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Preparation and characterization of organosilicon thin films for selective adhesion of *Yarrowia lipolytica* yeast cells

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Abstract: The adhesion of *Yarrowia lipolytica* and *Saccharomyces cerevisiae* on organosilicon thin films deposited on polycarbonate substrates was investigated through a series of adhesion tests in order to obtain a selective substrate for *Y. lipolytica* cell adhesion. Organosilicon thin films were prepared using atmospheric pressure surface barrier discharge. The surface was characterized by its total surface energy with its components calculated using the acid-base theory. Assessment of adhesion via cell surface coverage was obtained by standard tests and also through image analysis of yeast cells on the organosilicon layers. The results show that it is possible to create organosilicon thin films for a selective adhesion of *Y. lipolytica* face to *S. cerevisiae* behaviour, for potential use in fixed bed biofilm reactors for industrial purposes involving enzyme-based biotransformations.
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Keywords: *Yarrowia lipolytica*; *Saccharomyces cerevisiae*; plasma deposition; cell adhesion; biomaterials; thin films; barrier discharge

INTRODUCTION

The study of microbial adhesion onto solid or liquid surfaces is extremely important for many practical and natural processes such as filtration, water treatment, paper making, painting, printing, thrombosis, microbial and enzyme immobilization, biofouling, biomineralization, etc.

The advantages of cell immobilization in nonporous carriers result from a lower mass transfer limitation of substrates and products, due to direct contact between cells and bulk liquid, and also in the simplicity of the immobilization on these substrates. The main risk of this method is biofilm detachment induced by changes in cell environment. Nevertheless, adhesion can be intensified through short-distance forces as hydrophobic interactions, polar interactions, hydrogen bonds, and specific molecular interaction.[1]

Yeast selective adhesion onto solid/liquid interfaces has not been widely exploited. This is due to the fact that the yeast cell wall is structurally and chemically more complex and heterogeneous than the surface of synthetic colloidal particles, which has a strong impact on its adhesion onto surfaces.

Yarrowia lipolytica is one of the most studied non-conventional yeasts for use in enzyme production as well as a host for genetic engineering purposes.[2–4] It is a lipase-producing strictly aerobic micro-organism and it has been observed that the cell immobilization is extremely faithful to the underlying lipid template indicating potential use in tissue engineering as well as materials applications involving specific enzyme-based biotransformations.[5] Nevertheless under certain situations *Y. lipolytica* may not be able to cope with competition from other types of micro-organisms present simultaneously in the natural reaction substrate.[6] Therefore to selectively hold *Y. lipolytica* on the surface of a fixed bed reactor in the presence of other types of cells that could also be present in the medium, a surface with selective affinity to *Y. lipolytica* was prepared. This surface is based on an organosilicon thin film[7] that was deposited onto virgin synthetic polycarbonate substrates. The preparation of such a surface with selective affinity for *Y. lipolytica* was a challenging task due to the similar size and surface charge of many cell systems. There are, however, other factors influencing yeast adhesion onto surfaces, e.g. geometrical irregularities, non-uniformity of surface charge, steric hindrance, hydration layers, hydrogen bond formation, etc.[8]

While the nature of the cell surface of yeast plays an important role in adhesion, the role of the substrate's surface is equally important. Due to the fact that most

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synthetic polymer materials have sufficient mechanical stability, elasticity, as well as chemical and thermal stability towards degradation, and are non-toxic, they are frequently used as anchoring substrates for cell adhesion. However, the interaction between synthetic polymers and cells is generally inadequate.[9] Thus, surface treatment of synthetic polymers is required for adequate interaction with foreign cells.[10–14]

In the present study, atmospheric pressure surface barrier discharge in N_2 with admixtures of hexamethyldisiloxane on polycarbonate surfaces was applied to obtain uniform thin organosilicon films for subsequent cell adhesion tests. Optical emission spectroscopy measurements were used to monitor and optimize the conditions for film preparation. The sessile drop contact angle method was used for the characterization of the wettability of these films, and the surface energy was calculated using the acid-base model. The surface chemical composition was studied by Fourier Transform Infrared (FTIR) spectroscopy to assess the presence of the deposited organosilicon film. Yeast adhesion conditions were monitored using zeta potential measurements followed by standard adhesion tests performed on different organosilicon-modified polycarbonate substrates prepared using a variety of plasma gas feed rates and different deposition times. The materials were visualized by both optical and scanning electron microscopy (SEM). The images obtained by optical microscopy were subjected to image analysis[15] to quantify the fraction of adhered cells in terms of relative surface coverage values.

