

NITROGEN STARVATION AFFECTS BACTERIAL ADHESION TO SOIL

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ABSTRACT

One of the main factors limiting the bioremediation of subsoil environments based on bioaugmentation is the transport of selected microorganisms to the contaminated zones. The characterization of the physiological responses of the inoculated microorganisms to starvation, especially the evaluation of characteristics that affect the adhesion of the cells to soil particles, is fundamental to anticipate the success or failure of bioaugmentation. The objective of this study was to investigate the effect of nitrogen starvation on cell surface hydrophobicity and cell adhesion to soil particles by bacterial strains previously characterized as able to use benzene, toluene or xilenes as carbon and energy sources. The strains LBBMA 18-T (non-identified), *Arthrobacter aurescens* LBBMA 98, *Arthrobacter oxydans* LBBMA 201, and *Klebsiella* sp. LBBMA 204-1 were used in the experiments. Cultivation of the cells in nitrogen-deficient medium caused a significant reduction of the adhesion to soil particles by all the four strains. Nitrogen starvation also reduced significantly the strength of cell adhesion to the soil particles, except for *Klebsiella* sp. LBBMA 204-1. Two of the four strains showed significant reduction in cell surface hydrophobicity. It is inferred that the efficiency of bacterial transport through soils might be potentially increased by nitrogen starvation.

Key-words: hydrophobicity; bacterial adhesion; nitrogen starvation

INTRODUCTION

The subsurface contamination by hydrocarbon fuels is a frequent problem that results from leaking underground storage tanks. Depending on the characteristics of the contaminants and of the contaminated soil, contamination of deeper zones, including the saturated zone, may occur. The toxic substances that compose these fuels also have the potential to eliminate many of the microbial populations found in the contaminated zones. In the worst case, the contamination may reach the aquifer, posing a serious threat to the biological systems that make use of this resource.

The natural decontamination of these areas, without anthropic intervention, generally occurs at slow rates. The rate of *in situ* biological decontamination can be increased by optimizing the environmental conditions that control the activity of microbial populations, so that they can be stimulated to degrade the contaminants (3). Where microbial populations able

to degrade the contaminants are absent or in low numbers, this adequacy may be followed by the introduction of selected microorganisms in the contaminated zones, a technique known as bioaugmentation (3).

The efficiency of biodegradation increases with the reduction of the contaminant flow (35). It is possible to diminish the hydraulic conductivity of a porous system by stimulating the formation of biofilms (11,15,32). The use of microorganisms to clog the pores is known as biobarrier, which is based on the formation of a subsurface barrier capable of controlling the percolation of the contaminants (15). The screening of microorganisms for use in bioremediation processes based on the formation of biobarriers takes into account cell surface characteristics and the biodegradation potential of the contaminants by the selected microorganisms.

To be successful, the introduction of microorganisms must be accompanied by the transport of the cells to the contaminated spots. During transport through the soil, the bacteria can lose

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viability due to nutrient limitation, predation, cell lysis or parasitism (9). Bacterial hydrophobicity has been cited as a criterion to select strains for injection in porous matrices, since it will determine which strain will remain suspended for a long period, facilitating its transport into the porous medium (21). Besides, the cells may adsorb to soil surfaces, making their transport difficult through a porous matrix.

Some characteristics of the cell surface interferes with cell adhesion to soil particles. According to Ling *et al.* (2002) (18), bacterial adsorption to solid surfaces is a complex phenomenon, which depends on the characteristics of the microorganism, the characteristics of the solid surfaces and of the liquid phase in which the bacteria are injected. Previous works also mention the hydrophobicity of the cellular surface (26), the superficial charges (8) and the physiological state of the cells (2,30) as factors that may affect the attachment of bacteria to surfaces.

Kim & Fogler (1999) (16) state that once the cells are injected into the subsurface, they generally are subjected to starvation. The starvation may affect many characteristics and factors of the bacteria, such as cell size (3,16,36), cell viability (1,23), resistance to environmental stresses (10,19,23,27), cell surface hydrophobicity (5,29), cell adhesion (8,29,34,36) and the transport of the bacteria through the soil (3,29). Starvation can result in a significant reduction of cell size in bacteria, and sometimes leads to the formation of ultra-micro bacteria (UMB) (3). Marouga & Kjelleberg (1996) (19) showed that bacteria survival under nutrient limitation is coupled to the expression of certain genes that enable the cells to enter into a state of induced adaptation to starvation and to resist to a variety of physical and chemical stresses. Another important effect of nutrient starvation is the reduction of cell surface hydrophobicity when bacteria are cultivated under nitrogen limitation, apart from other alterations that may result in the reduction of cell adsorption to soil surfaces (29). Cell surface charges also exert great influence on cell adhesion, and this characteristic may also be modified during nutrient starvation (8,34). A decrease of cell dimension and changes in composition of cell membrane, following a long period of nutrient starvation, may equally affect cell adhesion (36). Finally, some works affirm that the adhesion influence on the transport of the cells through the soil is higher than the influence of other surface properties (4,34).

