

## **Effects of DNP on cell surface properties of marine bacteria and its implication for adhesion to surfaces**

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### **Abstract**

The effect of 2, 4-dinitrophenol (DNP) on extracellular polysaccharides (EPS), cell surface charge, and hydrophobicity of six marine bacterial cultures was studied, and its influence on attachment of these bacteria to glass and polystyrene was evaluated. Interestingly, after DNP treatment there was a significant decrease in the hydrophobicity of both hydrophobic ( $p = 0.01$ ) and hydrophilic ( $p = 0.05$ ) cultures, whereas no significant change was observed in the cell surface charge and EPS production of these cultures. DNP treatment resulted in significant reduction in the attachment of all the six cultures to glass ( $p = 0.02$ ) and polystyrene ( $p = 0.03$ ). Moreover, hydrophobicity but not the cell surface charge and EPS production, influenced bacterial cells attachment to glass and polystyrene. From this study, it was evident that DNP treatment influenced bacterial cell surface hydrophobicity, which in turn, reduced bacterial adhesion to surfaces.

*Keywords: Biofilm, 2, 4-dinitrophenol, hydrophobicity, surface charge, EPS, cell attachment, glass and polystyrene panels*

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## Introduction

Bacterial adhesion to submerged surfaces in marine water is considered as a primary step in surface colonization by microorganisms in the biofouling process (Callow, 1986). Biofouling of surfaces cause serious economic problems such as increase in fuel consumption by ships, impair heat transfer and may induce and/or inhibit metal corrosion (Walker *et al.*, 1998). Thus, initial bacterial adhesion to surfaces is probably the key to understand the fouling process. Such understanding will help in developing suitable techniques to control the problem of biofouling.

To prevent bacterial adhesion, marine structures are treated with toxic chemicals known as antifoulants. The use of antifoulants since few decades has been considered as a most effective method to prevent bacterial attachment to submerged surfaces (Townsin, 2003). Each antifoulants inhibits bacterial attachment by certain mechanisms of action. The most important mechanism of action are inactivation of essential enzyme (Chapman, 2003 & Cross *et al.*, 2003), oxidation of organic constituents in cells (Chapman, 2003), destabilization of cell membranes and cell lysis (Chapman, 2003 & Walsh *et al.*, 2003), and uncoupling the membrane potential (Zhao *et al.*, 1998). Interestingly, it appears that most of the antifoulants reduce (affects) cellular metabolism and growth thereby reducing the number of viable bacterial cells available for adhesion to surfaces (Haque *et al.*, 2005). However, there is a lack of information on the effect of antifoulants on other factors such as cell hydrophobicity, cell surface charge, and EPS production that are involved in bacterial adhesion to surfaces. Thus, understanding the effect of antifoulants on cell surface properties and EPS production may provide a better insight to the mechanism of inhibition of bacterial adhesion to surfaces that will help us in designing appropriate antifouling strategies.

The 2,4-dinitrophenol (DNP), a un-coupler of oxidative phosphorylation, is known to inhibit the bacterial adhesion to surfaces (Fletcher, 1980 & Paul, 1984). Previous study by Eighmy *et al.*, (1992) reported that low release rate ( $0.5$  to  $4.0 \mu\text{g cm}^{-2} \text{d}^{-1}$ ) of DNP from simple copolymer film inhibits bacterial activity, whereas, high release rate ( $15$  to  $70 \mu\text{g cm}^{-2} \text{d}^{-1}$ ) inhibits both activity as well as attachment. Recently, it was reported that DNP inhibits biofilm accumulation by blocking EPS synthesis (Cammarota, 1998 & Masak *et al.*, 2002). However, it is well documented that initial bacterial attachment to surfaces is influenced by several other factors such as cell surface hydrophobicity and cell surface charge (Fletcher, 1985; Handley *et al.*, 1987; Van loosdrecht *et al.*, 1987; Stenstrom, 1989 & Sorongon *et al.*, 1991). Therefore, the aim of this research was to assess the effect of the DNP on bacterial cell surface hydrophobicity, cell surface charge, and EPS production, and its affect on bacterial attachment to glass and polystyrene.

