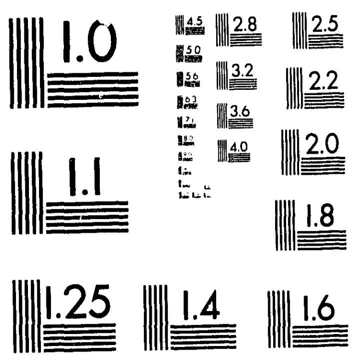
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MICROBIAL REDUCTION OF SO_2 AND NO_x AS A MEANS OF BY-PRODUCT RECOVERY/DISPOSAL FROM REGENERABLE PROCESSES FOR THE DESULFURIZATION OF FLUE GAS

Project No. DE-FG22-90PC90096

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Technical Progress Report

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1. INTRODUCTION

1.1 Statement of the Problem

With the continual increase in the utilization of high sulfur and high nitrogen containing fossil fuels (particularly coal and sour petroleum crudes), the release of airborne pollutants into the environment has become a critical problem. The bulk of the fuel sulfur is converted to SO_2 during combustion. Fuel nitrogen and a fraction of the nitrogen from the combustion air are converted to nitric oxide and nitrogen dioxide, NO_x. A typical 1000 MW boiler, for example, burning 3.5% sulfur coal will emit approximately 600 tons of SO_2 and 100 tons of NO_x per day. Sulfur dioxide and oxides of nitrogen react photochemically or catalytically with other atmospheric contaminants to produce smog and the principal components of "acid rain". Acid rain is rapidly becoming an alarming problem, especially in the northeastern part of the United States and in Canada.

There are several engineering solutions to this problem, although none alone satisfy all of the desired technical and economic requirements. There are two basic approaches to addressing the problem of SO_2 and NO_x emissions: (1) desulfurize (and denitrogenate) the feedstock prior to or during combustion; or (2) scrub the resultant SO_2 and oxides of nitrogen from the boiler flue gases. Although feedstock desulfurization and allied technologies (e.g., coal liquefaction) are of considerable interest, the flue gas processing alternative has been addressed in this project.

The most commercially important flue gas desulfurization technology at present is the use of solid, throwaway adsorbents such as limestones and dolomites which have affinity for acid gases like

SO_2. This type of process results in the production of large amounts of calcium sulfate ($CaSO_4$) which can represent a significant disposal problem. In addition, little or no NO_x removal is achieved.

Several of the more promising technologies under development combine SO_2 and NO_x removal. These include radiation-initiated processes, low-temperature, dry-scrubbing and regenerable, dry-scrubbing. The irradiation of flue gases with electron beams or microwaves can result in the oxidation of SO_2 and NO_x to their respective acids under the proper conditions of temperature and moisture. The process requires the addition of large quantities of ammonia to retard the formation of corrosive sulfuric acid. The oxidation products are recovered as ammonium sulfate and ammonium nitrate. In low-temperature, dry-scrubbing processes a lime sorbent is sprayed into the flue gases at 300-400 F. A dry waste of $CaSO_4$ and unreacted sorbent is produced. No NO_x removal is obtained without additives to the sorbent. In regenerable, dry-scrubbing processes, as the name implies, flue gas is contacted with a dry sorbent resulting in the chemisorption of SO_2. The sulfated sorbent is subsequently regenerated using a reducing gas such as hydrogen, carbon monoxide or methane. The two major regenerable, dry-scrubbing processes under development are the copper oxide process and the NOXSO process. In the copper oxide process NO_x is catalytically reduced to elemental nitrogen with ammonia. Regeneration of the copper oxide sorbent produces a concentrated stream of sulfur dioxide. In the NOXSO process, the sorbent consists of sodium aluminate ($NaAlO_2$) on gamma alumina. The sorbent also adsorbs or chemisorbs NO_x from flue gas. The NO_x chemisorption product is unstable above 400 C. During regeneration, heating the sorbent in air to 600 C produces a concentrated NO_x stream. Subsequent treatment of the sorbent with a reducing gas produces a mixture of SO_2, H_2S and elemental sulfur (1-3).

1.2 A Microbiological Contribution to the Problem of Flue Gas Desulfurization and NO_x Removal

For the past five years Combustion Engineering (now Asea Brown Boveri or ABB) and, since 1986, the University of Tulsa (TU) have been investigating the oxidation of H_2S by the facultatively anaerobic and autotrophic bacterium *Thiobacillus denitrificans* and have developed a process concept for the microbial removal of H_2S from a gas stream (4-7). K. Sublette has been the principal investigator. In 1987/88 we (K. Sublette and student Badri Dasu) demonstrated that the sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, can be grown anaerobically in mixed, septic cultures using SO_2 as terminal electron acceptor and glucose as the sole carbon and energy source (8). In these cultures SO_2 was completely reduced to H_2S with contact times of 1-2 s. This work was funded by ABB. In 1988/89, under DOE contract number DE-FG22-88PC88945, the simultaneous removal of SO_2 and NO by *D. desulfuricans* and *T. denitrificans* co-cultures and cultures-in-series was demonstrated. However, these systems could not be sustained due to NO inhibition of *D. desulfuricans*. These observations and others to be detailed subsequently led to the conclusion that simultaneous removal of SO_2 and NO_x by direct contact of cooled flue gases with microbial cultures is not feasible at this time. However, a preliminary economic analysis has shown that microbial reduction of SO_2 to H_2S with subsequent conversion to elemental sulfur by the Claus process is both technically and economically feasible if a less expensive carbon and/or energy source can be found compared to glucose. Therefore, microbial reduction of SO_2 is a viable process concept for by-product recovery from regenerable flue gas desulfurization processes which produce concentrated streams of SO_2.

In addition, under the above named DOE contract, it has also been demonstrated that *T. denitrificans* can be grown anaerobically on NO(g) as a terminal electron acceptor with reduction to elemental nitrogen. This capability may be common to facultatively anaerobic bacteria which can use nitrate as a terminal electron acceptor. Therefore, microbial reduction of NO_x is a viable process concept for the disposal of concentrated streams of NO_x as may be produced by certain regenerable processes for the removal of SO_2 and NO_x from flue gas.

Previous work with the microbial reduction of SO_2 and NO is reviewed in Section 2.

2. REVIEW OF WORK PREVIOUS TO THE CURRENT PROJECT

2.1 Sulfate-Reducing Bacteria

Sulfur compounds are an essential component of most living things. However, the oxidized forms of sulfur (sulfates and SO_x) must be reduced in order to be mobilized for biological use. The biological reduction of sulfates for incorporation into cellular material is called assimilatory sulfate reduction. Some microorganisms are also capable of utilizing sulfates as terminal electron acceptors with reduction to sulfide. This process is called dissimilatory sulfate reduction and is a unique characteristic of sulfate-reducing bacteria (SRB). Most of the sulfide produced by this process accumulates outside of the cell and is eventually hydrolyzed to form free H_2S and released into the environment. The SRB comprise the following genera: *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum*, *Desulfobacter*, *Desulfobacterium*, *Desulfobulbus*, *Desulfonema*, *Desulfosarcina* and *Thermodesulfobacterium*. As seen in Figure 1, the SRB play a key role in the sulfur cycle in nature.

The SRB are typically strict anaerobes; mere exclusion of oxygen from culturing media is insufficient to support growth in pure cultures. Redox-poising agents are generally required to maintain a redox potential in the medium of -150 to -200 mV. Most SRB are nutritionally restricted to certain mono- and dicarboxylic acids (acetate, pyruvate, lactate, etc.), alcohols (ethanol, butanol, etc.) and certain amino acids as carbon and energy sources. These compounds are recognized as fermentation products of other heterotrophic bacteria (9).

Most strains of SRB can utilize sulfite as well as sulfate as a terminal electron acceptor (9). For example, in a chemostat study, Hill *et al* (10) cultivated *Desulfovibrio* with sulfite and sulfate as terminal electron acceptors using lactate as an electron donor and carbon source. The yield of biomass per mole of electron acceptor reduced was greater for sulfite (9.2 g/mole) than for sulfate (6.3 g/mole). The greater yield on sulfite was attributed to the fact that no ATP is expended in its activation, unlike sulfate (9).

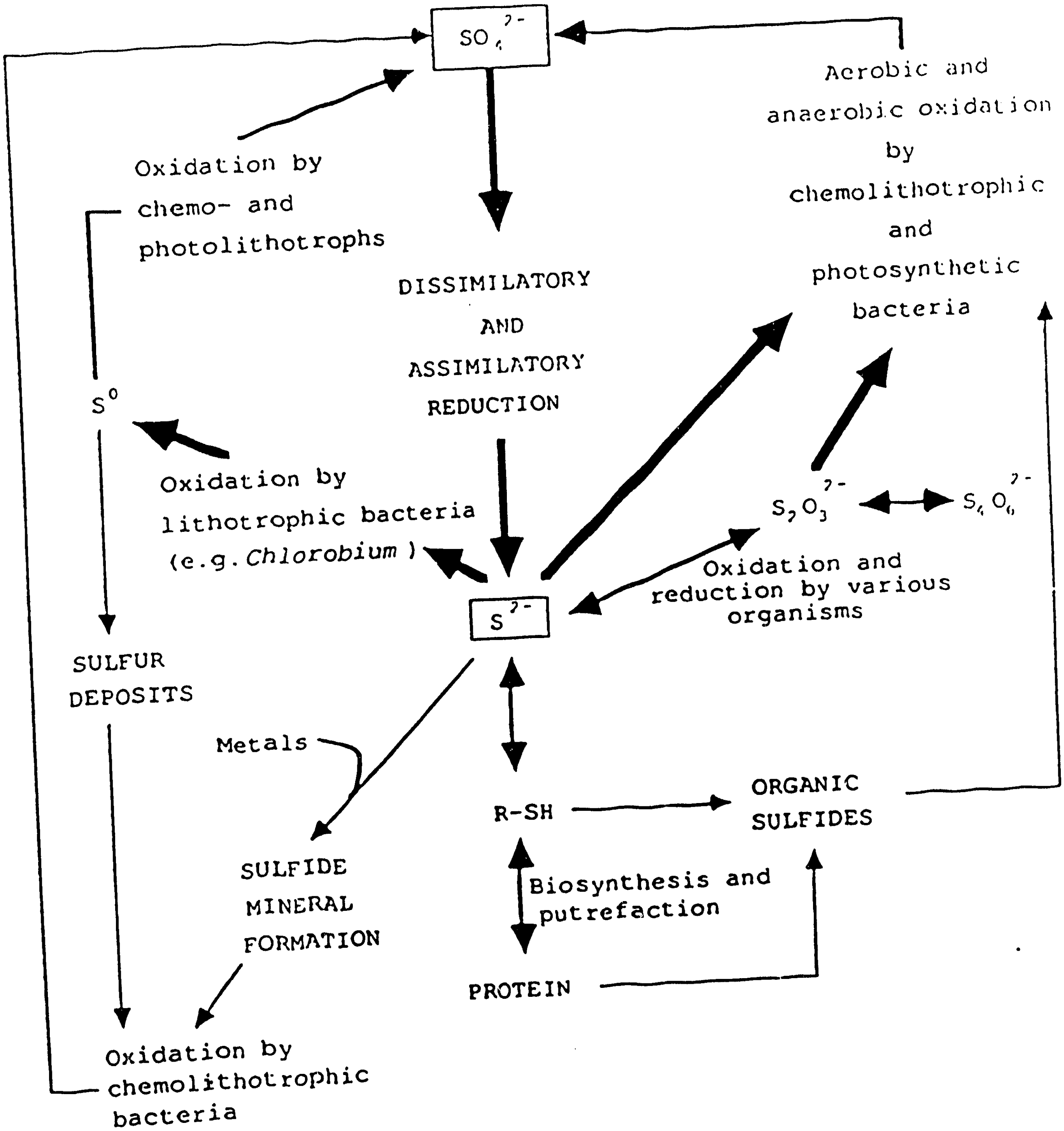


Figure 1. The essential features of the global biological Sulfur Cycle.

2.2 Microbial Reduction of SO_2

2.2.1 Microbial Removal of SO_2 From a Gas - Work Previous to DOE Contract

As noted previously, in 1987/88, a microbial process was developed on the bench scale to remove SO_2 from a gas stream with reduction to H_2S or net oxidation to sulfate. Reduction of SO_2 to H_2S was accomplished by contact of the gas with a co-culture of the SRB *D. desulfuricans* and mixed non-SRB, fermentative heterotrophs. Working cultures of *D. desulfuricans* were developed as follows. *D. desulfuricans* was grown septically in a complex glucose medium in a 2-L bench scale fermenter at pH 7.0 and 30 C. Sulfate was the terminal electron acceptor. The culture was purged with about 300 mL/min of nitrogen to strip H_2S. Septic operation resulted in the development of a large population of non-*Desulfovibrio*, heterotrophic bacteria. After 24 hr, cells were harvested by centrifugation, the supernatant discarded and cells resuspended in a glucose minimal medium, again with sulfate as the terminal electron acceptor. The resuspended cells were transferred back to the fermenter and grown in this medium for another 24 hr to acclimate cells to the minimal medium prior to the introduction of SO_2. At the end of this incubation cells were once again harvested by the method described above, then resuspended in the same minimal medium without sulfate and transferred back to the fermenter. At this time a gas mixture containing 0.99 mole % SO_2, 5% CO_2 and balance nitrogen was introduced at 34 mL/min corresponding to a molar SO_2 feed rate of 0.78 mmoles/hr. The culture also continued to receive a nitrogen purge of about 300 mL/min. Ten g/L glucose was added every 24 hr. The outlet gas from the fermenter was transferred to a culture of *T. denitrificans* which served to trap H_2S.

As noted in Section 2.1, sulfate-reducing bacteria do not use simple sugars (such as glucose) as carbon and energy sources. However, in septic cultures which utilized glucose as the sole carbon source, vigorous growth of *D. desulfuricans* was observed. Working cultures containing greater than 5 x 10^8 cells/mL were approximately 50% *D. desulfuricans*. Apparently, the fermentative heterotrophs which developed in the cultures as a result of septic operation utilized glucose and produced fermentative end products which then served as carbon and energy sources for *D. desulfuricans*. It should also be noted that the *D. desulfuricans* working cultures used in these experiments did not require redox-poising agents. Apparently, the mixed non-SRB heterotrophs in the cultures scavenged oxidants and thus kept the redox potential sufficiently negative to favor the growth of *D. desulfuricans*.

In a typical experiment in which SO_2 served as the terminal electron acceptor for *D. desulfuricans* (Exp. #062), SO_2 was fed to the reactor at a molar flow rate of 0.78 mmoles SO_2/hr. Under SO_2-limiting conditions no sulfite, sulfide or elemental sulfur could be detected in the culture medium. Complete removal of SO_2 from the feed gas was evidenced by the lack of sulfite accumulation in the *T. denitrificans* culture downstream. (Previous experiments demonstrated that sulfite immediately accumulates in the medium of a *T. denitrificans* culture when SO_2 is introduced.) Analysis of the off-gas from the *D. desulfuricans* reactor showed a steady concentration of H_2S of approximately 800 ppmv. The results of a sulfur balance performed on the *D. desulfuricans* reactor in three experiments of this type are given in Table 1. For the specific experiment under discussion here (Exp. #062), 69.0 mmoles of SO_2 were fed to the reactor and 63.7 mmoles of H_2S were detected in the off-gas. In these three experiments the ratio of H_2S produced to SO_2 consumed averaged 0.95. The detection limit for the gas chromatograph used to analyze the offgas for H_2S was 50 ppmv (thermal conductivity detector). Therefore, the H_2S analyses used in these calculations may have slightly underestimated the actual H_2S concentration. The true H_2S/SO_2 ratio is, therefore, very likely 1.0. In other words, all SO_2 was converted to H_2S.

As SO_2 was removed from the feed gas, the total biomass protein concentration and the *D. desulfuricans* and total non-SRB heterotroph counts increased. In Exp. #062, the aforementioned reduction of 69.0 mmoles of SO_2 was accompanied by the production of 0.79 g of biomass protein. The average ratio of biomass protein produced to SO_2 reduced was 11.4 g/mole. Assuming *D. desulfuricans* and the non-SRB heterotrophs to be approximately 50% protein by dry weight and noting that working cultures were approximately 50% *D. desulfuricans*, this yield is seen to be comparable to that reported by Hill *et al* (10) for growth of *Desulfovibrio* on lactate and sulfite.

As noted above, H_2S produced in the *D. desulfuricans* reactor was stripped with nitrogen and fed to a second stage containing a mixed culture of *Thiobacillus denitrificans* and various heterotrophs. In this stage H_2S was completely oxidized to sulfate anaerobically with nitrate as terminal electron acceptor. With gas-liquid contact times of 1-2 seconds the H_2S concentration in the feed gas (about 800 ppmv)

TABLE 1. Sulfur Balances on *D. desulfuricans* Reactors.

	Exp. # 062	Exp. # 090	Exp. # 091
mmoles SO_2 consumed	69.0	90.9	61.6
mmoles H_2S produced	63.7	86.2	60.4
H_2S/SO_2 (mole/mole)	0.92	0.95	0.98

was reduced to less than 1 ppmv. The oxidation of H_2S by *T. denitrificans* has been described in detail by Sublette and Sylvester elsewhere (4-7).

In each experiment described above, the SO_2 feed rate was always less than the maximum specific activity of the biomass for SO_2 reduction (SO_2-limiting conditions). A study was also conducted to investigate the effects of excess SO_2 feed and determine the maximum specific activity of the *D. desulfuricans* biomass for SO_2 reduction. Specifically, the SO_2 feed rate to working cultures developed as described above was increased in a stepwise manner. The H_2S concentration in the off-gas and the sulfite concentration in the culture medium were monitored accordingly. The stepwise increase in the SO_2 feed rate was continued until sulfite began to accumulate in the culture medium and further increases in the SO_2 feed rate resulted in a disproportionate increase in the H_2S concentration in the off gas. This was considered to be an upset condition. The molar SO_2 feed rate at this point was considered the maximum rate of SO_2 reduction by the SRB cells in the culture. The *D. desulfuricans* count was then determined and the maximum specific activity calculated as the ratio of the maximum SO_2 feed rate to the total SRB cells in the culture. The maximum specific activity of *D. desulfuricans* for SO_2 reduction was estimated to be 1.69 $\pm$ 0.04 mmoles SO_2/hr-10^{11} cells.

2.2.2 Microbial Removal of SO_2 From a Gas - Work Performed Under DOE Contract No. DE-FG22-88PC88945

2.2.2.1 Growth of *D. desulfuricans* and Mixed Heterotrophs on Molasses With Reduction of SO_2 to H_2S

In the United States the cost of starch hydrolysate, sucrose and cane molasses are comparable when compared on a $/ton of carbohydrate basis (11). Starch hydrolysate and sucrose in bulk represent relatively pure sources of easily fermentable sugars. On the other hand the composition of cane or beet molasses depends on several factors including location of cultivation, soil type, climate, and processing. In addition to glucose, fructose and sucrose, molasses also contain a high concentration of organic non-sugars. These factors combine to make molasses an undesirable feed stock for fermentations in this country. However, in Europe the cost of starch hydrolysate and sucrose are much higher than that of molasses resulting in much greater use of molasses as a feed stock (11). For this reason the reduction of SO_2 to H_2S by cultures of *D. desulfuricans* and mixed heterotrophs in which molasses was used as the ultimate source of carbon and energy was investigated.

The methodology of these experiments was much the same as that reported in Section 2.2.1 except that molasses (30 g/L) was substituted for glucose. It was observed that working cultures of *D. desulfuricans* could be prepared with molasses in a manner identical to that used with glucose. Vigorous growth of *D. desulfuricans* was observed in either complex or minimal medium with molasses as the source of carbohydrate. Cultures (1.5 L) were maintained for up to two weeks batch-wise with daily addition of 30 g/L molasses at an SO_2 feed rate of 0.78 mmoles/hr. Complete reduction of SO_2 to H_2S was indicated. Interestingly, however, greater than stoichiometric production of H_2S was observed. The production of H_2S surged after each addition of molasses and did not return to normal (stoichiometric) for 20-24 hr. This has been attributed to the presence of sulfate-S in the molasses which provided a source of terminal electron acceptor in addition to the SO_2 feed. Although difficult to analyze turbidometrically because of their dark color, aqueous solutions of the molasses feedstock (Plantation brand blackstrap molasses) were found to contain significant amounts of material precipitated by $BaCl_2$.

A number of experiments were conducted in which after 2-3 days of operation on SO_2, molasses addition was terminated and the behavior of the reactor monitored. One purpose of these experiments was to relate the utilization of the molasses sugars to time course of SO_2 reduction. The second purpose was to estimate the total SO_2 reduced per unit weight of sugar. The total sugars were essentially depleted 10 hr after the last molasses addition. However, the reactor continued to reduce SO_2 to H_2S with no sulfite accumulation for an additional 93 hr after the terminal addition of molasses. The probable explanation is that the sugars were metabolized by the mixed non-SRB heterotrophs much faster than the *D. desulfuricans* could utilize the end products of the fermentation of those sugars. Therefore, the *D. desulfuricans* continued to have these fermentative end products available as carbon and energy sources long after the sugars disappeared from the medium. The approximate amount of SO_2 which can be reduced per unit weight of sugar in molasses cultures was estimated from the amount of SO_2 reduced from the time of terminal molasses addition until sulfite began to accumulate in the medium. This was found to be approximately 0.22 g SO_2/g of sugar.

2.2.2.2 Material Balances for SO_2 Reduction in Glucose-Fed *D. desulfuricans* Reactors

In order to evaluate the microbial reduction of SO_2 to H_2S from both a technical and economics point of view, it was necessary to understand the flow of carbon from glucose (or other carbohydrate) to the fermentative non-SRB heterotrophs in the process culture, to the sulfate-reducing bacterium (*D. desulfuricans*) and finally to the end products of the process.

Working cultures of *D. desulfuricans* were prepared on glucose as described in Section 2.2.1. During growth on SO_2 the culture medium was sampled periodically and analyzed by gas chromatography. The following metabolites of glucose were identified: ethanol, lactic acid, acetate, propionate, butyrate and isobutyrate. Ethanol and lactic acid are common end products of the anaerobic fermentation of glucose. Lactic acid and ethanol are also recognized as carbon and energy sources for *D. desulfuricans*. Acetate, propionate, isobutyrate and butyrate have also been observed as end products of the oxidation of lactic acid and ethanol by *D. desulfuricans*. (Carbon dioxide is also formed from lactate.)

A marked increase in ethanol, lactate, acetate and butyrate concentrations followed fed-batch glucose additions. In some cases, glucose addition was terminated and the behavior of the reactor monitored until an upset condition was produced. In these experiments the lactic acid concentration fell essentially to zero within 24 hr of the last glucose addition. The ethanol concentration declined more gradually from 2-3 g/L to zero in about 96-120 hr. When ethanol was depleted, sulfite began to accumulate in the reactor medium and the

H_2S concentration in the off-gas declined indicating less than stoichiometric conversion of SO_2 to H_2S. Acetate accumulated while ethanol was depleted.

Material balances for two such experiments are given in Table 2. In summary, it appears that in these cultures, ethanol and to a lesser extent, lactic acid, were produced from the fermentation of glucose by the non-SRB heterotrophs in the cultures. Ethanol and lactic acid were then used as carbon and energy sources by *D. desulfuricans* with oxidation to acetate which accumulated in the medium. Acetate may also have been produced by glucose fermentation by the non-SRB heterotrophs. The exact role of propionate, butyrate and isobutyrate are uncertain. As seen in Table 2, the observed SO_2/glucose ratio was 0.5 mole/mole. If glucose were oxidized completely to CO_2 and H_2O this ratio would theoretically be 4.0 (with no allowances made for reducing equivalents for biosynthesis). Therefore greater SO_2 reduction per mole of carbohydrate could be obtained by incorporating SRB which can utilize acetate as a carbon and energy source with oxidation to CO_2.

2.2.2.3 Effect of O_2 on SO_2 Reduction by *D. desulfuricans* in Mixed Culture

Excess air in the combustion process results in oxygen in the flue gas. Therefore, any microbial process in which flue gases may be directly contacted with the culture must be resistant to oxygen. Given the strict requirements of SRB for a reducing environment the effects of O_2 on SO_2 reduction by *D. desulfuricans* were investigated.

The effect of oxygen on the reduction of SO_2 by *D. desulfuricans* in mixed culture was investigated utilizing glucose-fed cultures

TABLE 2. Material Balances for SO_2 Reduction in Glucose Fed *D. desulfuricans* Reactors

	Exp A	Exp B
Glucose utilized (g)	45.0	30.0
SO_2 reduced (g)	7.9	5.6
Acetate produced (g)	15.7	11.1
Propionate produced (g)	3.6	3.3
Butyrate produced (g)	1.7	0.5
Isobutyrate produced (g)	0.12	0.08
SO_2/Glucose (g/g)	0.18	0.19
SO_2/Glucose (mole/mole)	0.49	0.52
Acetate/Glucose (g/g)	0.35	0.37
Acetate/Glucose (mole/mole)	1.04	1.06

developed as described in Section 2.2.1. Air was introduced to cultures operating with an SO_2 feed and the redox-potential and sulfite concentration in the medium and the H_2S concentration in the outlet gas monitored. In one experiment, air was introduced stepwise from an inlet concentration of 0.9% to 6.3% O_2 over a period of 24 hr. The redox-potential increased accordingly, as expected. Sulfite began to accumulate in the medium at an inlet concentration of 4.5% O_2.

