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Adhesion of the marine bacterium *Pseudomonas* sp. NCIMB 2021 to different hydrogel surfaces

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Abstract

Adhesion of *Pseudomonas* sp. NCIMB 2021 was tested on different non-solid hydrogel surfaces under different shear conditions. Gels consisting of alginate (highly anionic), chitosan (highly cationic), modified polyvinyl alcohol PVA-SbQ (very low cationic) and agarose (neutral) were casted in moulds custom-made for a rotating annular biofilm reactor. Cells were stained with SYBR^R Green I nucleic acid gel stain, and images were collected using a confocal laser scanning microscope. Relative adhesion was quantified by determining percent cell coverage using image analysis. Bacterial adhesion on gels decreased at higher shear rates. At low shear rates, adhesion varied significantly between different gels, in the following descending order: alginate > agarose > chitosan > PVA-SbQ. Only adhesion to alginate remained significantly higher than to the others at high shear rates. Lowest cell coverage at all shear rates was recorded on PVA-SbQ gels. Clearly, the macroscopic hydrophobicity of the hydrogel surfaces did not enhance adhesion as observed for solid surfaces. A 5% PVA-SbQ gel showed the most promising antifouling properties.

Keywords: Biofouling; Non-solid surfaces; Biofilm reactor; Image analysis

1. Introduction

Submerged surfaces, including ships' hulls, are quickly covered with microfoulers such as bacteria, diatoms and protozoa, as well as macrofoulers such as barnacles and mussels, causing increased frictional resistance and biodeterioration [1]. The importance of bacteria in marine colonization of surfaces has been known for a long time [2]. A marine bacterium that has been studied intensively for the purpose of solving problems related to biofouling is *Pseudomonas* sp. NCIMB 2021 first described by Fletcher and Floodgate [3].

It is generally believed that the extracellular polymeric substances (EPS) play an important role in the adhesion of bacteria to surfaces. Considerable work has been done exploring the adhesion mechanisms of *P.* sp. NCIMB 2021 and ways to prevent it from developing a biofilm [3–12]). Fletcher [4] showed that the adhesion rate to polystyrene Petri dishes decreased in cultures passing from the exponential growth phase, through the stationary phase to the death phase. One explanation for this phenomenon was given by Christensen et al. [6] who showed that this organism produced one polysaccharide in the exponential growth phase and a different polysaccharide in the stationary phase. Fletcher and Loeb [7] compared directly the adhesion of this marine pseudomonad to hydrophobic and hydrophilic surfaces. Bacteria were most abundant on hydrophobic surfaces, and the number of attached cells increased inversely with the wettability of those surfaces. The surface charge seemed to be a crucial factor for adhesion to hydrophilic surfaces, where moderate numbers of cells attached to metals with a positive or neutral charge (platinum and germanium), and very few bacteria were detected on

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negatively charged surfaces (glass, mica, and oxidized plastics). Fletcher [5] concluded that bacteria have both active and passive attachment mechanisms.

As illustrated above, bacterial adhesion to solid surfaces has been studied intensively. Less attention has been given to non-solid surfaces such as hydrogels, consisting of 90–99% water. It has already been shown that hydrogels may be suitable for incorporating bioactive materials [13–16]. Cook et al. [17] found that the amount of *Pseudomonas aeruginosa* adhering to poly hydroxyethyl methacrylate (PHEMA) based hydrogels decreased with increasing water content of the gel. Preliminary experiments showed that PVA-SbQ gels revealed antiadhesive effects towards *P.* sp. NCIMB 2021 compared to glass slides [18]. Recently, we have shown that non-solid surfaces did not represent an absolute obstacle to settling and growth of the diatom *Amphora coffeaeformis* [19]. However, clearly reduced attachment was observed on gels with low charge density, particularly at high shear. The same gels also inhibited settlement of *B. amphitrite* cypris larvae compared to polystyrene controls [20]. Also in the case of barnacle settlement, gels with low charge density revealed the most promising antifouling properties.

