Global Journal of Biotechnology & Biochemistry 3 (2): 69-78, 2008
ISSN 2078-466X

Biofilm Formation by Endospore-forming *Bacilli* on Plastic Surface under Some Food-related and Environmental Stress Conditions

Hesham M. Elhariry

Department of Food Science, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

Abstract: The effect of some food-related and environmental stress conditions (glucose or NaCl, nutrient starvation, heating, UV, agitation and temperature) on the ability of biofilm formation on polystyrene surface by vegetative cells of four *Bacillus* species was studied. The final concentration of glucose or NaCl in TSB was adjusted within the range relevant for the food industry (1 to 7%). The optimum temperature for the biofilm formation by *Bacillus* species was the optimum growth temperature. The ability of cells to form biofilm was enhanced under the mimic food industry conditions (nutrient starvation). Addition of NaCl or glucose to TSB led to extreme changes in the behavior of biofilm formation by *B. subtilis* and *B. cereus*. The determined hydrophobicity of *Bacillus* spores was considerably greater than those of vegetative cells. The role of outer cellular membrane and hydrophobicity of the vegetative and spores of *Bacillus* species was also studied by applying different treatments included; 2% SDS at 100°C for 10 min; 1% trypsin at 37°C for 24h; 1% lysozyme at 37°C for 1h. Treatment with SDS or trypsin appeared to be more effective for reducing spore hydrophobicity compared with lysozyme treatment. Such treatments indicated that the hydrophobic interactions may play a role in the adhesion to polystyrene surface. The obtained results point out that strong biofilm formers exist among food-related *Bacillus* species. Moreover, the regulation pattern of biofilm formation under food-related and environmental stress conditions by *Bacillus* species is very complex. Therefore, understanding the mechanism of biofilm formation not only under optimum but also under suboptimum conditions should be involved in the hygienic control systems of food industry.

Key words: Microbial biofilm · *Bacillus* · Hydrophobicity · Polystyrene

INTRODUCTION

Biofilms are architecturally complex communities of microorganisms in which the cells are held together by an extracellular matrix, typically containing exopolysaccharides (EPS), proteins and even nucleic acids [1]. Bacteria in biofilm formed in food processing environments are more resistant to cleaning and sanitation than their planktonic (unattached) counterparts. Surfaces of equipment used for food handling and processing are recognized as sources of microbial contamination and recontamination as well as transmission of diseases, especially when improperly cleaned and sanitized [2, 3]. The closed systems such as pipes, valves and pumps or open systems such as conveyors are regularly found to be contaminated by microorganisms such as *Bacillus* spp., *E. coli* or *Listeria monocytogenes* [4, 5]. Therefore, evaluating and understanding the formation of microbial biofilm are essential components of HACCP system for food and processing industry [6].

Biofilms of *Bacillus* spp. are important contaminants in many food industry settings and are recognized as a serious problem [7]. For example, spore-forming bacteria survive and accumulate on pipelines and joint in the processing environment. Also, biofilm on surfaces of bakery equipment have been implicated as site for contamination of bread products with spoilage *Bacillus* [8]. The wild strains of endospore-forming (e.g. *B. subtilis*) form robust biofilm both at liquid/air interfaces and on solid surfaces. Therefore, understanding the surface properties of bacterial spores and their interactions with inanimate substrata is important for selecting packaging materials and for evaluation of the surface sterilization procedures used in the packaging of food [9, 10].

Spores are highly resilient dormant cell types that are able to withstand extremes of temperature, radiation and

Corresponding Author: Dr. Hesham M. Elhariry, Department of Food Science, Faculty of Agriculture, Ain Shams University, POB 68- Hadayek Shoubra, 11241, Cairo, Egypt

chemical assault [11]. Upon the return of favorable environmental conditions, spores can readily convert to actively growing vegetative cells through the germination process. These abilities enable spores not only to survive in extreme conditions but, in some species, to cause significant disease. If spores were produced with a biofilm matrix on food contact surfaces, those spores may have greater resistance to environmental stresses, including sanitizers [12].

Microbial attachment and biofilm formation are influenced by number of factors including biological factors [13] hydrodynamic features [14] and environmental conditions [15]. Removing irreversibly attached cells is difficult and requires application of strong shear force (scrubbing or scraping) or chemical breaking of the attachment forces through the application of enzymes, detergents, surfactants, sanitizers and/or heat [16, 17].

Many of bacteria including *Bacillus, Salmonella, Listeria, Staphylococcus* and *Escherichia* are capable of adhering and easily form biofilm on metal, glass or rubber surfaces [17,18]. Now, plastic materials are more widely used in food industry for the construction of accessories and cutting surfaces [19]. The natural transparency and brilliancy of polystyrene are two assets for an excellent presentation of foodstuffs at the point of sale. Today's stylists and designers have been won over by polystyrene. It allows them to fully express their creativity, because the very nature of polystyrene makes it greatly superior to other materials when it comes to the creation of shapes and colors. Therefore, polystyrene have been widely used in modern food packaging, particularly for meat, poultry, fish, fruits, vegetables and dried food. Although several studies have been made on the mechanism of microbial attachment and biofilm formation on plastic surfaces by nonspore-forming bacteria e.g. *Salmonella* and *Listeria monocytogenes*, little is known about spore-forming Gram-positive bacteria. For the food industry, it is important to identify the conditions, under which the spore-forming bacteria are able to survive and multiply with regard to food processing. Therefore, the objective of this work is to study some factors affecting attachment of spore-forming *Bacillus* to plastic surface under food-related and/or environmental stress conditions.

