A study of cultivation methods of sulphur bacteria found in thermal waters of Yellowstone National Park
by Henry R Kathrein

A THESIS Submitted to the Graduate Committee in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University

Abstract:
A chronological review of the literature on sulphur bacteria was made. The term "sulphur bacteria" has been used to include both the non-pigmented chemosynthetic types and the pigmented photosynthetic types. Both groups are able to oxidize hydrogen sulphide to sulphur, and store the sulphur in the form of granules within the cells.

Various cultivation technics for these sulphur organisms were tried and it was found that sodium sulphide is an essential metabolite for their growth and cultivation.

Attempts were made to obtain pure cultures by a series of dilutions and transfers, but with no success. In all instances, other forms of microflora overcrowded the sulphur organisms which were being studied. In further attempts to obtain pure cultures, a number of representative chemicals which are known to be inhibitory to certain bacteria were used. None of these chemicals proved to be inhibitory to the other forms present.

The organisms studied in this investigation appear to belong to the order Eubacteriales, suborder Rhodobacteriineae, family Thiorhodaceae.

Organisms collected from thermal waters of Yellowstone National Park were used in the present investigation.

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A STUDY OF CULTIVATION METHODS OF SULPHUR BACTERIA FOUND IN THERMAL WATERS OF YELLOWSTONE NATIONAL PARK

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ABSTRACT

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INTRODUCTION

Since so little work has been done with the sulphur organisms found in thermal waters of Yellowstone National Park, it was the purpose of the writer to determine a satisfactory method of cultivating these organisms in order that further studies might be facilitated.

REVIEW OF LITERATURE

The study of sulphur bacteria is a comparatively recent study of microorganisms. Although casual observations of composite sulphur bacteria in hot springs were made as early as 1860, studies as to their isolation and pure culture, morphology and physiology were not made until several years later. Cramer (1870) was the first to suggest that granules in Beggiatoa, a genus of the sulphur bacteria, consisted of sulphur. From investigations carried out in 1869-71 on the vegetation of the Yellowstone Hot Springs, Harshbarger (1897) reported the presence of bacteria able to deposit sulphur as granules within their cells. Cohn (1875) then postulated the theory that the beggiatoa and the purple bacteria produce hydrogen sulphide by reduction of sulphates. Weed (1889) in his paper entitled "The Vegetation of Hot Springs" shows that "travertine" is the result of sulphur deposition by the beggiatoa. This work was substantiated by Davis (1897). His

observations were that "travertine" and "felt", a closely woven mass of filamentous bacteria in which crystals of calcium carbonate were imbedded, were responsible for many of the colored deposits in the park. Controversy as to the origin of organisms in sulphur springs was the result of investigations carried out by Setchell (1903). His contention was that no organisms were found in strictly thermal waters nor in springs which were reputed to have a decided acid reaction.

Engelmann (1887) first postulated that purple and green sulphur bacteria belonged to the photosynthetic group of organisms. This theory was strongly opposed by Winogradsky (1888) who proposed the theory of chemosynthetic metabolism. In these processes, the energy supply of the organism is not furnished by decomposition of organic matter, but by the oxidation of inorganic substances. In these processes, also, hydrogen sulphide is oxidized by the organism to sulphuric acid. Molisch (1907) published his monograph on the purple bacteria in which he concluded that purple bacteria assimilate organic compounds in the light. This was his attempt to defeat the theory of an autotrophic mode of life for these organisms, as outlined by previous investigators. Such a view was in direct support of the work of Nadson (1903) who stated that hydrogen sulphide is not required for nutrition, and sulphur is not accumulated. Buder (1919), in

discussing the value of the various theories presented up until this time, was inclined to believe that the metabolism of the purple bacteria should be considered as a combination of photosynthetic and chemosynthetic modes of life, independent of each other, but providing the organisms with the faculty to live and thrive under divergent conditions. This idea is called "only a well founded assumption" but even at the present time, we have come no further in our knowledge of these metabolic processes.

Warming (1875) and Lankester (1876), in their early investigations, drew the conclusion that all the various forms and shapes of colored organisms with droplets inside the cells represented different developmental stages or "phases of growth" of one species. This idea was attacked by Cohn (1875) who held to the monomorphistic viewpoint, as did Winogradsky (1887). Such a viewpoint stressed the fact that distinct variations were characteristic of different species. On this basis, Winogradsky established an elaborate system of classification of the sulphur bacteria based upon the shape and size of the cells, as well as upon their mode of colony formation. This system has been retained--with only minor modifications--to the present day. Van Niel (1930), through extensive investigation, has concluded that variations as to size, shape, and growth are often encountered within a pure culture, and are the result of environmental effects such as

hydrogen sulphide concentration, pH of the medium, age of the culture, and presence of free oxygen.

