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Cell Surface Hydrophobicity of *Bacillus* spp. as a Function of Nutrient Supply and Lipopeptides Biosynthesis and its Role in Adhesion

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A b s t r a c t

Cell surface hydrophobicity (CSH) is recognised as a important factor in microbial adhesion to solid surfaces. Growth conditions have been found to determine the synthesis of extracellular molecules by microorganisms. It has major consequences in modification of bacterial surface properties and consequently, in bacterial adhesion to solid surfaces. In this paper, CSH properties of *Bacillus* spp. depending on the nutrient supply and lipopeptide biosynthesis and its role in bacterial adhesion to solid surfaces were investigated. The obtained results indicate that the examined factors (nitrogen and carbon availability) influence the CSH of *Bacillus* spp. cells. In most variants of the experiments the role of nutrient supply in adhesion process was characteristic for species. The strongest effect was observed for peptone concentration ($P<0.001$). A decrease of CSH was noticed in optimal nitrogen availability (10 g/l) and it was connected with maximum yield of surfactin biosynthesis. The highest values of CSH of examined *Bacillus* spp. strains were observed under nitrogen starvation and in excess of carbon source. In these conditions the adhesion to stainless steel surface was more extensive.

K e y w o r d s: *Bacillus* spp., adhesion, hydrophobicity, lipopeptides, nutrient availability

Introduction

Hydrophobic interactions have frequently been pointed out as an important factor in the control of interactions between microorganisms and interfaces. Microbial CSH is one of the surfaces properties influencing nutrient transport in heterogeneous media (Valcarce *et al*., 2002; Cunningham *et al*., 2007). It plays a major role in many biological systems such as assembly of phospholipids layers, micelle formation, phagocytosis and protein adsorption (Doyle, 2000). Bacterial CSH is recognised as one of the determinants in microbial adhesion to abiotic and biological surfaces (McNamara *et al*., 1997; Ahimou *et al*., 2001; Ly *et al*., 2006). CSH participation in adhesion has been explained based on the Derjaguin, Landau, Verwey, Overbeek (DLVO) theory of colloidal stability (Norde and Lyklema, 1989; Azeredo *et al*., 1999). This theory summarises the van der Waals and electrostatic contributions to the interfacial interaction energy between two interacting surfaces. It has been demonstrated to be useful in explaining most bacterial adhesion results (Vadillo-Rodriguez *et al*., 2005). Deviations from the DLVO theory have been reported in some cases, being mainly related to the occurrence of specific interactions at very short separation distance (Busalmen and Sanchez, 2001; Jacobs *et al*., 2007).

One of main aspects of interactions between microorganisms and surfaces is bacterial hydrophobicity. The accumulation of surfactants in the culture medium induces changes in the CSH of producing strains (Ahimou *et al*., 2000; Mukherjee and Das, 2005). Surfactants are amphipathic molecules consisting of both hydrophobic and hydrophilic moieties that partition preferentially at the interface between fluid phases having different degrees of polarity and hydrogen bonding. Surfactants of biological origin, referred to as biosurfactants, are produced by a wide variety of microorganisms. Some species of *Bacillus* genus (*Bacillus subtilis*, *Bacillus megaterium*, *Bacillus circulans*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus pumilus*) synthesize lipopeptides, which exhibit antibiotic and surface active properties (Razafindralambo *et al*., 1998; Ahimou *et al*., 2000; Ahimou *et al*., 2001; Youssef *et al*., 2004; Mulligan, 2005; Nitschke and Costa, 2007).

Biosurfactants produced by microorganisms are able to modify bacterial surface hydrophobicity and

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consequently, bacterial adhesion to solid surfaces. Adhesion of bacteria to solid surfaces is a general phenomenon which is recognised as the first step in the development of biofilms. Microbial colonization and biofilm formation have important detrimental consequences in medicine (contamination of prostheses, catheters, artificial organs, lenses) and in many economic fields (biofouling of marine materials, contamination of food product lines) (Jones *et al*., 1997; Peng *et al*., 2001; Planchon *et al.*, 2007). The contamination of abiotic surfaces by spoilage and pathogenic microorganisms is a serious problem in the food industry. In this way studies on the attachment of microorganisms to abiotic surfaces represent important aspects in the establishment of decontamination procedures directed at minimalizing health hazards.