Hydrogels are polymer networks with the ability to bind large amounts of water. Typical pore diameters may be in the range of 50–1500 Å, as observed for Ca-alginate gels [21]. On microscale, small molecules may therefore diffuse freely in and out of the gel matrix. On macroscale, however, these gels may be recognized as surfaces or barriers by particles and molecules larger than the pore size. Large structures such as bacteria, at the size of 1 μm = 10,000 Å, may sense and respond to the average macroscopic properties of this apparent surface, properties such as average charge density or hydrophobicity. Even larger structures, such as a drop of liquid at the size of 1 mm, may be applied to test the apparent wettability or macroscopic hydrophobicity of such a gel surface.

The aim of this work was to test bacterial adhesion to non-solid surfaces in relation to the physical and chemical properties of the gels. In order to reveal possible interactions between the bacteria and the polymeric gel network, cationic, anionic as well as neutral gelling substances were included. Restricting the study to biocompatible gel formation systems, alginate (highly anionic), chitosan (highly cationic), modified polyvinyl alcohol PVA-SbQ (very low cationic) and agarose (neutral) were therefore chosen for the test program. Glass served as a solid surface reference.

2. Materials and methods

2.1. Culture

A pure culture of *P.* sp. NCIMB 2021 (National Collections of Industrial Marine, Food and Industrial Bacteria, Aberdeen, Scotland) was grown at 20°C in the medium applied by Christensen et al. [8] with the following modifications: KH_2PO_4 and K_2HPO_4 were exchanged with 10 mM BIS-TRIS (Sigma Chemicals Co., USA) and 6.2 μM $NaH_2PO_4 \times H_2O$. In addition, the yeast extract applied in our work was not dialyzed. The pH was adjusted to 7.2. Cell densities were monitored by measuring the optical density (OD) at 660 nm using a UV/visible spectrophotometer (Ultraspec. 2000, Pharmacia Biotech).

2.2. Test surfaces

As summarized in Table 1, the following gels were tested for bacterial adhesion: agarose (Sigma Chemicals Co., USA), polyvinyl alcohol substituted with light-sensitive stilbazolium groups (PVA-SbQ, Toyo Gosei Kogyo Co., Ltd., Japan), alginate (LF10/60, Pronova Biopolymer A/S, Norway), and chitosan (F_A 0.17, Pronova Biopolymer A/S, Norway). Regular window glass (Float glass, Pilkington, Norway) was used as a

Table 1
Description of the test surfaces applied

Surface type	Surface material	Type	Standard concentration (%,wt/wt)	Gel strength, E (N/m^2)	Contact angle (degrees ± s.d.) (number of replicates)	Gelling principle
Solid reference	Glass	—	—	—	n.d.	—
Test gel	Agarose	Neutral	1.0	38 000	49 ± 7 (10)	Thermic; cooling
Test gel	PVA-SbQ	Low cationic	5.0	6 400	92 ± 3 (12)	Photoinduced crosslinking
Test gel	Alginate	Anionic	1.0	6 800	24 ± 2 (24)	Ca^{2+} crosslinking
Test gel	Chitosan	Cationic	1.0	1 600	67 ± 5 (20)	Glutaraldehyde crosslinking

solid surface reference material. The choice of polymer test concentration and the preparation of the gels has been treated in detail elsewhere [19]. Gelation occurred directly in moulds custom-made for the reactor. PVA-SbQ and chitosan gels were leached in PBS for 48 h, whilst agarose and alginate gels were leached for 24 h to remove soluble components [20]. Glass slides were washed in a 10% HCl solution and then in 70% ethanol. They were rinsed and stored in distilled water prior to use.

Gel strength was determined by calculating the Young's modulus (E) from the initial slope of the force versus deformation curve [22] measured by a Stable Micro Systems TA-XT2 Texture analyzer (Stable Micro Systems, England). At such low forces, the fluid will remain within the polymer network.