Adhesion behaviour of *Y. lipolytica* and *S. cerevisiae*, herein used as model for competitive eukaryotic microorganisms, on organosilicon modified polycarbonate substrates is discussed with respect to their potential application in fixed bed biofilm reactors.

EXPERIMENTAL

Plasma deposition

The deposition of thin films was carried out by barrier surface discharge at atmospheric pressure and an operating frequency of 5 kHz. Following the deposition procedure previously described in the literature,[16,17] the surface discharge was created on the surface of the insulating glass plate, which was on one side fully covered with a metal electrode. On the other side of the glass plate the metal electrode consisted of nine connected rotating rods 6 cm long and with 9 mm spacing. The surface power density was kept at $0.4\,W\,cm^{-2}$ in all cases. Films were deposited from mixtures of hexamethyldisiloxane ($C_6H_{18}Si_2O$; HMDSO) with nitrogen. In order to optimize the coating properties, different values of Q_{N_2}/Q_{org} (from 0.01 to 0.2) were tested, using different values of HMDSO flow rate (Q_{org}) while keeping the flow rate of nitrogen (Q_{N_2}) constant at $6\,L\,min^{-1}$.

The discharge was studied by means of optical emission spectroscopy. Plasma emitted spectra were recorded using a Jobin-Yvon TRIAX 550 monochromator (Horiba, Kyoto, Japan), equipped with a CCD detector cooled by liquid nitrogen.

Cell culture preparation

A strain of *S. cerevisiae* (ATCC 32 167) herein employed was kept at 4 °C on agar. A wild type strain of *Y. lipolytica* (IMUFRJ 50 682) was selected from an estuary in the vicinity of Rio de Janeiro, Brazil and was kept at 4 °C on agar.[18] Both yeasts were separately cultivated at 28 °C in a rotary shaker in 500 mL flasks containing 200 mL YPD (yeast extract, peptone and dextrose) medium (w/v: yeast extract, 1%; peptone (from casein), 0.64%; glucose, 2%). Pre-cultures were grown at 160 rpm for 48 h. Cells were harvested at their stationary phase of growth by centrifugation at 3000*g* for 10 min and washed twice with distilled water.

Adhesion tests

Following the procedure described by Rosenberg,[19] the cells were re-suspended in $0.1\,mol\,L^{-1}$ phosphate buffer (pH = 3.0, 5.0, 7.0 and 9.0) so that the optical density was 0.70 at 570 nm. Following this, 1 mL of the cell suspension at a given pH and concentration was then placed on a modified substrate (test plate) and left to settle for 24 h. The liquid and the non-adhered cells were removed by insertion of the test plate in a 2 L beaker with 1.5 L of distilled water vigorously stirred at 1000 rpm. After drying for 2 h the adhered cells were observed on an Olympus (Tokyo, Japan) optical microscope BX 51 equipped with a digital camera. The images obtained were treated by image analysis to determine cell surface coverage values.

Thin film characterization

The film total surface energy was investigated by the sessile drop technique using the Surface Energy Evaluation System (SEE System). Contact angles were measured directly from images of the solid–liquid meniscus of a liquid drop set on a solid using a CCD camera. The Acid Base Regression method[20] was used to calculate the total surface free energy γ and its components (Lifshitz-van der Walls γ^{LW}, acid-base γ^{AB}, acid part γ^{+} and base part γ^{-}) from contact angles measured with six different test liquids. Ten separate readings were averaged to obtain one representative value of contact angle for each surface studied when the volume of each drop was 10.0 µL. The liquids used and their parameters are listed in Table 1. The chemical composition of the films was studied by FTIR spectroscopy using a Nicolet IMPACT 400 spectrometer (Thermo Electron Corp., Madison, WI, USA).

Yeast characterization

Electro-kinetic zeta potential was studied using a Coulter Delsa 440SX type instrument (Beckman Coulter, Fullerton, CA, USA). Measurements were performed at an ionic strength corresponding to $0.1\,mol\,L^{-1}$ phosphate buffer. The pH was adjusted by adding $0.01\,mol\,L^{-1}$ HCl/NaOH aqueous solution.