The aim of our experiments was to investigate the CSH properties of *Bacillus* spp. depending on nutrient supply and lipopeptide biosynthesis and significance of CSH in bacterial adhesion to solid surfaces.

Experimental

Materials and Methods

Bacterial strains and growth conditions. Four bacterial strains, *Bacillus coagulans* (B6), *Bacillus megaterium* (B4), *Bacillus circulans* (B7) *and Bacillus brevis* (B1), were isolated from an industrial plant processing a variety of wastes from the food industry (Cibis *et al.*, 2004). They were identified using standard methods, based on Bergey's key (Claus and Berkeley, 1986) and API 50 CHB tests (Logan and Berkeley, 1984). Microorganisms were grown for 48 h at 37°C with shaking (100 rpm). Basic nutrient media for *Bacillus* spp. growth was the following composition: glucose 5 g/l; peptone K 10 g/l (Merck, Germany). The pH of the media was 7.0. Composition of the nutrient solution was changed according to model of experiments.

Test for cell surface hydrophobicity. The cell surface hydrophobicity of the examined strains of bacteria was determined using the bacterial adhesion to hydrocarbons (BATH) test. Cultures were centrifuged (3000×g for 10 min) and the cells were resuspended in PBS solution (pH 7.2) to the OD of 1.2–1.6. Cell suspensions (3 ml) were added to octane, xylene or hexadecane (1 ml) and mixed briefly on a vortex mixer (30 s). The absorbance of the aqueous phase was measured at 540 nm after standing for 30 min at ambient temperature to allow phase separation. The percentage hydrophobicity was determined from the initial OD of the bacterial suspension (A_i) and the OD of the aqueous phase after separation (A_s) using the formula $(A_i - A_s)/A_i \times 100$ (%) (Jordan *et al*., 1994; Flint *et al*., 1997). Experiments were repeated at twice for every strain and determination of CSH was done in triplicate for every hydrocarbon.

Extraction and analysis of lipopeptides. The culture medium (50 ml) was centrifuged at 10 000×g for 25 min at 4°C to remove the cells. The supernatant was applied to Bond Elut C_{18} (Agilent Technologies, USA). The cartridge, which retained lipopeptides, was rinsed successively with 20 ml water and 40 ml 50% aqueous methanol, and finally lipopeptides were eluted from the cartridge with 20 ml of methanol. The eluate was evaporated and the crude extract was dissolved in 1 ml of methanol (Razafindralambo *et al*., 1998).

Determinations of surfactin and iturin A were carried out on MERCK-HITACHI system consisting of autosampler (model L-7250), pump (model L-7100) and DAD (model L-7455) set at 205 nm. Analyses were performed isocratically at flow rate 1 ml/min, at 30ºC, on column ODS-Hypersil (200×4.6 mm; 5 mm), Hewlett-Packard. Acetonitryle and 3.8 mM trifluoroacetic acid (80:20) or acetonitryle and 10 mM ammonium acetate as mobile phases were used (for surfactin and iturin, respectively). Samples were filtered (0.22 m, Millex-GS, Millipore), the volume injected was 50 l. Standards were used to identify peaks in chromatograms, and the peak area was used to determine the samples' concentrations. The identity of each peak was confirmed by comparing the spectrum of the standard with that of the presumptive positive peak in the sample after normalization. This was done by computer integration (Chromatography Data Station Software, MERCK-HITACHI) operated in the mode of external standard (Wei and Chu, 1998).

Bacterial adhesion analysis. Stainless 1 cm×6.5 cm ×1 mm steel plates (type 304L) were treated with 50% solution of HNO_3 for 10 min at 70°C. After soaking under distilled water the plates were put into glass containers and sterilized at 121°C for 15 min (Parkar *et al*., 2001).

