Sporicidal Properties of Hydrogen Peroxide Against Food Spoilage Organisms

R. T. TOLEDO, F. E. ESCHER, AND J. C. AYRES
Department of Food Science, University of Georgia, Athens, Georgia 30602

Received for publication 2 July 1973

The sporicidal properties of hydrogen peroxide were evaluated at concentrations of 10 to 41% and at temperatures of 24 to 76°C. The organisms tested and their relative resistance at 24°C to 25.8% H₂O₂ were: Bacillus subtilis var. globigii > B. coagulans > B. steatothermophilus > Clostridium sp. putrefactive anaerobe 3679 > S. aureus, with “D” values of 7.3, 2, 1.8, 1.5, 0.8, and 0.2 min, respectively. Heat shocking spores prior to hydrogen peroxide treatment decreased their resistance. Wet spores were more resistant than dry spores when good mixing was achieved during hydrogen peroxide treatment. Inactivation curves followed first-order kinetics except for a lag period where the inactivation rate was very slow. Increasing the H₂O₂ concentration and the temperature reduced the lag period.

Packaging systems that utilize paper or plastic-based packaging materials are used increasingly by the food industry because of the simplicity of operation, the lower raw-material costs, and the easier disposal of the empty container. According to Hsu (4), the most widely accepted of these systems are suitable for aseptic operation, but the nature of the packaging material excludes the use of heat as a sterilizing agent.

Among the chemical sterilants available, hydrogen peroxide (H₂O₂) appears to be the most suitable because it does not impart an off-flavor to the product and small residues on the packaging material can be tolerated without adverse effects. The use of H₂O₂ for the sterilization of pipes, filters, etc., in the food industry has been reported as early as 1916, viz: Schumb et al., (5). Swartling and Lindgren (6) observed slow sporicidal activity of 11 to 22% H₂O₂ at room temperature on spores of Bacillus subtilis and Bacillus cereus var. mycoides which were inoculated on glass and polyethylene surfaces. However, exposure for 15 s to 22% H₂O₂ at room temperature, followed by hot-air heating at 125°C for 10 s, substantially reduced the numbers of survivors. Cerf and Hermier (2) reported that 3.5 min of exposure to 15% H₂O₂ at 80°C was necessary to achieve four-decimal reductions in populations of the most resistant of 21 Bacillus strains tested.

von Bockelmann and von Bockelmann (8)

1 Present address: Department of Agricultural Chemistry, Swiss Federal Institute of Technology, Zurich, Switzerland.

reviewed the literature on the sporicidal properties of H₂O₂. Many of the studies referred to the use of 15 to 20% H₂O₂. The absence of data on H₂O₂ inactivation of dry spores and the apparent differences in results by using different techniques were discussed.

The present study was undertaken to establish the relative resistance of food spoilage organisms to inactivation with H₂O₂ and to identify the factors that affect spore inactivation by H₂O₂ during aseptic packaging.

MATERIALS AND METHODS

Cultures. The organisms used were: B. subtilis var. globigii ATCC 9372; B. coagulans 56186 A (National Canners Association, NCA); B. steatothermophilus 1518 (NCA Flat Sour Organism); B. subtilis SA 22 (NCA #72-52); Clostridium sp. 3679 (NCA Putrefactive Anaerobe #3679); and Staphylococcus aureus ATCC 6538.

Preparation of test suspensions. B. subtilis var. globigii and B. coagulans were grown in tryptic soy broth (Difco) and incubated at 37 and 55°C for 4 to 6 days respectively. The spores were harvested by centrifugation, washed three times, suspended in physiological saline, and stored at 4°C until used.

B. steatothermophilus and B. subtilis SA 22 were grown on nutrient agar slants (Difco) in 900-ml screw-capped prescription bottles incubated at 55 and 37°C respectively. After 5 days, the spores were recovered from the slants by washing the surface with saline and collecting the suspended cells.

S. aureus ATCC 6538 was grown in micrococcus medium (peptone, 5.0 g; yeast extract, 3.0 g; beef extract, 1.5 g; glucose, 1 g; in 1 liter of distilled water, pH 7.4), and incubated at 37°C. Samples from the
actively growing cell suspension were used in the tests.

