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GROWTH OF *THIOBACILLUS THIOOXIDANS* IN SHAKEN CULTURE[1]

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ABSTRACT

COOK, THOMAS M. (Rutgers, The State University, New Brunswick, N.J.). Growth of *Thiobacillus thiooxidans* in shaken culture. J. Bacteriol. 88:620-623. 1964.—When flasks of sulfur-containing medium were inoculated with *Thiobacillus thiooxidans* and placed on a shaking machine immediately after inoculation, growth was markedly inhibited. If various wetting agents were provided, or if the ratio of inoculum to sulfur was increased, growth under shaking conditions was possible. Evidence cited shows that the natural wetting agent (or other externally supplied surfactants) is necessary to effect a bacteria-sulfur contact which is stable to shaking conditions.

Earlier studies on the growth of the chemoautotrophic bacterium, *Thiobacillus thiooxidans*, generally employed stationary cultures (Waksman and Joffe, 1922; Waksman and Starkey, 1923; Vogler and Umbreit, 1941). It was further originally claimed that sulfur had to be in direct contact with the bacteria before sulfur oxidation could occur (Vogler and Umbreit, 1941), and that shaking disturbed the contact and resulted in decreased sulfur oxidation. However, subsequent studies showed that growth on shaking machines was not only possible but also markedly enhanced growth (Newburgh, 1954; Starkey, Jones, and Frederick, 1956). To resolve these differences of opinion, we restudied the effect of sulfur-bacterial contact and shaking in some detail, and we believe that we have found the reason why some investigators find shaking very useful while others find it inhibitory.

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MATERIALS AND METHODS

The culture of *T. thiooxidans* used was originally obtained from R. L. Starkey, Department of Agricultural Microbiology, Rutgers, The State University, New Brunswick, N.J. This culture was maintained on the medium of Vogler and Umbreit (1941), which had the following composition: NH_4Cl, 0.3 g; KH_2PO_4, 3.0 g; $CaCl_2$, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4$, 0.01 g; distilled water, 1,000 ml (initial pH 4.5). Elemental sulfur, sublimed (Merck, Sharp and Dohme & Co., Inc., Rahway, N. J.), was included as the energy source.

The following standard conditions were adopted for the present growth experiments. Quantities of 100 ml of the basal medium were dispensed in 250-ml Erlenmeyer flasks; sulfur was weighed out and added before sterilization by intermittent steaming. Additions of sterile supplements were then made as desired, and the flasks were inoculated. The standard inoculum was 1.0 ml of a twice-washed cell suspension from an 8-day-old culture (from which the sulfur had been removed by repeated centrifugation at low speeds) adjusted to an optical density (600 mμ) of 0.400. This inoculum contained approximately 95 μg of protein, as determined by using the Folin-Ciocalteau reagent (Lowry et al., 1951), and contained approximately 10^9 cells, as determined by microscopic count. Flasks were incubated at room temperature (23 C). Except where otherwise specified, shaken flasks were shaken at 240 rev/min on a rotary shaker describing a circle 3 cm in diameter. Titratable acidity, expressed as normality of acid present, was followed as an index of growth of the culture. All treatments were run in duplicate.

Radioactivity of samples dried in aluminum planchets was measured with a gas-flow scaler (Compumatic V, Tracerlab Inc., Waltham, Mass.).

The following materials were purchased from

Nutritional Biochemicals Corp., Cleveland, Ohio: glutamic acid hydrochloride, glutathione (reduced), Tween 80, inositol phosphatide, α-lecithin (synthetic), α-cephalin (synthetic), and vegetable lecithin (purified).

RESULTS

With the standard growth conditions described, flasks receiving 1.0 g of sulfur with a low inoculum showed that shaking immediately after inoculation was distinctly unfavorable, but, if the flasks were given a preliminary 3-day stationary incubation, then shaking was definitely stimulatory. The effect depended upon the amount of sulfur supplied (Fig. 1). In stationary cultures, the growth was proportional to the amount of sulfur added up to approximately 0.5 g. With cultures shaken after 3 days of stationary incubation, growth was also correlated with the amount of sulfur added. At any given level of sulfur, the acid formed was considerably higher in the shaken flasks, reflecting a faster growth rate; but flasks shaken immediately after inoculation showed a quite different response to increasing levels of sulfur. Up to 0.5 g of sulfur, the growth response was similar to that of cultures given an initial stationary incubation before shaking. Beyond this point, higher levels of sulfur became distinctly inhibitory. Complete suppression of growth occurred with 2.0 g or more of sulfur.

It was found also that increasing the sulfur to 10 and 25 g per flask had no inhibitory effect on stationary cultures, but was clearly inhibitory to cultures shaken after 3 days of stationary incubation.