The stainless steel plates were put into *Bacillus* spp. cultures (48 h) and after 1, 2 and 4 hours the plates were removed and washed with PBS solution (pH 7.2) in order to remove unattached cells from their surfaces. The plates were stained with 0.01% solution of acridine orange (2 min at room temperature). For observation of bacteria adhering to the stainless steel surface a fluorescence microscope was used (CARL-ZEISS, Axiovert 200, Germany). To determine the level of *Bacillus* spp. adhesion to the surface of stainless steel the method described by Le Thi *et al.* (2001) was used. This technique is based on the estimation of 50 visual fields according to a 9-degree scale:

- 1st degree: from 0 to 5 bacteria cells in visual field;
- 2nd degree: from 5 to 50 bacteria cells in visual field;

3rd degree: only single bacteria cells (above 50 bacteria cells in visual field), no microcolonies;
4th degree: single bacteria cells + microcolonies;
5th degree: large but not confluent microcolonies + single bacteria cells;
6th degree: confluent microcolonies + single bacteria cells;
7th degree: ¼ visual field covered by the biofilm;
8th degree: ½ visual field covered by the biofilm;
9th degree: visual field totally covered by the biofilm.

Each experimental variant was repeated three times.

Design of experiments. To estimate the effects of nutrient supply on the cell surface hydrophobicity of *Bacillus* spp., the experiments were designed as a factorial search with three levels for each variable – Box-Behnken scheme (using computer program Design of Experiments version 6.02, Stat-Easy, Minneapolis, USA) and response surface method was used. The crucial factors involved in the study and their concentration are given in Table I. A total of 17 runs were carried out simultaneously, with runs 13–17 as three replications. The following empirical model was used for the determination of linear, interaction and curvature effects of the tested variables.

$$Z = b_o + b_1x_1 + \ldots + b_ix_i + b_{11}x_1^2 + \ldots + b_{ii}x_i^2 \quad (1)$$

where Z is the desired response; b_0 the regression coefficient at center point; b_1, b_i the linear coefficients, x_1, x_2 independent variables; and b_{11}, b_{ii} quadratic coefficients.

Table I
Process variables and level in the three-factor, three-level response surface design of cell surface hydrophobicity

Factors	Coded values		
	–1	0	1
	Actual values		
Peptone (g/l)	0	10	20
Glucose (g/l)	0	5	10
pH	5	7	9

All chemicals used were of the highest purity and, unless otherwise stated, were purchased from Sigma-Aldrich or Fluka

Results

Cell surface hydrophobicirty (CSH) of *Bacillus* spp. In the first step of these examinations CSH was measured with the BATH method, and octane, xylene and hexadecane, as hydrocarbons, were used. For further investigations octane was selected, with regard to repeatability of research results. Some solvents are usually toxic towards cells and octane was used to evaluate their adhesion to this solvent. The adhesions to octane of four examined *Bacillus* strains depending on nutrient availability (pH 7.0) are presented in Fig. 1. The obtained results indicate that the examined factors (nitrogen and carbon supply) have an influence on CSH of *Bacillus* spp. cells, but its role is characteristic for species.

In the case of *B. coagulans* peptone and glucose supply the strongest effect on CSH ($p = 0.0004$). Detailed analysis indicated that carbon source availability in culture medium stimulated the increase of CSH in the entire range of examined pH values. On the other hand, the presence of peptone caused decrease of CSH.

CSH of *B. megaterium* cells depends mostly on glucose supply in medium. The lowest effect was noticed for peptone concentration. Statistical analysis of results (Design of experiments) indicated strong interactions between peptone supply – pH ($p = 0.0004$) and glucose supply – pH ($p = 0.0002$).

The obtained results for *B. circulans* CSH showed that all examined factors (pH, peptone and glucose concentrations) have an influence on this feature. Strong interactions between peptone supply – pH ($p = 0.0003$) and glucose – peptone supply – pH ($p = 0.002$) were also observed.

CSH of *B. brevis* depends on peptone supply ($p < 0.0001$) and pH ($p = 0.0004$). An increase of CSH was observed together with increase of carbon source in the culture medium. It was indicated complex interaction between the examined factors.

