



Removability of bacterial spores made adherent to solid surfaces from suspension with and without drying

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ABSTRACT

Spores of *Geobacillus stearothermophilus* and *Bacillus subtilis* adhering to the surface of stainless steel (SS) and polypropylene (PP) were subjected to rinsing in water or NaOH solution to study their removability. The spores were made adherent through deposition from suspension under humid and dry conditions. The humid condition kept the surface wet throughout the deposition, whereas the dry condition made them dry up finally. Experimental results demonstrated that drying significantly enhanced both quantity and strength of the spore adhesion for both types of surface. No removal of the spores adhering to surfaces exposed to dry environment was observed through water rinsing with shear force application. Even with 0.2% NaOH, they were hardly removed irrespective of temperature. Although 1% NaOH removed or inactivated most of spores on SS surface, it left considerable viable spores on PP surface. Thus enhanced alkali tolerance was indicated for spores adhering to surfaces, especially made of PP, exposed to dry environment.

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1. Introduction

Adhesion of microbial cells to surfaces of food processing equipment or containers may lead to contamination of products. In particular, bacterial spores are often a concern because they are more resistant to such lethal factors as heat and sanitizing agents than vegetative cells. For keeping a hygienic process, it is important to avoid the adhesion of bacterial spores and to remove or inactivate any spores that do adhere. Thus information on adhesion and removal characteristics of bacterial spores on various types of food contact surfaces is desired for basic understanding.

Several studies have been conducted on adhesion of spores onto stainless steel surfaces. For example, Peng, Tsai, and Chou (2001) showed that the adhesion of *Bacillus cereus* spores from their suspensions (10^8 CFU/cm³) in phosphate-buffered saline (PBS), milk, or sterile water caused a significant level of contamination (around 10^5 CFU/cm²) on the surface of type 304 stainless steel plate. Parker, Flint, Palmer, and Brooks (2001) reported that spores of various *Bacillus* species suspended in distilled water adhered more favorably than vegetative cells to type 316 stainless steel surface. Information on removability of adherent spores from solid surfaces is also important from a hygienic point of view. In this context, cleanability of stainless steel equipment surfaces artificially soiled with a model food containing *B. cereus* spores was also studied for

assessing efficiency of cleaning-in-place (CIP) procedures, focusing on geometry or arrangement of the equipment (Blel, Bénézech, Legentilhomme, Legrand, & Le Gentil-Lelièvre 2007; Bénézech, Lelièvre, Membré, Viet, & Faille, 2002). In those studies, spores remained on the equipment surface after rinsing under any conditions tested. Some studies focused on the surface properties of spores and substrata as factors of adhesion and removability. Faille et al. (2002) studied the ability of bacterial spores to adhere to surfaces of six different materials including stainless steel. They found that *B. cereus* spores with more hydrophobic nature showed higher level and higher strength of adhesion than *Bacillus subtilis* spores for surfaces of the same material. They also found that hydrophobic material surfaces were harder to clean. Faille, Tauveron, Le Gentil-Lelièvre, and Slomianny (2007) reported the effects of exosporium of *B. cereus* on their adhesion ability and resistance to cleaning; damage to exosporium induced a decrease in the number of adhering spores and an increase in percentage of residual spores after CIP. Jullien et al. (2008) showed that conditioning (repetition of soiling and cleaning steps) of stainless steel surfaces affected the number of residual adhering *B. cereus* spores after CIP. In spite of those studies, reports on the effects of adhesion conditions on removability of spores are scarce. Information on adhesive strength or removability of spores adhering to food contact surfaces is still desired for compilation.

