Physicochemical and biochemical interactions in yeast immobilization by adhesion to a cellulose based support

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An important quality of yeast cell wall is the ability to adhere to other cell walls or solid surfaces. This feature of yeast is responsible for technologically important phenomena such as flocculation at the end of beer fermentation and cell adhesion to immobilization supports e.g. spent grains, DEAE-cellulose etc. Physicochemical properties of yeast surfaces, e.g. hydrophobicity and surface charge, have a substantial impact on cell adhesion and flocculation. The interaction energies calculated according to DLVO theory and interfacial free energies were compared with yeast adhesion experiments carried out in continuous gas-lift reactor. Four different brewing yeast strains (*Saccharomyces cerevisiae*) were tested for their adhesion onto spent grain particles. The role of physicochemical surface properties in cell-cell and cell-carrier interactions was evaluated by comparing the computed predictions with experimental results. In view of the somewhat contradictory results, the importance of specific biological interactions is outlined. Preliminary results on the presence of FLO 11 gene in studied yeast stains are presented.

INTRODUCTION

Microbial cell adsorption/adhesion onto the surface of a solid porous or non-porous support material is a traditional immobilization method. On porous carriers cells accumulate mainly due to steric retention in pores and cavities. Conversely the adhesion of living cells to non-porous supports is considered to have physicochemical character (electrostatic, hydrophobic). The main advantage of cell immobilization to (non)-porous carriers consist in lower mass transfer limitation of substrates and products due to the absence of a diffusion barrier between cells and bulk liquid. The main disadvantage of this method is the risk of biofilm detachment induced by changes in cell environment (Mozes and Rouxhet, 1990).

A microorganism tends to adhere to solid surfaces to minimize the free energy of interaction with its vicinity (Chamberlain, 1992). The cell-cell and cell-carrier interactions start when two surfaces approach each other. When long range attractive forces (van der Waals) overcome electrostatic repulsive forces a weak reversible attachment between surfaces is established (secondary minimum) as a result of a favorable energetic balance. The surface interaction (adhesion) can be further strengthened through short distance forces as hydrophobic and polar interactions, hydrogen bonds and specific molecular interaction (Boonaert et al., 1999).

Various industrially important processes take advantage of the surface properties of microbial cells e.g. brewing yeast flocculation, acetic bacteria adhesion and activated sludge formation. Flocculation of brewing yeast is probably the most extensively studied system concerning cell-cell interactions (Jin and Speers, 1998). Among several models proposed to explain this phenomenon, however, the most widespread is based on calcium dependent lectin-mannan interactions (Miki et al., 1982). Nevertheless, the role of the cell surface

physicochemical properties (composition, charge, hydrophobicity) has to be taken into account too. Hydrophobic interactions have been considered by some authors the major factors responsible for the flocculation of brewing yeast (Smit et al., 1992; Straver et al., 1993), while other studies challenged their importance (van Hamersveld et al., 1994; Suzzi et al., 1994). The role of hydrophobic interactions was found crucial in bakers' yeast attachment to plastics and mat formation, requiring a fungal cell surface glycoprotein encoded by FLO11 gene. The bakers' yeast strain possessing FLO11 gene was found more hydrophobic than the ΔFLO11 strain poorly adhering to polystyrene (Reynolds and Fink, 2001).

The interaction energies calculated according to DLVO theory and interfacial free energies were compared with yeast adhesion experiments carried out in continuous gas-lift reactor. Four different brewing yeast strains (*Saccharomyces cerevisiae*) were tested for their adhesion onto spent grain particles, a brewing by-product (Brányik et al., 2001). This biocatalyst consisting of brewing yeast immobilized onto a cellulose based carrier obtained from spent grains has been successfully applied in primary fermentation of lager beer (Brányik et al., 2002) and shows interesting features in terms of carrier costs. The role of physicochemical surface properties in cell-cell and cell-carrier interactions was evaluated by comparing the computed predictions with experimental results. Preliminary results supporting the hypothesis that the physicochemical interactions can predict cell adhesion to solid support are presented.

Besides the physicochemical aspect of cell adhesion, the contribution of specific biochemical interactions will play a significant role on yeast adhesion too. In the yeast *Saccharomyces cerevisiae*, a group of structurally related, cell-wall associated proteins encoded by the FLO gene family are directly responsible for many of the cellular adhesion phenotypes displayed by this organism. The role of FLO11 gene, which encodes a cell surface protein (Flo11p), in cell-solid surface interaction was investigated by PCR method.

EXPERIMENTAL

Microorganisms and Medium. The brewing yeast strains *Saccharomyces cerevisiae* were used for this part of research work:

-Top fermenting *Saccharomyces cerevisiae* strains 128 and 7 from the collection of Research Institute of Brewing and Malting Prague, Czech republic.