In a similar experiment, air was introduced at a sufficient rate to give an inlet O_2 concentration of 1.7% and held at this level for 5 days. During this time, the redox-potential was never higher than -130 mV, stoichiometric conversion of SO_2 to H_2S was observed and no sulfite accumulated in the medium.

If oxygen becomes limiting in the combustion process, carbon monoxide will be formed. As a footnote it was also of interest to investigate the possible effects of a transient exposure to CO on SO_2 reduction by *D. desulfuricans*. It was observed that a CO partial pressure of 25 mm Hg resulted in sulfite accumulation and less than stoichiometric production of H_2S indicating CO inhibition. However, a partial pressure of 12 mm Hg could be tolerated at least 8-10 hr.

2.2.2.4 Identification of Non-SRB Heterotrophs in *D. desulfuricans* Cultures

As noted previously, the non-SRB heterotrophs responsible for carbohydrate fermentation and the production of carbon and energy sources suitable for the sulfate reducing bacteria arise in working cultures simply from septic operation of the reactor. Obviously, in order to avoid variability it will eventually be necessary to use a standard inoculum for these cultures. Therefore, we identified several non-SRB heterotrophs which developed in these reactors. Those bacterial identified thus far are listed in Table 3. The relative importance of each of these species will be determined from its metabolic capabilities compared to the nature of the carbon and energy sources found in *D. desulfuricans* reactors as they are ultimately defined.

2.2.2.5 Effect of NO on SO_2 Reduction by *D. desulfuricans* Cultures

As noted in Section 1.3 it is conceivable that both SO_2 and NO_x can be removed from a gas stream by contact with *D. desulfuricans* and *T. denitrificans* cultures in series or co-cultures containing both organisms and mixed fermentative heterotrophs. Sulfite is inhibitory to *T. denitrificans;* therefore, with reactors-in-series the first stage would be a *Desulfovibrio* reactor operated under SO_2-limiting conditions. For this option to be viable the population in the first stage would need to be tolerant of NO_x. Therefore in preparation for a demonstration of simultaneous SO_2/NO_x removal from a gas described in a later section, the effect of NO on SO_2 reduction by *D. desulfuricans* was investigated.

The effect of NO on SO_2 reduction by *D. desulfuricans* was investigated by utilizing glucose-fed cultures developed as described in Section 2.2.1. In a typical experiment SO_2 was fed to the *D. desulfuricans* reactor at a molar feed rate of 0.78 mmoles/hr together with N_2 purge. The reactor was maintained at these conditions for 48 hr. During this time sulfite was undetectable in the liquid phase and stoichiometric production of H_2S in the outlet gas was observed. NO (0.49% NO, balance N_2) was then introduced.

TABLE 3. Non-SRB Heterotrophs Found in *D. desulfuricans* Reactors Operating With a SO_2 Feed and Their End Products of the Fermentation of Glucose

Organism	End Products
Enterococcus faecium	lactate, ethanol, acetate
Escherichia coli	lactate, acetate, formate
Citrobacter freundi	lactate, acetate, formate
Citrobacter diversus	lactate, acetate, formate
Klebsiella pneumoniae	2,3-butanediol, lactate, acetate, ethanol, formate
Klebsiella pneumoniae ozaenae	2,3-butanediol, lactate, acetate, ethanol, formate
Enterobacter agglomerana	2,3-butanediol, lactate, acetate, ethanol, formate
Clostridium bifermentaus	isobutyrate, isovalerate, isocaprate, butyrate, ethanol, propanol, isobutanol
Salmonella arizonae	lactate, acetate, formate
Enterobacter cloacae	2,3-butanediol, lactate, acetate, ethanol, formate

The results of these experiments are summarized in Table 4. At NO concentrations in the feed gas of 1500 ppmv, sulfite began to accumulate in the culture medium and less than stoichiometric conversion of SO_2 to H_2S was observed indicating inhibition of SO_2 reduction by *D. desulfuricans*. During the entire course of these experiments the redox-potential of the culture medium remained essentially constant at -150 mV. This observation suggests a specific toxic effect of NO rather than inhibition due to loss of a reducing environment.

2.3 Reduction of NO to N_2 by *Thiobacillus denitrificans* - Work Performed Under DOE Contract No. DE-FG22-88PC88945

2.3.1 Removal of NO From a Gas

As noted above, *T. denitrificans* is a facultative anaerobe which can utilize nitrate as an oxidant in the absence of oxygen with reduction to elemental nitrogen. Nitric oxide has been shown to be an intermediate in the reduction of nitrate to elemental nitrogen in *T. denitrificans*. Ishaque and Aleem (12) and Baldensperger and Garcia (13) have demonstrated that whole cells of *T. denitrificans* will catalyze the reduction of nitric oxide to elemental nitrogen with a concomitant oxidation of thiosulfate (electron donor). However, these experiments utilized "resting cells"; that is, the cells were not actively growing and reproducing. Prior to this work it was unknown whether nitric oxide would support the anaerobic growth of *T. denitrificans*.

Working cultures of *T. denitrificans* were prepared as follows. *T. denitrificans* was grown in thiosulfate medium (4) under anaerobic conditions at 30 C and pH 7.0. This medium contained thiosulfate as

TABLE 4. Effect of NO on SO_2 Reduction by *D. desulfuricans*

NO Feedrate[1] (ml/min)	Total Gas Feed[2] (ml/min)	[NO] (ppmv)	Stoichiometric[3] $[H_2S]$, ppmv	Exp. 1 $[H_2S]$, ppmv	Exp. 2 $[H_2S]$, ppmv
0	388	0	800		
36	424	420	732	700	
71	460	760	675	650	700
107	495	1060	626	600	
143	531	1320	584		600
179	567	1550	547	400[4]	
214	602	1740	515		400[4]

1 0.49% NO in N_2; 24 hr exposure at each feedrate

2 Total gas feed consisted of SO_2 feed gas, N_2 purge and NO feed gas

3 The stoichiometric concentration of H_2S declines as the gas is diluted by NO feed gas

4 Sulfite accumulation in the medium

an energy source, nitrate as a terminal electron acceptor, ammonium ion as a source of reduced nitrogen, a phosphate buffer and trace minerals. Carbon dioxide (5% CO_2, balance N_2) was bubbled through the reactor as a carbon source. When the culture reached an OD_{460} of approximately 0.8 (10^8-10^9 cells/mL), cells were harvested by centrifugation, washed and resuspended in the fresh thiosulfate medium without nitrate. At this time a gas feed of 0.49% NO, 5% CO_2, balance N_2 was initiated at approximately 8 L/hr (B. Braun Biostat M, 1.5 L culture, agitation rate 500-900 rpm). During batch fermentations of up to 6 days, NO was continually removed from the feed gas to produce an outlet concentration of 200-300 ppmv (94-96% removal).

As NO was removed from the feed gas the concentration of thiosulfate and ammonium ion declined in the culture medium with a corresponding increase in the optical density and the biomass protein and sulfate concentrations. Little or no elemental sulfur was observed to accumulate in the medium. Growth of *T. denitrificans* on thiosulfate as energy source and NO as terminal electron acceptor was clearly indicated. In a typical experiment the oxidation of 45.8 mmoles of thiosulfate was accompanied by the reduction of 190.1 mmoles of NO, the utilization of 47 mmoles of NH_4^+, the production of 188 mg of biomass protein and the accumulation of 90 mmoles of sulfate. The ratio of sulfate produced to thiosulfate consumed was 1.97.

The purely chemical reduction of NO by $S_2O_3^{2-}$ would be given by the equation below.

$$S_2O_3^{2-} + 4NO \longrightarrow 2SO_4^{2-} + 2N_2$$

Therefore, the $NO/S_2O_3^{2-}$ ratio for pure chemical reaction would be 4.0. However, if NO was used to support growth of *T. denitrificans* as a terminal electron acceptor, a $NO/S_2O_3^{2-}$ ratio of less than 4.0 was expected since some of the electrons derived from the oxidation of $S_2O_3^{2-}$ would be used as reducing equivalents for biosynthesis (growth). The stoichiometry of NO reduction by *T. denitrificans* with thiosulfate as energy source from four duplicate experiments is given in Table 5. An average $NO/S_2O_3^{2-}$ ratio of 4.1 was obtained. The discrepancy between this analysis and the data presented in Table 5 has been attributed to errors in gas analysis.

In control experiments without biomass, NO broke through almost immediately at concentrations comparable to that of the feed gas. No thiosulfate oxidation was observed.

2.3.2 Effect of Nitrate on NO Reduction by *T. denitrificans*

In a series reactor scheme for the simultaneous removal of SO_2 and NO_x from a gas as described in previous sections, the second stage containing the *T. denitrificans* culture would need to be operated on a sulfide-limiting basis. Otherwise sulfide would accumulate to toxic levels in the liquid phase (4). The same is basically true for a single stage co-culture of *D. desulfuricans* and *T. denitrificans;* that is, the reactor must be sulfide-limiting with respect to *T. denitrificans*. In either case, the terminal electron acceptor for *T. denitrificans* (NO, NO_2, NO_3^-) must be in stoichiometric excess. In most cases this would require supplementing the culture with nitrate. With NO_x from the gas competing with the intermediates of nitrate reduction for access to the appropriate enzymes in the biomass, complete removal of NO_x may not be possible. Accordingly the effects of nitrate on NO reduction by *T. denitrificans* was investigated.

In these experiments *T. denitrificans* working cultures were

TABLE 5. Stoichiometry of NO Reduction by *T. denitrificans* With Thiosulfate as Electron Donor

Exp. #	$NO/S_2O_3^{2-}$
5NA	3.6
6NA	4.2
7A	4.4
9A	4.2
Average	4.1

prepared as described in the previous section. A NO feed was initiated at molar flow rate of 1.3 mmoles/hr at a concentration of 4900 ppmv following the medium changeover and maintained at these conditions for 48 hr. During this time, about 90% removal of NO was observed with concommitant decrease in the concentration of thiosulfate and production of sulfate.

After 48 hr of operation, potassium nitrate (5 g/L) was added to the culture. A few minutes after the addition of nitrate the NO concentration in the outlet gas began to rise. At an elapsed time of 28 hr after the addition of nitrate the NO removal was down to 65% and remained at this level for the duration of the experiment (76 hr). With the addition of nitrate the rates of thiosulfate utilization and the sulfate accumulation increased significantly. Similarly, the optical density of the culture also increased indicating an increase in the rate of growth of *T. denitrificans*.

These observations support the hypothesis that nitrate in the culture medium will suppress the utilization of NO(g) as a terminal electron acceptor by *T. denitrificans*.

2.4 Simultaneous Removal of SO_2 and NO From a Gas - Work Performed Under DOE Contract No. DE-FG22-88PC88945

2.4.1 SO_2/NO Removal by *D. desulfuricans* and *T. denitrificans* Reactors-in-Series

As noted previously there are various ways in which a microbial process could potentially impact on the overall problem of flue gas desulfurization and NO_x removal. One of the more attractive options is simultaneous removal of SO_2 and NO_x directly from flue gas. This process could utilize *D. desulfuricans* and *T. denitrificans* reactors-in-series or mixed cultures in a single stage.

The simultaneous removal of SO_2 and NO by reactors-in-series was investigated as follows. *D. desulfuricans* cultures were developed as described in Section 2.2.1. After the cells were harvested and resuspended in minimal glucose medium with SO_2 feed, the reactor was operated for an additional 24 hr before connecting the outlet gas to the *T. denitrificans* reactor described below. The purpose of this incubation period was to ensure that all SO_2 in the feed gas was being reduced to H_2S, and to acclimate the cells to utilizing SO_2 as terminal electron acceptor. Glucose (10 g/L) was added daily.

T. denitrificans was grown on thiosulfate in the B. Braun Biostat M fermenter as described in Section 2.3.1. After the cells were resuspended in thiosulfate maintenance medium without thiosulfate, a gas mixture of 1% H_2S, 5% CO_2 and balance nitrogen (35-70 mL/min) was supplied to the fermenter under sulfide-limiting conditions. After 24 hr of operation, the H_2S feed was removed and the off gas of *D. desulfuricans* reactor was connected to the fermenter in its place. The reactors were then operated in series for 24-48 hr. During this time stoichiometric production of H_2S was observed from the *D. desulfuricans* reactor and no sulfite accumulated in the medium. Sulfate accumulated in the medium of *T. denitrificans* reactor and only trace amounts of H_2S were detected in the off-gas of the second stage.

When stable operation in series was demonstrated with respect to SO_2 reduction in the *D. desulfuricans* reactor and H_2S oxidation in the *T. denitrificans* reactor, NO (0.49%, 5% CO_2, balance N_2) was added to the feed gas of the first stage (*D. desulfuricans* reactor). The initial NO concentration in the feed gas was 760 ppmv at a molar feed rate of 0.86 mmoles/hr. (The SO_2 feed rate remained constant at 0.78

mmoles/hr.) After 24 hr the NO feed rate was increased to 1.3 mmoles/hr (1060 ppmv) and finally after another 24 hr the feed rate was increased to 1.72 mmoles/hr (1320 ppmv) and maintained at this level throughout the remainder of the experiment.

Sulfite began to accumulate in *D. desulfuricans* reactor at NO concentration of 1060 ppmv about 48 hr after the initiation of NO feed. As the sulfite concentration became inhibitory less than stoichiometric production of H_2S was observed in the outlet of the reactor. Dasu and Sublette (14) had previously operated *D. desulfuricans* and *T. denitrificans* reactors-in-series with SO_2 feed for over 100 hr without problems. Therefore, the upset condition in the *D. desulfuricans* reactor was apparently due to NO inhibition of SO_2 reduction. As SO_2 was removed from the feed gas of *D. desulfuricans* reactor sulfate accumulated in the *T. denitrificans* reactor. The total biomass protein and the optical density of each culture also increased with time. Growth of *T. denitrificans* was also indicated by the consumption of nitrate and ammonium ion.

Analysis of the off-gas from each reactor for NO by gas chromatography revealed that about 26% of the NO in the feed to the first stage (*D. desulfuricans* reactor) was removed by contact with that culture. Presumably facultatively anaerobic non-SRB heterotrophs in that culture were able to use NO as a terminal electron acceptor. This observation suggests that reduction of NO may be a common activity in denitrifying bacteria. More interesting, only 20-25% of the remaining NO was removed by the second stage (*T. denitrificans* reactor). This was a much lower removal efficiency than observed in *T. denitrificans* reactor when NO was the only terminal electron acceptor. These observations confirm again the hypothesis that nitrate in the culture medium suppresses the utilization of NO as a terminal electron acceptor (see Section 2.3.2).

This experiment was repeated with similar results.

2.4.2 SO_2/NO Removal by *D. desulfuricans, T. denitrificans* and Mixed Fermentative Heterotrophs in Co-Culture

In this series of experiments *D. desulfuricans* and *T. denitrificans* working cultures were developed on SO_2 and H_2S feeds as described in the previous section. After *D. desulfuricans* cells were resuspended in the minimal glucose medium with SO_2 feed, the 2-L culture was allowed to operate for an additional 24 hr while monitoring H_2S production to ensure that the culture was "healthy". The culture was then supplemented with components of thiosulfate maintenance medium which were not present in the minimal glucose medium (primarily sources of Mg^{+2}, Fe^{+3}, Ca^{+2}, Mn^{+2} and KNO_3 but no thiosulfate). A slurry (50 mL) of *T. denitrificans* cells which were previously grown to a cell density of 10^9 cells/mL in thiosulfte medium (1.5 L) was then added. Within one hour of the addition of *T. denitrificans* cells the H_2S concentration in the outlet gas of the reactor was reduced from 800 ppmv to trace levels. Therefore, the H_2S produced from SO_2 reduction by *D. desulfuricans* was immediately oxidized by *T. denitrificans* to sulfate which was observed to accumulate in the culture medium. No sulfide, sulfite and elemental sulfur were detected during this time.

After 24 hr operation in this mode, a NO(g) feed (0.49% NO, 5% CO_2, balance N_2) was introduced along with the SO_2 feed and N_2 purge at a concentration of 760-910 ppmv. On the average, sulfite began to accumulate in the culture medium within 12 hr of the introduction of NO. Once again NO inhibition of SO_2 reduction by *D. desulfuricans* was indicated.

Another interesting observation in these experiments was the tremendous amount of nitrate utilized. The ratio of nitrate consumed to H_2S oxidized in these cultures was approximately 4. This is more than twice that observed in anaerobic cultures of *T. denitrificans* growing on H_2S (4). In the *T. denitrificans* reactor heterotrophs grow only at the expense of waste products of *T. denitrificans* or products of cell lysis. Therefore, heterotroph growth rates and nitrate utilization rates by heterotrophs are low. This has been experimentally confirmed repeatedly in our laboratory. In the single-stage, mixed culture used here to simultaneously remove SO_2 and NO, anaerobic growth of mixed heterotrophs on carbohydrates was used to provide an inexpensive source of carbon and electron donors for the *Desulfovibrio.* Certain of the heterotrophs in this population probably were able to utilize nitrate as an electron acceptor. Therefore, in co-culture nitrate was consumed in the oxidation of H_2S by *T. denitrificans* <u>and</u> the oxidation of glucose by some fraction of the non-SRB heterotrophs in the culture.

2.5 Conclusions

2.5.1 Simultaneous SO_2/NO_x Removal From Flue Gas

Based on the work described in the previous sections simultaneous SO_2/NO_x removal from flue gas based on direct contact of the gas with SRB and *T. denitrificans* co-cultures or cultures-in-series has been eliminated as a viable process concept at this time. The technical reasons are as follows:

1) *NO inhibition of SO_2 reduction by D. desulfuricans* - Although the NO concentrations used in the experiments described above are somewhat higher than that found in a typical flue gas, it is quite possible that at lower NO concentrations (or partial pressures) the inhibiting effects will simply take longer to become apparent. One interpretation of these experiments is that NO transferred into the liquid phase inhibited (or killed) *D. desulfuricans* cells one by one until there was insufficient "active sites" available to reduce SO_2 as fast as it was sparged into the culture. At this point sulfite began to accumulate in the liquid phase, further inhibiting the biomass. At best NO inhibition imparts a borderline stability on microbial SO_2 reduction.

2) *Nitrate suppression of NO removal* - As noted previously, the cultivation of *T. denitrificans* in a microbial flue gas treatment system (either one or two stages) would require sulfide-limiting conditions. Therefore, the electron acceptor must be in excess, requiring nitrate in the *T. denitrificans* process culture. As shown in experiments described above, nitrate significantly suppresses the removal of NO from a feed gas making simultaneous SO_2/NO_x removal impractical by microbial means.

3) *O_2 inhibition of SO_2 and NO reduction* - It has been demonstrated that *D. desulfuricans* working cultures are tolerant of up to 1.7% O_2 in the feed gas. Apparently at low O_2 feed rates facultatively anaerobic non-SRB heterotrophs in the culture scavenge O_2 keeping the redox-potential sufficiently low to favor growth of *D. desulfuricans* (and SO_2 reduction). However, further

increases in the O_2 partial pressure in the feed gas resulted in O_2 inhibition of SO_2 reduction. These inhibiting levels of O_2 are comparable to those concentrations found in flue gases (3). Therefore, in any process in which raw flue gas contacts a *D. desulfuricans* culture marginal stability at best can be expected.

Oxygen in the feed gas will also produce a suppression in NO removal similar to the effect of nitrate. It has been observed in our laboratories that O_2 will completely inhibit nitrate reduction by *T. denitrificans.* Under aerobic conditions O_2 is the preferred terminal electron acceptor. It can be anticipated that O_2 will also be "preferred" over NO as a terminal electron acceptor resulting in reduced NO removal in the presence of O_2.

One last comment on process economics is appropriate. The microbial processes for simultaneous SO_2/NO_x removal described in the previous sections effect the net oxidation of SO_2 to sulfate. The recovery of this sulfate salt and its disposal or utilization as a by-product has not been specifically addressed here. Various options exist. As with processes which use throwaway adsorbents, disposal of the sulfate can have a negative impact on process economics. Unless SO_2 and NO_x removal from flue gas can be combined, it would be unlikely that a microbial process could offer a major advancement in the state of the art over the limestone scrubbing process.

2.5.2 By-Product Recovery

The technical problems which confront the simultaneous removal of SO_2 and NO_x from a flue gas by microbial means are for the most part eliminated if the two reactions of interest, SO_2 reduction to H_2S and

NO_x reduction to N_2, are decoupled. In the absence of NO_x (and O_2) SO_2 reduction by SRB working cultures proceeds rapidly and efficiently. If a noninhibitory energy source can be used (such as thiosulfate), a *T. denitrificans* culture can be operated on a terminal electron acceptor limiting basis. If that electron acceptor is NO(g), high removal efficiences from a gas can be expected. Therefore, by-product recovery/disposal from regenerable processes for flue gas desulfurization and NO_x removal remains a viable process option. It is the further technical development of SO_2 and NO_x reduction as independent processes that constitutes the current project.

3. PROJECT OBJECTIVES

3.1 Microbial Reduction of SO_2

As noted in Section 2.5.2, by-product recovery from regenerable scrubbing processes has been identified as the most viable means by which a microbial process can potentially impact on the overall problem of flue gas desulfurization and NO_x removal. Accordingly, at the conclusion of the previous DOE contract, an economic evaluation of the microbial reduction of SO_2 was performed comparing the microbial process to a conventional catalytic SO_2 hydrogenation (with H_2 generation from methane). The process design basis with respect to feed gas composition and source is given by Table 6. The design parameters for the bioreactors are given by Table 7. A comparative cost summary is given in Table 8. As seen in Table 8, microbial SO_2 reduction and conventional SO_2 hydrogenation were estimated to have similar costs in terms of capital investment. However, annual operating costs for the microbial process were much higher than the conventional process, due primarily to the cost of raw materials. Of the \$23.6 x 10^6/yr required for raw materials in the microbial process, \$22.9 x 10^6/yr was the cost of DE95 Corn Hydrolysate, the source of glucose. As noted in Table 7 a theoretical "yield" of 4 moles SO_2 reduced per mole of glucose oxidized was assumed. Therefore, under the best possible conditions with respect to the stoichiometry, microbial reduction of SO_2 with glucose as the electron donor is prohibitively expensive. If microbial reduction of SO_2 is to be economically viable, another less expensive electron donor must be found. The evaluation of alternative electron donors, namely municipal sewage sludges and elemental hydrogen, forms the basis of part of the current project.

3.1.1 Sewage Sludge as a Carbon and Energy Source in SO_2-Reducing Cultures

Excess sludges are produced in sewage-treatment processes at several stages including waste particulates removed in screening and primary sedimentation units and sludge produced in the secondary biological oxidation process. Sewage typically contains about 300 mg/L of suspended solids, much of which is cellulose. The largest and most dense particulates are removed in a primary settling basin simply by gravity settling. The soluble and colloidal components of the sewage (primarily fatty acids, carbohydrates and proteins, in that order) are sent to an aerobic biological reactor where they are oxidized to CO_2 and H_2O by a heterogeneous population of flocculated microorganisms. The product of this secondary treatment is biomass or activated sludge. This sludge (see Table 9) together with that obtained from the primary sedimentation units represents a significant disposal problem.

