Iron Availability in Mixed Cultures of Sulfate-Reducing Bacteria

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INTRODUCTION

The serious and costly problems of odor, corrosion, and toxicity arising from the reduction of sulfate to sulfide by sulfate-reducing bacteria have been examined in detail by sewage authorities in America and Australia.[1,2] Despite these and numerous other studies, the fundamental processes involved are not properly understood and there is a lack of quantitative data on the kinetics of bacterial sulfate reduction.

As part of our attempts to quantify the sulfide generation process in sewers, mixed microbial cultures of sewage origin were grown in suspended culture using synthetic media. Initial studies were carried out using some of the media formulations commonly recommended in the microbiological literature (i.e., Postgate's formulations[3]). Using these formulations, meaningful kinetic data could not be obtained. Consequently, the investigations of growth media reported below were carried out.

EXPERIMENTAL

Cultures were grown using both batch and continuous operation in 1-L glass fermentors. Temperature was controlled at 32 ± 0.2°C and pH at 7.2 ± 0.1. Generally, pH increased during culture growth as a result of the degassing of H_2S, and so acid additions only were necessary (2*N* HCl). High-purity nitrogen, at a flowrate of 60 L/day, was used to continuously purge the fermentor of H_2S. Mixing was effected by a vibrating plate that also served to disperse the nitrogen bubbles and ensure adequate removal of H_2S. The composition of the growth media were as follows: Postgate's medium C[3]: KH_2PO_4, 0.5 g/L; NH_4Cl, 1.0 g/L; $CaCl_2 \cdot 2H_2O$, 0.06 g/L; $MgSO_4 \cdot 7H_2O$, 0.06 g/L; $FeSO_4 \cdot 7H_2O$, 4 mg/L; sodium lactate (70% solution), 6.0 g/L; Na_2SO_4, 4.5 g/L; sodium citrate $2H_2O$, 0.3 g/L. Chemically defined medium: KH_2PO_4, 0.5 g/L; NH_4Cl, 1.0 g/L; $CaCl_2 \cdot 2H_2O$, 0.08 g/L; $MgSO_4 \cdot 7H_2O$, 0.05 g/L; $FeSO_4 \cdot 7H_2O$, 7 mg/L; sodium lactate (70% solution), 6.0 g/L; Na_2SO_4, 4.5 g/L; Na_2EDTA, 0.1 g/L; trace elements (B, Co, Cu, Mn, Zn), 0.05 mg/L each. Distilled water was used for both media preparations and the pH was adjusted to 7.2 with sodium hydroxide. The media were heat-sterilized prior to use. All chemicals were of reagent grade.

Culture growth was monitored by measuring turbidity, the production of sulfide, and sulfate removal. Turbidity was measured using a HIAC NT 260 Turbidimeter, after calibration of the instrument with a 0.14 FTU standard. Concurrent measurements of turbidity and suspended solids (using 0.45 μm membrane filters) gave a linear calibration for the range 5–400 mg dry weight cells/L. The calibration factor was 2.5 ± 0.5 mg dry weight cells/FTU.

Measurements of the concentrations of sulfate and dissolved sulfide were obtained by withdrawing 10-mL samples from the fermentor into plastic syringes, each containing 0.1 mL of 13 wt% zinc acetate solution. Suspended solids and precipitated zinc sulfide were then filtered out using 0.45 μm membrane filters. Sulfate analysis was carried out on the filtrate using the standard turbidimetric method.[4,6] Precipitated sulfide was measured using a modified iodometric method similar to that of Grasshof.[5] The same technique was used to measure H_2S evolved from the culture. The H_2S was trapped in a known volume of zinc acetate, and following vigorous shaking, an aliquot was taken for analysis. Lactate analyses were carried out using the spectrophotometric method of Lawrence.[7] With the exception of the method for lactate, the coefficient of variation of the analytical procedures was less than 5%.

The original source of the cultures was raw sewage. Postgate's medium B[3] was used to obtain an enriched culture of sulfate-reducing bacteria. Examination by microscope showed the culture to be predominantly motile, Gram-negative, rod-shaped bacteria of approximately 1–2 μm in length. Prior to inoculation of cultures, 1 L of medium was placed in the fermentor, and then sufficient sodium sulfide was added to raise the sulfide concentration to about 1m*M*. After 1–2 h the nitrogen purging reduced the concentration to about 0.1m*M*. This procedure ensured that oxygen was completely removed from the reactor and that the E_h was

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less than −100 mV. An active culture of sulfate-reducing bacteria was maintained under continuous culture conditions. Batch cultures were inoculated with a 30-mL sample from this culture.

