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Adhesion of anaerobic beer spoilage bacteria *Megasphaera cerevisiae* and *Pectinatus frisingensis* to stainless steel

Milan Bittner [a], Angelica Cristina de Souza [b], Martina Brozova [a], Dagmar Matoulkova [c], Disney Ribeiro Dias [d], Tomas Branyik [a, *]

[a] Department of Biotechnology, University of Chemistry and Technology Prague, Technicka 5, 166 28 Prague, Czech Republic
[b] Department of Biology, Federal University of Lavras (UFLA), Campus Universitário, 37.200-000, Lavras, MG, Brazil
[c] Department of Microbiology, Research Institute of Brewing and Malting, Lipova 15, 120 44 Prague, Czech Republic
[d] Department of Food Science, Federal University of Lavras (UFLA), Campus Universitário, 37.200-000, Lavras, MG, Brazil

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ABSTRACT

Although adhesion of acetic and lactic acid bacteria and yeasts have been extensively studied in a wide range of experimental and theoretical approaches, no attention has been paid to adhesion of anaerobic beer spoilage bacteria to solid materials. This work focuses on physicochemical aspects of adhesion of *Megasphaera cerevisiae* and *Pectinatus frisingensis* to stainless steel. The results, based on experimental characterization of surface properties, contact angle and zeta potential measurements, are used in modeling the surface interactions (thermodynamic and colloidal models) resulting in a quantitative prediction of interactions. The model predictions are compared with experimental adhesion tests in order to identify physicochemical forces controlling adhesion. The results revealed that the most significant adhesion occurred at a low ionic strength (10 mM) and an acidic to neutral pH. Under these conditions cell/stainless steel interactions were more pronounced for *M. cerevisiae* than for *P. frisingensis* and were influenced mostly by electrostatic attractions between surfaces. Overall, the lowest rate of adhesion between cells and stainless steel was observed at a low ionic strength and an alkaline pH. At high ionic strength (500 mM), adhesion was more pronounced for *P. frisingensis*, demonstrating the importance of Liftshitz-van der Waals interactions.

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1. Introduction

It has been claimed that improvements in processing technology, especially the reduction in oxygen levels in packaged beer and replacement of bottle pasteurisation by microfiltration, has enabled growth of strictly anaerobic bacteria in beer (Vaughan, Sullivan, & van Sinderen, 2005). Therefore anaerobic bacteria have attracted significant attention in recent decades. Gram-negative, strictly anaerobic beer-spoilage bacteria are currently identified as belonging to the genera *Megasphaera*, *Pectinatus*, *Selenomonas*, or *Zymophilus* (Juvonen, Koivula, & Haikara, 2008; Vavrova, Matoulkova, Balazova, & Sedo, 2014). Phylogenetic analyses placed these bacteria in class *Negativicutes* of the class *Clostridia* in the phylum *Firmicutes* (Marchandin et al., 2010). It was estimated that the taxons responsible for ca. 30% of all spoilage incidents of finished and packaged beer worldwide were *Pectinatus* and *Megasphaera* (Vaughan et al., 2005).

There are three species in the *Pectinatus* genus associated with beer spoilage: *P. cerevisiiphilus*, *Pectinatus frisingensis* and *P. haikarae*. *Pectinatus* mainly spoils unpasteurised beers by producing turbidity and the smell of rotten eggs (H_2S) (Suzuki, 2011). The beer spoilage group *Megasphaera* includes three species *Megasphaera cerevisiae*, *Megasphaera paucivorans* and *Megasphaera sueciensis*. *Megasphaera* mainly affects unpasteurised low-alcohol beers by producing C5 and C6 fatty acids, H_2S and turbidity. Both genera (*Pectinatus*, *Megasphaera*) have comparable tolerances to hop bitter acids (Back, 2005; Noack, Knödl, & Lachenmeier, 2008).

The occurrence of beer-spoilage *Pectinatus* and *Megasphaera* is largely unknown outside of the beer brewing environment, while they appear to be common inhabitants of biofilms in brewery bottling halls (Matoulková & Kubizniaková, 2014; Suzuki, 2011). Because of this, knowledge of the adhesive properties of *Pectinatus*

* Corresponding author.
E-mail address: tomas.branyik@vscht.cz (T. Branyik).

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and *Megasphaera* species to different solid materials (filling and packaging machinery, pipelines, conveyor belts etc.) could help to identify risks associated with biofilm deposition. One of the most common construction materials in brewery bottling halls is stainless steel.

Biofilm formation is a complex and dynamic process, for which the initial adhesion of cells to solid surfaces is a prerequisite (Simoes, Simoes, & Vieira, 2010). Cell-to-solid surface adhesion is influenced by the surface properties of the interacting entities, their motility and environmental characteristics (nutrient availability, ionic strength, pH, shear stress etc.) (Diao, Taran, Mahler, & Nguyen, 2014). In order to understand and evaluate the intensity of cell-solid surface interactions, different physicochemical models such as thermodynamic model (van Oss, 1995), Derjaguin-Landau-Verwey-Overbeek (DLVO) (Bos, van der Mei, & Busscher, 1999) and extended DLVO theory (van Oss, 2003) can be applied.

The goal of this work was to characterize the surface properties of *M. cerevisiae* DSM 20461 and *P. frisingensis* DSM 20465 and to study their interactions with highly corrosion-resistant stainless steel (AISI 316L), both experimentally and theoretically, using physicochemical models. Actual adhesion experiments were compared with model predictions of XDLVO theory in order to identify the main driving forces of cell adhesion.