Objective of this study is to clarify behaviors of bacterial spores directly adhering to solid surfaces against rinsing and cleaning, using spores of *Geobacillus stearothermophilus* and *B. subtilis* as test

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spores. Thermophilic *G. stearothermophilus* can cause souring with no or little gas production (flat sour spoilage) in food products. Its adhesion to stainless steel and subsequent growth appear to be a likely cause of contamination of manufactured dairy products (Flint, Palmer, Bloemen, Brooks, & Crawford, 2001). Mesophilic *B. subtilis* is commonly found in soil and also causes food spoilage, ropiness in bread (Rosenkvist & Hansen, 1995) for example. We focused on the effect of presence of water around the spores on their adhesive strength; the bacterial spores were made adherent through deposition by contacting their suspension with solid surfaces under humid and dry environments. Plates of stainless steel and polypropylene were used as substrate surfaces since they are major materials for food processing equipment and containers.

2. Material and methods

2.1. Preparation of spore suspension

G. stearothermophilus JCM12216 and *B. subtilis* NBRC3134 were inoculated onto Trypticase Soy Agar (TSA) (Becton, Dickinson and Co., Maryland, USA) and incubated for 6 days at 58 °C and 37 °C, respectively. Spores grown on TSA were harvested and suspended in 1 ml of sterile distilled water. The spore suspension was heated at 100 °C for 10 min to inactivate vegetative cells, and centrifuged at 8700 × g for 30 s. After the supernatant was discarded, 1 ml of sterile water was added to the spores, and centrifuged again. This rinsing procedure was repeated two more times. Finally, the spores thus collected were resuspended in 1 ml of sterile distilled water and stored at 4 °C until use.

2.2. Pre-treatment of test surfaces

Coupons (50 mm × 50 mm) of type 304 stainless steel (SS) were purchased from Toste Co. (Osaka, Japan). Commercially available polypropylene (PP) sheets were cut into square coupons (50 mm × 50 mm) for use as another type of test surfaces. These test coupons were soaked in alkaline detergent (SCAT 20-X, Dai-ichi Kogyo Seiyaku Co. Ltd., Kyoto, Japan) for 24 h, and rinsed with distilled water. To avoid microbial contamination, the coupons were then autoclaved at 121 °C for 20 min and kept in 70% ethanol until use. Just before use in every experimental run, the test coupons were dried under UV light in a clean bench for 1 h.

2.3. Spore adhesion

A hundred microliters of spore suspension (approximately 200 CFU) was inoculated onto the surface of each test coupon and spread as uniformly as possible. The coupons with spore suspension on them were left in a clean bench for 0–60 min. The amount of water on the coupons decreased with time due to evaporation, and almost all of the water disappeared after 60 min. The artificially contaminated coupons thus obtained were soaked in sterile distilled water for 10 min to remove loosely adherent spores and then subjected to enumeration of adherent spores as described in Section 2.5.

In some experiments, to study the effect of presence of water on the adhesion, the coupons with spore suspension on them were left in vessels covered with wet tissue paper under a sterile condition for 0–60 min. In such a humid condition, water remained on the coupons still after 60 min. The coupons were soaked in sterile distilled water for 10 min to remove loosely adherent spores. Then the number of adherent spores was determined as described in Section 2.5.

2.4. Removal experiment

The coupons artificially contaminated with 60 min exposure to dry or humid environment as described in Section 2.3 were subjected to rinsing experiments to investigate the removability of adherent spores. Sterile distilled water or NaOH solution (0.2% or 1.0%) was used as a rinsing medium. Throughout the rinsing experiment, shear force was continuously applied using the following experimental set-up. A stainless steel vessel (135 mm in diameter, 148 mm in height) containing 1 l of a rinsing medium was placed in a water bath kept at 25 °C or 75 °C. The contaminated coupon and a 4-blade impeller were placed in the rinsing medium so that the distance between the coupon perpendicularly kept and the edge of impeller was 45 mm. The rinsing medium was stirred at 2000 rpm for 10 min to apply shear force to the contaminated surface. The geometry of the set-up was carefully confirmed in each experimental run to avoid alteration of a shear force distribution on the coupon. The number of spores remaining on the coupon was determined as described in Section 2.5.