-Bottom fermenting *Saccharomyces carlsbergensis* strain 96 from the collection of Research Institute of Brewing and Malting Prague, Czech republic.

-*Saccharomyces cerevisiae* CCMI 890 (Culture collection of industrial microorganism of INETI, Lisbon, Portugal) supplied by DEB Universidade do Minho Braga, Portugal.

The yeast were cultivated either in a complex medium (CM) or all malt wort (12 % w/w). The composition of CM was (in g L^{-1}): 5, KH_2PO_4; 2, $(NH_4)_2SO_4$; 0.04, $MgSO_4.7H_2O$; 2, yeast extract (Merck, Darmstadt, Germany); 20, glucose. Barrels with 50 litres of CM were sterilized by autoclaving at 121ºC, 100 kPa for 60 min. Antifoam A (Fluka Chemie, Steinheim, Switzerland) was added to CM prior to sterilization (0.05 mL). All malt wort was obtained from a local Czech brewery.

Continuous gas-lift reactor (CGLR). The reactor used in this work to study the yeast immobilization rate was of the concentric draught tube type with an enlarged top section for degassing and a total working volume of 6 L. For detailed description of CGLR see Brányik et al., 2006. The desired gas flow (250 mL min^{-1} air) was adjusted with a mass flow controller (Aalborg GFC17, Aalborg Instruments, Orangeburg, New York, USA). Dry spent grains were cleaned by acidic hydrolysis (3 vol % HCl) followed by a delignification in 2 % (w/v) NaOH. Prior to use, the carrier was washed several times with water (until neutral pH) and dried (Brányik et at., 2001). Subsequently the GLR was charged with complex medium and then

inoculated with 1.0 L of yeast cell suspension. After 24 h of batch growth the start up period with continuous CM feed was initiated. Starting and operating of GLR is described in Bányik et al., 2004.The temperature in CGLR (15°C) was maintained by a cooling coil linked with a thermostat. The CM was fed into CGLR at a dilution rate D = 0.1 h^{-1} during the whole experiment. The continuous system was considered to be in steady state conditions after a period of 5 residence times (Rt = 1/D).

Immobilized biomass determination. A sample containing approximately 0.4 g of dry biocatalyst (carrier + immobilized cells) was taken from the reactor. The bulk liquid was removed with a syringe and the carrier was washed with 2 x 100 mL of distilled water. The biocatalyst was then filtered and washed with 400 ml of distilled water on a paper filter in order to remove the components of the medium from the sample. Then the biocatalyst together with the biomass was removed from the filter, homogenized and dried at 105°C for 12 hours. An amount of approximately 0.2 g dry biocatalyst was weighed into an Erlenmeyer flask with 50 ml of 3 % (w/v) NaOH solution and was shaken at 120 rpm for 24 h. During this time the immobilized cells were completely removed from the carrier, as was verified under the microscope. The cell free carrier was filtered and after being carefully washed on the filter with 400 ml of distilled water it was dried at 105°C for 5 hours. The amount of immobilized yeast biomass was determined from the weight difference before and after the treatment with caustic. Corrections of the biomass weight for the losses of carrier itself were carried out by blank experiments with clean carrier.

Contact angle measurement. Cells were harvested by centrifugation (6 000 rpm, 8ºC for 5 minutes) and then washed with increasing concentrations of ethanol (10, 20 and 50%). The cell suspension in 50% vol. ethanol was adjusted to 1.0 g L^{-1} dry cell weight. A solution of 20 g L^{-1} of agar and 10% of glycerol was cast into a Petri dish and was allowed to solidify. An aliquot of 20 ml of the yeast suspension was spread uniformly over the solidified agar layer and was let to dry at 25°C for approximately 24 hours. Previous to contact angle measurements, base treated carrier particles were fixed on a microscopic slide by an adhesive tape. Contact angles were measured by the sessile drop technique (drop volume of ca. 3 μL) on the cell lawns and carrier particles using a contact angle measurement apparatus (OCA 20, Dataphysics, Germany). The measurements were performed at 15°C using three different liquids: water, formamide and α-bromonaphthalene. At least 20 readings of contact angles per sample were carried out for each liquid. The total surface tension (γ^{tot}) and its components (γ^{LW}, γ^{+}, γ^{-}, γ^{AB}), the values of the free energy of interaction between cells and water and the free energy of interaction between cells and carrier in water were calculated according to van Oss et al., 1995.

