Deposition of *Yarrowia lipolytica* on plasma prepared teflonlike thin films

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The adhesion of *Yarrowia lipolytica* to teflonlike thin films deposited by plasma on polycarbonate substrates was investigated through a series of tests in order to develop a substrate for strong and selective adhesion of *Yarrowia lipolytica* cells. Teflonlike thin films were prepared using atmospheric pressure surface barrier discharge with mixtures of octafluorocyclobutane (C_4F_8) and nitrogen as plasma gas. A variety of plasma gas feedrates and different deposition times were studied. The films were characterised by Fourier transform infrared and contact angle measurements using the sessile drop technique. Total surface energy and its components were calculated using the acid base theory. Attachment of the yeast cells was assessed by optical and scanning electron microscopy. The optimal deposition conditions for cell adhesion were determined using standard adhesion tests.

Keywords: Yarrowia lipolytica, Plasma deposition, Cell adhesion, Thin films, Barrier discharge, Oil degradation

Introduction

During the last few decades, there has been an increasing interest in developing new coatings to improve biocompatibility and to prepare biomaterial surfaces that can either resist or enhance cellular adhesion by mimicking extracellular matrix components. However, selective deposition of yeasts on solid/liquid interfaces has not been much exploited because 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. Moreover, in the case of cells, processing factors such as pH, temperature and ionic strength must be considered while studying non-thermal processes to properly define the process conditions.[1] Because of the unfavourable cell deactivation or protein denaturation, temperature and pH can not be shifted in a broad range. However, it is possible to adjust the ionic strength I of the cell suspension to optimise the cell adhesion to surfaces. Besides these factors the nature of the substrate plays a key role. While synthetic polymers offer a wide range of properties to be used as cell supports such as mechanical, thermal and chemical stability, their surface characteristics are generally inadequate to promote cell adhesion unless they are submitted to surface treatments.[2]

Further to the authors' previous work regarding the development of a surface with affinity to *Yarrowia lipolytica* via the preparation of organosilicon thin films,[3] in the present work the authors investigate the preparation of teflonlike thin films in order to further increase the hydrophobicity of the surface. The effect of I is used to assess the *Yarrowia lipolytica* adhesion to this type of surface.

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.[4–6] It is a lipase producing strictly aerobic micro-organism and it has been observed that the cell immobilisation is extremely faithful to the underlying lipid template indicating potential use in tissue engineering as well as materials applications involving specific enzyme based biotransformations.[7] However, this yeast may not be able to cope with competition from other types of microorganisms present simultaneously in the natural reaction substrate.[8]

As *Yarrowia lipolytica* is one of the few amphiphilic yeasts that are able to attach to very hydrophobic surfaces[3,9] and fluorocarbon compounds are known to be water and oil repellent, due to their lower surface energies, a superhydrophobic surface such as teflonlike thin films was expected to be particularly selective to this type cell preventing competition from other cells that may simultaneously be present in the fermentation media.

As suggested in the literature[10,11] teflonlike thin films were prepared by atmospheric pressure surface barrier discharge using an admixture of octafluorocyclobutane (C_4F_8) and N_2 as plasma gas on polycarbonate surfaces to obtain uniformly thin teflonlike films for subsequent cell adhesion tests.

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Optical emission spectroscopy measurements were used to monitor and thus optimise the conditions for film preparation. Sessile drop contact angle method was used to characterise the hydrophobicity/hydrophilicity of the modified surfaces. The surface energy was calculated using the acid base model for these layers. The surface chemical composition was studied by Fourier transform infrared (FTIR) spectroscopy to assess the presence of the deposited teflonlike film.

For potential application in bioreactors cell adhesion must be strongly resistive to the liquid flow conditions present in the bioreactor and have a satisfactory cellular surface coverage of active cells. To achieve this, a variety of plasma gas feedrates and different deposition times were tested to prepare teflonlike modified polycarbonate substrates. Yeast deposition conditions were monitored using zeta potential measurements following standard adhesion tests. The effect of electrolyte concentration on the yeast cells' adhesion was tested using phosphate buffers with two concentrations. Yeast cells attached to the substrates were assessed by optical and scanning electron microscopy (SEM), and image analysis[12] was used to quantify adhesion efficiency in terms of relative surface coverage values. Attachment/detachment behaviour of *Yarrowia lipolytica* on teflonlike modified polycarbonate substrates is discussed with respect to their potential application in fixed bed biofilm reactors.

