Spore age and its effect on thermal resistance of *Bacillus coagulans* and *Bacillus macerans* in acid ripe plantain puree[1]

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ABSTRACT

Decimal reduction times (D values) at 100° C in sterile acid ripe plantain puree were calculated for 2-, 10- and 16-month old spores of *Bacillus coagulans* and *Bacillus macerans*. Results indicated that the $D_{100° C}$ values obtained were, respectively, 8.4, 13.2 and 9.2 min for *B. coagulans* and 9.7, 9.7 and 9.7 min for *B. macerans*. According to these data, spore age has no effect on heat resistance for *B. macerans*. For *B. coagulans*, however, spore age appears to have an effect on thermal resistance. When 10-month old *B. coagulans* spores were used, the $D_{100° C}$ values obtained were significantly higher ($P \leq 0.01$) than the ones for either 2- or 16-month old spores. This possible fluctuation in heat resistance with age of spore may introduce an element of variability that should not be overlooked when *B. coagulans* is used to determine the adequacy of a thermal process in acid or acidified foods. Since under certain conditions the spores of *B. macerans* could be more, or at least equally, heat resistant than those of *B. coagulans*, the question arises whether *B. macerans* could or should be used in lieu of *B. coagulans* in determining the adequacy of thermal processes in acid or acidified foods.

RESUMEN

Edad y resistencia al calor de las esporas de *Bacillus coagulans* y *B. macerans* en un puré ácido de plátano maduro

Se calculó el tiempo de reducción decimal (valor D) a 100° C. en puré ácido de plátano maduro estéril para esporas de *Bacillus coagulans* y *Bacillus macerans* de 2, 10 y 16 meses de edad. Los resultados indicaron que los valores D obtenidos fueron, respectivamente, de 8.4, 13.2 y 9.2 min. para *B. coagulans* y de 9.7, 9.7 y 9.7 m. para *B. macerans*. Según estos datos, en *B. macerans* la edad de la espora no tiene ningún efecto sobre su resistencia al calor. En el caso de *B. coagulans*, sin embargo, la edad de la espora parece tener un efecto sobre su resistencia térmica. Cuando se usaron esporas de *B. coagulans* de 10 meses de edad, los valores $D_{100° C}$ obtiendos fueron mayores significativamente ($P \leq 0.01$) que los de las esporas de 2 ó 16 meses de edad. Esta posible fluctuación en la resistencia térmica con la edad de la espora podría introducir un elemento de variabilidad que no se debería pasar por alto cuando se use *B. coagulans* para determinar la suficiencia de unproceso térmico para un alimento ácido o acidificado. Dado que bajo ciertas condiciones las esporas de *B. macerans* pueden ser más resistentes significativamente ($P \leq 0.05$) al calor, o por lo menos no ser diferentes significativamente ($P > 0.05$) a las de *B. coagulans*,

[1]Manuscript submitted to Editorial Board 29 May 1990.

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surge la interrogante de que si se podría o debería usar a *B. macerans* en vez de a *B. coagulans* para determinar la suficiencia de procesos térmicos en alimentos ácidos o acidificados.

INTRODUCTION

Since federal regulations do not require it, acid and acidified foods in hermetically sealed containers are not thermally processed to destroy spores of microorganisms that could survive and grow in the product (6). Both spore-producing facultative and obligate anaerobes play an important role in the spoilage of these foods. Of the facultative anaerobes, *Bacillus coagulans* is the most important, particularly in the spoilage of tomatoes and tomato products. Since the spores of the obligate anaerobes are usually less heat resistant than the spores of *B. coagulans*, thermal processes designed to free acid and acidified foods of this organism are generally adequate to free them of the obligate anaerobes (17).

Research studies have indicated that growth of *Bacillus licheniformis* (11) and *B. coagulans* (2) could raise the pH of tomato products, thus posing a botulinal risk in these water bath-processed foods if the spores of such organisms are not destroyed through the thermal process applied. An acid or acidified food is safe from *Clostridium botulinum* if the heat process kills all organisms capable of growing at pH 4.6 and there is no post-process contamination (14).

Fernández-Coll and Rodríguez-Toro (5) studied factors that increased the thermal resistance of spores of both *B. coagulans* and *B. macerans*. They reported that growing these organisms in nutrient agar modified with 50 p/m $MnSO_4$, pH 6.8 and incubated at 50° C produced spores with increased heat resistance in both bacteria. Previously, incubation temperature has been raised and divalent cations have been added to the culture medium to increase bacterial spore heat resistance (1, 3, 4, 8, 9, 10, 18). Heat resistance can also be increased by factors operating in the suspending medium during thermal processing, such as reduced water activity and high concentration of sugars (7). We have found, however, no information regarding the effect of age on the spore's heat resistance as applied to the commercial sterility of acid and acidified foods.