## **Materials and methods**

### *Panel preparation*

Panels (3.5 x 1.5 cm<sup>2</sup>) were cut (Engineering workshop, NIO, Goa, India) from autoclavable polystyrene petriplates (Hi media, India) and glass slides (corning, USA). Panels were prepared according to modified method of Sonak and Bhosle (1995). Briefly, panels were treated with liquid detergent (Labolene; Qualigens fine chemicals, India), washed thoroughly with distilled water and air dried. The panels were then covered with aluminum foil and sterilized at 121 °C for 15 minutes.

### *Growth media, bacterial strains and inoculum preparation*

Throughout the course of this study a modified basal salt solution (BSS) medium was used. The medium contains (g L<sup>-1</sup>): 25.0 NaCl, 0.75 KCl, 7.0 MgSO<sub>4</sub>, 0.9 NH<sub>4</sub>Cl, 0.2 CaCl<sub>2</sub>, 7.0 K<sub>2</sub>HPO<sub>4</sub>, 3.0 KH<sub>2</sub>PO<sub>4</sub>, 1.0 glucose and 1.0 ml/L trace metal solution (Bhosle, 1981). All media were sterilized as above.

Six bacterial cultures used in the present study were collected from the culture collection of the MCMRD, NIO, Goa (India). The cultures were isolated from the biofilm developed on panels (Steel, Titanium and Copper) immersed in the surface coastal waters of the Dona Paula Bay, west coast of India. Isolated cultures were morphological and biochemically identified (Table I). These cultures were maintained on Zobell marine agar (ZMA) slants at 4 °C. For inoculum preparation, a loop full of culture from slant was subcultured using BSS medium for 3 days at room temperature (28 ± 2°C). This step was repeated once more and the culture was used as a source of inoculum for the experiments describe below.

### *Minimum inhibitory concentration (MIC) of DNP*

The minimum inhibitory concentration of DNP was determined separately for each of the six cultures. A series of test tubes containing 5 ml of BSS medium and various concentrations (4 x 10<sup>-3</sup>M to 6 x 10<sup>-4</sup>M) of DNP were inoculated with the individual culture prepared as above. The tubes were incubated for 72 hours in dark at room temperature. After incubation period the absorbance of each culture was measured at 540 nm using a UV- VIS spectrophotometer (Shimadzu Model 1601).

### *DNP treatment and preparation of bacterial suspension*

For assessing the effect of DNP on growth, EPS production, bacterial cell surface properties, and attachment, each culture was grown separately in 150 ml BSS medium in 500 ml conical flasks, with and without 25% of the respective MIC of the DNP. The flasks were incubated for 72 hours in dark at room temperature. At the end of incubation period, two subsamples of 500 µl

each were removed, to estimate viable cell count and EPS production as describe below. The remaining culture broth was centrifuged at 5000 rpm for 20 minutes. The cell pellet obtained after centrifugation was washed thrice with phosphate buffer saline (PBS) (NaCl 2.5%) and resuspended in the same buffer so as to obtain  $OD_{540} = 0.2$ , using UV- visible spectrophotometer. This Cell suspension was used to evaluate effect of DNP on cell surface properties and attachment to glass and polystyrene, as describe below.

#### *Viable cell counts*

In order to enumerate viable counts, an aliquot of 500  $\mu$ l from culture describe above was serially diluted using sterile seawater. The appropriate dilutions were spread on ZMA containing petri plates. The plates were incubated at room temperature ( $28\text{ }^{\circ}\text{C} \pm 2$ ) for 24 hours, and colonies were counted manually.

#### *Estimation of extracellular polysaccharides (EPS)*

Amount of EPS produced by the cultures grown as above was estimated by the modified phenol- sulphuric acid method, using glucose as a calibration standard (Dubois *et al.*, 1956). In brief, 1 ml culture broth was centrifuged and supernatant was filtered through 0.22  $\mu$ m (Millipore filter). Filtered sample was then dialyzed against distilled water for 48 hours, using 13000 MW dialysis bags. After dialysis the sample was made to known volume. A known aliquot (500  $\mu$ l) of the sample was removed in a test tube and 1 ml phenol (2.5%) followed by 2.5 ml concentrated sulphuric acid was added. The absorbance of the sample was read at 490 nm using a spectrophotometer