The study of bacterial starvation on the efficiency of bioremediation is relevant for two main reasons: injecting starved bacteria might be a strategy to diminish bacterial adhesion to soil particles (29,34) and because, during bacterial transport, these cells suffer effects of starvation with relevant consequences, such as a decrease in cell viability. In this paper, we focused on some effects of starving bacterial cells in solutions without nitrogen, specially on the survival, surface hydrophobicity and adhesion to soil particles. To predict the effects of this strategy on bacterial transport, future works must be conducted.

MATERIALS AND METHODS

Microorganisms

The microbial strains were selected amongst those pertaining to the culture collection of the Laboratory of Environmental Biotechnology and Biodiversity, BIOAGRO/DMB/UFV, according to their growth capacity on benzene, toluene or xylenes as the only carbon sources. The microbial strains used in this work were *Arthrobacter aureescens* LBBMA 98, *Arthrobacter oxydans* LBBMA 201, *Klebsiella* sp. LBBMA 204-1 and LBBMA 18-T, which doesn't pertain to the library TSBA40 of Midi System (Microbial Identification, Newark, DE, USA).

Cell starvation experiments

Frozen-preserved bacterial strains were activated in R2A (28) at 150 rpm and 30°C for 15h. The medium was removed by centrifugation at 6,100 X G for 10 min and the cells were washed three times in 10mL of NaCl 0.85%. The cells were resuspended in R2A and incubated at 150 rpm and 30°C until the middle of the log phase. For starved-cells preparation, the washed cell pellet was resuspended in N-limiting mineral medium (NLMM) containing (g L⁻¹): Na₂HPO₄, 2.8; yeast extract, 0.01; Ca (NO₃)₂·4H₂O, 0.05; ferric ammonium citrate, 0.01; MgSO₄·7H₂O, 0.02; glucose, 15.0; KH₂PO₄, 2.0. An aliquot of cell suspension was transferred to an Erlenmeyer flask containing 150mL of NLMM, and nitrogen starvation was initiated with a cell concentration equivalent to an optic density of 600 nm (OD₆₀₀) of 0.4. Cell concentration (OD₆₀₀) was monitored daily through a seven day incubation at 150 rpm and 30°C.

Cell Surface Hydrophobicity

Cell surface hydrophobicity was determined according to Sanin *et al.* (2003) (29), with modifications. Suspended cells were washed three times in NaCl 0.85%, after centrifugation at 6,100 X G for 10 min. Washed cells were resuspended in PBS, the OD₆₀₀ was adjusted to 0,3 and 3mL aliquots were transferred to 10 x 100 mm round bottom glass tubes and the initial optical density (OD₁) was measured at 600 nm. After that, 0,3 mL of n-hexadecane was added to the suspension and the mixture was mixed vigorously for 2 min. After standing for 15 min, the water phase was removed and its OD₆₀₀ was measured (OD₂). The cell surface hydrophobicity was calculated as:

$$\text{Hydrophobicity (\%)} = (1 - \text{OD}_2 / \text{OD}_1) \times 100 \quad (6)$$

Where: OD₁ = Optical density at 600 nm before the addition of n-hexadecane;

OD₂ = Optical density at 600 nm 15 min after the addition of the n-haxadecane.

Analysis of bacterial adhesion

The soil used for the study of bacterial adhesion was collected in the Federal University of Viçosa, Brazil, at 3 m depth and was composed of 58% clay, 22% silt and 20% sand.

Bacterial adhesion to soil particles was evaluated by a modification of the differential centrifugation technique proposed by Ling *et al.* (2002) (18). Log phase non-starved cells or starved cells were centrifuged four times at 6,100 X G for 10 min and resuspended in NaCl 0.85%. The last resuspension was prepared in a phosphate-buffered saline (PBS) and the OD₆₀₀ was adjusted to the corresponding value of 10⁸ CFU mL⁻¹.

