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Determination of Growth of *Sphaerotilus discophorus* in the Presence of Manganese

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Manganese interferes strongly with most chemical methods of biomass determination. However, the biomass of manganese-encrusted *Sphaerotilus discophorus* can be determined after removal of the MnO_2 with trichloroacetic acid and oxalic acid. Evidence which indicates that manganese inhibits the heterotrophic growth of *S. discophorus* and that the cells only oxidize manganese late in the growth curve is presented.

Since their discovery, the question of autotrophy of iron- and manganese-oxidizing sheathed bacteria has been controversial (3, 8, 9). The controversial nature is due in part to the lack of an adequate determination of cell growth in the presence of large accumulations of iron and manganese oxides (3). Recently, Ali and Stokes (1) have claimed to observe autotrophic growth of *Sphaerotilus discophorus*. This claim has been questioned by van Veen (13) who argues that the growth obtained seemed high relative to the theoretical amount of energy (manganese) input.

S. discophorus is a particularly good organism to use for these studies since it readily oxidizes manganous ions, which compared to ferrous ions are relatively stable at physiological pH's. However, under alkaline conditions ferrous ions will readily oxidize.

In shaking liquid culture, *S. discophorus* grows as visible clumps with some growth adhering to the walls of the vessel in an otherwise clear broth. Most common bacteriological methods of measuring cell growth (plate counts, turbidity) are inaccurate with *S. discophorus* cultures. Therefore, this paper describes a simple method devised to treat a portion of culture so that all manganese (as MnO_2) is removed yet all the original organic material remains and may then subsequently be determined. This provides a chemical method of biomass determination; recently *S. discophorus* biomass has been estimated by [^{3}H]alanine incorporation (11). We have also tested the methods used by Ali and Stokes (1) and have found them to be greatly affected by the presence of manganese. We also present data that demonstrate that manganese oxidation is not concomitant with heterotrophic growth but occurs late in the growth cycle.

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MATERIALS AND METHODS

Strain. A rough, i.e., filamentous, colony-forming strain, designated *S. discophorus* 35R was the gift of J. L. Stokes.

Culture. The growth medium consisted of: peptone, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.02%; $CaCl_2 \cdot 2H_2O$, 0.005%; thiamine, 0.2 μg/ml; and biotin, 0.02 μg/ml. When manganese was included in the medium it was used as $MnSO_4$. Stock cultures were maintained on a solid medium having the above constituents plus agar, 1.5%; $MnSO_4$ (anhydrous), 0.005%; and ferric ammonium citrate, 0.05%. Glass-distilled water was used to make all media which were adjusted to pH 7.2 prior to autoclaving. This is a modification of the medium of Rouf and Stokes (12).

Manganese determination. Manganese as either manganese ion or insoluble MnO_2 was measured by the formaldoxime method of Morgan and Stumm (8). Mixing experiments showed that the presence of whole cells did not affect the results as they completely dissolved in the reagents (within 1 h) and did not affect the color value of standard manganese solutions. Anhydrous $MnCl_2$ was used as a standard.

DNA determination. Cellular deoxyribonucleic acid (DNA) was determined by the diphenylamine method (5). The DNA standard was DNA (Difco) that required solubilization in hot 40% trichloroacetic acid filtration (pore size, 0.45 μm; Millipore Corp.) to remove impurities. The actual concentration of the standard DNA solution was calculated from its absorbance at 260 nm.

Protein and nitrogen determinations. Nitrogen was determined by direct Nesslerization of Kjeldhal digests (2) using ammonium chloride as a standard. Cell protein was determined with the biuret reagent (4) using bovine serum albumin as a standard. The Lowry method (7) cannot be used because extremely small amounts of manganese as either MnO_2 (data not shown) or as Mn^{2+} strongly interfere with the color reaction (see below).

Growth studies. In liquid culture, with aeration,

the rough forms of *S. discophorus* grow as clumps free and attached to the walls of the culture vessel. Consequently equal portions from one large culture cannot be obtained. Rather, the growth studies must necessarily be done in multiple flasks each of which is inoculated with identical portions of an inoculum that has been aseptically washed and homogenized with sterile distilled water. From these multiple cultures, the growth was quantitatively removed with the aid of a rubber policeman and several washes of distilled water. For convenience we used cultures of 10 ml in 50-ml Erlenmeyer flasks. At each time interval two flasks were removed, and the growth in each was determined; replication between flasks was always within 10%. Inoculum volumes were either 0.1 or 0.5 ml.

