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Effect of Phenol on *Bacillus subtilis* Spores at Elevated Temperatures

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ABSTRACT

RUSSELL, A. D. (Welsh College of Advanced Technology, Cardiff, Wales, Great Britain), AND MURIEL LOOSEMORE. Effect of phenol on *Bacillus subtilis* spores at elevated temperatures. Appl. Microbiol. **12**:403-406. 1964.—The nature of the recovery medium is shown to influence the number of *Bacillus subtilis* spores which, after exposure to 2.5 or 5% phenol at high temperatures, can produce a visible colony. Higher survivor counts were obtained in nutrient agar containing L-alanine and D-glucose than in plain nutrient agar.

Until recently, comparatively few investigations have been made into the sporicidal activity of phenol. Davies, Wyss, and Williams (1948) showed that a heat-resistant strain of *Bacillus globigii* survived longer in 5% (w/v) aqueous solution of phenol at room temperature than did a heat-sensitive strain. Reddish (1950) found that *B. cereus* spores exposed to an initial heat treatment at 80 C for 15 min did not survive subsequent treatment with 5% phenol for more than 1 to 3 hr, whereas spores which had not been initially heat-shocked remained viable after 3 to 5 days of exposure under the same conditions.

Berry, Jensen, and Siller (1938) investigated the effect of certain phenolic, and organic mercury, compounds on *B. subtilis* spores at elevated temperatures. Phenol itself was shown to be rapidly lethal under such conditions. However, in view of the recent finding (Loosemore and Russell, 1963; J. Appl. Bacteriol., *in press*) that survivor counts of phenol-treated *B. subtilis* spores were significantly higher in nutrient agar containing the germination stimulants, L-alanine and D-glucose, than in plain nutrient agar, we decided to study the recovery of spores subjected to phenol at elevated temperatures.

MATERIALS AND METHODS

Media. Nutrient agar and nutrient broth were prepared from the respective Oxoid granules (Oxoid Division of Oxo, Ltd., London, England.)

After sterilization by autoclaving, the pH of all media was 7.4.

Water. Water was obtained from an all-glass still.

Chemicals. Phenol was purchased from Hopkins and Williams, Ltd., Chadwell Heath, England; L-α-alanine and D(+) glucose were purchased from British Drug Houses, Ltd., London, England. All chemicals were of analytical reagent quality.

Spore suspensions. The organism was a laboratory strain of *B. subtilis*. A 48-hr culture of the organism at 37 C was washed from the surface of an agar plate with sterile water, and the suspension was centrifuged for 20 min at 2,000 rev/min. The pellet was washed twice with sterile water, and shaken with sterile glass beads to break up any clumps. The opacity was adjusted to give approximately 1.5×10^8 spores per ml, and the suspension was heat-shocked at 75 C for 20 min to destroy vegetative organisms.

Viable counts. Viable counts were carried out by serial dilution, followed by plating of 0.1-ml volumes into nutrient agar (containing 5 mM L-alanine and 10 mM D-glucose where necessary). In experiments employing phenol, care was taken to ensure that this substance was diluted well below its sporostatic concentration. Colonies were counted after incubation of plates at 37 C for 24 and 48 hr. An increased number of colonies was not obtained after additional incubation.

Sporicidal activity of phenol at elevated temperatures. A suspension of *B. subtilis* spores containing approximately 1.5×10^5 spores per ml, and a glass-stoppered tube containing 18 ml of phenol, were placed in a thermostatically controlled water bath at the desired temperature. When a temperature equilibrium had been attained, 2 ml of the spore suspension were added to the phenol (final concentration, 2.5 or 5%). Samples were removed at intervals, and surviving spores were determined by plating into nutrient agar or, where applicable, into agar containing L-alanine and D-glucose.

RESULTS

Viability of B. subtilis spores at elevated temperatures. Preliminary experiments in which spore suspensions (prepared with or without heat shock) were heated at various temperatures showed that (i) spores of this organism could withstand heating at temperatures up to 90 C for 30 min, and (ii) that spore suspensions subjected to an initial heating process to remove vegetative organisms were able to resist the same heat treatments as spore suspensions not previously heat-shocked.

Apparent sporicidal activity of 5% phenol at elevated temperatures. The results obtained when nutrient agar was employed as the recovery medium are shown for phenol-treated spores at 63 C (Table 1), 70 C (Table 2), and 80 C (Table 3). It appears that all spores were killed by 5% phenol within 55 to 65 min at 63 C, after approximately 30 min at 70 C, and within 5 min at 80 C.

Recovery of B. subtilis spores, after exposure to phenol at

elevated temperatures, by means of enriched recovery media. Previous investigations have demonstrated the fallacy of relying on nutrient agar for determining the numbers of spores surviving a damaging process (Curran and Evans, 1937; Schmidt, 1955; Loosemore and Russell, 1963; *in press*). Accordingly, further experiments were carried out in which, after treatment with 2.5 or 5% phenol at elevated temperatures, surviving spores were determined by serial dilution of samples in sterile water, followed by plating into nutrient agar or into agar containing 5 mM L-alanine and 10 mM D-glucose. The results are shown graphically in Fig. 1–5.