RESULTS AND DISCUSSION

Bacterial Growth Measurements on Postgate's Medium

Measurements of sulfate concentration, sulfide production, and turbidity were made during batch culture using Postgate's medium C as the growth medium. The results are plotted in Figure 1. Following inoculation of the medium, there was a lag phase of approximately 10–15 h duration. This was followed by two distinct growth phases and then, after approximately 45 h, retardation of the growth rate. The plots for turbidity and sulfide generation show a similar growth pattern. Corresponding behavior was demonstrated by the sulfate uptake plot. Measurements of the lactate concentration showed that termination of growth was associated with the depletion of this carbon source. A second experiment with Postgate's medium gave similar results to those shown in Figure 1. Thus both experiments showed that there was a distinct reduction in the rate of turbidity increase, and sulfate removal rate, after about 30 h of growth. This was followed by a second accelerated growth phase. These, and subsequent experiments, have indicated that the early increases in turbidity cannot be correlated with the sulfate uptake rate. It is likely that increasing concentrations of sulfide inhibited the growth of bacterial species other than sulfate reducers, causing a reduction in the overall growth rate of the culture.

The classical description of the growth of microbial cultures predicts exponential growth until nutrient concentrations become limiting.[8] Postgate's medium is designed to promote growth of sulfate-reducing bacteria with the limiting nutrient being either lactate or sulfate.[3] The concentration of these nutrients was in excess of 500 mg/L at the point where the second lag phase occurred, and it seems unlikely that they would become limiting at this concentration. With mixed microbial populations two-phase growth may result from a change in the predominant bacterial species. For this to account for the change in the rate of sulfate uptake, a population shift would have to be from one species of sulfate-reducing bacteria to another.

An investigation of the literature showed that two-phase growth has also been observed by a number of other workers using both mixed microbial populations containing sulfate-reducing bacterial[9,10] as well as a pure strain of *Desulfovibrio*.[11] Leban et al.[11] suggested that the two-phase growth obtained in their experiments was due to the early depletion of an unidentified growth factor that was subsequently self-synthesized by the growing *Desulfovibrio*. Leban et al. also found that concentrations of sulfide in excess of 10m*M* inhibited growth of their cultures. Measurements of the sulfide concentration during the second batch culture described above yielded values of up to 0.7m*M*. At the time of the second lag phase, the sulfide concentration was 0.1m*M*.

The Availability of Iron

Postgate[12] noted that linear growth (rather than exponential growth) was observed during some of his studies of pure cultures of sulfate-reducing bacteria. He postulated that the reason for linear growth might include a direct inhibitory effect of sulfide, an indirect effect of sulfide formation and evolution causing changes in pH, and iron sulfide precipitation resulting in a loss of soluble iron and also interfering with suspended solids measurements. Sulfate-reducing bacteria appear to have a relatively high requirement for iron[3]; and Postgate noted that exponential growth of sulfate-reducing bacteria was observed when iron complexing agents such as citrate and EDTA are added to media.[12] To avoid the possible limitation of growth due to depletion of iron by precipitation, Postgate included citrate in his medium C.[3] During our experiments, blackening of the culture was not observed—indicating that iron

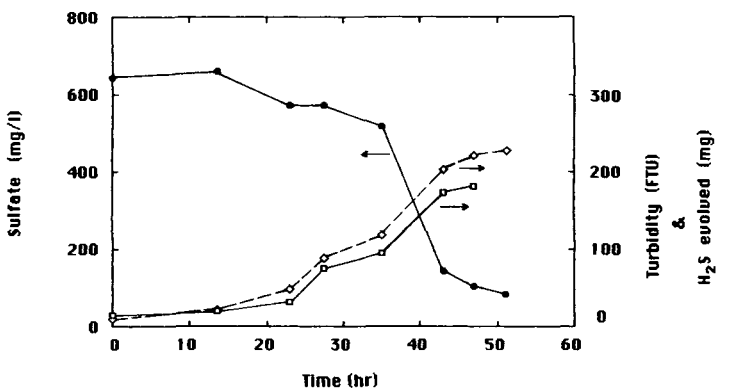

Figure 1. Turbidity (-□-) and sulfide (– –) generation, and sulfate removal (-●-) during batch culture with Postgate's medium C.

sulfide precipitation did not occur. However, this does not preclude the possible formation of colloidal iron sulfide.