Frobisher (1944) places all the sulphur bacteria in the order Thiobacteriales. Criteria for further subdivision were the presence of photosynthetic pigments and the presence of free sulphur as granules within the cell walls. Those organisms which possessed photosynthetic pigments and store sulphur within their cell walls were classified under the family Thiorhodaceae. Those organisms which possessed photosynthetic pigments and did not store sulphur within their cell walls were classified under the family Athiorhodaceae. Those organisms which possessed no photosynthetic pigments but stored sulphur within their cell walls were divided into the filamentous organisms under the family Beggiatoaceae, and the non-filamentous organisms under the family Achromatiaceae. The sulphur bacteria have been reclassified, however, in the sixth edition of Bergey's Manual. In this classification, the sulphur bacteria which resemble true bacteria in morphology have been placed under Order I, Eubacteriales, Suborder III, Rhodobacteriineae, and the sulphur bacteria which resemble algae in morphology but do not possess pigments under Order III, Chlamydobacteriales.

Ellis (1932) in his investigations on the sulphur bacteria was the first to use a chemical compound to show the existence of sulphur granules by color indication. When a smear of the

sulphur containing organisms was treated with a concentrated solution of sodium nitroprusside ($Na_2FeNO(CN)_5$), the rings of sulphur assumed a blood red color. This procedure indicated the presence of sulphur granules; however, it was of no value in determining cell morphology since it failed to show the cell outline. Howard (1948) in a study of sulphur bacteria, developed a differential staining technic in which malachite green or methylene blue was used to stain the cell, and after mordanting with tannic acid, sodium nitroprusside was added to stain the sulphur granules.

REVIEW OF TECHNICS EMPLOYED

One of the greatest difficulties encountered by investigators in the field of sulphur bacteria has been the development of media and technics suitable for the isolation and cultivation of pure cultures. Molisch (1907) employed a solid medium containing river water, peptone, dextrin, and agar. However, this medium contained an excess of organic material which would not permit exact studies of autotrophic forms.

Kiel (1912) employed a strictly inorganic medium containing the following constituents:

$CaH_2(CO_3)_2$	0.34%	$Ca_3(PO_4)_2$	0.02%
$MgH_2(CO_3)_2$	0.27%	KCl	0.01%
$CaSO_4$	0.31%	K_2S	0.01%
$MgSO_4$	0.51%	FeS	0.01%
Na_2SO_4	0.21%	CaS	0.01%

A small amount of ammonium sulphate was added; also oxygen, hydrogen sulphide and carbon dioxide were introduced.

Van Niel (1930) obtained very satisfactory results with a medium of the following composition:

NH_4Cl----------0.1%

K_2HPO_4----------0.05%

$MgCl_2$----------0.02%

$NaHCO_3$----------0.1%

$Na_2S \cdot 9H_2O$----------0.1%

The medium was adjusted to a pH of 8.0-8.5 by the addition of sterile Na_2CO_3 or H_3PO_4. The use of $Na_2S \cdot 9H_2O$ by van Niel as a source of hydrogen sulphide was based on experimental evidence obtained which indicated that many sulphur bacteria were capable of oxidizing various inorganic sulphur compounds to sulphate with the simultaneous photoreduction of carbon dioxide.

Van Niel found that cultures of sulphur bacteria which developed under natural daylight conditions in the laboratory in three to four weeks showed the same growth in four to five days when they were illuminated continuously by an ordinary electric light bulb of 25-50 watts, placed at a distance of 20-30 cm from the cultures.

During investigations carried out by Howard (1948) using both Kiel's inorganic medium and van Niel's inorganic medium, it was found that Kiel's medium had little selective value for

sulphur bacteria since algae and diatoms also survived. However, van Niel's medium seemed to be specific for bacterial growth. Fewer number of algae and diatoms were observed in this type of medium. In neither medium was he able to carry a culture beyond the second transfer, indicating that some essential metabolite was missing.

Ten samples used for the present study of cultivation technics of sulphur bacteria were collected in Yellowstone National Park from thermal waters having a distinct odor of hydrogen sulphide. Data concerning the source, temperature at time of collection, and pH of samples are listed in Table I. The pH measurements were made by means of the Beckman pH meter upon returning to the laboratory.

