Thermal Resistance of *Bacillus stearothermophilus* Heated at High Temperatures in Different Substrates

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**ABSTRACT**

The effect of mushroom extract, with or without acidification with glucono-δ-lactone, and the overnight incubation of the spores in CaCl₂, on the heat resistance of *B. stearothermophilus* ATCC 12980 spores was studied. The temperature range considered was 121 to 140°C for mushroom extract and CaCl₂ and 121 to 145°C for double-distilled water as a reference substrate. The results indicated that mushroom extract without added acid significantly reduces the thermal resistance of the spores in comparison to the double-distilled water. Acidification of the mushroom extract reduces the heat resistance of the spores inoculated in the TTl when they were exposed at high temperatures, although studies carried out at low temperatures indicated that heat resistance was highest when the concentration of calcium ions in the protoplast of the spore was increased. A thorough understanding of the effect of different substrates on the heat resistance of these spores.

The thermal resistance of *B. stearothermophilus* spores has previously been determined in reference substrates with neutral pH and at temperatures under 125°C (8, 10). Recently, Fernandez et al. (11) published a systematic study describing the effect of the substrate and pH on the thermal resistance of these spores.

Regarding the temperatures frequently used in HTST sterilization processes, few data are available concerning the effect of the substrate on the heat resistance of *B. stearothermophilus* spores, and those which are available were obtained in substrates with neutral pH, or buffers at different pH values, the most recently published studies being those of David and Merson (8) and López et al. (13). Studies carried out at temperatures below 125°C using different microorganisms and substrates, indicated that it is possible to use the effect of the substrate or the pH in reducing the thermostability of the spores to design less severe thermal processes (6, 11), as these processes led to increased reductions in quality. López et al. (13) studied the heat resistance of *B. stearothermophilus* spores heated in McIlvain buffer at different pH levels, and found reductions in the thermal resistance to be less apparent at the highest temperatures studied (128 and 135°C). At present, there are acidulants available that do not confer such an acid taste as the traditional acidulants used in the vegetables canning industry, such as citric acid Glucono-δ-lactone, for example, could be considered one alternative (7, 16).

Regarding the gelation process necessary to prepare an alginate-based TTl, there is no information available concerning the effect of the CaCl₂ used in the gelation process on the heat resistance of the spores inoculated in the TTl when they are exposed at high temperatures, although studies carried out at low temperatures indicated that heat resistance was highest when the concentration of calcium ions in the protoplast of the spore was increased. A thorough understanding of the effect of different substrates on the heat resistance of these spores.
resistance of B. stearothermophilus spores is an essential first step to obtain a TTI which is capable of establishing and evaluating processes accurately.

In the present work the heat resistance of B. stearothermophilus spores heated at 121 to 145°C in distilled water was studied, as was the effect of the substrate (mushroom extract), acidification of the extract using glucono-δ-lactone at different pH levels, and overnight incubation in CaCl₂ on the thermal resistance of this microorganism heated over the temperature range 121 to 140°C.

MATERIALS AND METHODS

Preparation of the spore suspension

B. stearothermophilus ATCC 12980 was obtained from the National Culture Type Collection (Spain). The freeze-dried sample was hydrated and suspended in nutrient broth. Two colony variants were isolated, and the smooth variant only was used for sporulation in yeast-dextrose-tryptone agar medium plus 0.1% soluble starch (YDTAS) described by Brown et al. (4). A good spore yield of uniform physiological age was obtained by the synchronization procedure used by Kim and Naylor (12), which was slightly modified by increasing the incubation temperature from 52 to 60°C in order to increase thermal resistance. The spores were harvested by flooding the surface of the agar with sterile double-distilled water. Subsequently the spores were washed and centrifuged (at 1,000× g for 5 min) with double-deionized sterile water and then stored in double-distilled water at 4°C until use.

Preparation of the acidified mushroom extract

Fresh mushrooms (Agaricus bisporus) obtained from a commercial supplier (Cuenca, Spain) were washed, cut, sliced, and put into 445-cm³ glass jars (113 mm in height, 80.4 mm in diameter). Several aqueous solutions of glucono-δ-lactone (GDL) were prepared and added to the mushroom extract to obtain the preestablished pH levels (6.2 and 5.3). Samples were also prepared without acidification in order to have available mushroom extract with its natural pH. The solid-to-liquid ratio was always the same as that of the commercial canned product for the same jar capacity, as calculated in previous work (16). The jars were closed and thermally sterilized at 121°C for 20 min. The sterilized product was then homogenized and filtered through cheese cloth, and the pH was monitored. The extracts were put into glass tubes and sterilized again at 121°C for 20 min before being stored at 4°C. The final pH of the extracts respectively was the natural pH, 6.70, and 6.22 and 5.3.