When a drop of liquid is placed on a planar surface, and the surface tension of the liquid exceeds that of the surface, a definite contact angle may be identified between the droplet and the surface [23]. By applying a droplet of 4 μl water, corresponding to a spherical radius of 2 mm, on a horizontal planar gel, macroscopic or apparent static water contact angles of non-treated gel surfaces were determined in a Dynamic Contact Angle and Absorption Tester (DAT 1121, Fibro system AB, Sweden). The angle was recorded 10 s after the water drop had been applied on the surface. Data obtained by this operational definition are by us referred to as apparent contact angles, indicating macroscopic wettability or hydrophobic properties of the gel surface, that is properties of relevance for structures much larger then the pore size of the gel.

2.3. Experimental setup

The experimental setup is pictured in Fig. 1. A culture flask containing 100 ml of sterile medium was inoculated with a *P.* sp. NCIMB 2021 suspension stored at −80°C. After 24 h, 1 ml of the new culture was transferred to another culture flask containing 200 ml of medium. This culture was allowed to grow for 10 h before the OD was measured, and the whole culture was transferred to the sterilized rotating annular biofilm reactor (Biosurface Technologies Corporation, MT, USA). The reactor has been described elsewhere [19]. Briefly, the reactor consisted of two stationary outer cylinders, and a rotating inner cylinder. The inner cylinder had been modified to contain 16 removable casting moulds for gels. Water from a waterbath was recycled in the space between the two outer cylinders to maintain a constant temperature of 20°C. Three slides of each gel type and four glass slides were used in each experiment. Medium was recycled through a recycle loop containing a mixing chamber. A liquid sample was collected for pH and OD measurements after the reactor had been running for 1 h in batch mode. Afterwards, medium was continuously added at a dilution rate of $1\,h^{-1}$. The recirculation rate was approximately 10 times higher than the dilution rate to ensure a well-mixed system. Test surfaces were sampled after a total of 14 h in the reactor. The pH and OD were also measured at this time.

Slides were then submerged 3 times in PBS, 2 min in 96% ethanol [24], and finally stained for 15 min in a SYBR^{R} Green I nucleic acid gel stain solution (Molecular Probes, Leiden, The Netherlands) prepared by diluting a 10 000X concentrate 400 times [25] with PBS.

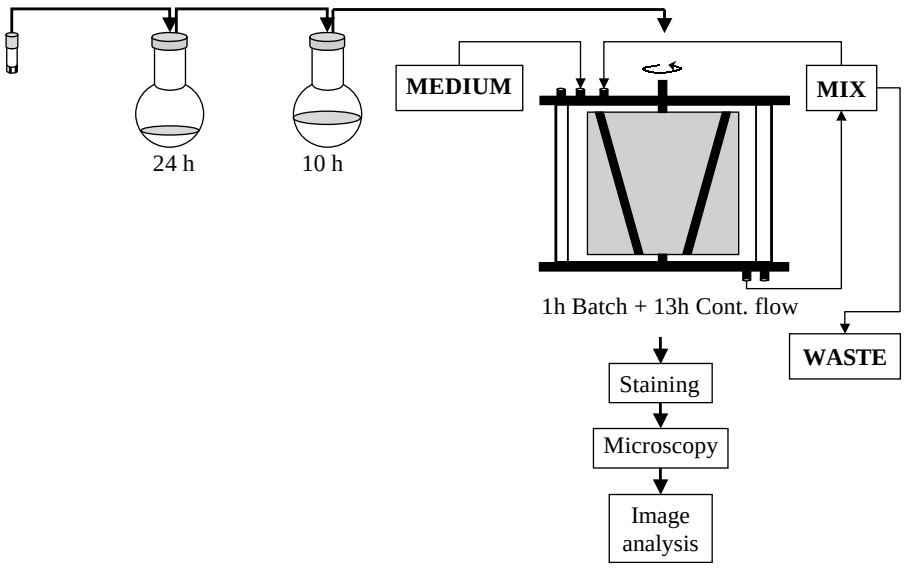

Fig. 1. Experimental setup.

Twelve images were randomly collected along the center-line of each slide using a confocal laser scanning microscope (MRC-600, BIO-RAD Microscience Division, England), see also Fig. 2. The percent coverage [26] by bacteria on a $64\,\mu m \times 64\,\mu m$ square of each image was determined using Matrox^{R} Inspector (version 3.0, Matrox Electronic Systems Ltd., USA). Experiments were performed once at four different shear rates, calculated from the angular velocity of the rotating inner cylinder.

