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Influence of surface characteristics on the adhesion of *Alcaligenes denitrificans* to polymeric substrates

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Abstract—The adhesion of *Alcaligenes denitrificans* to several polymeric materials was investigated. As the nature of the surfaces of the micro-organisms and the substrate materials is an important factor in the adhesion process, characteristics such as the electrokinetic potential and hydrophobicity were also determined and correlated with the capacity of bacterial cells to adhere to solid surfaces. The substrates used were high-density polyethylene (HDPE), polypropylene (PP), poly(vinyl chloride) (PVC), and poly(methyl methacrylate) (PMMA). The electrokinetic potential of the cells and the substrates was determined by measurements of electrophoretic mobility and the hydrophobicity was determined by contact angle measurements. All the substrates studied as well as the bacterial strain have a negative zeta potential, which means that adhesion is not mediated by electrostatic interactions. As far as hydrophobicity is concerned, PP is the most hydrophobic material, PMMA is the least hydrophobic, whereas HDPE and PVC present an intermediate behavior. As bacteria cells are hydrophilic, adhesion is favored to PP; therefore, this substrate material seems to be the one that promotes a stronger adhesion and the development of the most stable biofilm for use as a biomass carrier in denitrifying inverse fluidized bed reactors. This was confirmed by the results of adhesion tests. In this way, adhesion seems to be dominated by hydrophobic interactions.

Keywords: Initial adhesion; polymeric substrates; hydrophobicity; zeta potential; micro-organism adhesion; acid–base.

1. INTRODUCTION

One way to avoid the wash-out limitation in continuous biological systems and to increase productivity is to retain the cells inside the reactor via immobilization onto a support material. From a physical-chemical point of view, the adhesion of cells to surfaces is determined by the interplay of electrostatic and hydrophobic interactions. Thus, the nature of the surfaces of the micro-organisms and supports is a determining factor. The two characteristics of relevance are electrokinetic

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potential and hydrophobicity. Through contact angle measurements and using the approach of van Oss *et al.* [1–3], it is possible to evaluate surface characteristics such as the surface free energy and hydrophobicity. This approach considers the surface free energy, γ, of the solid or a liquid as the sum of apolar Lifshitz–van der Waals, γ^{LW}, and polar acid–base interactions, γ^{AB}:

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

The apolar interactions consist mainly of the London dispersion interactions, but induction (Debye) and orientation (Keesom) interactions may also be involved [3]. In many cases, the polar acid–base interactions are entirely due to hydrogen bonding, and in the most general sense, they are electron-donor, γ^-, and electron-acceptor interactions, γ^+. The total interfacial free energy between phases i and j can be expressed as [1–3]

$$\gamma_{ij}^{TOT} = \gamma_i^{LW} + \gamma_j^{LW} - 2\sqrt{\gamma_i^{LW}\gamma_j^{LW}} + 2\left(\sqrt{\gamma_i^+\gamma_i^-} + \sqrt{\gamma_j^+\gamma_j^-} - \sqrt{\gamma_i^+\gamma_j^-} - \sqrt{\gamma_i^-\gamma_j^+}\right). \quad (2)$$

To determine the surface free energy components of a solid, the contact angles of three different liquids (for which apolar and polar components are known) need to be measured. Thereafter, three forms of the following equation, resulting from Young's equation, can be solved simultaneously using the contact angle values:

$$(1 + \cos\theta)\gamma_l^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right), \quad (3)$$

where subscripts s and l mean solid and liquid, respectively.

