Note

Thermal Death of *Bacillus subtilis* Spores in Oil-Water Systems

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The thermal death of the spores of *Bacillus subtilis* 168 in oil-water systems including emulsions and separated layers consisting of phosphate buffer and soybean oil or \(n\)-hexadecane was investigated. The resultant survivor curve consisted of two phases, an initial rapid reduction followed by a slow reduction, possibly as reflected by the death in the water phase and the oil phase, respectively. The concentration of oil in the system strikingly affected the pattern of thermal death. These results suggest that the spore location in the oil-water system may be a critical factor in determining the heat resistance.

Key words: Thermal death / *Bacillus subtilis* / Bacterial spore / Oil-water system.

Bacterial spores are highly resistant to various kinds of stress including heat, UV irradiation and disinfectants (Russell, 1982; Setlow and Johnson, 1997). In the heat sterilization of food, therefore, their high resistance is a serious problem and it is important to develop a useful and effective way of inactivating these spores. In addition, some food components including edible oils may raise the resistance of spores to heat and, therefore, sterilization of foods containing such oils is difficult (Nakagawa et al., 1998). Many kinds of oil-containing food products have been commercialized and therefore their thermal processing should be carefully designed, considering this increased resistance of spores in oil.

The increased resistance to heat of spores in oil has been reported by several investigators (Ababouch and Busta, 1987; Ababouch et al., 1987 and 1995; Gaze, 1985; LaRock, 1975; Molin and Snygg, 1967; Nakagawa et al., 1998; Senhaji, 1977; Senhaji and Loncin, 1977; Severance and LaRock, 1973; Thuillot et al., 1968). We have previously indicated that the \(D\) value of *Bacillus subtilis* 168 spores heated in soybean oil at 98.4°C was seven times greater than that in 50 mM potassium phosphate buffer (KPB) at pH 7.0 (Nakagawa et al., 1998). In addition, the oil was found to increase the \(z\) value by 6.5 times, indicating a marked reduction in the temperature dependency of the death rate (Nakagawa et al., 1998).

Although the high resistance of the spore itself has been attributed to a low content of water in the spore core (Marquis et al., 1994), the contribution of the oil outside the spore to its increased resistance has not been clearly understood. From the above studies, it has been suggested that the water level in both the oil phase and spore core may profoundly affect the heat stability of spores (Ababouch et al., 1987; Nakagawa et al., 1998). Furthermore, in different oil-water systems, including oil-in-water (o/w) and water-in-oil (w/o) emulsions and a two separately layered system of oil and water, where the spore is present, namely, in the oil phase, the aqueous phase, or their interface, should also be of importance. However, to our knowledge no detailed studies have been published on this subject to date.

*Bacillus subtilis* 168 trpC2 was used throughout this study. Cells were grown at 37°C for 16 h in
Trypticase soy broth (Becton Dickinson and Co., Cockeysville, Maryland, U.S.A.), and then inoculated on nutrient agar plates (16 g l⁻¹; Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 0.01% (w/v) MnCl₂·4H₂O, and then the plates were cultivated at 37°C for a week (Nakagawa et al., 1998). Spores were harvested and then suspended in KPB. The spore suspension was treated with lysozyme (Seikagaku Corp., Tokyo, Japan) at a concentration of 1 mg ml⁻¹ at 37°C for 2 h, and the spores were washed and then lyophilized (Nakagawa et al., 1998). The spores were stored at room temperature in a desiccator containing silica gel. Before use, spores were suspended in either KPB or oil, and then the suspension was homogenized with a sonifier (US-1, AS ONE Corp, Osaka, Japan) at 38kHz for 2 min. As oil samples, soybean oil (Ajinomoto Co., Tokyo) and n-hexadecane (Wako Pure Chemical Co., Osaka) were used. The latter oil was used as a reference and an inert oil, and, contrary to the former, contained toxic free fatty acids which may possibly affect the heat resistance of spores.

The spore suspension in either KPB or oil was mixed with the oil or KPB, respectively, to prepare required oil concentrations in a test tube (18mm × 180 mm for the measurement of optical density at 660 nm, OD₆₆₀, or 12 mm × 90 mm for the heat resistance test) at different volume ratios to give a total volume of 3.0 ml for OD₆₆₀ measurement or 1.0 ml for the heat resistance test. The mixture was either homogenized by using the sonifier or a vortex mixer for 1 min. The formation of the o/w emulsion and the location of spores in the phases were confirmed by observation with an optical microscope (BH-2, Olympus, Tokyo) with a magnification of 600×.

