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Letters to the Editors

The Lysozyme Susceptibility of *Bacillus cereus* No. 2 and Its Relation to the Phase of Growth

Salton (1957) showed that *B. cereus* is resistant to lysozyme and thus no protoplasts are obtained by treating the cells with this particular enzyme. Recently Repaske (1956) described a method for preparing protoplasts from some gram negative bacteria using a combination of lysozyme and EDTA in a medium maintained at an alkaline pH by tris buffer. By applying the above method modified by use of polyethylene glycol (Carbowax m.w.: 4,000) as a hypertonic agent, we have succeeded to obtain protoplast from *B. cereus* No. 2. During a study of sporulation of this organism, we found that, neither the mature spores, nor germinating spores are susceptible to lysozyme. It was also found that protoplasts can be prepared from the cells both during their exponential and declining growth phase but that no protoplast is obtainable from cells forming endospores or during the lag phase.

Stock strains of *B. cereus* No. 2 were used. Spores were harvested from a nutrient agar culture after 7 days incubation at 35°C and washed thrice in cold distilled water. They were stored after drying *in vacuo* over P_2O_5. Suspensions of the dried spores were heat-shocked at 65°C for 15 minutes just before inoculation. CYG medium composed of 0.5 per cent casein hydrolysate, 0.5 per cent yeast extract, and 0.5 per cent glucose was employed at pH 7.0. Spore suspensions equivalent to 50 mg. dried weight were inoculated into 100 ml. of the medium in a 500 ml. flask and incubated at 35°C with shaking at 120 strokes per minutes. For studying the lysozyme susceptibility of the spores during the germinating process, the spore suspensions were incubated at 25°C with shaking so that the process could be analysed precisely by lowering the rate of growth.

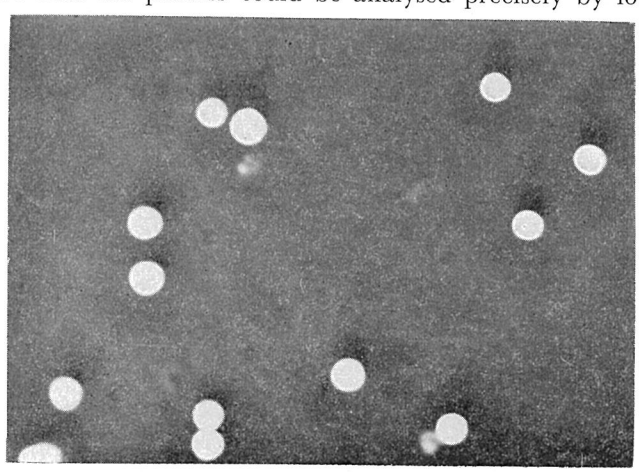

Fig. 1 Protoplasts of *Bacillus cereus* No. 2 Observed by the phase contrast microscope, ×1375

For preparation of protoplasts, vegetative cells of *B. cereus* No. 2 harvested from a 4 hour culture were usually employed. The harvested cell paste was washed twice with cold distilled water and resuspended in tris buffer of pH 7.8 containing 10 per cent Carbowax and 0.004 M $MgCl_2$. EDTA and lysozyme were then added to the buffer at final concentrations of 0.005 per cent and 0.05 per cent

respectively. The suspension was incubated at 35°C. Under these conditions, protoplast formation proceeded rather slowly and was complete after 30 minutes. The protoplasts thus obtained are shown in Fig. 1.

Since the method was found reproducible for preparation of protoplast from cells of *B. cereus* No. 2 cultured for 4 hours, lysozyme susceptibility of the cells at various stages of growth, including germination, was studied under the conditions mentioned above. Table 1 shows the varying susceptibility to lysozme of the cells and spores harvested at various stages of growth. Both free spores and germinating spores were entirely insensitive to lysozyme, whereas vegetative cells are susceptible to the enzyme and are either completely lysed or protoplasts are formed. Once endospores are formed, the vegetative cells tend to be easily lysed by this treatment. Whether this lysis is due to lysozyme treatment or to autolysis is not yet clear.

The fact that the vegetative cells harvested in early lag phase can be completely lysed without any formation of protoplasts suggests that formation of the cytoplasmic membrane of the cell may still be incomplete.

Table 1. Lysozyme susceptibility of the cells in various growing stages

Growing stage	Incubation Time	Temperature	Lysozyme Subsceptibility	Protoplast Formation
Germinating stage	0—30 min.		(—)	(—)
		25°C		
Lag phase	30—90 ,,		(+)	(—)
Logarithmic phase	1.5—4 hrs.		(+)	(+)
Phase of declining growth rate	4— 8 ,,		(+)	(+)
Stationary phase	8—18 ,,	35°C	(+)	(+)
Shortly before sporulation	20—22 ,,		(+)	(—)
Spore	24 ,,		(—)	(—)

Condition; Harvested cell paste from various stages culture was washed twice with distilled water in the cold, and suspended in a tris buffer, pH 7.8, containing 10 per cent Carbowax and 0.004 M $MgCl_2$, EDTA and lysozyme were then added to make a final concentration of 0.005 per cent and 0.05 per cent respectively to this hypertonic suspension, and incubated at 30°C. Under these conditions, lysozyme susceptibility and protoplast forming ability of the cells in various stages were determined by the phase contrast microscopic observation.

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