Table 1. Values of the surface energy components of the test liquids[20]

Test liquid	γ^{TOT} [mJ m^{-2}]	γ^{LW} [mJ m^{-2}]	γ^{AB} [mJ m^{-2}]	γ^{+} [mJ m^{-2}]	γ^{-} [mJ m^{-2}]
Water	72.8	21.8	51.0	25.5	25.5
Glycerol	64.0	34.0	30.0	3.92	57.4
Formamide	58.0	39.0	19.0	2.28	39.6
Ethylene glycol	48.0	29.0	19.0	1.92	47.0
Methylene iodide	50.8	50.8	0	0	0
α-Bromonaphtalene	44.4	43.4	≈0	0	0

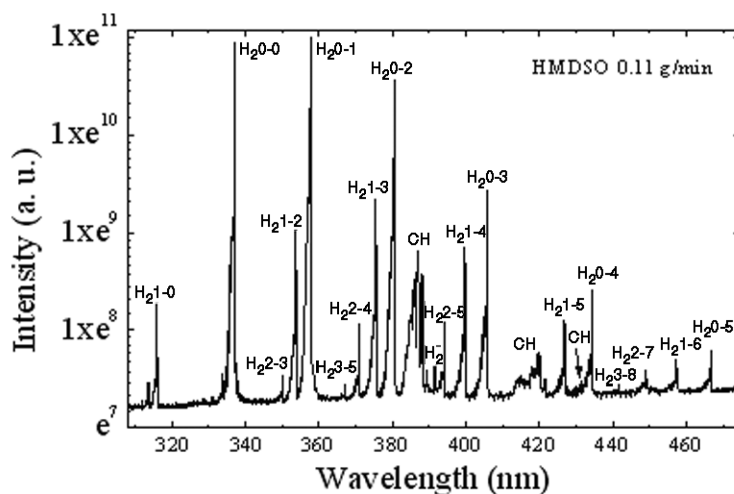


Figure 1. Emission spectrum of the surface discharge in nitrogen with HMDSO admixture.

The surface morphology of the cells was observed by SEM using a Hitachi (Tokyo, Japan) S4100 microscope operating at 25 kV.

RESULTS AND DISCUSSION

Discharge characterization

A typical emission spectrum of the nitrogen discharge with small admixture of HMDSO is displayed in Fig. 1. Emission spectra of the discharge were recorded in the 300–800 nm range, however, above 500 nm, only the second spectral order was registered. Thus the spectrum is plotted only in the range 300–500 nm. The spectrum consists of the molecular bands of the second positive system of nitrogen ($C\ ^3\Pi_u \rightarrow B\ ^3\Pi_g$). When organosilicon monomer was mixed with nitrogen, intensive bands of the CN violet system ($^2\Pi \rightarrow ^2\Sigma$) at 388 nm and 422 nm were observed. The weak band at 431 nm probably belongs to the CH system ($^2\Delta \rightarrow ^2\Pi$). The intensity of N_2 and the CN system depends on the concentration of HMDSO in nitrogen. Therefore, the integrated intensity of the CN band at 388 nm and the integrated intensity of the N_2 system was calculated. The ratio of integrated intensities of CN and N_2 bands as a function of HMDSO flow rate is shown in Fig. 2. The CN/N_2 ratio initially increases with increasing flow rate of organosilicon, and then it decreases.

The vibrational temperature calculated from the bands of the second positive system of N_2 0–2, N_2 1–3 and N_2 2–4 was at about 1800 K and the value of the vibrational temperature varied only slightly with concentration of HMDSO in nitrogen.

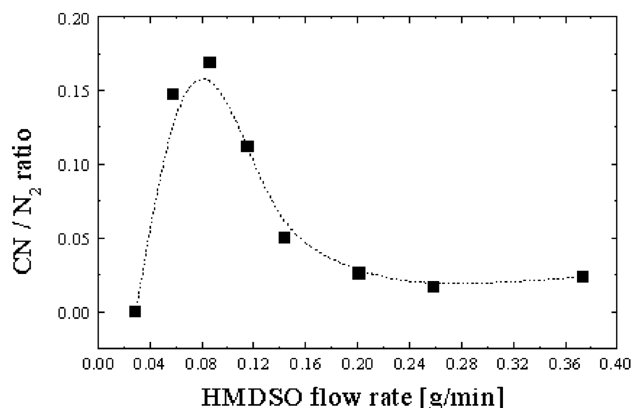


Figure 2. Ratio of integrated intensities of CN and N_2 bands for different HMDSO flow rates.