Zeta potential measurement. Cells were harvested and suspended in the mineral medium MM (in g L^{-1}, 5.0 KH_2PO_4, 2.0 $(NH_4)_2SO4$, 0.4 $MgSO_4$) to a concentration of 0.1 g L^{-1} dry cell weight. The acid/base treated carrier (0.5 g in dry state) was triturated in a mortar and then suspended in 100 ml of MM. The suspension of carrier particles was filtered through a polyester mesh (Estal mono PE 18, Seidengazefabrik AG Thal, Switzerland) with mesh openings of 18×18 μm. The pH of all suspensions was adjusted to vary over the range 3-6 by addition of HCl or KOH (Dengis et al., 1995). The electrophoretic mobility of the yeast from the reactor outflow was determined with Zetasizer Nano-ZS (Particle Sizer, Malvern, UK) at an applied electric field of 50 V using the Helmholtz-Smoluchowski equation at 15°C. Either the cell suspension or the carrier particles suspension was filled into the electrophoresis cell and after at least 30 electrophoretic mobility readings the average zeta potential was calculated by the apparatus automatically.

Theory. Yeast cells used in this work are charged particles with a diameter of approximately 10μm, thus they resemble colloids. Consequently, the DLVO theory (Derjaguin-Landau-Verwey-Overbeek) can be used to investigate their adhesion to solid surfaces (sphere-flat plate interaction), based on electrostatic (repulsive) and attractive van der Waals energy balances. According to Derjaguin's approximation (DA) the van der Waals interaction energy (VDW) can be calculated as:

$$U_{VDW}^{DA} = -\frac{A_H \cdot a}{6 \cdot D} \qquad [2]$$

where A_H is the effective Hamaker constant (kT) of the interacting media, a is the cell radius (m) and D is the distance of closest approach between two surfaces (m).

The repulsive energy of the electrostatic double layer (EDL) can be described by a formula derived by Derjaguin:

$$U_{EDL}^{DA} = 64a\pi\varepsilon_0\varepsilon_r\gamma_1\gamma_2\left(\frac{kT}{\nu e}\right)^2 \exp(-\kappa D) \qquad [3]$$

where γ_i is defined as $\gamma_i = \tanh(\Psi_i/4)$ and $\Psi_i = \nu e\psi_i/kT$, ψ_i - surface potentials of sphere and plate, ε_0 is the permittivity of a vacuum (8.8542×10^{-12} F m^{-1}), ε_r - dielectric constant of water at 15°C (78.54), k - Boltzmann konstant (1.38×10^{-23} J K^{-1}), T – absolute temperature (K), ν - charge number, e – electronic charge (1.6×10^{-19} C), κ - inverse Debye screening length ($\kappa = 2{,}32 \cdot 10^9 \cdot \sqrt{2I}$), I – ionic strength (0,075 M). The DLVO theory was used to calculate the changes of the total interaction energy $G_{TOT} = U_{EDL}^{DA} + U_{VDW}^{DA}$ on the separation distance between the cell surfaces. The probability of an attachment after a collision of two cells is characterized by the height of the potential barrier.

An approach based on a balance of cell-liquid, support-liquid and cell-support interfacial free energies was used to estimate whether the physicochemical surface properties of cells and support (carrier) would lead to adhesion. The total surface tension (γ^{tot}) and its components ($\gamma^{LW}, \gamma^+, \gamma^-, \gamma^{AB}$) were determined by contact (θ) measurements using Young's equation:

$$(1+\cos\theta)\cdot\gamma_L = 2\cdot\left(\sqrt{\gamma_S^{LW}\cdot\gamma_L^{LW}} + \sqrt{\gamma_S^+\cdot\gamma_L^-} + \sqrt{\gamma_S^-\cdot\gamma_L^+}\right) \qquad [4]$$

where L stands for contact angle liquid and S for the solid.

The values of the free energy of interaction for systems cell-water-cell, cell-water-support (ΔG^{tot}), and its components ($\Delta G^{LW}, \Delta G^{AB}$), were calculated according to (van Oss et al., 1995).

Microbial adhesion to solvents test (MATS). The cells from CGLR were harvested by centrifugation at 6 000 rpm, 8 ºC for 3 minutes. The immobilized cells (ca. 0.5 g biocatalyst in dry state) were removed from the carrier by vigorous mixing (600 rpm, 2 cm magnetic bar, 100 ml 150 mM NaCl) and then harvested daily by centrifugation (6 000 rpm, 8 ºC for 3 minutes). After discarding the supernatant the cells were re-suspended in 150 mM NaCl solution (pH 5.4) at a concentration of about 7 x 10^8 cells/mL. Apply the same procedure three times. At the end, the absorbance (A_0) of the suspension was measured at 400 nm wavelength ($A_0 \sim 0.5$). Then 3 mL of washed yeast suspension was vortexed for 60 seconds with 0.5 mL of the organic solvent (chloroform, hexadecane, ethyl acetate, and decane). To ensure the complete separation of the two phases the mixture was kept still for 10 min. Then 2 mL sample from the aqueous phase were removed and measured the absorbance (A) at 400 nm. The cells affinity for each solvent was calculated using the fowling equation:

