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Physico-chemical surface characterization of *Bacillus cereus* spores isolated from an Algerian dairy plant

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***Bacillus cereus* is an endospore-forming bacterium frequently found in dairy products and dairy environment. In this study, the hydrophobicity and surface electrical charge of spores from fourteen (14) *Bacillus cereus* strains isolated from a dairy plant located in north-western Algeria (were studied using microbial adhesion to hydrocarbon (MATH) method, and zeta potential measurements, respectively. Spores of eleven (11) strains presented a hydrophilic character and three (3) a hydrophobic one. The spore zeta potential values for all strains were between 12.28 and −44, 51 mV. Four spore morphologies were investigated by transmission electron microscopy (TEM) after negative staining. This allowed the clear observation of an exosporium surrounding all *B .cereus* spores. The ability of spores to adhere to stainless steel was also studied and varied among strains. The presence of an exosporium was not sufficient to explain the ability of spores to adhere to stainless steel surfaces. When physico-chemical surface characters of *B. cereus* spores were compared: the hydrophobicity, the appendages length, the surface of spore and exosporium were found as the significant adhesion parameters.**

Key words: Bacterial spore, hydrophobicity, electrical charge, adhesion, transmission electron microscopy (TEM).

INTRODUCTION

Bacillus is ubiquitously present in nature, and can easily spread through food production systems. In dairy environments, *Bacillus* is part of the most commonly encountered bacteria (Salo et al., 2006; Sharma and Anand, 2002; Waak et al., 2002). Furthermore, *Bacillus cereus* is widely reported as responsible for food spoilage, and is occasionally an opportunistic human pathogen (Schoeni and Wong, 2005; Lindsay et al., 2000).

B. cereus spores are highly resistant to a large number of stresses (Lindsay et al., 2000); they have been found to account for 12.4% of constitutive biofilm microflora in a dairy plant (Matz et al., 1970; Sharma and Anand, 2002). In fact, *B. cereus* adheres easily to a range of surfaces and readily forms biofilms on food processing equipments (Faille, 2010c).

In general, *B. cereus* spores share common properties

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Table 1. Main Biochemical characteristics of *Bacillus cereus* strains used in this work.

Strains	Origin [a]	ADH [b]	GEL [c]	GLU [d]	SAC [e]	AMY [f]	Amylase	Lecithinase	Resistance to βLactams				
									CAZ	AMP	OX	KF	CRO
B. cereus 14	1	-	+	+	-	-	+	+	R	R	R	R	R
B. cereus 18	5	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 44	5	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 80	3	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 82	4	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 100	4	+	+	+	+	+	+	+	R	R	R	R	R
B. cereus 107	6	+	+	±	+	+	+	+	R	R	R	R	R
B. cereus 109	2	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 110	7	-	+	+	-	-	+	+	R	R	R	R	R
B. cereus 120	3	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 123	6	+	+	±	-	+	+	+	R	R	R	R	R
B. cereus 89	2	-	+	+	-	-	+	+	R	R	R	R	R
B. cereus103	1	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus126	8	+	±	+	-	+	+	+	R	R	R	R	R

(+) positive reaction, (-) negative reaction. [a]Sources: (1): Pasteurized milk storage tank, (2): Pasteurized recombinated milk storage tank (3): Raw recombinated milk storage tank, (4): raw milk storage tank, (5): canalization of pasteurized milk; (6): canalization of pasteurized recombinated milk; (7): Canalization of raw recombinated milk; (8): Canalization of raw milk. [b] ADH: Arginine dihydrolase, [c] GEL: Gelatinase production, [d]GLU: D-Glucose utilization, [e] SAC: D-saccharose utilization,[f]AMY: Amygdalin utilization.

such as hydrophobicity and electronegativity (Andersson et al., 1998), however, some differences have been reported within the *B. cereus* group. Some spores of this group are hydrophilic (Andersson and Rönner, 1998; Tauveron et al., 2006) and the exosporium size and the length of the hair-like nap can be very different (Sylvestre et al., 2003; Tauveron et al., 2006).

