APPLIED MICROBIOLOGY, Nov., 1966
Vol. 14, No. 6

Anaerobic Production of *Thiobacillus denitrificans* for the Enzyme Rhodanese

K. SARGEANT, P. W. BUCK, J. W. S. FORD, AND R. G. YEO
Microbiological Research Establishment, Porton, Wiltshire, United Kingdom

Received for publication 7 July 1966

ABSTRACT

SARGEANT, K. (Microbiological Research Establishment, Porton, Wiltshire, U.K.), P. W. BUCK, J. W. S. FORD, AND R. G. YEO. Anaerobic production of *Thiobacillus denitrificans* for the enzyme rhodanese. Appl. Microbiol. **14**:998–1003. 1966. —A method for the anaerobic growth of *Thiobacillus denitrificans* in a 140-liter (total capacity) stainless-steel culture vessel is described. As a result of controlling the *p*H value of cultures, and of ensuring that certain essential nutrients were in excess, cell yields approaching 700 mg (dry weight) per liter were obtained. These were over threefold higher than the best yields hitherto reported. The average rhodanese content of the cells from four cultures was 176,000 units per gram (dry weight). Adenosine-5′-phosphosulfate reductase (average content, 238 units per gram dry weight) and adenylate kinase (average content, 15,300 units per gram, dry weight) were also present.

Earlier methods of cultivating *Thiobacillus denitrificans* under anaerobic conditions were based on use of the medium of Baalsrud and Baalsrud (2), which contains: NH_4Cl, 0.05%; $Mg_2Cl_2.6H_2O$, 0.05%, $Na_2S_2O_3.5H_2O$, 1.0%; KNO_3, 0.4%; KH_2PO_4, 0.5%; $NaHCO_3$, 0.2%; $FeSO_4$, 0.001%; in tap water. Energy is derived from the oxidation of thiosulfate to sulfate or to intermediate products, and, under anaerobic conditions, nitrate, which is reduced to nitrogen, is the essential terminal respiratory electron acceptor.

Baalsrud and Baalsrud (3) showed that the $Na_2S_2O_3 \cdot 5H_2O$-KNO_3 ratio had to be at least 5:2 by weight, so that the excess of thiosulfate could prevent the accumulation of nitrite. They showed that nitrite is toxic to the nitrate-reducing enzyme system, thus inhibiting the growth of the organism.

The essential nitrogen source is ammonium ion, and the organism is an obligate chemoautotroph which uses carbon dioxide as its source of carbon.

Baalsrud and Baalsrud did not give values for the cell yield in terms of dry weight per liter of culture, but Trudinger (9), using the same medium, with 0.002% $FeSO_4$ instead of 0.001%, obtained up to 200 mg (dry weight) per liter in cultures grown in 5-liter conical flasks. Aubert, Millet, and Milhaud (1) used a medium based on that of Baalsrud and Baalsrud (2), but the medium was adjusted to *p*H 7.0 to 7.2 and had other minor changes. They obtained about 170 mg per liter of acetone-dried cells from 150-liter cultures.

Recently, Bowen, Butler, and Happold (4) reported cell yields of only 1 g (wet weight) per 9 liters from cultures grown on a modified Baalsrud medium in 10-liter flasks. This is equivalent to about 30 mg (dry weight) per liter.

The earlier workers did not report on the rhodanese content of their cells, but Bowen, Butler, and Happold (*personal communication*) found about 70,000 units of rhodanese per gram (dry weight). The purpose of the present study was to discover a reliable method for producing cells with a high rhodanese content.

MATERIALS AND METHODS

Temperature. All cultures were grown at 30 C.

Determinations. Sodium thiosulfate was determined by treating samples of centrifuged culture supernatant fluid with an excess of standard iodine-potassium iodide solution and titrating the excess iodine against standard sodium thiosulfate solution.

Ammonium ion was liberated as ammonia by distillation in the presence of excess sodium hydroxide. The ammonia was collected in excess boric acid and titrated against standard hydrochloric acid.

