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Culturability of *Clostridium botulinum* Spores under Different Germination Conditions, Sublethal Heat Treatments, and in the Presence of Nisin

Yoon-Kyung Chung† and Ahmed E. Yousef

Department of Food Science and Technology, The Ohio State University, Columbus, Ohio 43210-1007, USA

Abstract

Thermal resistance and heat activation characteristics of *Clostridium botulinum* ATCC 25763 spores were evaluated. The effects of nisin and pH on the activation and subsequent germination were also investigated. Spores of *C. botulinum* were not inactivated by heat treatments up to 92°C for 2 hr. Heat treatment at 85°C for 90 min was selected as the optimal activation condition based on monitoring subsequent germination. L-alanine alone or in combination with L-cysteine was not sufficient to germinate the spores of this strain. Tryptone-Peptone-Glucose-Yeast extract (TPGY) broth supplemented with L-alanine was used as a suitable germination medium. Decreasing pH of activation suspension increased the degree of phase darkening, i.e., germination. In addition, the presence of nisin during activation increased the degree of phase darkening. The majority of spore populations were dormant at a pH of less than 2.8, and these populations required heat activation to increase the culturability on TPGY agar medium. However, extended heating in the presence of nisin at pH 2.8 decreased the spore count; however, heat activation was less necessary at pH 3.4, compared at pH 2.8.

Key words: *Clostridium botulinum*, spore culturability, germination, nisin

INTRODUCTION

Spores can remain in a viable dormant state for extremely long periods of time. It is still not understood clearly how the spores are able to remain dormant for such long periods of time; nor has the initial trigger for breaking their dormancy been identified. This lack of understanding is of particular concern for the food industry because it requires harsh processing conditions to ensure that any spores are destroyed. A better understanding of the spore germination will help to develop novel milder processing conditions while maintaining microbiological safety. However, spore populations vary in the degree of dormancy, with some being more dormant than others. Typical levels of germination for the *Bacillus subtilis* PSB 357 strain was reported as approximately 50% (1). These researchers stated that this spore strain is generally assumed to have a high proportion of what are referred to as 'super dormant' spores. This explains the difficulties in using germination as a reliable control method for spores (2). Therefore, maximizing attempts for the activation of dormant spore population and subsequent germination is critical for controlling spores.

Activation describes changes in spores, which has been subjected to various pretreatments that alter the rate and extent of germination (3). Activation is most commonly achieved by sublethal heat treatment, although several other treatments, such as extremes of pH, reducing agents, $CaCl_2$, and ethanol, have been used (4-9). Heat activation results in an increased extent of germination as well as simplification of the requirements for the initiation of germination. However, when the heat treatment is not carefully controlled, low spore counts will be obtained due to inadequate heat activation or excessive spore destruction (8). Extreme dormancy can result in an apparent reduction in resistance while leaving most spore components undamaged and the spore thus potentially viable (10). It is therefore important to be able to predict the ability of the spores to survive heat treatment and the effect of environmental conditions on their subsequent growth in foods (11). This approach is especially important for pathogenic spores such as *C. botulinum*, and the development of improved practical control procedures is needed.

One possible method for enhancing the effectiveness of thermal processes while using less severe heat treatments is through the addition of a bacteriocin such as nisin to lower the thermal resistance of bacterial spores so that survivors of nisin treatment will be more susceptible to thermal processes. Therefore, this study was conducted to investigate the optimal heat activation conditions of *C. botulinum* Type A spores. In addition, the effect of nisin and pH during heat activation of spores

†Corresponding author. E-mail: Chung.115@osu.edu
Phone: +1-614-247-6831, Fax: +1-614-292-0218

was examined.