One mililiter of each sample was inoculated into 1.5 by 15 cm tubes containing 10 ml portions of van Niel's medium. In an attempt to simulate natural environmental conditions, samples 1, 2, 3, and 6, having a pH range of 8.1-8.4 were inoculated into this medium adjusted to pH 8.3. Samples 4, 5, 9, and 10, having a pH range of 7.2-7.8 were inoculated into this same basic medium adjusted to pH 7.5, and samples 7 and 8, having a pH range of 6.2-6.4, were inoculated into the basic medium adjusted to pH 6.3. Phosphoric acid was used to adjust the pH levels.

Primary inoculations were made in duplicate and will be labeled hereafter Transfer No. 1. Both sets of cultures

TABLE I

Samples Collected From Thermal Waters of Yellowstone National Park Showing Temperatures and pH of the Samples for the Different Locations

Sample No.	Location	Temp. C	pH
1.	Mirror Pool, Biscuit Basin - South edge of pool.	60	8.5
2.	Mirror Pool, Biscuit Basin - East edge of pool.	69	8.5
3.	Mirror Pool, Biscuit Basin - North edge of pool. Drainage area.	29	8.1
4.	Mirror Pool, Biscuit Basin - West edge of pool. Drainage area.	12	7.8
5.	Mirror Pool, Biscuit Basin - North edge of pool.	56	7.7
6.	Cauliflower Pool, Biscuit Basin - East edge of pool. Drainage area.	29	8.1
7.	Terrace Road, Mammoth Terraces, Formation Loop Road. Small pool around H_2S bubbling hole.	29	6.4
8.	Terrace Road, Mammoth Terraces, Formation Loop Road, Small pool around H_2S bubbling hole.	39	6.2
9.	Formation Loop Road, Mammoth Terraces Orange Mound Spring	55	7.2
10.	Formation Loop Road, Mammoth Terraces Orange Mound Spring	58	7.3

received an initial amount of $Na_2S \cdot 9H_2O$ (0.01 g). One set of cultures received no additional supply of sodium sulphide; however, the other set of cultures received supplemental amounts of 0.01 g $Na_2S \cdot 9H_2O$ at three day intervals. All cultures were incubated aerobically in a cabinet illuminated by two 25 watt bulbs and maintained at a temperature of 40 C. Samples 1, 5, and 7 were selected for further study because they represented the three different pH ranges used in these investigations, namely 8.3, 7.5, and 6.3. Counts of the number of organisms present were made on these samples at the time of the initial inoculation, and thereafter at 2 day intervals. A Breed milk pipette that delivers 0.01 ml was employed and the counts were made by spreading 0.01 ml portions of the sample over an area of one square centimeter on a clean glass slide. The smears were then fixed by heat, stained with carbol fuchsin, washed, dried and observed under the oil immersion objective. Counts were made only of typical sulphur organisms of one morphological type, which had been established as sulphur bacteria by means of the sodium nitroprusside stain developed by Howard (1948). Twenty fields were counted and the average of the number of such organisms per field was multiplied by the microscopic factor (565,000) that had been determined previously.

A compilation of counts and the logarithms of the counts on Transfer No. 1 are shown in Table II. Graphs showing the

TABLE II

Transfer No. 1 Counts of Sulphur Organisms Showing Effect of $Na_2S \cdot 9H_2O$ on growth

Date	Sample 1		Sample 5		Sample 7	
	Number x 10^3	Log.	Number x 10^3	Log.	Number x 10^3	Log.
Cultures receiving $Na_2S \cdot 9H_2O$ at 3 day intervals						
9/27	932	5.9	621	5.8	297	5.5
9/29	1,469	6.2	1,186	6.1	508	5.7
10/2	2,429	6.3	2,994	6.5	1,143	6.1
10/4	4,350	6.6	4,415	6.6	1,186	6.1
10/6	6,045	6.7	8,757	6.9	1,299	6.1
10/9	8,418	6.9	12,612	7.1	1,299	6.1
10/11	11,582	7.1	18,944	7.2	2,881	6.5
10/13	11,305	7.1	22,769	7.4	5,706	6.8
10/15	8,927	6.9	17,679	7.2	5,719	6.8
10/17	6,152	6.8	10,678	7.0	4,576	6.7
Cultures receiving $Na_2S \cdot 9H_2O$ only at start						
9/27	706	5.8	565	5.8	169	5.2
9/29	1,243	6.1	1,356	6.1	734	5.9
10/2	1,129	6.1	1,412	6.1	621	5.8
10/4	1,015	6.0	1,469	6.2	678	5.8
10/6	791	5.9	1,173	6.1	621	5.7
10/9	678	5.8	960	6.0	508	5.7
10/11	452	5.7	565	5.8	339	5.5
10/13	282	5.4	339	5.5	226	5.4
10/15	169	5.2	282	5.5	113	5.1
10/17	113	5.1	226	5.4	56	4.8

