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# Adhesion of *Alcaligenes denitrificans* to Polymeric Materials: The Effect of Divalent Cations

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The influence of surface characteristics of the microorganisms and the substrate materials, such as electrokinetic potential and hydrophobicity, in the adhesion process was investigated in a previous work [1]. As neither surface charge nor hydrophobicity can fully explain the process of bacterial adhesion, the effect of divalent cations was experimentally studied. Adhesion assays were performed in sodium phosphate buffer saline (PBS) medium and in a medium with the same ionic strength containing  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Fe}^{2+}$ . In the presence of divalent cations, contrary to what happened in PBS, adhesion was more favourable to the more negatively charged polymeric material. This points out a strong contribution of ion bridging in culture medium. In the absence of divalent cations, a higher hydrophobicity of the support is required in the process of adhesion.

*Keywords:* Bacterial adhesion; DLVO-theory; Ion bonding; XDLVO-theory

## 1. INTRODUCTION

The adhesion of microorganisms to solid surfaces has been described in the past, by the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloidal stability [2, 3], as a balance between the van der Waals force of attraction and the electrostatic double-layer force of repulsion. Later, this model was extended by the inclusion of acid/base (hydrophobic) interactions and is now generally known as

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extended-DLVO (XDLVO) theory [4]. In both theories, microorganisms are considered as colloidal particles, namely, with a perfectly smooth surface. Nevertheless, they have to be regarded as “living colloids” with their adaptable and varied nature, with heterogeneous cell surfaces, being capable of very special types of interactions such as steric interactions established by their exopolymers [5]. Surface structures such as fimbriae, lipopolysaccharides (LPS), capsule material and flagella are also believed to be involved in bacterial adhesion [6]. The effect of such structures is not accounted for in either the DLVO or the XDLVO theory.

Positively-charged ions, particularly divalent ones, can act as bridging agents between two negatively-charged surfaces. Divalent cations, such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Fe}^{2+}$ , are considered the most effective ion binders in biological systems [5]. van Oss *et al.* [7] proposed that  $\text{Ca}^{2+}$  can depress the monopolar electron donor parameter of the surface tension of the interacting species, depressing their capacity for mutual repulsion and the respective degree of hydration, resulting in a decrease of the hydration pressure. Other cations can also act as ion binders but their effect is not yet very well understood. The aim of the present study is to determine the effect of divalent cations on the adhesion of the bacterium *Alcaligenes denitrificans* to different polymeric materials.

In addition in order to verify if this effect could be explained by the DLVO theory or its recent extension (XDLVO), the experimental data were interpreted *via* both theories. The ultimate objective of this study was the selection of the best conditions to promote adhesion and also the selection of the most appropriate polymeric material to be used as a carrier in a biological denitrifying reactor.

## 2. MATERIALS AND METHODS

### 2.1. Microorganism

*Alcaligenes denitrificans* ATCC 15173, is a Gram negative, rod-shaped bacterium, capable of denitrifying in anoxic conditions.

In the present study, *Alcaligenes denitrificans* was grown in a medium consisting of 0.2448 g  $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ , 0.289 g  $\text{KNO}_3$ ,

0.93 g  $K_2HPO_4$ , 0.18 g  $KH_2PO_4$ , 0.0242 g  $NaMoO_4 \cdot 2H_2O$ , 0.0056 g  $FeSO_4 \cdot 7H_2O$ , 0.00081 g  $MnCl_2 \cdot 2H_2O$ , 0.0515 g  $CaCl_2 \cdot 2H_2O$  and 0.4092 g  $MgSO_4 \cdot 7H_2O$  in 1 litre of distilled water. The cultures were grown in batch for 3 days at 27°C and with orbital shaking at 150 rpm. To obtain the desired biomass concentration, cells were harvested by centrifugation (5 min at 5000 rpm), washed twice in sodium phosphate buffer saline (PBS) (0.29 g  $KH_2PO_4$ , 1.19 g  $K_2HPO_4$ , 4.93 g NaCl in 1 litre of distilled water), twice in distilled water and finally resuspended in the culture medium. The concentration of the cellular suspension was determined by direct count in a Neubauer chamber.