Characterization of organosilicon layers

The chemical composition of plasma deposited organosilicon films was studied by FTIR spectroscopy. The bands at 450 cm^{-1} $\nu(Si_3N)$, 801 and 845 cm^{-1} $\nu(SiC_x)$ and the broad band 980–1230 cm^{-1} (Si–O–C) vibration confirm the presence of the thin film in agreement with detailed descriptions in the literature.[7]

Another widely used method to characterize surface properties is the determination of wettability by contact angle using the sessile drop technique. The acid-base theory, which was used to calculate the surface energy of the samples is summarized below. This theory enables determination of the polar and apolar contributions to the total surface free energy as well as the electron-donor and electron-acceptor

components of the polar part of the surface free energy. According to this theory

$$\gamma^{TOT} = \gamma^{LW} + \gamma^{AB} \quad (1)$$

where γ^{TOT} is the total surface energy, the superscript LW denotes the total apolar (dispersion) Lifshitz–van der Walls interaction and AB refers to the acid-base interaction. Lewis suggested that the acid-base interaction can be determined using

$$\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}} \quad (2)$$

where γ^{+} is the electron donor and γ^{-} is the electron acceptor component of the acid-base part of the surface energy.

The surface free energy γ^{TOT} can be calculated using the Young–Dupré equation:

$$(1+\cos\Theta_i)\gamma_i = 2\left(\sqrt{\gamma_i^{LW}\gamma_j^{LW}} + \sqrt{\gamma_i^{+}\gamma_j^{-}} + \sqrt{\gamma_i^{-}\gamma_j^{+}}\right). \quad (3)$$

Here j refers to the material studied, i the test liquid, and Θ is the measured contact angle. The liquids used in our experiments and their characteristic parameters are listed in Table 1.

In Table 2 the sample abbreviations, the surface free energy and the corresponding contributions calculated using Eqn. (3) are given. In comparison to the virgin polymer substrate ($S_{(initial)}$), for a feed rate of 0.064 g min^{-1} it is possible to observe a significant decrease in the total surface energy from approximately 47 mJ m^{-2} to 29 mJ m^{-2} with increasing deposition time. For higher feed rates the value around 30 mJ m^{-2} indicates the creation of a multilayer coating. This indicates that short deposition times and small feed rates are enough to significantly increase the hydrophobicity of the initial polycarbonate samples. According to the results obtained, longer deposition times influence mainly the polar component, in particular its electron-acceptor contribution. This allows the preparation of materials that elicit little or no response. Alternatively, materials that elicit specific responses, such as ingrowth and adhesion of specific types of cells, depending on the electrokinetic behaviour of the cells and on the cell–cell interaction, can be prepared. Differences in the electron-acceptor contribution of the substrate are used to prepare *S. cerevisiae* 'inert' or 'non-interactive' biomaterials, which in turn can interact with more hydrophobic cells such as *Y. lipolytica*. The differences between micro-organism hydrophobicities herein used must be considered. Surface studies considering the contact angle measurement in water and other methods are described in Mozes and Rouxhet[21] for *S. cerevisiae* and in Amaral *et al.*[22] for *Y. lipolytica*.

Cell and cell-adhesion characterization

Electrokinetic zeta potential measurements of the cell suspension were used to monitor deposition conditions. Cells of *Y. lipolytica* and *S. cerevisiae* were studied using constant ionic strength aqueous solution equivalent to a 0.1 mol L^{-1} phosphate buffer. The zeta potential results of the cell suspension as a function of the pH shown in Fig. 3 indicate that the isoelectric point was approximately at pH 2.3 for both types of cells indicating that the surface charge of these cells is almost identical. This could imply a similar adhesion behaviour regardless of the surface type. However, this phenomenon is more complex because it depends on other electro-kinetic parameters.