Experimental

Plasma deposition

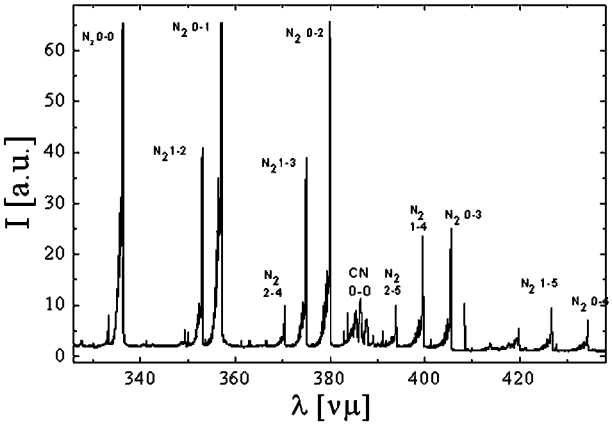
The deposition of thin films was carried out by barrier surface discharge at atmospheric pressure at an operation frequency of 5 kHz following the deposition procedure previously described elsewhere.[3] The films were deposited from mixtures of C_4F_8 with nitrogen. In the present case the surface power density was kept at 1·5 W cm^{-2} in all cases.

In order to optimise the coating properties different values of $Q_{C_4F_8}/Q_{N_2}$ have been tested. This was achieved using different values of C_4F_8 flow rate $Q_{C_4F_8}$ (from 0·2 to 0·6 L min^{-1}) while keeping the flow rate of nitrogen Q_{N_2} constant at 6 L min^{-1}.

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

Polymer surface characterisation

The chemical composition of the films was studied by FTIR spectroscopy using Nicolet IMPACT 400 spectrometer. The film total surface energy was investigated by the sessile drop technique using the surface energy evaluation system. Contact angles were measured directly from the images of the solid–liquid meniscus of a liquid drop set on a solid using a CCD camera. The so called acid base regression method[13] was used to calculate the total surface free energy γ^{TOT} 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 testing liquids. Ten separated readings were averaged to obtain one representative value of contact angle for each studied surface. Water, glycerol, formamide, ethylene glycol, methylene iodide and α-bromnaphthalene were the liquids used in the authors' study. Their tabular parameters for surface energy evaluation were taken from the literature.[14]



1 Emission spectrum of surface discharge in nitrogen with admixture of C_4F_8

Adhesion tests

The adhesion tests to surfaces here carried out are an adaptation of the method proposed by Rosenberg.[15]

As in the authors' previous work[3] a wild type strain of *Yarrowia lipolytica* (IMUFRJ 50682) was here used. A volume of 1 mL of the yeast suspension at a given pH and concentration was placed on a test surface and left to settle for 24 h. The supernatant was removed by insertion of the test plate in a 2 L beaker with 1·5 L of distilled water vigorously stirred at 1000 rev min^{-1}. After 2 h of drying the attached cells were observed on an Olympus optical microscope BX 51 equipped with digital camera. The images obtained were treated by image analysis to determine cell surface coverage values.

Detachment experiments

Detachment of previously adhered cells was studied by placing separately each sample under laminar stream of water for 10 h to simulate similar flow conditions to those encountered on an industrial fixed bed biofilm reactor. Upon drying images taken by optical microscope were treated by image analysis as described in the section 'Adhesion tests'.