Clostridium sp. NCA 3679 was grown in thioglycolate broth (Difco), sealed with mineral oil, and incubated at 37°C. Spores were recovered by successive centrifugation and suspension in saline as was previously discussed.

Cultures were tested for the extent of sporulation before harvesting by staining smears with malachite green. Counts taken of samples from spore suspensions plated directly from stock and those plated after heating at 80°C for 20 min showed no significant differences, indicating that the stock suspensions were essentially all spores.

Enumeration of viable organisms. Standard plate count techniques were used. B. subtilis var. globigi, B. stearothermophilus, S. aureus, and B. coagulans were plated on plate count agar (Difco) and incubated at their optimal growth temperature. B. subtilis spores were plated on tryptone glucose extract agar (Difco). Clostridium sp. 3679 spores were plated on thioglycolate agar and incubated at 35°C in an anaerobic chamber.

Initial spore counts were made by plating a sample of the test spore suspension that had been heated for 20 min at 80°C. Spores treated with H₂O₂ were not heat shocked so that they were able to retain maximum resistance to H₂O₂.

H₂O₂ treatment: (i) Wet spores at room temperature. A 1-ml amount of spore suspension and 4 ml of H₂O₂ were drawn into a 5-ml disposable plastic syringe. H₂O₂ solutions of various concentrations were prepared by diluting either a 35 or 50% stabilized food grade H₂O₂ solution (Food Machinery Corp., BECCO Division). All H₂O₂ concentrations were calculated according to the 4 to 1 mixing ratio with spore suspensions. Efficient mixing was achieved by the turbulence induced during suction. After the desired contact time, 1 ml of the mixture was discharged into 99 ml of saline containing 0.5 mg of catalase per ml (activity: 100 mg of H₂O₂ decomposed per minute per milligram of catalase) and mixed. Plate counts were taken of the survivors. Aqueous solutions of freeze-dried beef liver catalase (Nutritional Biochemicals Corp., Cleveland, Ohio) were sterilized by using a presterilized filter unit (Falcon 7102 filter, 0.22 μm; Falcon Plastics, Los Angeles, Calif.). The efficiency of the removal of H₂O₂ by catalase was tested by adding 1 ml of 41% H₂O₂ to 99 ml of the saline with 0.5 mg per ml of catalase and by testing for residual H₂O₂ by adding 5 ml of 1 N KI solution and 1 ml of 1% soluble starch solution after 1 min. The solution remained colorless, indicating the absence of H₂O₂. Diluted spore suspensions were occasionally tested for the presence of H₂O₂ prior to plating by using KI and starch.

Heavy metals were excluded from the system (except for that present in the H₂O₂ that the manufacturer certified as FDA-approved food grade H₂O₂) to prevent the H₂O₂ from decomposing rapidly and to evaluate the effect of H₂O₂ by itself on bacterial spores.

(ii) Wet spores at elevated temperatures. Sterile test tubes containing 4 ml of the H₂O₂ solution were immersed in a water bath until the H₂O₂ reached water bath temperature. One ml of spore suspension at room temperature was then introduced to the test tubes rapidly by using a 5-ml plastic disposable syringe fitted with a cannula. After the desired contact time, 1 ml of the mixture was removed by using a sterile syringe and cannula and immediately discharged into the catalase solution, and the numbers of survivors were determined. Average temperatures to which the spores were actually exposed after mixing the cold spore suspension with hot H₂O₂ were determined by recording the temperature change in control test tubes by using H₂O₂ and water.

(iii) Dry spores. A 1-ml amount of spore suspension was introduced into sterile 25- by 150-mm culture tubes and dried over CaCl₂ in a desiccator at 25°C for 72 h. No evidence of spore injury due to drying was found as shown by similar counts obtained from the dried spores and that from the same volume of wet spores. H₂O₂ was diluted with distilled water to the same concentration used for the wet-spore treatments after the 4 to 1 ratio mixing and added to the dry spores in the culture tube. A small, sterile, magnetic stirring bar was then introduced, and the mixture was agitated continuously over a magnetic stir plate. At appropriate time intervals, 1-ml samples were removed, and the numbers of survivors were determined, as was previously described for the wet spores.