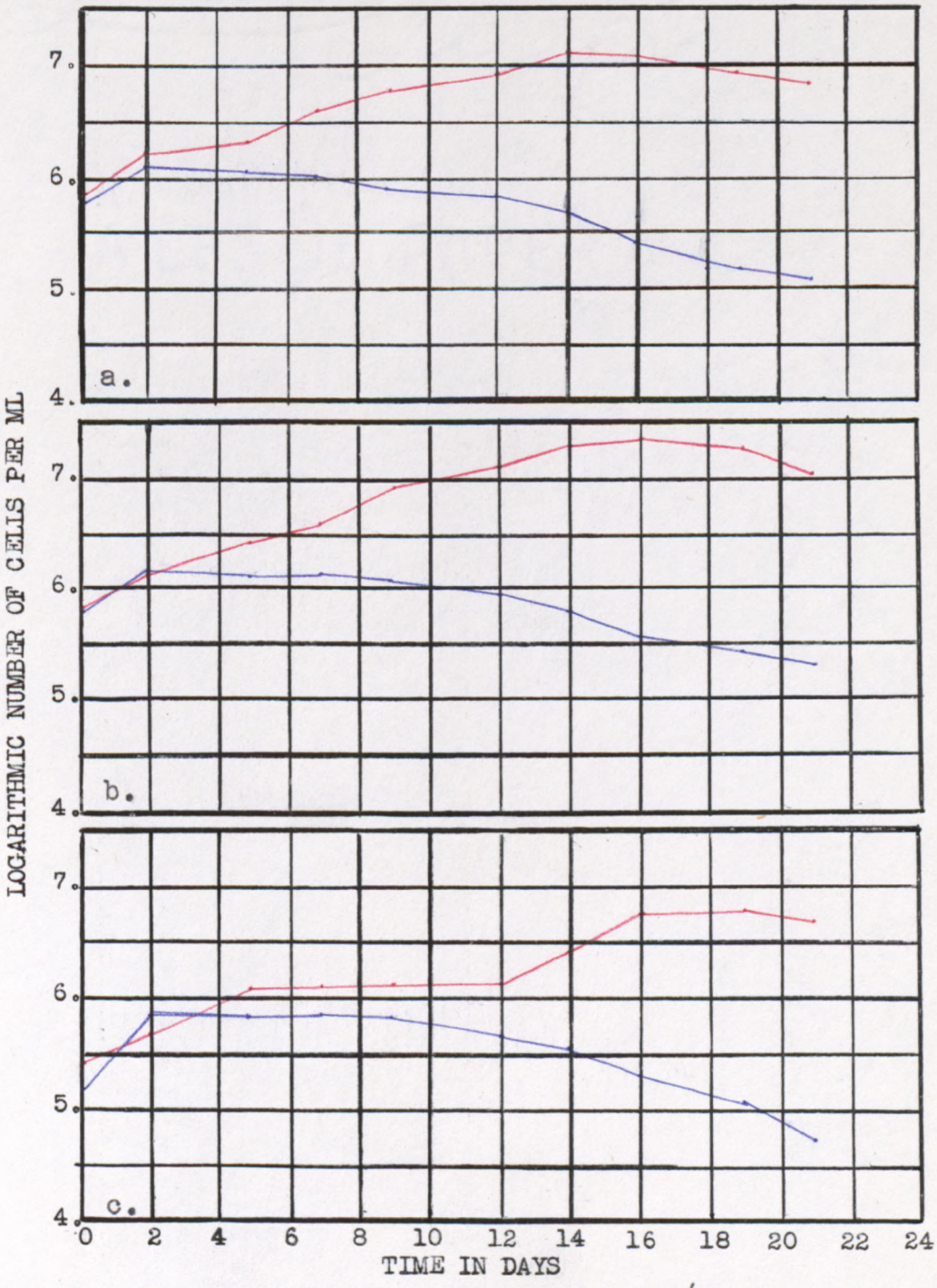

Figure 1. Transfer No. 1 Effect of $Na_2S/9H_2O$ on growth of sulphur bacteria in an inorganic medium. Red indicates cultures receiving Na_2S at three day intervals. Blue indicates cultures receiving Na_2S only at start.

effect of $Na_2S \cdot 9H_2O$ on the growth of sulphur bacteria are included in Figure 1. Figure 1 (a) represents sample No. 1 collected from Mirror Pool and having a pH of 8.4. Figure 1 (b) represents sample No. 5, also collected at Mirror Pool and having a pH of 7.7; and Figure 1 (c) represents sample No. 7 collected at Mammoth terraces, having a pH of 6.4. Red lines indicate cultures which received $Na_2S \cdot 9H_2O$ at three day intervals; blue lines indicate cultures which received only an initial supply of $Na_2S \cdot 9H_2O$.