$$\%\ affinity = 100 \times \left(1 - \frac{A}{A_0}\right) \qquad [1]$$

Microorganisms and isolation of their DNA. The sequence of FLO11 reveals a 4104 base-pairs open reading frame on chromosome IX in *S. cerevisiae* ∑*1278b* genetic background (Wan-Sheng et al., 1996). The presence of FLO11 gene on DNA was examined on all tested yeast strains. As the positive control of FLO11 gene presence has been considered the strain *Saccharomyces cerevisiae* BY4741 haploid, as the negative yeast *Kluyveromyces lactis* (KL CBS2359) and *Kluyveromyces marxianus* (KM CBS 3665), supplied by DEB Universidade do Minho Braga, Portugal.
Standard YPD plates were used to cultivate yeast for 2 days at 25 °C prior the isolation of their DNAs. The isolation of DNA was performed according to the method in *Current Protocols in Molecular Biology* (1997) 13.11.1-13.11.4.

PCR reaction and primers. The operational conditions of the PCR reaction in a thermocycler are listed in Table I. The following oligonucleotide primers were used to amplify the FLO11 sequence; Fprobe:5´-CACGACGGCTATTCCAACC-3´;Rprobe:5´-TTAGAATACAACTGGAAGAGCGAG-3´. The amplified PCR product corresponds to the nucleotides +3700 to +4104 of the FLO11 open reading frame.

Table I. Operational conditions in a thermocycler during PCR reaction.

		number of cycles	time	temperature
initial denaturation		1	5 min	95°C
main reaction	denaturation	30	30 s	95°C
	annealing		30 s	45°C
	elongation		30 s	72°C
final elongation		1	10 min	72°C

Determination of the PCR reaction products. The products of PCR reaction were separated using 1.7 % agarose gel electrophoresis, subsequently visualized using loading dye and DNA size marker (100-5000 base pairs, Fermentas) under UV lamp.

Other analytical methods. The size of free cells from CGLR was analyzed by an automatic image analyzer program LUCIA (Laboratory Imaging s.r.o., Czech republic) calculating the average diameter of different brewing yeast strains from the outflow of CGLR. Flocculation tests of brewing yeast strains were carried out according to absorbance method (ASBC Methods, 2006) after cultivation in all malt wort.

RESULTS AND DISCUSSION

Surface characteristics of brewing yeast cells. MATS tests based on partitioning of cells between water and solvent phase have been carried out in order to evaluate the relative surface properties of the studied yeast strains. The following pairs of solvents were used: on the one hand chloroform, an electron acceptor solvent, and hexadecane, a nonpolar solvent, and on the other hand ethyl acetate, a strong electron donor solvent, and decane, a nonpolar solvent. Due to the surface tension properties of these solvents, differences between the results obtained with chloroform and hexadecane and the results obtained with ethyl acetate and decane indicated that there were electron donor/electron acceptor interactions at the yeast cell surface and revealed hydrophobic and hydrophilic properties. The MATS results for the yeast, suspended in a 150 mM NaCl solution (pH 5.4), are displayed in Fig. 1. All yeast strains show higher affinity to chloroform (an electron acceptor solvent) than to ethyl acetate (an electron donor solvent). The differences in affinity between these two solvents were due

to Lewis acid–base interactions, i.e., electron donor/electron acceptor interactions resulting from the electron donor nature of the yeast. Results of MATS test seem to be in accordance also with the balance of interfacial free energies. These suggest that the strain 96 more readily provides nonpolar (hydrophobic) interactions as manifested both by higher affinity to nonpolar solvents (Fig. 1.) and the most favorable interaction energy (average of 2nd and 12th day) with hydrophobic carrier surface (Table III).