An electrokinetic mechanism that is mainly involved in the adhesion of cells to substrates is based on the cell blocking behaviour. There are two

Table 2. Sample abbreviations, total surface free energy values and its contributions

Sample abbreviation	HMDSO feed rate [g min^{-1}]	Deposition time [s]	γ^{TOT} [mJ m^{-2}]	γ^{LW} [mJ m^{-2}]	γ^{AB} [mJ m^{-2}]	γ^{+} [mJ m^{-2}]	γ^{-} [mJ m^{-2}]
$S_{(initial)}$	0	0	45.8	42.9	2.9	0.7	3.3
$S_{(a,30)}$	0.064	30	47.3	44.6	2.7	1.1	1.6
$S_{(a,60)}$	0.064	60	27.6	26.0	1.6	0.5	1.2
$S_{(a,90)}$	0.064	90	28.2	24.2	4.0	0.7	5.9
$S_{(a,120)}$	0.064	120	28.6	26.4	2.2	0.7	1.7
$S_{(b,30)}$	0.087	30	34.3	29.2	5.1	–	–
$S_{(b,60)}$	0.087	60	22.6	21.9	0.7	0.3	0.5
$S_{(b,90)}$	0.087	90	34.9	30.5	4.4	2.3	2.1
$S_{(b,120)}$	0.087	120	35.0	28.2	6.8	–	–
$S_{(c,30)}$	0.115	30	34.7	34.1	0.6	0.02	4.6
$S_{(c,60)}$	0.115	60	32.6	32.2	0.4	–	–
$S_{(c,90)}$	0.115	90	31.3	31.3	0.02	0	0.01
$S_{(c,120)}$	0.115	120	32.2	30.1	2.1	0.4	2.5
$S_{(d,30)}$	0.17	30	27.0	26.6	0.4	0.5	0.07
$S_{(d,60)}$	0.17	60	31.4	26.8	4.6	0.95	5.7
$S_{(d,90)}$	0.17	90	27.8	27.7	0.11	0.05	0.06
$S_{(d,120)}$	0.17	120	30.8	29.1	1.7	0.2	3.4

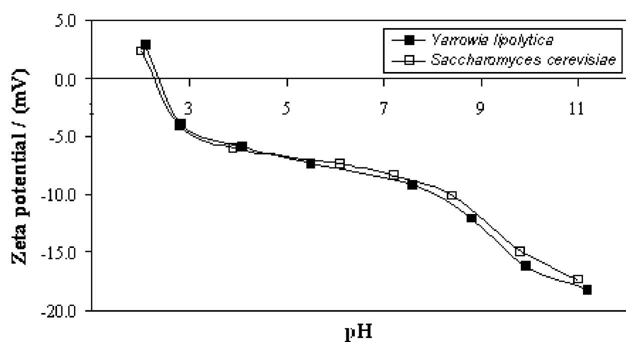
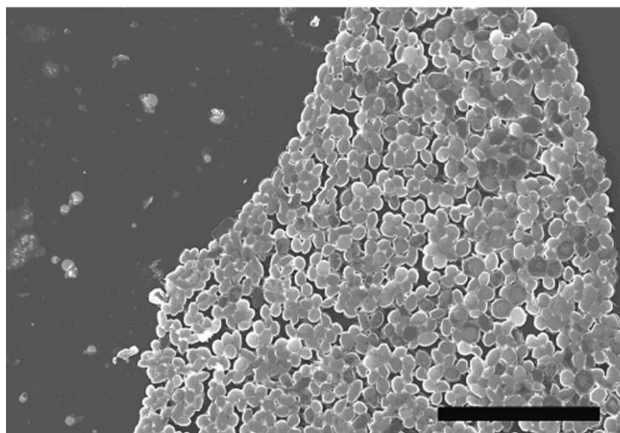


Figure 3. Influence of pH of the cell suspension on zeta potential for both *Y. lipolytica* and *S. cerevisiae* yeast cells.