2.5. Enumeration of spores adhering to test coupon

The number of spores adhering to each coupon was determined as follows. Each SS coupon was placed in a plastic dish with the artificially contaminated side up and covered by Modified Shapton and Hindes Agar (MSHA) (Shapton & Hindes, 1963). In the case of PP coupon, the test coupon was sandwiched with MSHA by two step solidification of the medium to assure that the test plate was covered by MSHA. After 20 h incubation at 58 °C for *G. stearothermophilus* or at 37 °C for *B. subtilis*, the number of colonies grown on the coupon was counted and taken as the number of adherent spores.

2.6. Viability of spores suspended in alkali solution

Lethality of alkaline solutions was studied as follows. Spores collected by centrifugation (8700 × g for 30 s) were suspended in 1.0 ml of 0.2% or 1.0% NaOH, and incubated at 25 °C or 75 °C for 10 min. The alkaline suspension was then neutralized by adding aliquot of 10% acetic acid, and adequately diluted. The number of viable spores was determined by colony count on MSHA plate.

2.7. Scanning electron microscopy (SEM)

PP coupons after alkaline cleaning were subjected to SEM observation as follows. PP coupons (10 × 10 mm) contaminated with approximately 1000 CFU spores were subjected to whirlpool rinsing with 1.0% NaOH solution for 10 min as described in Section 2.4. Each coupon was treated with 2.0% glutaraldehyde, and then with 2-methyl-2-propanol. After being freeze-dried (JED-310, JEOL, Tokyo, Japan), the plate was mounted on aluminum stub, coated with Pt/Pd for 90 s (Ion sputter coater E-1030, Hitachi, Tokyo, Japan), and subject to observation with a scanning electron microscope (S-4000, Hitachi).

2.8. Data analysis

All experiments were done in triplicate. Data sets obtained were subjected to statistical analysis by Student's *t*-test or analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

3. Results

3.1. Adhesion of spores to solid surfaces

Numbers of spores adhering to each type of test coupon exposed to dry and humid environments for 0–60 min are shown

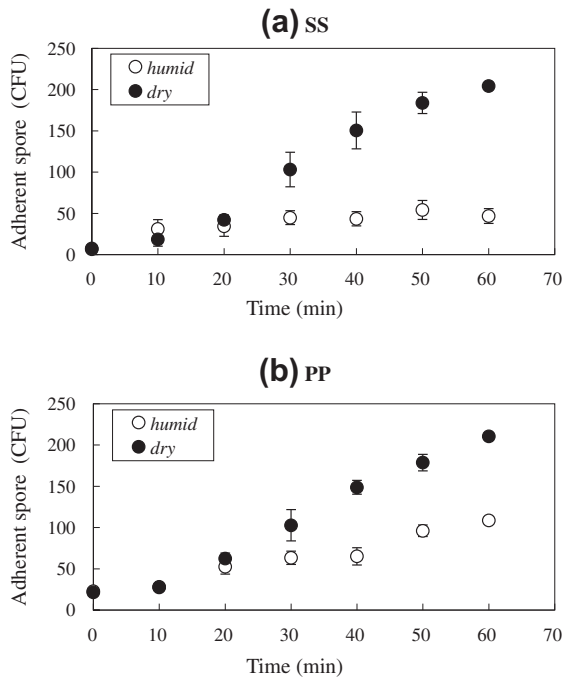


Fig. 1. Adhesion of *G. stearothermophilus* spore from its suspension to (a) SS and (b) PP coupons with exposure to humid or dry environment. Bars show standard deviation ($n = 3$).