#### *Estimation of cell surface charge*

Cell surface charge of bacteria was determined using the method of Pedersen (1980). In short, Pasteur pipettes of 15 cm length and 5 mm inner diameter were plugged with glass wool and washed with PBS. Anion exchange resin Dowex 1 x 8 or cation exchange resin Dowex 50W x 8, (100 – 200  $\mu$ m), was suspended in PBS (1 g/ml). Each ion exchange resin in PBS was packed separately in Pasteur pipettes. Prior to use column was washed with 5 ml PBS. One ml of the bacterial suspension was added to the column and the cells were eluted with 5 ml PBS. The affinity of bacteria for ion exchange column was calculated as below

$$\text{Relative surface charge (r/e)} = (1 - X / X_0)$$

where X is the OD (540 nm) of the eluant, and  $X_0$  is the OD (540 nm) of cell suspension before it was loaded on the column.

#### *Measurement of hydrophobicity*

Hydrophobicity of the bacterial cell suspension prepared as above was evaluated using BATHS

assay following the procedure described by Rosenberg *et al* (1980). In brief, 100 µl of *n*-hexadecane (Sigma, USA) was added to 3 ml bacterial cell suspension ( $OD_{540} = 0.2$ ) prepared as above. The suspension was vortexed for 1 minute to ensure mixing and then left to stand for 15 minutes to allow separation of the two phases. The absorbance of aqueous phase was taken at 400 nm using Spectrophotometer using 1 ml polystyrene cuvetts (Brand, USA), and the percentage of the cells bound to *n* hexadecane was calculated using the following formula:

$$\text{Bacterial cell Adherence to hexadecane (\%)} = (1 - A / A_0) \times 100$$

where  $A_0$  is the OD (400 nm) of the aqueous cell suspension before mixing hexadecane and  $A$  is the OD (400 nm) after mixing.

#### *Bacterial attachment assay*

Cell attachment assay was performed by immersing glass and polystyrene panels in a beaker (50 ml) containing 40 ml bacterial suspension ( $OD_{540} = 0.2$ ) prepared above. Panels were removed after 2 hours of immersion and rinsed thrice with PBS to remove the loosely attached bacterial cells. Enumerations of attached cells were done by modified method of Sonak and Bhosle (1995). In brief, panels were stained with 0.1% crystal violet for 15 minutes and rinsed with distilled water. Attached bacteria were counted under oil immersion 100 x objectives in 10 randomly chosen fields, using Olympus, upright light microscope.

#### *Statistical analysis*

Each experiment was done twice using duplicate samples. One-way analysis of variance (ANOVA) was used to evaluate differences between cultures, and the effect of DNP. A simple linear regression analysis was carried out to assess the relationships between cell surface hydrophobicity, cell surface charge, extracellular polysaccharides, and cell adhesion to glass and polystyrene. All calculations were performed using XLSTAT2006 (version 2006.3).

## **Results**

#### *MIC of DNP*

In the present study, the MIC defined as the minimum concentration of DNP at which bacterial growth was inhibited. For the six bacterial cultures MIC varied between  $3 \times 10^{-4}$  to  $6 \times 10^{-4}$  M (Table II).

#### **Effects of DNP**

##### *Bacterial growth*

We found that, at 25% of MIC of DNP the growth of all six cultures were inhibited. However, the percentage decrease in growth varied from 10% to 30%. (Figure 1a).

### *EPS production*

The extracellular polysaccharide production by six marine isolates in presence and absence of DNP varies from 0.5 to 3.8  $\mu\text{g} / \log \text{CFU}$  and from 0.46 to 3.3  $\mu\text{g} / \log \text{CFU}$ , respectively (Figure 1b). Out of six cultures three cultures (Ti 2, SS 15 and CE- 2) showed decrease in EPS production. One-way ANOVA for EPS production showed that there is no significant change in EPS production of cultures grown in presence of DNP ( $p = 0.188$ ) (Table III).

### *Hydrophobicity*

Based on the values of percentage adhesion of cells to hexadecane, we found that three out of six strains were moderately hydrophobic (Ti 2, SS 10 and Ti 6), while the other three were strongly hydrophilic (SS 15, CE-2 and CE-10). Out of six cultures two cultures (Ti2 and SS10) showed maximum decrease in hydrophobicity after DNP treatment (Figure 2 c). Results from the one-way analysis of variance (ANOVA) for moderately hydrophobic and strongly hydrophilic cultures showed a significant decrease in hydrophobicity after DNP treatment (Table III). Logarithmic regression analysis showed a significant positive linear correlation between hydrophobicity of cultures and cell attachment to glass ( $p > 0.01$ ) as well as polystyrene ( $p > 0.05$ ) (Figure 2 a & b) (Table V).