In this paper, the physico-chemical characterization of fourteen (14) spores has been carried. These spores come from our *B. cereus* collection isolated from dairy equipment surfaces of a dairy plant located in Tlemcen (north-western of Algeria). The method of microbial adhesion to hydrocarbon (MATH) was used to examine the hydrophobic characteristics of *B. cereus* spores and the spore zeta potential was also measured.

On the other hand, we investigated if the exosporium and spore surfaces, the length and the number of appendages were important for spore adhesion to the stainless steel surface. This work deals with the optimization of cleaning procedures and thermochemical disinfection using detergents and disinfectants already marketed in Algeria.

MATERIALS AND METHODS

Origin of *B. cereus* strains and stock spore production

Samples came from inner tanks surfaces of pasteurized and unpasteurized local milk, tanks of pasteurized and unpasteurized recombined milk and from packaging lines.

Fourteen *B. cereus* strains from our collection of 155 strains isolated in 2010-2012 from dairy plant processing lines located in Tlemcen (north-western of Algeria) were analyzed in this study (Table 1). All the equipment was sampled after the cleaning and sanitizing procedures.

Biochemical identification of *B. cereus* was done by determination of respiratory enzymes: catalase, cytochrome-oxidase (TMPD test) and the reduction of nitrate. Additional biochemical tests for β-galactosidase (ONPG), ornithine decarboxylase (ODC), lysine decarboxylase (LDC) and the arginine-dihydrolase (ADH) activity, production of H_2S, use of the citrate, production of indole and Voges-Proskauer reaction, gelatin liquefaction and degradation of some sugars were performed. These tests were done using the API20E plate (bioMerieux SA, Lyon, France, test kit) (EL Sersy and Mohamed, 2011).

We also looked for: Extracellular hydrolytic activity as for amylolytic and proteolytic activity:, namely the search of the caseinase activity, and the determination of lipolytic activity (lecithinase test). Resistance to Four β-lactam antibiotics: ceftazidime (CAZ), ampicilline (AMP), céfalotine (KF), oxacilline (OX) and ceftriaxone(CRO) (Bio-Rad- Exosporium structure was observed by transmission electron microscopy (Table 1).

Sporulation was induced by adding $MgSO_4$ (40 ppm w/v) and $CaCl_2$ (100 ppm w/v) in nutrient agar, and followed by microscopic observations. When at least 90% of spores were observed (in general after 4 to 6 days at 37°C), the culture was harvested and subsequently washed with sterile distiller water (three times) then centrifuged (4000 rev/min) for 15 min in an Eppendorf Centrifuge 5810 R (Leguerinel et al., 2000).

The spore suspensions were stored at 4°C in sterile distiller water until use. Before each experiment two additional washes with sterile distiller water were performed.

Determination of physico-chemical properties of spores

In order to characterize the spore hydrophobic property, a MATH

partitioning method was used, based on the affinity of spores to an apolar solvent, that is, hexadecane (Sigma). The surface hydrophobicity of bacterial cells has been previously determined by several methods based on the precipitation of cells by salts (Leguerinel et al., 2000), hydrophobic interaction chromatography (Doyle et al., 1984; Smyth et al., 1978), and adherence to various liquid hydrocarbons including hexadecane (Craven and Blankenship, 1987; Kutima and Foegeding, 1987; Doyle et al., 1984; Rosenberg et al., 1980) but the hexadecane-aqueous partition system used in our work is one of the simplest and fastest methods described.

Spore suspensions in a saline solution (0.85% NaCl solution) were adjusted to an absorbance of 0.5 to 0.6 at 600 nm (A_0) in glass tubes (10 x 75 mm). Three milliliter aliquots of each spore suspension and 500 µL of hexadecane were vortexed four times ranging from 5 to 150 s and left to settle for 30 min, to allow complete separation into two phases. The absorbance at 600 nm of the aqueous phase was measured (A_t), and then $\frac{At}{A0} \times 100$ was plotted against the vortexing time (s). The initial slope, giving the initial removal rate (R_0) from the aqueous suspension, is related to the hydrophilic/hydrophobic spore character. A spore was considered to be hydrophobic when (R_0) fell between −4.0 and −6.0 and to be highly hydrophobic for lower values.