## 2.2. Supports

Four different types of materials were used as biomass carriers; high-density polyethylene (HDPE), polypropylene (PP), polyvinylchloride (PVC) and polymethylmethacrylate (PMMA). Each polymer, in the form of a slide of 2 cm × 2 cm, was carefully washed with detergent, followed by rinsing with ethanol and finally with sterile water, before being utilised.

## 2.3. Surface Characteristics

The surface tension components were obtained from contact angle measurements in a standard contact angle apparatus (Kruss-GmbH). The measurements were performed automatically with the aid of an image analysis system (G2/G40) installed in the apparatus. The images were transmitted by a video camera to a 486 DX4 100 MHz personal computer for evaluation. All the measurements were performed at room temperature. In the case of bacterial cells the measurements were performed on bacterial layers deposited on membrane filters, according to a method described by Busscher *et al.* [8]. The probe liquids used were ultra-pure water, di-iodomethane and glycerol, both of analytical grade. Their total surface tensions were measured with a K6 tensiometer (Kruss-Hamburg). The values thus obtained were in accordance with those reported in the literature. Therefore, expecting no differences, we used the literature values of the components of the surface tension for each liquid [9].

The zeta potential of the bacteria and the polymers was determined by measuring the electrophoretic mobility in a Zeta-Meter 3.0 + operating at 100 V. The measurements were performed in culture medium (described above) adjusted to different pH values between 5.9 and 9.0 with HCl or NaOH. A soaking period of 24 hours in the culture medium was allowed in order to establish equilibrium between the particles and the liquid.

## 2.4. Adhesion Assays

Two types of initial adhesion tests were performed, differing in the liquid medium used: culture medium and phosphate buffer saline (PBS). The ionic strength was the same in both cases ( $42.55 \times 10^{-3}$  M). In each triplicate assay ten slides of each type of polymer were immersed horizontally in a sterile glass dish containing 100 ml of liquid medium with a bacterial concentration of  $0.5 \times 10^8$  cell/ml. After 2 hours of incubation at 27°C and orbital shaking at 90 rpm, the slides were rinsed with sterilised water according to the procedure described in Ref. [10]. They were then covered with a 0.1% acridine orange solution and observed under an epifluorescence microscope. Photographic images were taken through a microscope and then digitised. The number of bacteria per square mm was enumerated by image analysis [11]. In each slide an average area of 2 cm<sup>2</sup> was characterized.

## 2.5. DLVO and XDLVO Analysis

The adhesion results were interpreted *via* DLVO and XDLVO theories. In this context, for model calculation, bacteria are considered as spherical particles of radius,  $R$ , located at a distance,  $H$ , with respect to the surface of adhesion (flat plate). In the Hamaker constant of the combination of the materials involved,  $A_{132}$ ,  $A_{ii}$  is the Hamaker constant of the material (i) and is obtained from the apolar component of the surface tension ( $LW$ ), taking the separation distance between two semi-finite slabs equal to 0.157 nm [12] (Tab. I). The bacteria average equivalent diameter is 1.4  $\mu$ m.  $\lambda$  is the decay-length of water ( $\approx 1.0$  nm) [12]. The electrostatic energy of interaction,  $\Delta G_H^{EL}$  (sp), between a sphere and a plate as a function of their separation distance,  $H$ , can be calculated on the basis of three different models: constant

TABLE I Values of Hamaker constants  $A_{ii}$  and  $A_{132}$  (1 = bacteria, 2 = polymeric material and 3 = water) in  $10^{-20}$  J

Component	$A_{ii}$	$A_{132}$
Bacteria	4.44	—
PP	7.49	0.07
HDPE	7.33	0.07
PVC	6.98	0.06
PMMA	8.08	0.08
Water	4.05	—

TABLE II The Lifshitz-van der Waals free energy of interaction,  $\Delta G_H^{LW}$  (sp), the electrostatic energy of interaction,  $\Delta G_H^{EL}$  (sp) and polar interaction energy,  $\Delta G_H^{AB}$  (sp), as a function of the separation distance