### *Cell surface charge*

The relative surface charge value ( $r/e$ ) of six marine isolates, grown in presence and absence of DNP, shows high anion  $r/e$  values then cation  $r/e$  values (Table IV). Results from the one-way ANOVA, shows no significant change ( $p = 0.79$ ) in the cell surface charge values of cultures after DNP treatment (Table III). Logarithmic regression analysis, showed no significant correlation between cell surface charge of cultures and bacterial cell attached to glass ( $p > 0.1$ ) and polystyrene ( $p = 0$ ) (Table IV).

### *Bacterial cell attachment*

Out of six bacterial cultures two cultures (Ti2 and SS 15), showed maximum decrease in attachment, after growth on DNP, to both the surfaces (Figure 1d & 1e). Also, all the six cultures after DNP treatment, showed almost same amount of decrease in attachment to glass and polystyrene (except Ti 2 and SS 15). Results from one-way ANOVA for cell attachment showed that there is a significant decrease in cell attachment of all the cultures grown in presence of DNP, to glass ( $p > 0.01$ ) as well as polystyrene ( $p > 0.02$ ) (Table III).

## **Discussion**

For the six bacterial cultures MIC varied between  $3 \times 10^{-4}$  to  $6 \times 10^{-4}$  M (Table II). However, the effect of DNP on bacterial cultures was evaluated using 25% of their respective MIC as done

earlier workers (Paul, 1984). At the 25% MIC, small inhibition (10 to 30 %) in the growth of each culture was observed (Figure 1a). This corroborated with earlier observation suggesting 10 to 30 % inhibition in the growth of an estuarine bacterium *Vibrio* sp at  $1.2 \times 10^{-4}$  M DNP (Paul, 1984). DNP at 25 % of the MIC concentrations was used to evaluate its effect on EPS production, cell surface charge, and hydrophobicity of the six bacterial cultures.

Production of EPS by the bacterial cultures in the presence and absence of DNP is shown Figure 1b. However, from our results it was evident that there was variable effect of DNP treatment on EPS production by these cultures. For example, EPS production by three cultures (Ti 2, SS 15 and CE-2) decreased while EPS production by other cultures was not influenced. It was reported earlier that DNP inhibits EPS production in attached cells (biofilm) and not in planktonic cells (Cammarota, 1998 & Masak *et al.*, 2002). Moreover, EPS production did not correlate with bacterial attachment to both glass and polystyrene. Therefore it can be concluded that polymeric interaction is not involved in attachment of these cultures to glass as well as polystyrene.

The relative surface charge values (*r/e*) of six cultures, grown with and without DNP, showed high anion (*r/e*) values compared to the cation (*r/e*) values, indicating a overall net negative surface charge on the cell surface of each bacterium (Table IV). Similar results were reported for marine bacteria cell surface charge by Pedersen (1980). However, anion *r/e* values obtained for all six isolates appear to be less, suggesting low negative charge on cell surface. There was no significant difference ( $p = 0.79$ ) in the cell surface charge values of 6 cultures after DNP treatment (Table III). On the other hand, cell surface charge did not show significant correlation with the bacterial cell attachment to both glass and polystyrene (Table V). From these results it was evident that DNP does not influence the cell surface charge and electrostatic interaction did not play a major role in the attachment of these bacteria to the glass as well as polystyrene.

There was a significant positive correlation between bacterial cell surface hydrophobicity and cell adhesion (Fig. 2a-b). This suggests the importance of hydrophobic interactions in the attachment of these cultures to glass and polystyrene. The hydrophobicity of both, moderately hydrophobic ( $p = 0.01$ ) and strongly hydrophilic ( $p = 0.05$ ) cultures showed significant decrease after DNP treatment (Table III). It is difficult to explain the observed decrease in bacterial hydrophobicity and adhesion to surfaces as little is known about the effect of antifouling compounds on bacterial cell surface hydrophobicity. Nevertheless, Teo *et al* (1998) suggested that DNP treatment destabilizes sludge granules due to decrease in hydrophobic interaction. These authors proposed that metabolic inhibitor like DNP can cause inhibition of proton translocation across the bacterial cell surface. This results in decreased dehydration of cell

surface leading to decrease in hydrophobicity. This may be the reason for observed decrease in the hydrophobicity, and subsequent bacterial adhesion to surfaces.