MATERIALS AND METHODS

Bacterial strains: *Bacillus cereus* DSMZ 345, *B. coagulans* DSMZ 1, *B. stearothermophilus* DSMZ 297 and *B. subtilis* ATCC 14593 were obtained from stock cultures maintained in the microbial culture collection, Department of Food Science, Faculty of Agriculture, Ain-Shams University, Cairo, Egypt. The vegetative cells were harvested from an overnight culture of cells in nutrient broth (Oxoid) at 37°C and 100 rpm. The harvested cells were washed twice in phosphate buffer (0.1 M, pH 6.8) and then resuspended in the same buffer to about 1.0×10^6 cfu per ml.

Spore preparation: Sporulation was induced by adding 10 mg $MnSO_4.H_2O$ per litter of nutrient broth [20] and followed by using a phase-contrast microscope (Olympus, Japan). When at least 95% spores were presented (typically 3 to 5 days) the culture was harvested and subsequently washed by repeated centrifugation (4000 x g for 20 min) and resuspension in sterile distilled water at 4°C [21]. Spore suspensions were treated with 50% ethanol for 1h to kill any remained vegetative cells. After three times of washing with the P-buffer, the spore pellet was resuspended in P-buffer (7-10 ml) to obtain about 1.0×10^6 spores per ml and then stored at 4°C.

Quantification of biofilm formation by vegetative cells on polystyrene: The commonly used microtitre-plates method [22] for determining bacterial adhesion to plastic surface was applied in the present study. Briefly, the wells of sterile 96-well polystyrene microtitre-plates (Minitek, USA) were filled with 230 µl of tryptone soy broth (TSB). A quantity of 20 µl of each culture was added into each well. The negative control wells contained TSB only. The plates were incubated aerobically for 24h at tested temperatures (30, 37, 45 and 55 °C). The content of the microtitre-plates was poured off and the wells were washed three times with 300 µl of P-buffer. The remaining attached bacteria were fixed with 250 µl of methanol per well. After 15 min microtitre-plates were emptied and air dried. The microtitre-plates were stained with 250 µl per well of 1% crystal violet used for Gram staining (Merck, Germany) for 5 min. The excess of stain was rinsed off by placing the microtitre-plates under running tap water. After the microtitre-plates were air dried, the dye bound to the adherent cells was extracted with 250 µl of 33% (v/v) glacial acetic acid per well. The absorbance of each well was measured at 570 nm using a UV/Visible spectrophotometer (6105-Jenway, U.K.). Based on the absorbance (A_{570nm}) produced by bacterial films, strains were classified into four categories according to the classification of Christensen and others [23] which modified by Stepanovic and others [24]. Briefly, the cut-off absorbance (Ac) was the mean absorbance of

the negative control. Strains were classified as follows: $A = Ac$ = no biofilm producer (0); $Ac < A = (2 \times Ac)$ = weak biofilm producer (+); $(2 \times Ac) < A = (4 \times Ac)$ = moderate biofilm producer (++); $(4 \times Ac) < A$ = strong biofilm producer (+++). All tests were carried out in triplicate and the results were averaged.

Influence of food-related stress conditions on the production of biofilm: The concentrations of glucose and NaCl used in this study are within the range relevant for the food industry [22]. In addition to glucose (0.25%) and NaCl (0.5%) contents of TSB medium, different concentrations (1, 2, 3, 4, 5, 6 and 7%) of glucose or NaCl were added. Biofilm formation under these food-related stress conditions was examined at different incubation temperatures (30, 37, 45 and 55°C).

Influence of environmental stress conditions on the production of biofilm: The ability of vegetative cells to form biofilm was studied after heat treatment (100°C for 10 min) or after exposure to U.V. light (254 nm) for 10 min in sterilized plates opened in a laminar flow [25]. Biofilm formation was also studied under dynamic conditions by incubating the microtitre-plates in at 150 rpm. A diluted TSB medium [26] was used as mimic food industry conditions. This medium was prepared by mixing TSB with sterilized distilled water (1:20, v:v). In these experiments, biofilm formation was studied at the optimum growth temperature for each tested bacterium.

Influence of destroying and removing surface layers on biofilm formation: Surface layers of the vegetative cells were removed or destroyed [25]. Briefly, stock of either cells or spores were centrifuged (1000 g for 10 min) and then resuspended to the original volume in one of the following solutions: 2% sodium dodecyl sulphate (SDS, Aldrich) at 100°C for 10 min or 1% trypsin (Sigma) at 37°C for 24h; 1% lysozyme (Sigma) at 37°C for 1h. A control preparation was made in distilled water. After treatment, the cells or spores were centrifuged (1000 g for 10 min), resuspended to the original volume in P-buffer and stored at 4°C. After aforementioned treatments, biofilm formation by vegetative cells and attachment of spores to substratum were carried out at the optimum growth temperature of each tested bacterium.