The spore zeta potential was measured using a zetameter (ZetaCompact, CAD Instrumentation, France). This was determined from the electrophoretic mobility using Helmholtz–Smoluchowski equation. For this purpose, spores were suspended in 1 mM KNO_3 to obtain around 50 spores per analysis. The pH was adjusted to values ranging from 3 to 9, with HNO_3 1 mM or KOH 1 mM. Trials at pH 2.86 were performed directly in HNO_3 1 mM. Each sample was analyzed in duplicate (10).

Transmission electron microscopy (TEM)

Spores were adsorbed onto Formvar-coated grids (EMS, 22400) and examined after negative staining with 2% w/v uranyl acetate (EMS, G100H-Cu) on a Hitachi H7500 electron microscope at an accelerated voltage of 80 kV. About 50 TEM pictures were taken for each spore.

Test of spore adhesion to stainless steel coupons

In order to determine the relationship between the physico-chemical properties (hydrophobicity and electrophoretic mobility) and adhesion, spores were analyzed for their ability to adhere to stainless steel coupons in static conditions.

The adhesion of spores from four selected *B. cereus* strains to stainless steel was tested on coupons (15 × 45 mm, AISI 304 L, bright annealed), which were filled-up by vertical immersion for 4 h in an aqueous spore suspension (10^5 spores/mL), and then quickly rinsed with sterile water. The fouled coupons were subjected to ultrasonication in 10 mL Tween 80 2% (v/v) during 5 min, (Ultrasonic bath, Deltasonic, France). The detached spores following sonication were enumerated on nutrient agar (Bio-Rad Laboratories, France), after 48 h at 30°C (Faille et al., 2013). All experiments were repeated three times

RESULTS AND DISCUSSION

In the present study, we evaluated the surface physico-chemical properties of spores from fourteen *B. cereus* strains and the adhesion abilities of 4 representative isolates (2 with hydrophobic and 2 with hydrophilic spores) on the stainless steel surface.

Table 2. Hydrophobicity and zeta potential of fourteen *Bacillus cereus* spores isolated from an Algerian dairy plant.

Strains	Hydrophobicity (s^{-1})	Zeta potential (mV)
B. cereus 014	2.34±0,58	-26.630±1,99
B. cereus 018	3.53±0,08	-26.990±2,47
B. cereus 044	2.16±0,70	-19.360±3,96
B. cereus 100	2.10±0,38	-37.055±1,067
B. cereus 107	1.79±0,95	-32.300±1,51
B. cereus 109	0.107±0,20	-20.806±0,42
B. cereus 110	5.32±1,07	-28.085±10,38
B. cereus 120	1.34±1,28	-31.225±3,32
B. cereus 123	4.34±0,83	-26.590±4,46
B. cereus 80	1.17±0,67	-19.315±0,091
B. cereus 82	2.72±0,59	-20.910±3.74
B. cereus 89	2.85±1,02	-27.065±4,49
*B. cereus*103	2.05±0,1	-12.285±1,18
*B. cereus*126	0.250±1,58	-44.510±7,28

The results on the spores hydrophobic/hydrophilic character estimated by MATH assay and their zeta potential are given in Table 2, Figures 1 and 2. From the values obtained in this work, the isolates were classified in three groups:

Group 1: Highly hydrophilic spores (14.29%) including *B. cereus* 109 spores with an initial removal rate of −0.107 s^{-1} and *B. cereus* 126 with initial removal rates around −0.25 s^{-1}.
Group 2: Moderate hydrophilic spores (64.29%) including spores from 9 B. *cereus* strains with initial removal rates between 2.05 s^{-1} (*B. cereus* 103) and 2.85 s^{-1} (*B. cereus* 89).
Group 3: moderately hydrophobic (21.43%) including spores from 3 *B. cereus* strains as indicated by the initial removal rate ranging from −3.53 s^{-1} (*B. cereus* 18) to −5.32 s^{-1} (*B. cereus* 110).