In polypropylene centrifuge tubes, 6 g of soil and 6 mL of suspended cells were mixed, following a vigorous mixing for 1 min. After five minutes, the tubes were centrifuged for 3 min at 48 g, and the supernatant was collected for cell concentration analysis. To each tube, 15 mL of PBS were added, following a new mixing for 3 min. The samples were centrifuged at 200 g during 15 min, and the supernatant was collected for cell concentration analysis by the micro drop technique on R2A. The cells that remained attached to the soil after the first centrifugation were considered to represent both a weakly and a strongly adhered population, and those that remained adhered to the soil particles after the second centrifugation, as a population of strongly adhered cells.

The data was used to calculate the distribution coefficient, expressed as:

$$Kd\ tot\ (mL\ g^{-1}) = Cs / Cw ,\ \text{where} \quad (1)$$

Cs and Cw = cell concentration in solid phase (CFU g⁻¹) and in liquid (CFU ml⁻¹), respectively, and

$$Cs = [Nt - (Ns)] / W \quad (2)$$

$$Cw = (Ns) / V \quad (3)$$

$$Ns = Nw + N1, \text{ where} \quad (4)$$

- Nt = number of cells added to the soil (CFU)
- Ns = number of cells in the supernatant (CFU)
- Nw = number of cells in the supernatant after the first washing (CFU)
- N1 = number of cells in the supernatant after the second washing (CFU)
- W = soil weight (g)
- V = volume of liquid in the mixture (mL).

In this study, two coefficients of distribution were calculated: Kd1, relative to cells weakly adhered to the soil particles and Kd2, relative to cells strongly adhered to the soil particles. These coefficients indicates the ratio of bacterial cells adhered to the soil particles per unit of bacterial cells that entered in suspension after each centrifugation.

The Adhesion Ration (AR) is a new variable proposed to indicate if the bacterial strain has a strong (AR >1,0) or weak adhesion to the soil particles (0 < AR ≈ 1,0). AR was calculated as:

$$AR = Kd2 / Kd1 \quad (5)$$

Statistical Analysis

The experiments were conducted using an entirely random design, with three replications. The data was submitted for analysis of variance and the averages were compared by the Tukey's test at 5%. The correlations between superficial cell surface hydrophobicity and adhesion of bacterial cells to soil particles were evaluated by Pearson's coefficient at 10% of probability.

RESULTS

The cell concentration curves (expressed as ln OD₆₀₀) (Fig. 1) and the concentration of viable cells (Fig. 2) indicated distinct behavior of the bacterial strains during nitrogen restriction. The optical density (OD₆₀₀) increased for all bacterial strains during the first hours under nitrogen starvation. *A. oxydans* LBBMA 201 and *Klebsiella* sp. LBBMA 204-1 showed a sharp increase of the OD₆₀₀ until approximately 10 h under N-starvation, while LBBMA 18-T and *A. aurescens* LBBMA 98 showed a more significant increase of OD only after 40h under N-starvation.

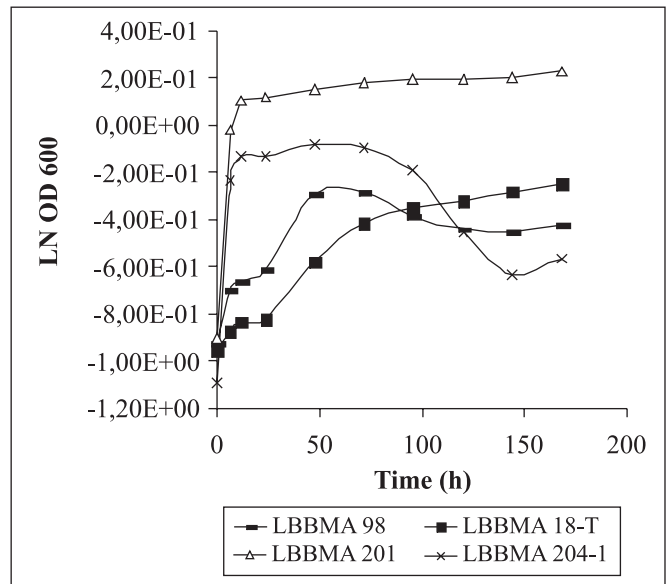


Figure 1. OD 600 nm during bacterial starvation in N-limiting mineral medium. LBBMA 18-T (non-identified), *Arthrobacter aurescens* LBBMA 98, *Arthrobacter oxydans* LBBMA 201, *Klebsiella* sp. LBBMA 204-1