Vosjan[13] demonstrated an iron deficiency in continuous cultures of sulfate-reducing bacteria using a media formulation similar to that of Postgate. He showed that the deficiency was still present at high dilution rates, despite the addition of iron in the form of an iron–citrate complex. Consideration of the chemical equilibria involved shows that, under the conditions that typically arise in cultures of sulfate-reducing bacteria, citrate is in fact fairly ineffective as a complexing agent. The major equilibria that need to be considered are the acid–base equilibria of sulfide and citrate, the complexes that are formed between iron and citrate, and the solubility of FeS. Equilibrium constants for these reactions may be obtained from the compiled data presented by Sillen and Martell[14] and Martell and Smith.[15] At a pH of 7.0 and under conditions where the concentration of citrate is much greater than that of iron (as is the case for Postgate's medium), it may be shown that the concentration of uncomplexed iron (mM) is approximately given by

$$Fe^{2+} = \frac{T_{Fe}}{1 + T_c \cdot 10^{4.4}} \qquad (1)$$

where T_{Fe} represents the total iron concentration (mM) and T_c represents the total citrate concentration (M). The allowable concentration of sulfide (total of all dissolved species, mM), before precipitation will occur, may be calculated using this expression, together with the solubility product for iron sulfide. For the concentrations of iron and citrate in Postgate's medium (total citrate, 1.2mM; total iron, 0.025mM), this allowable sulfide concentration is 0.01mM or 0.3 mg/L. Measurement of total sulfide concentrations during the active growth phases of our cultures has yielded values in the range 0.1–3mM. The average value maintained was approximately 1mM. This was despite vigorous sparging with nitrogen to remove H_2S from solution. Accordingly, citrate would not be expected to prevent the precipitation of iron under normal culture conditions.

Equation (1) may also be used to calculate the total dissolved iron concentration under conditions where the allowable level of sulfide is exceeded. For a total dissolved sulfide concentration of 1mM, the total iron concentration in Postgate's medium is calculated to be 3.1×10^{-4}mM. This is only about 1% of the concentration initially added.

Exponential growth of pure cultures of sulfate-reducing bacteria was observed by Cappenberg,[16] who included EDTA in his growth medium. The same calculations may be carried out for EDTA as a complexing agent in place of citrate. The corresponding expression for the free iron concentration is

$$Fe^{2+} = \frac{T_{Fe}}{T_L \cdot 7.6 \times 10^{10}} \qquad (2)$$

Where T_L represents the total concentration of EDTA (M). At an EDTA concentration of 0.25mM (the concentration in the medium recommended in this article), the allowable concentration of sulfide is calculated to be 8M. Thus, at sulfide concentrations of about 1mM, EDTA would be expected to totally prohibit the precipitation of ferrous sulfide.

The Role of Yeast Extract

Another component of Postgate's medium which might inhibit iron precipitation is yeast extract. Postgate suggested that improved growth yields and growth rates, associated with the addition of yeast extract to media, were probably due to the presence of organic compounds such as amino acids, which have an ability to complex iron and thus make it more readily available.[12] Because it is not possible to calculate the effect of yeast extract on the availability of iron, the effectiveness of yeast extract as an "iron carrier" was investigated experimentally.

Various amounts of yeast extract were added to solutions containing 1.0mM sodium sulfide and 5.0mM KH_2PO_4. The pH was adjusted to 7.0 with HCl, and then aliquots of 50mM ferrous amonium sulfate were progressively added. The absorbance of the solutions was measured periodically using a UV–Visible spectrophotometer set at 570 nm. The 570-nm wavelength is commonly used in bacterial optical density measurements. The procedure was repeated using EDTA in place of yeast extract in order to compare the relative effect of these agents. Calibration of the procedure, using solutions containing no added complexing agent, showed that absorbance measurements were linear with respect to the amount of precipitated sulfide. The results are illustrated in Figure 2. Absorbance measurements were corrected for non-iron sulfide absorbance by subtracting the absorbance reading obtained prior to the addition of iron. The change in the solution volume, due to the addition of the iron, was