The spore electric charge characterized by the zeta potential indicated a clear electronegative character of all strains at pH 7.0. However wide variations were observed between strains (zeta potential ranging from −12.28 to −44, 51 mV). The less negative charge was -12.28 (strain 103). In conclusion, this data set showed no correlation between the hydrophilic/hydrophobic character and spore electric charge (R^2=0.0137).

In this study hydrophobicity and surface electrical properties of *B. cereus* spores were in the range or lower, than that observed in previously published data. Indeed, Ankolekar and Labbe (2010) found that the values of hydrophobicity ranged from 55.6 to 14.1% and those for Zeta potential from -8.18 to -26.8. Instead Faille et al. (2010 a), found that the values of hydrophobicity ranged from 9 (≈45%) to 0.5 (≈2.5%) and Zeta potential from

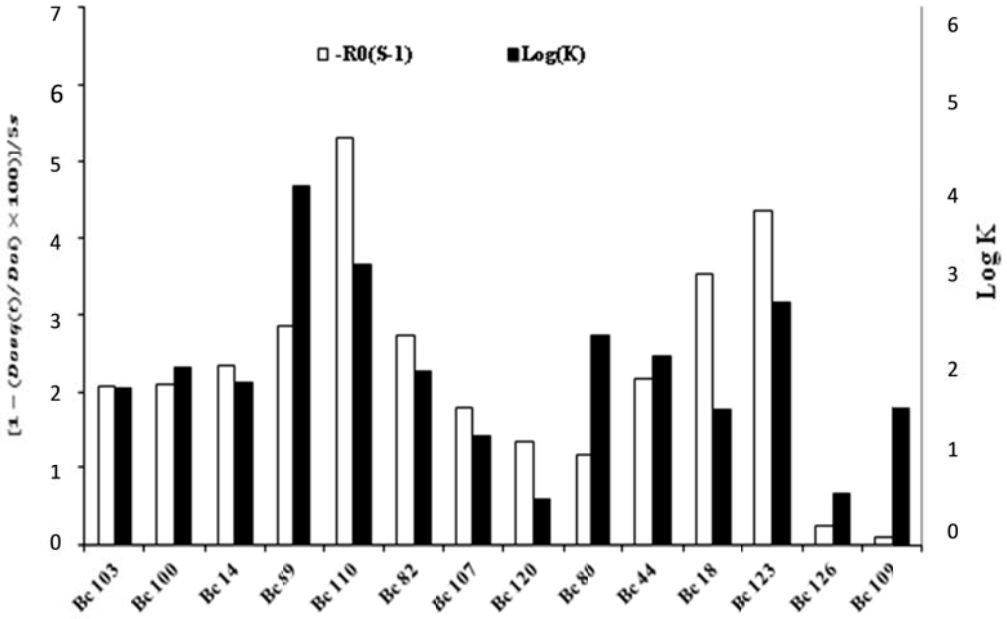

Figure 1. Affinity for hexadecane of spores of fourteen *B. cereus* strains isolated from an Algerian dairy plant. Left axis represent the initial removal rate R0 from the aqueous suspension and right axis represent the energy of affinity of hexadecane.

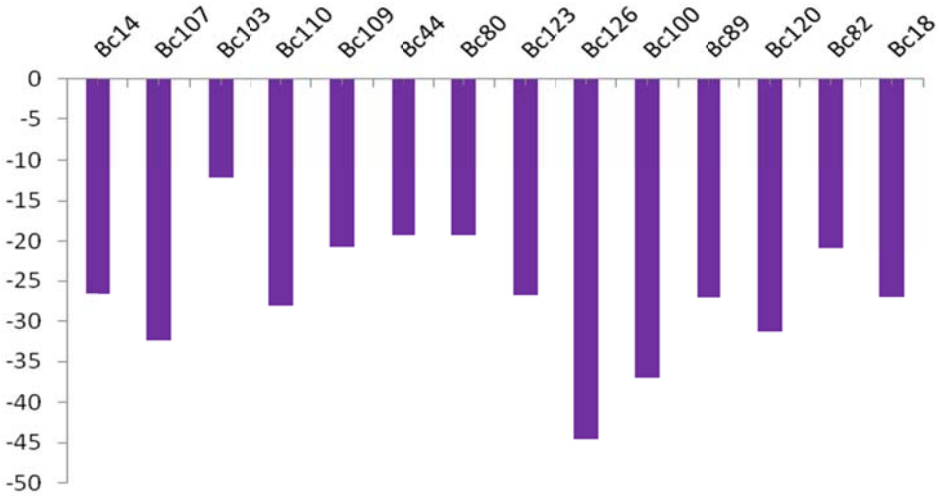

Figure 2. Zeta potential of spores of fourteen *B. cereus* strains isolated from an Algerian dairy plant.