Interaction	Equation	Reference
$\Delta G_H^{LW}$ (sp)	$-(A_{132}R/6H)$	[12]
$\Delta G_H^{EL}$ (sp) (c. charge)	$-\varepsilon\pi R\{(\Psi_{o1} + \Psi_{o2})^2 \ln[1 - \exp(-\kappa H)] + (\Psi_{o1} - \Psi_{o2})^2 \ln[1 + \exp(-\kappa H)]\}$	[14]
(c. potential)	$\varepsilon\pi R\{(\Psi_{o1} + \Psi_{o2})^2 \ln[1 + \exp(-\kappa H)] + (\Psi_{o1} - \Psi_{o2})^2 \ln[1 - \exp(-\kappa H)]\}$	[15]
(mixed)	$\varepsilon\pi R\{2\Psi_{o1}\Psi_{o2}[\pi/2 - \tan^{-1} \sinh(\kappa H)] - (\Psi_{o1} - \Psi_{o2})^2 \ln[1 + \exp(-2\kappa H)]\}$	[16]
$\Delta G_H^{AB}$ (sp)	$2\pi R\lambda\Delta G_{H=1_o}^{AB} \exp[1_o - H/\lambda]$	[12]

surface potential, constant charge or mixed mode (the bacterial surface is at constant charge while the surface of the support material is at constant potential). Table II summarises the equations used for the calculations.

### 3. RESULTS AND DISCUSSION

The results of the adhesion assays are presented in Figure 1. In culture medium there is a clear preference of the bacteria for PP, whereas they have hardly any affinity for PMMA. In sodium phosphate buffer the higher affinity is between bacteria and HDPE, while PMMA is again the material showing less capability for bacterial adhesion.

The values of the contact angles obtained with the various liquids employed for bacteria and support materials, as well as the surface tension components, are summarised in Table III. The values obtained

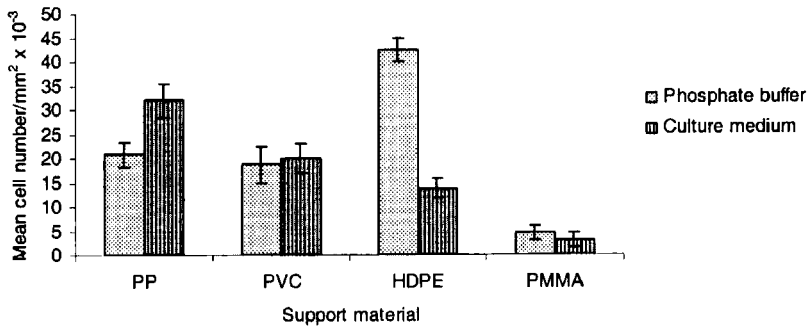


FIGURE 1 Number of bacteria per square mm of the polymer surface.

TABLE III Contact angles (degrees) and surface tension components of *Alcaligenes denitrificans* and supporting materials (mJ/m<sup>2</sup>)

Material	Contact angle			Surface tension components				
	Wat.	Glyc.	Diiod.	$\gamma_s^{LW}$	$\gamma_s^+$	$\gamma_s^-$	$\gamma_s^{AB}$	$\gamma_s^{TOT}$
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 ± 1.7	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

Wat. - water, Glyc. - glycerol, Diio. - Diiodomethane.

for *Alcaligenes denitrificans* reveal the highly hydrophilic nature of this bacterial strain. Although the contact angle measurements were performed after 3 hours of air drying, the ratio  $\gamma^-/\gamma^+ \approx 7$  is indicative of a still significant amount of water of hydration, which is substantially oriented [13]. The hydration of the bacterial cells is responsible for the very small order of magnitude of the Hamaker constants, resulting in negligible van der Waals attraction. That is to say that according to DLVO theory a high energy barrier is expected near the support surface.