2.4. Statistical methods

Analysis of variance (ANOVA) was performed on adhesion data using MINITAB^{TM} (version 13.1, Minitab Inc.). A multiple comparison of different gels for each shear rate was done using Tukey's test [27].

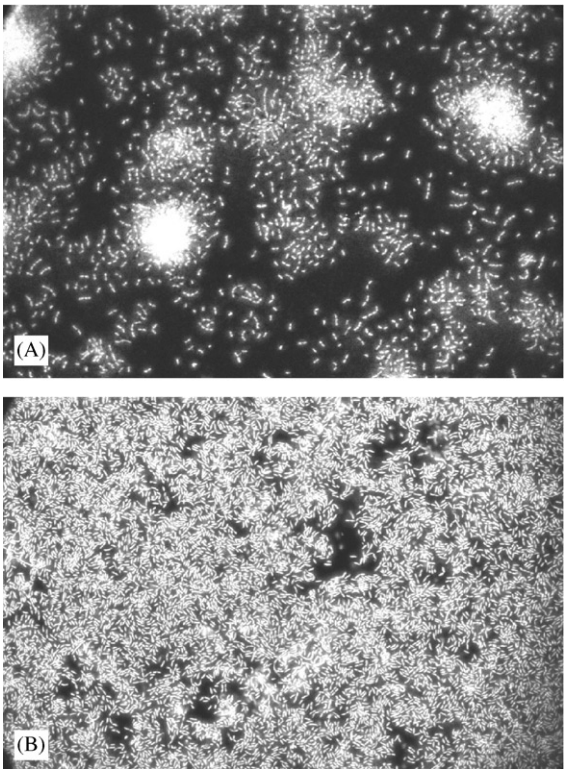

Fig. 2. Growth patterns recorded on (A) a PVA-SbQ gel and (B) glass tested at a shear rate of $5.80\,s^{-1}$.

3. Results and discussion

In order to get an indication of the macroscopic hydrophobicity of the gels, apparent water contact angles were measured. Results in Table 1 show that the PVA-SBQ appeared as more hydrophobic than the chitosan gel. The alginate gel was the most hydrophilic gel, followed by agarose.

Results from pH and OD measurements for each experiment are presented in Table 2, showing that the cell densities and growth conditions of the experiments were reproducible. A separate growth experiment in batch showed that logarithmic growth phase ceased when the OD exceeded 0.05 (results not included). Hence, cells in the inoculum were in the transition phase between exponential growth and stationary phase. In spite of a dilution rate of $1\,h^{-1}$, that is higher than the maximal specific growth rate observed in batch ($0.5–0.7\,h^{-1}$), the cell density increased in the reactor during the continuous flow mode. However, the OD did not exceed 0.05, and the cells were thus maintained in the log phase.

Fig. 2 shows a typical example of growth patterns recorded on glass and a PVA-SbQ gel tested at the lowest shear rate ($5.8\,s^{-1}$). Cell coverage on glass was clearly higher than on the PVA-SbQ gel. Another difference between the two surfaces was the presence of cell clusters on PVA-SbQ as visualized. However, cell clustering was also observed on the other gels, and even on glass at higher shear rates.

Relative adhesion of *P.* sp. 2021 to different gels and glass as a function of shear rate is summarized in Fig. 3. Cell coverage decreased at higher shear rates. ANOVA, performed as described in the Methods section, indicated significant difference in adhesion to all gels at a shear rate of $5.8\,s^{-1}$. Adhesion was lowest on PVA-SbQ followed in increasing order by chitosan, agarose, and alginate. At the highest shear rate tested, $28\,s^{-1}$, only adhesion to alginate was significantly higher than to the other three gels. There was no significant difference in