RESULTS AND DISCUSSION

Comparative resistance of different microorganisms to H₂O₂. The resistance of various microorganisms to 25.8% H₂O₂ at 24°C is shown in Fig. 1. As expected, S. aureus was less resistant than any of the spore forms requiring only 1 min of exposure to reduce populations by

![Fig. 1. Comparative survival curves of microorganisms exposed to 25.8% H₂O₂ at 24°C. 1. B. subtilis SA; 2. B. subtilis var. globigi; 3. B. coagulans; 4. B. stearothermophilus; 5. Clostridium sp. 3679; 6. S. aureus.](image-url)
six log cycles. Thus, the most rigorous treatment necessary to eliminate spore-forming spoilage organisms should eliminate S. aureus as well. Data reported by Dittmar (3) indicated a very low resistance of this microorganism to H$_2$O$_2$, requiring only 10 min in 0.05% H$_2$O$_2$ for inactivation. Amin and Olson (1) worked with more resistant strains of S. aureus and reported as much as 171 min of exposure to 0.05% H$_2$O$_2$ for 99.9% destruction of cells in 250 ml of a 10$^6$ suspension per ml. Both groups of investigators used 0.05% H$_2$O$_2$, a much lower concentration than the 25.8% H$_2$O$_2$ used in the present investigation.

Survival curves for most of the spore-forming organisms showed a lag period after initial exposure where there was a slow reduction in count, followed by a rapid rate of inactivation representative of first-order reaction kinetics. The less the resistance of the organism, the shorter the lag period, and for the two least resistant organisms, the survival curve followed first-order kinetics from the initial exposure. If there was a lag period for these organisms, it was not observed after the initial 30-s interval. The shapes of the survival curves were the same as those reported by Swartling and Lindgren (8) for B. subtilis ATCC 95244 spores in 10% H$_2$O$_2$. No tailing was observed in the survival curves of the organisms tested. Cerf and Hermier (2) reported a tail in the survival curve of a Bacillus strain isolated from milk when it was exposed to 23% H$_2$O$_2$ at pH 7.7 and 26 C. According to their data, the tail appeared to be less pronounced at pH 2.9. The present study was conducted by using stabilized H$_2$O$_2$ (pH 3.8) without adjustment of pH. Schumb (5) points out that H$_2$O$_2$ has maximal stability at pH 3.5 to 4.0, but becomes increasingly unstable at very low and very high pH values. The presence of the tail in Cerf and Hermier's survival curve could be due to decomposition of H$_2$O$_2$, resulting in decreased activity at the later stages of exposure.

Figure 1 shows that the order of resistance was B. subtilis SA 22 > B. subtilis var. globigii > B. coagulans > B. stearothermophilus. The “D” values determined from the straight line portion of the inactivation curves were 7.3, 2, 1.8, and 1.5 min, respectively, in 25.8% H$_2$O$_2$ at 24 C. Clostridium sp. 3679 and S. aureus showed very low resistance to H$_2$O$_2$. By comparison, Swartling and Lindgren (7) reported a “D” value of 2.3 min for B. subtilis ATCC 95244 in 20% H$_2$O$_2$, and Cerf and Hermier (2) reported a “D” value of 3.5 min in 23% H$_2$O$_2$ at pH 4.6 for the most resistant Bacillus strain isolated from milk. Thus, B. subtilis SA 22 is much more resistant than the organisms previously tested by other investigators. The resistance of B. subtilis var. globigii is comparable to organisms previously tested.

**Effect of combined heat and H$_2$O$_2$ treatment on spore survival.** Heat shock of spores at 80 C for 20 min prior to H$_2$O$_2$ treatment enhanced their destruction. Because the heat-shocked spores were quickly cooled in ice water and immediately treated with H$_2$O$_2$, the possibility of germinated spores being responsible for the decreased resistance to H$_2$O$_2$ was eliminated. The inactivation curve for heat-shocked spores of B. subtilis var. globigii (Fig. 2) showed a rapid rate of destruction after 4 min of exposure to H$_2$O$_2$ compared with untreated spores. This is evidenced by the “D” values of the straight line portion of the curves which were 0.5 and 2 min, respectively. The mild heat treatment alone did not significantly reduce the spore count, but it was sufficient to decrease their resistance to H$_2$O$_2$.