MATERIALS AND METHODS

Bacterial strains and preparation of spore suspensions

Proteolytic *C. botulinum* Type A ATCC 25763 was obtained from the American Type Culture Collection (Manassas, VA). Culture was grown in Tryptone-Peptone-Glucose-Yeast extract (TPGY) broth and kept frozen at -80°C with 40% (v/v) sterile glycerol added to the broth. Spores of *C. botulinum* were formed on the surface of TPGY agar plate. Overnight culture was spread onto TPGY agar and incubated at 37°C in an anaerobic chamber for 7～10 days until more than 90% of population was sporulated. Spores were harvested by adding 10 mL of sterile cold deionized water, releasing the colonies containing spores from the surface of the agar with the use of a sterile disposable inoculating loop. Spores were collected by centrifugation at 4,000×*g* for 10 min at 4°C and the pellet was washed in sterile cold deionized water, and centrifuged again at 10,000×*g* for 10 min at 4°C. Spore suspensions were then enzymatically cleaned by the methods of Billon et al. (12). The cleaning process consisted of aseptically treating each pellet with 100 mL of lysozyme solution (200 μg/mL of lysozyme in 0.05 M potassium phosphate buffer [KH_2PO_4 and K_2HPO_4], pH 8.1) for 30 min at 45°C. Then 100 mL trypsin solution (100 μg/mL trypsin in 0.05 M potassium phosphate buffer [KH_2PO_4 and K_2HPO_4], pH 8.1) was added and the mixture was incubated for 2 hr at 45°C. Spore crops, then, were washed ten times in sterile deionized water by centrifugation at 10,000×*g* for 10 min at 4°C. The final pellets were resuspended in sterile cold deionized water and kept refrigerated. Microscopic observations were made to confirm the purity of spore suspensions.

Germination media

Various germination buffers were tested to determine which germinants were needed for *C. botulinum* ATCC 25763 spores. Two germination buffers were used: citrate-phosphate buffer at pH 7 or 2-(*N*-morpholino)-ethanesulfonate (MES) buffer at pH 6. Germinants such as L-alanine (100 mM or 200 mM) and/or L-cysteine (50 mM) were also included in the germination buffer. In addition, TPGY broth containing 200 mM L-alanine was tested as a germination medium. Germination media are listed in Table 1. Spore suspension (containing approximately 10^7 spores/mL in deionized water) was dispensed in each 200-μL thin wall microtest tubes (Biorad Laboratories, Hercules, CA) and submerged and heated at 80°C for 15 min (13,14) in a circulating water bath (Haake, Germany). Activated spores were transferred into the anaerobic chamber, added into the different germination buffers, and incubated at 37°C for germination. Samples of spore-buffer mixture were taken periodically, and phase-change of the spores in germination medium was examined under a phase-contrast microscope (Bausch and Lomb, Rochester, NY). The numbers of phase-bright (ungerminated) spores and phase-dark (germinated) spores were counted.

Measuring degree of germination

Spore germination was measured by determining loss of heat resistance and observing phase darkening of spores. For monitoring germination, spore suspensions were heat activated at various temperatures, and then transferred to the germination buffer inside the anaerobic chamber. Spores in germination buffer were incubated at 37°C, and aliquots were withdrawn at predetermined intervals. Aliquots were spread-plated in TPGY agar without reheating, or heated at 80°C for 30 min and surviving spores were enumerated using TPGY agar medium. Samples of the incubated mixture were also examined by phase-contrast microscopy (Bausch and Lomb, Rochester, NY) at a magnification of 1,000X. The numbers of phase-bright (ungerminated) spores and phase-dark (germinated) spores were counted. All media and reagents were prepared at least 24 hr before use and kept at 22～25°C in the anaerobic chamber (15).

Optimal heat activation treatment

The heat resistance was assayed at various temperatures ranging from 80 to 100°C to investigate the lethal and sublethal temperatures for *C. botulinum* ATCC 25763 spores. Aliquots of 200 μL (containing approx-

Table 1. Media used in this study for germination of *Clostridium botulinum* spores

Germination medium	pH	Concentration of germinant	Reference
Citrate-phosphate buffer	7.0	100 mM L-alanine	13, 14
		200 mM L-alanine	25
		100 mM L-alanine + 50 mM L-cysteine	
MES buffer[1)]	6.1	100 mM L-alanine + 50 mM L-cysteine	15
TPGY broth[2)]	6.8	200 mM L-alanine	

[1)]MES: 2-(*N*-morpholino)-ethanesulfonate. [2)]TPGY: Tryptone-Peptone-Glucose-Yeast extract.

imately 10^7 spores/mL in deionized water) were dispensed in 200 μL thin wall microtest tubes (Biorad Laboratories, Hercules, CA) and submerged and heated in a circulating water bath (Haake, Germany). Each sample tube was withdrawn at predetermined intervals, kept on ice, and then transferred into the anaerobic chamber. Spore suspensions were serially diluted in 0.1% peptone water and were enumerated in TPGY agar after incubation at 37°C for 24～48 hr under anaerobic conditions.