The purpose of this study is to determine to what extent the age of the spore contributes to the thermal resistance of *B. coagulans* and *B. macerans* in acid or acidified foods. This information, together with that already obtained (5), would serve as a starting point for thermal death time (TDT) determinations to establish thermal processes for acid or acidified foods in hermetically sealed containers.

MATERIALS AND METHODS

Preparation and standardization of spore suspensions

Cultures of *B. coagulans* (ATCC 8038) and *B. macerans* (ATCC 7069) were grown under conditions that enhance spore heat resistance (5),

namely inoculation onto Nutrient Agar Slants (Difco Laboratories, Detroit, Michigan)[4] modified with 50 p/m $MnSO_4$, pH 6.8 and incubated at 50° C. Cultures were harvested after 4 days' incubation and suspended in sterile phosphate buffer (0.07 M KH_2PO_4 and 0.07 NA_2HPO_4 solutions mixed to pH 7) to which 340μg lysozyme (41,100 units/mg, Grade I, Sigma Chemical Co., St. Louis, Mo) per ml suspension were added to destroy vegetative cells. Suspensions with added lysozyme were held at 35° C until microscopic examination showed absence of vegetative cells. Suspensions were stored at 7° C until needed. Before using the suspensions, the spore concentration of each one was determined by diluting it in sterile phosphate buffer as before; it was then plated in duplicate with Plate Count Agar (Difco Laboratories, Detroit, Michigan) and incubated at 37° C for 3 days.

Growth feasibility and decimal reduction times determination

Ripe plantains were sliced, fried in corn oil at 177° C for 5 minutes and microwaved at high setting for 1.5 additional min. A 30° Brix syrup was made with sucrose and water. A puree was prepared by blending one part plantain with four parts syrup in a Waring Blender. The puree was transferred to glass flasks and autoclaved at 121° C for 15 min. Sterility was checked by making aerobic plate counts with Plate Count Agar incubated at 37° C for 3 days. The final pH of the sterile puree was 4.15. The sterile puree was stored at 7° C until needed.

To assess growth feasibility of the bacteria in the product we inoculated 12 tubes, each with 10 ml of puree, with 0.1 ml of the *B. coagulans* spore suspension and 12 tubes with 0.1 ml of the *B. macerans* spore suspension. Six tubes with each type of spore were incubated at 37° C and six tubes at 50° C. At 0, 1, 2, 3, 4, and 5 days' incubation, aerobic plate counts were performed with Plate Count Agar incubated at 37° C for 3 days.

For Decimal Reduction Times (D values) determinations, the flask method (13) was used with slight modifications. Instead of using a 3-neck flask immersed in a hot water bath, we used a 2-neck flask placed inside a Heating Mantle (Glas-Col Apparatus Co., Terre Haute, Indiana). The heating mantle was in turn placed on top of a magnetic stirrer. Three hundred ml of the sterile puree was aseptically poured into the sterile flask, which already contained a sterile magnet. A disinfected thermometer embedded in a rubber stopper was firmly held in place through one of the necks of the flask while the other neck was kept covered with a

[4]Trade names in this publication are used only to provide specific information. Mention of a trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the Uiversity of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

sterile glass stopper. The glass stopper was moved every time a sample had to be retrieved and replaced afterwards. The puree was heated with constant stirring until it reached and maintained a temperature of 100° C. At this point, the puree was inoculated with the corresponding standardized spore suspension to give an initial theoretical concentration of 5.0×10^5 spores/ml puree. Every 2 min for up to 50 min, a 1 ml sample of the puree was aseptically withdrawn, diluted in sterile phosphate buffer, pour plated in duplicate with Plate Count Agar and incubated at 37° C for 3 days. After this incubation period, the average number of colony forming units (CFU) was determined for each time period assayed.

$D_{100° C}$ value determinations were performed with 2-, 10- and 16-month old *B. coagulans* and *B. macerans* spores. In addition, each given run was repeated on a different day to account for possible spore variation (7, 16).

Data on thermal death of spores were submitted to linear regression analyses and the D values obtained as the reciprocal of the regression coefficient (15).

RESULTS AND DISCUSSION

Results on growth feasibility of *B. coagulans* and *B. macerans* spores in the sterile ripe plantain puree appear in table 1. Both bacteria exhibited slight growth in the puree at 37° C and at 50° C. This growth was observed during the first day of incubation (for both bacteria at both temperatures), but generally started to diminish at day 2 and stabilized thereafter throughout day 5. Should spores of the microorganisms survive the thermal process given to such a product, subsequent growth is possible. It should be noted that commercial production of canned acid or acidified ripe plantain in Puerto Rico has been mainly for institutional purposes, particularly the School Lunch Program.