According to van Oss and Giese [4], the boundary between hydrophobicity and hydrophilicity occurs when the difference between the apolar attraction and the polar repulsion between molecules or particles of material (1) immersed in water (w) is equal to the cohesive polar attraction between the water molecules. When the total interfacial free energy of interaction between particles of 1 immersed in water, ΔG_{lwl}, is attractive, i.e. has a negative sign, the material is 'hydrophobic'. When this value is positive, the material is 'hydrophilic'. This interfacial free energy is expressed as

$$\Delta G_{lwl} = \Delta G_{lwl}^{LW} + \Delta G_{lwl}^{AB} \quad (4)$$

or

$$\Delta G_{lwl} = -2\left(\sqrt{\gamma_l^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_l^+\gamma_w^-} + \sqrt{\gamma_l^-\gamma_w^+} - \sqrt{\gamma_l^+\gamma_l^-} - \sqrt{\gamma_w^+\gamma_w^-}\right). \quad (5)$$

The aim of this study was to determine which type of material promoted a stronger adhesion and whether electrostatic interactions or hydrophobicity prevailed in the initial adhesion.

2. MATERIALS AND METHODS

2.1. Micro-organisms

Alcaligenes denitrificans was grown in a citrate minimal medium containing 0.2448 g of $C_6H_5Na_3O_7{\cdot}2H_2O$, 0.289 g of KNO_3, 0.93 g of K_2HPO_4, 0.18 g of KH_2PO_4, 0.0242 g of $NaMoO_4{\cdot}2H_2O$, 0.0056 g of $FeSO_4{\cdot}7H_2O$, 0.00081 g of $MnCl_2{\cdot}2H_2O$, 0.0515 g of $CaCl_2{\cdot}2H_2O$, and 0.4092 g of $MgSO_4{\cdot}7H_2O$ in 1 l of distilled water. The cultures were performed in batches for 3 days. Cells were harvested by centrifugation (5 min at 5000 rpm), washed twice in phosphate buffer saline (PBS), twice in distilled water, and finally resuspended in culture medium. The concentration of the cellular suspension was determined by a direct count in a Neubauer chamber.

2.2. Substrates

Four different types of materials were compared: high-density polyethylene (HDPE), polypropylene (PP), poly(vinyl chloride) (PVC), and poly(methyl methacrylate) (PMMA). Substrate slides of 2 cm $\times$ 2 cm were carefully washed with detergent, ethanol, and in sterile water baths, before being used in the adhesion assays.

2.3. Surface characteristics

The surface energy components were determined from contact angle measurements (25 determinations for each material) in a standard contact angle apparatus (Kruss-GmbH, Hamburg). The determinations of the contact angles were performed automatically, with the aid of an image analysis system (Kruss-GmbH, Hamburg). The images were received by a video camera connected to a 486 DX4 100 MHz personal computer, with an automatic measuring system (G2/G40) installed. All the measurements were made at room temperature and the three different liquids used were water (Chromasolv), diiodomethane (Ridel-de Haen), and glycerol (Merck). In the case of bacterial cells, the measurements were performed on bacterial layers deposited on membrane filters, according to the method described by Busscher *et al.* [5].

The zeta potential of the bacterial cells was determined by measuring the electrophoretic mobility in a Zeta-Meter 3.0+ (Zeta-Meter Inc.) operating at 100 V. The determinations were performed in the culture medium adjusted to different pH values between 5.9 and 9.0 with NaOH or HCl, as necessary, and after a soaking period of 24 h in the culture medium to establish an equilibrium between the cells and the liquid. The same procedure was followed to obtain the zeta potential of the polymeric materials. In this case, the polymers were previously reduced to small particles using a steel file.

2.4. Adhesion tests

Initial adhesion tests were performed with four slides of each type of substrate placed horizontally in a sterile Petri dish and covered with 100 ml of culture medium with a bacterial concentration of 0.5×10^8 cell/ml. After 2 h of incubation at 27°C and 90 rpm, the slides were rinsed with sterilized water. They were then covered with a 0.1% acridine orange solution and observed under an epifluorescence microscope. The images were recorded by microscope photography and then digitized. The number of bacteria per mm^2 was enumerated by image analysis [6].

3. RESULTS AND DISCUSSION

The results of the adhesion assays of *Alcaligenes denitrificans* to the polymeric substrates are presented in Fig. 1. As can be seen, adhesion occurs to the greatest extent to PP, followed by PVC, HDPE, and PMMA.