−17.61 to −46.81, and Buhr et al. (2008) found that the values of hydrophobicity ranged from 92.2 to 12.7. (Ankolekar and Labbe, 2010; Buhr et al., 2008; Faille et al., 2010b).

The observation of whole spores from 4 strains (*B. cereus* 110 and *B. cereus* 123 had hydrophobic spores and *B. cereus* 82 and *B. cereus* 109 had hydrophilic ones) by electron microscopy and the examination of the pictures for each spore showed that *B. cereus* whole spores shared a common architecture such as the presence of an exosporium surrounding the spores. The presence of appendages on spores was also examined (Figure 3).

For the strain *B. cereus* 110, the hydrophobic character was found to be linked to the presence of an exosporium in agreement with the work of Koshikawa et al. (1989). However, our results indicated that *B. cereus* 109 and *B. cereus* 82 spores, being hydrophilic, were surrounded by

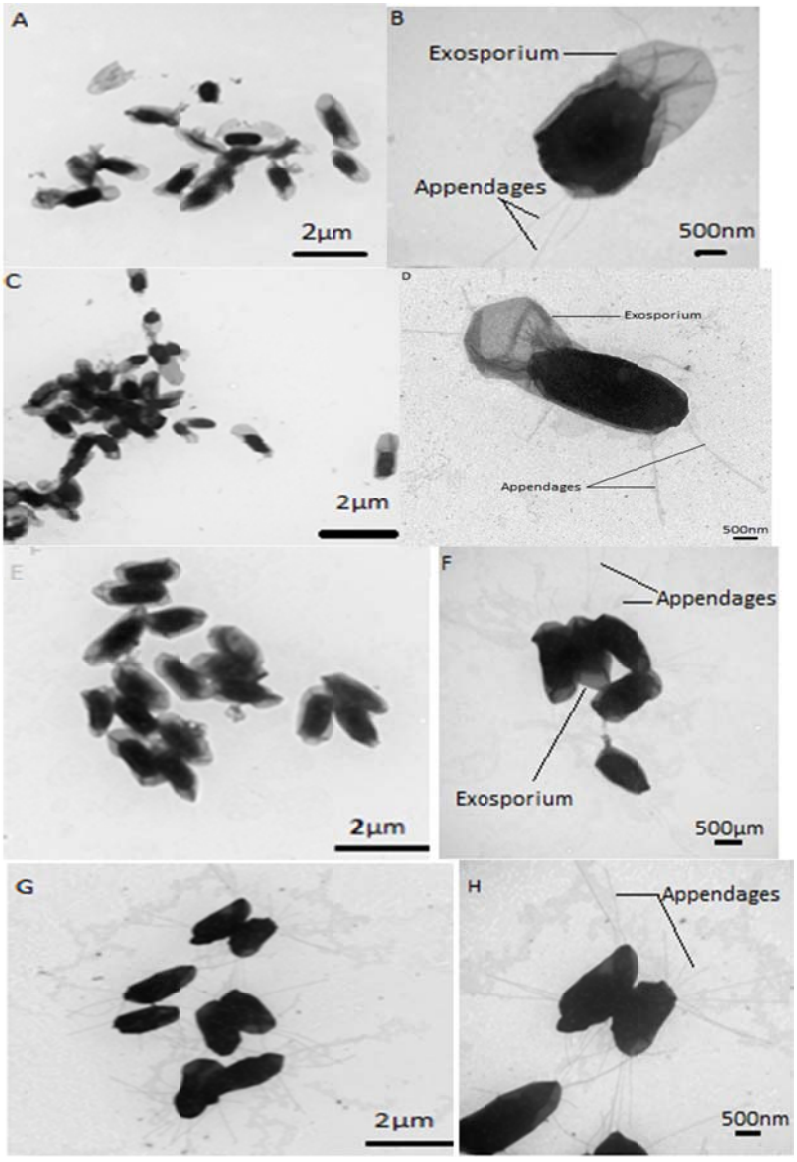

Figure 3. Transmission electron microscopy of spores after negative staining. (A, B): *B. cereus 82*; (C, D): *B. cereus109*; (E, F): *B. cereus 110*; (G, H): *B. cereus 123*.

an exosporium. It has been suggested that the increased hydrophobicity of bacterial spores is due to the relative abundance of proteins in the outer coats and exosporium when compared with peptidoglycan on Gram-positive vegetative cell surfaces (Henriques and Moran, 2007; Doyle et al., 1984; Takumi et al., 1979; Matz et al., 1970).