Dissolved carbon dioxide was determined by measuring the gas evolved when a sample of centrifuged culture supernatant fluid was acidified with citric acid in a Warburg apparatus.

Bacterial dry weights were determined by heating a sample of wet cells at 105 C to constant weight.

Nitrogen determinations on dried cells were made by use of the Kjeldahl method. After digestion, the ammonium ion liberated was estimated as above.

T. denitrificans was the "Oslo" strain supplied by F. C. Happold of the University of Leeds, who originally obtained it from K. S. Baalsrud.

Gas evolution was measured in smaller cultures (up to 4 liters) by collecting the evolved gas in a measuring cylinder via a pneumatic water trough. With larger cultures, gas evolution was measured by means of a Parkinson "P.13" test meter. Because the appreciably water-soluble carbon dioxide is a component of the evolved gas, the values obtained by the two methods were not the same.

Culture maintenance and seed production. The culture medium contained (per liter): $Na_2S_2O_3 \cdot 5H_2O$, 5 g; KNO_3, 2 g; NH_4Cl, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; KH_2PO_4, 2 g; $NaHCO_3$, 1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g. The first four materials were sterilized together in about 800 ml of water (15 psi, 15 min). The phosphate and bicarbonate were each dissolved in water and sterilized separately under similar conditions. The ferrous sulfate was made up as a 1% solution of $FeSO_4 \cdot 7H_2O$ in 0.1 N HCl, and was also similarly sterilized; 1 ml of the resultant solution was used per liter of medium. The phosphate and sulfate were added to the bulk of medium, the *p*H was adjusted with 1 N NaOH to 6.5, and the bicarbonate was added to give a final *p*H of 6.9 in 1 liter of medium (volume adjusted if necessary with sterile distilled water). Analysis demonstrated that the bicarbonate was not decomposed under the conditions used for its sterilization.

The culture supplied was in sealed 1-oz bottles; it had been grown for 3 days on a medium similar to the above, and stored at 0 C. At first, our own cultures were grown in 1-oz bottles, but growth was variable. We therefore turned to larger containers and used gas evolution as the growth parameter.

Three or more 1-oz cultures were transferred to a bottle containing 4 liters of medium and connected to a pneumatic water trough containing an inverted, water-filled, measuring cylinder. Gas evolution was measured, and, after a suitable growth period, approximately 700 ml of the culture was used to inoculate 4 liters of fresh medium in a second 4-liter bottle (nominal capacity; actual capacity, about 4.7 liters), similarly equipped for gas measurement. (The bottle was completely filled.) Therefore, culture maintenance was by serial transfer, by use of about 700 ml of a growing culture as inoculum for 4 liters of fresh medium.

The seeds used for larger cultures eventually consisted of the contents of several 4-liter bottles, which had been grown as described above. Alternatively, 16 liters of medium in a 20-liter bottle (nominal capacity) was inoculated with the entire contents of a growing 4-liter bottle culture. After growth, the resultant culture was used for the inoculation of a 100-liter batch. The 100-liter cultures (approximate volume) were grown in a stirred, jacketted culture vessel, 140 liters in total capacity and 21 inches in diameter. It had provision for automatic temperature and *p*H control, contained four equally spaced, full-length radial wall baffles 1.5 inches wide, and was similar to the 20-liter vessel described by Elsworth, Williams, and Harris-Smith (5). The stirrer was operated at 630 rev/min, and the impeller had the following dimensions (inches): A, 4; B (eight vanes), 1⅜; C, ⅝; D, ¼.