(a)



(b)

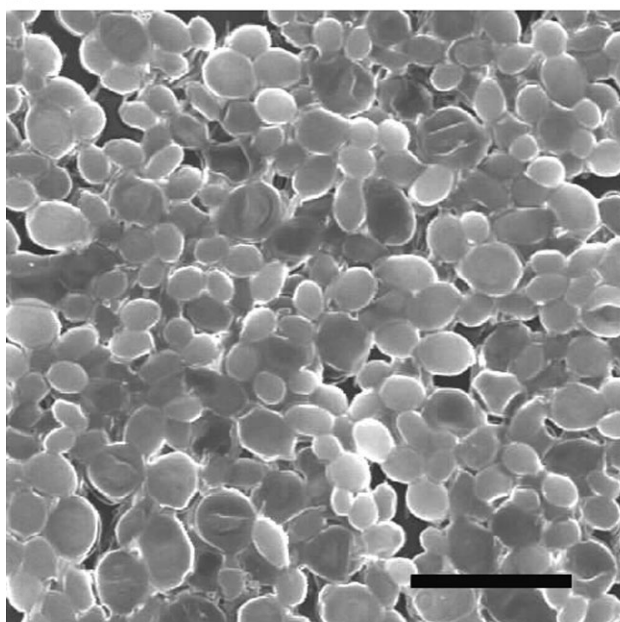


Figure 4. SEM image of *S. cerevisiae* deposited on $S_{(a,30)}$ sample. (a) bar size 30 µm; (b) bar size 3 µm.

general possibilities: either the cells are strongly blocking, forming monolayers, or the cells are weakly blocking, forming multilayer adhesion and pore-clogging.[23]

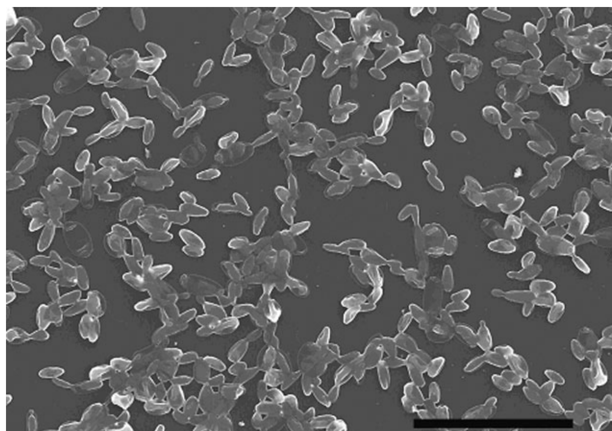


Figure 5. SEM image of *Y. lipolytica* deposited on $S_{(a,30)}$ sample (bar size 30 µm).

This phenomenon seems to play the most important role in our cell systems and may explain the different adhesion behaviour on plasma deposited thin organosilicon layers. The difference is illustrated by SEM images of the two yeasts studied (Figs 4 and 5). *S. cerevisiae* formed local aggregates of cell multilayers on ($S_{(a,30)}$) the surface (Fig. 4(a), (b)) while *Y. lipolytica* creates a randomly oriented homogeneous cell monolayer (Fig. 5).

Besides electrokinetic zeta potential measurements, there are other methods available to assess the potential of cell surface adhesion, such as adhesion tests to polystyrene, which is a standard method for the determination of optimal adhesion conditions.[24] This method was used in the present study. The cells adhered to polystyrene surfaces were observed with an optical microscope. Each image frame obtained was subjected to a digital image analysis procedure described by Freire *et al.*[15] to obtain surface coverage values. As shown by Amaral *et al.*[22] *Y. lipolytica* cells are well spread forming a monolayer and the fraction of adhered cells increases for pH values up to 7, after which the coverage does not seem to change further. This result is in agreement with the zeta potential measurements previously discussed and shown in Fig. 3.

S. cerevisiae cells formed aggregates mainly for pH below 7. This can be explained by the fact that in this pH range the adhesion between the cell and surface is weaker than the adhesion between cells themselves. At pH 7 and 9 respectively, the cells are well spread on the surface and no significant aggregates were observed. However, the fraction of adhered cells is low. The results from *S. cerevisiae* adhesion test are also in agreement with the electrokinetic zeta potential results previously discussed.

Due to these results for *Y. lipolytica* and *S. cerevisiae* and to the fact that pH 7 is approximately the optimum pH for both yeast growths, this pH was considered as an optimal value for adhesion tests on plasma deposited organosilicon layers.