Heat treatments at selected sublethal temperatures were compared for their ability to enhance spore germination. Variable heating periods, i.e., 15, 30, 45, 60, or 90 min, at three different temperatures, i.e., 80, 85, or 90°C, were selected and germination was monitored by the methods described earlier.

Spore germination in different nisin preparations

Nisaplin (10^6 IU of nisin/g), a commercial nisin concentrate composed of 2.5% nisin in denatured milk solids and sodium chloride, or pure nisin was obtained from Alpine and Barrette Ltd. (Trowbridge, UK). Stock solutions of nisin containing 10^5 IU/mL in 0.02 N HCl was prepared as described by Scott and Taylor (16). The pH was adjusted to 2～3 with HCl solution and the mixture was autoclaved (10 min at 121°C) and stored at 4°C.

Different nisin preparations (Nisaplin or pure nisin) were added to the spore suspension to achieve a concentration of 250 μg/mL. The addition of nisin preparation lowered the pH of the activation medium (i.e., mixture of nisin stock solution and spores in deionized water), since the pHs of stock solutions of nisin preparations were pH 2.0 and pH 2.5, for Nisaplin and pure nisin, respectively. Thus, resulting pH values of activation medium were 2.8 and 3.4 for Nisaplin and pure nisin-containing solution, respectively. The pH value of the spore suspension without nisin preparation was 4.9. Peptone water adjusted pH to 2.8 and 3.4 was used as control, to differentiate the effect of pH and nisin. Different heat activation treatments, in the absence or presence of nisin, under two pHs (2.8 and 3.4), were applied as follows: (i) no heat; (ii) heating at 85°C for 30 min; (iii) heating at 85°C for 90 min; (iv) heating at 85°C for 90 min, followed by 5 min of cooling, then reheating at 80°C for 30 min, and enumerated cultivable spores on TPGY agar. Phase change of spores was also examined before and after heat activation treatment in the presence of nisin.

RESULTS AND DISCUSSION

Germination media

Different media were compared for their capabilities for germinating *C. botulinum* spores (Table 1). Examination of phase change of spores showed that germination occurred in TPGY broth, but not in other buffer media in the presence of germinant (Data not shown). After heat activation at 80°C for 15 min, spores in the buffer media containing L-alanine and/or L-cysteine remained phase-bright (ungerminated) after incubating at 37°C overnight (Data not shown). Therefore, L-alanine alone or in combination with L-cysteine was not sufficient to induce spore germination. It is apparent that ingredient(s) in TPGY broth aided germination, but this was not investigated in this study. Pol et al. (17) also observed similar results with *Bacillus cereus* spores. These researchers monitored the germination process in BHI broth and HEPES buffer containing L-alanine (10 mM) as a germinant. Only 35% of the spores germinated in HEPES buffer containing L-alanine, while more than 95% of the spores germinated in BHI broth.

Generally, the germination requirement of *Clostridium* species is complex, requiring a combination of several ingredients, compared to *Bacillus* species, in which a single compound, e.g. L-alanine, often initiates germination. Germination of spores of proteolytic *C. botulinum* type A and B (strains 62A, 190, B-aphis, Ba410, Beans) was triggered by a defined three-component mixture comprising L-alanine (or L-cysteine)/L-lactate (or sodium thioglycollate)/ sodium bicarbonate in buffer at a neutral pH (18-20). Similarly, optimum germination of spores of non-proteolytic *C. botulinum* types B, E, F occurred in L-alanine/L-lactate, L-cysteine/L-lactate, and L-serine/L-lactate containing phosphate buffer (21). Uehara and Frank (22) also found that germination of spores of *C. sporogenes* PA 3679h was stimulated by thioglycollate (0.1%) in L-alanine + pyrophosphate system, in which the two latter components were essential. Amino acids alone did not stimulate the germination of spores of *C. bifermentans*, and the minimum requirement of compounds needed for the germination was the presence of L-alanine, L-phenylalanine and lactate (23). In addition, an unknown factor present in yeast extract was suggested for enhanced germination (23). Treadwell et al. (24) also indicated that yeast extract and sodium bicarbonate were necessary for rapid germination of *C. botulinum* 62A spores. Therefore, it is also probable that some ingredient(s) in the yeast extract, included in TPGY agar, was essential for the germination of *C. botulinum* spores used in our study.