Determination of hydrophobicity: Percentage of hydrophobicity was determined using the Microorganism Adhesion To Hydrocarbon (MATH) test [27]. An overnight-grown culture the prepared spore suspension was centrifuged at 3000 g for 10 min and the pellet was resuspended in sterile distilled water to an $A_{600\ nm}$ of 1.2 - 1.6. Samples of each suspension (3 ml) were added to 3 ml hexadecane, mixed on a vortex mixer for 1 min, incubated at 30°C for 10 min and then vigorously remixed again on a vortex mixer at ambient temperature for 2 min. The absorbance of the aqueous phase was measured at 600 nm after standing at ambient temperature for 20 min. The percentage of hydrophobicity (%h) was determined from the absorbance of the initial bacterial cell, or spore suspensions (*Ai*) and the absorbance of the aqueous phase after mixing with hexadecane (*Af*) using the following equation: $\%\ h = [(Ai - Af) \times 100] / Ai$

Adhesion of *Bacillus* spores to plastic surface: The wells of sterile polystyrene microplate were filled with 230 µl of P-buffer (0.1 M, pH 6). A quantity of 20 µl of each treated spore stock was added into each well. The microtitre-plates were incubated aerobically for 2h at the examined temperatures [28] and after that, the wells were washed with sterile distilled water. To determine the number of adhering spores, fouled wells were washed with 100 µL of 2% tween 80. The detached spores were enumerated on nutrient agar after 48h at the optimum growth temperature of each tested bacterium.

Statistical analysis: Data were analyzed with SAS software [29] using SAS analysis of variance (PROC ANOVA). Significant differences between means were determined by the least significant difference test (P = 0.05).

RESULTS

Effect of incubation temperature on biofilm formation: At the optimum growth temperature (30°C), *B. subtilis* and *B. cereus* had maximum ability to form biofilm (Table 1). These species still moderate biofilm producers (++) above their optimum growth temperature (37°C). The same behavior was noticed by *B. coagulans* at 37 and 45°C. The incubation temperature 45°C was the optimum temperature for biofilm formation by

Table 1: Ability of the vegetative cells of four *Bacillus* species to form biofilm on polystyrene surface at different incubation temperatures

	Incubation temperature (°C)			
Strain	30	37	45	55
B. subtilis ATCC 14085	++	++	+	+
B. cereus DSMZ 345	++	++	+	0
B. coagulans DSMZ 1	+	++	++	0
B. stearothermophilus DSMZ 297	+	+	++	+

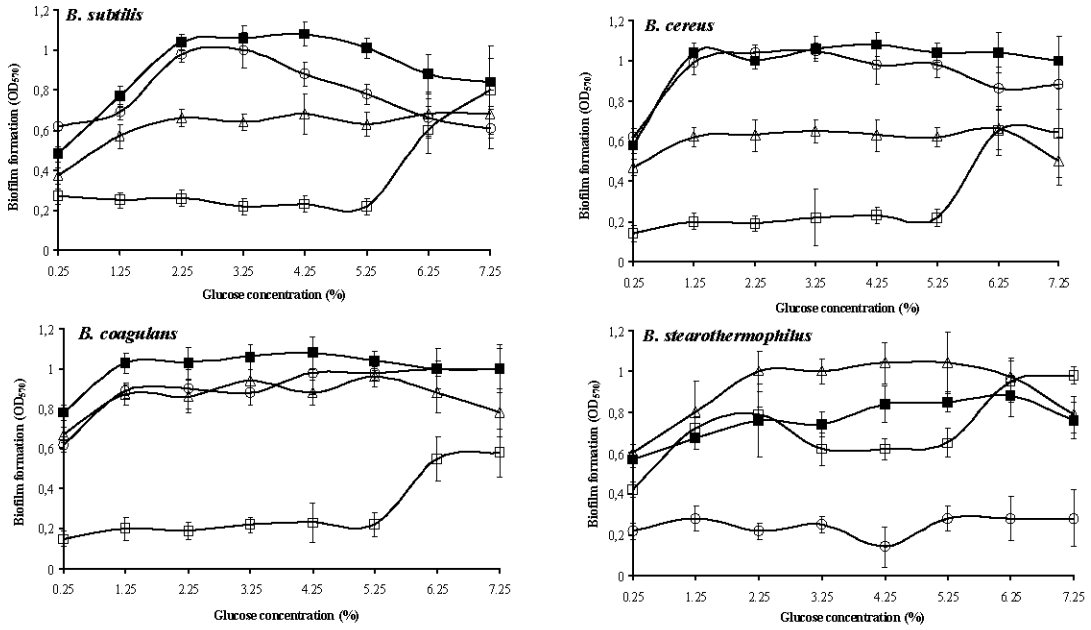

Fig. 1: The effect of glucose concentration on the formation of biofilm by vegetative cells of *Bacillus* spp. on polystyrene surface at 30°C (o), 37°C (■), 45°C (Δ) and 55°C (□)

Table 2: Biofilm formation on polystyrene surface by vegetative cells of four *Bacillus* species before and after exposure to different environmental stress conditions

		Stress parameters				
Strain	Incubation Temp.	Control	Nutrients starvation (TSB 1:20)	Heating (100°C/10 min)	UV-treatment (254nm/10min)	Dynamic incubation (150 rpm)
B. subtilis ATCC 14085	30	++	+++	+	+	0
B. cereus DSMZ 345	30	++	+++	+	+	0
B. coagulans DSMZ 1	37	++	+++	+	+	0
B. stearothermophilus DSMZ 297	55	+	+	+	+	0

B. stearothermophilus. Moreover, *B. subtilis* and *B. stearothermophilus* had moderate ability to form biofilm at 55°C. On the other hand, *B.* cereus and *B. coagulans* lost their abilities to form biofilm gradually by increasing incubation temperature up to 55°C (Table 1).