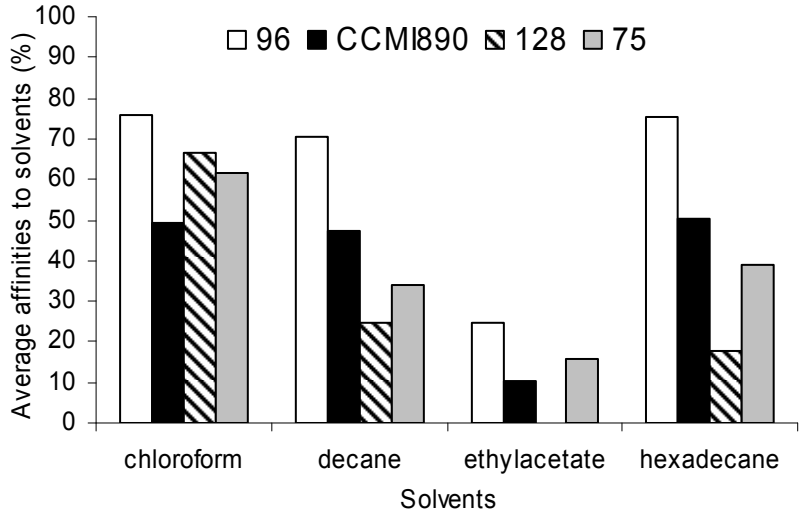

Fig. 1. Average affinities of free cells during continuous cultivation for the four solvents used in the MATS analysis (C: chloroform, HD: hexadecane, EA: ethyl acetate, D: decane).

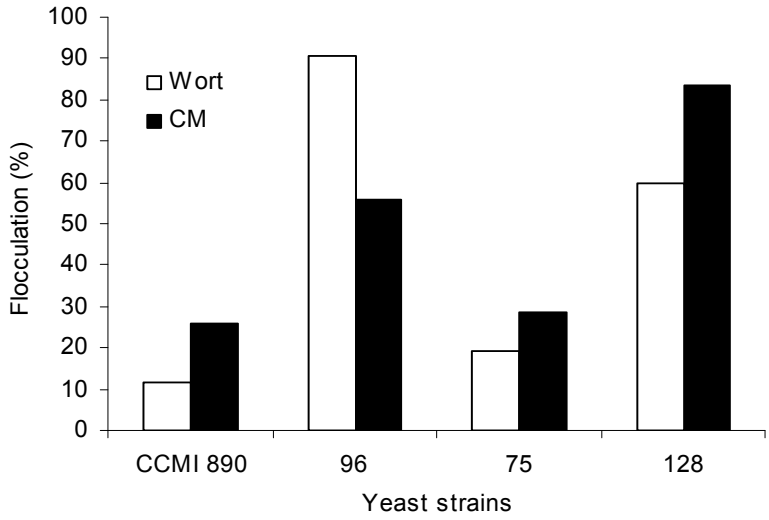

Fig. 2. Flocculation characteristic of yeast strains in stationary growth phase cultivated on brewery wort and complex medium (CM)

Difference between the studied yeast strains was found also in terms of their flocculence. After cultivation in all malt wort the strains 96 and 128 showed strong and medium flocculation ability, respectively. The flocculation values of strain CCMI 890 and 75 are ranking them among non-flocculent yeast (Fig. 2). Although experiments repeated with yeast grown on complex medium, from stationary growth phase, resulted in slightly different values, the flocculence of the strains 96 and 1287 remained the highest (Fig. 2). The same situation repeated with free cell isolated from the outflow of the continuous gas-lift reactor

during adhesion experiments. While the free cell population of the strain 96 and 128 in CGLR showed significant flocculation, cells of the strain 75 and CCMI 890 did not flocculate (data not shown).

Surface characteristics of immobilization support (spent grain particles). The contact angle measurements on the surface of solid particles required a sufficiently large and flat area that would not absorb the drop of the test liquid. While the measurements on acid/base treated carrier failed due to the highly wettable character of the carrier, in the case of the base treated spent grains there was a portion of sufficiently large and hydrophobic carrier particles allowing the placing of the test drop. The average contact angles and of the base treated spent grain particles are presented in Table II. Although the calculated surface tension values (data not shown) of the base treated carrier cannot be regarded as an average value for the overall carrier surface, they proved the presence of very hydrophobic areas and moieties on the surface of the spent grains.