The medium was prepared as follows. $Na_2S_2O_3 \cdot 5H_2O$ (1,500 g), KNO_3 (600 g), $MgCl_2 \cdot 6H_2O$ (50 g), and NH_4Cl (100 g) were made up to about 95 liters with tap water in the culture vessel, heated to 95 C by applying steam to the jacket, and sterilized by passing in steam and maintaining 15 psi excess pressure for 15 min. The vessel was cooled to 30 C and so maintained automatically, with continuous stirring. Nitrogen was passed over the charge at the rate of 1 to 5 liters per min from the time when the excess pressure was discharged during cooling. KH_2PO_4, 200 g in 1 liter of water, and $FeSO_4 \cdot 7H_2O$, 1 g in 100 ml of 1 N hydrochloric acid, were sterilized separately (15 psi excess pressure, 15 min) and added to the culture vessel contents. $NaHCO_3$ (1,500 g) was made up to 20 liters in tap water and sterilized (15 psi excess pressure, 15 min). The culture medium was adjusted to *p*H 6.3 by the addition of sterile 1 N NaOH and brought to 6.7 or 6.9, depending on the experiment, by the addition of $NaHCO_3$ solution under automatic control. The final volume was about 100 liters.

A supply of bottles containing $Na_2S_2O_3 \cdot 5H_2O$ (1,500 g) and KNO_3 (600 g) made up to 4 liters in tap water was sterilized separately (15 psi excess pressure, 15 min).

Stirred cultures were initiated by inoculating with the seed, either grown in 4-liter bottles or in a 20-liter bottle (12 to 20 liters total). The flow of nitrogen was stopped, and the culture vessel was sealed except for a single outlet via the gas meter, and dual inlets supplying sodium bicarbonate as demanded by the automatic *p*H control and the thiosulfate-nitrate solution under manual control. Gas evolution was measured and samples were taken at intervals. In all cultures except the first, when the $Na_2S_2O_3 \cdot 5H_2O$ concentration had fallen appreciably (the exact figures are given in the Results section), a feed of thiosulfate-nitrate was started, and maintained ideally at a rate such that the thiosulfate concentration did not fall further. The progress of cultures was followed by the gas evolution and by the thiosulfate consumption.

The contents of the culture vessel were cooled to 4 C before the cells were recovered.

Recovery of the cells was in a Laval type 1700 centrifuge with a flow rate of 1 liter per min. Cells were stored frozen at −20 C, prior to further use.

Enzyme assays. Assays were carried out at the University of Leeds by P. J. Butler and B. Taylor. Rhodanese, which catalyzes the reaction thiosulfate + cyanide → sulfite + thiocyanate, was determined by the method of Bowen et al. (4), by use of suspensions of thawed whole cells. One unit of rhodanese produces 1 mμmole of KCNS per min.

Adenosine-5′-phosphosulfate reductase, which catalyzes the reduction of sulfate in this form to sulfite, was determined by the method of Peck (8), by use of Hughes press extracts. (For experimental details, *see* reference 4*a*). One unit of adenosine-5′-phosphosulfate reductase reduces 1 μmole of $K_3Fe(CN)_6$ per min.

Adenylate kinase, which catalyzes the reaction 2-adenosine diphosphate → adenosine triphosphate + adenosine-5′-phosphate, was determined by a modification of the method of Oliver (7), also by use of Hughes press extracts. One unit of adenylate kinase produces 1 μmole of adenosine triphosphate per hr.

The following notes on the adenylate kinase assay were kindly supplied by B. Taylor. The yeast preparation, which Oliver (7) used as a source of hexokinase and glucose 6-phosphate dehydrogenase, was replaced by commercial preparations (Sigma Chemical Co., St. Louis, Mo.) of these enzymes (Patterson, Ph.D. Thesis, Univ. Leeds, 1964) dissolved in 0.1% (w/v) aqueous bovine serum albumin (British Drug Houses Ltd. Poole, Dorset, U.K.) to give two solutions, each containing about 23 enzyme units per ml (1 unit causes the turnover of 1 μmole of substrate per min). The assay solution contained: glucose, 0.05 M; KCl, 0.10 M; $MgCl_2$, 0.01 M; adenosine diphosphate, 0.001 M; nicotinamide adenine dinucleotide phosphate, 0.0001 M; in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer, *p*H 8.0. The assay was performed at 25 C with water in the blank cuvette. The reaction cuvette (optical depth, 1 cm) contained 2.5 ml of the assay solution, to which was added at zero-time 0.05 ml of both the hexokinase and the glucose 6-phosphate dehydrogenase solutions. Initially, there was a sharp but small rise in extinction at 340 mμ, but after about 5 min a low steady blank-rate resulted. The extract to be assayed was added at 10 to 15 min, and, after a lag of about 2 min, the extinction at 340 mμ increased at a uniform rate which was measured.