The combined effect of temperature and glucose or NaCl on biofilm formation: The ability of all tested *Bacillus* species to form biofilm on polystyrene surfaces was enhanced by increasing glucose concentration in TSB up to 5% (Fig. 1). *B subtilis* and *B. cereus* became strong biofilm producers at 30 and 37°C and moderate biofilm producer at 45°C, when the glucose concentration ranged between 1.25 to 5.25%. At this range of glucose concentration, *B. coagulans* was strong producer at 30, 37 and 45°C. A noticeable enhancement in biofilm formation by *B subtilis*, *B. cereus* and *B. coagulans* was recorded at 55°C in the presence of high glucose concentrations (Fig. 1). Noticeably, The optimum temperature for biofilm formation was changed by *B. subtlis* and *B. cereus* due to addition of glucose to TSB. On the other hand, slight enhancement in biofilm formation by all studied strains was obtained by increasing NaCl concentration up to 2.5% (Fig. 2). However, sharp reduction in the ability of biofilm formation was noticed by increasing the concentration of NaCl from 2.5 to 7.5%.

Effect of environmental stress on biofilm formation: The effect of the environmental stress on the ability of *Bacillus* species to form biofilm on polystyrene surface was studied at the optimum growth temperature of each tested bacterium (Table 2). Under conditions of nutrient starvation (diluted TSB 1:20), the ability to produce biofilm on plastic surface was significantly enhanced by

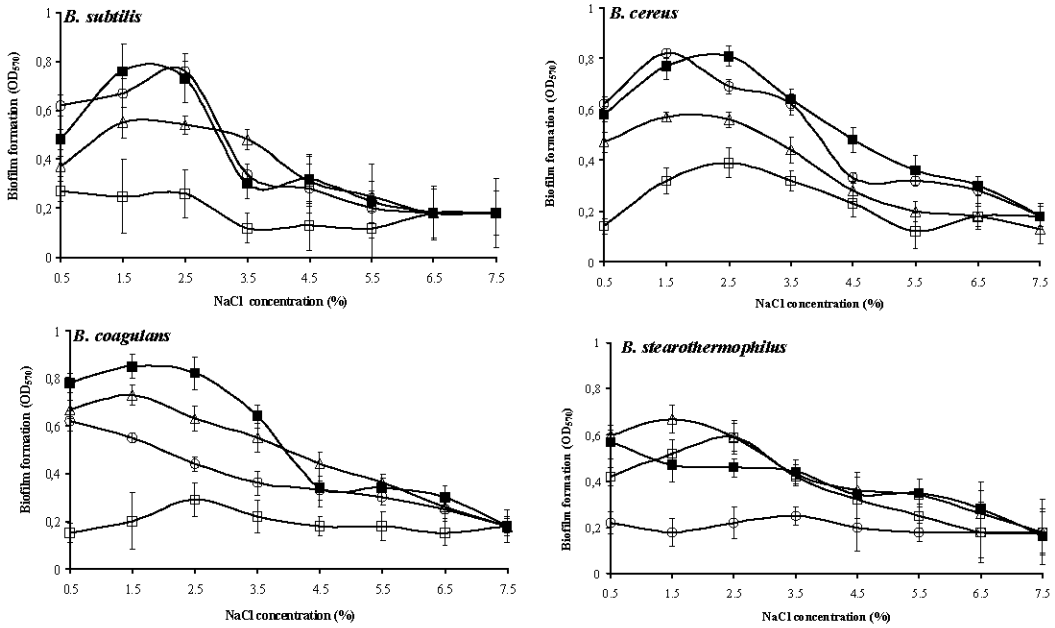

Fig. 2: The effect of NaCl concentration on the formation of biofilm by vegetative cells of *Bacillus* spp. on polystyrene surface at 30°C (○), 37°C (■), 45°C (Δ) and 55°C (□).

Table 3: Biofilm formation on polystyrene surface by vegetative cells of four *Bacillus* species before and after treatment with SDS, trypsin and lysozyme

		Treatment			
Strain	Incubation Temp.	Control	SDS	Trypsin	Lysozyme
B. subtilis ATCC 14085	30	++	+	+	0
B. cereus DSMZ 345	30	++	0	0	+
B. coagulans DSMZ 1	37	++	+	+	0
B. stearothermophilus DSMZ 297	55	+	0	0	0

all tested species except *B. stearothermophilus* which still weak biofilm producer (Table 2). In contrast, significant reduction was recorded by all tested species after heating (100°C for 10 min) the cell suspension or after exposure to UV (254nm/10min). Under dynamic conditions (150 rpm) all tested *Bacillus* species lost their abilities to produce biofilm (0).

Role of outer layers of vegetative cells in adhesion to polystyrene: The ability of *Bacillus* cells to form biofilm on polystyrene surface was dramatically decreased after treatment with 1% lysozyme (no biofilm producer) except *B. cereus* which became weak biofilm producer (Table 3). *B. cereus* and *B. stearothermophilus* became no biofilm producer (0) after treatment with either SDS or trypsin. Also, the ability of *B. subtilis* and *B. coagulans* to form biofilm on polystyrene was reduced to be weak biofilm producer by treatment with SDS or trypsin.

Role of hydrophobicity of vegetative cells and spores in attachment: The hydrophobicity of *Bacillus* cells and spores was determined, expressed as hydrophobicity percentage and illustrated in Fig. 3. Surface hydrophobicity of untreated *Bacillus* spores (ranged between 56±1.3 and 94±2%) was higher than those of untreated vegetative cells (ranged between 4±1.1 and 15±1.7%). The treatment with SDS led to extreme reduction on the hydrophobicity of all studied vegetative *Bacillus* cells (Fig. 3a). On the other hand, treatment with trypsin or lysozyme led to significant reduction in the hydrophobicity of all tested *Bacillus* cells compared with the hydrophobicity of untreated cells. However, no significant difference was recorded between reduction effects caused by trypsin and lysozyme (Fig. 3a). The highest hydrophobicity percentage (94±2%) of untreated spores was recorded when *B. cereus* was examined whereas the lowest one (56±1.3%) was obtained by untreated spores of *B stearothermophilus* (Fig. 3, b). All treatments applied in the present study (SDS, trypsin and lysozyme) caused significant reduction in the hydrophobicity of all tested spores compared with untreated spores. However, SDS and trypsin were more effective for reducing hydrophobicity of the studied *Bacillus* spores compared with reduction effect of lysozyme.