This is shown in Figure 2, which indicates that the bacteria should be repelled in almost all cases by the polymeric materials at any distance of separation ( $H$ ) above 1 nm, except in the mixed interaction mode. Even in this situation, irreversible adhesion (in a primary minimum) should not be expected due to the energy barriers arising near the surface. But a reversible adhesion is possible in the secondary

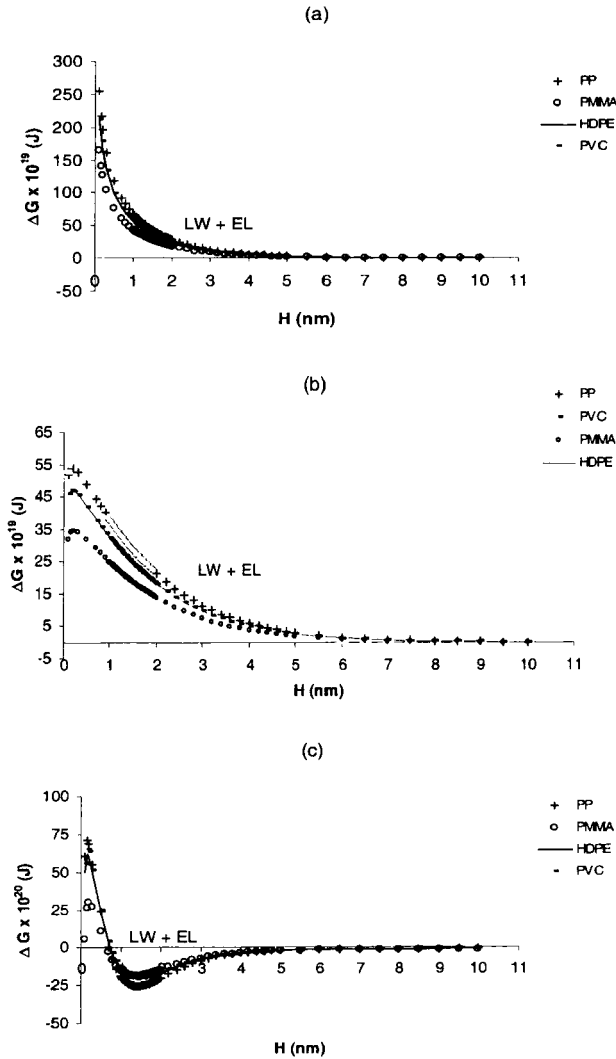


FIGURE 2 DLVO-plots: The total free energy of interaction as a function of the separation distance,  $H$ , under the condition of constant charge (a), constant potential (b) and mixed case (c).

minima formed at 1–2 nm from the surface. If this is the case, the cells would adhere more strongly to PP than to the other polymers, because this one shows the deepest secondary minimum. As far as XDLVO



theory is concerned (Fig. 3), adhesion would only be favourable to HDPE, especially in the mixed mode interaction, because all the other materials show energy barriers near the surface and no secondary