The zeta potential of the bacterial cells and substrates was measured via electrophoretic mobility. Figures 2 and 3 show the average values of the corresponding results, obtained from 20 measurements with standard deviations ranging between 2% and 5%. In the pH range 6–9, and when immersed in the culture medium, all the polymeric materials as well as the bacterial cells studied were found to have

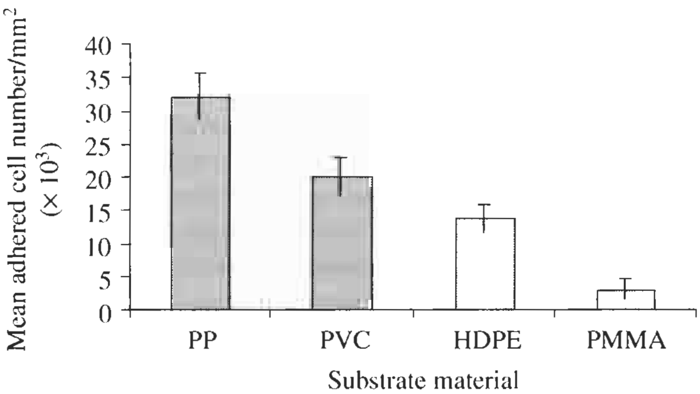

Figure 1. Number of adhered cells per mm^2 of the polymer surface.

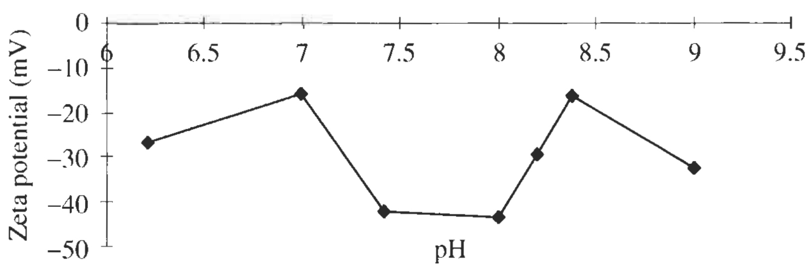

Figure 2. pH dependence of the zeta potential of the bacterial cells.

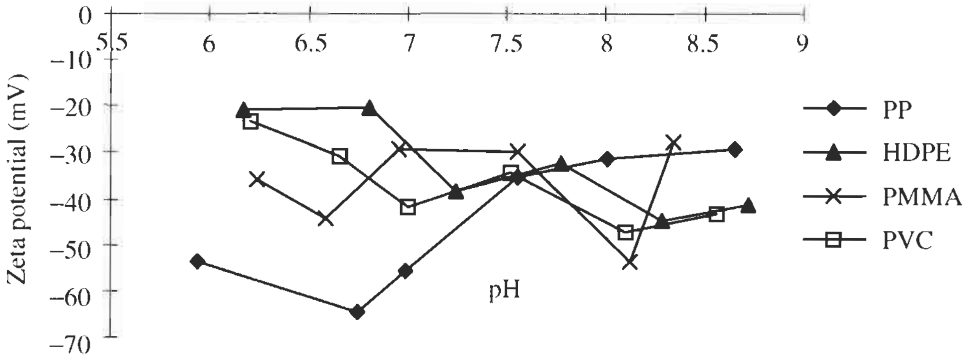

Figure 3. pH dependence of the zeta potential of the substrate material.