Number of spores attached to the polystyrene surface was dramatically affected by the hydrophobicity

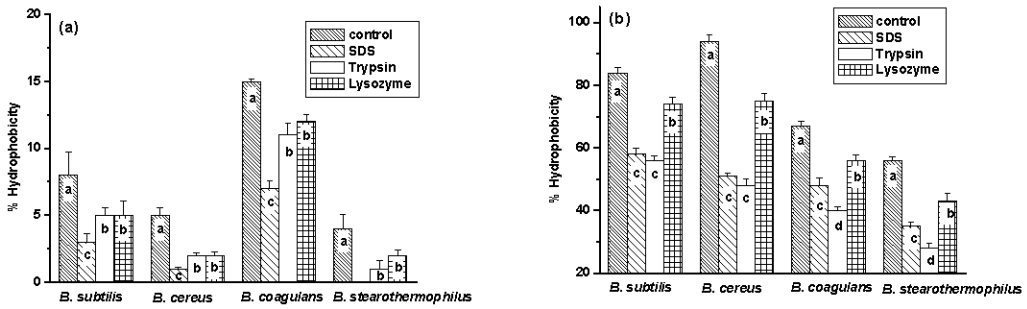

Fig. 3: Effect of treatment with SDS, trypsin, or lysozyme on the hydrophobicity of vegetative cells (a) and spores (b) of four *Bacillus* spp. Small letters on the same column group indicate the degree of significant effect (p = 0.05) on hydrophobicity of each examined strain due to treatments

Table 4: Relationship between spore hydrophobicity (h%) and spore numbers of *Bacillus* species attached to polystyrene surface before and after treatment with SDS, trypsin or lysozyme

		Treatment			
Strain		Control	SDS	Trypsin	Lysozyme
B. subtilis	Spore No.	$256^{a}\pm7$	$138^{b}\pm4$	$128^{c}\pm10$	$257^{a}\pm11$
ATCC 14085	% h	$84^{a}\pm1.8$	$58^{c}\pm1.8$	$58^{c}\pm1.5$	$74^{b}\pm2.3$
B. cereus	Spore No.	$251^{a}\pm12$	$169^{b}\pm10$	$120^{d}\pm4$	$228^{a}\pm21$
DSMZ 345	% h	$94^{a}\pm2.0$	$51^{c}\pm1.0$	$48^{c}\pm2.2$	$65^{b}\pm2.5$
B. coagulans	Spore No.	$122^{a}\pm5$	$122^{a}\pm7$	$118^{a}\pm6$	$124^{a}\pm8$
DSMZ 1	% h	$67^{a}\pm1.5$	$48^{c}\pm2.4$	$46^{c}\pm1.3$	$56^{b}\pm1.7$
B. stearothermophilus	Spore No.	$120^{a}\pm10$	$56^{b}\pm5$	$56^{b}\pm5$	$118^{a}\pm5$
DSMZ 297	% h	$56^{a}\pm1.3$	$35^{c}\pm1.4$	$32^{c}\pm1.6$	$43^{b}\pm2.6$

Values followed by the same letter on the same row are not significantly different ($P < 0.05$)

of *Bacillus* spores (Table 4). The highest numbers of attached spores were observed by untreated spores of *B. subtilis* and *B. cereus*. According to the number of attached spores (Table 4), it can be stated that treatment of *B. subtilis*, *B. cereus* and *B. stearothermophilus* with 2% SDS or 1% trypsin led to significant reduction in their abilities to attach to polystyrene substratum. On the other hand, all examined treatments had insignificant effect on the ability of *B. coagulans* spores to attach to polystyrene substratum, although these treatments led to significant reduction in spore hydrophobicity. From data in Table 4 and Fig. 3, it can be concluded that there is strong relation between biofilm formation by *Bacillus* species and their hydrophobicity.

DISCUSSION

Effect of incubation temperature on biofilm formation: The obtained results demonstrated that the optimum temperature for biofilm formation by *Bacillus* species on polystyrene was the optimum growth temperature of each tested bacterium. This could be explained by the strong relation between the bacterial attachment conditions and the optimum metabolic activity, which achieved at the optimum growth temperature [30]. The ability of cells to attach to polystyrene surface at high temperature, above the optimum temperature (Table 1), may be due to the production of heat stress proteins associated with the cell surface as stated by Briandet and others [31]. They suggested also that attachment ability is controlled by surface proteins. On the other hand, the reduction in biofilm formation by *B. coagluans* and *B. stearothermophilus* at suboptimal temperature may be due to increase the cell hydrophilic properties. In this respect, Chavant and others [32] demonstrated that low temperatures increase the cell hydrophilic properties and alter the ability of Gram-negative bacteria such as *Listeria monocytogenes* to adhere to hydrophobic materials like polystyrene.