RESULTS

Culture maintenance and seed production. There was an induction period of several days before 4-liter cultures, inoculated from 1-oz bottles, began to grow and evolve gas. Once growth had started, it was usually complete in 2 to 3 days. When the larger inocula available from 4-liter bottles were used to initiate other 4-liter cultures, the induction period was reduced and growth was more rapid. Results were not uniform, but Table 1 summarizes those obtained during an experimental period.

Culture 1 was derived from seed grown in three 1-oz bottles (each containing 30 ml), and stored at 0 C. The induction period before gas

TABLE 1. *Gas-evolution data and final pH values for 4-liter seed cultures*

Culture no.	Inoculum size	Induction period before gas evolution	Total growth period	Total vol of gas evolved (collected over water)	Final *p*H value
	ml	*hr*	*hr*	*ml*	
1	3 × 30	48	100	620	6.05
2	750	14	73	1,010[a]	6.0
3	750	13	67	1,020[a]	6.0
4	750	14	47	780	6.0
5	750	11	28	500	6.1
6	750	9	50	645	5.95
7	750	17	49	865	6.15
8	750	15	49	510	6.2
9	750	24	48	610	—[b]
10	750	7	43	830	6.2
11	750	41	68	300	6.1
12	750	14	50	700	6.4
13	750	13	46	360	6.5
14	750	3	45	970	6.1

[a] Gas evolution was virtually complete in these cultures only.

[b] Not measured.

TABLE 2. *Effect of pH control on growth in culture 1*[a]

Time	$S_2O_3^{-2}$ (g of $Na_2S_2O_3 \cdot 5H_2O$/liter)	Dissolved CO_2 (mg of $NaHCO_3$/liter)	Vol of gas evolved
hr			*liters*
0	12.2	135	0
12	12.2	130	1.8
24	11.7	200	4.8
36	9.9	330	13.8
48	7.5	1,050	39.2
60	3.1	2,050	71.6
65 (harvested)	2.4	2,340	80.0

[a] The culture was harvested when gas evolution had ceased. Final culture volume, 116 liters; wet weight of cells, 132 g; dry weight of cells, 31 g; initial soluble N, 248 mg per liter (as NH_4^+); final soluble N, 188 mg per liter (as NH_4^+); volume of 7.5% $NaHCO_3$ used in *p*H control, 8.7 liters $Na_2S_2O_3 \cdot 5H_2O$ utilized, 1,190 g.

TABLE 3. *Effect of pH control and increased energy source on growth in culture 2*[a]

Time	$S_2O_3^{-2}$ (g of $Na_2S_2O_3 \cdot 5H_2O$/liter)	Dissolved CO_2 (mg of $NaHCO_3$/liter)	Vol of gas evolved
hr			*liters*
0	13.0	180	0
12	12.6	—	0
24	12.0	435	3.0
36	—	—	10.0
48	9.2	560	27.0
60	7.7	—	54.1
72	8.2	530	83.0
84	7.9	—	114.5
96 (harvested)	8.9	930	145.9

[a] The culture was harvested when gas evolution was still vigorous. Final culture volume, 123 liters; wet weight of cells, 205 g; dry weight of cells, 51 g; initial soluble N, 245 mg per liter (as NH_4^+); final soluble N, 167 mg per liter (as NH_4^+); volume of 7.5% $NaHCO_3$ used in *p*H control, 14.0 liters; volume of solution containing 37.5% $Na_2S_2O_3 \cdot 5H_2O$ and 15% KNO_3 supplied after 48 hr, 3.5 liters; $Na_2S_2O_3 \cdot 5H_2O$ utilized, 1,710 g.