Results of *Y. lipolytica* cell adhesion are expressed in terms of coverage Θ.[12] Coverage is a dimensionless value that characterizes the ratio of surface covered

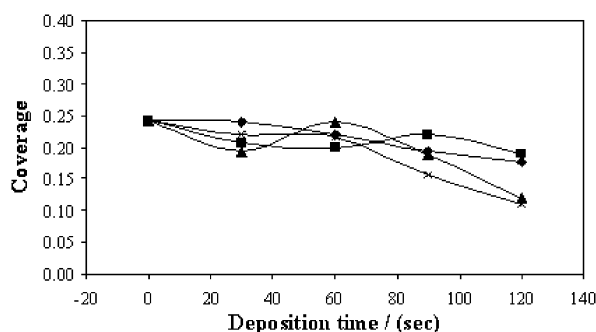


Figure 6. Influence of plasma deposition time on coverage by *Y. lipolytica* (HMDSO feed rates: diamonds 0.064, rectangles 0.087, triangles 0.115 and stars 0.170 g s^{-1}).

by cells to the whole observed surface. For spherical particles this can be calculated as

$$\Theta = \frac{\pi r^2 N}{S_{TOT}} \quad (4)$$

where r is the radius of the adhered particles, N is their number and S_{TOT} is the total area of the surface under observation. In the case of cell dimorphism this equation is modified to

$$\Theta = \frac{sN}{S_{TOT}} \quad (5)$$

where s denotes the mean area of a single particle present in the system. Hence, it is possible to obtain values of surface occupied by the particles on the image frame, S_p, determined by image analysis. Then the following equation describes coverage of the cell system used on the tested surfaces:

$$\Theta = \frac{S_p}{S_{TOT}} \quad (6)$$

The image analysis results for *Y. lipolytica* type cells are shown in Fig 6. These results suggest that the deposition time and feed rate used for the preparation of organosilicon layers have a slight influence on the adhesion of *Y. lipolytica* cells onto these types of films compared to the virgin polycarbonate substrate. However, the cells never exceed monolayer formation, as they are randomly spread and no formation of aggregates was observed, indicating that *Y. lipolytica* has a strong blocking behaviour on this kind of surface.

On the other hand, the results of adhesion tests of *S. cerevisiae* cells show weak blocking behaviour. The coverage values of *S. cerevisiae* deposited onto some organosilicon layers was: $S_{(a,30)} = 0.23$; $S_{(b,60)} = 0.11$; $S_{(c,90)} = 0.02$ and $S_{(d,120)}. = 0.00$. This shows that increasing the HMDSO feed rate during the plasma process, as well as the treatment time, have a significant effect on adhesion. Higher HDMSO feed rates combined with longer plasma treatment times led to very weak or no deposition of *S. cerevisiae* cells.

To evaluate the performance of the prepared material on the selective adhesion of a mixed culture a test was carried out using a suspension with both types of cells in the same concentration on a $S_{(d,120)}$ surface. The results of image analysis show a coverage value of 0.10. The images obtained (data not shown) show that only cells with ellipsoidal shape, characteristic of *Y. lipolytica*, remain adhered to the $S_{(d,120)}$ surface. No spherical *S. cerevisiae* cells were found. The circularity of the cells was calculated as the maximum diameter divided by the minimum diameter. The diameter is given by the distance between two parallel tangents in any given direction.[15] The circularity of the mixed culture on a $S_{(d,120)}$ surface was 2.15. The circularity for a pure culture of *S. cerevisiae* was 1.69 and for *Y. lipolytica* 2.26. This test clearly shows that the modified surface can promote selective adhesion of one type of cells even in the presence of a competing cell if the surface is adequately designed for that purpose.

Summarizing, surfaces prepared using higher feed rates of HMDSO and longer plasmochemical process times avoid possible interactions with *S. cerevisiae* while retaining high adhesion capacity towards *Y. lipolytica*. Hence, this kind of surface treatment could be used to prepare supporting surfaces in fixed bed biofilm reactors for industrial purposes such as enzyme production, where the active bio-component is based on *Y. lipolytica* cells.

CONCLUSIONS

The set of experiments carried out in this study shows that the deposition of organosilicon thin films by atmospheric pressure surface barrier discharge plasma on virgin polycarbonate produce a marginal decrease in the compatibility of this polymeric substrate with *Y. lipolytica*. In contrast, higher deposition times change the physicochemical composition of the final surface layer preventing any adhesion of the competing *S. cerevisiae* cells. Hence, despite the identical surface charge of both cell systems used, this surface modification procedure yields surfaces with selective adhesion for *Y. lipolytica* yeast cells. Further studies are under current investigation, regarding cell concentration and ionic strength to obtain higher coverage of *Y. lipolytica* on polymeric supports.

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