When the unheated spores were exposed to 25.8% H$_2$O$_2$ for 4 min at 24 C and then, after destroying the H$_2$O$_2$ by catalase, heated at 80 C in a water bath for 20 min, a six-log cycle reduction in count was observed. The 4 min of treatment with H$_2$O$_2$ by itself did not reduce the spore count by more than 1 log cycle (Fig. 2), and the heat treatment by itself did not signifi-
cantly alter the count. Yet, the \( \text{H}_2\text{O}_2 \) treatment considerably reduced the resistance of spores so that a mild heat treatment inactivated the injured spores. This result is in agreement with data given by Swartling and Lindgren (6) who showed that 11 s of exposure to 22% \( \text{H}_2\text{O}_2 \) at room temperature did not inactivate spores of \( \text{B. subtilis} \), but that exposure of the spores to hot air at 125 °C for 8 to 10 s following the \( \text{H}_2\text{O}_2 \) treatment resulted in a 99.2% destruction of spore population.

**Effect of \( \text{H}_2\text{O}_2 \) concentration.** Increasing the concentration of \( \text{H}_2\text{O}_2 \) increased its sporicidal properties. The survival curves of \( \text{B. subtilis} \) var. \( \text{globigii} \) in varying concentrations of \( \text{H}_2\text{O}_2 \) at 24 °C (Fig. 3) showed that increasing \( \text{H}_2\text{O}_2 \) concentrations lowered the time during which the curves showed the initial change in slope, reduced the exposure time, and also decreased the "D" value. Ten minutes of exposure at 10 and 20% was insufficient to start the curve on an exponential decline of surviving spore populations, whereas 6, 4, and 1 min were sufficient when 25.8, 35, and 41% \( \text{H}_2\text{O}_2 \), respectively, were applied. "D" values were 2, 1.5, and 0.75 min for 25.8, 35, and 41% \( \text{H}_2\text{O}_2 \), respectively.

**Effect of temperature.** The temperature at which \( \text{H}_2\text{O}_2 \) was incorporated has a very marked effect on spore inactivation. Figure 4 shows that the rate of inactivation in 25.8% \( \text{H}_2\text{O}_2 \) increased with increasing temperature.

Examination of the inactivation curves shown in Fig. 1 through 4 reveals that simple multiplication of the "D" value by the number of log cycles for the spore inactivation desired is insufficient to obtain the required exposure time for inactivation because of the initial persistence of spores, particularly those of \( \text{B. subtilis} \) SA 22 and \( \text{B. subtilis} \) var. \( \text{globigii} \). However, in most of the curves, the lag does not persist for more than one log cycle of initial decrease in spore population. Thus, the "D" concept utilized in heat sterilization can also be utilized for \( \text{H}_2\text{O}_2 \) sterilization if, in addition to the "D" value, the time required for the first log cycle reduction is incorporated into the calculation.

In Fig. 5, the "D" value and time required to achieve the first log-cycle reduction in spore population of \( \text{B. subtilis} \) var. \( \text{globigii} \) are plotted as a function of temperature. The "D" value decreased exponentially with increasing temperatures. At temperatures of less than 60 °C, the time required for the first log cycle reduction was higher than the "D" value; but as the temperature increased, the two curves converged. Above 60 °C, the exponential inactivation started from initial exposure of the spores to \( \text{H}_2\text{O}_2 \). The required exposure time for inactivation can therefore be calculated by adding the time required for the first log cycle of inactivation to the product of the "D" value and the desired number of log cycle reductions, minus one. Thus, at 40 °C, a 12-log cycle inactivation of \( \text{B. subtilis} \) var. \( \text{globigii} \) would require 11 min of
exposure (1.6 min + 11 x 0.85 min) to 25.8% H₂O₂.

Based on the "D" value, the "z" value for B. subtilis var globigii in 25.8% H₂O₂ is 40 °C. This compares with "z" values of 46 °C, 52 °C, and 47 °C determined from the data reported by Swartling and Lindgren (7) for B. subtilis in 10%, 15%, and 20% H₂O₂, respectively. The "z" value is the temperature change required to bring about a 10-fold change in the "D" value.

Comparative resistance of wet and dry spores. In a well-mixed system, dry spores are less resistant to H₂O₂ (viz. Fig. 6). Spores of B. subtilis SA 22 in 25.8% H₂O₂ at 24 °C had a first log-cycle inactivation time and a "D" value of 8.5 and 7.3 min respectively when wet, compared to 4.8 and 4.7 min respectively when dry. Spore inactivation started immediately after contact of dry spores with H₂O₂, whereas a short induction period was required for wet spores. Because the wet-spore suspension was replaced by water in the H₂O₂ solution used for the dry-spore treatment, the actual H₂O₂ concentration in both treatments was the same.