TABLE 4. *Effect of pH control and increased energy source on growth in culture 3*[a]

Time	$S_2O_3^{-2}$ (g of $Na_2S_2O_3 \cdot 5H_2O$/liter)	Dissolved CO_2 (mg of $NaHCO_3$/liter)	Vol of gas evolved
hr			*liters*
0	13.3	60	0
12	13.2	—	1.8
24	13.1	710	3.8
36	12.6	—	4.8
48	11.0	300	11.6
60	8.7	—	32.0
72	5.4	700	61.9
84	4.1	—	94.0
96	4.5	1,330	127.4
108	5.1	—	159.2
120	5.6	1,560	182.9
132	8.7	—	197.4
139 (harvested)	8.7	2,400	201.8

[a] The culture was harvested when gas evolution had almost ceased. Final culture volume, 110 liters; wet weight of cells, 283 g; dry weight of cells, 76 g; initial soluble N, 245 mg per liter (as NH_4^+); final soluble N, 140 mg per liter (as NH_4^+); volume of 7.5% $NaHCO_3$ used in *p*H control, 15.5 liters; volume of solution containing 37.5% $Na_2S_2O_3 \cdot 5H_2O$ and 15% KNO_3 supplied after 70.5 hr, 5 liters; $Na_2S_2O_3 \cdot 5H_2O$ utilized, 2,420 g.

TABLE 5. *Effect of pH control and increased energy source on growth in culture 4*[a]

Time	$S_2O_3^{-2}$ (g of $Na_2S_2O_3 \cdot 5H_2O$/liter)	Dissolved CO_2 (mg of $NaHCO_3$/liter)	Vol of gas evolved
hr			*liters*
0	12.5	8	0
12	12.8	—	4.4
24	12.8	700	4.5
36	11.7	—	5.8
48	10.2	390	12.4
60	7.1	—	31.7
72	4.0	170	66.1
84	4.3	—	97.6
96	4.9	750	132.1
108	4.4	—	163.0
122 (harvested)	4.9	890	182.4

[a] The culture was harvested when gas evolution had almost ceased. Final culture volume, 108 liters; wet weight of cells, 304 g; dry weight of cells, 73 g; initial soluble N, 266 mg per liter (as NH_4^+); final soluble N, 154 mg per liter (as NH_4^+); volume of 7.5% $NaHCO_3$ used in *p*H control, 12.8 liters; volume of solution containing 37.5% $Na_2S_2O_3 \cdot 5H_2O$ and 15% KNO_3 supplied after 70 hr, 4 liters; $Na_2S_2O_3 \cdot 5H_2O$ utilized, 2,480 g.

evolution started was 48 hr, and even after 100 hr gas evolution was incomplete. Cultures 2 and 3, inoculated with 700 ml from an earlier 4-liter culture, were allowed to proceed to completion, and gas evolution had almost ceased in these cases after about 70 hr. The other cultures listed were interrupted before gas evolution was complete, and the arbitrary criterion which was finally used in deciding that a culture was suitable for use as an inoculum was that more than 500 ml of gas was evolved during the first 48 hr. When possible, inocula were used fresh, but satisfactory results were obtained when some or all of the inoculum had been stored at 0 C for up to 2 weeks after growth for 48 hr.

Description of 100-liter cultures. Four such cultures are described. In the first one, the *p*H was maintained at 6.9 and no additional thiosulfate and nitrate were added. The culture was harvested after 65 hr, when gas evolution ceased (80 liters evolved in all) and 1,190 g of $Na_2S_2O_3 \cdot 5H_2O$ was utilized. The yield of cells was 270 mg (dry weight) per liter. The results given in Table 2 show that when the culture was harvested there was an excess of both ammonium ion and dissolved carbon

TABLE 6. *Rhodanese, adenosine-5′-phosphosulfate reductase, and adenylate kinase contents of Thiobacillus denitrificans cells*[a]