In conclusion, evidence from previous studies suggests that L-alanine is essential for the germination of clostridial spores, however, additional germinants were also required for enhanced germination. Therefore, TPGY

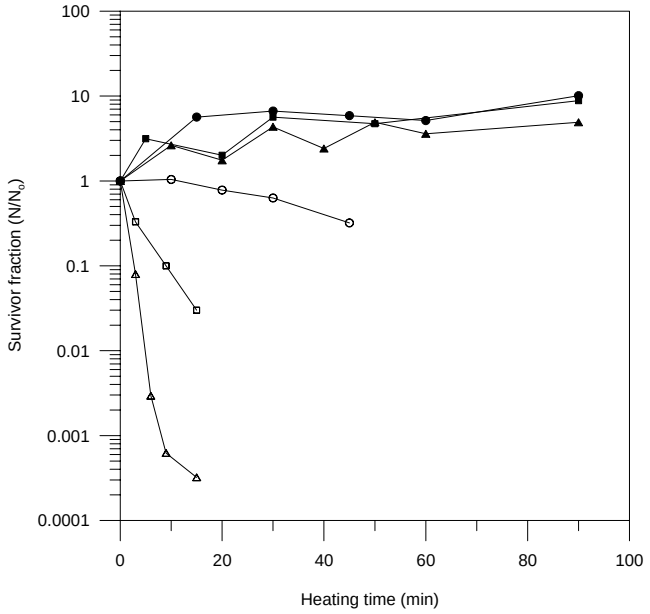

Fig. 1. Representative survivor curves for *Clostridium botulinum* ATCC 25763 spores suspended in deionized water. (●) 80°C; (■) 85°C; (▲) 92°C; (○) 94.5°C; (□) 97.5°C; (△) 100°C.

broth supplemented with L-alanine (200 mM) was selected as the germination medium for the strain used in this study.

Optimal heat activation treatment

To determine optimal activation treatment, spores of *C. botulinum* were heated at 80°C to 100°C for up to 90 min. Culturable spore population of this strain were not decreased by heat treatments up to 92°C for 90 min; actually, culturability increased with extended heating (Fig. 1). This indicates that considerable activation occurred and inactivation did not occur during 90 min heating at 80～92°C. Different sublethal heating treatments, 80°C for 15 min, 85°C for 15 min, and 85°C for 90 min, were compared for optimal activation and subsequent germination. Loss of heat resistance was not observed during 3 hr period of germination incubation at 37°C after heat activation at 80°C for 15 min, and the counts of heat-treated samples were even higher than control samples (Fig. 2a). These results suggest that heat activation at 80°C for 15 min did not fully activate the spores, so the spores did not germinate readily, and further activation, which occurred during reheating (80°C for 30 min), increased the count. Generally, heat activation treatment at 80°C for 10～15 min is used for the proteolytic clostridial spores in most other studies (13,15,25,26). However, this treatment was not sufficient to activate the spores of this strain. Therefore, a higher degree of heat treatment was tested. Heat treatments for 15 and 90 min at 85°C increased heat sensitivity of