In summary, our results indicate that in addition to EPS production DNP affects the marine bacterial cell surface hydrophobicity, thereby reducing bacterial adhesion to glass and polystyrene.

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Table I Biochemical identification of six marine bacterial isolates used in the present study.

<b>Culture</b>	<b>CE-2</b>	<b>CE-10</b>	<b>Ti 2</b>	<b>Ti 6</b>	<b>SS10</b>	<b>SS15</b>
Grams	–	–	+	+	+	–
Grams character	Thin long rods	Small rods	Short rods in chain	Long rods	Thin rods	Short rods
Motility	+	+	+	+	+	+
Gelatin	+	–	+	–	–	+
Starch	+	–	+	–	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Nitrate	+	+	–	–	+	+
Citrate	+	+	+	+	–	+
Hugh-lesifson	F	O	O	F	NC	NC
Indole	–	–	–	–	–	–
Glucose	+	– <sub>A</sub>	+	+	+	+
Sucrose	+	– <sub>A</sub>	+	+	+	+
Inositol	+	– <sub>A</sub>	+	+ <sub>S</sub>	+	+
Galactose	– <sub>A</sub>	– <sub>A</sub>	+	+	+	+
Lactose	– <sub>A</sub>	– <sub>A</sub>	– <sub>A</sub>	+ <sub>S</sub>	+	+ <sub>S</sub>
<b>Tentative Genus</b>	Pseudomonas	Pseudomonas	Bacillus	Bacillus	Bacillus	Pseudomonas

NC = No change  
 F = Fermentative  
 O = Oxidative  
 –<sub>A</sub> = Alkaline  
 +<sub>S</sub> = Slightly alkaline

Table II Minimum inhibitory concentration (MIC) for six bacterial isolates.

Culture	Minimum inhibitory concentration (MIC) (M)	25 % MIC (M)
Ti 2	$4.0 \times 10^{-4}$	$1.0 \times 10^{-4}$
SS 10	$6.0 \times 10^{-4}$	$1.5 \times 10^{-4}$
Ti 6	$4.0 \times 10^{-4}$	$1.0 \times 10^{-4}$
SS 15	$6.0 \times 10^{-4}$	$1.5 \times 10^{-4}$
CE 2	$6.0 \times 10^{-4}$	$1.5 \times 10^{-4}$
CE 10	$3.0 \times 10^{-4}$	$0.7 \times 10^{-4}$

Table III One-way analysis of variance (ANOVA) (*p* values) for hydrophobicity, surface charge and EPS production, of six bacterial cultures and their attachment to glass and polystyrene.

Culture	Hydrophobicity	Surface charge	EPS production	Attachment	
				Glass	polystyrene
Moderately hydrophobic cultures	$p = 0.01^*$	$p = 0.89$	$p = 0.33$	$p = 0.05^*$	$p = 0.07$
Highly Hydrophilic cultures	$p = 0.05^*$	$p = 0.34$	$p = 0.32$	$p = 0.03^*$	$p = 0.22$
All six isolates	$p = 0.104$	$p=0.794$	$p = 0.188$	$p = 0.02^*$	$p = 0.03^*$

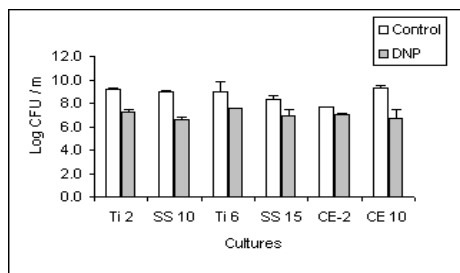
\* The difference was considered as significant at  $p \leq 0.05$

Table IV Cell surface charge (ESIC r/e values) of six cultures in control and DNP treatment