TABLE 6. Process Design Bases for SO_2 Reduction Processes

Design Basis Parameter	
Flue Gas Source	Coal Fired Power Plant
Power Plant Capacity, MW_e	1,000
Ultimate Feed Coal Analysis C/H/O/N/S, wt%	78.7/5.5/10.9/1.4/3.5
Feed Gas Source for SO_2 Reduction	Regenerator Off-Gas From Copper Oxide Process (90% SO_2/NO_x removal)
Reduction Feed Gas Rate, mol/hr	2,115
Reduction Feed Gas Composition $SO_2/CO_2/H_2O/CH_4$, mol %	33/22/44/1
Ultimate Product Gas for Comparison	Balanced H_2S/SO_2 Feed Gas to Claus Unit
Ultimate Product Gas H_2S/SO_2 mol Ratio	2.0

TABLE 7. Design Parameters for SO_2 Reduction Bio-Reactors (1,000 MW_e Equivalent Capacity)

Reactor Type	Agitated stirred tank with internal cooling coil
Reactor Temperature, C	30
Reactor Pressure In/Out, psia	35/17
Agitation Intensity, HP/1000 gal	2
Total Cell Density, gm/liter	25
Desulfo/Heterotroph Cell wt. ratio	1/1
Total Cell Count, cells/liter	6×10^{11}
Individual Cell Weight, gm/cell	0.1375×10^{-11}
Desulfuricans Specific Activity, lbmol SO_2/hr-cell	3.72×10^{-17} $\frac{1.69 \text{ mmol } SO_2}{\text{hr-}10^{11} \text{ cells}}$
Total Reactant Feed Gas Rate, mol/hr	818.9
Total SO_2 Feed Rate, mol/hr	465.2
SO_2 Conversion to H_2S, % per pass	100^-
Total Reactor(s) Cell Inventory, lb	84,720
Total Reactor(s) Oper. Volume, gal	407,000
Total Reactor(s) Des. Volume, gal	512,000 (w/20% free board)
Number of Reactors Required	8 (in parallel)
Des. Volume per Bio-Reactor, gal	64,000
Reactor Dimensions, ft dia. x ft (T/T)	14 x 55
Nutrient Source	DE95 corn hydrolysate 68% glucose)
Glucose Consumption, mol glucose/mol SO_2 reduced	0.25

TABLE 8. Comparative Production Cost Summary for Bio-Chemical and Conventional SO_2 Reduction Processes

(1,000 MW_e Equivalent Capacity)

	$MM	
Investment (3rd Qtr. 1989)	Bio-Chemical SO_2 Reduction	Conventional SO_2 Reduction
Inside Battery Limits (ISBL)	19.75	19.25
Outside Battery Limits (OSBL) @ 30% ISBL	5.92	5.78
Total Fixed (TFI)	25.67	25.03
Production Cost	$MM/yr	
Raw Materials	23.60	3.55
Utilities	3.21	(0.91)
Labor, For, Supvn L,F,S	0.69	0.63
Maintenance, Material and Labor @ 4% ISBL	0.79	0.77
Direct Overhead @ 45% L,F,S	0.31	0.28
General Plant Overhead @ 65% Oper. Cost (L,F,S + M,M,L)	0.96	0.91
Insurance, Prop. Taxes @ 1.5% TFI	0.38	0.38
By-Product Credit/Debit	0	excl*
Cash Cost of Production	29.94	5.61

*Waste water treatment costs excluded

TABLE 9. Composition of Municipal Sewage Sludge[19]

Fraction	Raw Sludge
Ether-soluble	34.4
Water-soluble	9.5
Alcohol-soluble	2.5
Hemicellulose	3.2
Cellulose	3.8
Lignin	5.8
Protein	27.1
Ash	24.1

A common treatment of this excess sludge is anaerobic digestion. The overall mechanism of anaerobic digestion is as follows:

```
                   liquefaction by
Insoluble biomass  -------------->
                   extracellular
                   hydrolytic
                   enzymes

                                             |----> cells
                            acid producing   |
          Soluble organics -------------->   |----> other products
                            bacteria         |
                                             |----> volatile acids
                                                   + CO2 + H2
                                                       |
                                         gasification by|
                                         CH4-producing  |
                                         bacteria       |
                                                        |
                                                CH4 + CO2 + cells
```

Anaerobic digestion reduces the organic solids content of the sludge by 50-60% and produces a product which is more easily dewatered than the original sludge.

We propose that with the introduction of the appropriate sulfate-reducing bacteria, an anaerobic reactor could be operated with a feed of municipal sewage sludge for the reduction of SO_2 to H_2S. Municipal sewage sludge is readily available in large quantities in urban areas, is relatively consistent in composition (Table 9) and is available at a negative or near zero cost. This type of reactor would operate in much the same way as a glucose-fed system. Mixed non-SRB heterotrophic bacteria would convert the sewage sludge by liquefaction and fermentation into end products suitable as carbon and energy sources for sulfate-reducing bacteria which would use SO_2 as a terminal electron acceptor with reduction to H_2S . However, instead of a single SRB being used, a consortium of SRB would be utilized to effectively use a maximum amount of the fermentation end products while giving high SO_2-reducing activity. Sulfate-reducing bacteria are readily available from commercial and private collections which utilize lactate, ethanol, CO_2/H_2, as well as acetate and other short chain alcohols and carboxylic acids as carbon and energy sources (see Table 10 for a partial listing). Although acetate is a common end product of the oxidation of many of these species by certain SRB, other species are capable of complete oxidation of acetate to CO_2. Therefore, complete mineralization by SRB of many, if not all, of the major end products of the fermentation of sewage sludge by non-SRB heterotrophs is possible in SO_2-reducing cultures.

The non-SRB heterotrophs required will likely already be present in the sewage sludge with one possible exception. It will be advantageous for the biomass in SO_2-reducing cultures to be flocculated so that biomass can be retained or readily recycled in a continuous reactor. We have previously demonstrated that *T. denitrificans* can be immobilized by co-culture with floc-forming heterotrophs under aerobic conditions (15). We propose that the incorporation of anaerobic floc-forming organisms in SO_2-reducing cultures can lead to immobilization of SRB's and associated fermentative heterotrophs. Floc-formers will be obtained from anaerobic digesters.

3.1.2 CO_2/H_2 as Carbon and Energy Sources in SO_2-Reducing Cultures

Elemental hydrogen generated on site from natural gas represents another potentially economical energy source or electron donor for SRB reduction of SO_2 to H_2S. Several SRB including (but not limited to)

TABLE 10. Carbon and Energy Sources for Sulfate Reducing Bacteria

Growth with:	Lactate + sulphate	Pyruvate + sulphate	Formate + sulphate	Acetate + sulphate	Glucose + sulphate	Malate + sulphate	Benzoate + sulphate	Other
Desulfovibrio								
desulfuricans	+	+	±	–	v	+	–	
vulgaris	+	+	±	–	–	–	–	
gigas	+	+	±	–	ND	–	–	
africanus	+	+	±	–	ND	+	–	
salexigens	+	+	±	–	ND	+	–	
thermophilus	+	+	±	–	ND	–	ND	
buculatus	+	+	±	–	–	+	ND	
baarsii	–	–	+	+	–	–	ND	Fatty acids to C_{18}
sapovorans	+	+	–	–	–	–	ND	C_{odd} fatty acids
Desulfobacter postgatei	(±)	–	–	+	–	–	–	Vitamins required
Desulfobulbus propionicus	+	+	–	–	–	–	–	Propionate
Desulfococcus multivorans	+	+	+	+	–	–	+	
Desulfonema								
limicola	+	+	+	+	–	–	+	
magnum	–	–	±	+	–	+	+	
Desulfosarcina variabilis	+	+	+	+	–	–	–	
Desulfotomaculum								
nigrificans	+	+	–	–	v	ND	ND	
orientis	+	+	–	–	–	ND	ND	
ruminis	+	+	+	–	–	ND	ND	
acetoxidans	–	–	±	+	–	–	–	Vitamins required
antarcticum	+	ND	–	–	+	–	ND	

v signifies variable; some strains +, some –
ND no data

Desulfobacter hydrogenophilus, *Desulfotomaculum orientis*, *Desulfobacterium autotrophicum* and some strains of *Desulfovibrio vulgaris* are capable of autotrophic growth oxidizing H_2 as an energy source and using CO_2 as a carbon source (16). (*D. vulgaris* requires a small amount of acetate in the culture medium.) Other less defined species are available from private collections.

We propose that with the appropriate choice of SRB, an anaerobic reactor can be developed for the reduction of SO_2 to H_2S which would operate on a feed of $H_2(g)$, $CO_2(g)$ and possibly trace organics. The SRB would be composed of one or more strains of autotrophic or mixotrophic SRB. Fermentative heterotrophs would result in these cultures as a result of septic operation even if the medium was autotrophic. We have observed, for example, that heterotrophic contamination will develop in cultures of the autotroph *T. denitrificans* under septic conditions deriving carbon and energy sources from waste products and products of cell lysis from the autotroph. We have shown that these heterotrophs have no effect on growth of *T. denitrificans* on reduced sulfur compounds (7). It is likely that heterotrophs will also have no effect on SO_2 reduction by autotrophic SRB. In fact trace organics required by mixotrophic SRB could be provided by cross-feeding from the heterotrophs. If these heterotrophs are anaerobic floc-formers, immobilization of autotrophic SRB by adsorption and entrapment in the floc will likely occur. As noted previously, the autotroph *T. denitrificans* has been immobilized in this manner in cultures operating with an H_2S feed.

3.2 Microbial Reduction of NO_x

As noted in Section 2.5.2 disposal of concentrated streams of NO_x by microbial reduction to N_2 has been identified as a potentially viable process option. Many bacteria are known to use nitrate as a terminal electron acceptor under anaerobic conditions with reduction to N_2. Nitric oxide (NO) has been identified in many of these organisms as an intermediate in the reduction of NO_3^- (17). Like *T. denitrificans*, these organisms may be capable of growth using NO as a terminal electron acceptor with reduction to N_2. (Recall the removal of NO from the feed gas to SO_2-reducing cultures.) *T. denitrificans* is a chemoautotroph which derives energy from the oxidation of reduced sulfur compounds. Most other denitrifying organisms are heterotrophs requiring organic compounds as carbon and energy sources. These organisms include (but are not limited to) the following:

E. coli K12
Enterobacter aerogenes
Proteus mirabilis
Micrococcus denitrificans
Bacillus licheniformis
Bacillus stearothermophilus
Bacillus cereus
Pseudomonas stutzeri
Pseudomonas perfectomarinus
Pseudomonas aeroginosa
Pseudomonas mirabilis
Pseudomonas denitrificans

The reduction of NO (and NO_2) by *T. denitrificans* requires further study in order to evaluate the process both technically and economically. In addition, other denitrifying organisms, such as those listed above, should be screened to determine the best possible candidate for an NO_x-reducing organism.

4. SPECIFIC WORK PLAN

4.1 SO_2 Reduction

The following work plan is proposed for an investigation of the use of municipal sewage sludge as a carbon and energy source for SO_2-reducing cultures:

Task I *Develop a consortium of SRB, fermentative heterotrophs and floc-forming heterotrophs which will use municipal sewage sludge as a carbon and energy source and SO_2 as a terminal electron acceptor. Demonstrate flocculation of the process culture and stability of the population dynamics with respect to SO_2 reduction and efficient utilization of carbon and energy sources under septic conditions. (6-12 months)*

Some trial and error and enrichment will be necessary in the development of these cultures. However, in short we will optimize cross-feeding in the culture to maximize the specific activity of the SRB for SO_2 reduction while minimizing the BOD of the reactor effluent and maintaining the biomass in a flocculated state.

Task II *Investigate important design parameters for a continuous SO_2-reducing system with municipal sewage sludge feed. Perform preliminary cost analysis. (6 months)*

The stoichiometry (C-economy, biomass yield, end products) and kinetics of SO_2 reduction in optimized mixed and flocculated cultures will be determined in the course of Task I. However, before proceeding to a conceptual design the maximum volumetric productivity of a continuous reactor (CSTR, bubble column, etc.) must be determined. Since these systems must be operated on an SO_2-limiting basis, volumetric productivity will largely be determined by the biomass concentration that can be maintained in the reactor. However, increasing biomass concentrations will have a negative effect on effluent BOD and SO_2 mass transfer. Upon completion of Task II a preliminary cost analysis of a full-scale system will be performed.

The following work plan is proposed for an investigation of the use of CO_2/H_2 as carbon and energy sources for SO_2-reducing cultures:

Task III *Screen sulfate reducing bacteria for the capability of growing on CO_2/H_2 as carbon and energy sources and SO_2 as a terminal electron acceptor. (6-12 months).*

The following sub-tasks will make up the screening methodology:

IIIa *Demonstrate growth of SRB on H_2, CO_2 and SO_2 in mixed, septic cultures.*

IIIb *Estimate the specific activity of SRB for SO_2 reduction given optimum growth conditions with respect to the carbon and energy source.*

Task IV *Develop optimum SO_2-reducing culture operating on CO_2/H_2 feed. Demonstrate flocculation of the biomass and culture stability under septic conditions. (6-12 months).*

The best SO_2-reducing SRB (identified under Task III) in terms of specific activity for SO_2 reduction and general growth characteristics will be chosen for further study.

Flocculation will be achieved by addition of anaerobic floc-forming bacteria to the SRB cultures. Floc-formers will be obtained from an anaerobic digester and may require an external organic carbon source. If so, incomplete oxidation of that carbon source is likely under anaerobic conditions. Judicious choice of the SRB or a consortium of SRB including autotrophic and heterotrophic SRB may allow those end products to be oxidized to CO_2, improving the quality of the effluent produced by a continuous process. In short, we will attempt to optimize cross-feeding in the culture to minimize cost of raw materials, maximize specific activity for SO_2 reduction and minimize the BOD of the reactor effluent.

Task V *Investigate important design parameters for continuous SO_2-reducing system with H_2-feed. Perform preliminary cost analysis. (6 months).*

This task is similar to Task II described above with the exception of optimizing the feed gas condition and gas-liquid mass transfer. As noted above these cultures must be operated on an SO_2-limiting basis; therefore, H_2 and CO_2 must be provided in excess. This fact coupled with the low solubility of H_2 in water dictate incomplete removal of H_2 (and CO_2) from the feed gas. Fractionation of the outlet gas with recycle of H_2 and CO_2 would be anticipated in a full scale operation. The feed gas composition and feed rate and gas-liquid contacting must be optimized with respect to H_2 (and CO_2) flux into the culture medium.

4.2 NO_x Reduction

The following work plan is proposed for an investigation of microbial reduction of NO_x to N_2:

Task I *Screen denitrifying bacteria for NO and NO_2 activity. (12 months).*

The organisms listed above and species isolated by enrichment from mixed anaerobic populations will be utilized. The following subtasks will make up the screening methodology:

Ia *Demonstrate growth on NO as terminal electron acceptor with best known carbon and energy source (glucose in most cases) in pure cultures and septic or mixed cultures. In the latter case a brief study of population dynamics will be made.*

Ib *Demonstrate growth on NO with less defined carbon and energy sources (municipal sewage sludge, for example). Identify end products of fermentation.*

Ic *Estimate specific activity of organism for NO reduction with both refined and crude carbon and energy sources. (This remains to be done for T. denitrificans as well.)*

Task II *Develop optimum NO-reducing culture. Demonstrate flocculation of the biomass and culture stability under septic conditions. (6-12 months).*

The best NO-reducing organisms (as identified under Task I) in terms of specific activity for NO-reduction and septic growth on low cost carbon and energy sources will be chosen for further study. In the case of a waste material, like municipal sewage sludge as a carbon and energy source, a co-culture of two or more NO-reducing organisms may be desirable to enhance the extent of oxidation of the feed. More oxidation of the feed will result in a lower BOD effluent from the reactor. Once an optimum culture has been identified the culture will be flocculated by incorporating anaerobic floc-forming heterotrophs into the culture as described previously. A likely source of these organisms will be an anaerobic digester. The success or failure of flocculation may depend on culturing conditions, carbon and energy sources available for the floc-formers, etc. Therefore, flocculation may influence the choice of NO-reducing organisms in the culture.

Some amount of trial and error or enrichment may be required to produce the optimum, flocculated NO-reducing culture.

<u>Task III</u> *Investigate important design parameters for a continuous system. Perform preliminary cost analysis. (6-12 months).*

In order to facilitate the design of a pilot or demonstration scale NO-reducing reactor and perform a preliminary cost analysis, the important design parameters for a continuous system must be investigated. This will include a study of the kinetics of NO-reduction, stoichiometry (C-economy, biomass yield, end products, BOD of effluent) and an estimate of the maximum volumetric productivity for a CSTR or bubble column reactor each with biomass recycle. The volumetric productivity will largely be determined by the biomass concentration that can be maintained in the reactor. However, increasing biomass concentrations will have negative effects on effluent BOD and NO-mass transfer. Upon completion of Task III, a preliminary cost analysis of a full-scale system will be performed.

4.3 Tentative Time Table

TASK	Year 1	Year 2	Year 3
SO_2 - I		←———-------→	
SO_2 - II			←——→
SO_2 - III	←———-------→		
SO_2 - IV		←———-------→	
SO_2 - V			←——→
NO - I	←——————→		
NO - II		←———-------→	
NO - III			←———-------→

5. WORK COMPLETED DURING PREVIOUS REPORTING PERIODS

5.1 Municipal Sewage Sludge as a Carbon and Energy Source for Mixed Cultures of *D. desulfuricans*

5.1.1 Raw Sludge Supplemented with Yeast Extract

A number of different batch experiments were conducted using *D. desulfuricans* working cultures developed as described in Section 2.2 with raw municipal sludge as a carbon and energy source (rather than glucose) and sulfate as the terminal electron acceptor. (Municipal sludge was obtained from the recycle from the secondary settler of an activated sludge treatment system of a municipal waste treatment plant in Tulsa, OK.) Under these conditions very little reduction of sulfate (and production of H_2S) was observed indicating that the sludge was possibly nutrient deficient for either *D. desulfuricans* or the mixed heterotrophs responsible for generating the specific carbon and energy sources required for the SRB. Alternatively, the mixed heterotroph population which developed in these cultures as a result of septic operation, simply did not contain organisms capable of liquefaction of the complex biomolecules which compose the municipal sludge. The possibility of a simple nutrient deficiency was investigated first.

It was found that addition of yeast extract to batch cultures operated with a feed of raw municipal sludge would stimulate H_2S production. Figure 2 shows the pattern of H_2S production in one of these batch reactors. The H_2S concentration was determined chromophorically using Gas Tech gas analysis tubes (Yokahama, Japan) The accuracy claimed for these tubes is $\pm$ 25%. In the experiment represented here 100 g of wet-packed sludge was suspended in 1.6 L of the minimal medium described by Table 11 without glucose. Sulfate was the terminal electron acceptor. The sludge suspension was incubated at 30°C and pH 7.0 in a B. Braun Biostat M fermenter. The culture was sparged with 300 mL/min of nitrogen. A small amount of H_2S production was noted (60-80 ppm in the outlet gas) in the absence of an SRB inoculum. The culture was inoculated with *D. desulfuricans* after 48 hrs. H_2S production remained low for another three days. On day 5, 4.5 g of yeast extract was added with a resulting large increase in H_2S production as shown in Figure 2. Forty-eight hours after addition of yeast extract H_2S production was still quite high (6000 ppmv in the outlet gas). At this time the total biomass was recovered by centrifugation and resuspended in an equal volume of fresh minimal medium (Table 11) without glucose. When the N_2 purge was re-established H_2S production was greatly reduced (400 ppmv in the outlet

Table 11. Minimal Glucose Maintenance Medium for *D. desulfuricans* with Sulfate as Terminal Electron Acceptor

Component	one liter
Na_2HPO_4	1.2 g
KH_2PO_4	1.8 g
$MgSO_4$	1.5 g
Na_2SO_4	1.5 g
$Fe(NH_4)_2(SO_4)_2$	0.14 g
Glucose	5.0 g
Balch Vitamin Solution (Table 12)	2.0 mL
Heavy Metal Solution (Table 13)	15.0 mL
Mineral Water	50.0 mL

Table 12. Balch Vitamin Solution

Component	mg/L
Biotin	2.0
Folic Acid	2.0
Pyridoxine Hydrochloride	10.0
Thiamine Hydrochloride	5.0
Riboflavin	5.0
Nicotinic Acid	5.0
DL-Calcium Pantothenate	5.0
Vitamin B_{12}	0.1
p-Aminobenzoic	5.0
Lipoic Acid	5.0

Table 13. Heavy Metal Solution

Component	amount/L
EDTA (Ethylenediaminetetraacetic acid)	1.5 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
Trace element solution (Table 14)	6.0 mL

Table 14. Trace Element Solution Used in the Preparation of Heavy Metal Solution

Component	g/L
$AlCl_3$	0.507
KI	0.139
KBr	0.139
LiCl	0.139
H_3BO_3	3.060
$ZnCl_2$	0.280
$CuCl_2 \cdot 2H_2O$	0.326
$NiCl_2 \cdot 6H_2O$	0.513
$CoCl_2 \cdot 6H_2O$	0.513
$SnCl_2 \cdot 2H_2O$	0.139
$BaCl_2 \cdot 2H_2O$	0.163
$Na_2MoO_4 \cdot 2H_2O$	0.139
$CuSeO_4 \cdot 5H_2O$	0.139
$NaVO_3$	0.024

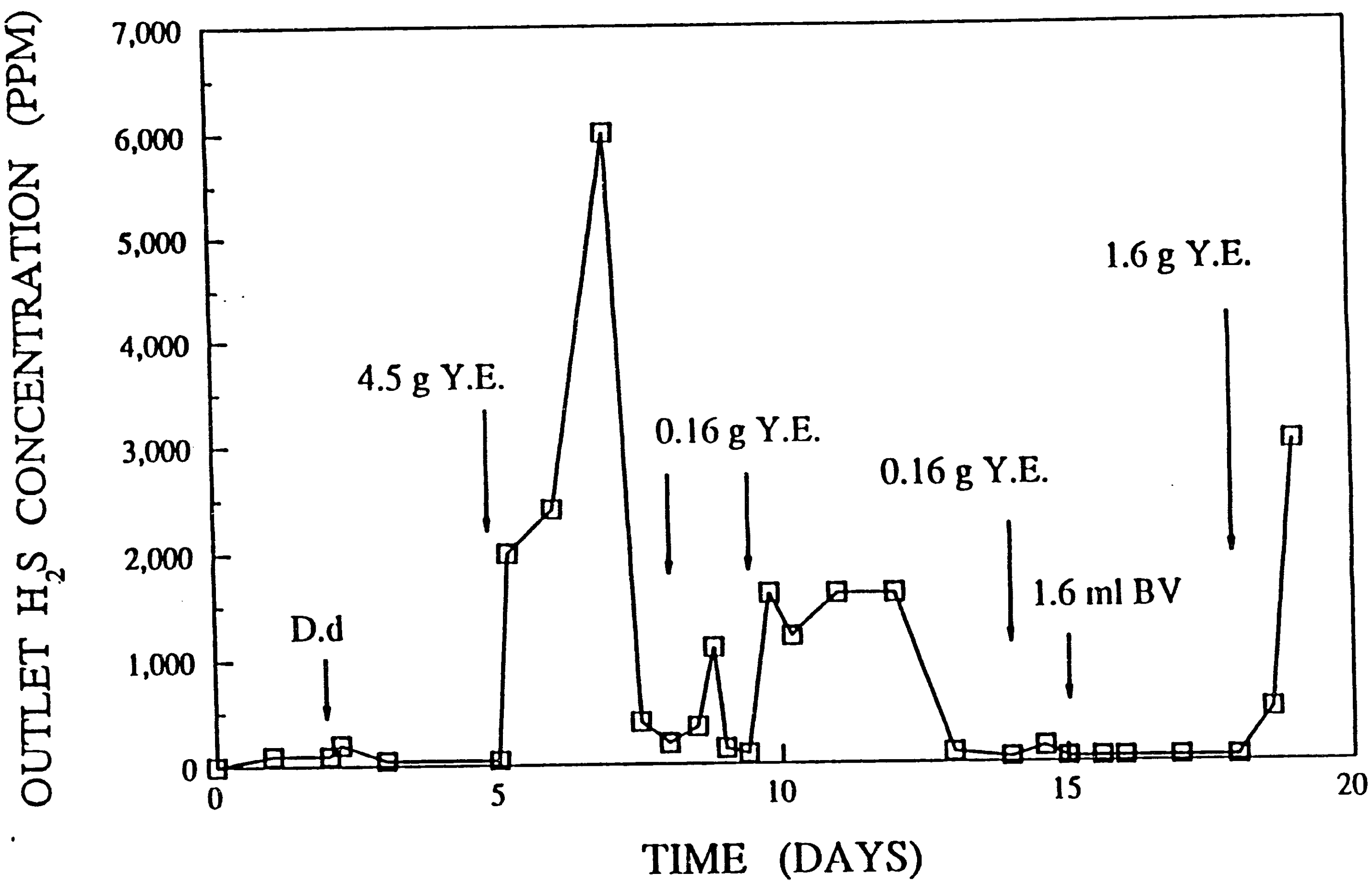


Figure 2. Effect of additions of yeast extract and vitamins on H_2S production from working cultures of *D. desulfuricans* with municipal sludge as the principal carbon and energy source. YE = yeast extract; D.d. = inoculation with *D. desulfuricans*; BV = Balch vitamin solution.

gas). On the 8th day the H_2S concentration in the outlet gas was 200 ppm. As shown in Figure 2, two further additions of yeast extract (0.16 g) produced a surge in H_2S production. A third addition of 0.16 g yeast extract on the 14th day or the addition of 1.6 mL of Balch vitamin solution (Table 12) failed to stimulate H_2S production. However, a larger addition of yeast extract (1.6 g) produced a surge in H_2S production on the 18th day of the experiment.

The yeast extract can potentially stimulate H_2S production by two mechanisms. First the components of yeast extract can serve directly as carbon and energy sources for the SRB or act as easily fermentable substrates for the mixed non-SRB heterotrophs in the culture. Secondly, the yeast extract may provide growth factors lacking in the sludge which are required by the SRB and/or the non-SRB heterotrophs in order for components of the sludge to be used as carbon and energy sources. The experiment described above seems to suggest that the former mechanism predominates when large amounts of yeast extract are added and the latter mechanism predominates when small amounts are added. The failure to produce a stimulation of H_2S production when 0.16 g of yeast extract was added on the 14th day indicates a possible depletion of fermentable substrate in the sludge.