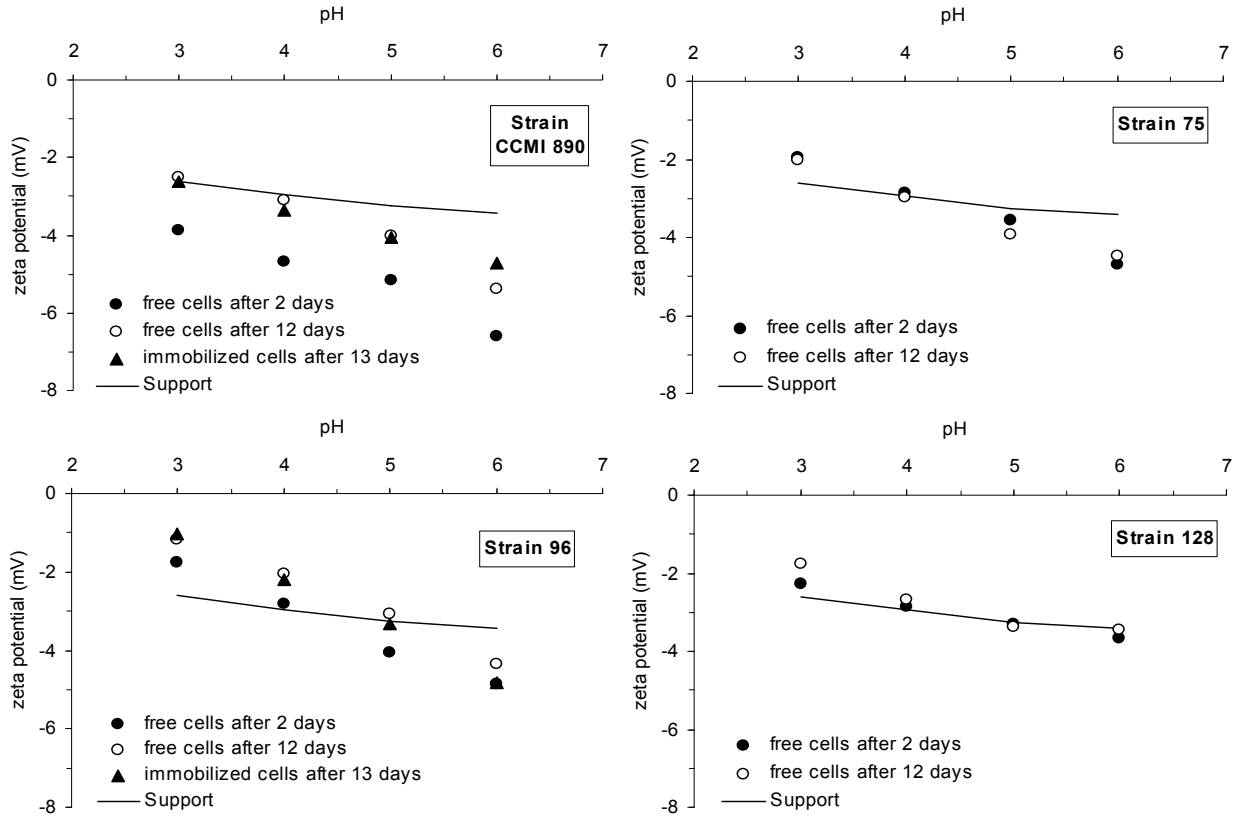

Fig. 3. Values of zeta potential as a function of pH for yeast cells and support (acid/base treated carrier particles) in mineral medium (MM).

The zeta potential of the acid/base treated spent grains particles was measured after filtering the triturated carrier powder, suspended in mineral medium (MM) through a polyester mesh with mesh size of 18×18 μm. The carrier particles, smaller than 30 μm in diameter, had a negative zeta potential in the studied pH range (Fig. 3). Apart from the physicochemical interactions between yeast cell and carrier particle surfaces, the spatial retention of yeasts in various shelters (crevices, pores, tangled threads) on the carrier surface can result in local biomass accumulations.

The DLVO (Derjaguin-Landau-Verwey-Overbeek) theory. It allows the computation of the interaction potential energy between two spherical particles (cell-cell) or between a spherical particle and a flat plate (cell-support) approaching each other by taking into consideration the dispersive and the electrostatic interactions (Bhattacharjee and Elimelech, 1997). This approach was applied here, using the Hamaker constant (0.8 kT), as estimated for biological particles in water (Hamersveld et al., 1994), ionic strength of 55 mM calculated for complex medium (Boonaert et al., 1999), and the zeta potentials of cells and spent grains particles at the pH of the continuous fermentation (pH = 4) as were found from Fig. 3. Furthermore, the cell radius (ca. 3.5 μm), as determined by image analysis, was used in calculations. The results show that at an ionic strength of a common microbial media with the corresponding zeta potentials of cells and support particles, there were no potential barriers between colliding particles to overcome (data not shown). It means that under real fermentation conditions the close contact between cells and carrier particles will not be hindered by any repulsive electrostatic interactions.

Table II. Average contact angles and respective standard deviations of three probe liquids over a lawn of brewing yeast strains and on the surface of support (base treated spent grain particles).