Removal of manganese from cells. Cells from cultures were quantitatively rinsed into centrifuge tubes with the aid of a rubber policeman and sedimented by centrifugation at 10,000 rpm for 10 min at 4°C. They were washed with ice-cold distilled water two times. Under these conditions there is no lysis (J. C. Makemson, Ph.D. thesis, Washington State Univ., Pullman, 1970). The resultant cell pellet was resuspended in a small volume (usually 1 ml) of ice-cold distilled water to which 1 volume of ice-cold 40% trichloroacetic acid and 2 volumes of ice-cold 1.5 N oxalic acid were added. Incubation in ice for 10 min results in 98 to 99% solubilization of cellular accumulations of MnO_2. The cells were sedimented again and washed two more times with the same combination of trichloroacetic and oxalic acids. Then the cell pellet was suspended in distilled water with glass homogenization for determination of organic biomass (protein, DNA).

RESULTS AND DISCUSSION

Quantitative determinations. The effects of manganese on the diphenylamine reaction, Nesslerization, Lowry protein determination, and the biuret reaction are significant (Table 1). The Lowry determination and Nesslerization should be avoided, as they are affected by very small amounts of manganese; the biuret and diphenylamine reactions are not so severely affected and, therefore, were used as the more reliable measures of biomass and should preclude arguments that trace amounts of manganese may remain in the cells no matter how rigorously they were washed free of manganese.

In the cell washing procedure to remove manganese, cold H_2SO_4 at the same concentration as trichloroacetic acid was found to require a seven-times-longer incubation period to achieve the same degree of manganese removal. Therefore, trichloroacetic acid was retained as the organic (macromolecule) precipitant. To test the reliability of the method in retaining a soluble protein, a portion of soluble bovine serum albumin was treated with the combination of trichloroacetic and oxalic acids through three washes and incubations (Table 2), and virtually none was lost to the washings. Further, the procedure was tested with cells of *S. discophorus* that were grown in the growth medium without added iron or manganese. Cells at mid-logarithmic growth were harvested and washed. The cell pellet was resus-

TABLE 1. *Effect of manganese on biomass determinations*

Method	Absorbance[a]	% Error[b]
Diphenylamine (DNA)		
0.70 μg of DNA standard	0.54	–
0.70 μg of DNA standard + 10 μmol of Mn^{2+}	0.46	8.0 (–)[d]
Nesslerization (NH_4^+)[c]		
0.2 μmol of NH_4^+	0.11	–
0.2 μmol of NH_4^+ + 0.2 μmol of Mn^{2+}	0.29	163 (+)
Lowry (protein)		
50 μg of BSA[e]	0.242	–
50 μg of BSA + 0.2 μmol of Mn^{2+}	0.365	40 (+)
50 μg of BSA + 1.0 μmol of Mn^{2+}	0.96	380 (+)
Biuret (protein)		
0.6 mg of BSA standard	0.370	–
0.6 mg of BSA standard + 6 μmol of Mn^{2+}	0.461	28 (+)

[a] Absorbance at 600 nm (diphenylamine reaction), 490 nm (Nesslerization), 660 nm (Lowry), and 540 nm (biuret).

[b] Percent error is the absorbance with manganese less that without divided by the absorbance without manganese times 100.

[c] Assay performed in the presence of Kjeldahl digestion mixture.

[d] Symbols: +, Increases color value; –, decreases color value.

[e] BSA, Bovine serum albumin.

TABLE 2. *Effect of two tricholoracetic acid-oxalic acid washes on solubilized BSA as measured by the biuret reaction*[a]

Amt of BSA	Treatment	Abosrbance (540 nm)
6.0 mg	None	0.372
	Trichloroacetic acid + oxalic acid[b]	0.375
	Oxalic + trichloroacetic acid[c]	0.371
4.8 mg	None	0.300
	Trichloroacetic acid + oxalic acid	0.310
	Oxalic + trichloroacetic acid	0.305

[a] BSA, Bovine serum albumin.