In another batch experiment illustrated by Figure 3, a *D. desulfuricans* working culture was developed as described in Section 2.2 using the complex medium given in Table 15. No municipal sludge was present. When H_2S production was indicated the biomass was harvested by centrifugation and resuspended in minimal medium (Table 11) without glucose. As seen in Figure 3, H_2S production declined markedly following the medium changeover. Three additions of yeast

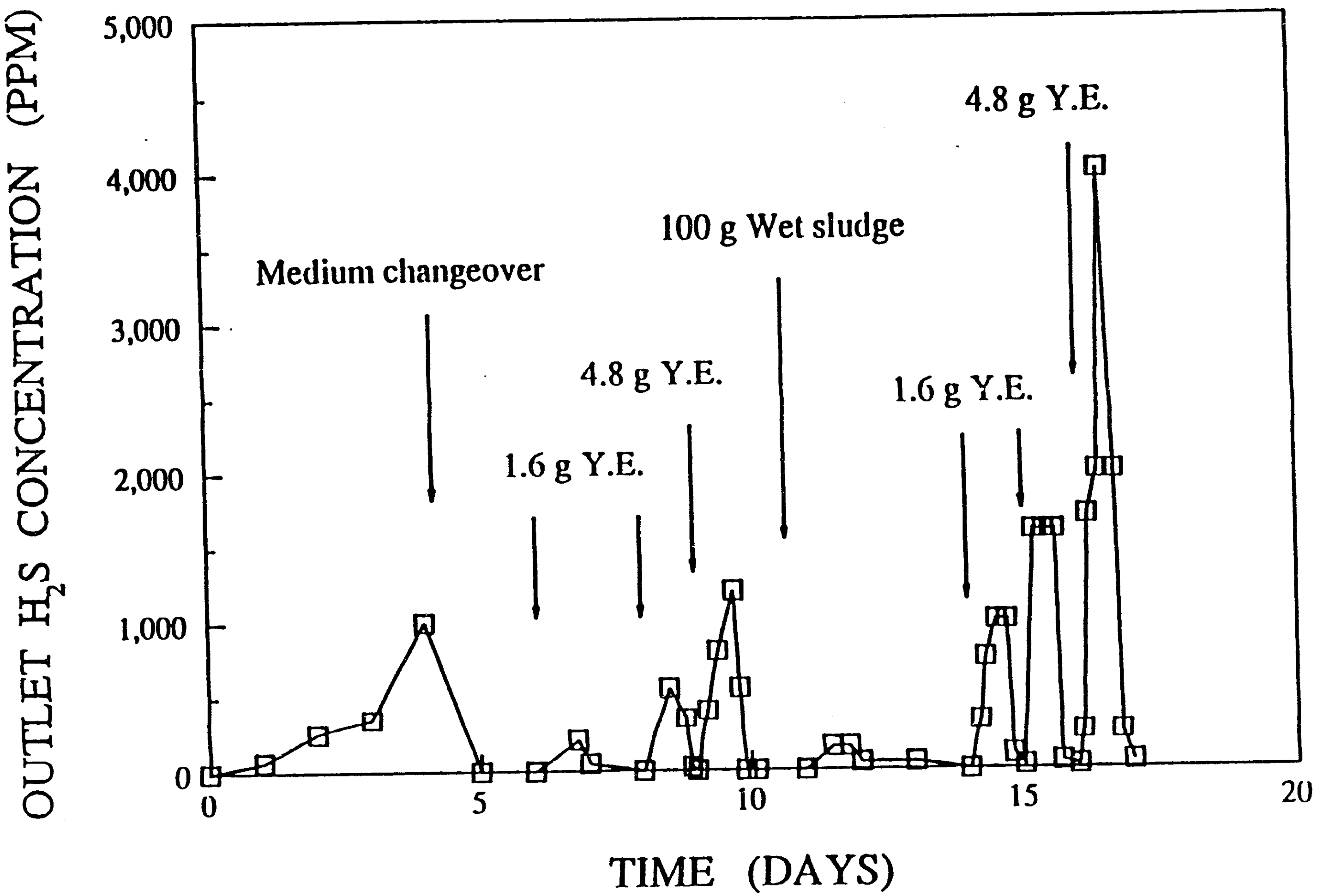


Figure 3. Effect of additions of yeast extract on H_2S production in working cultures of *D. desulfuricans* in the presence and absence of municipal sludge. YE = yeast extract.

Table 15. Complex Glucose Medium for Growth of *D. desulfuricans* in Mixed Heterotrophic Culture.

Component	g/L
Peptone	5.0
Beef Extract	3.0
Yeast Extract	0.2
$MgSO_4 \cdot 7H_2O$	2.9
Na_2SO_4	1.5
$Fe(NH_4)_2(SO_4)_2$	0.1
Glucose	10.0

extract at 6, 8 and 9 days produced only modest stimulation of H_2S production. On the 11th day 100 g of wet-pack municipal sludge was added to the culture (1.6 L). Further additions of yeast extract, using the same pattern of addition, produced greater production of H_2S indicating stimulation of the utilization of some component(s) of the sludge to support sulfate reduction.

A continuous culture of *D. desulfuricans* and mixed heterotrophs was being operated on a feed of minimal medium (Table 11) without glucose, yeast extract and 50 g wet-packed sludge/L in a B. Braun Biostat M at pH 7.0 and 30°C. The culture volume was 1.6 L. The volumetric feed rate was 12 mL/hr giving a dilution rate of 0.18 d^{-1}.

The H_2S concentration in the outlet gas from this continuous system is given by Figure 4. Initially the yeast extract delivery rate was 3 g/L-d giving an H_2S concentration in the outlet of 1000-1200 ppm (with 300 mL/min N_2 purge). The H_2S production remained fairly steady when the yeast extract delivery rate was decreased to 2 g/L-d (by decreasing the yeast extract concentration in the feed). During this time complete utilization of the sulfate in the feed was observed; therefore, the culture was sulfate limiting with regard to H_2S production. When the yeast extract delivery rate was reduced to 1 g/L-d the culture became yeast extract limiting and the delivery rate was increased again to 2 g/L-d.

On the 28th day after the initiation of continuous flow conditions the SO_4^{-2} molar feed rate was increased to 0.42 mmole/hr by increasing the concentration of Na_2SO_4 in the feed from 1.5 g/L to 3.0 g/L. The result was a large increase in H_2S production as seen in

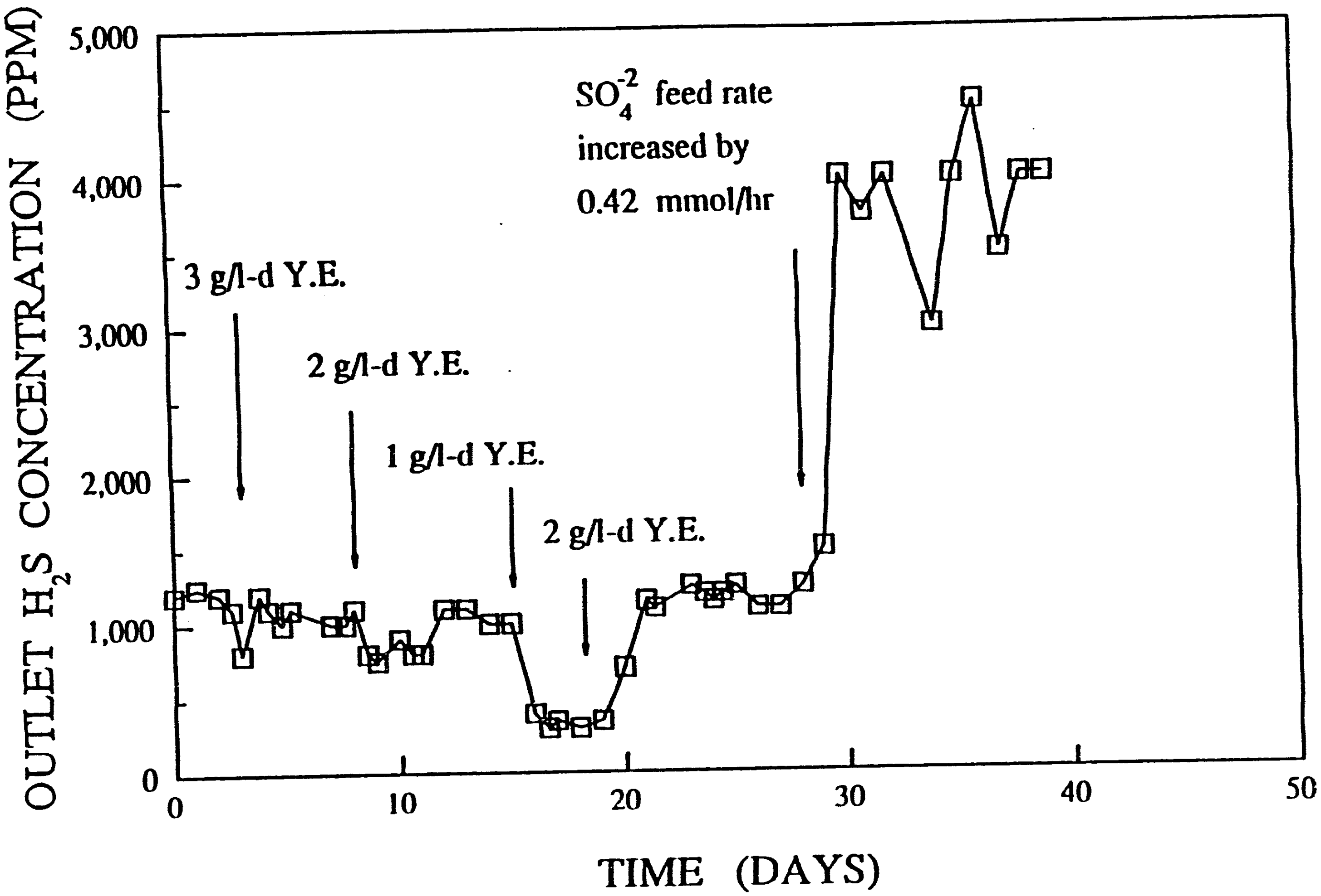


Figure 4. H_2S production from a continuous working culture of *D. desulfuricans* receiving a feed of yeast extract and municipal sludge in minimal medium. The initial sulfate feed rate was 0.29 mmoles/hr. $D = 0.18\ d^{-1}$; YE = yeast extract.

Figure 4. Little or no sulfate was found in the effluent; therefore, the culture again became sulfate-limiting.

This continuous culture was maintained under the operating conditions summarized in Table 16 until which time more personnel could be added to the project. With the addition of more personnel, much more thorough analyses of the feed, culture and off-gases was accomplished. This basic study was extended for approximately 50 days. It was anticipated that some source of pretreatment would be required to fully utilize the potentially fermentable carbon in the sludge. Therefore, the processing of raw sludge represents a base case. Fresh sludge was obtained on a weekly basis from the municipal waste treatment facility. Given the variability inherent in activated sludge systems due to operating conditions, weather, etc., it was important to operate the bioreactor over a period of several weeks.

Once this documentation was obtained under conditions described in Table 16; the yeast extract in the feed was reduced to 5.6 g/l for two purposes. First, it was desirable to determine the response of the bioreactor with regard to factors other than H_2S production which was examined in the earlier phases of this work. Secondly, the yeast extract concentration was reduced to put the SRB in the bioreactor in a carbon-limiting condition in preparation for sludge pretreatment experiments.

When more thorough analysis techniques were introduced, H_2S was determined in the outlet gas by gas chromatography (see Table 17). Previously chromophoric Gas Tech gas analysis tubes were used for H_2S analysis. It was quickly shown that this method had greatly overestimated H_2S concentrations, more so than expected based on

Table 16. Operating Conditions for Continuous Working Culture of *D. desulfuricans* with a Municipal Sludge Feed

Fermenter:	B. Braun Biostat M (culture volume 1.5 L)
Feed:	Minimal Medium with 6.0 g/L Na_2SO_4
	+ 11.2 g/L yeast extract
	+ 100 g wet-packed sludge/L
Volumetric Feed Rate:	12 mL/hr (dilution rate 0.19 d^{-1})
pH:	7.0
Agitation Rate:	200 rpm
N_2 Purge:	308 mL/min

Table 17. Chromatographic Conditions for Analysis of H_2S in Reactor Outlet Gas*

Instrument:	Hewlett Packard 5890
Column:	10'x 1/8" ID Teflon, 80/100 Porapak QS
Carrier Gas and Flow Rate:	He, 30 mL/min
Oven Temperature:	90°C
Injection Oven and Detector Temperature:	120°C
Detector:	Thermal Conductivity Detector

*Standard employed was a Matheson Gas Co. primary standard containing 1.001% H_2S by volume.

manufacturer's claims. However, it was assumed that each determination was proportional by some constant factor to the actual concentration. Therefore, the relative relationships are still valid.

The sulfate concentration in the feed and effluent of the bioreactor are shown in Figure 5. With 11.2 g/L yeast extract in the feed, most of the sulfate in the feed was removed. However, when the yeast extract concentration in the feed was reduced to 5.6 g/L, the sulfate concentrations in the reactor and effluent were seen to rise to about half of that in the feed. In other words, when the yeast extract concentration was halved, the difference in influent and effluent sulfate concentrations was approximately cut in half.

The H_2S concentration in the outlet gas during the latter part of this study is shown in Figure 6. A relatively constant rate of H_2S production (and concentration in the outlet gas) was observed prior to the reduction in the feed yeast extract concentration. When the yeast extract was halved the H_2S concentration in the outlet gas was similarly reduced about 50%.

The results of sulfur balances performed before and after the reduction in yeast extract concentration in the feed are given in Table 18. In almost every case the sulfur balances could be closed to within 2-4%, indicating that all of the H_2S produced by the SRB was being stripped and could be accounted for.

The biomass that composed the municipal sludge as well as the biomass in the reactor effluent was flocculated. Therefore mixed liquor suspended solids (MLSS) measurements were used as one measure of biomass concentration. The MLSS in the influent and effluent of the SRB-bioreactor are shown in Figure 7. There is seen in Figure 7 a

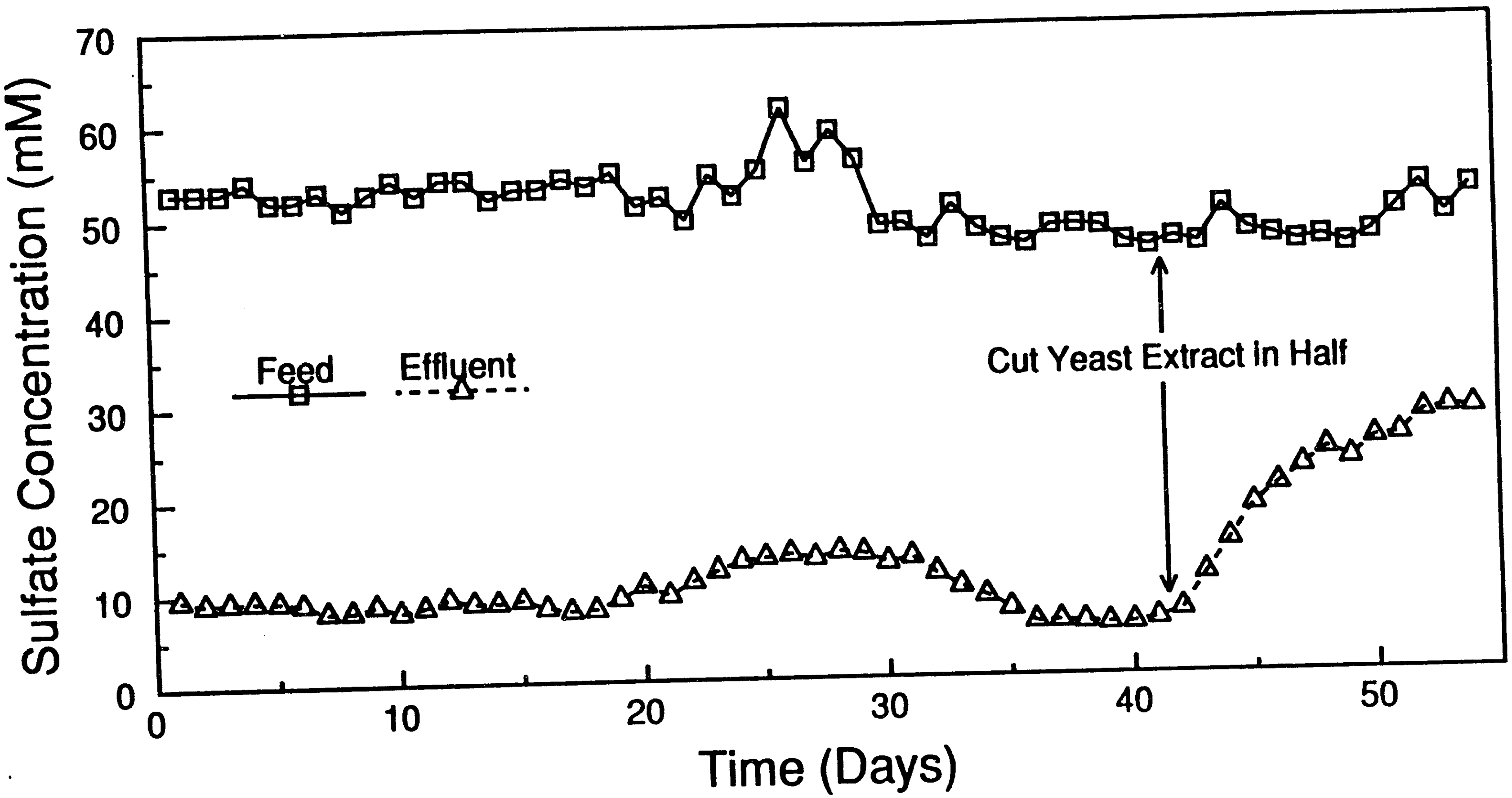


Figure 5. Influent and effluent sulfate concentrations in SRB-bioreactor operating with a feed of municipal sludge and yeast extract.

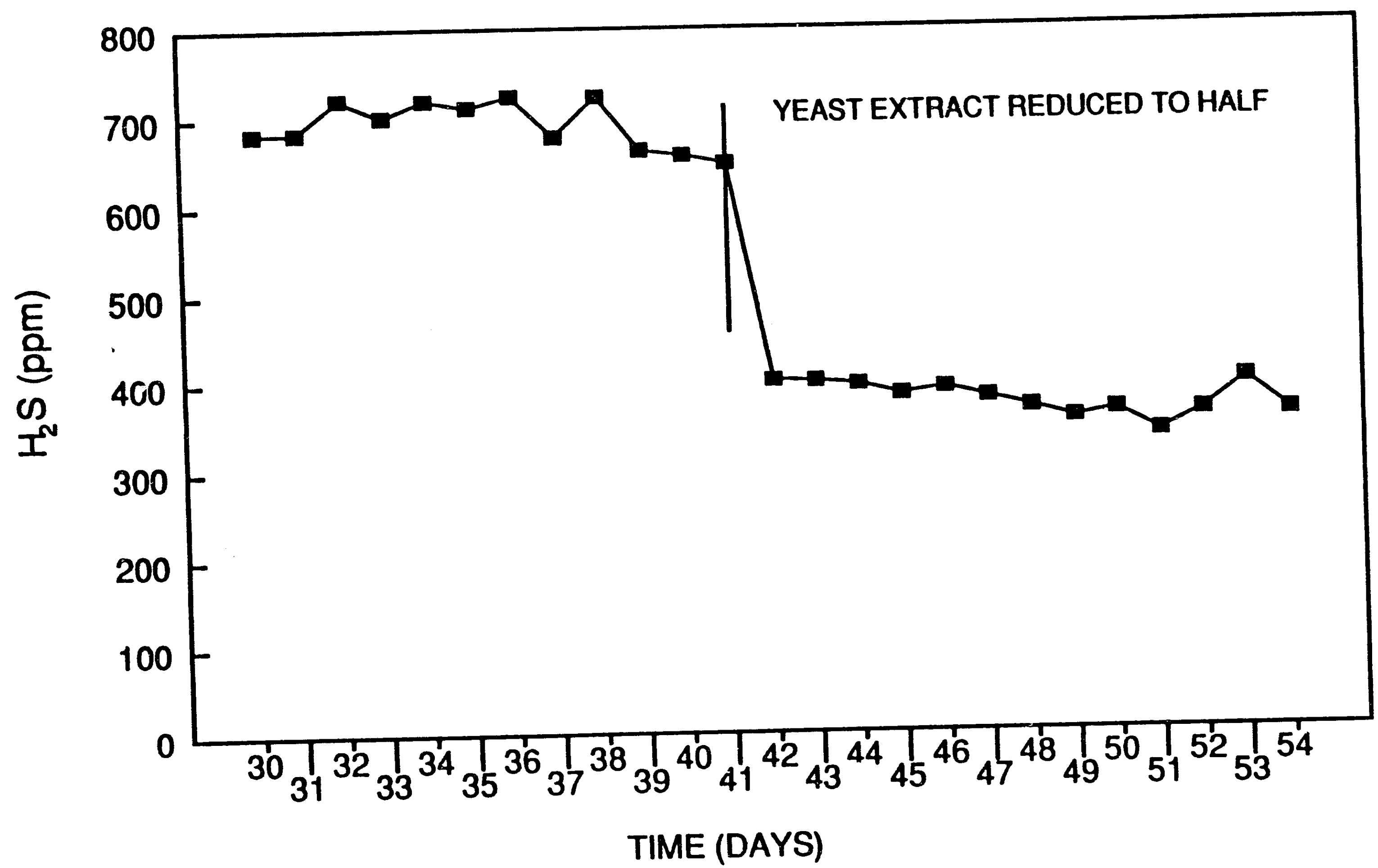


Figure 6. Outlet gas H_2S concentration from SRB-bioreactor operating with a feed of municipal sludge and yeast extract.

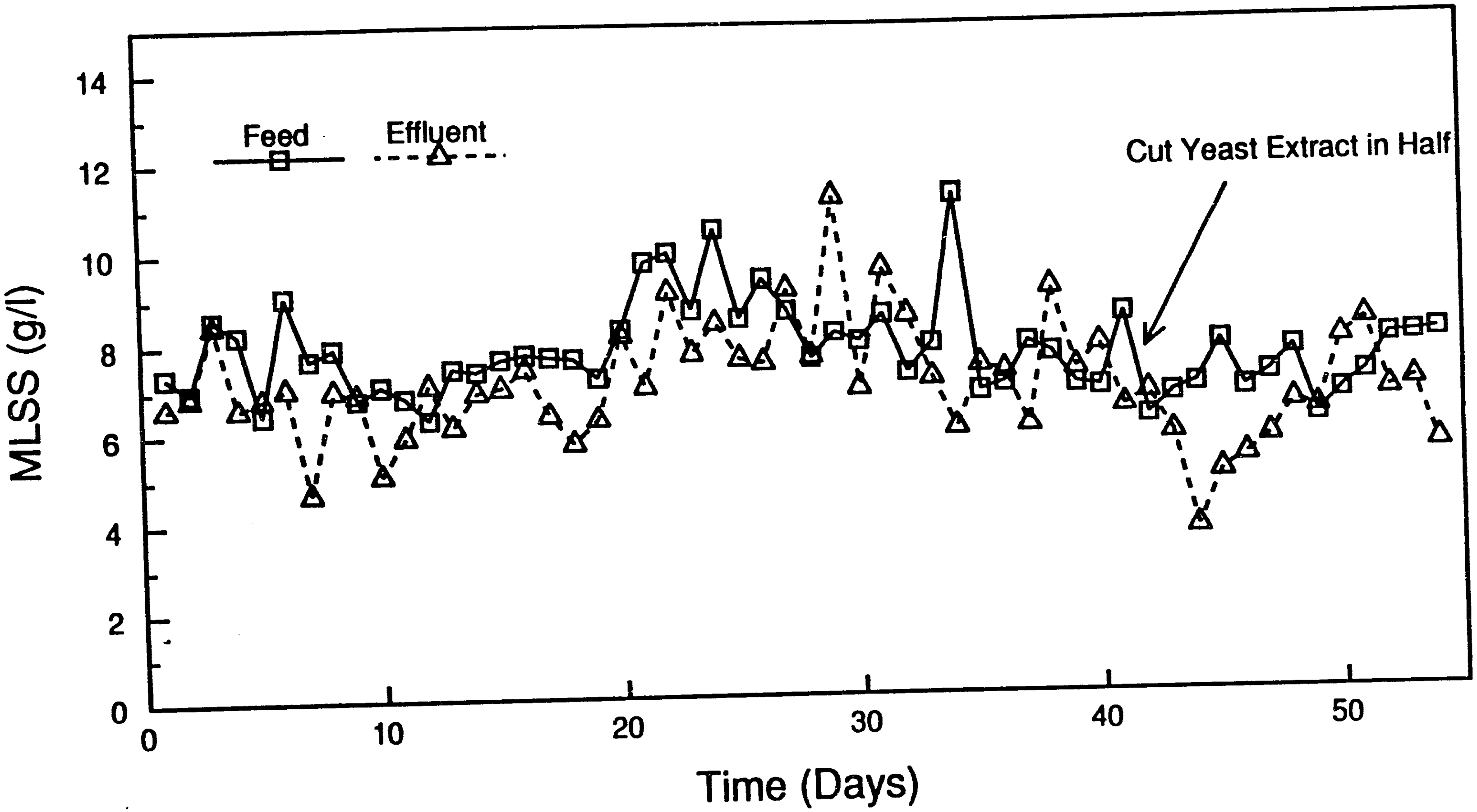


Figure 7. Mixed liquor suspended solids (MLSS) concentration in the influent and effluent of the SRB-bioreactor operating with a feed of municipal sludge and yeast extract.