The diameter of an oil particle in the o/w emulsion was measured by using a Coulter Counter (Coulter Multisizer II, Coulter Electronics Ltd., Luton, England) equipped with an aperture tube having a slit diameter of 30 µm. In this case, the emulsification by sonication was performed for 5 min in a 300 ml beaker containing a total volume of 150 ml of the mixture. The average size of oil-particles in the emulsion was confirmed with a microscope to be identical to that of the particles obtained in the test tube system described above.

To know the heat stability of the o/w emulsion, we prepared a 5% (v/v) n-hexadecane emulsion by sonication for 1 min and the emulsion was heated at 60 and 98.4°C for 20 min before the measurement of the diameter of oil particles. Furthermore, to examine the influence of total interfacial areas between the oil phase and the aqueous phase in the mixture systems on the apparent heat resistance of spores, we prepared the following two types of the mixture, both consisting of 5% (v/v) n-hexadecane and 95% (v/v) KPB (pH 7.0) with different total interfacial areas between oil and aqueous phases. One was the o/w emulsion prepared by sonication for 1 min and the other was a mixture of two separate phases with a smaller interfacial area prepared by vortexing. The spore suspension in KPB was injected into both mixtures and the suspensions were then vortexed for 10 s for dispersion.

The spores were heated in a test tube (12mm × 90 mm) with an aluminum cap in the unsealed system as described previously (Nakagawa et al., 1998). A portion (0.9ml) of the emulsion, the non-miscible liquid of oil and KPB, or KPB alone was poured into a test tube for each heating time. A portion (0.1ml) of spore suspension (ca. 10⁶ml⁻¹) prepared by the method described above was poured into each test tube and then vortexed. The tubes were transferred into an aluminum block heater (TAITEC, TAH-1G) preheated to give a temperature of the liquid inside the test tube of 98.4°C. It took 2.0 min for the liquid to reach that temperature. At intervals, test tubes were withdrawn and then cooled in ice-water.

Spore viability was determined by the colony count method by plating the heated sample on nutrient agar followed by overnight incubation at 37°C (Nakagawa et al., 1998). When oil was contained in the sample, polyoxyethylene (20) sorbitan monooleate (Tween 80) at a final concentration of 0.1% (w/v) was added to a diluent to prevent spores from clumping and to emulsify oil.

To measure the spore surface hydrophobicity, the following two methods were employed. One was the method of Rosenberg et al. (1980). Spores were suspended in saline to give an OD₆₆₀ of 0.4 to 0.5. To 2.7 ml of this suspension 0.3 ml of n-hexadecane was added and then the mixture was vortexed for 1 min. After the mixture was kept for 15 min at room temperature, an appropriate amount of the aqueous layer was withdrawn and its OD₆₆₀ was measured. The spore surface hydrophobicity was expressed by the percentage of the reduction in OD₆₆₀ relative to the initial OD₆₆₀. The other method was the aqueous two-phase partition method (Yano et al., 1994). The spore suspension (0.1 ml containing 10⁶ spores) was mixed with 4 ml of 30 mM Tris-HCl buffer (pH 7.0) containing polyethylene glycol (PEG) 6000 (Wako Pure Chemicals Ind., Osaka, Japan) and dextran (M. W. 200,000 - 300,000, Sigma Chemical Co., St. Louis, MO, U.S.A.) at concentrations of 4.4 and 6.2% (w/v), respectively. After the two phases were separated, the spore count in each layer was estimated by colony counting. The percentage of the count in the
upper (PEG) layer to the total count was calculated and used as an index of hydrophobicity.

When *B. subtilis* spores were heated at 98.4°C in 5% (v/v) soybean oil or 5% (v/v) n-hexadecane, they demonstrated a biphasic death pattern consisting of rapid death and slow death (Fig. 1). The spores heated in KPB at pH 7.0 demonstrated a linear death pattern of the first order kinetics, as described by Nakagawa et al. (1998).

With an increase in the concentration of soybean oil up to 80% (v/v), there was an increase in the survivors at the time of shift from the first phase to the second phase in the above death pattern. However, the comparison of the slopes would confirm that the apparent death rate in the second phase seemed to be similar, irrespective of oil concentration (Fig. 1a).

In 100% (v/v) oil spores were killed at a rate similar to that of the above second phase from the commencement of heating. In the n-hexadecane emulsion, similar patterns of spore death were obtained, but a markedly high resistance was observed even at a concentration of 5% (v/v) (Fig. 1b).