2. Materials and methods

2.1. Microorganisms and cultivation

The anaerobic beer spoiling bacteria *M. cerevisiae* DSM 20461 and *P. frisingensis* DSM 20465 used in this study were obtained from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). Both microorganisms were cultivated in 52.2 g/L MRS broth medium (Merck, Darmstadt, Germany) with a typical composition (g/L): peptone from casein 10.0, meat extract 8.0, yeast extract 4.0, D(+)-glucose 20.0, dipotassium hydrogen phosphate 2.0, Tween 80 1.0, diammonium hydrogen citrate 2.0, sodium acetate 5.0, magnesium sulfate 0.2, manganese sulfate 0.04. The MRS broth was further supplemented with (g/L) (R)-(+)-Cysteine hydrochloride hydrate 0.25 and sodium thioglycolate 0.25. Cultivations were carried out in microbiology test tubes (10 ml of medium in each tube), which, after autoclaving, were cooled in an anaerobic incubator (30 °C, 12 h, N_2 atmosphere). After inoculation (1 ml of inoculum) subsequent cultivations were also carried out in an anaerobic incubator (30 °C, 24 h, N_2 atmosphere).

2.2. Surface characterization of cells and stainless steel particles

The surface properties of stainless steel particles (SSP) (AISI 316L, 60–80 nm, SkySpring Nanomaterials, Inc., TX, USA) in the form of compressed pellets were characterized by contact angles (CA). Pellets were prepared from 1 g of SSP by pressing (7 MPa, evacuable pellet press 13 mm, Pike Technologies). The surface properties of cells and SSP, in the form of layers on membrane filters, were also characterized by CA. In order to create flat layers, the cells and SSP were deposited on a filter (cellulose nitrate membrane, 0.45 μm pore size, 47 mm diameter, Whatman, Germany) under negative pressure. The cell suspension was highly concentrated (cell concentration determined using a Bürker chamber, Brand GmbH, Wertheim, Germany) in order to obtain 7×10^6 cells/mm^2 on the filter. The bacterial lawns were then deposited on agar plates to stabilize their moisture content, fixed on a microscopy glass slide, and allowed to dry for 40 min at 25 °C (Sharma & Hanumantha Rao, 2002). The CA measurements on cell/SSP lawns and SSP pellets were carried out by the sessile drop technique (volume ≈ 3 μL) using a CAM 200 goniometer (KSV Instruments, Finland). Measurements were performed at 25 °C with three test liquids (water, formamide, 1-bromnaphthalene), readings were taken after 0.5 s of deposition, and each sample was tested ten times with each test liquid.

The zeta potentials (ZP) of cells and SSP in contact with electrolyte (10 and 500 mM KCl, pH 3–12) were measured at 25 °C using a Zetasizer Nano-ZS (Malvern, UK). The cell and nanoparticle suspensions had an absorbance of 0.1 (600 nm). All samples were measured three times with an experimental error ±10%.

The size distribution of SSP (4950 readings per sample) in contact with electrolyte was determined microscopically (Nikon Eclipse E400, Japan) using image analysis software (NIS-Elements).

2.3. Thermodynamic and XDLVO model calculations

The total surface tension and its components were calculated from CA employing the extended Young's equation. The values of free energy of interactions between cells and SSP in water were calculated according to van Oss (1995). The extended DLVO (XDLVO) theory was used to model interactions between SSP and anaerobic beer spoilage bacteria (van Oss, 2003). Simulations according to the XDLVO theory were carried out in symmetrical electrolytes (KCl, 10 and 500 mM), the Hamaker constant was estimated from the ΔG^{LW}_{mwp} values (Table 2), the characteristic decay length for acid-base (AB) interactions was 0.6 nm (van Oss, 2006, chap. 7), and the intensity of AB interactions was expressed using ΔG^{AB}_{mwp} values (Table 2). For calculations of $\Delta G^{LW/AB}_{mwp}$ values, CA data for compressed pellets were used (Table 1).

2.4. Oxygen resistance test

Aliquots of *M. cerevisiae* or *P. frisingensis* suspensions grown in MRS broth medium under anaerobic conditions were transferred to 10 mM KCl (pH 7) to form a suspension with an absorbance (600 nm) of 0.6. The suspension was constantly aerated and the dissolved oxygen concentration (6.2 ± 0.8 mg/L) was monitored (InPro 6800, Mettler Toledo, Switzerland). Samples were taken to determine the intracellular ATP content with a control kit (CCK-4, Hygiena, UK) and luminometer (Pi-102, Hygiena, Germany). Similarly, the effect of dissolved oxygen on ZP of cell envelopes was monitored with a Zetasizer Nano-ZS (Malvern, UK).

2.5. Adhesion tests

Adhesion between SSP and microorganisms was tested in defined model environments similar to a previously described procedure (Prochazkova, Podolova, Safarik, Zachleder, & Branyik, 2013). Cell suspensions (10 ml) of a defined concentration (0.3 ± 0.1 g/L) in de-aerated electrolyte (10 and 500 mM KCl, pH 3–12) were mixed (15 rpm, orbital mode, Hulamixer, Invitrogen, USA) with specific amounts of SSP for 10 min in plastic test tubes. Cell-SSP aggregates were then exposed to an external magnetic field (NdFeB magnets, 25 × 10 mm, Neomag, Czech Republic) for 10 min and subsequently the absorbance of the supernatant was measured at 600 nm. Adhesion between bacteria and SSP (AI, %) was calculated as follows: $AI = [(A_0\text{-}A_1)/A_0] \times 100$, where A_0 is the initial absorbance (600 nm) of the bacterial suspension and A_1 the absorbance (600 nm) of the supernatant after separation of cell-SSP aggregates. All experiments were performed in triplicate and results are presented as mean values.

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