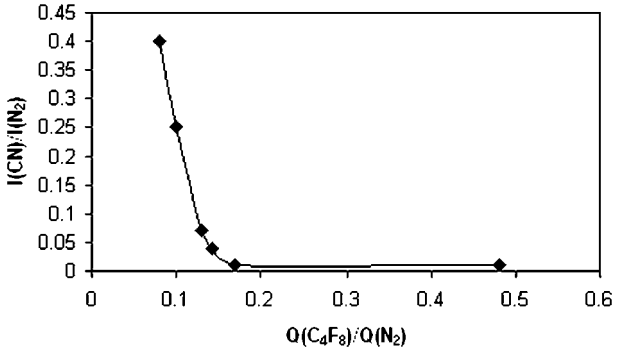
Cell surface characterisation

Electrokinetic zeta potential of the cells was studied using a Coulter delsa 440SX type instrument. The pH was adjusted by adding 0·01M HCl/NaOH aqueous solutions. The cells surface morphology was characterised by SEM using a Hitachi S4100 microscope operating at 25 kV. Samples were covered with an ultra thin layer of graphite.

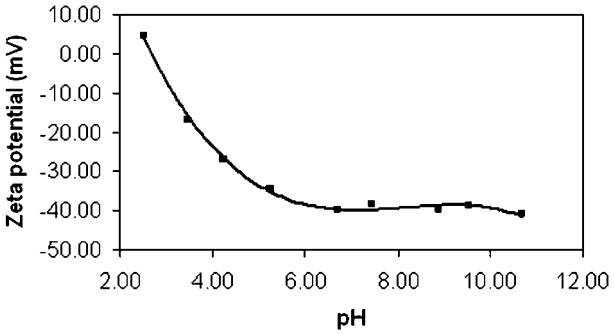
Results and discussion

Discharge characterisation

A typical spectrum of the discharge created in a mixture of nitrogen and C_4F_8 is shown in Fig. 1. Emission spectra of the discharge were recorded in the 300–800 nm range, however, >500 nm, only the second spectral order was registered. Thus the spectrum is plotted only in the range 300–500 nm. The spectra consist of the molecular bands of the second positive system of nitrogen (C $^3\Pi_u \rightarrow$ B $^3\Pi_g$). When octafluorbutane was mixed into nitrogen, intensive bands of CN



2 Ratio of integrated intensities of CN and N_2 bands for different C_4F_8 flows



3 Influence of pH of cell suspension on zeta potential for *Yarrowia lipolytica* yeast cells

violet system ($^2\Pi \rightarrow {}^2\Sigma$) at 388 and 422 nm were observed. Intensity of N_2 and CN system depends on the octafluorbutane to nitrogen flow rate ratio $Q_{C_4F_8}/Q_{N_2}$. Therefore, the integrated intensity of the CN band at 388 nm and that of N_2 system were calculated. The ratio of integrated intensities of CN and N_2 bands as a function of C_4F_8 to nitrogen flow rate ratio $Q_{C_4F_8}/Q_{N_2}$ is shown in Fig. 2. The CN/N_2 ratio increases with decreasing C_4F_8 concentration.

The vibrational temperature was calculated from the bands of second positive system of nitrogen N_2 0–2, N_2 1–3 and N_2 2–4. The value of the vibrational temperature varied only slightly with the flowrate of octafluorbutane admixed to pure nitrogen, and its value was at ~2100 K in all cases.

Characterisation of teflonlike layers

The chemical composition of plasma deposited teflonlike films was studied by FTIR spectroscopy. The bands at 1152, 1262 (CF_2 symmetric) and 1465 cm^{-1} (CF_2 asymmetric) vibrations confirm the presence of the thin film with a teflonlike structure.

Wettability of the surface was characterised by contact angle using the sessile drop technique. The acid base theory was used to calculate the surface energy of the samples using the Young–Dupré equation

$$(1+\cos\Theta_i)\gamma_i = 2\left[\left(\gamma_i^{LW}\gamma_j^{LW}\right)^{1/2} + \left(\gamma_i^{+}\gamma_j^{-}\right)^{1/2} + \left(\gamma_i^{-}\gamma_j^{+}\right)^{1/2}\right] \quad (1)$$

where j refers to the studied material, i the testing liquid, Θ is the measured contact angle, γ^{LW} corresponds to the apolar component (Lifshitz–van der Walls interaction), γ^+ is the electron donor and γ^- is the electron acceptor component of the acid base part of the surface energy.[13] The samples abbreviations, the surface free energy and the corresponding contributions calculated using equation (1) are given in Table 1.