Table 18. Sulfur Balances in SRB-Bioreactor Operating with a Feed of Raw Municipal Sludge and Yeast Extract

Day	SO_4^{-2} (mmole/hr) in	out	H_2S produced (mmole/hr)	SO_4^{-2} Removed (mmole/hr)	H_2S/SO_4^{-2}
33	0.609	0.118	0.531	0.491	1.08
137	0.581	0.070	0.514	0.511	1.01
39	0.579	0.066	0.502	0.513	0.98
41	0.552	0.072	0.490	0.480	1.02
46	0.564	0.243	0.296	0.321	0.92
49	0.551	0.274	0.269	0.277	0.97
50	0.565	0.300	0.275	0.265	1.04

small but consistent reduction in the MLSS in the bioreactor. This loss in filterable solids could represent the utilization of a small fraction of the municipal sludge as carbon and energy sources for mixed heterotrophs in the culture.

It has previously been observed that in *D. desulfuricans* working cultures in which glucose or molasses sugars served as the ultimate carbon and energy sources, the utilization of the end products of the anaerobic fermentation of these sugars by *D. desulfuricans* was somewhat slower than the rate of their production. Therefore, ethanol and, to a lesser extent, lactic acid, were observed to transiently accumulate in the medium. Small molecular weight carboxylic acids, primarily acetate, were also seen to accumulate. Acetate was shown to be the end product of ethanol oxidation by *D. desulfuricans.* The culture medium of the SRB-bioreactor operating with a feed of municipal sludge was analyzed for ethanol, lactic acid, and small molecular weight carboxylic acid in order to relate the decrease in MLSS to the accumulation of either end products of heterotrophic fermentation or end products of oxidation of these species by *D. desulfuricans.* Ethanol and lactic acid were both determined by Sigma assay kits. The ethanol assay was based on the oxidation of ethanol by nicotinamide adenine dinucleotide (NAD^+) catalyzed by alcohol dehydrogenase. The lactic acid assay was based on the oxidation of lactate by NAD^+ catalyzed by lactate dehydrogenase. Small molecular weight carboxylic acids were determined by gas chromatograph as described in Table 19. The highest lactic acid concentration found during the course of the experiment was 50 mg/L. The highest ethanol concentration was 7 mg/L. Acetate was found occasionally in the

Table 19. Chromatographic Conditions for Analysis of Carboxylic Acids in Bioreactor Medium

Instrument:	Hewlett Packard 5840
Column:	2 m x 1.8 mm ID glass, 80/120 Carbopack B-DA/ 4% Carbowax 20 M
Carrier Gas and Flow Rate:	N_2, 24 mL/min
Oven Temperature:	175°C
Injection Oven and Detector Oven:	200°C
Detector:	Flame Ionization Detector

culture medium, but the concentration never exceeded 20 mg/L. No other carboxylic acids were found. It is likely that the ethanol and lactic acid are end products of fermentation of feed components by the mixed heterotrophs in the culture. However, it is difficult to determine, given the small quantities, where these arise from fermentation of sludge solids or yeast extract. The acetate is probably the oxidation product of lactate and ethanol by *D. desulfuricans*.

Total protein was also estimated in both the influent and effluent of the SRB-bioreactor by the Bradford method using bovine serum albumin as a standard. Samples were diluted and sonicated prior to analysis to break microbial cells. Generally the total protein concentration in the effluent was about 20% higher on the average than that of the influent or feed (Figure 8). These observations indicate net growth of microorganisms in the bioreactor. However, the bulk of this growth seems to have been at the expense of yeast extract since the protein concentration in the effluent dropped after the yeast extract concentration in the feed was reduced. These observations also serve to illustrate that the feed contains significant protein which is not utilized in the bioreactor. Some sort of pre-treatment is needed to make this protein more accessible.

Chemical oxygen demand (COD) measurement of the feed and effluent confirm that most of the microbial activity in the bioreactor is attributable to the yeast extract. Figure 9 shows the total COD of feed and effluent. Figure 10 gives the COD of filterable solids in the feed and effluent. (Here filterable solids are defined as solids measured as MLSS.) Comparison of these figures shows that about 60%

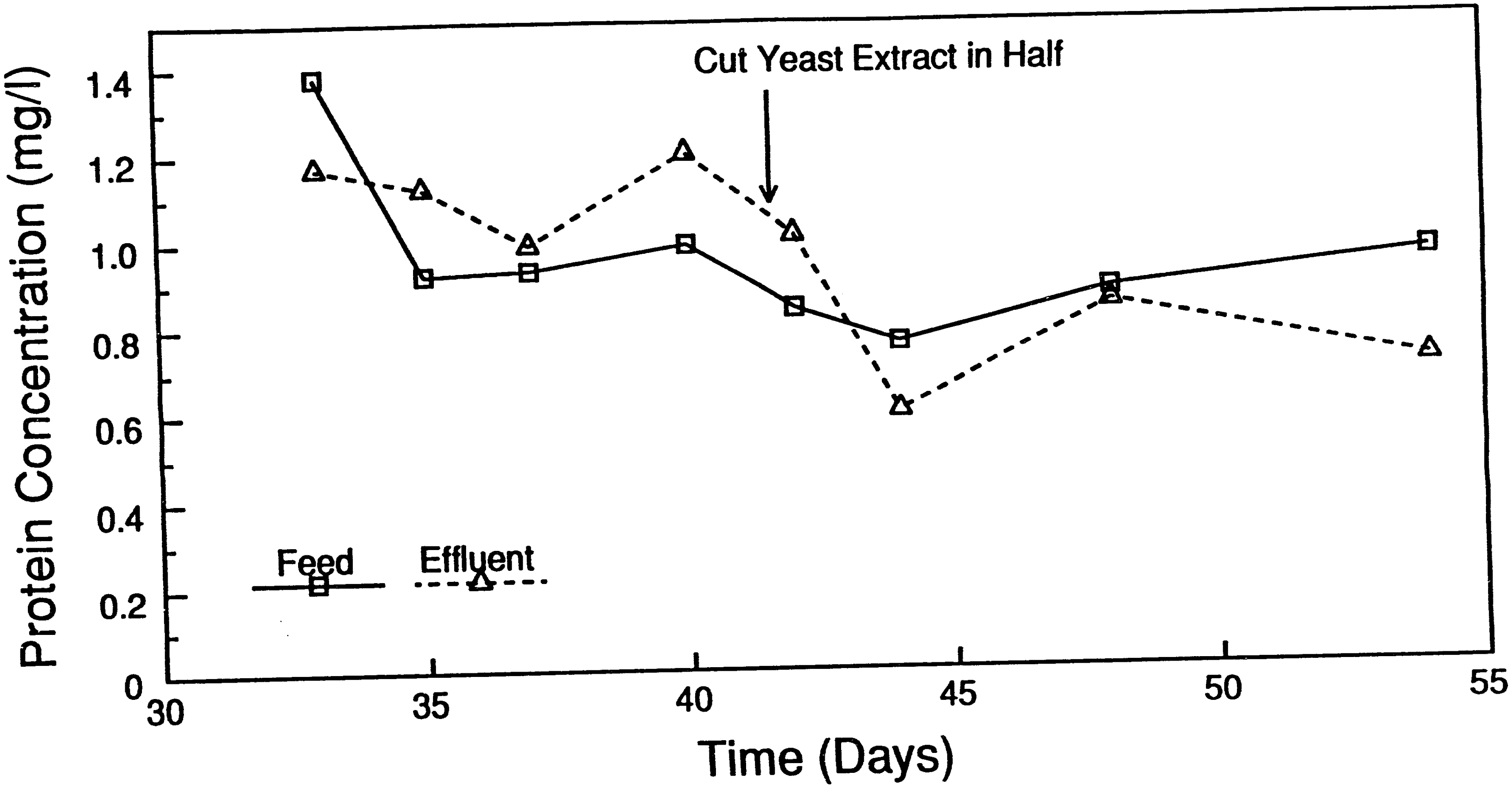


Figure 8. Influent and effluent protein concentrations in the SRB-bioreactor operating with a feed of municipal sewage sludge and yeast extract.

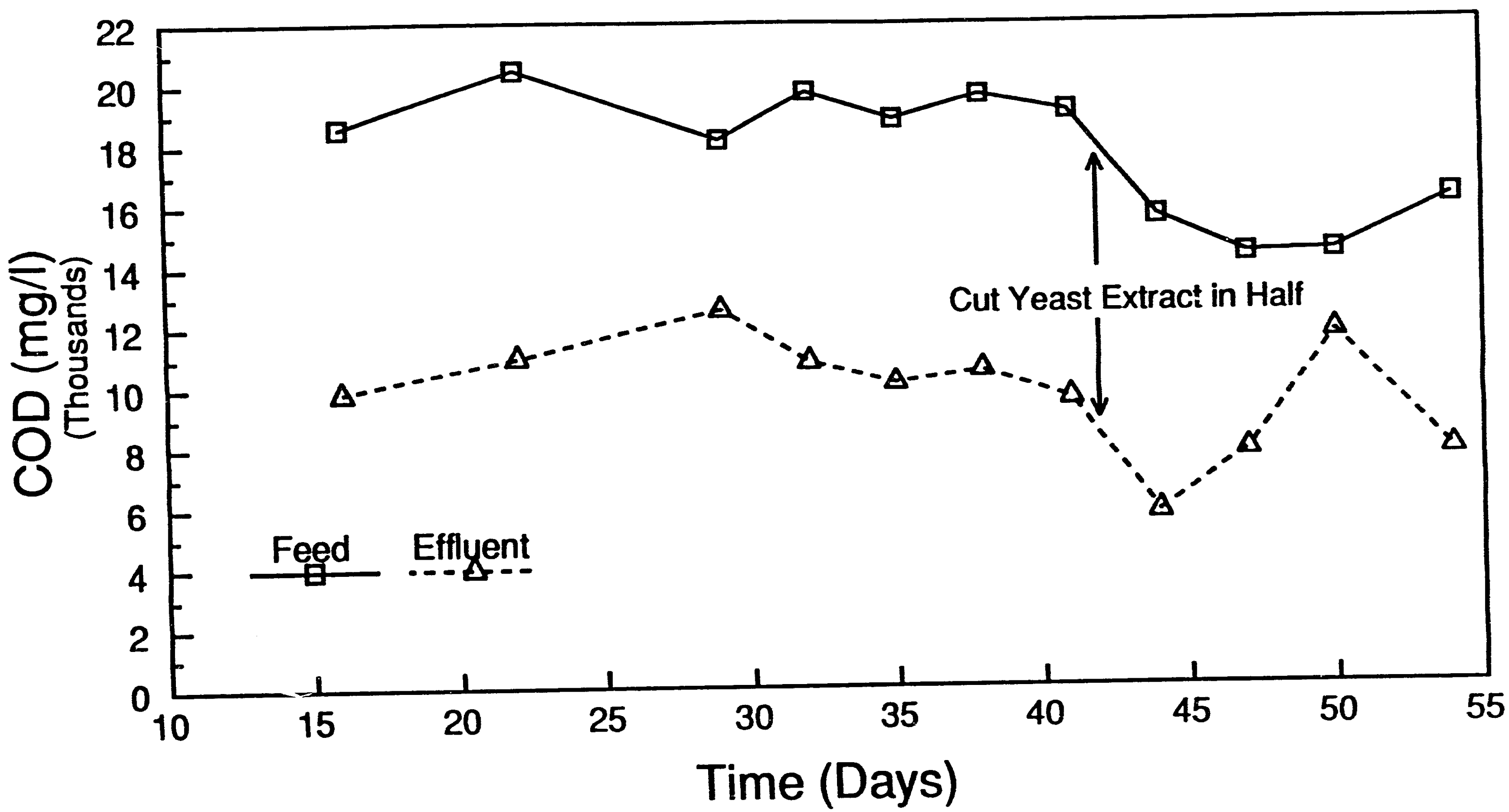


Figure 9. Total COD in influent and effluent of SRB-bioreactor operating with a feed of municipal sewage sludge and yeast extract.

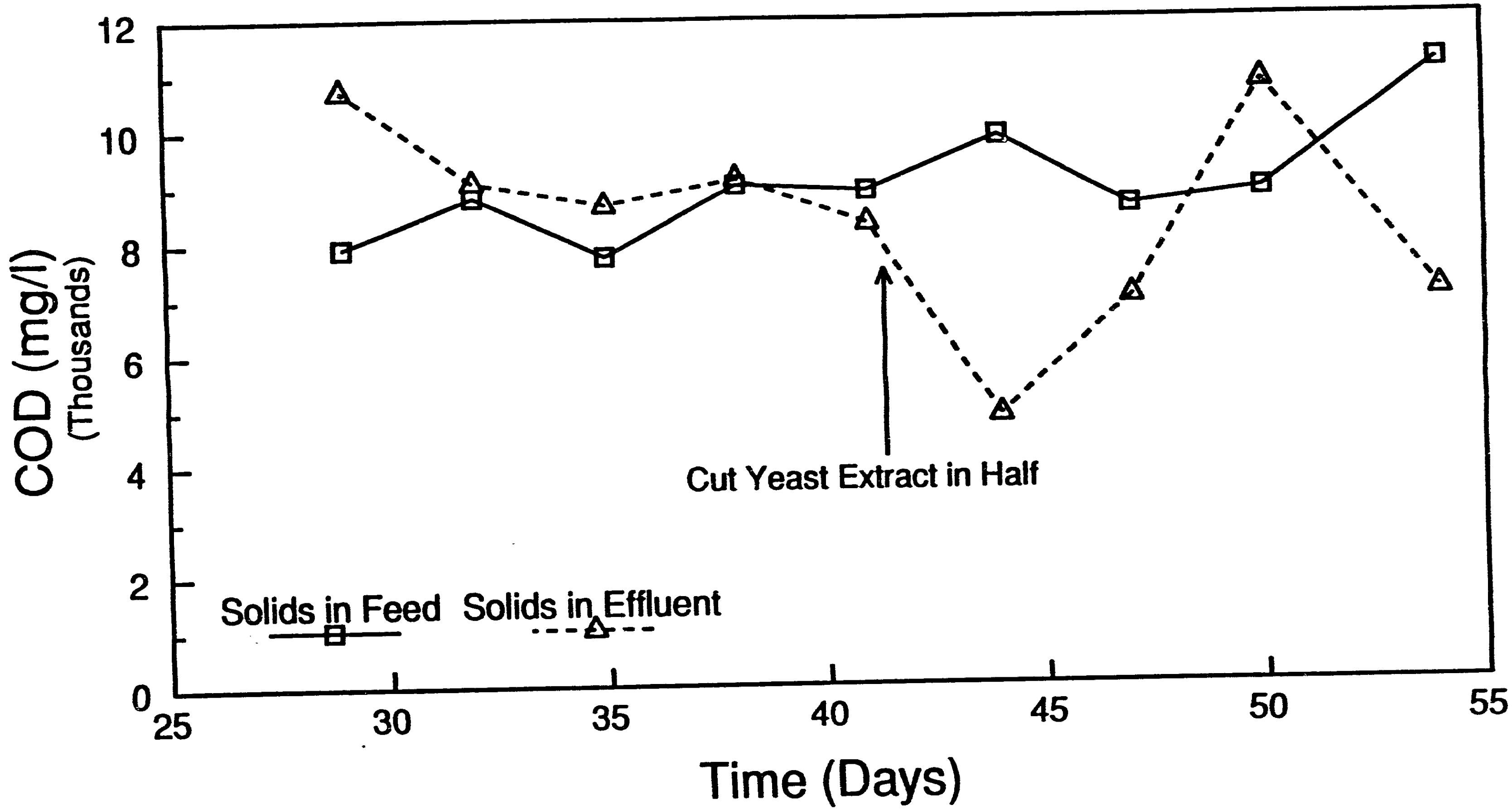


Figure 10. Solids COD in influent and effluent of SRB-bioreactor operating with a feed of municipal sewage sludge and yeast extract.

of the feed COD is in the yeast extract (and reduced salts in the minimal medium). The effluent COD is only slightly higher than the solids COD of the feed. The solids COD of the effluent was greater than the solids COD of the feed, again indicating net growth of microorganisms in the reactor. However, once again we see that this growth was primarily produced through the use of the components of yeast extract as carbon and energy sources. The high COD of the effluent solids confirms that most of the feed biomass has remained non-degraded.

Figure 11 shows typical settling curves for the feed and effluent solids (compared at the same concentrations) during operation at the higher yeast extract concentration. Effluent solids were seen to settle faster than feed solids. This is probably attributable to the production of new cells in the bioreactor and subsequently biopolymers which enhanced flocculation.

In conclusion, it is readily apparent that the carbon and energy resources of the raw municipal sludge have been utilized in the bioreactor to only a small extent in this base case. The effects of various pretreatments (or pre-digestions) on utilization of of the municipal sludge solids are described below. These pretreatments include (1) heat treatment, (2) alkali treatment, (3) heat and alkali treatment, (4) treatment with lipase, and (5) treatment with a protease. Heat and/or alkali treatment should hydrolyze many of the glycosidic and peptide linkages responsible for holding monomeric units of complex carbohydrates and proteins together. These monomeric units will be more fermentable. Municipal sludge consists of about 34% ether-soluble material (Table 9). Pretreatment with lipase (with

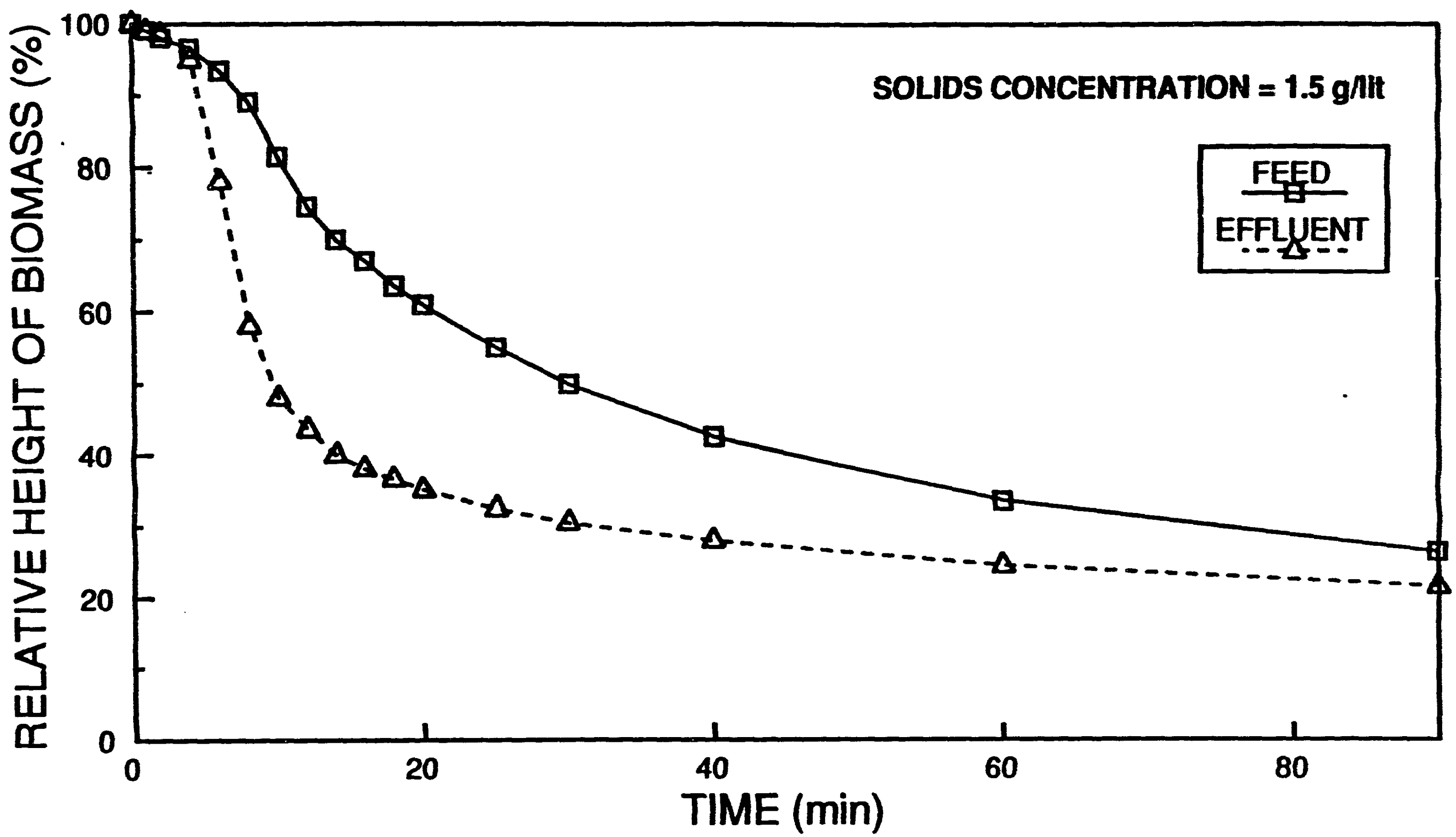


Figure 11. Settling properties of influent and effluent solids of SRB-bioreactor operating with a feed of municipal sewage sludge and yeast extract.

or without heat and/or alkali treatment) could make some of this material more available as a substrate for fermentation by hydrolyzing the ester linkages of fats and phospholipids. Similarly proteases could break down the 24% protein in municipal sluuges producing amino acids and short peptides to serve as substrates for fermentation. The fermentation of these "depolymerized" biopolymers will in turn produce carbon and energy sources for the SRB.

5.1.2 Pretreatment of Raw Municipal Sewage Solids

5.1.2.1 Heat /Alkali Treatment

As noted above, with raw sewage solids as a feed, the carbon and energy resources of municipal sludge are utilized to only a small extent in an SO_2-reducing bioreactor. Some type of pretreatment or predigestion will be necessary in order for the SRB and non-SRB organisms in the SO_2-reducing cultures to be able to utilize these substrates efficiently. Microbial cells which make up much of the sewage solids must be broken open liberating fermentable material and biopolymers must be "depolymerized" to make carbon and energy sources available.

The first pretreatment examined was heat under alkaline conitions. Municipal sewage solids were suspended in the medium described by Table 20 to a concentration of 100 g wet-packed sewage solids per liter. At this time 100-mL samples of this suspension were adjusted to pH 10, 11 or 12 with 10N NaOH with duplicate samples autoclaved at 121°C for 30 or 60 minutes. Suspensions were then cooled to room temperature and the pH adjusted to 7.0 with 6N HCl. Control suspensions received no treatment. Each sample was analyzed to determine the MLSS and the soluble COD and soluble protein

Table 20. Sulfate-Free Minimal Medium for *D. desulfuricans*

Component	Quantity/L
Na_2HPO_4	1.2 g
KH_2PO_4	1.8 g
$MgCl_2$	0.7 g
NH_4Cl	0.2 g
$FeCl_3$	0.04 g
Batch Vitamin Solution (Table 12)	2.0 mL
Heavy Metal Solution (Table 13)	15.0 mL
Mineral Water	50.0 mL

concentrations in the filtrate. Results are shown in Figures 12-14. Figure 12 shows that the MLSS concentration declined with increasing pH of incubation and increasing heating time. The MLSS level in the control was 5800 mg/L. These results suggest that some fraction of the biosolids of the raw municipal sewage is solubilized by heat/alkali treatment. This is confirmed by Figure 13 which shows increasing filtrate COD with increasing pH and heating time and Figure 14 which shows increasing concentrations of soluble protein under the same conditions. The COD and soluble protein concentrations in the untreated controls were 700 mg/L and 0.024 g/L, respectively.