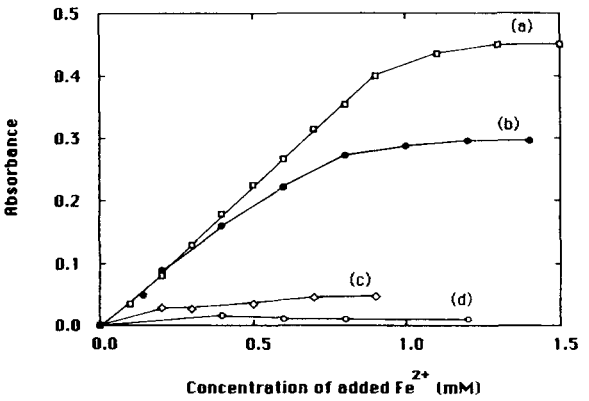

Figure 2. Absorbance measurements for solutions containing 1.0mM sulfide following the addition of increasing amounts of ferrous ion [(a) blank solution; (b) 0.5% w/v yeast extract; (c) 2.0% w/v yeast extract (d) 0.25mM EDTA].

negligible; and so it was not necessary to correct the absorbance readings for the dilution effect.

In Figure 2 it can be seen that the effect of yeast extract and EDTA reduces the total amount of ferrous sulfide precipitated. The total amount of FeS precipitated is given by the maximum absorbance level measured. The presence of 0.25m*M* EDTA has a greater effect than 2.0% w/v yeast extract, which in turn has a greater effect than 0.5% w/v yeast extract. Postgate's medium C contains only 0.1% w/v yeast extract, and so these results demonstrate that yeast extract has a marginal effect on the precipitation of iron when compared with 0.25 m*M* EDTA.

The small amount of precipitation that was observed in the presence of EDTA was found to be the result of adding the iron quickly. If the additions were made slowly, while agitating the solution, very little precipitation occurred. Iron sulfide precipitation in the presence of EDTA appears to be a kinetic effect resulting from the competition between the rate of iron sulfide formation and the rate of formation of the iron—EDTA complex. Following the addition of iron, the absorbance tended to decrease, indicating dissolution of the FeS precipitate. The same effect was noted when sodium sulfide was added to the culture media containing EDTA. After immediate formation of a black precipitate of FeS, the solution slowly cleared over a period of hours. This illustrates the fact that the FeS precipitate cannot be regarded as "insoluble." It will gradually redissolve so as to maintain the equilibrium that exists in solution between complexed iron and free iron.

It should be noted that EDTA, citrate, and yeast extract will form complexes with other cations besides ferrous ion and that there are numerous other chemical equilibria that should be considered.[17] The results presented above only illustrate the relative complexing ability of EDTA, citrate, and yeast extract under idealized conditions. Ions such as calcium and magnesium will reduce the effectiveness of complexing agents by forming complexes in competition with iron. Thus, under normal culture conditions, precipitation is likely to occur at lower sulfide concentrations than those calculated above. Nevertheless, the results presented clearly illustrate the relative complexing abilities of EDTA, citrate, and yeast extract.

Experiments in this laboratory, using the chemically defined media and EDTA, indicate that total sulfide concentrations of up to 10m*M* (320 mg/L) are possible without either observable iron precipitation or an adverse effect on the rate of sulfate reduction. This is coincidentally the concentration Leban et al.[11] found inhibitory to growth. An apparent inhibitory effect of lowered redox potential (E_h) has been reported by Brown et al.[18] and by Cappenberg.[16] In both of these studies, elevated sulfide concentrations were used as a means of decreasing the E_h. It is difficult to establish whether these inhibitory effects were a direct effect of sulfide or E_h on the bacteria or an indirect effect of iron sulfide precipitation.