In comparison to the virgin polymer substrate ($S_{(0;0)}$), for a feedrate of 0·2 L min^{-1} it is possible to observe a significant decrease in the total surface energy from approximately 45 nearly to 21 mJ m^{-2} with increasing deposition time. For higher feedrates the value ~23 mJ m^{-2} indicates the creation of a multilayer coating. This indicates that short deposition times and small feedrates are enough to significantly hydrophobise the initial polycarbonate samples. Accordingly, longer deposition times influence mainly the polar component, in particular its electronacceptor contribution.

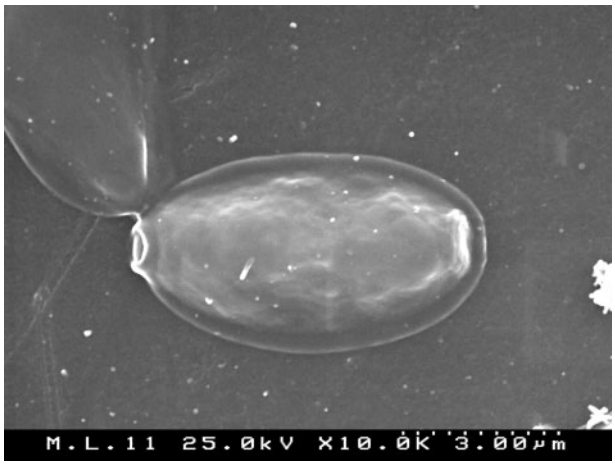
Cell and cell adhesion characterisation

Electrokinetic zeta potential measurements of the cell suspension were used to monitor deposition conditions. The results of the zeta potential of the cell suspension shown in Fig. 3 indicate that the most suitable region for *Yarrowia lipolytica* cell deposition is neutral or basic pH. Near pH 6 the zeta potential reaches values in the order of −40 mV which remain approximately constant for higher pH values. The isoelectric point was found to be ~2·7.

Besides electrokinetic zeta potential measurements, the adhesion efficiency was assessed by adhesion tests to

Table 1 Samples abbreviations, total surface free energy values and its contributions

Sample	Flow rate C_4F_8, L min^{-1}	Dep. 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}
$S_{(0;0)}$	0	0	45·79	42·85	2·94	0·65	3·32
$S_{(30;0·2)}$	0·2	30	30·95	20·8	10·15	6·76	3·81
$S_{(60;0·2)}$	0·2	60	25·47	23·23	2·25	4·33	0·29
$S_{(90;0·2)}$	0·2	90	22·17	21·05	1·12	4·34	0·07
$S_{(120;0·2)}$	0·2	120	21·07	20·21	0·86	4·24	0·04
$S_{(30;0·3)}$	0·3	30	32·79	23·52	9·28	1·33	16·18
$S_{(60;0·3)}$	0·3	60	26·66	26·52	0·14	2·34	0·001
$S_{(90;0·3)}$	0·3	90	22·07	20·5	1·58	6·24	0·1
$S_{(120;0·3)}$	0·3	120	23·76	21·55	2·21	4·66	0·26
$S_{(30;0·4)}$	0·4	30	33·90	23·54	10·36	1·11	24·5
$S_{(60;0·4)}$	0·4	60	22·95	21·74	1·21	5·99	0·06
$S_{(90;0·4)}$	0·4	90	23·58	21·63	1·95	5·51	0·17
$S_{(120;0·4)}$	0·4	120	23·09	21·64	1·45	3·74	0·14
$S_{(30;0·6)}$	0·6	30	32·78	22·43	10·35	6·4	4·18
$S_{(60;0·6)}$	0·6	60	27·54	22·73	4·81	9·33	0·62
$S_{(90;0·6)}$	0·6	90	22·53	21·66	0·87	5·19	0·04
$S_{(120;0·6)}$	0·6	120	23·09	21·29	1·82	0·21	2·94



4 Detailed SEM image of single *Yarrowia lipolytica* cell

polystyrene which is a standard method for the evaluation of adhesion and determination of the optimal adhesion conditions.[15] The cells attached onto polystyrene surfaces were observed by SEM and by polarised light microscopy (*see* Figs. 4 and 5). *Yarrowia lipolytica* cells are well spread forming a monolayer. Adhesion efficiency increases up to pH 7 upon which the coverage does not seem to change further as reported in previous studies.[3] This result is in full agreement with the zeta potential measurements previously discussed and shown in Fig. 3. These results suggest that pH 7 is an adequate condition to study the adhesion of *Yarrowia lipolytica* to surfaces. Moreover this being closely to the optimum pH for yeast growth was adopted for the subsequent adhesion tests to the surfaces prepared in the present work.