Strain/ Support	Probe liquid*	Free cells		Immobilized cells
		Day of fermentation		
		2	12	13
CCMI 890	W	12.4 ± 0.5	9.3 ± 0.6	8.4 ± 0.2
	F	11.7 ± 0.8	8.8 ± 1.2	10.0 ± 1.1
	BR	67.3 ± 0.5	60.3 ± 0.8	62.0 ± 0.8
96	W	21.0 ± 1.6	24.2 ± 2.6	30.7 ± 2.7
	F	17.5 ± 1.0	19.1 ± 2.8	22.3 ± 4.8
	BR	55.4 ± 1.1	52.5 ± 4.0	54.6 ± 2.0
75	W	15.9 ± 1.6	9.2 ± 1.5	-
	F	11.0 ± 0.3	9.0 ± 1.4	-
	BR	53.5 ± 1.4	65.0 ± 4.1	-
128	W	6.3 ± 0.5	5.2 ± 0.5	-
	F	6.0 ± 0.2	5.0 ± 0.5	-
	BR	59.1 ± 1.4	58.9 ± 2.1	
Support	W	83.7 ± 6.7	-	-
	F	63 ± 5	-	-
	BR	25 ± 2	-	

*W- water. F – formamide. BR - α-bromonaphthalene

Balance of interfacial free energies. Since the DLVO theory counts only with long-range forces and does not allow predictions of interaction energy to be made at short distances. Therefore, an approach based on a balance of cell-liquid, carrier-liquid and cell-carrier interfacial free energies was used to estimate whether the physicochemical surface properties of cells and carrier would lead to adhesion. It was found that the carrier contains hydrophobic particles, namely among the base treated carrier, showing very negative values of the free energy of interaction between two support surfaces in water ΔG^{tot} = -65.8 mJ m^{-2}. It can be assumed that either the outmost waxy layer of the barley husks and/or lignin, a common part of natural materials, might serve as a hydrophobic interaction site for yeast adhesion. In consequence of this, the total free energy of interaction between the brewing yeast strains and

the base treated carrier particles in water (cell-water-support) is close to negative and depends on prevailing hydrophobic or hydrophilic nature of yeast surface.

Comparing all tested strains it can be seen that according to the initial (2nd day) total free energy of interaction (ΔG^{tot}) for the system cell-water-support, only the strains 75 and 96 should show adhesion onto spent grain particles (Table III). However, in the case of the strain 75, the total interaction energy increased, which is less favorable for cell adhesion, at the end of the continuous cultivation. An opposite tendency, namely more negative and decreasing ΔG^{tot}, was observed for strains 96 and CCMI 890, respectively (Table III). It can be speculated, that the selection pressure exerted by the continuously operating bioreactor leads to separation of sub-populations with different surface properties. Whether these alterations will favor the cell-support adhesion depends most probably on cultivation conditions and genetic potential of the yeast cell.

Table III. Free energy of interaction and their components between yeast and support (base treated carrier) in water (cell-water-support).

Interacting system*	2nd day, free cells			12th day, free cells			13th day, immobilized cells		
	ΔG^{AB}	ΔG^{LW}	ΔG^{TOT}	ΔG^{AB}	ΔG^{LW}	ΔG^{TOT}	ΔG^{AB}	ΔG^{LW}	ΔG^{TOT}
C-W-S (CCMI890)	7.02	0.18	7.20±0.15	5.68	-1.08	4.59±0.29	6.41	-0.78	5.62±0.40
C-W-S (Strain 96)	0.94	-1.92	0.94±0.21	-1.29	-2.39	-3.68±0.51	-4.45	-2.05	-6.50±0.36
C-W-S (Strain 75)	2.07	-2.23	-0.16±0.02	7.01	-0.24	6.76±1.66	-	-	-
C-W-S (Strain 128)	5.82	-1.29	4.53±0.55	5.84	-1.33	4.51±0.93	-	-	-

*C – yeast cell, W – water, S – support

Adhesion of brewing yeast to support (spent grains). The immobilization of the brewing yeast onto carrier particles was characterized by an initial slow yeast accumulation (lag phase) followed by an increased biomass accumulation rate (Fig. 4). The time corresponding to the beginning of the massive carrier colonization and the time course of cell adhesion was similar for both adhering yeast strains. However, there was a difference observed in the final maximum immobilized biomass load, which was significantly higher for strain 96 (Fig. 4). This difference may be ascribed to the more distinct multi-layer cell immobilization of the strain 96 thanks to its higher flocculation ability (Fig. 2).

The remaining two brewing yeast strains (75 and 128, both top fermenting) did not show any adhesion to spent grain particles under the conditions of the experiment (Fig. 4). It is surprising since their initial total free energy of interaction (ΔG^{tot}) was more favorable for adhesion than of the strain CCMI 890. Moreover the strain 75 had initially (2nd day) the only negative ΔG^{tot} among all studied strains (Fig. 4). Apparently, the physicochemical approach to yeast adhesion to spent grain particles has only a limited ability to explain the observed phenomena. In order to provide a more reliable insight into the process of microbial adhesion, the biochemical aspects of this process have to be taken into account.