Kozuka and Tochikubo (1985) reported chemical properties of the main component of *B. cereus* IAM1110 appendages. They suggested the appendages may not have strong hydrophobic properties in comparison with the exosporium.

Appendages have a spiral structure (Faille et al., 2010a) and are a common but not an universal feature of a *B. cereus* group. The number and length of spore appendages of the *B. cereus* group is species-associated. Wijman et al. (2007) mention that the variation of number and length of spore appendages can be due to their fragility and loss during the preparation operations (Wijman et al., 2007). We observed such appendages on the surface of spores of every strain. Yet, the number of appendages observed in our work varied among strains ranging from 5.32±2.76 to 7.81±4.24 (means of 50 pictures of each strain) and the length varied from 0.50 to 3.74 µm as determined by Image J software (rsbweb.nih.gov/ij/download.html). Similar values have been reported by Ankolekar and Labbé

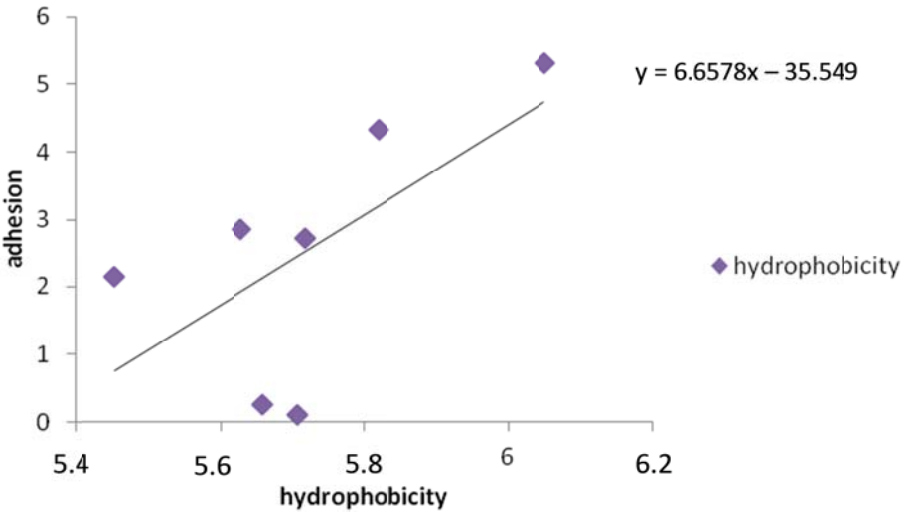

Figure 4. Relation between bacterial adhesion and hydrophobicity of spores from seven *B .cereus* strains isolated from an Algerian dairy plant (spores are: *B. cereus* 107, *B. cereus* 109, *B. cereus* 110, *B. cereus* 120, *B. cereus* 123**,** *B. cereus* 082 and *B. cereus* 018 and *B. cereus*103).