The results suggest that nonexponential growth of cultures of sulfate-reducing bacteria may be attributed to the loss of readily available iron from solution. Under conditions where the dissolved iron concentration is very low, sulfate-reducing bacteria may obtain iron from the ferrous sulfide precipitate using highly specific chelating agents. These agents could be located on the surface of the cell or released into solution. Evidence for the former mechanism is contained in the observation that freshly isolated cultures of sulfate-reducing bacteria exhibit "tachophily,"[3] that is, a tendency to adhere to surfaces. This may be a genetic by-product of the stresses associated with a low-iron environment, which will inevitably arise from the continued release of sulfide. If the extraction of iron for growth is indeed a surface reaction, then under certain circumstances the rate of sulfate reduction may be limited by factors such as the rate of attachment to the precipitate, the available surface area, and the rate of incorporation of iron into the cell. It is possible that the second lag phase observed in this study reflects a period of attachment to ferrous sulfide, following the depletion of soluble iron. Further investigations are necessary for the mechanisms of these processes to be established.

Investigations of the nutritional requirements of sulfate-reducing bacteria of the genus *Desulfovibrio* have shown that yeast extract is not necessary for the growth of the bacteria[19,20] if sulfate and lactate are present. Macpherson and Miller[20] also demonstrated the growth of pure cultures of *Desulfovibrio* with yeast extract as the sole source of organic carbon. The carbon sources within yeast extract that are utilized by sulfate-reducing bacteria have not been identified. Their presence, however, gives rise to the possibility of diauxic growth. Two-phase growth has previously been observed in association with diauxic growth,[8] with the intermediate lag phase occurring as the culture adjusts from one carbon source to another. The two-phase growth observed in this and other studies[9–11] is possibly a result of diauxic growth of sulfate-reducing bacteria.

Diauxic growth of cultures complicates the study of the growth kinetics considerably. It is therefore desirable to use chemically defined media with a single carbon source. This applies to both pure and mixed microbial cultures. If bacteria have specific requirements for organic compounds other than the primary carbon source, then it is preferable to add these compounds individually rather than using a complex organic mixture such as yeast extract. For the case of sulfate-reducing bacteria of the very common genus *Desulfovibrio,* most species do not require any special organic factors for growth.[3] Thus, there seems to be little point in complicating kinetic studies by including yeast extract in the growth medium.

Bacterial Growth on Chemically Defined Media with EDTA

Regardless of reasons for nonexponential growth, there is still a need to establish the quantitative effects of nutrients such as lactate and sulfate on the growth rate of sulfate-reducing bacteria. Much of the available data on the kinetics of bacterial sulfate reduction has been obtained without consideration of the factors discussed in this article. To obtain meaningful data for the evaluation of the effects of a particular nutrient on the growth rate, it is necessary to ensure that iron precipitation is minimized and that the nutrient in question is the sole limiting substrate. Accordingly, a chemically defined medium, which included EDTA as an iron complexing agent, was developed. The medium was based on that of Macpherson and Miller.[20]

Using the chemically defined medium, single-phase, exponential growth was observed in batch cultures. Figure 3 shows a plot of the sulfate uptake and turbidity generation of a typical batch culture with the chemically defined medium. After an initial lag phase of about 10 h duration, growth proceeded in a smooth manner until sulfate was depleted. The time required to completely reduce 500 mg/L of sulfate was about 15 h. This compares with a corresponding period of about 30 h for cultures growing on Postgate's medium. The specific growth rate was determined by plotting the natural logarithm of the sulfate uptake as a function of time.

Exponential growth was evident from a semi-log plot of sulfate uptake versus time, and also from a semi-log plot of turbidity versus time (Fig. 4). The former plot was obtained by subtracting the sulfate concentration at time t (S_t) from the initial sulfate concentration (S_0). This plot is probably more significant since it relates directly to the activity of sulfate-reducing bacteria. The estimated error due to the analytical procedures (2σ) was only significant for the early concentration measurements, where there is relatively small difference between S_0 and S_t. For those cases where

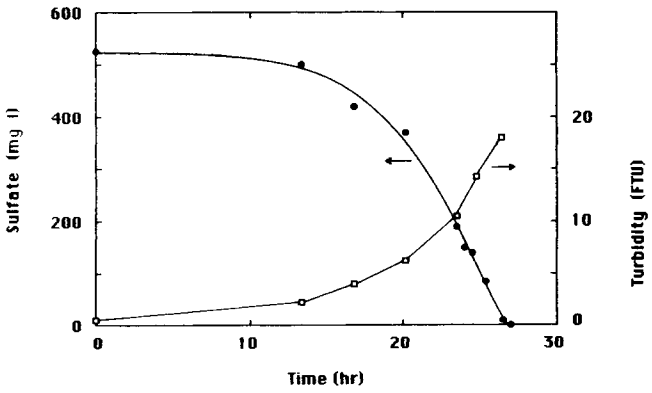

Figure 3. Turbidity generation (-□-) and sulfate removal (-●-) during batch culture with chemically defined media incorporating EDTA.