[b] Treated as described in Materials and Methods.

[c] Treated as in footnote *b* but by adding oxalic acid before trichloroacetic acid.

pended in distilled water with the aid of a small glass homogenizer. To half of the suspension 100 mg of MnO_2 was added. The MnO_2-containing suspension was then treated with three washes and incubations with trichloroacetic and oxalic acids. Manganese was not detected in either sample. The protein values obtained from portions were almost identical (Fig. 1).

Preliminary experiments showed that peptone at concentrations manyfold greater than that used in the growth medium and concentrations of cells grown without manganese when added with standard manganese did not affect the Mn^{2+} color value in the formaldoxime method. We, therefore, tested the recovery of manganese from cultures grown in the presence of manganese in which part of the manganese was oxidized to insoluble MnO_2. Table 3 shows that all but 3.9% of the total manganese could be accounted for, and insignificant fractions of the manganese added remained on the culture flask walls or in the distilled water washings of the cells.

The manganese determination does not take into account the oxidation state of manganese (8). Manganous ions adsorb to MnO_2 precipitates (W. L. Van Veen, personal communication), and the amount of manganese determined on the cells represents both MnO_2 and Mn^{2+} adsorbed.

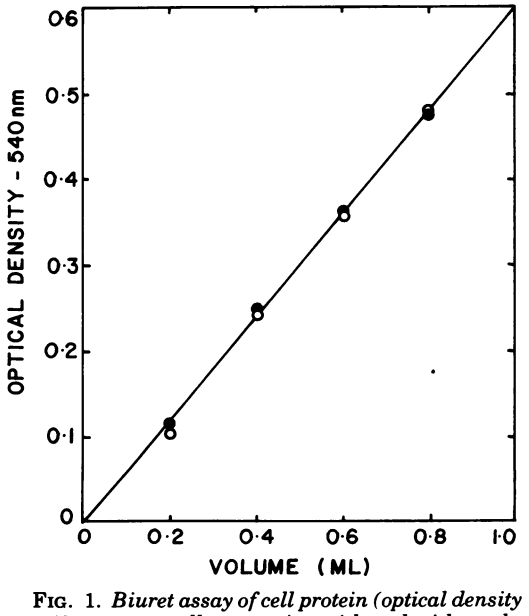

FIG. 1. *Biuret assay of cell protein (optical density at 540 nm) on a cell suspension with and without the presence of added MnO_2. Symbols: Untreated cell suspension (○); cell suspension treated with 100 mg of MnO_2 and then subjected to three trichloroacetic acid-oxalic washes (●).*

Test washings using the Hurlbert method (1) on manganese-encrusted cells left in all cases greater than 90% of the encrusted manganese in the cell fraction. This suggests that most cell preparations made by Ali and Stokes contained significant manganese contamination; their values for protein (biuret) and DNA are probably over- and underestimated, respectively.

Growth studies. The effect of manganese on the heterotrophic growth of *S. discophorus* is shown in Fig. 2. Manganese lowered the growth rate and the overall yield. Cells growing either with or without manganese contained nearly identical DNA/protein ratios. Consequently, in most work cellular protein is taken as a measure of growth. The addition of 0.1% glucose stimulated manganese oxidation and accumulation (Fig. 3). However, increasing

TABLE 3. *Recovery of manganese from an S. discophorus culture encrusted with MnO_2*

Fraction	Mn (μmol)	% Total
Cells[a]	2.42	38.5
Distilled-water washes of cells	0.04	0.6
Spent medium	3.60	57.5
Sides of the flask[b]	0.03	0.5

[a] Cells were washed twice with ice-cold distilled water by centrifugation at 10,000 rpm for 10 min each at 4°C.

[b] Sides of flask were rinsed with 1.5 N oxalic acid at room temperature for 10 min.

