of this study was to produce a model biofilm with *R. mucilaginosa* for testing the removal efficiency of washing devices (eg household washing machines). A model was developed to form biofilms that were stable with a sufficient amount of viable cells, protein and polysaccharides. In this study yeast biofilms were grown in a repeatable manner in a modified bench-top bioreactor after 6 days. Yeast biofilms grown on smooth PP surfaces were similar in terms of the amount of organic matter and viable cell number in all five independent reactor experiments. Moreover, it was demonstrated that surface modification (roughening) increased the surface area for attachment but concomitantly also increased the variability of all measured parameters compared to the smooth coupons. This study also demonstrated that neither roughness alone nor the surface hydrophilicity is decisive for cell attachment and consequent biofilm formation by *R. mucilaginosa*. However, for yeast biofilms and their cultivation, more fundamental knowledge needs to be acquired. In particular, yeast cells do not have completely identical biofilm formation behaviour as bacteria. Also cell attachment, cell–cell communication and expression profile during biofilm formation are still largely unknown. Therefore, the relevance and benefit of yeast biofilms need to be further explored.

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