This experiment was repeated with a wider range of heating times in order to better document the effect of heating time at 121°C on solubilization of sewage solids. Municipal sewage solids were suspended in the medium described in Table 20 as described above to a concentration of 100 g wet-packed sewage solids per liter. At this time 100-mL samples of this suspension were adjusted to pH 11 or 12 with 10N NaOH with duplicate samples autoclaved at 121°C for 15, 30, 45 and 60 min. Suspensions were then cooled to room temperature and the pH adjusted to 7.0 with 6N HCl. Control suspensions received no treatment. Each sample was analyzed to determine the MLSS and the soluble COD an soluble protein concentratoins in the filtrate. Results are shown in Figures 15-17. Figure 15 shows that the MLSS concentration declined with increasing pH and heating time at 121°C. The MLSS in the control was again 5800 mg/L. It was anticipated that solubilization of the sewage solids might level off at longer heating times under the assumption that not all of the solids are subject to solubilization. This was not the case up to 60 min of heating at

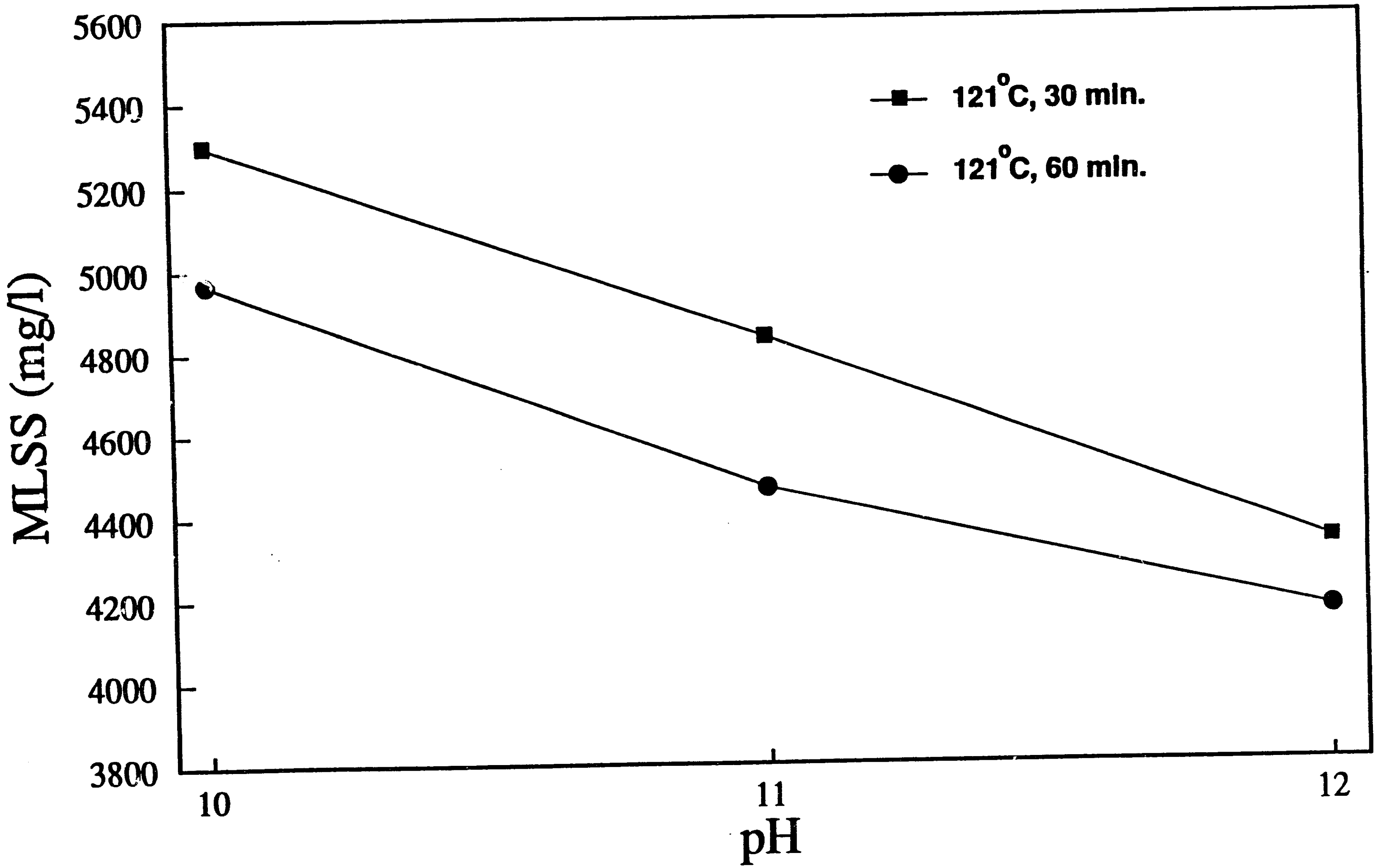


Figure 12. Effect of pH and heating time on MLSS concentration in a suspension of municipal sewage solids. (The initial MLSS concentration, without treatment was 5800 mg/L.)

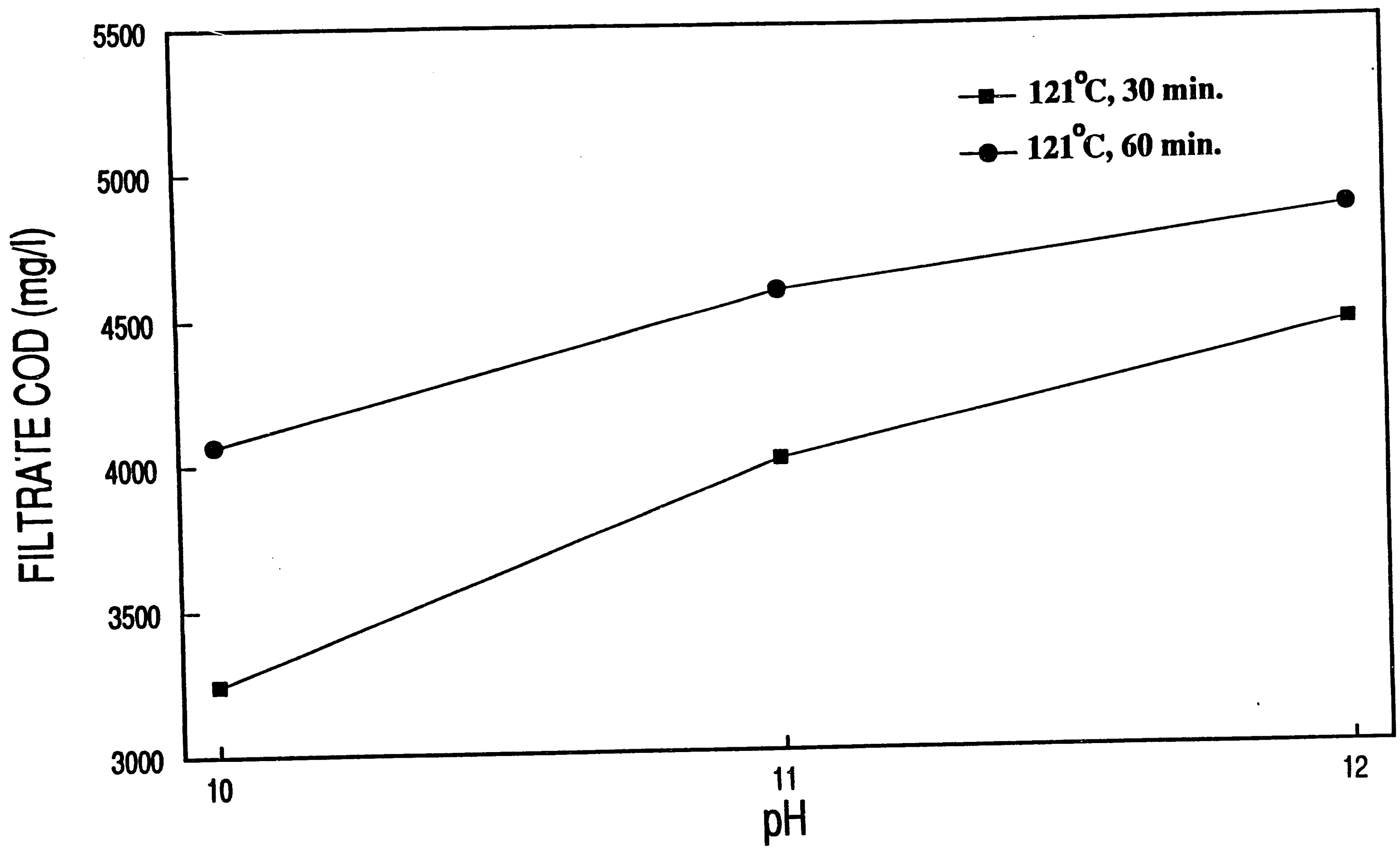


Figure 13. Effect of pH and heating time on filtrate COD in a suspension of municipal sewage solids. (The filtrate COD in untreated samples was 700 mg/L.)

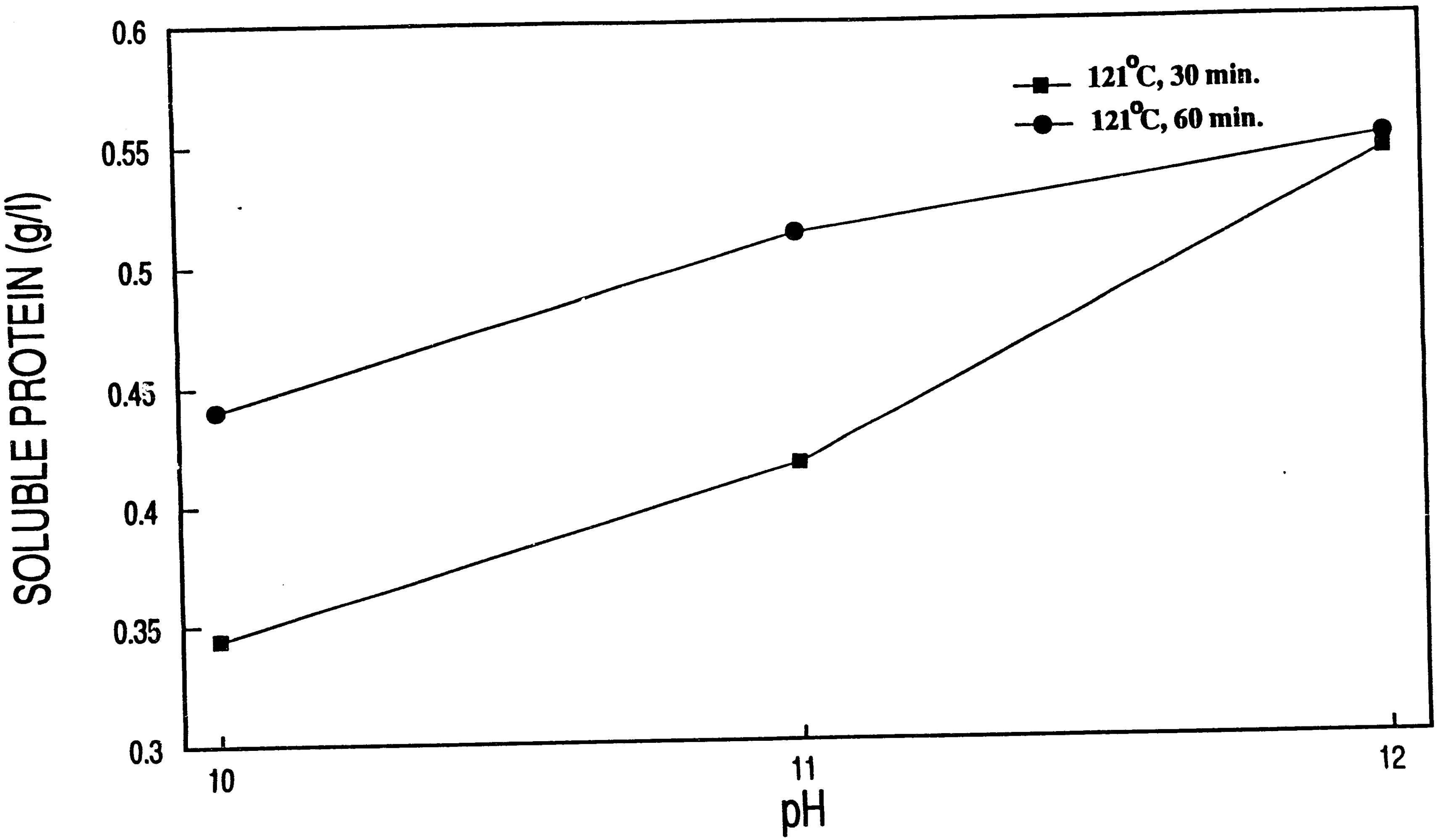


Figure 14. Effect of pH and heating time on soluble protein concentration in a suspension of municipal sewage solids. (The soluble protein concentration in untreated samples was 24 mg/L).

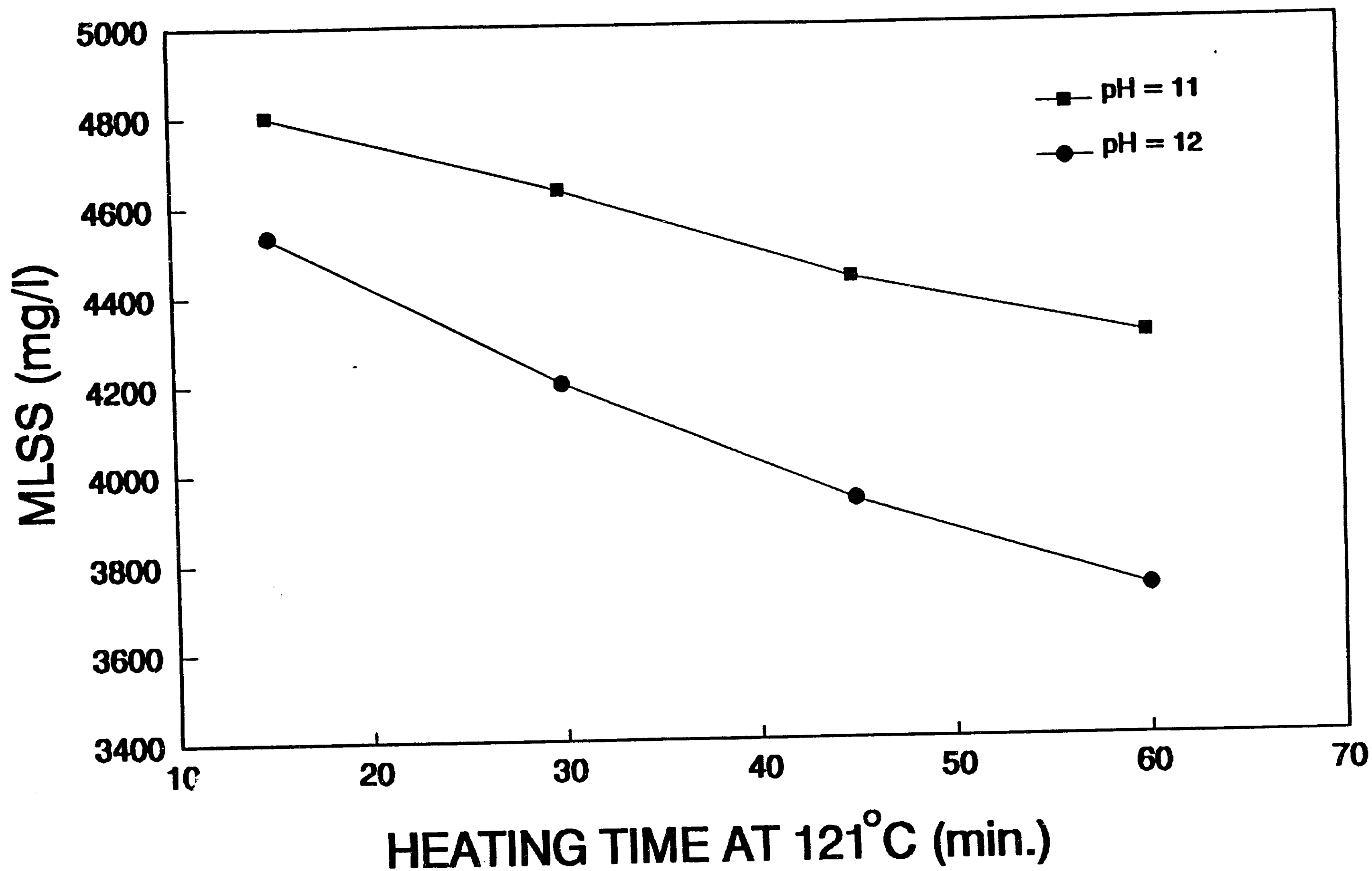


Figure 15 .. Mixed liquor suspended solids (MLSS) in a suspension of sewage sludge solids heated at 121°C at pH 11 and 12. (MLSS in the untreated sample was 5800 mg/L)

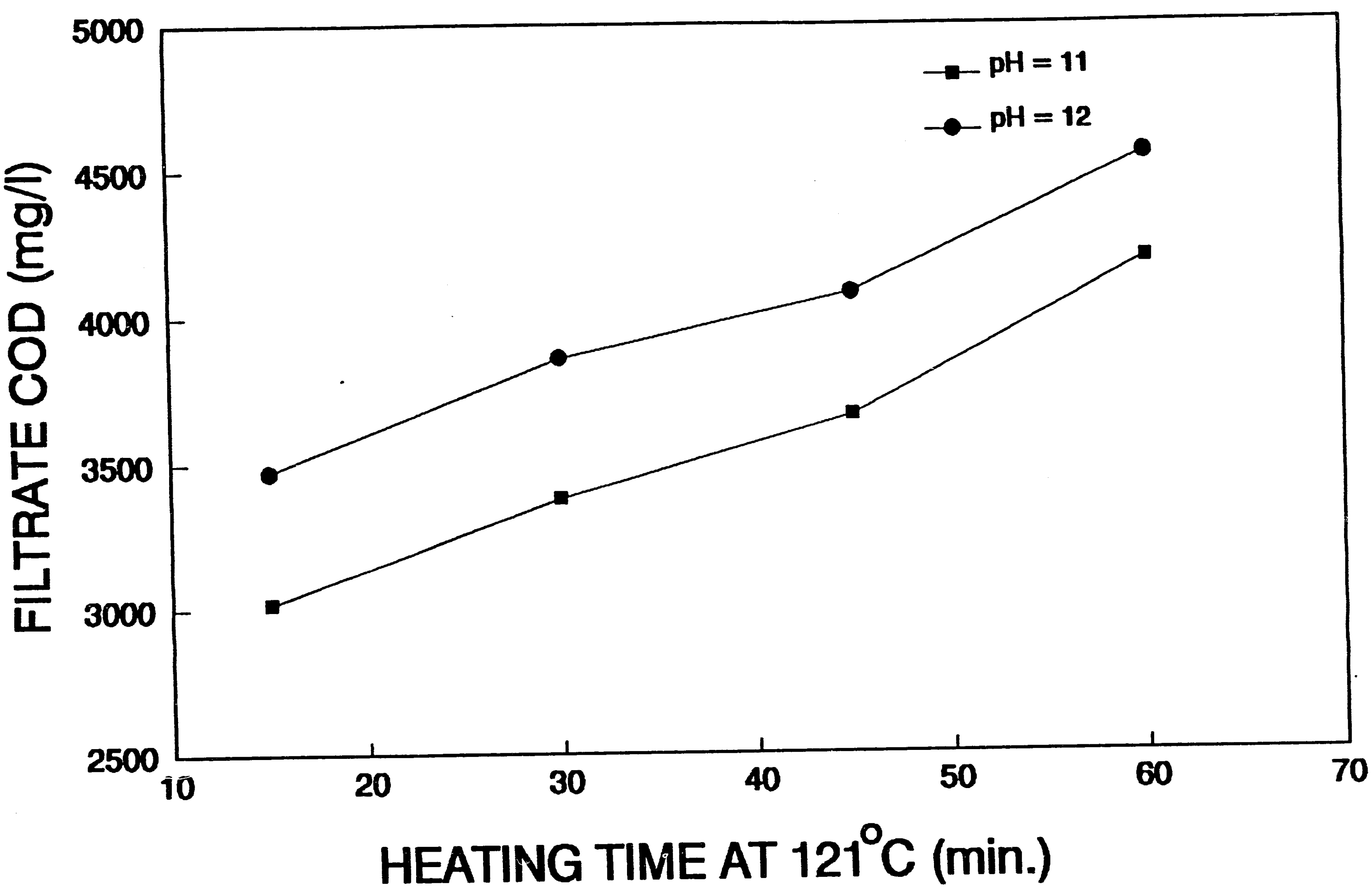


Figure 16. Soluble COD in a suspension of sewage sludge solids heated at 121°C at pH 11 and 12. (Soluble COD in the untreated sample was 80 mg/L)

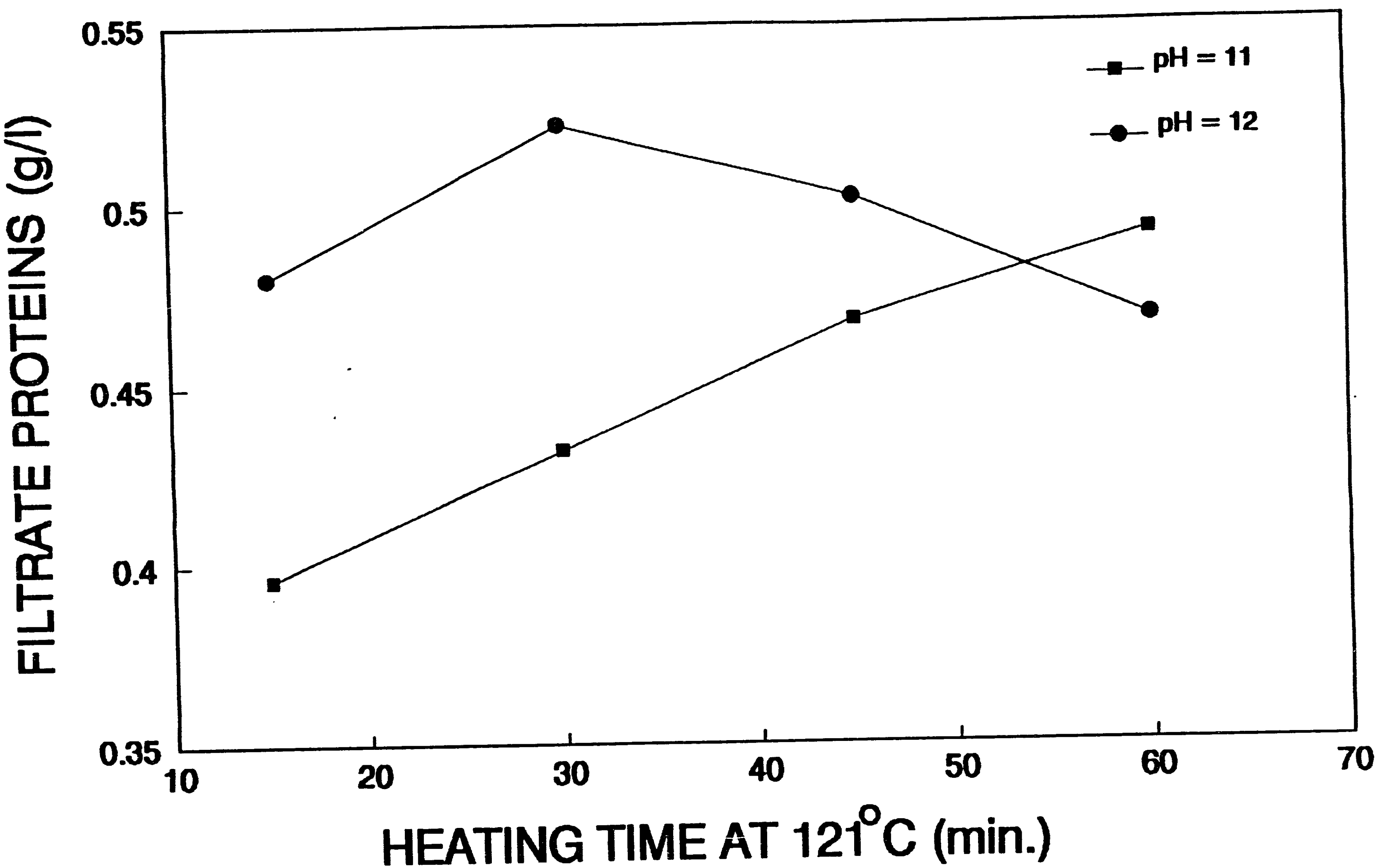


Figure 17. Soluble protein in a suspension of sewage sludge solids heated at 121°C at pH 11 and 12. (Soluble protein in the untreated sample was 0.04 g/L)

121°C. Figure 16 also indicates significant solubilization throughout the range of heating times. As seen in Figure 17, soluble protein was seen to decrease with increasing heating time at pH 12 above 30 min. This was likely due to hydrolysis of solubilized proteins to produce peptides and amino acids which did not respond to the Bradford analysis method for protein.

The effect of heating temperature at pH 11 and 12 on solubilization of sewage solids was also investigated. Municipal sewage solids were again suspended in the medium described in Table 20 to a concentration of 100 g wet-packed solids per liter. At this time 100-mL samples of this suspension were adjusted to pH 11 or 12 with 10N NaOH with duplicate samples incubated at room temperature (25°C), 50°C, 80°C and 121°C (autoclave) for 60 minutes. Suspensions were then cooled to room temperature and the pH adjusted to 7.0 with 6N HCl. Control suspensions received no treatment. Each sample was analyzed to determine the MLSS and the soluble COD and soluble protein in the filtrate. Results are shown in Figures 18-20. As seen in Figure 18 the MLSS declined with increasing temperature of incubation at pH 11 and pH 12. However, sisgnificant solubilization (35%) was indicated only at 121°C and pH 12. It is interesting that the filtrate COD (Figure 19) did not follow the same trend in that the filtrate COD at pH 11 and 12 were similar. As seen in Figure 20 significantly more protein was solubilized at pH 12 than pH 11 at 25-80°C. At 121°C the soluble protein at pH 12 is actually lower than that at pH 11. This was probably due to hydrolysis of proteins at pH 12 and 121°C producing amino acids and short peptides which do not respond to the Bradford method of protein analysis.