In general, exploration of the interdependence of these factors and high values of determination coefficients ($P \leq 0.003$) indicate complex interactions between the variables. In all experiments, the strongest effect was observed for peptone concentration ($P < 0.001$). The lowest CSH values were observed with optimal nitrogen supply (10 g/l).

Similar results were observed in *Bacillus* spp. growing in pH 5 and pH 9 (data not presented). The highest values of CSH of *Bacillus* spp. were observed under low concentration of peptone and in excess of glucose as carbon source.

Lipopeptides biosynthesis. Production of lipopeptides (surfactin and iturin A) by examined *Bacillus* spp. strain under optimal culture conditions (peptone 10 g/l, glucose 5 g/l, pH 7) is shown in Table II.

Table II
Surfactin and iturin A biosynthesis by examined species of *Bacillus* spp.

	Species	
	Surfactin (mg/l)	Iturin A (mg/l)
B. coagulans	21.231 ± 1.811	0.138 ± 0.009
B. megaterium	7.795 ± 1.212	0.351 ± 0.020
B. circulans	34.216 ± 2.112	0.128 ± 0.008
B. brevis	12.601 ± 1.010	0.222 ± 0.019

± – standard deviation

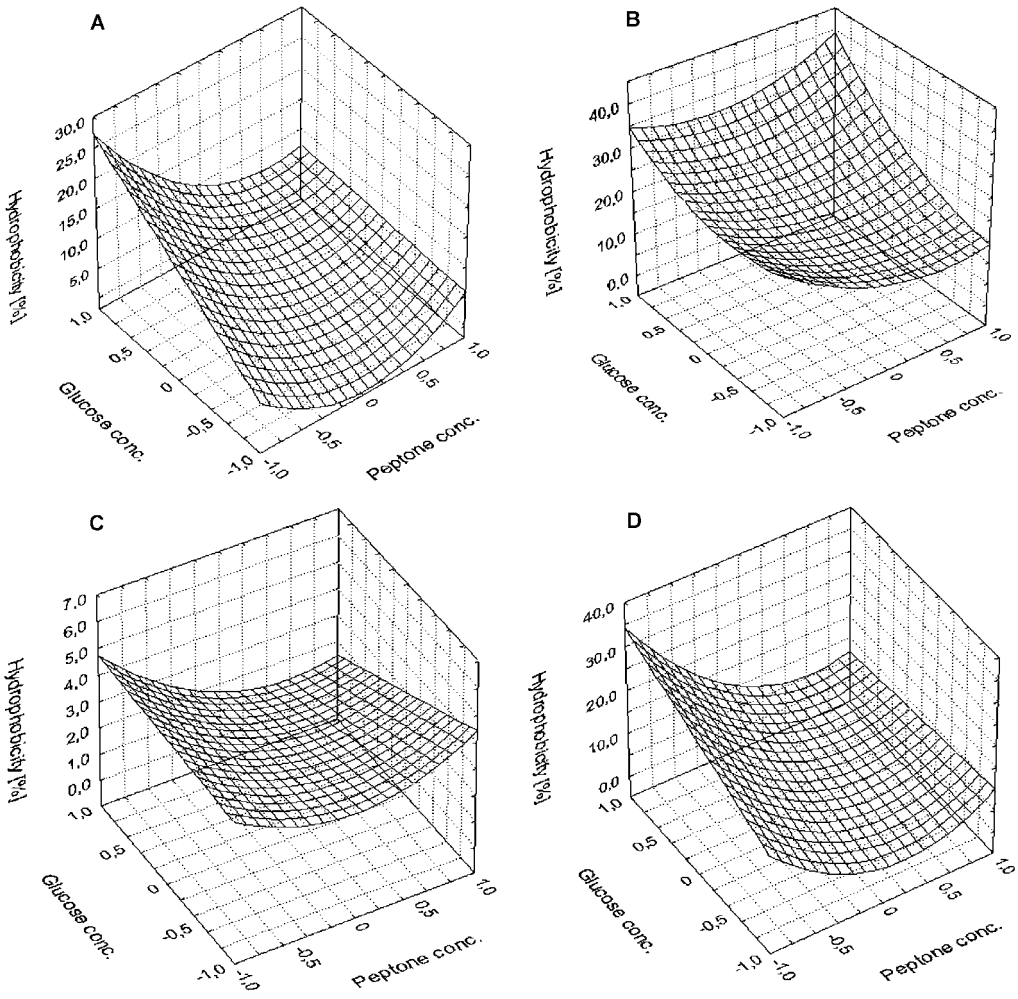

Fig. 1. Relationship between CSH of *Bacillus* spp., glucose and peptone concentration in culture medium for: A – *Bacillus coagulans*, B – *Bacillus megaterium*, C – *Bacillus circulans*, D – *Bacillus brevis* (glucose and peptone concentration, respectively, were expressed by coded values)

All strains synthetized both lipopeptide types, but biosynthesis of surfactin was on higher than iturin A. Under these conditions the highest production of surfactin was observed for *B. circulans* (34.216 mg/l). Peptone supplied as a source of nitrogen in culture medium influenced essentially surfactin production in all examined *Bacillus* species. Figure 2 shows the influence of nitrogen source availability in culture medium on surfactin biosynthesis. Maximum biosurfactant production was observed when the peptone concentration was between 10 and 12 g/l. These strains were able to produce surfactants (at a similar level) in the entire tested pH range (data not shown). The influence of carbon source on surfactin biosynthesis by examined *Bacillus* spp. strains was statistically insignificant.

Bacterial adhesion. The results of the influence of nitrogen source availability on the attachment of *Bacillus* spp. cells to stainless steel (type 304L) are presented in Table III. Approximately 10^6–10^7 cfu/ml bacterial cells were present in the culture medium dur-

Table III
Bacillus spp. adhesion to the stainless steel (304L) surface in dependence on nitrogen source availability in medium (glucose concentration 5 g/l, pH = 7)

Species		Peptone concentration (g/l)					
		0		10		20	
		D	H	D	H	D	H
B. coagulans	1 h	4; 2	5	1; 2	–	4; 2	–
	2 h	4; 5	5; 6	2; 1	–	4; 2	5
	4 h	4; 5; 2	5; 6	2; 4	5	1; 2	–
B. megaterium	1 h	4; 3	5	1; 2	–	2; 4	–
	2 h	5; 4	5; 6	2; 1	–	4; 2	5; 6
	4 h	4; 2	5; 6	4; 2	6; 5	2; 1	–
B. circulans	1h	2; 4	5; 6	2; 1	–	4; 1	–
	2 h	4; 2	5; 6; 7	2; 1	–	2; 4	5
	4 h	1; 2	–	4; 2	5, 6	4; 2	5
B. brevis	1.h	4; 1	5	1; 2	–	2; 1	–
	2 h	4; 5	5; 6	1; 2	–	4; 2	5; 6
	4 h	5; 4; 2	5; 6; 7	4; 1	5	1; 2	–

D – **D**ominant adhesion degrees
H – Appearance of **H**igher adhesion degrees

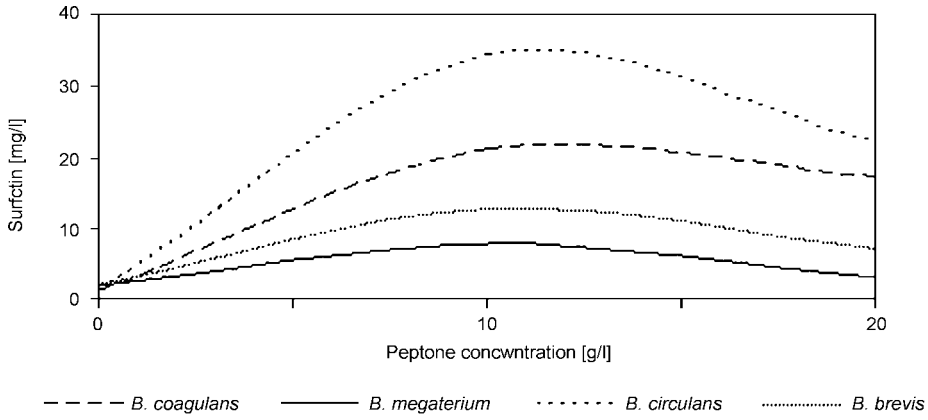

Fig. 2. Effect of peptone concentration in growth medium on surfactin biosynthesis by *Bacillus* spp.

ing experiments. When a particular degree of adhesion occurred with a minimum amount of 20% that degree was described as dominant. Appearance of higher adhesion degrees was also noticed.