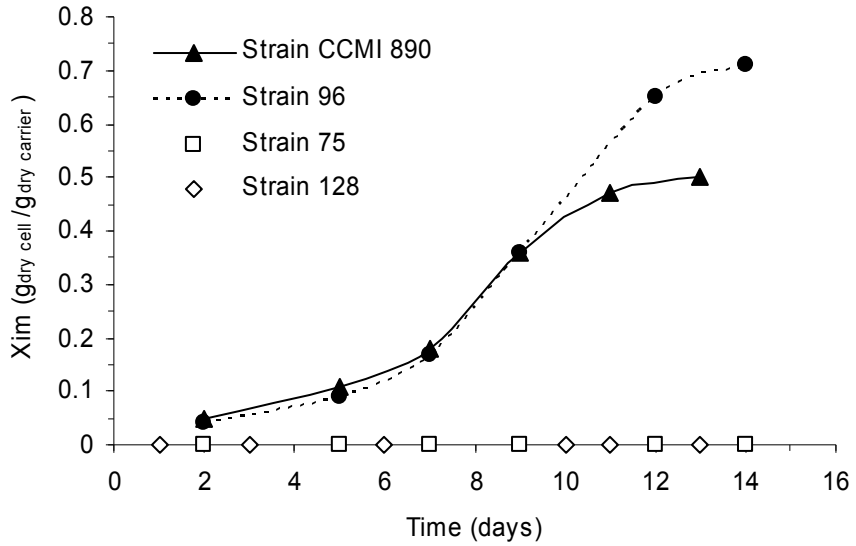

Fig. 4. The development of the immobilized biomass (X_{im} - g dry cell/gdry carrier) formed by yeast strains on the surface of spent grain particles in a CGLR at dilution rate D = 0.1 h^{-1}.

Determination of FLO11 gene. Bakers' yeast attachment to plastics and mat formation, requiring a fungal cell surface glycoprotein encoded by FLO11 gene, is also indicating to the role of hydrophobic interactions. The *Saccharomyces cerevisiae* FLO11 bakers' yeast strain was found more hydrophobic than the strain poorly adhering to polystyrene and lacking FLO11 gene (Reynolds and Fink, 2001). The PCR reaction product determined using gel analysis corresponds to the size of 404 base pairs of DNA and was amplified with the used primers (Fig. 5). This approach resulted in confirmation of the presence of FLO11 gene in all studied strains DNA (1 bottom fermenting, 2 top fermenting and a non-flocculating CCMI 890). The method was confirmed by both negative and positive control. For further understanding of the FLO11 contribution to immobilization process, the quantitative expression of the corresponding protein (Flo11p) under different culture conditions will have to be tested in the future.

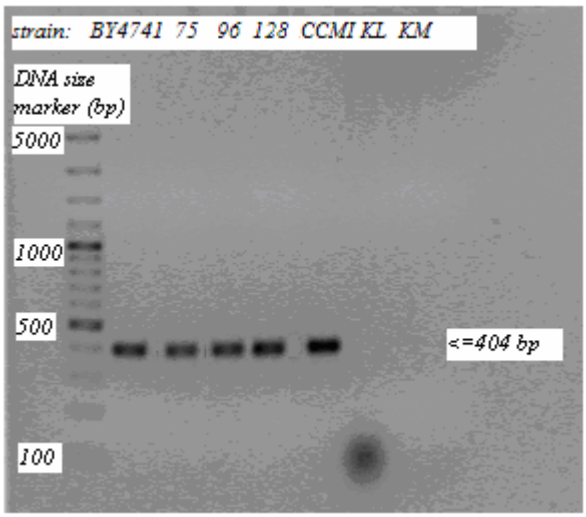

Fig. 5. Gel analysis of the PCR reaction products. KL and KM are strains for negative control *Kluyveromyces lactis* and *Kluyveromyces marxianus*, respectively. S*accharomyces cerevisiae* BY4741 was used as a positive control to confirm the presence of FLO11 gene.

CONCLUSIONS

The physicochemical aspects of brewing yeast adhesion to carrier particles obtained from spent grains were evaluated by two approaches. The calculations based on the DLVO theory showed no significant potential energy barrier that would prevent the cell deposition on the surface of the carrier. The energy balance of interfacial interactions was used to predict the possibility of a stable cell-support adhesion. The predictions made for the bottom fermenting brewing yeast strain (96) were in accordance with adhesion experiments. However, the conclusions from the other tested strains were slightly contradictory. Therefore, in order to verify the reliability of predictions based on physicochemical properties of interacting surfaces, more yeast strains with different surface properties will have to be tested in the future. Moreover, both of the physicochemical approaches (DLVO and interfacial free energies) require simplifying assumptions and therefore it is imperative to avoid quantitative conclusions and to consider only trends obtained in the data. Besides the physicochemical aspect of cell adhesion, the contribution of specific biochemical interactions will play a significant role on yeast adhesion too.

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