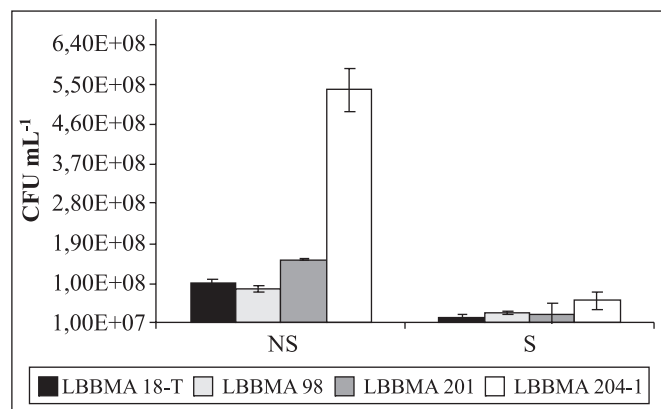


Figure 2. Concentration of viable cells (CFU mL⁻¹) in suspensions of non-starved (NS) and of seven days nitrogen-starved cells (S). LBBMA 18-T (non-identified), *Arthrobacter aurescens* LBBMA 98, *Arthrobacter oxydans* LBBMA 201, *Klebsiella* sp. LBBMA 204-1

There was a significant reduction in the concentration of viable cells in suspension after 7 days of starvation, which ranged from 11% to 39% of the initial values, depending on the strain (Fig. 2).

Nitrogen starvation caused a drop in the cell surface hydrophobicity of two bacterial strains, LBBMA 18-T and *Arthrobacter oxydans* LBBMA 201 and a small increase in the hydrophobicity of *Arthrobacter aurescens* LBBMA 98. The cell surface hydrophobicity of *Klebsiella* sp. LBBMA 204-1 was not affected by N-starvation (Table 1).

The four bacterial strains presented significant reduction of the adhesion to soil particles by the end of the seven day N-starvation (Table 1). The largest decreases of cell adhesion were observed for LBBMA 18-T and *Arthrobacter aurescens*

LBBMA 98. A significant decrease of the strong to weak adhesion ratio of these strains was also evidenced (AR) after the seven day incubation under nitrogen limitation (Table 1).

Nitrogen starvation caused a reduction of the coefficient of strong adhesion to soil particles (Kd2) in the four bacterial strains (Table 1). Significant change of the coefficient of weak adhesion, however, was observed only with *Arthrobacter aurescens* LBBMA 98 (Table 1). This demonstrates that the observed reduction of the adhesion ratio (AR) after N-starvation (Table 1) was caused mainly by the reduction of the strong adhesion of the cells to the soil particles, and not by an increase of the weak adhesion (Table 1).

A negative correlation between the coefficients of weak and strong adhesion of non-starved cells was observed (-0.858, $p < 0.01$). An inverse result was observed for the cells submitted to nitrogen starvation (0.754, $p < 0.01$), evidencing that some important cell surface characteristics that affect the adhesion of these bacteria to soil particles might have been altered by nitrogen-starvation.

DISCUSSION

The concentration of viable cells in suspension diminished after 7 days of incubation in N starvation medium (Fig. 2). However, it is possible to observe an increase in the number of cells during the first hours of starvation (DO600 curves, Fig. 1), followed by the maintenance of cell concentration for some strains and decrease for others. This initial growth was probably sustained by the consumption of cell nitrogen reserves. The cells were grown initially in a rich medium (R2A), in which the bacteria may have accumulated some reserves, including nitrogen.