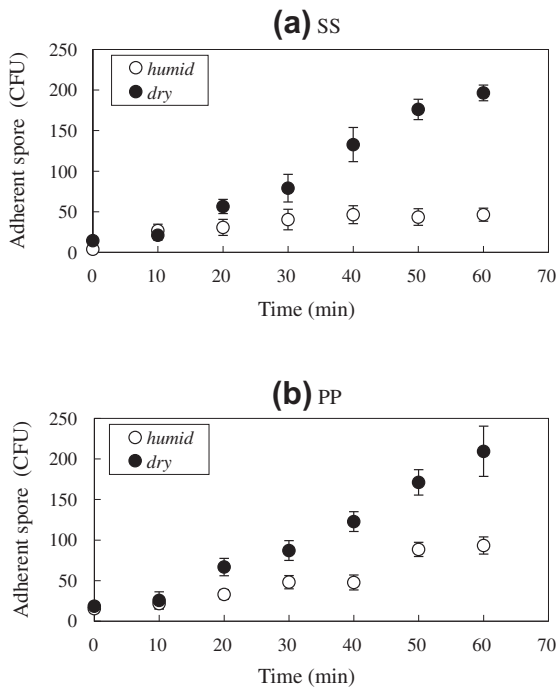


Fig. 2. Adhesion of *B. subtilis* spore from its suspension to (a) SS and (b) PP coupons with exposure to humid or dry environment. Bars show standard deviation ($n = 3$).

in Fig. 1 for *G. stearothermophilus* and in Fig. 2 for *B. subtilis*. In general, similar trends of adhesion were observed for the spores of both species. For SS surface exposed to the humid environment, the number of adherent spores increased with time initially for about 30 min. However the increase ceased thereafter, resulting in approximately 50 CFU per coupon as the final number of adherent spores. For PP surface exposed to the humid environment, the

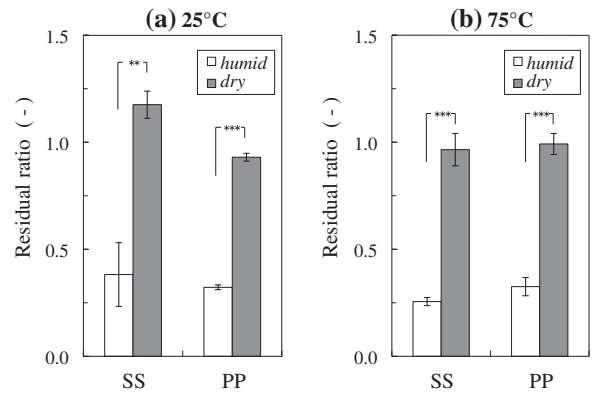


Fig. 3. Residual ratios after water rinsing for *G. stearothermophilus* spore on SS and PP coupons. Each coupon was contaminated with spore suspension and exposed to humid or dry environment for 60 min prior to water rinsing. Water rinsing was conducted for 10 min with 2000 rpm agitation at 25 °C (a) or 75 °C (b). Bars show standard deviation ($n = 3$). ** $P < 0.01$, **** $P < 0.001$.

number of adherent spores increased with time and finally reached around 100 CFU per coupon, the final number being significantly higher than that on SS surface for each species ($P < 0.01$). This suggests that PP is more attractive for the spores than SS. As for SS and PP coupons exposed to the dry environment, the numbers of adherent spores increased steadily with time and reached around 200 CFU per coupon after 60 min of exposure. This number means that almost all of spores contained in the initial suspension adhered to the surface. For each type of surface and each species, 30 min of exposure was enough for the number of adherent spores in dry environment to become significantly different ($P < 0.05$) from that in humid environment. The difference is highly significant ($P < 0.01$) for the exposure time longer than 40 min. Thus exposure to the dry environment greatly promoted adhesion of spores by removing surrounding water through evaporation, irrespective of surface material type.