TABLE 1. *Apparent sporicidal activity of 5% phenol against Bacillus subtilis spores at 63 C*

Time	Expt no.		
	1	2	3
min			
0	100*	100	100
5	52.0	51.4	52.0
10	34.4	33.8	42.3
15	31.6	34.4	40.4
20	19.8	22.8	29.8
25	11.9	16.9	14.8
30	6.2	12.2	13.9
35	5.1	10.6	9.6
45	1.7	3.7	4.8
55	<1	1.6	3.8
65	<1	<1	<1

* Figures represent percentage of viable spores at 0 min. Viable counts were made in nutrient agar.

TABLE 2. *Apparent sporicidal activity of 5% phenol against Bacillus subtilis spores at 70 C*

Time	Expt no.		
	1	2	3
min			
0	100*	100	100
5	44.6	43.6	36.4
10	16.7	14.5	13.0
15	11.8	16.4	9.8
20	4.9	1.8	3.3
30	<1	3.6	1.9
40	<1	<1	<1

* Figures represent percentage of viable spores at 0 min. Viable counts were made in nutrient agar.

TABLE 3. *Apparent sporicidal activity of 5% phenol against Bacillus subtilis spores at 80 C*

Time	5% Phenol present		Phenol absent	
	Expt 1	Expt 2	Expt 1	Expt 2
min				
0	203*	203	203	204
5	0	0	202	202
10	0	0	—	—
15	0	0	204	190

* Figures represent colony counts in nutrient agar.

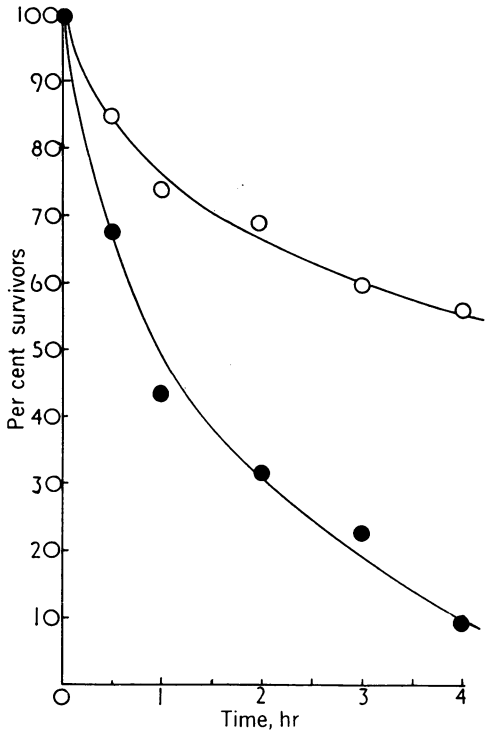

FIG. 1. *Effect of 2.5% phenol on Bacillus subtilis spores at 63 C. Colony counts in nutrient agar (●). Colony counts in agar containing 5 mM L-alanine and 10 mM D-glucose (○).*

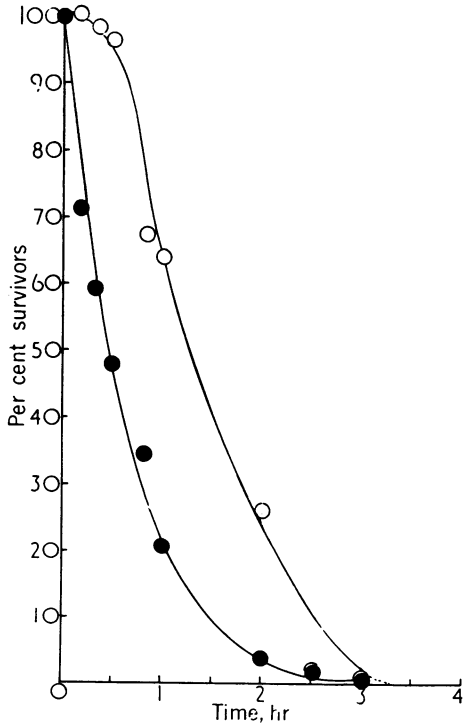

FIG. 2. *Effect of 2.5% phenol on Bacillus subtilis spores at 70 C. Colony counts in nutrient agar (●). Colony counts in agar containing 5 mM L-alanine and 10 mM D-glucose (○).*