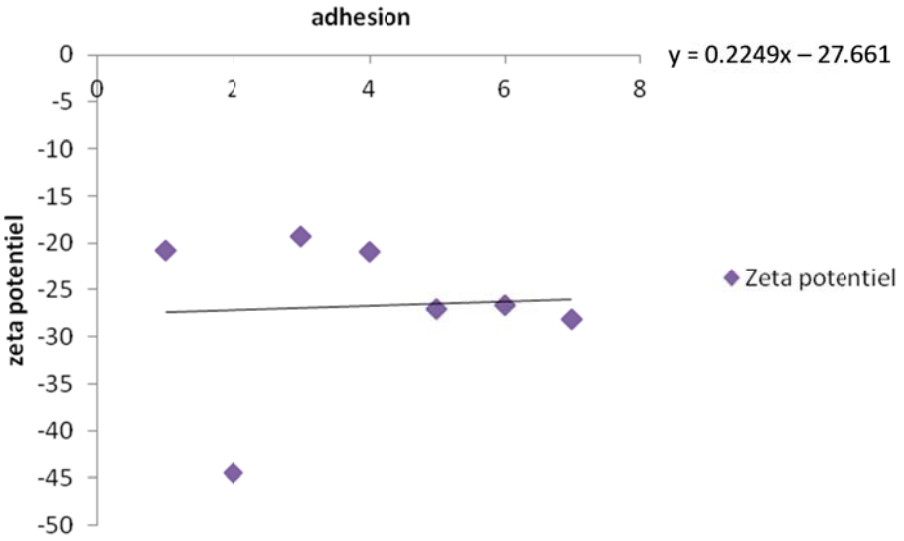

Figure 5. Relation between electrophoretic mobility and adhesion capacity of spores from seven *B. cereus* strains isolated from an Algerian dairy plant.

(2010) and Faille et al. (2010b). For each strain the appendages were peritrichous.

Our results indicated that the physical-chemical characteristics of *B. cereus* are independent on the source sampling (Table 1 and Figures 4 to 6).

The correlation between bacterial cell surface hydrophobicity and adhesion capacity of spores is given in Figure 4. A linear correlation was found between bacterial hydrophobicity and adhesion (y=6.66x + 35.55).

The relation between bacterial cell surface charge and bacterial adhesion capacity of spores is given in Figure 5. No correlation was found between bacterial zeta potential and adhesion (y = 0.22x – 27.66).

Figure 6 illustrates the relation between bacterial electrophoretic mobility and spores hydrophobicity and shows also that no correlation was found between bacterial zeta potential and hydrophobicity (y = 0.66 x – 28.17).

In accordance with previous works, spores with lower charge have a higher adhering ability to surfaces

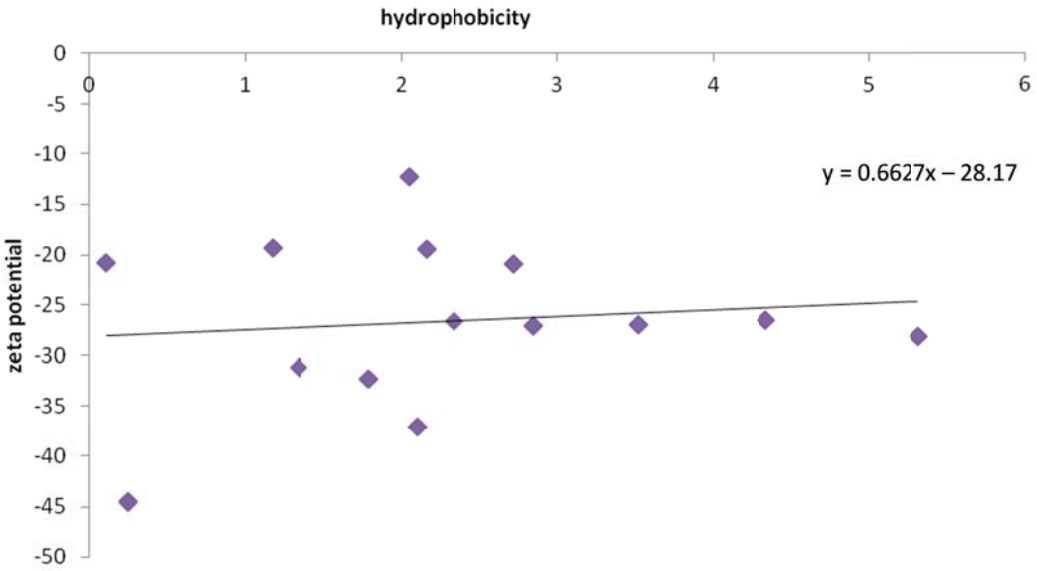

Figure 6. Relation between hydrophobicity and zeta potential of *B. cereus* spores from fourteen strains isolated from an Algerian dairy plant.