3.2. Removability of adherent spores in water rinsing treatment

The contaminated SS and PP coupons exposed to dry and humid environments for 60 min and pre-rinsed by water soaking were subjected to water rinsing to study their removability under shear force. Fig. 3 shows the ratio of number of *G. stearothermophilus* spore remaining on each surface still after 10 min water rinsing to that before rinsing (referred to as residual ratio, hereafter). The residual ratio of spores adhered in the humid environment was below 0.4 irrespective of rinsing temperature for the both types of surface. In contrast, the residual ratio of spores adhered in the dry environment was around one; no removal by water rinsing was suggested for the both types of surface irrespective of rinsing temperature. The residual ratios above one may be ascribed to possible deviation in the number of spores initially loaded on coupons. Thus exposure to the dry environment during adhesion, or removing water around spores by evaporation, not only increased the number of adherent spore but also made the spore adhesion firm enough to endure shear force applied in the water rinsing experiment. These results also showed that rinsing temperature had little effect on the removability of spores. Similar results were obtained for *B. subtilis* as shown in Fig. 4.

3.3. Effect of alkali treatment on residual ratio of spores

Contaminated SS and PP coupons exposed to dry environments for 60 min and pre-rinsed by water soaking were subjected to alkali treatment in 0.2% or 1.0% NaOH with agitation. Table 1 shows

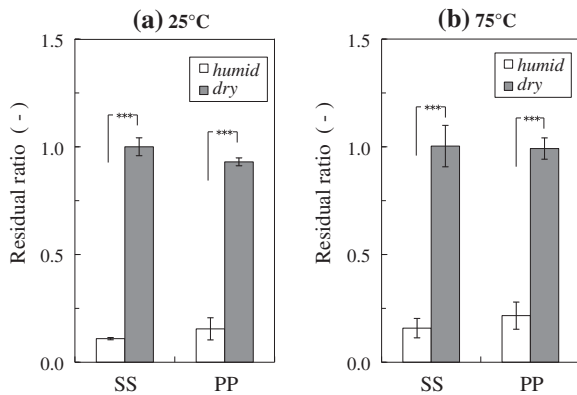


Fig. 4. Residual ratio after water rinsing for *B. subtilis* spore on SS and PP coupons. Each coupon was contaminated with spore suspension and exposed to humid or dry environment for 60 min prior to water rinsing. Water rinsing was conducted for 10 min with 2000 rpm agitation at 25 °C (a) or 75 °C (b). Bars show standard deviation ($n = 3$). *** $P < 0.001$.

Table 1

Residual ratios of bacterial spores on SS and PP coupons^A after 10 min alkali treatment with 2000 rpm agitation at 25 °C or 75 °C in comparison with survival ratios of spores suspended for 10 min in alkali solution.

Conditions		Residual ratio of adherent spore ^B		Survival ratio of suspended spore ^B
NaOH (%)	Temp. (°C)	On SS	On PP	
<i>(A) G. stearothermophilus</i>				
0.2	25	1.12 ± 0.07 ^a	0.97 ± 0.18 ^a	0.56 ± 0.08 ^b
0.2	75	1.07 ± 0.15 ^a	0.88 ± 0.04 ^a	0.46 ± 0.11 ^b
1.0	25	0.01 ± 0.01 ^b	0.50 ± 0.22 ^a	0.02 ± 0.01 ^b
1.0	75	0.01 ± 0.01 ^b	0.46 ± 0.18 ^a	0.01 ± 0.01 ^b
<i>(B) B. subtilis</i>				
0.2	25	0.79 ± 0.07 ^a	0.98 ± 0.03 ^a	0.45 ± 0.12 ^b
0.2	75	0.63 ± 0.02 ^a	0.87 ± 0.23 ^a	0.17 ± 0.05 ^b
1.0	25	0.01 ± 0.01 ^b	0.72 ± 0.07 ^a	0.01 ± 0.01 ^b
1.0	75	ND	0.34 ± 0.01	ND

ND: Viable spores not detected.

^A Each coupon was contaminated with spore suspension and exposed to dry environment for 60 min prior to alkali treatment.

^B Values are expressed as mean ± standard deviation ($n = 3$).