It has been indicated that the heat resistance of *B. subtilis* spores increased in oils (Ababouch et al., 1987; Nakagawa et al., 1998). As a hypothesis, therefore, the most likely factor responsible for the above characteristic death pattern may be the varied localization of spores in the oil-water system. The initial rapid reduction in viability may be due to the killing of the spore fraction present in the aqueous phase, while the subsequent slow reduction may come from the death of spores present in the oil or oil-water interface. The translocation of spores from the water phase to the oil phase might also occur during the heating period. No spore clumping was observed in the emulsion.

The effect of spore location in the o/w emulsion upon the spore death was examined. Figure 2 demonstrates the death pattern of spores suspended in 40% (v/v) soybean oil during the heat treatment at 98.4°C. At this concentration, the mixture of oil and water did not form a homogeneous emulsion, separating into two phases immediately after mixing. Samples were removed from each phase. The viable spore count detected in the aqueous phase decreased rapidly by four log cycles during the first 20 min of heating and subsequently at a much reduced rate. On the other hand, the survivors in the oil phase were decreased by one log cycle after 20 min, and then gradually decreased. The results obtained seem to be explained by the heat resistance in each liquid phase as indicated in our previous paper (Nakagawa et al., 1998). A small volume of water containing a low count of spores contaminating the oil phase may be responsible for this initial reduction. Likewise, the survivors remaining in the aqueous phase during the prolonged heating period seemed to be derived from the contaminated oil.

It is likely that the location of spores may change during the heating period between oil and water phases in the emulsion. Most oil particles had a diameter of 4-5 μm with S. D. of 0.20 to 0.23 μm before heating, but the number of particles was markedly decreased after heat treatment. Particle size increased over the upper limit of measurable size, indicating that the particles may fuse to each other. After being heated at 60°C and 98.4°C for 20 min, due to the extensive fusion, the o/w emulsion was destabilized and separated to two layers (Fig. 3). It may be possible therefore that such a physicochemical change of liquid phase affects the location and resultant heat resistance of spores.
The heat resistance of spores at 98.4°C was compared between two types of the n-hexadecane-KPB system prepared by sonication (forming an o/w emulsion) and vortexing (forming two separate layers) as described above. As a result, no difference in the heat resistance of spores was found between these liquid systems, suggesting that the survivors in the oil phase may not depend upon whether oil is in an emulsion having a relatively larger interfacial area or it is present as a massive layer (Fig. 4).

We examined the effect of the period of vortexing on the spore location as reflected by percentage of the OD_{450} of the aqueous phase after vortexing to the initial OD_{450}. As a result, these percentages were 18.4, 32.6, 36.6, and 33.3% for 10, 30, 60, and 120 s, respectively, indicating that it reached a plateau after 30 s of vortexing and about 30% of spores moved into the oil phase. Based upon this result, the vortexing was carried out for 1 min in this study.

We also examined the possibility of the translocation of spores from the oil phase to water phase. The oil suspension of spores was mixed with KPB to give a final concentration of 10% (v/v) of oil. The vortexing of the mixture for at least 2 min caused no increase in the ratio of translocated spores, being constant at 0.08% for 10% (v/v) n-hexadecane emulsion. This result indicated that the spore translocation from oil phase to aqueous phase was extremely difficult, compared to that from the aqueous phase to oil phase. The heat resistance data in Fig. 2 supported this observation, demonstrating that the survivor curve leveled off at a relatively high count. Therefore, in the heat sterilization of foods containing oil emulsions, it seems very important whether contaminating spores are located in the aqueous phase or oil phase. If spores are located in the latter phase, the sterilization may be extremely difficult.

From the above experiments, the location of spores in the o/w emulsion seemed to be important for the spore survival in the thermal processing. Since, in the spore translocation, the surface hydrophobicity of spores is supposed to be greatly involved, we examined it by two methods, the method of Rosenberg et al. (1980) and the aqueous two-phase partition method. The values of surface hydrophobicity of intact spores obtained as the mean values of each three independent experiments were 30 and 33%, respectively, for the above two methods under the tested conditions. When spores were heated at 98.4°C for 30 min, their surface hydrophobicity measured by the former method, increased to 63%, indicating that the surface of heated spores become more hydrophobic. With the aqueous two-phase partition method, no reliable value of hydrophobicity was obtained for the heated spores, possibly due to the toxic effect of PEG on the heat-damaged spores.

Based upon these results, it is likely therefore that spores present in water phase translocate into the oil phase during the heating period, probably due to an increased hydrophobic interaction at high temperatures between the surfaces of the spore and oil and also possibly due to the damage to the spore surface. These results may provide us with useful information for understanding the behaviors of the heat resistance of bacterial spores in the heat sterilization of
oil-containing foods.

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REFERENCES