Nitrogen-starvation resulted in a small decrease in cell surface hydrophobicity of both strains of *Arthrobacter* (LBBMA 98 and LBBMA 201). No effect of N-starvation on the

Table 1. Cell surface hydrophobicity and parameters of cell adhesion to soil particles of non-starved and nitrogen-starved cells

MICROBIAL STRAIN**	HFB(%) ⁽¹⁾		K _{d tot} ⁽²⁾		AR ⁽³⁾		Kd1 _{ns} ⁽⁴⁾		Kd2 _{ns} ⁽⁵⁾		Kd1 _s ⁽⁶⁾		Kd2 _s ⁽⁷⁾	
	To	T7	To	T7	To	T7	To	T7	To	T7	To	T7	To	T7
LBBMA 18-T	84.5A	8.8B	2,804A	16.4B	3,240A	3.3B	2.0(0.7)	6,367(414)	6.8(0.5)	22.5(8.4)				
LBBMA 98	7.4A	9.0B	5,572A	65.2B	14,954A	1.2B	1.2(0.1)	17,664(5,327)	56.1(26)	68.7(3.3)				
LBBMA 201	2.5A	1.6B	43.4A	5.0B	15.3A	2.3B	4.5(0.2)	67.9(6.5)	3.6(0.05)	8.5(3.5)				
LBBMA 204-1	0.6A	1.8A	30.6A	18.2B	14.1A	16.4A	3.7(0.7)	51.9(4.0)	2.3(0.6)	37.9(6.2)				

(1) Cell surface hydrophobicity; (2) Total adhesion coefficient; (3) Adhesion ratio (strong adhesion coefficient / weak adhesion coefficient); (4) Kd1_{ns}: Coefficient of distribution (Kd) related to weak adhesion of non-starved cells; (5) Kd2_{ns}: Coefficient of distribution (Kd) related to strong adhesion of non-starved cells; (6) Kd1_s: Coefficient of distribution (Kd) related to weak adhesion of nitrogen-starved cells; (7) Kd2_s: Coefficient of distribution (Kd) related to strong adhesion of nitrogen-starved cells.

* Averages followed by the same letter, in the line, do not differ by Turkey's test at 5%;

** LBBMA 18-T (non-identified), *Arthrobacter aurescens* LBBMA 98, *Arthrobacter oxydans* LBBMA 201, *Klebsiella* sp. LBBMA 204-1.

hydrophobicity of *Klebsiella* sp LBBMA 204-1 was observed. However, the LBBMA 18-T strain showed a steep decrease of the cell surface hydrophobicity after nitrogen starvation. Sanin *et al.* (2002) (29) also observed a significant decrease in cell surface hydrophobicity after nitrogen starvation. On the other hand, some authors notified an increase in surface hydrophobicity of the starved cells (31,37), suggesting that this effect might be specie or even strain-specific.

Jones *et al.* (1996) (13) defined only three groups of hydrophobicity to characterize the bacteria according to the effect of this variable on the interaction between bacterial cells and surfaces. Those authors considered cells with hydrophobicity percentage greater than 70% as highly hydrophobic; from 70% to 30% as weakly hydrophobic and those with hydrophobicity lower than 30% as highly hydrophilic (13). According to this proposal, the LBBMA 18-T strain showed significant change of cell surface hydrophobicity after N-starvation, moving from the highly hydrophobic ($H = 84.5\%$) to the highly hydrophilic group ($H = 8.8\%$). The other bacterial strains maintained their highly hydrophilic cell surface after seven days of nitrogen restriction (Table 1).

The lack of some nutrients results in the reduction of the cell size (1,37), loss of flagella (1), modification of cell shape (37) and change in the cell surface hydrophobicity (29,37), among others. Cell starvation causes changes in protein concentration at the cell membrane (5) and in the types of proteins found in the cell surface (20). Alterations of some of these characteristics can be responsible for changes in cell adhesion to surfaces (29). The cell surface hydrophobicity of some bacterial strains increased in carbon-limited medium (29,37), decreased in nitrogen-limited medium (5,29) and decreased slowly in PBS (12). Sanin *et al.* (2003) (29) argues that the excess of carbon in the nitrogen-limited medium is partially used by the bacteria for the production of extracellular carbohydrates, whose hydrophilic groups cover the bacterial surface, diminishing its hydrophobicity.

The data on cell adhesion obtained in this work allow us to infer about the potential of the transport of these bacterial isolates through the soil, since the adhesion to the solid surfaces diminishes cell transport through porous media (34). According to Camper *et al.* (1993) (4), the potential of microbial transport can be determined with greater accuracy by the analysis of its adhesion to glass surfaces than by the analysis of individual characteristics, such as cell dimension and motility. It is also reported that inhibition of pili formation due to starvation impairs the adherence of *Myxococcus xanthus* to polystyrene surfaces (14). Strains of *Rhizobium meliloti* submitted to nutrient starvation showed a gradual loss of flagella and of motility (38). The rate in which non-flagellated cells adhere to and accumulate in a substratum is lower than that of cells with these structures (22). In *Vibrio cholerae*, a protein of the flagellum participates effectively in the process of cell adhesion (7).