By holding the sulfur constant at 1.0 g, it was found that something on the order of 10 to 50 times as much inoculum was required to obtain growth when the flasks were shaken immediately as when similar flasks were held for 3 days in the stationary condition before shaking (Fig. 2). Since the addition of 0.0005% Tween 80 markedly reduced the inoculum requirement and brought both the flasks shaken immediately and those stationary for 3 days to a comparable level, it appeared that the difference in inoculum requirement was somehow related to a wetting agent. Starkey et al. (1956) had reported Tween 80 to be stimulatory in shaken cultures, but results were variable until it was realized that its stimulatory effect depended upon the relation between

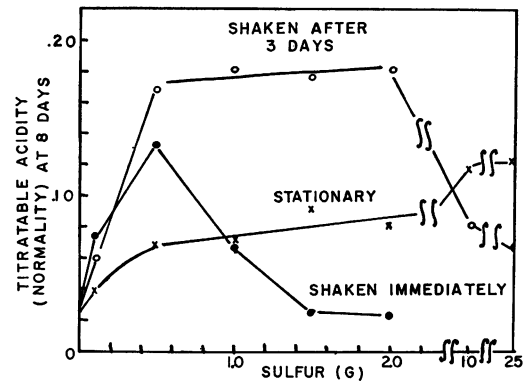

FIG. 1. *Effect of sulfur level on the growth of Thiobacillus thiooxidans in shaken and stationary cultures.*

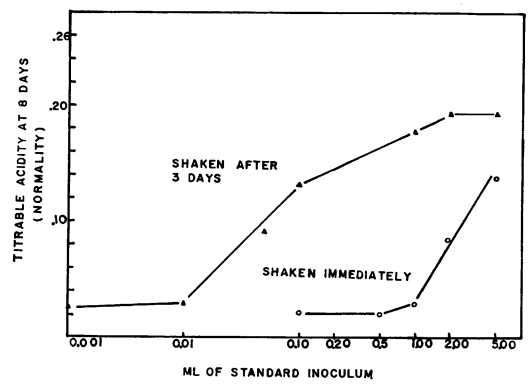

FIG. 2. *Effect of inoculum size on growth of Thiobacillus thiooxidans in shaken and stationary cultures with 2 g of sulfur.*

size of inoculum, amount of sulfur, and whether shaking was immediate or not.

It then was found that, under conditions of high sulfur content and low inoculum, inhibition of sulfur oxidation by shaking was apparently a matter of initial dispersion rather than continuous disturbance of the sulfur-bacterial contact. As little as 30 min of shaking immediately after inoculation with static incubation for 3 days before continuous shaking caused complete inhibition of growth for as long as 11 days. So long as the sulfur content is below 0.4 g per flask (with the standard inoculum employed), continuous shaking from the start of the experiment does not cause inhibition, but if 2 g of sulfur are supplied, inhibition is complete for a period of 8 to 10 days (Fig. 1). If 0.1 g of sulfur is inocu-

TABLE 1. *Restoration of growth of Thiobacillus thiooxidans in cultures inhibited by shaking immediately after inoculation**

Expt	Addition	Final concn (w/v)	Growth (as acid-produced)
		%	N
1	None	—	0.042
	Thiobacillus crude lipid	0.001	0.175
	α-Lecithin	0.001	0.157
	α-Cephalin	0.001	0.104
	Gelatin	0.001	0.194
	L-Glutamic acid·HCl	1.5	0.093
2	None	—	0
	Tween 80	0.0001	0.341
	Inositol phosphatide	0.001	0.318
	Vegetable lecithin	0.001	0.137

* Incubation period was 7 days for experiment 1 and 11 days for experiment 2.

lated with the standard inoculum and shaken immediately, with 2 g of sterile sulfur added at intervals thereafter, the addition of the extra sulfur results in inhibition when added at any time before 18 to 24 hr. But, after this time, no inhibition is observed. It seemed reasonable to suppose that the organism growing on the small amount of sulfur produced something which now enabled it to handle much larger quantities. Such a material could be demonstrated in "spent" medium, providing that medium had supported growth for an adequate period. However, the experiments showed that there was a more profound alteration in the sulfur rather than in the medium as such.

For example, the sulfur which had been removed from a 3-day growing culture was rinsed briefly with distilled water while on the filter and added to fresh basal medium without any additional inoculum or sulfur. These flasks showed growth when placed on the shaker immediately. Therefore, it was concluded that the changes occurring during the 3-day stationary incubation primarily involve the sulfur and its associated population of cells, rather than the medium.

Sulfur was removed from a 3-week-old culture by low-speed centrifugation, and the spent medium containing the free cells was discarded. The sulfur was resuspended in distilled water and again centrifuged at low speed (500 × *g* for 5 min). This washing procedure was repeated for a total of 12 times. The resulting well-washed sulfur was found to serve as an efficient inoculum when a small portion was added to fresh medium plus 2.0 g of sulfur and the flasks were shaken immediately.