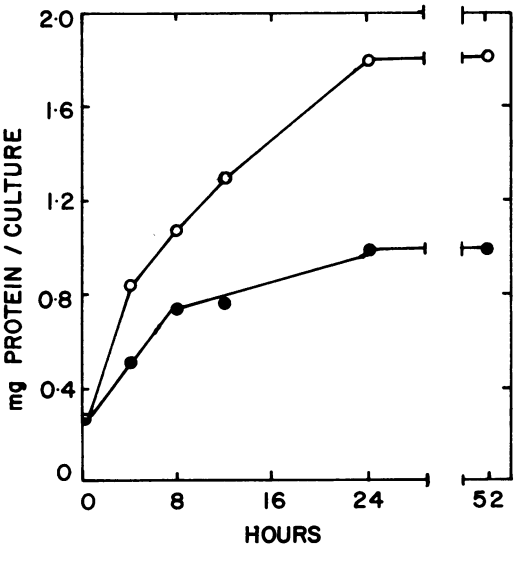

FIG. 2. *Effect of manganese on the heterotrophic growth of S. discophorus. Symbols: Cultures with 0.005% $MnSO_4$ (●); cultures with no added manganese (○).*

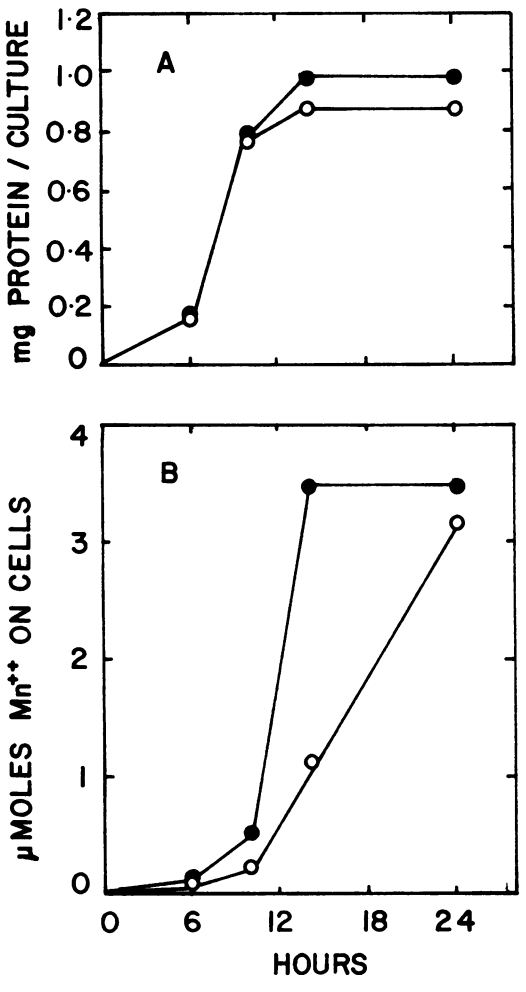

FIG. 3. *Effect of glucose on growth and manganese oxidation of S. discophorus in the peptone medium containing 6.3 μmol of manganese per culture. (A) Growth. (B) Manganese oxidation. Symbols: Cultures with 0.1% glucose (●); cultures without added glucose* (○).

the glucose concentration to 0.5% did not increase the onset or extent of manganese oxidation. In this and all other cases, manganese oxidation occurred only during the latter portion of logarithmic growth and into the stationary phase. Thus, manganese oxidation is similar to iron oxidation which, when measured by ^{59}Fe incorporation, occurred late in the growth curve (11). All these results (those presented here and elsewhere) must be viewed with some caution, since minor fluctuations in pH and chemical changes in the medium were not monitored, all of which could affect manganese oxidation and accumulation. Moreover, there may be an induction lag, since some data suggest manganese oxidation to be inducible (1, 5) or that manganese oxidation is other than a first-order process.

These data indicate that manganese in peptone medium does not stimulate growth. However, Ali and Stokes (1) obtained growth stimulation of *S. discophorus* 36R by manganese (0.01 to 0.05%) in a Casamino Acids-mineral salts medium. Manganese oxidation under these conditions of growth probably does not serve as an energy source, but since the organism does oxidize and accumulate MnO_2, its biooxidation must have some other significance. Using the methods developed here and by others, studies may be carried out under nutritional regimes that approximate natural conditions; then the real significance and effect of manganese oxidation by *S. discophorus* may become apparent.

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