The combined effect of temperature and food-related stress conditions (glucose or NaCl) on biofilm formation: The present investigation indicated highly diverse and complex patterns of biofilm formation of four *Bacillus* species exposed to glucose and NaCl concentrations under various temperatures. This diversity demonstrated that caution must be exercised regarding biofilm formation knowledge from single strain studies under single incubation temperature or at different glucose and NaCl concentrations. Similar studies including the combined effect of temperature and some additives (glucose or NaCl) have not previously been undertaken for *Bacillus*. The mechanisms behind increasing the production of biofilm at optimum growth temperature were discussed earlier. At temperature higher than the optimum growth

temperature the ability of these species to form biofilm may be due to a heat protective effect of glucose. Such effect was demonstrated [22] for increasing biofilm formation by *Staphylococcus aureus* at 46°C comparing with those obtained at the optimum growth temperature (37°C). Where, an increased biofilm production at increased osmotic pressure was recorded [22] for several strains of *S. aureus* at high temperatures (46°C). The suddenly increase in the ability of *B. subtilis*, *B. cereus* and *B. coagulans* to produce biofilm at 55°C could be also referred to the protective effect of glucose at the concentration of 6.25 and 7.25%. In general, excess of glucose in the medium improves the growth of *Bacillus* cells in biofilm, where a link between availability of the nutrient and biofilm development was demonstrated [33].

Addition of NaCl to TSB led to extreme change in the behavior of biofilm formation by all studied *Bacillus* species. In this respect, the optimum temperature for biofilm formation was changed to be below or above the optimum growth temperature. Generally, biofilm formation by *Bacillus* species was significantly reduced by increasing NaCl concentration in TSB. This could be explained by those mentioned by Csonka and Epstein [34]. They reported that the high salt concentration (up to 10%) in growth medium resulted in a lower environmental a_w which is disruptive of normal cellular activities. Hence, this observation suggests that osmotic drift influences the response of the tested *Bacillus* species biofilm growth on polystyrene. Ngwai and others [35] obtained the same manner by the response of *Salmonella* Typhimurium and *S.* Enteritidis biofilm growth on polystyrene under osmotic stress generated by NaCl.

Effect of environmental stress on biofilm formation: Nutrient availability has a major influence on biofilm structure and composition of the microbial community. Bacteria in food processing environment may be exposed to different levels of nutrients, depending upon the location in the plant [2]. Also, the amount and the type of sugar provided influenced the development of biofilm. Initial stimulation of biofilm development was suggested at low phosphate levels on pure culture of *Listeria* [36]. Biofilm formation by mesophilic tested *Bacillus* species was remarkably enhanced under the conditions of nutrient starvation (diluted TSB). This notice is in accordance with those mentioned by Korber and others [37]. They stated that biofilm formation of many bacilli species is initiated by starvation and is assumed to be advantageous for bacteria in oligotrophic environments. Moreover, in spore forming bacteria nutrient starvation induces some genes encoding starvation-activated transcription factors, exopolysaccharide biosynthesis and surfactin production [38]. These alterations lead to alter bacterial surface characteristics such as hydrophobicity, charge and irreversible attachment, which are essential factors in biofilm formation (discussed later). On contrary, in Gram-negative bacteria, such as *Salmonella*, nutrient starvation leads to reduction in biofilm formation [35].

Changing the ability of Bacillus cells from moderate biofilm producer (control) to weak biofilm producer after heat- or UV treatment indicates that both treatments significantly affected cell viability but did not affect attachment of cells to polystyrene surface.

The dynamic conditions (150 rpm) had negative effect not only on attachment of Bacillus cells to polystyrene surface but also on biofilm development, where all tested strains were no biofilm producer (0). In accordance with Chmielewski and Frank [2], this finding emphasis that steady state conditions play an important role in the initial adhesion of bacteria to surfaces. They mentioned that, adhesion to a substratum can be active or passive depending on cell motility. The passive attachment is driven by diffusion and fluid dynamics.

Role of bacterial outer layers and hydrophobicity in attachment: The active attachment of bacterial cells is facilitated by cell surface properties such as adhesion proteins, capsules, surface charge, flagella and pili [39]. Any change in cell surface properties influences adhesion of the cells to the solid surfaces. Therefore, measurement of bacterial hydrophobicity can be of importance in many research areas, e.g. biofouling and industrial microbiology [3]. In the MATH-test, removing cells from the aqueous suspension depends on their adhesion to the hydrocarbon phase. Thus, this method is very sensitive to the amount of surface area created during mixing of the two liquid phases [27]. The hydrophobicity of studied *Bacillus* spores was considerably greater than those of their vegetative cells. Koshikawa and others [40] assured same observations and reported that the spores of *B. megaterium* QMB1551 were 80% more hydrophobic than the vegetative cells. Moreover, Wiencek and others [9] stated that *Bacillus* spores, which have a hydrophobic surface due to their outer coat proteins and exosporium, have enhanced attachment capability on hydrophobic surfaces compared to vegetative cells, which have peptidoglycan on their cell surface. Therefore, it can be concluded that the hydrophobicity of *Bacillus* spores plays an important role in the hydrophobic interactions in the adhesion of bacteria to the surfaces of inert materials.

This finding could be interpreted by those stated previously [9,41] where, substratum hydrophobicity and bacterial cell surface hydrophobicity, as well as the related parameters surface tension and surface free energy mediate a non-specific reversible interaction which can lead to permanent adhesion. Practically, Bower and others [42] assured that spores adhere better to food contact inert-surfaces, such as plastic surfaces, than vegetative cells due to the high hydrophobicity of their hairy surfaces. Although the SDS-treatment applied in the present study had significant reduction effect on the hydrophobicity of *Bacillus* spores, they could tightly attach to the plastic surface. This result is in agreement with those mentioned by Bower and others [42]. They demonstrated that *Bacillus* and other spore forming bacteria are resistant to several detergents used in cleaning processes.