The most advanced stages of biofilm formation were observed during nitrogen starvation. In these conditions the 4th degree of adhesion was dominant, and higher levels of adhesion (6th to 9th degrees) were also observed. *Bacillus* spp. cells grown in optimal conditions colonized the studied abiotic surface at the 1st, 2nd and 4th degree of adhesion and higher degrees appeared only after 4 hours.

The effect of carbon source on the adhesion of *Bacillus* spp. cells to stainless steel surface is presented in Table IV. Stainless steel (type 304L) was efficiently colonized by *Bacillus* spp. cells in excess of carbon source in growth medium. In these conditions the 4th degree of adhesion was dominant, and higher levels of adhesion were also observed (with the exception of *B. circulans*). The lowest stages of adhesion was observed in optimal growth conditions and higher degrees were appeared only after 4 hours. In the case of *B. brevis* the most advantages stages of adhesion were observed both in excess of carbon source and during carbon starvation.

Table IV
Bacillus spp. adhesion to the stainless steel (304L) surface in dependence on carbon source availability in medium (peptone concentration 10 g/l, pH = 7)

Species	Glucose concentration (g/l)					
	0		5		10	
	D	H	D	H	D	H
B. coagulans						
1 h	1; 2	–	1; 2	–	4; 2	6; 7
2 h	2; 1	–	2; 1	–	4; 3	5; 6
4 h	1; 2	–	2; 4	5	2; 4	5
B. megaterium						
1 h	2; 1	–	1; 2	–	4; 2	5; 6
2 h	2; 3	–	2; 1	–	4; 3	5; 6
4 h	2; 4	5; 6	4; 2	6; 5	4; 1; 6	5; 7
B. circulans						
1h	2; 1		–	2; 1	–	2; 1
2 h	2; 1	–	2; 1	–	2; 3; 1	–
4 h	4; 3; 2	5; 6	4; 2	5; 6	2; 1	–
B. brevis						
1.h	2; 1	5	1; 2	–	4; 3	6; 7
2 h	2; 3	6	1; 2	–	4; 5	6; 7
4 h	4; 2; 5	5; 6; 7	4; 1	5	4; 5	6; 7; 8

D – **D**ominant adhesion degrees
H – Appearance of **H**igher adhesion degrees

Discussion

Microbial adhesion to surfaces is a phenomenon commonly observed in natural and engineering systems. Although extensive work has been performed on microbial adhesion, many aspects of this process are still unclear, especially the forces that determine the interactions of microorganisms and support surfaces. Bacterial CSH is the most studied property of the cell surface with regard to adhesion to abiotic surfaces (Jones *et al*., 1997; Faille *et al*., 2002; Jullien *et al*., 2003). Microorganisms are able to biosynthesize certain enzymes responsible for selective intake of nutrients and synthesis of cell surface components. The production of exopolymers (extracellular proteins and extracellular polysaccharides) plays a important role in hydrophobic interactions between an organism and substratum (Uberos *et al*., 2001).

Extracellular polymeric substances (EPS) production is known to be affected by nutrient status of the growth medium. The general explanation is in such that the hydrophobicity increases slightly in the presence of nitrogen source in the culture medium (Sanin *et al*., 2003). Excess nitrogen channeled into protein ends up in the extracellular polymer matrix. It is known that proteins and amino acids are the hydrophobic components of EPS. Therefore the increase of these

compounds in cell surface causes increase of hydrophobicity (Doyle, 2000). On the other hand, when excess carbon is present in the medium, it is used for production of extracellular carbohydrates (more hydrophilic components). A different situation was observed in these experiments. In these investigations the highest values of CSH of examined *Bacillus* spp. strains, were observed upon nitrogen limitation and carbon excess. Increase of peptone concentration (near the optimal) caused change of the CSH of *Bacillus* spp. to more hydrophilic. There may be two reasons for these results. First, connected with hydrophobic or hydrophilic properties of EPS, and second – the ability of *Bacillus* spp. to synthesize surface active compounds and modify CSH.