TABLE 1.—*Growth[1] of* Bacillus coagulans *and* Bacillus macerans *spores in acid ripe plantain puree (pH 4.15) at two different temperatures*

Incubation (days)	*B. coagulans*		*B. macerans*	
	37° C	50° C	37° C	50° C
0	8.0×10^3	1.0×10^4	8.0×20^2 Est.	6.0×10^2 Est.
1	5.5×10^4	3.9×10^4	7.0×10^4	5.2×10^4
2	3.8×10^4	2.5×10^4	8.8×10^4	3.0×10^4
3	3.1×10^4	3.5×10^4	6.8×10^4	3.3×10^4
4	3.6×10^4	3.3×10^4	6.2×10^4	3.1×10^4
5	3.4×10^4	2.8×10^4	3.5×10^4	3.0×10^4

[1]CFU per ml puree. Counts represent the average of six tubes of puree for each bacterium at each incubation temperature.

Table 2 presents $D_{100° C}$ values. According to these results, age has no effect on the heat resistance of *B. macerans* spores, since all $D_{100° C}$ values obtained were exactly the same. For *B. coagulans*, however, spore age appears to have an effect on its heat resistance. When 10-month-old *B. coagulans* spores were used, $D_{100° C}$ values obtained were significantly higher ($P \leq 0.01$) than the ones for either 2- or 16-month old spores. No significant differences ($P > 0.05$) were found between $D_{100° C}$ values for 2- and 16-month old spores. These findings suggest that *B. coagulans* spores increase their heat resistance with time up to a certain limit, after which time this resistance diminishes to a point equivalent to that found initially. With respect to raising incubation temperature to increase spore heat resistance, previous reports (5, 18) indicate that there is an optimum incubation temperature for producing spores of highest heat resistance, below or above which this resistance diminishes. This also appears to be the case with spore age for *B. coagulans*.

The exact mechanism or mechanisms responsible for the resistance of bacterial spores to wet heat are still not fully known. There is no single chemical or determinant that can explain both the increase in heat resistance of the spores vs. the vegetative cell and the large range in heat resistance of different species of spores. Instead, the spore is a complex structure whose properties, and in particular thermal resistance, depend on the integrity and necessary complementation of many structural components and chemical constituents (16). Stabilization of the spore core components to heat may result from a reordering of the labile components into a stabilized matrix, where solvation water has been supplanted by peptide cross-linkages, diaminopimetic acid or cations, or from water reduction resulting from mechanical pressure, osmotic pressure or syneresis (7). At this moment, we could only speculate about the expla-

TABLE 2.—*Decimal reduction times[1] of different-age* B. coagulans *and* B. macerans *spores heated in acid ripe plantain puree*

Bacteria	Spore age (months)		
	2	10	16
B. coagulans	8.4 a[2]	13.2 b	9.2 a*[3]
B. macerans	9.7 c	9.7 c	90.7 c*

[1]$D_{100° C}$ values in minutes. These values are the average of two runs for each bacterium at each spore age and performed on two different days.

[2]Means within a given row (same organism) having the same attached letter are not significantly different ($P > 0.05$).

[3]Means within a given column (spore age) having an attached asterisk are not significantly different ($P > 0.05$).

nation for the heat resistance behavior of *B. coagulans* spores observed in this study.

When the $D_{100° C}$ values for 16-month old spores of *B. coagulans and B. macerans* are compared no significant difference ($P>0.05$) is observed. However, with 10-month-old spores $D_{100° C}$ values for *B. coagulans* are significantly higher ($P\leq0.01$) than for *B. macerans*. The opposite is true for 2-month-old spores ($P\leq0.05$).

The spores of *B. coagulans* were described by Stumbo (17) as more heat resistant than the spores of *B. macerans*. It was reported earlier (5) that under certain conditions the spores of *B. macerans* could be significantly more heat resistant ($P\leq0.05$) than, or at least not significantly different ($P>0.05$) from those of *B. coagulans*. The present results confirm this finding, since 2-month old *B. macerans* spores were more heat resistant than those of *B. coagulans*, and 16-month old spores of both bacteria were equally resistant. Only with 10-month old spores was *B. coagulans* more heat resistant than *B. macerans*.

B. coagulans has been used in determining the adequacy of thermal processes in acid and acidified foods (12, 13). Since the spores of this organism are considered the most heat resistant of those capable of growing in acid foods, our findings could have some implications. First, if the thermal resistance of *B. coagulans* spores could be altered with age, then this may introduce an element or variability that should be considered when using the spores of this bacterium to determine the adequacy of a thermal process. Second, if the above holds true in all circumstances, then before spores of *B. coagulans* reach their peak in heat resistance with age, the spores of *B. macerans* could be more, or at least equally, resistant to heat. This may raise the question of whether *B. macerans* spores could or should be used in lieu of those of *B. coagulans* in determining the adequacy of thermal processes in acid and acidified foods.

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