Lysozyme damages bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan [43]. All tested Gram-positive *Bacillus* species were sensitive to lysozyme treatment except *B. cereus*. Severin and others [44] reported that the peptidoglycan of *Bacillus cereus* has several distinguishing features: the overwhelming majority of cross-linked muropeptides are dimmers and virtually all muropeptides lack the *N*-acetyl group from glucosamine residues, thus explaining resistance of the cell walls to lysozyme. These features provide an acceptable explanation for the obtained biofilm on polystyrene by *B. cereus* even after lysozyme treatment. The highly sensitivity of *B. stearothermophilus* to lysozyme was previously stated [43]. The present data suggested that *B. subtilis* and *B. coagulans* were also sensitive to the treatment with lysozyme. All *Bacillus* spores showed relative resistance to lysozyme; therefore, hydrophobicity of the studied spores did not considerably affect by lysis with lysozyme. This may be explained by the chemical composition of cortex layer in the structure of *Bacillus* coat, which protects spores from lysozyme effect [45].

The capability of SDS to solubilized S-layer in the cell wall of most *Bacillus* species could be interpreted the extreme reduction of the hydrophobicity due to the treatment with SDS of all studied vegetative *Bacillus* cells. Treatment with trypsin or SDS appeared to be more effective as a reducer of spore hydrophobicity compared with lysozyme treatment. This finding goes in parallel with Cazemier and others [46]. They found that SDS-treatment was effective for obtaining coat-defective spores (more than 95% of the treated spores). The major protein released by the treatment with trypsin is highly hydrophobic proteins [47]. Therefore, the appreciable changes in number of attached spores may be referred to changes in spore hydrophobicity after treatment with SDS or trypsin.

REFERENCES

1. Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber and H.M. Lappin-Scott, 1995. Microbial biofilms. Annual Rev. Microbiol., 49: 711-745.
2. Chmielewski, R.A.N. and J.F. Frank, 2003. Biofilm formation and control in food processing facilities. Compr. Rev. Food Sci. Food Safety, 2: 22-32.
3. Davey, M.E. and G.A. O'Toole, 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev., 64: 847-67.
4. van den Eltzen, A.M. and J.M. Snjders, 1993. Critical point in meat production lines regarding the introduction of *Listeria monocytogenes*. Vet. Q., 15: 143-145.
5. Sammarco, M.L., G. Ripabelli, A. Ruberto, G. Iannitto and G.M. Grasso, 1997. Prevalence of *Salmonellae*, *Listeriae* and *Yersiniae* in the slaughterhouse environment and on work surfaces, equipment and workers. J. Food Prot., 60: 367-371.
6. Sharma, M. and S.K. Anand, 2002. Biofilms evaluation as an essential component of HACCP for food/dairy processing industry. Food Control, 13: 469-477.
7. Frank, J.F., 2000. Control of biofilm in the food and beverage industry. *In*: Walker J., S. Surman and J. Jass (eds). Industrial Biofouling. Chicester, N.Y.: John Wiley & Sons Ltd. pp: 205-224.
8. Bailey, C.P. and A. von Holy, 1993. *Bacillus* spore contamination associated with commercial bread manufacture. Food Microbiol., 10: 287-294.
9. Wiencek, K.M., N.A. Klapes and P.M. Foegeding, 1990. Hydrophobicity of Bacillus and Clostridium spores. Appl. Environ. Microbiol., 56: 2600-2605.
10. Branda, S.S., J.E. González-Pastor, S.R. Ben-Yehuda Losick and R. Kolter, 2001. Fruiting body formation by *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA, 98: 11621-11626.
11. Nicholson, W.L., N. Munakata, G. Horneck, H.J. Melosh and P. Setlow, 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev., 64: 548-572.