EPS is highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. EPS may be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic (Sutherland, 2001a). Excess available carbon and limitation of nitrogen promote EPS synthesis. Sutherland (2001b) noted that the composition and structure of the EPS determine its primary conformation. For example many bacterial EPS possess a backbone structure that contains 1,3- or 1,4-β-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble in water (hydrophobic) (Doyle, 2000; Sutherland, 2001a). The biosynthesis of EPS in biofilms is not generally uniform and may vary during cultivation. It is possible that under limited nitrogen conditions the configuration of cell surface proteins stay more hydrophobic and protects the cell from the loss of surface cell proteins (Doyle, 2000).

Cyclic lipopeptides including surfactin, iturin, fengycin and lichenisin, are the major classes of biosurfactants produced by *Bacillus* spp. Among the many classes of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antibiotic potential (Ahimou *et al*., 2000). The ability of microorganisms to produce lipopeptides is not dependent on bacterial hydrophobicity. However, after their excretion and accumulation in the culture medium changes in the CSH of the producing strain are induced (Ahimou *et al*., 2000). Our results showed that nutrient availability has an influence on surfactin biosynthesis by *Bacillus* spp. Availability of nitrogen source in culture medium influenced the surfactin production in all the species used. A decrease of cell surface hydrophobicity was noticed when surface-active compounds were produced. Of the two examined lipopeptides, the surfactin effect is more marked than that of iturin A, because the surfactin is able to cover more space than iturin A (larger molecular area) (Maget-Dana *et al*., 1992). Surfactin contains seven residues of α-amino acids and one residue of β-hydroxy fatty acid. When the bacterial cell surface is hydrophilic, lipopeptide molecules are probably oriented in such a way that the peptide cycles, as polar heads, are adsorbed onto surface and the hydrocarbon chains are exposed to the surrounding medium. Hence, the bacterial surface becomes more hydrophobic. The orientation is inverted for the hydrophobic bacterial surface (Ahimou *et al*., 2000). The hydrophobicity alternations suggested the important role of lipopeptide molecules to perform in *Bacillus* spp. adhesion mechanisms onto various surfaces by hydrophobic interaction (Ahimou *et al*., 2000; Ron and Rosenberg, 2001).

Studies of bacterial adhesion to solid surfaces showed that process was affected by environment (*eg* nutrient availability), harvesting time, topography and roughness of the substrata and by the morphology and surface properties of bacterial cells (Flint *et al*., 1997; Peng *et al*., 2001; Jefferson, 2004). Microbial surface properties are considered to play a major role in interactions between bacteria and their environment, especially in adhesion (Liu *et al*., 2004; Ly *et al*., 2006; Zikmanis *et al.*, 2007). Little is known about the effect of substances produced by these microorganisms on their surface properties. In this work the relationships between CSH of *Bacillus* spp. (as a function of nutrient availability and lipopeptide biosynthesis) and its adhesion to stainless steel were investigated. The highest biosurfactants biosynthesis was noticed when the peptone concentration was between 10 and 12 g/l (optimal nitrogen availability) and consequently resulting in decrease of CSH value. The highest values of CSH of examined *Bacillus* spp. strains were observed upon nitrogen limitation and carbon excess. In these conditions the most advanced stages of biofilm formation were observed. The weak adhesion was observed in optimal growth conditions.

In summary, our investigations show that nutrient availability and lipopeptide biosynthesis are able to modify bacterial surface hydrophobicity, which is involved in adhesion to stainless steel surface. A better understanding of the factors involved in the adhesion process will help in designing methods to control biofilms through the prevention of adhesion or by enhancing the removal of attached bacteria.

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