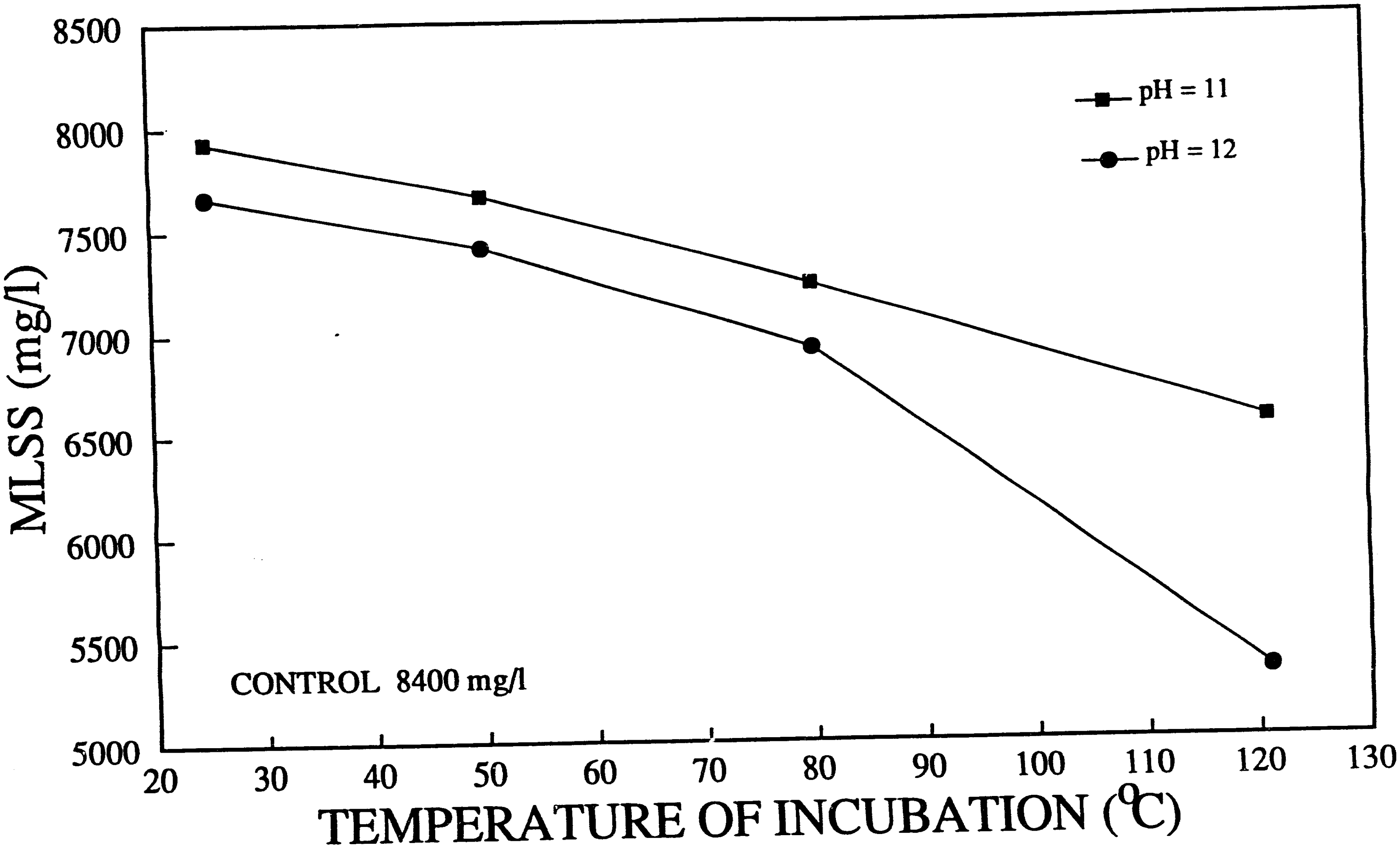


Figure 18. Effect of pH and incubation temperature on MLSS concentration in a suspension of municipal sewage solids.

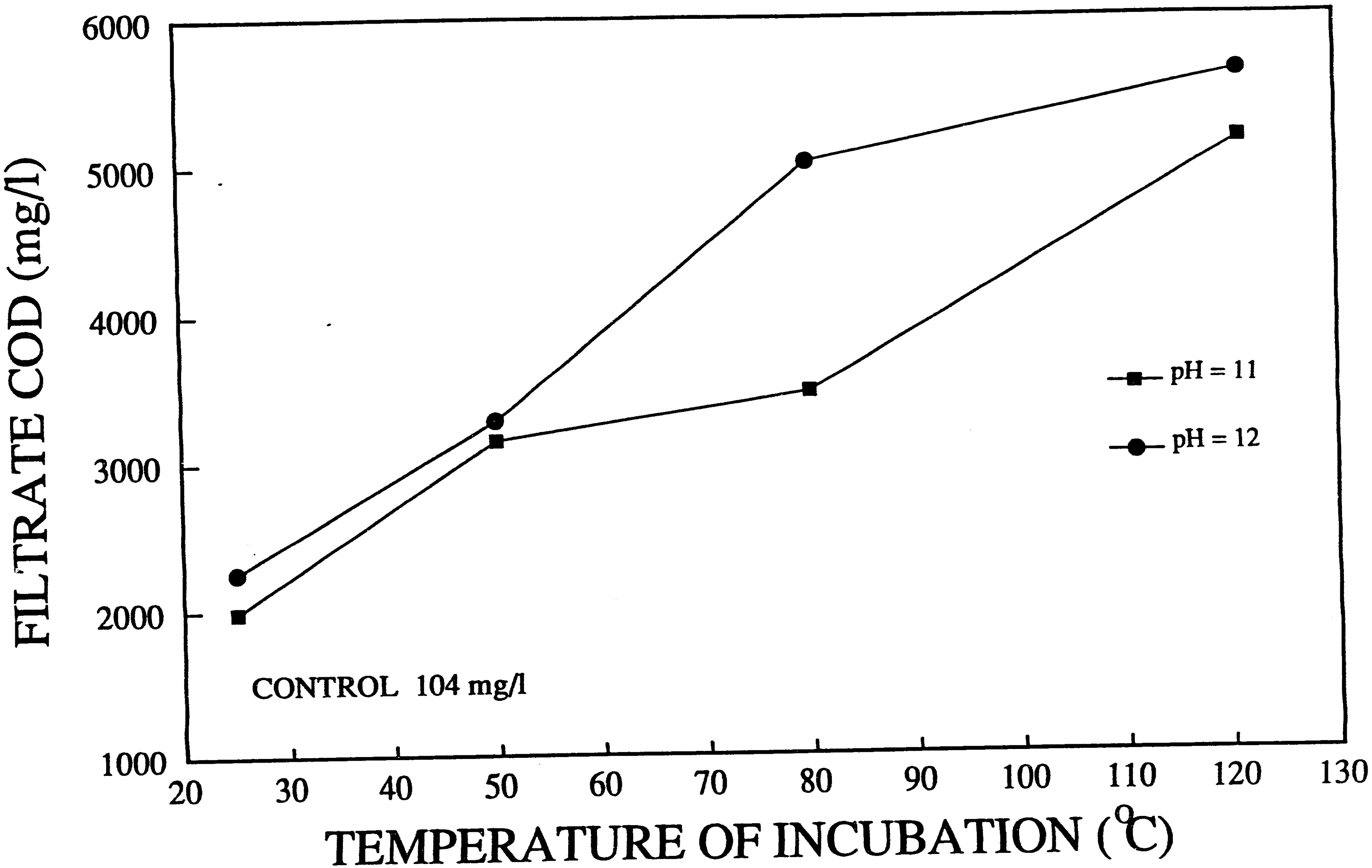


Figure 19. Effect of pH and incubation temperature on filtrate COD in a suspension of municipal sewage solids.

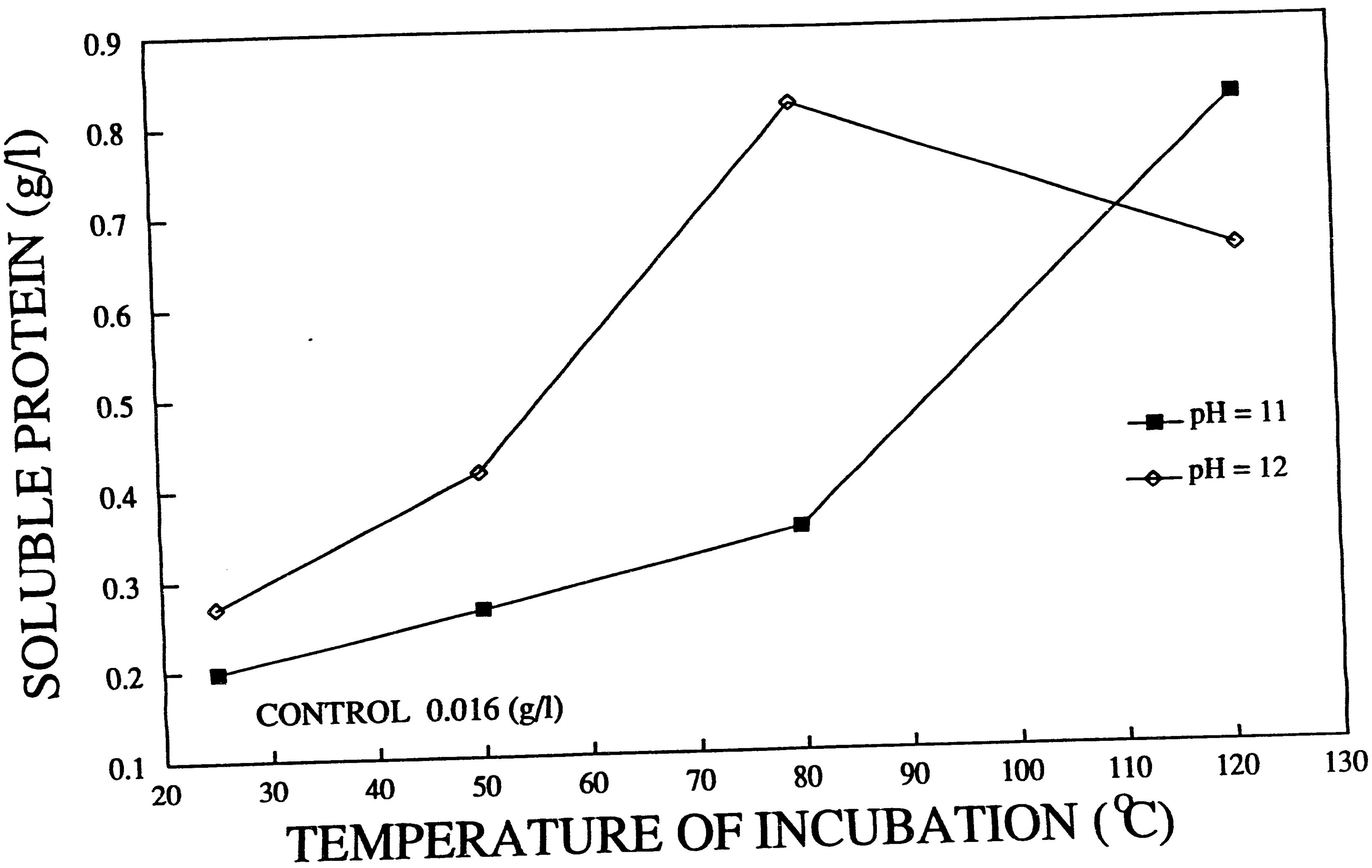


Figure 20. Effect of pH and incubation temperature on soluble protein concentration in a suspension of municipal sewage solids.

This experiment was repeated at incubation temperatures of 75°C, 85°C, 100°C and 121°C with similar results (Figures 21-23).

5.1.2.2 Enzyme Treatment

As noted in Section 5 enzymes can potentially be used to "depolymerize" biopolymers of municipal sewage solids and make fermentable substrates available in SO_2-reducing cultures. A preliminary investigation of the use of liquefaction enzymes was conducted as follows. Three enzymes were chosen for this initial study: lipase, pronase and lysozyme. Lipase (triacylglycerol acylhydrolase) hydrolyzes triglycerides liberating free fatty acids. Pronase is an unusually non-specific protease from *Streptomyces griseus*. Lysozyme is a mucopeptide N-acetylmuramoylhydrolase and is useful in fragmenting bacterial cell wall biopolymers.

Municipal sewage solids were suspended in the medium described in Table 20 to a concentration of 100 g wet-packed sludge solids per liter. 100-ml samples of this suspension were adjusted to pH 12 with 10N NaOH and autoclaved at 121°C for 30 min. Cooled suspensions were adjusted back to pH 7.0. Various suspensions were then treated with 1.0 mL of a 0.10 g/mL solution of pronase, lipase or lysozyme, mixed well and incubated at 37°C for 1 hour. (Controls were incubated without enzyme treatment.) At the end of this time suspensions were filtered and the filtrates analyzed for soluble COD and soluble protein. Appropriate dilutions of each enzyme solution were also analyzed in order to correct the COD and protein measurements for contributions from the enzyme preparations. Results are shown in Table 21. Table 21 indicates that soluble COD increaed by about 16% when heat/alkali treated sludge suspensions were treated with pronase

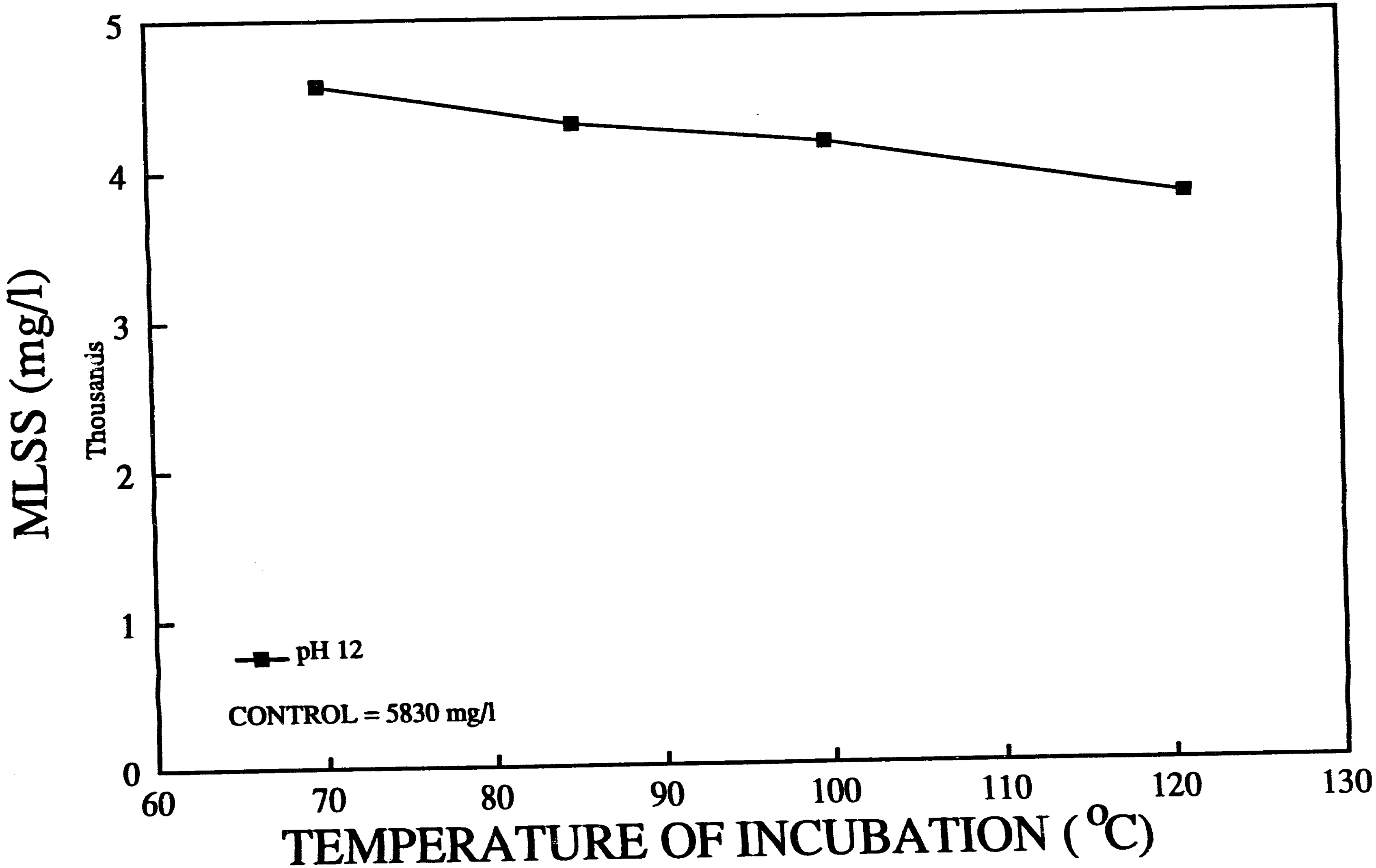


Figure 21. Effect of incubation temperature at pH 12 on MLSS concentration in a suspension of municipal sewage solids.

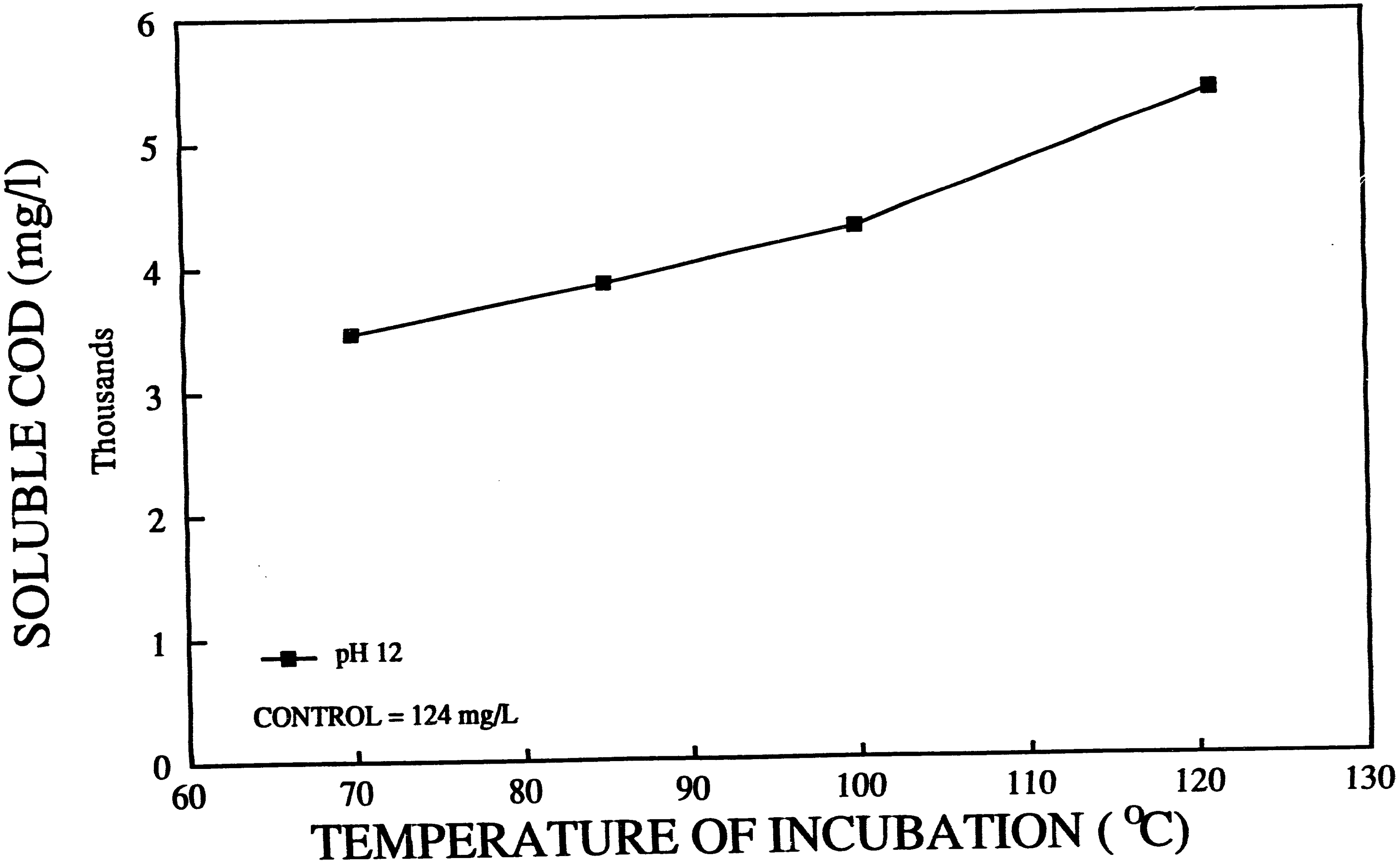
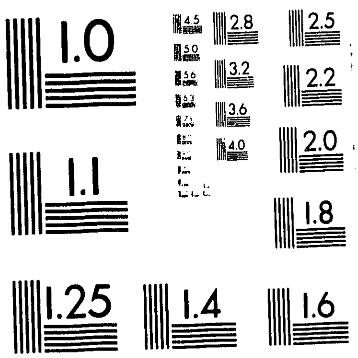


Figure 22. Effect of incubation temperature at pH 12 on filtrate COD concentration in a suspension of municipal sewage solids.


1.0
4.5
5.0
5.6
6.3
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.1
1.8
1.25
1.4
1.6

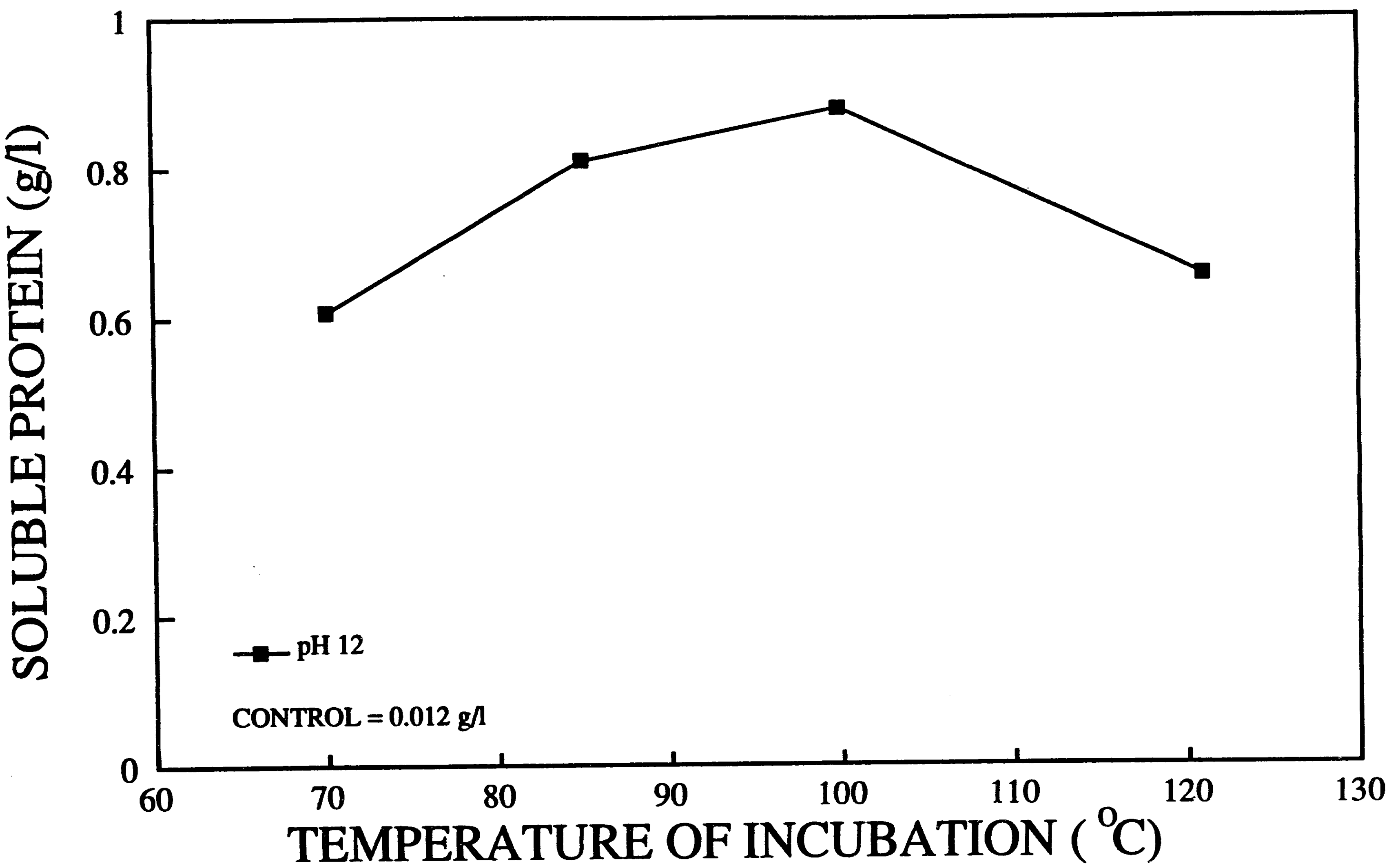


Figure 23. Effect of temperature at pH 12 on soluble protein concentration in a suspension of municipal sewage solids.

Table 21. Soluble COD and Protein Concentrations in Filtrates of Enzyme Treated Suspensions of Heat/Alkali Treated Sewage Solids

Enzyme Treatment	COD, mg/L*	Protein, mg/L*
Lipase	4521	0.41
Pronase	4542	0.17
Lysozyme	3345	0.33
None	3903	0.52

* Corrected for COD and protein contributions of enzyme preparations

or lipase. However, the soluble protein concentrations were seen to decrease in all enzyme-treated samples. A dramatic decrease was seen in pronase-treated samples. This is not unexpected since hydrolyzed proteins would be unreactive toward the reagent utilized for protein analysis (Bradford). The decrease in soluble protein concentration in the other enzyme-treated samples may be due to some contamination of these preparations with proteases.