Table 1.
Contact angle (in degrees) and surface energy components of bacterial cells and support materials (in mJ/m^2)

Material	Contact angle ± SD			Surface energy components				
	Water	Glycerol	Diiodomethane	γ_S^{LW}	γ_S^+	γ_S^-	γ_S^{AB}	γ_S^{TOT}
Bacteria	15.4 ± 3.3	17.6 ± 1.8	68.1 ± 2.0	23.9	7.1	48.6	37.1	61.0
PP	83.0 ± 2.5	70.6 ± 3.1	38.6 ± 3.0	40.3	0	4.0	0	40.3
HDPE	79.2 ± 3.1	54.9 ± 2.5	40.3 ± 3.1	39.5	2.2	1.6	3.8	43.2
PMMA	58.5 ± 1	56.3 ± 2.7	31.8 ± 2.9	43.5	0	21.0	0	43.5
PVC	67.7 ± 2.3	66.4 ± 3.1	44.0 ± 2.8	37.5	0	17.4	0	37.5

a negative zeta potential. This result could be anticipated, because most of the materials acquire a net negative electric charge when immersed in aqueous solutions with pH near neutrality. The importance of this fact is to provide evidence that in this case adhesion is not mediated by direct electrostatic interactions between the bacteria and substrates. Otherwise, PP, which is the material with the most negative zeta potential, would not show the highest number of adhered micro-organisms.

In biological systems, hydrophobic interactions are usually the strongest of all long-range non-covalent interactions [7]. This is the reason why the hydrophobicity of the interacting surfaces was determined.

The values of the contact angles with the various liquids employed and the surface energy components for the bacterial cells and substrates are summarized in Table 1. The surface energy components were obtained by solving equation (3) and using the surface tension components of the reference liquids reported in the literature [8], although considering diiodomethane completely apolar [9].

Analyzing the data of Table 1, it can be seen that *A. denitrificans* is predominantly electron-donating, since the component γ_s^- is much higher than γ_s^+. This polar character can be due to the presence of residual water of hydration or polar groups [10]. The values obtained for the free energy components of the substrate materials are in accordance with the results of van Oss *et al.* [11], who stated that

Table 2.
Interfacial free energy of interaction, ΔG_{lwl}, and its components between the material (l) immersed in water (w) and the free energy of hydration (ΔG_{lw}) (in mJ/m^2)

Material	$\Delta G_{\mathrm{lwl}}^{\mathrm{LW}}$	$\Delta G_{\mathrm{lwl}}^{\mathrm{AB}}$	ΔG_{lwl}	ΔG_{lw}
Bacterial cells	−0.10	18.33	18.23	−143.00
PP	−5.64	−61.6	−67.24	−79.49
HDPE	−5.2	−54.0	−59.2	−86.41
PMMA	−7.4	−9.41	−16.81	−107.85
PVC	−4.25	−17.79	−22.04	−99.32

most polymers have a $\gamma_{\mathrm{S}}^{\mathrm{LW}} \approx 40$ mJ/m^2 ± 10%. It must be noted that, with the exception of HDPE, all the other substrates are electron acceptors.

According to the more recent definition [4], hydrophobic materials are those substances which tend to aggregate in water having a $\Delta G_{\mathrm{lwl}} < 0$, while hydrophilic materials, for which $\Delta G_{\mathrm{lwl}} > 0$, repel each other when immersed in water. However, attraction between one hydrophobic and one hydrophilic site immersed in water is possible and close contact is attained when the water layer in between is removed. Based on the above criterion, *A. denitrificans* is hydrophilic and the substrate materials are hydrophobic (Table 2). It becomes clear that PP is the most hydrophobic material, PMMA the least hydrophobic, and PVC and HDPE present an intermediate behavior. Thus, as bacteria are hydrophilic, their adhesion is most favorable to PP and least favorable to PMMA, which is in accordance with the adhesion test results (Fig. 1). In HDPE, the presence of a non-negligible value for γ_{S}^{+} (Table 1) suggests the existence of water of hydration, which in the presence of a measurable γ_{S}^{-} value gives rise to a finite $\gamma_{\mathrm{S}}^{\mathrm{AB}}$. The value of $\gamma_{\mathrm{S}}^{\mathrm{AB}}$ is then a measure of the degree of residual hydration [9]. Thus, in spite of the intermediate hydrophobicity of HDPE, the adhesion of *Alcaligenes* is not favored, since the bound water layer has to be removed before complete contact and adhesion can occur.