^{a,b} Values in the same row followed by different superscript letters differ significantly.

residual ratios after 10 min alkali treatment for *G. stearothermophilus* spore and *B. subtilis* spore. Generally the residual ratio was lower at higher temperature and at higher NaOH concentration for both types of spores. Decrease in residual ratio may be not only due to removal but also due to inactivation of spores by alkali. Therefore survival of spores suspended in 0.2% or 1.0% NaOH for 10 min was studied for comparison. The results are also shown in Table 1. After alkali treatment at 25 °C, the residual ratio of spores on both types of surface was significantly higher than the survival ratio in suspension. This indicates at least that spores of the both species adhering to SS and PP surfaces had a higher tolerance to alkali than spores in suspension. After alkali treatment at 75 °C, the residual ratio of spores on PP surface was much higher than the survival ratio in suspension, though that of spores on SS surface was similar to the survival ratio. Thus the spores adhering to PP surface had significantly high tolerance to alkali.

Fig. 5 shows SEM images of PP surface with adherent spores before and after the treatment with 1.0% NaOH for 10 min at 75 °C. Spores were thus confirmed on PP surfaces. However, the SEM images showed larger numbers of spores on the surfaces than those expected from colony count data. Although a conclusion cannot be drawn because distribution of spores on the surfaces is

rarely uniform, the observation may reflect the presence of inactivated or non-culturable spores.

4. Discussion

This study showed that quantity and strength of spore adhesion to SS and PP surfaces were enhanced through evaporation of water around them. This may relate to hydrophobicity of spores. Hydrophobic interaction has been suggested as responsible for a wide range of adhesion, including bacterial adhesion (Hood & Zottola, 1995). Hydrophobicity of bacterial spores and vegetative cells has been evaluated by several methods; one of them is evaluation of partition to the interface between hydrocarbon (hexadecane or octane) and water (Rosenberg, 1984). Based on this method, Doyle, Nedjat-Haiem, and Singh (1984) and Wienczek, Klapes, and Foegeding (1990) showed that spores of *Bacillus* and *Clostridium* have higher hydrophobicity than their corresponding vegetative cells. Thus, if the surfaces have portions of hydrophobic nature, spores can strongly interact with those portions upon removal of water molecules covered them. Such interaction induced by drying may be a major cause for no removal in water rinsing even with shear force application, though further study is necessary for clarification. For removal of the spores from surfaces, a detergent may be essential.

Ortega, Hagiwara, Watanabe, and Sakiyama (2008, 2010) reported the removability of *Staphylococcus epidermidis* and *Escherichia coli* attached from their suspension to SS surface. The experimental set-up they employed allowed wet adhesion of those non-spore-forming bacteria. Thus their experimental condition is comparable to that of experimental runs with exposure to humid environment in this study. Residual ratios of those non-spore-forming bacteria after 10 min water rinsing were around 0.1 for *S. epidermidis* and 0.01 for *E. coli*, indicating that non-spore-forming bacteria, especially *E. coli*, tend to show high removability compared with the bacterial spores tested in this study. Considering that *E. coli* was reported as a highly hydrophilic microorganism (Gilbert, Evans, Duguid, & Brown, 1991; Strevett & Chen, 2003), the difference in removability may reflect hydrophobicity of bacterial cells.

Spores adhering to the surfaces, especially made of PP, showed significantly higher alkali tolerance than those in suspension. In general, bacteria in biofilm are known to show higher tolerance to environmental stresses, including chemical ones, than planktonic cells (Dykes, Mills, & Bell, 2001; Isobe 2001; Lerebour, Cupferman, & Bellon-Fontaine, 2004). Bacteria can be protected from chemical stresses by the biofilm surrounding them. In the case of this study, however, the spores were adherent to the surfaces without any protective materials to cover them. Although mechanism for enhanced alkali tolerance of adherent spores is unclear at present, hydroxyl ions might hardly reach the surface because of chemical characteristics of surfaces.