Table 2
Shear rate, pH- and optical density data for each experiment

Shear rate (s^{-1})	pH 1 h after incubation	pH 14 h after incubation	OD_{660} of inoculum	OD_{660} 1 h after incubation	OD_{660} 14 h after incubation
5.80	7.03	6.93	0.082	0.016	0.042
13.7	7.05	6.98	0.076	n.d.	0.044
20.6	7.06	7.01	0.081	0.016	0.046
27.5	7.04	6.99	0.070	0.017	0.042

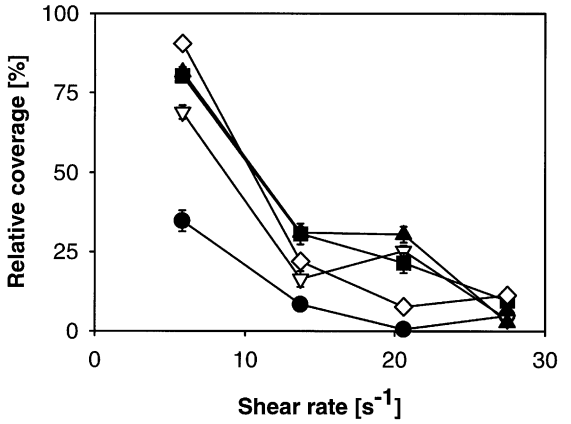

Fig. 3. Relative adhesion of *P.* sp. 2021 to (◇) glass, (■) agarose, (●) PVA-SbQ, (▲) alginate, and (▽) chitosan as a function of shear rate. Each data point represents the average relative coverage of three gel slides or four glass slides where 12 images were collected on each slide. Error bars represent the standard error of the mean.

adhesion to agarose, PVA-SbQ and chitosan gels at these conditions.

Earlier work including 2-h adhesion experiments has shown that *P.* sp. NCIMB 2021 prefer to adhere to hydrophobic rather than hydrophilic solid surfaces [7,10,11]). Cell coverage data presented in this paper is the result of both adhesion and growth. Hence, the results are not directly comparable with the results from the short-term adhesion experiments cited above. However, it is clear from Fig. 3 that other forces than the hydrophobic interactions are determining for cell coverage on non-solid surfaces. The most hydrophobic gel, PVA-SbQ, supported the lowest number of cells at all shear rates tested.

The surface charge was crucial for adhesion of the pseudomonad to hydrophilic surfaces in the work of Fletcher and Loeb [7]. Cells seemed to be electrostatically repelled by negatively charged surfaces. The polymer charge of the gels in our work seemed to have minor effect on adhesion and subsequent growth. Cell coverage on alginate (anionic) gels was higher than on chitosan (cationic) gels at 5.8 and 28 s^{-1}, it was the other way round at 21 s^{-1}, and there was no significant difference at 14 s^{-1}. However, it should be emphasized that the pK_a for chitosan is approximately 6.6, meaning that amino groups may be discharged at a pH ranging from 6.9 to 7.2. This may result in both a lower surface charge as well as a denser polymer network.

Both PVA-SbQ and agarose contain low amounts of charged groups. In addition, agarose is less hydrophobic than PVA-SbQ gels. However, three of our experiments show that cell coverage was significantly higher on agarose than on PVA-SbQ (Fig. 3). This should be related to the Young's moduli of the two gels (Table 1), revealing that 1% agarose gels were more rigid.

4. Conclusions

(1) Adhesion of *P.* sp. NCIMB 2021 to glass and all four gels tested was reduced at increased shear rates in the interval tested (5.80–27.5 s^{-1}).
(2) Adhesion to a 5% PVA-SbQ gel was generally lower than all other surfaces tested.
(3) At low shear rates adhesion varied significantly between different gels in the following descending order: alginate > agarose > chitosan > PVA-SbQ.
(4) At the highest shear rate tested (27.5 s^{-1}), only adhesion to alginate remained significantly higher than to the other gels.
(5) There was no direct correlation between the hydrophobicity of the hydrogel surfaces and adhesion of *P.* sp. NCIMB 2021 as observed previously for solid surfaces. On the contrary, the apparently most hydrophobic gel was the least attractive for cell adhesion.

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