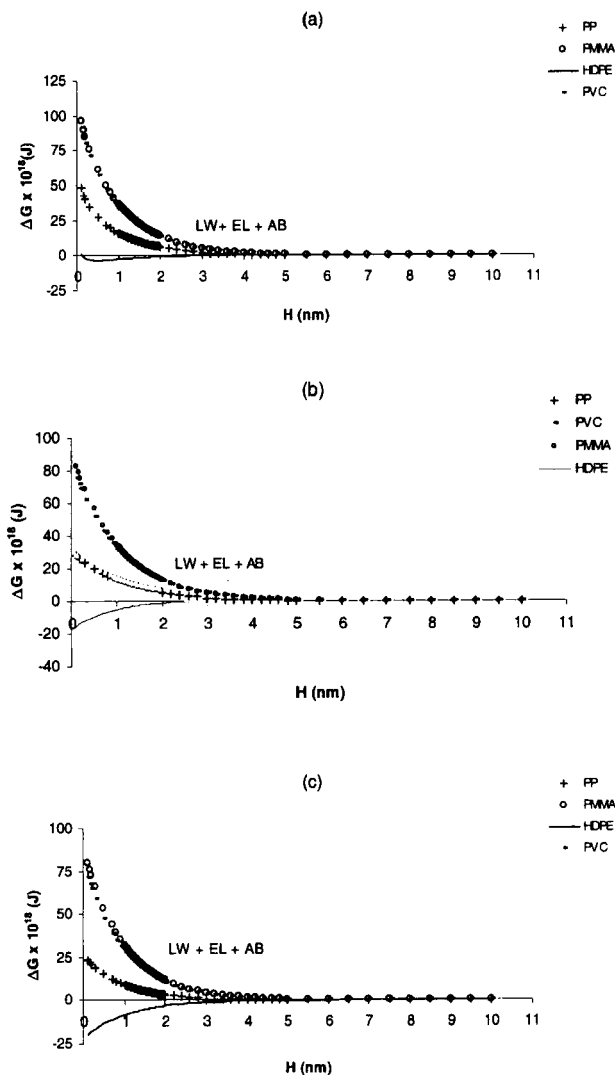


FIGURE 3 XDLVO-plots: Total free energy of interaction *versus* distance, including the effect of polar AB forces for sphere/plate geometry at constant charge (a), constant potential (b) and mixed case (c).

minimum. This means that the inclusion of polar interactions is not enough to explain the adhesion process. It must be noted that the energy profiles obtained for the interactions in PBS are the same as in culture medium, since the ionic strength is the same.

Figure 4 shows that adhesion taking place in culture medium is more favourable to the more negatively charged materials and reveals a linear dependence between zeta potential and the number of adhered cells. This points out the preferential establishment of ion bonds, especially with divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ), present in the medium. This hypothesis is validated by the adhesion tests performed in sodium phosphate buffer, in the absence of plurivalent cations.

A possible explanation for the higher ability of HDPE in PBS for bacterial adhesion arises from its  $\gamma^+ \neq 0$  ( $\gamma^-/\gamma^+ = 0.73$ ), which means that HDPE has a non-oriented hydration layer and is able to establish interactions by accepting electrons. From the data in Figure 1 and Table III it can be seen that in this medium the number of adhered cells decreases as the  $\gamma^-$  of the polymeric materials increases. The decrease in the number of cells adhered to HDPE, when immersed in culture medium, can be attributed to a preferential electron acceptance by plurivalent cations rather than by the polymeric surface. A higher value of  $\gamma^-$  gives rise to a strong orientation of the water molecules in the first layer adjoining the interface and to a lesser extent in subsequent layers. This might cause an increase of the repulsive "hydration pressure" along the distance from the interface. It should be kept in mind that polymeric interactions, established between

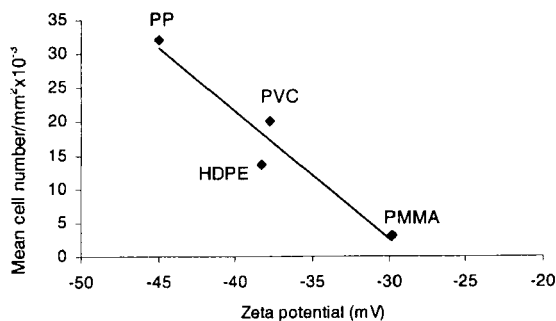


FIGURE 4 Mean cell number per square mm adhered to each polymeric material in culture medium as a function of zeta potential.

the bacterial exopolymers and the solid surfaces, can also mediate adhesion. An increase in surface hydrophobicity favours the repulsion of water molecules, promoting the approach of bacteria and the establishment of polymeric bridges with the hydrophobic sites of the bacterial exopolymeric extensions. This may explain why, with the exception of HDPE due to the reasons given above, adhesion in PBS increases with the increasing hydrophobicity (contact angle of water – Tab. III) of the polymeric materials.

#### 4. CONCLUSIONS

The adhesion of *Alcaligenes denitrificans* to the polymeric materials used is not totally explained by DLVO theory and the inclusion of polar interactions – XDLVO theory – only predicts favourable adhesion to one of the polymers (HDPE).

The results of the adhesion assays support the assertion that the interactions between bacteria and polymeric supports, when immersed in culture medium, are strongly influenced by ion bridging.

In the absence of plurivalent cations, the establishment of polymeric bridges and a higher hydrophobicity of the support seems to be required for the adhesion process.

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