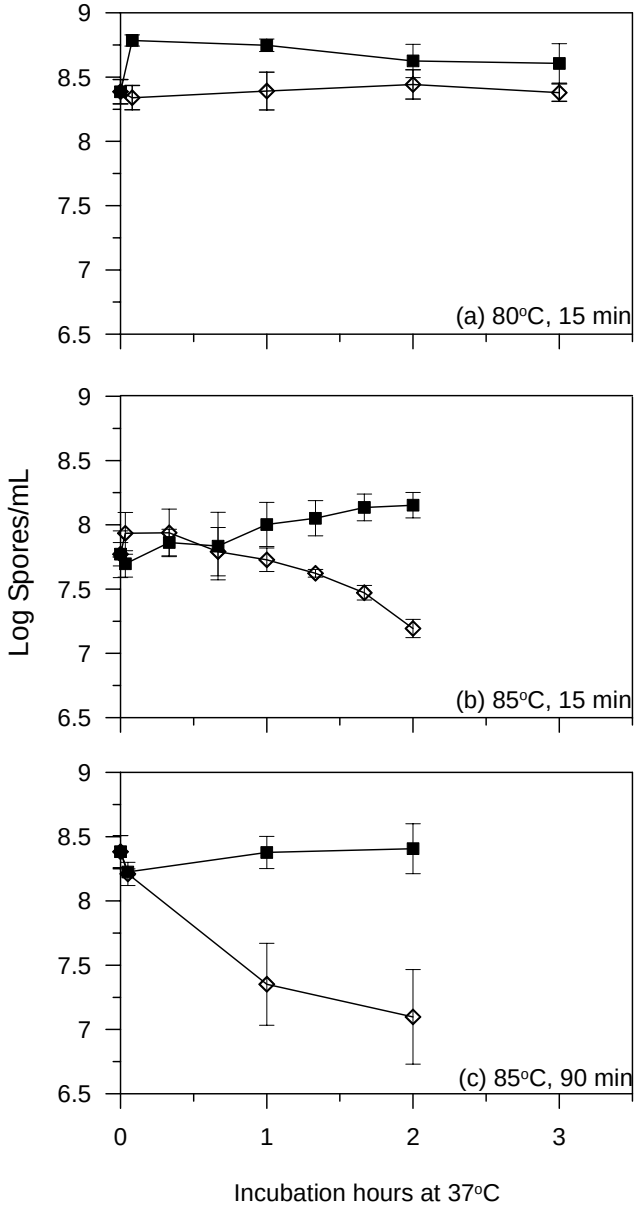

Fig. 2. Monitoring germination of *C. botulinum* spores. Spore suspension was heat activated at (a) 80°C for 15 min, (b) 85°C for 15 min, or (c) 85°C for 90 min, incubated at 37°C in the presence of germinant. Samples were taken at intervals, reheated at 80°C for 30 min and culturable spores were counted on TPGY agar. Non-reheated spores were also plated as a control treatment. Heat activation treatment: (■) no heating (control); (◇) reheating at 80°C for 30 min during sampling.

spores, spore counts decreased as the germination proceeded (Fig. 2b & 2c). Germination occurred more readily after heating for 90 min at 85°C than for 15 min heating. In addition, the effect of intermittent heating on the culturability of spore was examined after these three heat activation treatments (Fig. 3). Culturability was increased after all three heat activation (pre-heating) treatments, compared to non-heated spores. Reheating for 30 min at 80°C further increased the culturability

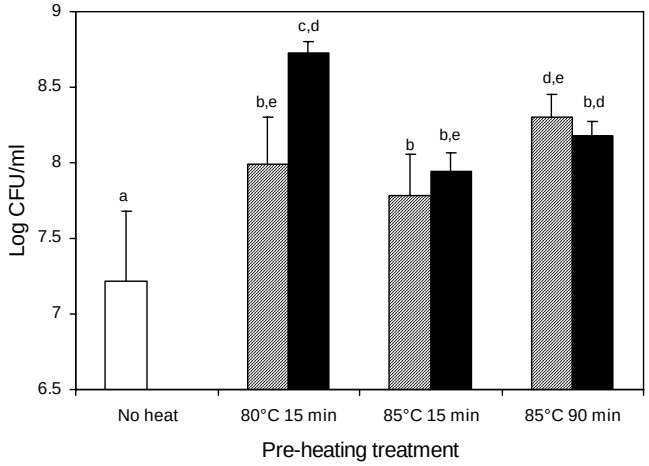

Fig. 3. Activation of *C. botulinum* spores by intermittent heating. Spore suspension was pre-heated at 80～85°C for 15～90 min and then reheated at 80°C for 30 min. (□) no pre-heating; (▨) pre-heated only; (■) pre-heated and reheated. Bars denoted by different letters indicate significant ($\alpha = 0.05$) differences as measured by ANOVA.