Considering only the polymers having a negligible polar component, the relation between the number of adhered cells and the degree of hydrophobicity, ΔG_{lwl}, is presented in Fig. 4.

Owing to the suggested importance of the water of hydration in the bacterial adhesion process, the values of the free energy of hydration (ΔG_{lw}) were calculated (Table 2). These values correlate almost linearly with the number of adhered cells, as can be seen in Fig. 5.

It is clear that hydrophobic interactions seem to be controlling the process of bacterial adhesion, although they cannot fully explain the phenomenon. Other interactions are due to bonding by plurivalent cations such as Ca^{2+}, Fe^{2+}, and Mg^{2+} that are part of the culture medium (results not presented). These cations have a hydrophobizing effect on negatively charged surfaces [10]. For instance, Ca^{2+} can establish preferential bonding between highly negatively charged surfaces. This

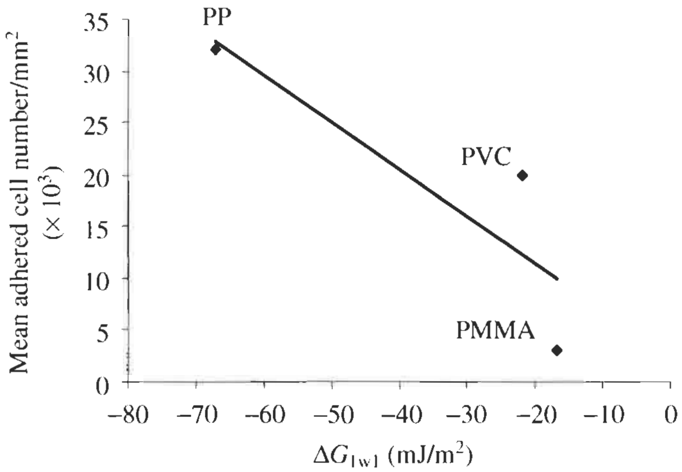

Figure 4. Number of adhered cells of *A. denitrificans* to each type of polymeric surface as a function of the degree of hydrophobicity given by ΔG_{lwl}. The more negative the value of ΔG_{lwl}, the higher the degree of hydrophobicity of the substrate material.

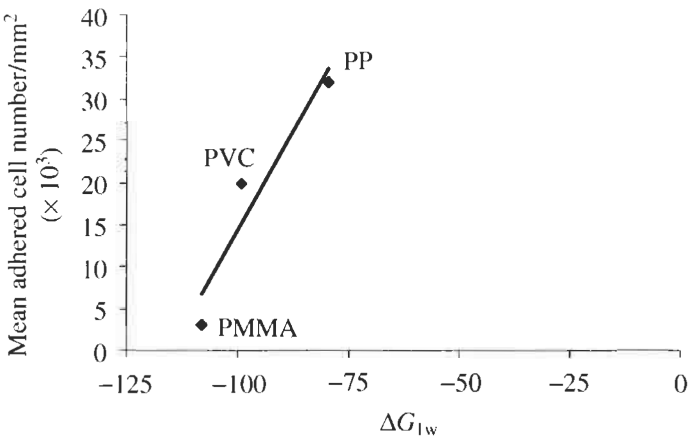

Figure 5. Number of adhered cells of *A. denitrificans* to each type of polymeric surface as a function of the free energy of hydration, ΔG_{lw}.

type of interaction is very important in biological systems. This helps to explain why the most negative substrate is the most favorable material for adhesion.

4. CONCLUSIONS

The following conclusions can be drawn from this study:

(1) From the adhesion assays, PP seems to be the most favorable material for the adhesion of *A. denitrificans*.

(2) Adhesion seems to be mediated by hydrophobic rather than electrostatic interactions.

(3) The presence of a non-negligible value for γ^+ in HDPE suggests the presence of water of hydration, promoting a decrease in cell adhesion.

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