Jones and Starkey (1961) reported the excretion of a sulfur "wetting agent" during growth of this organism on sulfur, and part of this material was identified by chromatographic methods as phosphatidyl inositol by Schaeffer and Umbreit (1963). It appeared reasonable to suppose that this material might affect the sulfur-bacteria contact and be involved in the inhibition due to immediate shaking when the sulfur level was high. Accordingly, the crude lipid fraction from *T. thiooxidans* culture was recovered by ether extraction of 6 liters of 2-week-old culture after removal of sulfur. This material was taken up in methanol and added to standard flasks containing 2 g of sulfur. Controls received an equal volume of methanol alone. It was found that the crude lipid material at a final concentration of 0.001% (but not 0.0001%) would allow growth in flasks shaken immediately after inoculation. There was little effect of crude lipid on growth in flasks given an initial 3-day stationary incubation before shaking. The same results were obtained when inositol phosphatide was used at 0.001% instead of crude lipid. It was found also that several phospholipids, including inositol phosphatide, α-lecithin (synthetic), vegetable lecithin, and α-cephalin (synthetic), would replace *Thiobacillus* lipid. Tween 80 and gelatin also proved to be effective. Glutamic acid was slightly active, but only at very high levels. Reduced glutathione, glucose, and glycerol were not active. These results are summarized in Table 1.

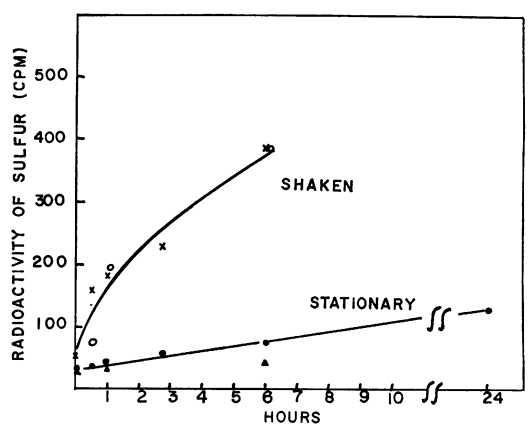

FIG. 3. *Changes in the radioactivity associated with the sulfur fraction during incubation with C^{14}-labeled cells of Thiobacillus thiooxidans.*

It appeared that the active agents were concerned only with the initiation of growth. Once growth was established, the maximal rate of growth was not significantly increased by addition of Tween 80, phospholipid, or the other surface-active agents.

Previous information has been compatible with the concept that the bacteria are attached firmly to sulfur, especially in the presence of wetting agents, and once this contact is established it is not destroyed by shaking. However, there is no direct evidence of such attachment. Therefore, an attempt was made to demonstrate it. A radioactive inoculum was prepared by exposure of an 8-day culture to $C^{14}O_2$ for 30 min prior to harvesting. The washed inoculum prepared from this culture was diluted to obtain 2,000 counts per min per ml. A 1.0-ml sample of this suspension was added to flasks containing 0.1 g of sulfur, and incubation was made at room temperature, with and without shaking. At various intervals, the sulfur was filtered off through coarse filter paper and vigorously washed with distilled water on the filter. After drying, the sulfur was transferred to planchets for counting of radioactivity.

During incubation there was a progressive increase in radioactivity associated with the sulfur fraction (Fig. 3). Shaking greatly accelerated this rate of increase. The increase in radioactivity associated with the sulfur fraction was taken to indicate attachment of radioactive cells to the sulfur particles.

DISCUSSION

These experiments establish two significant points concerning the growth of *T. thiooxidans* in shaken cultures. First, in cultures shaken immediately after inoculation with a given amount of inoculum, growth is related to the amount of sulfur added up to a critical point, beyond which additional sulfur becomes inhibitory. Inhibition by high levels of sulfur occurs only in shaken, not in stationary, cultures. Shaking for as little as 30 min is sufficient to cause the inhibition. Second, growth can be restored to such sulfur-inhibited cultures by increasing the inoculum size; or by allowing a preliminary stationary incubation before shaking; or by the addition of phospholipids, Tween 80, or other surface-active materials.

It therefore seems probable that the attachment to the sulfur particle is dependent upon a wetting agent. This wetting agent is formed by the organism itself or it may be added externally. When there is too much sulfur to be coated to a sufficient depth by the wetting agent provided in the inoculum, shaking, even for brief intervals, causes too wide a dispersion of the wetting agent, and growth is inhibited and may be stopped completely. We, as well as Jones and Starkey (1961), can find such a wetting agent in spent media and on the sulfur particles themselves. It appears that a certain level of the material is necessary before adequate sulfur-bacteria contact can be maintained while shaking the cultures.

ACKNOWLEDGMENTS

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