of spore samples, which were activated at 80°C for 15 min and 85°C for 15 min. The effect of double heating was greater with the lower degree of pre-heating treatment (80°C for 15 min), than 85°C for 15 min. This suggested that double-stage, intermittent heating with mild temperatures was very effective to increase the culturability (i.e., germinated population on the agar medium) of spores. The spore count after double-heating (pre-heated at 80°C for 15 min, and reheated at 80°C for 30 min), was comparable to the count obtained after single-heating at 85°C for 90 min. Cho et al. (27) also found that double-stage heating at lethal temperatures inactivated *B. subtilis* spores more effectively as compared to the single-stage heating, indicating intermittent heating effectively increased the degree of activation. On the other hand, reheating did not increase the culturability of spores, which was heat activated at 85°C for 90 min (Fig. 3). Therefore, treatment at 85°C for 90 min was sufficient to activate the spore populations fully. In other words, the best activation was achieved with a single heating treatment at this condition.

Phase contrast microscopy observation also showed similar results. Heating at 85°C for 90 min produced the greatest degree of phase change of spores, compared to the other heat activation treatments (Fig. 4). At 80°C, even for an extended period (90 min) of heating (single-stage heating), did not activate most of the spore population (Fig. 4a). Thus, less than 20% of spores were phase-darkened after 2 hr germination incubation. On the other hand, at 85°C and 90°C, the degree of germination increased in proportion to the heating time at each temperature (Fig. 4b & 4c). However, in general, germination occurred more readily in samples treated at 85°C than 90°C. Therefore, activation treatment at 85°C for 90 min was chosen for *C. botulinum* strain used in this study. Earlier studies indicated that the temperature and duration of heating for optimal activation of spores vary widely among different species and even among different spore preparations of the same strain (28). Gibbs (23) also reported that spores of *C. bifermentans* germinated readily after heat activation at 80～90°C (optimal at 85°C), but heating at temperatures ≤ 70°C or ≥ 90°C pre-

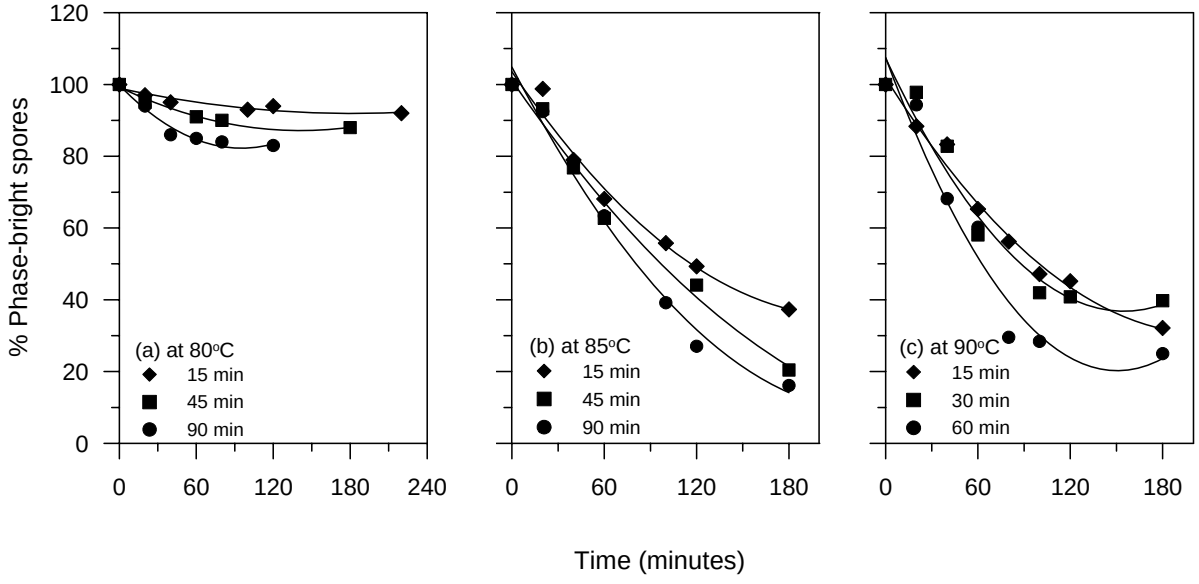

Fig. 4. Monitoring germination of *C. botulinum* spores by phase change observation. Spore suspension was heat activated at 80, 85, or 90°C for 15～90 min, and incubated at 37°C in the germination medium. Samples were examined under phase-contrast microscope at intervals.

vented subsequent germination.