Each image obtained was treated using the digital image analysis procedure described by Freire *et al.*[12] to obtain surface coverage values. Examples of the images before and after image treatment are presented in Fig. 5*a* and *b*. Results of ten images of each sample were averaged to obtain one representative coverage value.

Coverage Θ_c (Ref. 16) is a dimensionless value that characterises the ratio of covered surface by cells against the whole observed surface. In the case of cell dimorphism coverage can be calculated by equation (2) where S_{TOT} is the total

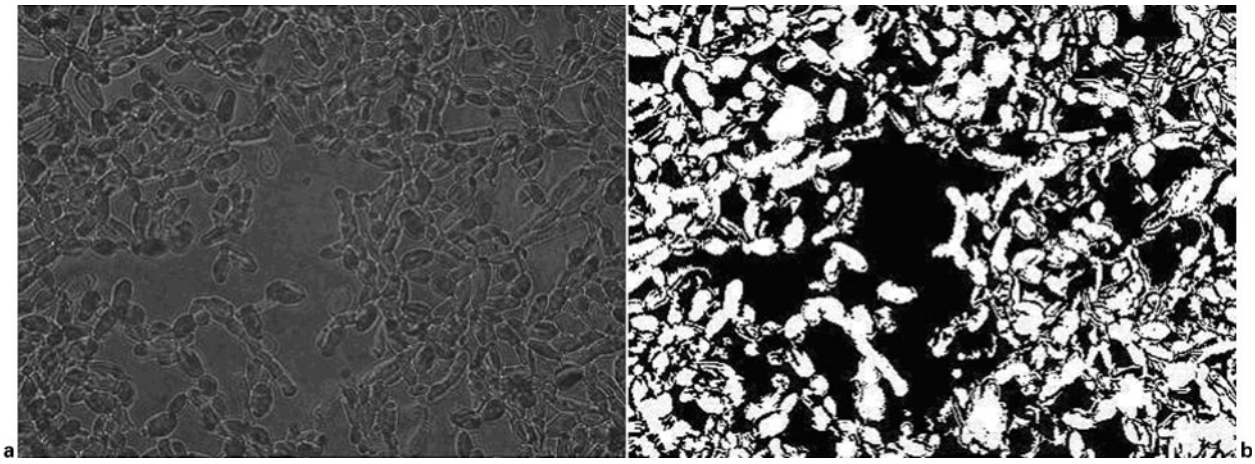
$$\Theta_c = \frac{S_p}{S_{TOT}} \tag{2}$$

area of the surface under observation and S_p is the surface occupied by the particles on the image frame determined by image analysis.

The results of image analysis of *Yarrowia lipolytica* type cells are summarised in Table 2. These results suggest that the deposition time and feedrates used for the preparation of teflonlike layers have a negative influence on the adhesion of *Yarrowia lipolytica* cells onto these type of films comparing to the to virgin polycarbonate substrate. However, the cells form monolayers, as they are randomly spread and no formation of aggregates was observed indicating that *Yarrowia lipolytica* has a strong blocking behaviour on this kind of surface. Moreover, their interaction with the surface seems to be stronger than those obtained for the virgin substrates.

Adhesion seems also to be dependent on the ionic strength of the cell suspension. As can be observed in Table 2, the coverage values for the cells deposited from a suspension containing phosphate buffer 0·01M are, in most cases, higher than the values obtained when a 0·1M phosphate buffer was used. This is also valid for the virgin polycarbonate surface. The best results were obtained for 90 s plasma treated samples using a 0·1M buffer suspension which reached deposition values close to those for virgin polycarbonate substrate. The same plasma treatment also shows the best deposition results for cell suspensions in a 0·01M buffer. Moreover, unlike what is observed for most surfaces the ionic strength of the medium has little influence on cell adhesion suggesting that the characteristics of the surface are determinant for a strong adhesion. Hence, deposition conditions can be tuned so that the ionic strength of the fluid flow promotes reversible attachment of the cells to the surface of the bioreactor.