Thermal resistance studies

Aqueous spore suspensions were shaken with glass beads in order to remove spore clumps and to provide a spore suspension with a standardized concentration, which was tested by plate counts. Aliquots of this suspension and the substrate were mixed thoroughly with a Gallencamp Spinmix mixer. The effect of CaCl₂ on the heat resistance of the bacterial spores was studied by holding them overnight at room temperature in a solution of 2% CaCl₂ and then heating the spores in the CaCl₂ solution. In all the thermal resistance studies, the spores were activated by heating at 100°C for 15 min.

The thermal resistance studies were carried out by using a computer-controlled thermoresistometer (CCFRA, Chipping-Campden, England) in the temperature ranges 121 to 140°C for extracts and CaCl₂ solutions and 121 to 145°C for distilled water. In all cases and for each time-temperature combination, 8 Millipore HA hydrophobic membrane filter disks (10 mm diameter, 0.45-μm pore size), inserted in a previously sterilized vial cap (11 mm diameter), were inoculated with 0.01 μl of spore-extract suspension (1:4, vol/vol) by using a 10-μl Hamilton syringe. Five samples (replications) were put in the thermoresistometer, which is specially designed to expose both sides of the filter paper and the 5 samples at once to the action of the steam. The 3 remaining samples were used as controls to ascertain the initial spore numbers. A computer-controlled piston pushed the samples into the treatment chamber, as described by Rodrigo et al. (15). At the end of the heating time, the samples were withdrawn to the discharge position and dropped into universal bottles containing glass beads and 10 ml of sterile distilled water. The vials were vigorously shaken to separate the microorganisms from the paper disk. The effect of substrate pH on the thermal resistance of the B. stearothermophilus spores was studied by heating the spores in mushroom extract acidified to the different pH levels, as indicated previously, using GDL as acidulant. After appropriate dilution, the spores were recovered on duplicate plates of the yeast-dextrose-tryptone agar medium plus 0.1% soluble starch (YDTAS) described by Brown et al. (4) for recovery. The same batch of recovery medium was used in the whole study. Three repetitions were carried out and all data were statistically analyzed by BMDP software (BMDP Statistical Software, Berkeley, CA USA).

RESULTS

Thermal inactivation

Survivor curves were constructed representing the colony-forming units per milliliter (CFU/ml) against the heating time. Figure 1 shows some examples of such survivor curves at 137°C. Regression lines were established for each temperature and substrate studied. The linear correlation coefficients were greater than 0.98 in all cases, with a significance of 99%. The D values for the individual experiments were obtained as the negative inverse of the slope of the regression line.

![FIGURE 1. Survivor curves of B. stearothermophilus spores treated at 137°C in different substrates; + Bidistilled water; △ mushroom extract, natural pH; ○ mushroom extract, pH 6.2; □ mushroom extract, pH 5.3; the last two acidified with glucono-δ-lactone.](image-url)
TABLE 1. Mean D values and coefficient of variation obtained by using the thermoresistometer of Bacillus stearothermophilus spores heated in different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>D value, min (CV, %)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>121</td>
</tr>
<tr>
<td>Double-distilled water</td>
<td>2.78 (5.0)</td>
</tr>
<tr>
<td>Mushroom extract, natural pH</td>
<td>1.59 (4.2)</td>
</tr>
<tr>
<td>Mushroom extract, pH 6.2</td>
<td>0.66 (7.3)</td>
</tr>
<tr>
<td>Mushroom extract, pH 5.3</td>
<td>0.66 (7.3)</td>
</tr>
<tr>
<td>CaCl₂ (2%)</td>
<td>5.50 (4.2)</td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
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* Values within columns followed by the same letter are not significantly different (P ≤ 0.05).
* NA, data not available.

Effect of the substrate on the thermal resistance of the spores

Table 1 shows the changes in the D values as a function of the substrate. It can be observed that at 121°C the substrate and pH have a substantial effect on the thermal resistance of the spores, which is much lower than that obtained in double-distilled water. However, at higher temperatures, no significant differences were observed between the D values for each pH value, or between the D values obtained using substrate at natural pH and pH 6.2.

Overnight incubation of the spores in CaCl₂ and subsequent heat treatment in this solution increased the heat resistance of the spores at 121 and 125°C, but did not at 130°C, in contrast to the results obtained in double-distilled water in this and other studies (11). This result is in agreement with the findings of Bender and Marquis (3), who state that metal ions have more influence on the thermal resistance of the spores when they are heated at lower temperatures.

The z value in the conditions studied can be observed in Table 2. Each value was obtained by plotting the D value against the heating temperature, as the negative inverse of the slope of the regression line. In all cases straight lines were obtained for the temperature intervals studied, with linear correlation coefficients greater than 0.99 (99% significance). All the z values obtained compare well with those in the literature (8, 13). However, slight variations with changes in substrate and pH were observed: the lower the pH, the smaller the variations in the z value, which had a tendency to increase as the pH fell.