Culture no.	Cells recovered (g, dry wt, per liter)	Nitrogen content of dry cells	Rhodanese (thousand units per liter of culture)	Rhodanese (thousand units per g of cells)	Adenosine-5′-phosphosulfate reductase (units per g of cells)	Adenylate kinase (thousand units per g of cells)
		%				
1	0.27	9.2	34	125	363	25.9
2	0.41	8.9	91	221	251	14.5
3	0.69	9.1	130	188	155	8.4
4	0.68	8.8	114	168	184	12.2

[a] One unit of rhodanese produces 1 mμmole of KCNS per min in the system described by Bowen et al. (4). One unit of adenosine-5′-phosphosulfate reductase reduces 1 μmole of $K_3Fe(CN)_6$ per min in the system described by Bowen, Happold, and Taylor (4*a*). One unit of adenylate kinase produces 1 μmole of adenosine triphosphate per hr in the system described in the text. The rhodanese assay was performed on suspensions of whole cells. The other two assays were performed on Hughes-press extracts of whole cells.

dioxide. It was concluded that growth ceased because nitrate was exhausted.

Culture 2 was grown under conditions similar to those used for culture 1, except that (i) the *p*H was controlled at 6.7 instead of at 6.9 (this still ensured an excess of dissolved carbon dioxide), and (ii) a solution of sodium thiosulfate (37.5%) and potassium nitrate (15%) was supplied to the growing culture during the later stages. This culture was harvested while the rates of thiosulfate consumption and gas evolution were still high. The experimental data summarized in Table 3 show that the cell yield [410 mg (dry weight) per liter] was higher than was obtained without the use of additional thiosulfate and nitrate in culture 1.

Cultures 3 and 4 were grown under the same conditions as were used for culture 2, except that harvesting was delayed until the thiosulfate-consumption rate and the gas-evolution rate had fallen off appreciably. The relevant data are recorded in Tables 4 and 5, and the respective yields of dry cells were 690 and 680 mg per liter.

The rhodanese content of the cells from all four cultures had an average value of 176,000 units per gram (dry weight). The average adenosine-5′-phosphosulfate reductase content was 238 units per gram (dry weight), and the average adenylate kinase content was 15,300 units per gram (dry weight). The enzyme assay results for individual cultures are given in Table 6.

DISCUSSION

Baalsrud and Baalsrud (2, 3) established that *T. denitrificans* could be grown anaerobically with ammonium ion as the nitrogen source, carbon dioxide as the carbon source, and thiosulfate and nitrate as the energy source.

In all of our culture media, ammonium chloride was incorporated at such a level that ammonium ion was still present when the cultures were harvested. Therefore, lack of nitrogen never limited growth.

We found that cultures grown in 4-liter bottles tarting at *p*H 6.9, but without *p*H control, had a snal *p*H value in the region of 6.0, and at the end fiontained no dissolved carbon dioxide. We therecore concluded that the low cell yields reported ffrom flask cultures by Bowen et al. (4), and confirmed by us, were caused by carbon limitation. This resulted from the drop in *p*H value which occurred during the growth of cultures and led to the evolution of carbon dioxide. To prevent this carbon limitation in our 100-liter cultures, automatic *p*H control was instituted by use of 7.5% sodium bicarbonate solution as the *p*H restoring fluid. This ensured that carbon dioxide was always in excess. In the first such culture, a *p*H value of 6.9 was maintained, and subsequent cultures were controlled at *p*H 6.7.

The first 100-liter culture gave a much improved yield of cells, but ceased growing, and it stopped evolving gas abruptly when 80 liters had been given off. At the end, there was so little thiosulfate remaining that it is presumed that the supply of nitrate was exhausted. Therefore, to increase further the yield of cells, subsequent cultures were supplied with additional thiosulfate and nitrate during growth. It was necessary to supply these additional nutrients in this way rather than to add them at the beginning, because initial ionic strengths much in excess of those used inhibited the growth of the organism. Culture 2 was harvested while it was still growing freely, and gave a higher cell yield. Cultures 3 and 4 had almost stopped growing when they were harvested, despite the fact that in both cases the nitrogen, carbon, and energy sources were all still present in excess. The cell yields obtained from these cultures [690 and 680 mg (dry weight) per liter, respectively] were, so far as we are aware, the highest ever reported for *T. denitrificans*.