Spore germination in different nisin preparations

Culturability of control samples (no nisin added) during activation at pH values of 2.8 and 3.4 was compared. Lower pH (2.8) suppressed the activation by heat (Fig. 5). In other words, heat activation treatment was necessary to increase the spore culturability under lower pH conditions. In addition, at pH 2.8, culturability was increased in proportion to the heating duration. At pH 2.8, similar spore counts on agar plates, as the counts obtained from control samples under pH 3.4, could be obtained only after double-stage, intermittent heating (85°C for 90 min + 80°C for 30 min). On the other hand, there was no measurable increase in spore culturability after heat treatments at pH 3.4. Thus, the majority of spore populations (more than 90% of maximum spore count) were dormant at a lower pH (pH 2.8), and these populations required heat activation treatment to germinate on the nutrient medium.

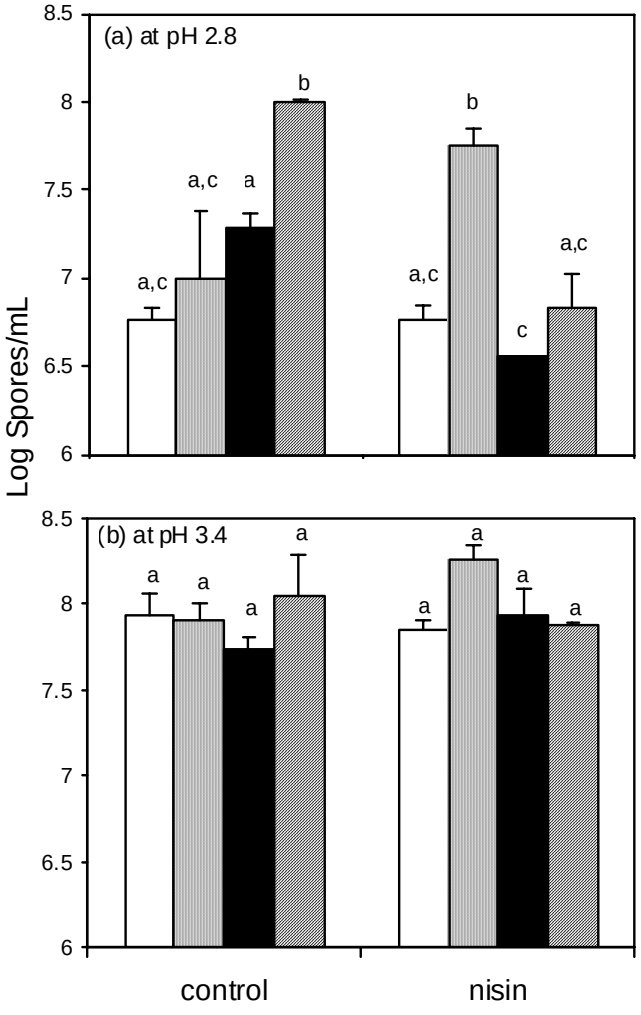

Fig. 5. Culturability of *C. botulinum* spores after heating at two different pH values and in the presence or absence of nisin (250 μg/mL). (□) no heat; (▨) heated at 85°C for 30 min; (■) heated at 85°C for 90 min; (▨) heated at 85°C for 90 min + reheated at 80°C 30 min.