TABLE 2. z values for Bacillus stearothermophilus spores heated in different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>z value (°C)</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-distilled water</td>
<td>9.00</td>
<td>0.992</td>
</tr>
<tr>
<td>Mushroom extract, natural pH</td>
<td>8.31</td>
<td>0.999</td>
</tr>
<tr>
<td>Mushroom extract, pH 6.2</td>
<td>9.30</td>
<td>0.993</td>
</tr>
<tr>
<td>Mushroom extract, pH 5.3</td>
<td>9.98</td>
<td>0.995</td>
</tr>
<tr>
<td>CaCl₂ (2%)</td>
<td>8.00</td>
<td>0.998</td>
</tr>
</tbody>
</table>

There is very little information in the literature concerning the thermal resistance of B. stearothermophilus heated at high temperatures. When our survivor curves are compared with those obtained by David and Merson (8) for a more resistant strain of B. stearothermophilus, it can be seen that there is not an initial shoulder in our case. They attributed the initial shoulder to the fact that at short heating times, there are more spores being activated than are dying. It is possible that, using spores with a lower thermal resistance, the activation treatment (15 min at 100°C) would be sufficient to activate all the spores.

With regard to the thermal death time (TDT) curves, the results obtained in this work by heating the spores in double-distilled water are comparable to those obtained by David and Merson (8), using a different thermoresistometer. No double slopes were observed in the temperature intervals studied (121 to 140°C in extract and 121 to 145°C in distilled water). However, these authors observed a tail at temperatures higher than 145°C. This was not observed in the present work, since the spores used were less heat resistant.

DISCUSSION

![FIGURE 2. Thermal death curves of B. stearothermophilus combining D values obtained with spores heated in capillary tubes (11) and in the thermoresisto-meter: + Bidistilled water; Δ mushroom extract, natural pH; ○ mushroom extract, pH 6.2; □ mushroom extract pH 5.3; the last two acidified with glucono-δ-lactone.](http://meridian.allenpress.com/jfp/article-pdf/60/2/144/1666412/0362-028x-60_2_144.pdf)
The slopes originating from previous studies carried out with capillary tubes heated to 125°C (11) were compared with those obtained in this work over the range 121 to 145°C for the same substrates. The differences observed do not allow one to affirm that there are two slopes for the interval 115 to 145°C. Despite the difference between the two techniques (capillary tubes and thermoressistometer), the D values obtained at each temperature could be plotted together (Figure 2). Straight lines were obtained in all cases with regression coefficients greater than 0.99 (99% significance).

The substrate with its natural pH of 6.7, as well as acidified, produced important reductions in the thermal resistance of B. stearothermophilus compared with that obtained using double-distilled water; the higher the acidity of the substrate, the greater the effect observed. However, this behavior was only found at 121°C, since at higher temperatures the decrease in pH was not accompanied by significant reductions in thermal resistance. In a previous study carried out with capillary tubes heated to 125°C (11), it was observed that at 125°C there were no significant differences between the acidified extracts. The results of the present work confirm this observation and go further, suggesting that this behavior is the same at other high temperatures, since it was observed at 130, 137 and 140°C.

This behavior does not appear to be substrate dependant, since it has also been observed in B. stearothermophilus when spores were heated in McIlvaine buffer at different pH values (13). Neither does it seem to depend on the microorganism studied, since a similar behavior has been observed for Clostridium sporogenes PA 3679 (15) and C. botulinum 213B (5) heated in mushroom extract at acid and natural pH of 6.7. One possible explanation might be that when the temperature is increased, the heating time is reduced with a consequent reduction in the period of contact between the acid and the spore, meaning that the acid cannot fully develop its action. El-Mabsout and Stevenson (9) observed that the effect of acid on the spore germination mechanism was less evident as the acid-spore contact time decreased. On the basis of these results, Fernandez et al. (11) suggested that the pH during the sterilization process might render the germination mechanisms more susceptible to the action of heat.

The results presented here indicate that no generalization can be made as regards the effect of the pH, and that in HTST sterilization the application of combined heat and pH processes need to be carried out with much care.

The gelation process to produce an inoculated particle for use as a TTI involves submerging the alginate-food puree mixture with the immobilized spores in a solution of 2% CaCl₂ for 24 h. Ocio et al. (14) observed that when spores immobilized in the gelled mixture were heated at temperature range of 115 to 130°C, the D values obtained were higher than those obtained in double-distilled water or in the nongelled mixture. It is known that calcium ions play a role in spore heat resistance (2). Results obtained in this work heating spores in CaCl₂ at high temperatures confirm these findings.

ACKNOWLEDGMENTS

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REFERENCES