Table 3. Relation between some spore surface characteristics and number of adherent spores of *B. cereus* (mean of three trials).

Strains (a)	Surface of exosporium (µm^2)	Surface of spore (µm^2)	Number of appendages	Length of appendages (µm)	Number of adherent spores/cm^2
B. cereus 110	0.48	0.39	6±3	0.58	1.12×10^6
B. cereus123	0.43	0.36	5 ±2	0.53	6.60×10^5
B. cereus 82	0.53	0.41	7± 2	3.74	5.23×10^5
B. cereus 109	0.56	0.43	8 ±4	0.50	5.10×10^5

[a]*B. cereus 110* and *B. cereus 123* are hydrophobic and *B. cereus 82* and *B. cereus 109* are hydrophilic.

(Hüsmark and Rönner, 1992; Giarouris et al., 2009), and hydrophobicity was shown to play a major role in spore adhesion (Faille et al., 2013). On the other hand, the spores are covered with long appendages and these promote adhesion (Stalheim and Granum, 2001; Smirnova et al., 1989). Husmark and Rönner (1992) found that adhesion of *B. cereus* IAM1110 spores after sonication, which removes the appendages is around 2.5 time less than adhesion of *B. cereus* whole spores.

Due to the relatively high hydrophobicity, spore adhesion is especially high to hydrophobic materials such as stainless steel, which is commonly used in dairy processing equipment. *B. cereus* spores present a remarkable ability to adhere firmly to various inert materials (Seale et al., 2008).

The work of Klavenes et al. (2002) on the attachment of *B. cereus* spores to stainless steel surfaces shows that in contrast with the results from the static conditions, the dynamic conditions gave unexpected results. One possible reason for this might be that the appendages promote the initial adhesion of the spores, but when finally attached, the appendages serve no further purpose and other adhesion mechanisms dominate. Another explanation could be that spores with appendages aggregate more easily or get scrambled into each other, making large clusters of spores which are more easily removed from the surface in dynamic conditions. Some controversy as to their role in adhesion persists (Seale et al., 2008). Cleaning agents that degrade appendages already exist and could possibly be developed further if the appendages are found to be critical in the adhesion phenomenon (Stalheim and Granum, 2001).

Results given in Table 3 indicated that the adhesion was affected by the length of appendages while Faille et al., (2010b) found that the adhesion of spores of *B. cereus* is due to the number of appendages. Over the spore surface and exosporium and little more, the adhesion is strong.

According to the work of Rönner et al. (1990), the most hydrophobic spores (measured by the hydrophobic interaction chromatography method) are able to adhere in a much larger extent to the hydrophobic surfaces (Rönner et al., 1990). *B. cereus* 110 and *B. cereus* 123 are hydrophobic and strongly adhering to the stainless steel.

Conclusion

The spores' surface characterization showed that two-thirds of our spores were moderate hydrophilic and the spore electric charge characterized by the zeta potential indicated a clear electronegative character of all strains at pH 7.0; however, huge variations were observed between strains.

Our results show also that there is no correlation between adhesion and Zeta potential characters. A weak correlation was found between bacterial hydrophobicity and Zeta potential and a real correlation was found between bacterial hydrophobicity and adhesion.

Also, the ability of spores to adhere to stainless steel surface was essentially related to the differences in the length of the appendages, the surface of the exosporium and spore and electrical charge.

These data are very important. In fact we can use chemical agents that degrade appendages or modify the surface properties (enzymes or surfactants). We can also try physical treatments as ultrasonic cleaners to improve cleaning and disinfection strategies.

Conflict of interest

The authors have not declared any conflict of interest.

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