On the other hand, presence of nisin decreased the heat activation requirement for the spores under low pH. In other words, short time, single-stage heating in the presence of nisin resulted in a similar degree of culturability as in spores treated with double-stage heating in the absence of nisin. However, extended heating at pH 2.8 in the presence of nisin decreased spore culturability. Steinbuch (7) also found that heat activation in acidic condition resulted in destruction of spores during the heat treatment. Activation of *B. stearothermophilus* spores in a suspension of pH 3.5 for 20 min at 100°C caused no destruction, however, intensification of heat activation to 15 min at 105°C or 10 min at 110°C, decreased spore counts by 1.5 and 3 logs, respectively (7). Beard et al. (29) reported that D-values of *B. licheniformis* spores were lower in the presence of nisin and this effect of nisin was further enhanced under lower pH.

High acidity during heat activation treatment increased the degree of phase darkening of spores, and nisin enhanced it synergistically (Fig. 6). Different pH of the heating suspension during activation at 85°C for 90 min affected phase-change of spores. Initially, % phase-dark spores in three different pH suspensions before heat activation were between 5～7%. After heat activation, lower pH (pH 2.8) enhanced phase darkening of spores, compared to higher pH values (pH 3.4 and 4.9). Heat activation in a suspension at pH 2.8 induced phase darkening to 17～18% of the spore population. Under similar pH values, degree of phase darkening was greater in the presence of nisin than its absence (Fig. 6). In addition,

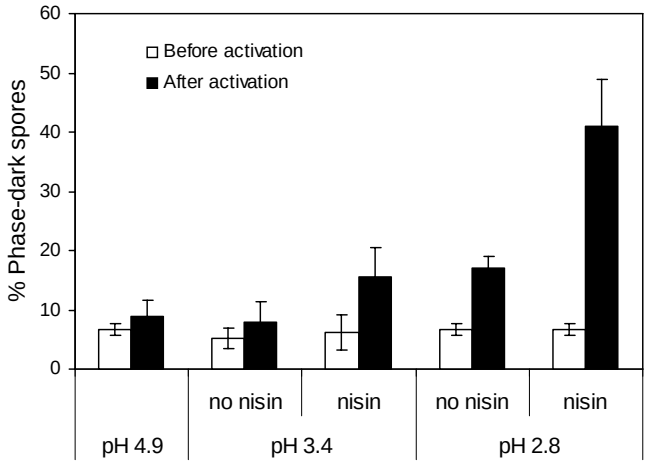

Fig. 6. Phase-change observation of *C. botulinum* spores before and after heat activation treatment (at 85°C for 90 min). Different activation conditions were compared under different pHs and in the presence or absence of nisin (250 μg/mL).

% phase-dark spores were higher (40%) when spores were activated at low pH (pH 2.8) in the presence of nisin, than in suspension at pH 3.4 (15%) in the presence of nisin (Fig. 6). Keynan et al. (28) also indicated that incubation of *B. cereus* spores in the presence of a reducing agent such as mercaptoethanol or thioglycollate or under low pH (less than 4.5) resulted in a partial activation of spores. Even though the incubation with reducing agents or under low pH did not replace the heat activation completely, it increased the germination rate considerably compared to unheated samples (28). Therefore, nisin and/or acidic environment activated spores, and furthermore some populations were germinated, by observing phase-darkening, without the addition of germinants.

It has been reported that nisin activity is directly related to the presence of dehydroalanine residues, which can readily react with sulfhydryl groups (30,31). Spore coats mainly consist of spore proteins, which contain high amount of cysteine (32), and it is likely that a coat protein rich in cysteine, stabilized by S-S linkages, is responsible for maintaining the dormant state (28). Therefore, the effect of nisin and pH on the activation of spores could be related to the reduction of disulfide bonds in spore protein. Hashimoto and Conti (33) observed the comb-like structures in the spore coat in heat-activated *B. cereus* spores. Their study indicated that these comb-like structures might represent cysteine-rich structural protein present in the spore coat and the rapid disappearance of this subcoat space occurred concomitantly with the loss of heat resistance. Srivastava and Fitz-James (34) also reported that heat activation (70^{o}C for 20 min) resulted in alteration in structural proteins and enzymes found in *B. cereus* spore coats. These researchers observed the changes, such as increased glycosylation of coat proteins, alteration in polypeptide patterns on sodium dodecyl sulfate-polyacylamide gels, and an increase in free SH groups of proteins. All of these studies suggested that spore coat or spore coat proteins are the possible targets for activation of germination by reducing agent, heat or pH.

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