In an unmixed system (treatment was similar to wet spores at room temperature except that spores were allowed to dry inside the syringe prior to H₂O₂ treatment), data scatter was very pronounced, and in most instances more of the dry spores survived the same H₂O₂ treatment than wet spores. Clumping and slow penetration of H₂O₂ into spores in the middle of a clump may have been responsible for this variation from the results of a well-mixed system.

H₂O₂ is an effective sporidical agent. Food-grade H₂O₂ can be used on packages and equipment in aseptic packaging if no H₂O₂ eventually appears in the packaged food and a reasonable margin of safety is allowed in the elimination of pathogenic and food-spoilage microorganisms. Acceptance of H₂O₂ has been slow because of uncertainties with regard to its sporidical properties and the lack of quantitative information necessary to evaluate the sterilizing effectiveness of a specific processing condition within a system.

The system described by Hsu (4) is now used primarily on refrigerated or on acid foods. Swartling and Lindgren (7) described microbiological studies on which treatments used in the above system were based. By using 10 to 20% H₂O₂, the conditions of exposure to H₂O₂ were sufficient to reduce the population of B. subtilis 95244 (the one species of spore-forming organisms studied) by four to six log cycles. Our results show that organisms exist that are more resistant than the B. subtilis used by Swartling and Lindgren (7) and may be used to evaluate
SPORICIDAL PROPERTIES OF H₂O₂

sterilizing treatments needed for paper-based packaging materials. The organisms in the present study were able to survive the treatments they recommended. A safer process would result if the most resistant organism in our study, Bacillus subtilis SA 22, is used as a basis for developing requirements for sterilization in H₂O₂.

Although an anaerobe, Clostridium sp. PA3679 appears to have very low resistance to H₂O₂, and the resistance of other anaerobes such as Clostridium botulinum to H₂O₂ should be further investigated. The danger from anaerobic organisms in aerobically packaged foods is minimized because of the permeability of these materials to oxygen and the minimal vacuum in the container.

Our results show that “D” and “z” value concepts used in determining inactivation times in heat sterilization could be applied to H₂O₂ if the time required for inactivating the first 90% of the population was used in addition to the “D” value. We have presented data on the “D” values of six microorganisms at 24°C and the “z” value of one organism in 25.8% H₂O₂. When more data are compiled on the “D” and “z” values of various microorganisms at different H₂O₂ concentrations, it should be possible to evaluate conditions in any aseptic packaging system in which H₂O₂ is used for sterilization.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of K. von Stetten and the cooperation and funding given by the Mead Packaging Corporation.

LITERATURE CITED

1. Amin, V. M., and N. F. Olson. 1968. Influence of catalase activity on resistance of coagulase-positive staphylococci to hydrogen peroxide. Appl. Microbiol. 16:267–270.
2. Cerf, O., and J. Hermier. 1972. Diversité de la résistance des spores de Bacillus a l’eau oxygénée. Lait 52:1–20.
3. Dittmar, H. R., I. L. Baldwin, and S. B. Miller. 1930. The influence of certain inorganic salts on the germicidal activity of hydrogen peroxide. J. Bacteriol. 19:203–211.
4. Hau, D. A. 1970. Ultra-high temperature processing and aseptic packaging of dairy products. Damana Tech. Inc., New York.
5. Schumb, W. C., C. N. Satterfield, and R. L. Wentworth. 1955. Hydrogen Peroxide. Reinhold Publishing Corp., New York.
6. Swartling, P., and B. Lindgren. 1962. Aseptic filling in Tetra Pak, sterilization of the paper. Milk Dairy Research report no. 66, ALNARP, Sweden.
7. Swartling, P., and B. Lindgren. 1968. The sterilizing effect against Bacillus subtilis spores of hydrogen peroxide at different temperatures and concentration. J. Dairy Res. 35:423–428.
8. von Bockelmann, I., and B. von Bockelmann. 1972. The sporicidal action of hydrogen peroxide. A literature review. Lebensm. Wissensch. Technol. 5:221–225.