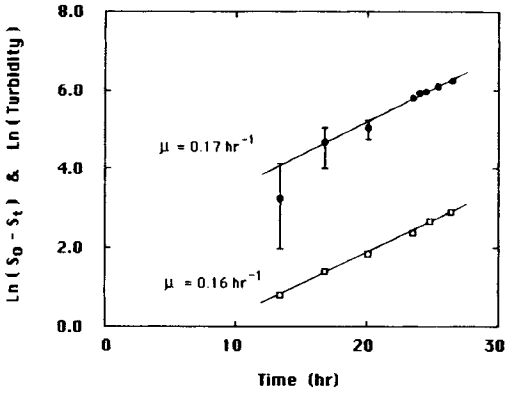

Figure 4. Semi-log plot of turbidity versus time (-□-) and sulfate uptake versus time (-●-) during batch culture with chemically defined media incorporating EDTA.

the error was significant, the standard error is given by the error bars illustrated in Figure 4.

The specific growth rate (μ) of the sulfate reducers may be obtained from the slope of the log-linear plot, if it is assumed that the yield of sulfate-reducing bacteria is constant with respect to sulfate uptake. For the data presented in Figure 4, μ was found to be 0.17 h^{-1}. This compares with a value of 0.16 h^{-1}, obtained from the bacterial density measurements. Values for the specific growth rate calculated in this manner were in the range 0.17–0.27 h^{-1}, for cultures grown under sulfate limitation. These results are comparable with specific growth rate measurements reported for pure cultures of sulfate-reducing bacteria grown on media containing yeast extract.[12,13,16,18,21]

Another feature of the growth on the chemically defined medium was the reduction in the overall cell yield. Cell yields with Postgate's medium were greater than 0.2 mg cells/mg sulfate utilized. When the chemically defined medium was used, the cell yield was approximately 0.1 mg dry weight cells/mg sulfate utilized. This reduction in yield is not a result of the prevention of iron sulfate precipitation. Measurements of the turbidity of solutions containing precipitated FeS showed that the turbidity decreased with increasing concentrations of FeS. The black precipitate tends to absorb the incident light, rather than scattering it. Since turbidity is a measure of the amount of scattered light, the presence of FeS causes a lower turbidity reading than would normally be expected.

The reduction in cell yield could result from increased energy requirements of sulfate-reducing bacteria, as a result of the need to synthesize organic components normally contained in yeast extract. Postgate[3] indicated that the expected yield for *Desulfovibrio* growing on medium C is about 0.13 mg dry weight cells/mg sulfate utilized. This is quite close to the yield obtained in the chemically defined medium and suggests that the chemically defined medium is simply more "selective" for bacteria with simple nutritional requirements (such

as *Desulfovibrio spp.*). Further evidence for this is given by the similarity between the values for the specific growth rate, as calculated from the sulfate uptake, and bacterial density data. The concordance between these values was found to be a consistent feature of batch cultures using the chemically defined media. It is probable that bacterial species with more complex nutritional requirements were disadvantaged by the removal of yeast extract from the medium.

CONCLUSIONS

1. Postgate's medium C was found to be unsuitable for quantitative studies of mixed microbial cultures containing sulfate-reducing bacteria.

2. Equilibrium calculations of iron concentration, along with the experimental observations show that precipitation of iron occurs in Postgate's medium, despite the presence of citrate and yeast extract.

3. The often-ascribed inhibitory effect of sulfide on the growth of sulfate-reducing bacteria may be largely due to the reduced availability of iron, as a result of the precipitation of ferrous sulfide.

4. Because it is essential to clearly define the growth-limiting nutrient when carrying out quantitative bacterial growth studies, yeast extract should be excluded from the culture medium.

5. Single-phase, exponential growth was observed for mixed cultures containing sulfate-reducing bacteria, using a chemically defined medium that incorporated the metal complexing agent EDTA.

6. When carrying out quantitative kinetic studies of sulfate-reducing bacteria, a chemically defined medium incorporating EDTA is recommended.

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