12. Paidhungat, M. and P. Setlow, 2002. Spore germination and outgrowth. *In*: Sonenshein, A.L., J.A. Hoch and R. Losick (eds.), *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C., pp: 527-536.
13. Danese, P.N., L.A. Pratt and R.S. Kolter, 2000. The outer membrane protein, Ag43, mediates cell-to-cell interactions in *E. coli* biofilms. Mol. Microbiol., 37: 424-432.
14. Mittelman, M.W., 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. J. Dairy Sci., 81: 2760-4.
15. Flemming, H.C., 1993. Biofilms and environmental protection. Water Sci. Technol., 27: 1-10.
16. Cunliffe, D., C.A. Smart, J. Tsibouklis, S. Young, C. Alexander and E.N. Vulfuson, 2000. Bacterial absorption to thermoresponsive polymer surfaces. Biotechnol. Lett., 22: 141-145.
17. Sinde, E. and J. Carballo, 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microbiol., 17: 439-47.
18. Ryu, J.H. and L.R. Beuchat, 2005. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide and a peroxyacetic acid-based sanitizer. J. Food Prot., 68: 2614-2622.
19. Pompermayer, D.M.C. and C.C. Gaylarde, 2000. The influence of temperature on the adhesion of mixed cultures of *Staphylococcus aureus* and *Escherichia coli* to polypropylene. Food Microbiol., 17: 361-365.
20. Rizk, I.R.S., M.A. El-Nawawy and H.M. Ebeid, 1989. The use of ethanol for the selective isolation of Bacillus strains originating from spores. Zentralbl. Mikrobiol., 144: 123-128.
21. Johnson, K.M., C.L. Nelson and F.F. Busta, 1982. Germination and heat resistance of *Bacillus cereus* spores from strains associated with diarrheal and emetic food-borne illnesses. J. Food Sci., 47: 1268-1271.
22. Rode, T.M., S. Langsrud, A. Holck and T. Møretrø, 2007. Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. Int. J. Food Microbiol., 30: 372-83.
23. Christensen, G.D., W.A. Simpson, J.J. Younger, L.M. Baddour, F.F. Barrett, D.M. Melton and E.H. Beachey. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol., 22: 996-1006.
24. Stepanovic, S., D. Vukovic, I.B. DakicSavic, M. Svabic-Vlahovic, 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods, 40: 175-179.
25. Parker, S.G., S.H. Flint, J.S. Palmer and J.D. Brooks, 2001. Factors influencing attachment of thermophilic bacilli to stainless steel. J. Appl. Microbiol., 90: 901-908.
26. Stepanovic, S., I.L. Cirkovic Ranin and M. Svabic-Vlahovic, 2004. Biofilm formation by *Salmonella* spp. And *Listeria monocytogenes* on plastic surface. Lett. Appl. Microbiol., 38: 428-432.
27. Crow, V.L. and B. Herndon, 1995. Cell structure differences of lactococcal strains. Int. Dairy J., 5: 45-68.
28. Faille, C., C. Jullien, F. Fontaine, M. Bellon-Fontaine, C. Slomianny and T. Benezech. 2002. Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. Can. J. Microbiol., 48: 728-738.
29. SAS Institute Inc. Co. 1997. SAS\STAT users guide; Ver. 6.12, Cary, NC: SAS institute, Inc.
30. Hood, S. and E. Zottola, 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. Int. J. Microbiol., 37: 145-53.
31. Briandet, R., T. Meylheue, C. Maher and M.N. Bellon-Fontaine, 1999. *Listeria monocytogenes* Scott A: cell surface charge, hydrophobicity, electron donor and acceptor characteristics under different environmental growth conditions. Appl. Environ. Microbiol., 65: 5328-33.
32. Chavant, P., B. Martinie, T. Meylheuc, M. Bellon-Fontaine and M. Hebraud, 2002. *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. Appl. Environ. Microbiol., 68: 728-737.
33. Kjelleberg, S., B. Humphrey and K. Marshall, 1983. Initial phases of starvation and activity of bacteria at surfaces. Appl. Environ. Microbiol., 46: 978-84.

34. Csonka, L.N. and W. Epstein, 1996. Osmoregulation. *In*: Neidhardt F.C., R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, *et al.* (Eds) *Escherichia* and *Salmonella*: Cellular and molecular biology, 2nd Ed., ASM Press, Washington, D.C., U.S.A., pp: 1210-1223.
35. Ngwai, Y.B., J.A. Onaolapo, J.O. Ehinmidu, Y. Adachi and Y. Ogawa, 2006. Interaction of *Salmonella typhimurium* and *Salmonella enteritidis* with polystyrene does not correlate with virulence in young chickens. African J. Biotechnol., 5: 1122-1130.
36. Kim, K.Y. and J.F. Frank, 1995. Effect of nutrients on biofilm formation by Listeria monocytogenes on stainless steel. J. Food Prot., 58: 24-8.
37. Korber, D.R., J.R. Lawrence, H.M. Lappin-Scott and J.W. Costerton, 1995. Growth of microorganisms on surfaces. *In*: Lappin-Scott H.M. and J.W. Costerton (eds), Microbial biofilms, Plant and microbial biotechnology research series: 5, University Press, Cambridge, UK., pp: 15-45.
38. Morikawa, M., 2006. Beneficial Biofilm Formation by Industrial Bacteria *Bacillus subtilis* and Related Species. J. Biosci. Bioeng., 101: 1-8.
39. Kumar, C.G. and S.K. Anand, 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol., 42: 9-27.
40. Koshikawa, T., M. Yamazaki, M. Yoshimi, S. Ogawa, A. Yamada, K. Watabe and P. Torii, 1989. Surface hydrophobicity of spores of *Bacillus* spp. J. Gen. Microbiol., 135: 2717-2722.
41. Kotiranta A., M. Haapasalo, K. Kari, E. Kerosuo, I. Olsen, T. Sorsa, J.H. Meurman and K. Lounatmaa, 1998. Surface structure, hydrophobicity, Phagocytosis and adherence to matrix proteins of *Bacillus cereus* cells with and without the crystalline surface protein layer. Infection and Immunity, 6: 4895-4902.
42. Bower, C.K., J. McGuire and M.A. Daeschel, 1996. The adhesion and detachment of bacteria and spores on food contact surfaces. Trends Food Sci. Technol., 7: 152-7.
43. Hughey, V.L. and E.A. Johnson, 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl. Environ. Microbiol., 53: 2165-2170.
44. Severin, A., K. Tabei and A. Tomasz, 2004. The structure of the cell wall peptidoglycan of *Bacillus cereus* RSVF1, a strain closely related to *Bacillus anthracis*. Microbial Drug Resistance, 10: 77-82.
45. Driks, A., 2002. Proteins of the spore core and coat, *In*: Sonenshein, A.L., Hoch, J.A., Losick, R. (eds.), *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C. pp: 527-536.
46. Cazemier, A.E., S.F.M. Wagenaars. and P.F. ter Steeg, 2001. Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli. J. Appl. Microbiol., 90: 761-770.
47. Yamamoto and Lampen, 1976. The hydrophobic membrane penicillinase of *Bacillus lichniformis* 749/C. J. Biological. Chem., 251: 4102-4110.