Increases in cell density during the growth of cultures were not determined, because the insoluble material was not composed exclusively of bacteria. A varying amount of elemental sulfur was also present (*see* 3). The nitrogen content of the dry cells recovered from the four cultures varied about 9.0% (*see* Table 6), and it is therefore unlikely that the content of elemental sulfur was high in the harvested cells.

The most likely cause of the slackening in growth rate in cultures 3 and 4 when the cell density approached 700 mg (dry weight) per liter was the buildup in ionic strength. It is probable that a further considerable improvement in yield could be obtained by growing cultures in a fermentor equipped for dialysis in fresh medium. A practical system of this type was described by Gallup and Gerhardt (6).

The rhodanese content of the cells from culture 2 (125,000 units per gram, dry weight) was somewhat higher than the 70,000 units per gram (dry weight) observed by Bowen, Butler, and Happold (Butler, *personal communication*) in cells obtained at 30 mg (dry weight) per liter from flask cultures. The other three cultures gave cells with a higher rhodanese content. Thus, cultures 3 and 4, which were grown under our best conditions and which gave high cell yields, averaged 122,000 units per liter compared with about 2,100 units per liter obtained from flask cultures. When this work was being carried out, we were unaware that the addition of potassium cyanide at 40 μM to 48-hr

flask cultures increased rhodanese activity threefold after a further 24 hr (4). The cells from such cultures had a rhodanese content of the same order as that of cells from our cultures 3 and 4. It is possible that we could have induced even higher yields of rhodanese by adding potassium cyanide to growing cultures at a suitable stage.

The harvested cells contained sufficient quantities of the three enzymes to allow studies of their properties to be made. Some properties of the rhodanese system were described by Bowen et al. (4), and studies on adenosine-5′-phosphosulfate reductase were reported by Bowen, Happold, and Taylor (4*a*).

LITERATURE CITED

1. AUBERT, J. P., J. MILLET, AND G. MILHAUD. 1959. Isolement et propriétés du cytochrome C de *Thiobacillus denitrificans*. Ann. Inst. Pasteur **96**: 559–576.
2. BAALSRUD, K., AND K. S. BAALSRUD. 1952. The role of phosphate in CO_2 assimilation of *thiobacilli*, p. 544–576. *In* W. D. McElroy and B. Glass [ed.], Phosphorus metabolism, vol. II. The Johns Hopkins Press, Baltimore.
3. BAALSRUD, K., AND K. S. BAALSRUD. 1954. Studies on *Thiobacillus denitrificans*. Arch. Mikrobiol. **20**:34–62.
4. BOWEN, T. J., P. J. BUTLER, AND F. C. HAPPOLD. 1965. Some properties of the rhodanese system of *Thiobacillus denitrificans*. Biochem. J. **97**: 651–657.

4*a*. BOWEN, T. J., F. C. HAPPOLD, AND B. F. TAYLOR. 1966. Studies on adenosine-5′-phosphosulphate reductase from *Thiobacillus denitrificans*. Biochim. Biophys. Acta **118**:566–576.

5. ELSWORTH, R., V. WILLIAMS, AND R. HARRIS-SMITH. 1957. A systematic assessment of dissolved oxygen supply in a 20 litre culture vessel. J. Appl. Chem. **7**:261–268.
6. GALLUP, D. M., AND P. GERHARDT. 1963. Dialysis fermentor systems for concentrated culture of microorganisms. Appl. Microbiol. **11**:506–512.
7. OLIVER, I. T. 1955. A spectrophotometric method for the determination of creative phosphokinase and myokinase. Biochem. J. **61**:116–122.
8. PECK, H. D., JR. 1961. Evidence for the reversibility of the reaction catalysed by adenosine-5′-phosphosulphate reductase. Biochim. Biophys. Acta **49**:621–624.
9. TRUDINGER, P. A. 1956. Fixation of carbon dioxide by extracts of the strict autotroph *Thiobacillus denitrificans*. Biochem. J. **64**:274–286.