From a hygienic point of view, it should be noted that around a half of spores on PP surface exposed to dry environment remained active even after 10 min treatment with 1.0% NaOH at 75 °C with shear force application. Sufficient care should be taken for food contact surfaces, especially made of PP, dried after water rinsing. SEM observation suggested the presence of inactivated or non-culturable spores on the PP surface. Inactivated spores may recover germination ability. For example, Setlow et al. (2002) reported that *B. subtilis* spores inactivated by alkali recovered germination ability by treatment with lysozyme, acting as a substitute for inactivated cortex-lytic enzymes, which are essential for germination (Ishikawa, Yamane, & Sekiguchi, 1998).

European Hygienic Engineering and Design Group (EHEDG) has proposed use of bacterial spores as a tracer in a standardized test

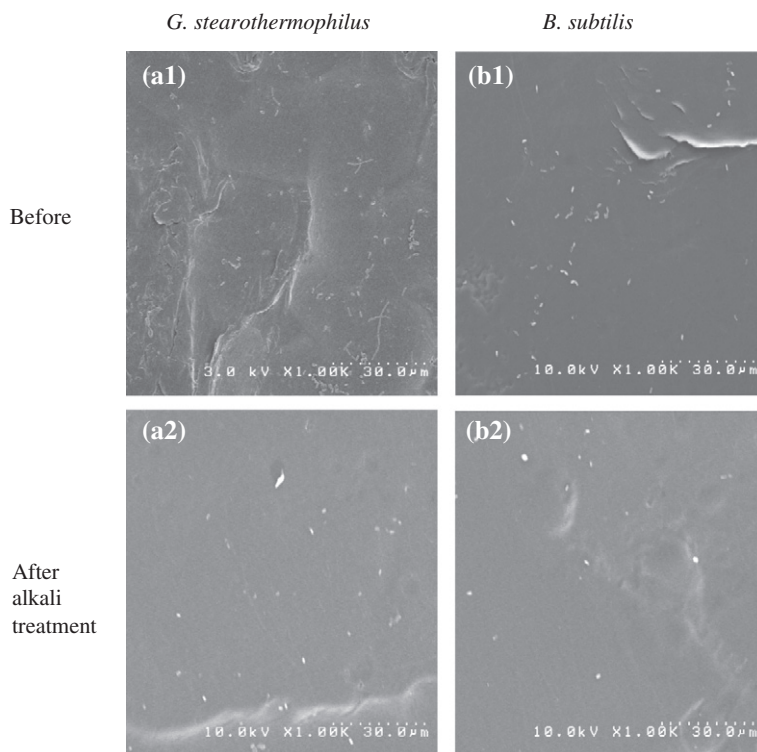


Fig. 5. Scanning electron microscope images of (a) *G. stearothermophilus* spores and (b) *B. subtilis* spores adherent to PP coupon before and after 10 min alkali treatment with 1.0% NaOH at 75 °C.

method for in-place cleanability of food processing equipment (EHEDG Update, 2007). In the test method, spores of *Bacillus stearothermophilus* var. *calidolactis* NIZO C953, the same strain as *G. stearothermophilus* employed in this study, are mixed with soured milk to soil a test section. After the soured milk is drained, the test section is dried by flushing with dry filtered air before cleaning tests. In this case, residue of the soured milk is dried with the bacterial spores on the surface of test section. Not only in this cleanability test, but also in most practical cases, bacterial spores can be left on equipment surface together with food components, though the concern of this study is limited to adhesion of spores suspended in water. Further study is necessary to investigate the effects of food components on spore adhesion to food contact surfaces.

In conclusion, this work has demonstrated that adhesion strength of bacterial spores can be significantly enhanced upon drying. Spores adherent to surfaces, particularly made of PP, after exposure to dry environment were hard to remove and/or inactivate even by alkali cleaning at an elevated temperature, indicating enhanced alkali tolerance of the adherent spores. These results provide essential knowledge for hygienic control of food contact surfaces.

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