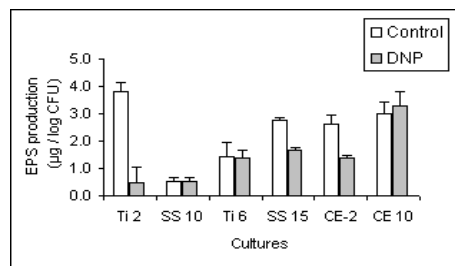
Cultures	ESIC (r/e values)			
	Control		DNP treatment	
	Anion value	Cation value	Anion value	Cation value
Ti 2	0.4 ± 0.1	0.3 ± 00	0.44 ± 0.02	0.26 ± 0.02
SS 10	0.07 ± 0.03	0.04 ± 0.01	0.09 ± 0.01	0.06 ± 0.01
Ti 6	0.4 ± 0.1	0.2 ± 0.01	0.41 ± 0.12	0.24 ± 0.03
SS 15	0.03 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
CE 2	0.2 ± 00	0.0	0.16 ± 0.08	0.07 ± 0.01
CE10	0.20 ± 0.02	0.0	0.02 ± 0.0	0.0

Table V Coefficients of correlation (r values) between cell adhesion and the hydrophobicity, cell surface charge and EPS production of six bacterial cultures.

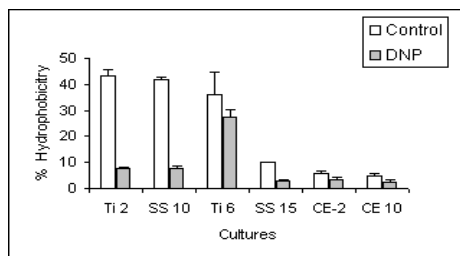
Parameters	Cell attachment			
	Control		DNP treatment	
	Glass	Polystyrene	Glass	polystyrene
Hydrophobicity	r = 0.86	r = 0.81	r = 0.73	r = 0.73
Surface charge	r = 0.66	r = 0.34	r = 0.63	r = 0.52
EPS production	r = 0.75	r = 0.014	r = 0.44	r = 0.18



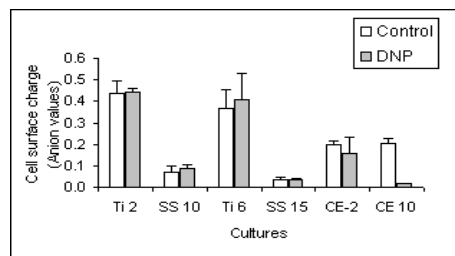
(a)



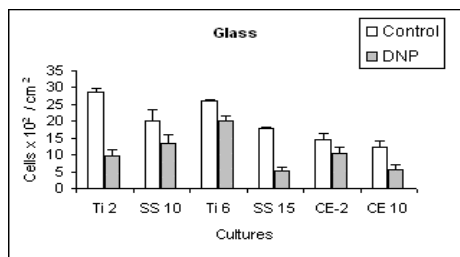
(b)



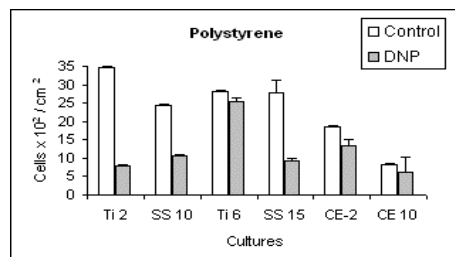
(c)



(d)

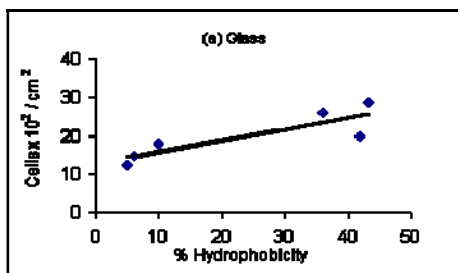


(e)

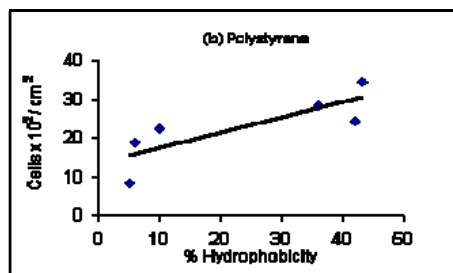


(f)

Figure 1 Effect of DNP on growth (a), EPS production (b), cell surface hydrophobicity (c), cell surface charge (d) and adhesion of six bacterial cultures in the presence of DNP and in the control.



(a)



(b)

Figure 2 Relationship between bacterial cell hydrophobicity and cell adhesion to glass (a) and polystyrene (b).