Detachment of previously adhered cells was carried out to evaluate the strength of the cell surface interaction. Comparing the results for adhesion and detachment experiments presented in Table 2 it is clearly visible the advantage of teflonlike surfaces. Cell coverage values for the untreated polycarbonate surface decreased dramatically when subjected to the shear stresses induced by the liquid flow used on the detachment studies. Comparing to surface energy values, significant decrease in the γ^{LW} dispersion forces for treated surfaces seems to play a key role for the description of



5 Optical microscopy of *Yarrowia lipolytica a* before and *b* after image analysis treatment procedure

Table 2 Coverage values of *Yarrowia lipolytica* on tested samples

			Adhesion		Detachment	
			Coverage, %	Coverage, %	Coverage, %	Coverage, %
Treatment time, s	Monomer feedrate, L min^{-1}	Abbreviation	0·1M buffer	0·01M buffer	0·1M buffer	0·01M buffer
0	0	$S_{(0;0)}$	0·518	0·722	0·125	0·145
30	0·2	$S_{(30;0·2)}$	0·105	0·43	0·091	0·384
30	0·3	$S_{(30;0·3)}$	0·284	0·312	0·263	0·288
30	0·4	$S_{(30;0·4)}$	0·244	0·514	0·24	0·476
30	0·6	$S_{(30;0·6)}$	0·144	0·424	0·127	0·399
60	0·2	$S_{(60;0·2)}$	0·379	0·291	0·361	0·274
60	0·3	$S_{(60;0·3)}$	0·252	0·39	0·239	0·367
60	0·4	$S_{(60;0·4)}$	0·224	0·413	0·213	0·394
60	0·6	$S_{(60;0·6)}$	0·177	0·5	0·162	0·466
90	0·2	$S_{(90;0·2)}$	0·468	0·47	0·428	0·451
90	0·3	$S_{(90;0·3)}$	0·401	0·467	0·382	0·446
90	0·4	$S_{(90;0·4)}$	0·496	0·476	0·459	0·461
90	0·6	$S_{(90;0·6)}$	0·166	0·427	0·158	0·408
120	0·2	$S_{(120;0·2)}$	0·166	0·314	0·152	0·301
120	0·3	$S_{(120;0·3)}$	0·147	0·482	0·129	0·458
120	0·4	$S_{(120;0·4)}$	0·31	0·526	0·284	0·489
120	0·6	$S_{(120;0·6)}$	0·252	0·366	0·239	0·351

cell surface interaction expressed in detachment coverage values.

In summary, as aimed in the present study, teflonlike surfaces lead to very small cell loss indicating much stronger interaction of *Yarrowia lipolytica* than with virgin polycarbonate substrates. However, cell coverage still needs to be improved.

The results obtained indicate that upon optimisation of surface treatment and deposition conditions can be used in fixed bed biofilm reactors for hydrocarbon degradation or oil mills wastewater treatment where the active biocomponent is based on *Yarrowia lipolytica* type cells. The only disadvantage of plasma prepared teflonlike layers is the fact that it is still difficult to prepare homogenous films at high industrial level. Nevertheless, this problem is under intensive investigation and is expected to be satisfactorily resolved in the near future. Then, this kind of materials will certainly have a high industrial importance due to their relative low cost and advanced properties.

Conclusions

The present work aimed at developing a surface with a strong adhesion towards *Yarrowia lipolytica* cells. Given the particular characteristic of this yeast it was shown that the deposition of teflonlike thin films by atmospheric pressure surface barrier discharge plasma on virgin PC produce a surface onto which the yeast adheres strongly. Although the surface coverage obtained for these modified surfaces is lower than that for the original virgin polymeric substrate, higher adhesion forces are observed with little loss of cells compared with the non treated surface. This enhanced adhesion is required for the application of these materials in fixed bed biofilm reactors where the cell should be able to withstand the shear stresses induced by liquid flow.

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