The strong adhesion coefficient of bacterial cells to the soil was higher than the weak adhesion coefficient. The same result was found by Ling *et al.* (2002) (18) for one of the soils they used. This effect may have been caused by the ionic interaction during cell washing in NaCl 0.85% which, instead of removing the cells adhered to the soil surfaces, may have increased the strength of cell adhesion (9). However, after nitrogen starvation, there was a reduction of the coefficient of strong adhesion to soil particles. For LBBMA 18-T and *Arthrobacter aurescens* LBBMA 98, a decline of the strong to weak adhesion ratio was evidenced; we did not find any reference in the consulted literature that could help explain this effect of N-starvation in the correlation between these two variables, though.

Changes in cell hydrophobicity during starvation, together with other factors, may be involved in the decreased bacterial adhesion to soil observed in this work. Oliveira *et al.* (2007) (25) reported that an increase in cell surface hydrophobicity would increase cell adhesion to surfaces; however, they didn't find a direct relationship between these two variables. In this study, we did not observe the existence of significant correlation between cell hydrophobicity and adhesion in non-starved cells, suggesting the existence of other factors more relevant for bacterial adhesion to soil particles. However, a significant correlation between hydrophobicity and cell adhesion to soil was found in nitrogen-starved cells (0.639, $p < 0.05$). It must be emphasized, however, that the existence of a significant correlation does not mean necessarily the existence of a direct relationship between the variables (24), which lead us to conclude that those variables may not present a cause-effect relationship, but instead may have been modified in a similar way in response to nitrogen starvation. Studies with bacterial species like *Staphylococcus aureus*, *Listeria monocytogenes*, and *Lactobacillus* sp. indicate the existence of a positive relationship between cell hydrophobicity and adhesion (6,8,33), while others did not show a correlation between these two variables (9,25,31).

The evaluation of the response of bacteria to nutrient starvation has implications on the efficiency of bioaugmentation operations, since a significant loss of cell viability can occur during transport of the cells through the soil matrix. Conditioning the microbial strains to nutrient scarcity and studying their response to starvation may allow screening for those that can adapt and survive for long periods under this condition, and if the starved cells can resume growth after environmental factors become favorable (1). The microbial survival after introduction into the soil is affected by the physiological state of the cells, and it is possible to improve the efficacy of microbial agents after their application in the environment by using small starvation periods (38). Besides, bacteria submitted to small periods of starvation are more efficient in degrading toxic compounds than the same bacteria grown in a rich medium (17).

Data from this study suggest that nitrogen-starvation may help to diminish the adhesion of bacterial cells to soil particles.

Studies of cell transport through soil will help to elucidate the correlation between cell surface characteristics, adhesion and cell transport efficiency.

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RESUMO

Adesão de bactérias desnutridas por nitrogênio a solo

Um dos principais fatores limitantes da biorremediação *in situ* de solos subterrâneos, baseada na bioaugmentação, é o transporte dos microrganismos selecionados até o local contaminado. A caracterização das respostas fisiológicas dos microrganismos introduzidos no subsolo a condições de escassez nutricional, notadamente a avaliação de características que afetam a adesão celular ao solo, é fundamental para se prever o sucesso da bioaugmentação. O objetivo deste trabalho foi determinar o efeito da desnutrição em meio com escassez de nitrogênio sobre a hidrofobicidade celular e a adesão ao solo de quatro isolados bacterianos previamente caracterizados como capazes de utilizar benzeno, tolueno ou xileno como fonte de carbono e energia. As linhagens LBBMA 18-T (não identificada), *Arthrobacter aurescens* LBBMA 98, *Arthrobacter oxydans* LBBMA 201, and *Klebsiella* sp. LBBMA 204-1 foram utilizadas nos experimentos. O cultivo das células em meio deficiente em nitrogênio causou uma redução significativa na força de adesão das células às partículas do solo, exceto para a *Klebsiella* sp. LBBMA 204-1. Dois dos quatro isolados estudados sofreram alteração significativa na hidrofobicidade celular. Os resultados sugerem que a eficiência do transporte bacteriano através do solo pode ser aumentada pela desnutrição celular.

Palavras chave: Hidrofobicidade; adesão bacteriana; desnutrição por nitrogênio

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