As can be seen from the counts in Table II and also from the graphical presentation of these counts in Figure 1, both sets of cultures exhibit approximately the same amount of growth during the first few days. However, after a period of three to four days after inoculation, those cultures which received supplemental increments of $Na_2S \cdot 9H_2O$ show a definite increase in number, whereas those cultures which received no additional amounts of $Na_2S \cdot 9H_2O$ exhibit a stationary phase or a phase of slight decrease in number. This difference is further accentuated after a growth period of 16 days. Those cultures receiving supplemental amounts of sodium sulphide show a 10-15 fold increase in number over those in the original inoculum, whereas in those cultures receiving no supplemental sodium sulphide, the numbers were reduced from two to three times the original number of organisms. These data indicate that the addition of sodium sulphide has a decided stimulatory

effect upon the growth of sulphur organisms.

Samples No. 1 and 5 tend to exhibit the same general growth pattern. However, it will be noticed that sample No. 7 did not grow as well, nor did it reach the maximum growth shown by samples 1 and 5, although the cells resemble those in 1 and 5 morphologically. Despite this fact, a sharp distinction is shown between the cultures receiving supplemental amounts of sodium sulphide and the cultures receiving no additional amounts of sodium sulphide.

After 14 days of incubation, the ten samples from Transfer No. 1 were inoculated into 10 ml portions of fresh medium, using an inoculum of one ml in each case. Again inoculations were made in duplicate, one set receiving only an initial supply of $Na_2S \cdot 9H_2O$ and the other set receiving supplemental increments of $Na_2S \cdot 9H_2O$ at three day intervals in addition to the initial supply. The same technics of inoculation and counting were carried out on Transfer No. 2 as for Transfer No. 1. Compilation of counts and the logarithms of the counts on Transfer No. 2 are shown in Table III. The effect of $Na_2S \cdot 9H_2O$ on the growth of sulphur bacteria is shown in Figure 2. Figure 2 (a) represents culture No. 1, Figure 2 (b), culture No. 5, and Figure 2 (c), culture No. 7.

After an incubation period of 14 days, the samples from Transfer No. 2 were re-inoculated into 10 ml portions of fresh medium, again using an inoculum of 1 ml. Transfer No. 3 was

TABLE III

Transfer No. 2 Counts of Sulphur Organisms Showing Effect of $Na_2S \cdot 9H_2O$ on Growth

Date	Sample 1		Sample 5		Sample 7	
	Number x 10^3	Log.	Number x 10^3	Log.	Number x 10^3	Log.
Cultures receiving $Na_2S \cdot 9H_2O$ at 3 day intervals						
10/11	113	5.1	254	5.4	56	4.7
10/13	395	5.6	960	5.9	169	5.2
10/15	1,695	6.2	1,751	6.2	565	5.7
10/18	2,429	6.4	2,655	6.4	1,186	6.1
10/20	3,616	6.6	4,463	6.6	2,034	6.3
10/22	5,189	6.7	6,554	6.8	3,107	6.5
10/25	5,254	6.7	6,497	6.8	3,164	6.5
10/27	5,367	6.7	6,045	6.8	3,051	6.5
10/29	4,689	6.7	5,367	6.7	2,938	6.5
11/1	4,520	6.6	5,254	6.7	2,825	6.4
11/3	4,068	6.6	4,972	6.7	2,429	6.4
Cultures receiving $Na_2S \cdot 9H_2O$ only at start						
10/11	141	5.1	282	5.5	28	4.5
10/13	508	5.7	524	5.7	282	5.5
10/15	678	5.8	734	5.9	226	5.4
10/18	282	5.4	339	5.5	56	4.7
10/20	226	5.4	339	5.5	28	4.5
10/22	226	5.4	282	5.4	-	-
10/25	169	5.2	282	5.4	-	-
10/27	56	4.6	169	5.2	-	-
10/29	56	4.6	113	5.1	-	-
11/1	56	4.6	56	4.6	-	-
11/3	28	4.4	56	4.6	-	-