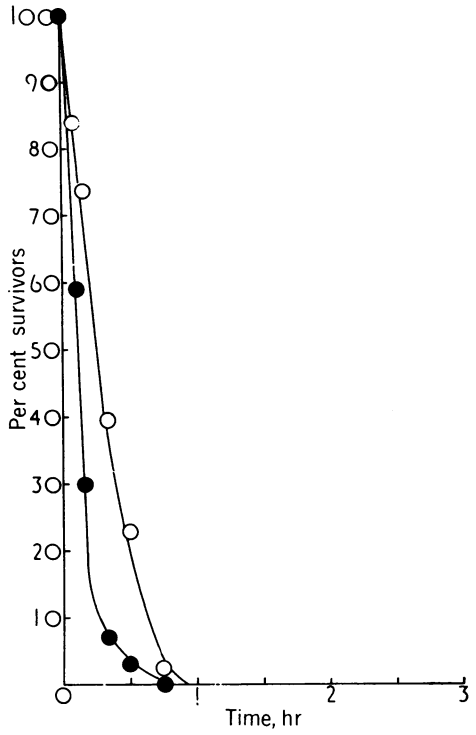

FIG. 3. *Effect of 2.5% phenol on Bacillus subtilis spores at 80 C. Colony counts in nutrient agar* (●). *Colony counts in agar containing 5 mM* L-*alanine and 10 mM* D-*glucose* (○).

DISCUSSION

Spores which are plated into a solid nutrient medium must germinate before the vegetative cells formed can multiply to produce a visible colony. It is conceivable that spores surviving a damaging treatment are more fastidious in their germination requirements than are untreated spores. This fact has been taken into account in determining a suitable medium for the recovery of *B. subtilis* spores treated with phenol at 37 C (Loosemore and Russell, 1963; *in press*). If nutrient agar is employed as the counting medium for assessing the number of spores which survive a phenol treatment, it may be assumed that 5% phenol has a lethal action within 65 min at 63 C (Table 1), after approximately 30 min at 70 C (Table 2), and within 5 min at 80 C (Table 3). Similarly, 2.5% phenol takes more than 4 hr at 63 C (Fig. 1), approximately 3 hr at 70 C (Fig. 2), and approximately 45 min at 80 C (Fig. 3) to bring about the same result.

The use of nutrient agar containing L-alanine and D-glucose as a recovery medium for phenol-treated spores increases the number of spores capable of forming colonies (Fig. 1–4). Where the treatment has been particularly harmful, however, such a medium has no special advantage over plain agar, e.g., for the recovery of *B. subtilis* spores exposed to 5% phenol at 70 C (Fig. 5) or 80 C; in the latter case, no colony counts in either medium could be

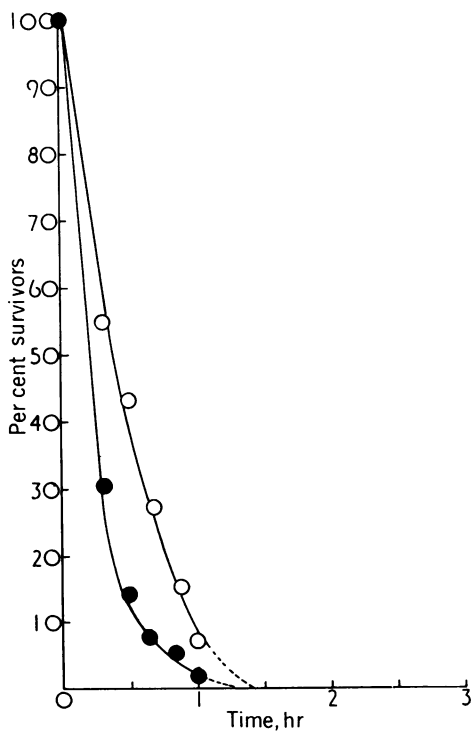

FIG. 4. *Effect of 5% phenol on Bacillus subtilis spores at 63 C. Colony counts in nutrient agar* (●). *Colony counts in agar containing 5 mM* L-*alanine and 10 mM* D-*glucose* (○).

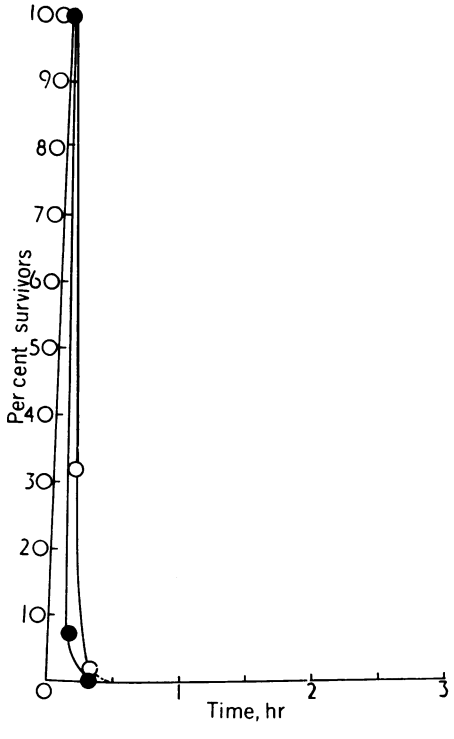

FIG. 5. *Effect of 5% phenol on Bacillus subtilis spores at 70 C. Colony counts in nutrient agar* (●). *Colony counts in agar containing 5 mM* L-*alanine and 10 mM* D-*glucose* (○).

obtained after a brief treatment of the spores with phenol. It may thus be assumed that this is owing to an irreversibly harmful effect of phenol on *B. subtilis* spores.

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