In conclusion, it appears that these enzymes can produce only modest increases in the concentration of fermentable substrates in the best cases. However, these and other enzymes may be investigated again at a later date.

5.1.3 Further Studies of a Continuous Sulfate-Reducing Mixed Culture of *D. desulfuricans* Operating with a Feed of Municipal Sewage Sludge

The continuous culture described in Section 5.1.1 was operated with sulfate as the terminal electron acceptor for an additional 60 days with various changes in the feed and operating conditions. At the beginning of this experiment, the operating conditions were essentially the same as those described in Table 16 except that the sulfate and yeast extract concentrations in the feed were 7.8 g/L (as Na_2SO_4) and 5.5 g/L, respectively. The following changes were made in the feed condition during the course of the experiment:

Day	Change
2	Feed autoclaved at 121°C for 30 min
26	pH of feed adjusted to 10 followed by autoclaving at 121°C for 30 min, and readjustment to pH 7.0
31-35	50 g wet-packed sludge from anaerobic digester added daily. (The purpose of this introduction of anaerobic sludge was an attempt to introduce bacteria capable of liquefaction of biopolymers).

35	Yeast extract concentration reduced from 5.5 g/L to 2.0 g/L
42	Feed autoclaved at 121°C for 60 min (at pH 10)
51	Yeast extract eliminated Feed autoclaved at 121°C for 30 min (at pH 12)

The MLSS concentrations in the feed and effluent from the reactor during this experiment are shown in Figures 24a and 24b. The total COD and filtrate COD (minus solids) of the feed and effluent are shown in Figures 25 and 26 respectively. The sulfate concentrations are shown in Figures 27a and 27b. Protein concentrations, total and feed filtrate, are shown in Figures 28a and 28b.

Examination of these figures leads to the following conclusions.

1) Autoclaving alone at 121°C for 30 min did not produce any appreciable reduction in MLSS of the feed and therefore little appearent solubilization of sewage solids. However, the effluent sulfate concentration was seen to decline in this period indicating that SRB activity was stimulated.

2) Heating feed to 121°C under alkaline conditions produced a decrease in MLSS of the feed resulting in greater availability of fermentable substrates. (Note increase in feed filtrate protein and COD).

3) The effluent MLSS was lower than the influent MLSS under all feed conditions indicating utilization of some solid components of the feed.

4) Addition of sludge from an anaerobic digester did not produce any appreciable increase in the soluble COD or protein in the reactor effluent or any decrease in the

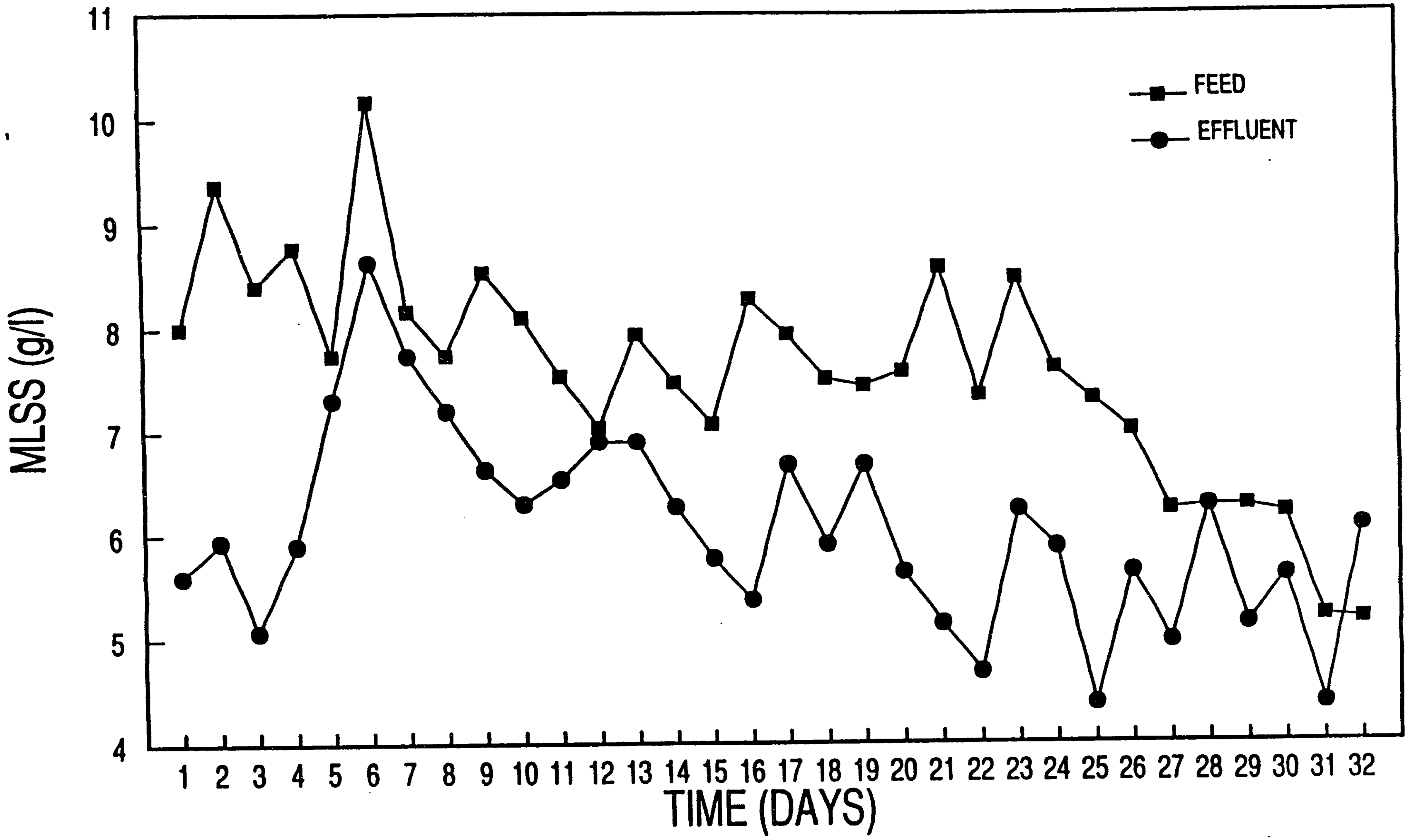


Figure 24a. MLSS concentrations in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

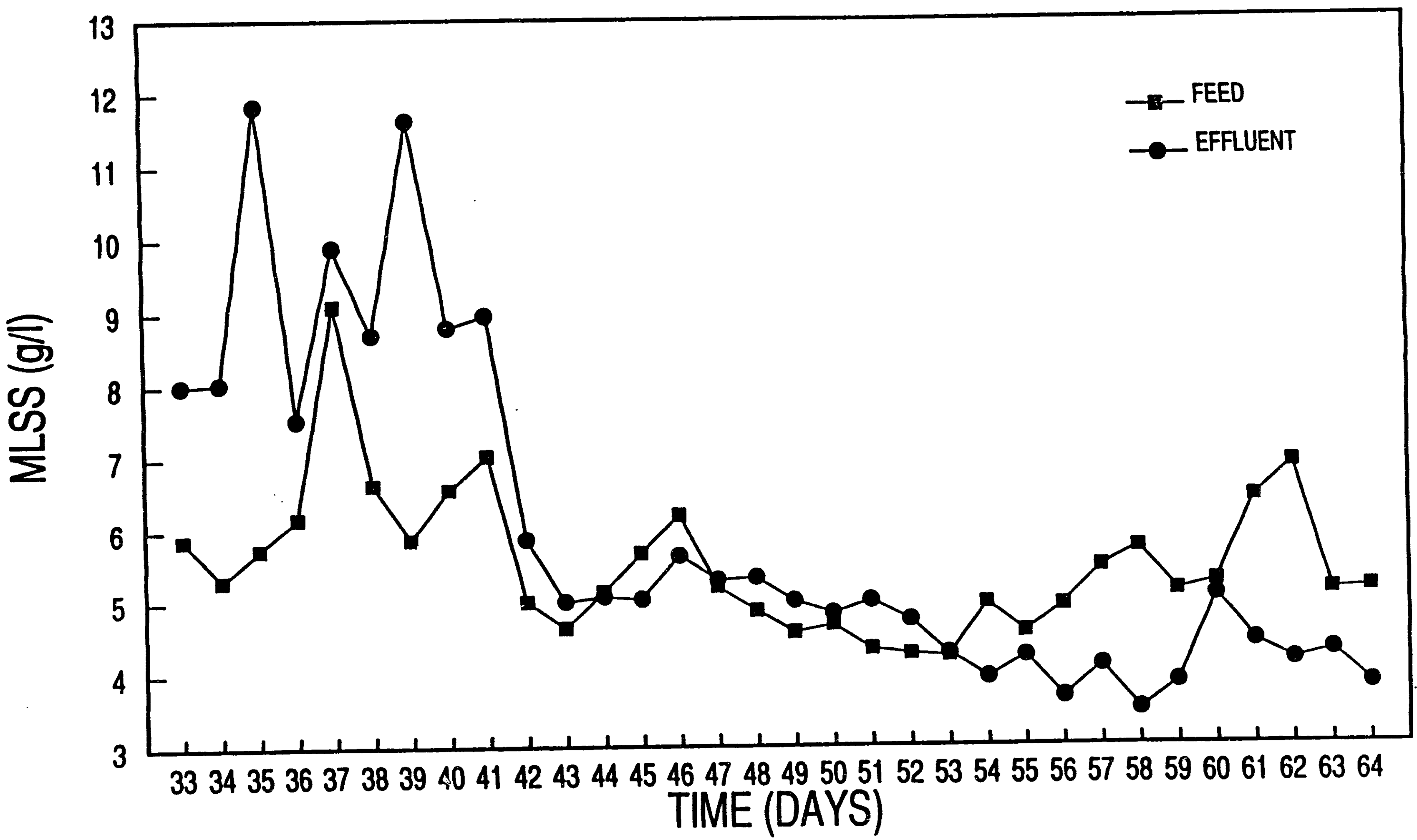


Figure 24b. MLSS concentrations in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

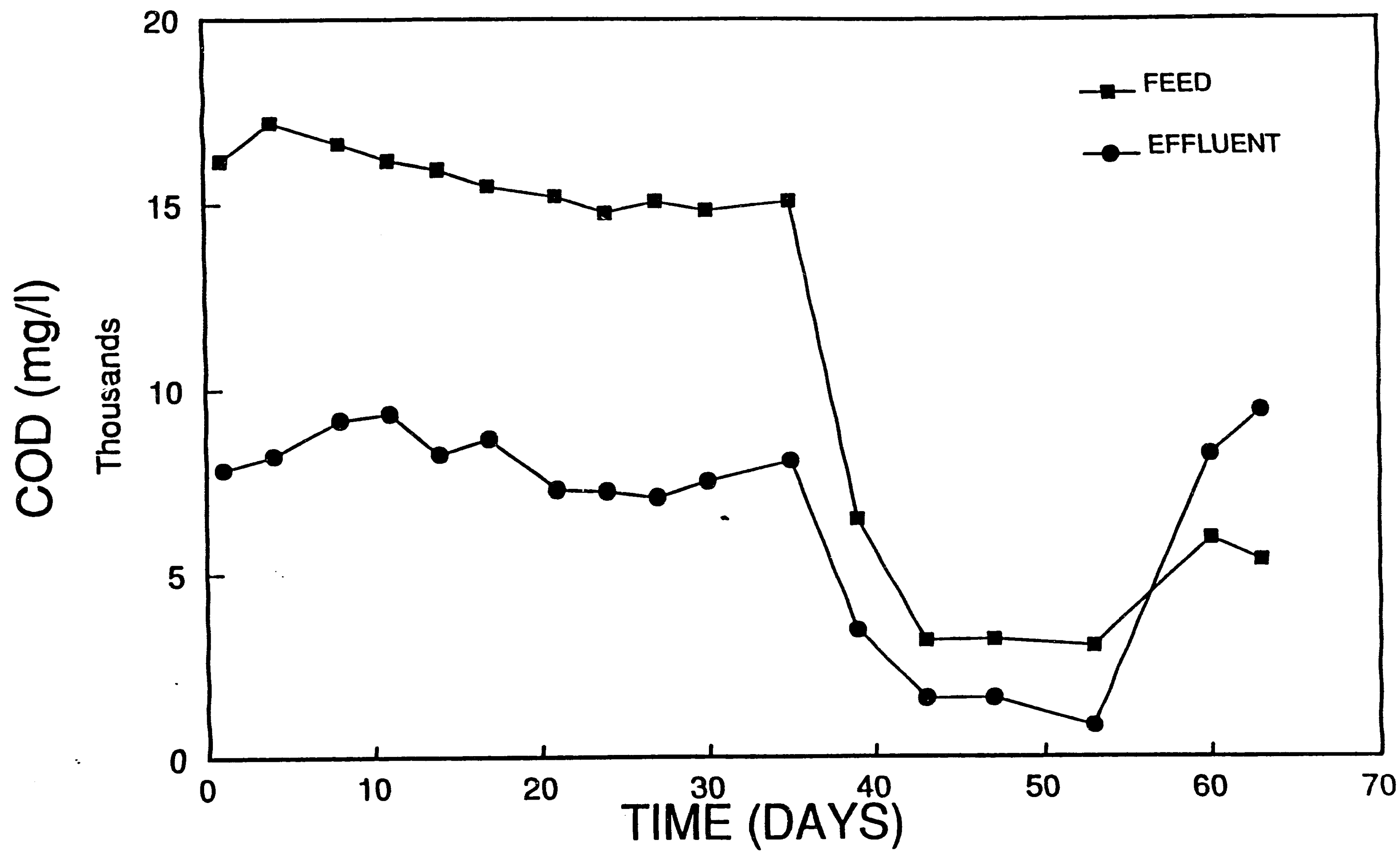


Figure 25. Total COD in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

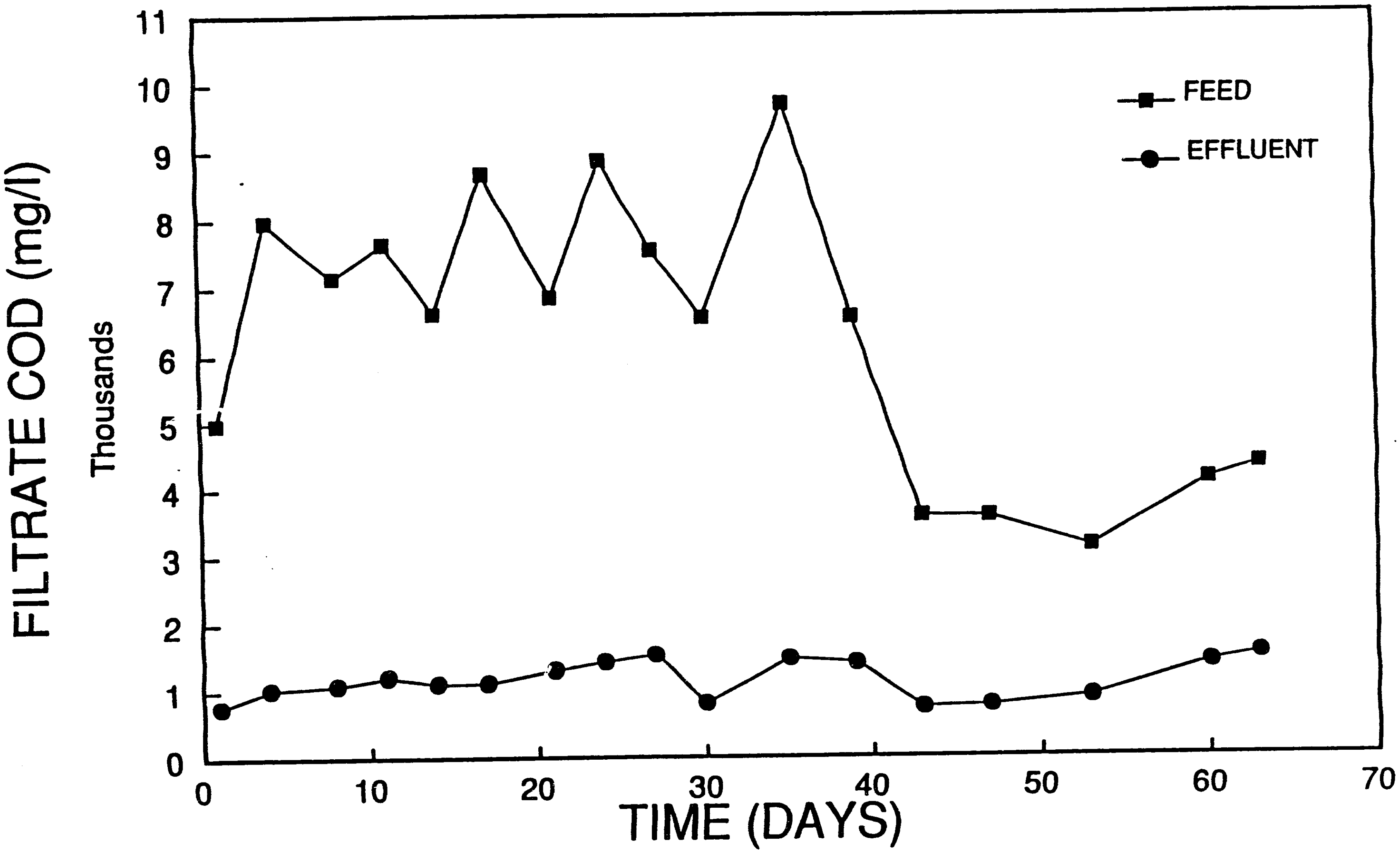


Figure 26. Filtrate or soluble COD in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

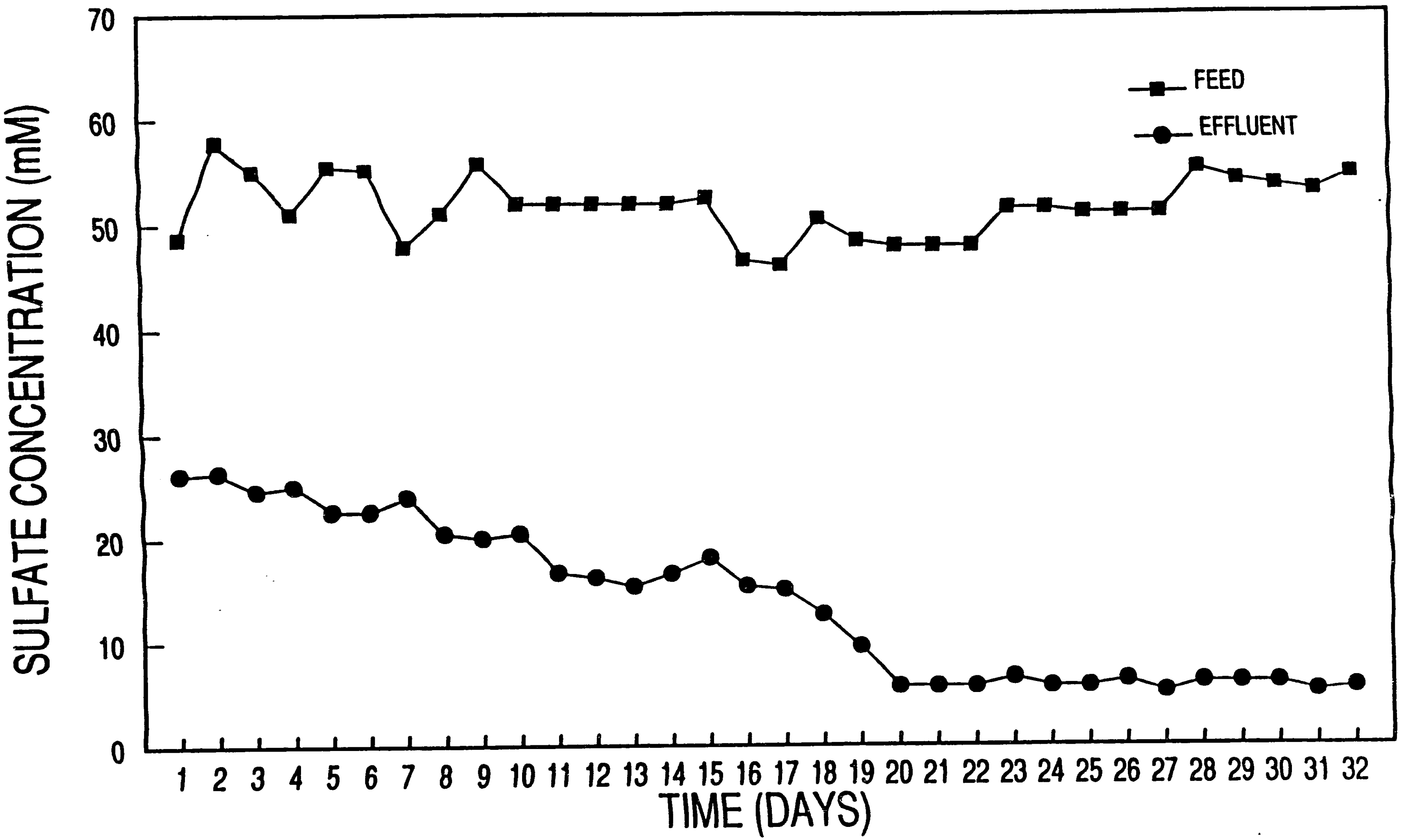


Figure 27a. Sulfate concentration in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

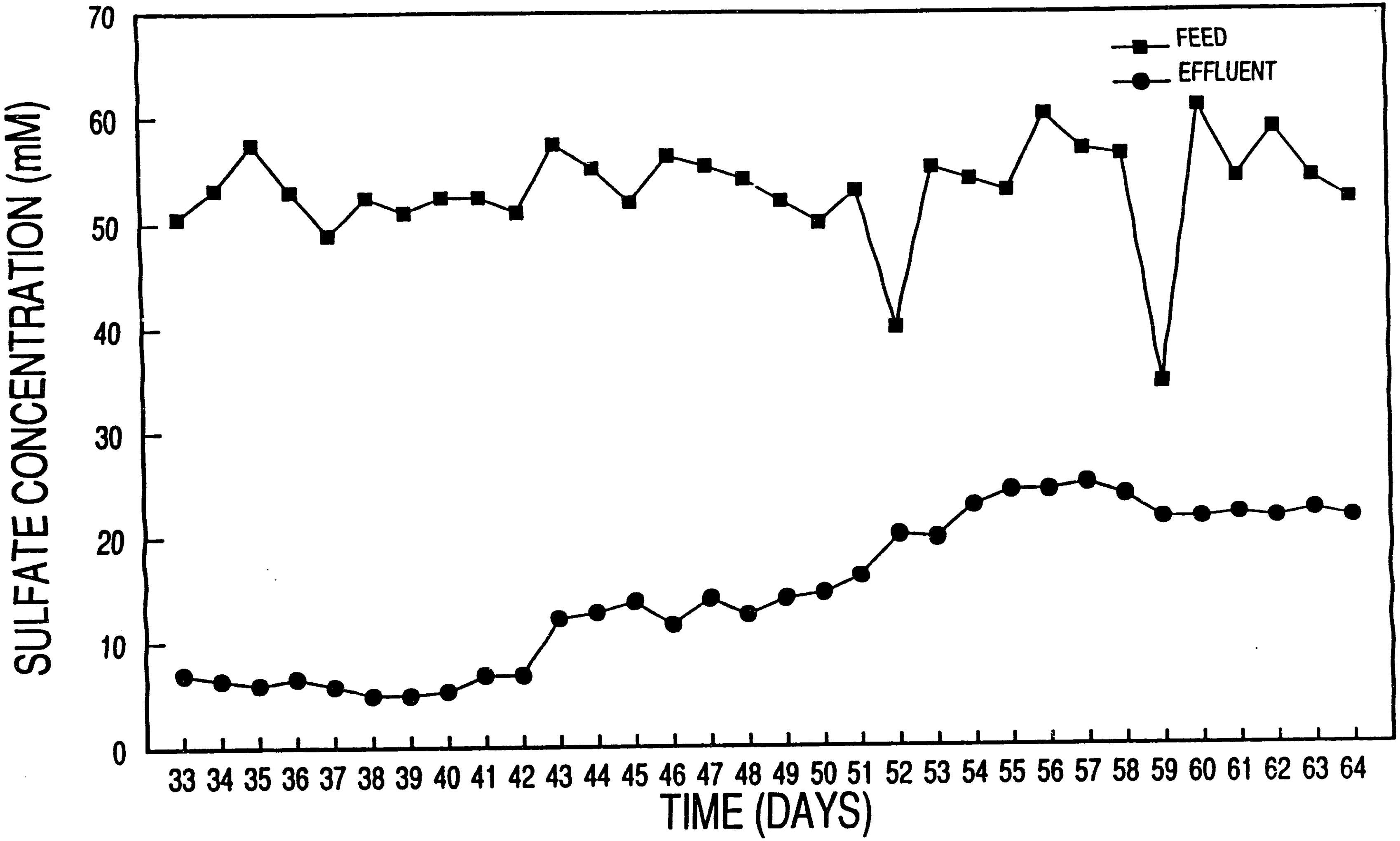


Figure 27b. Sulfate concentration in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

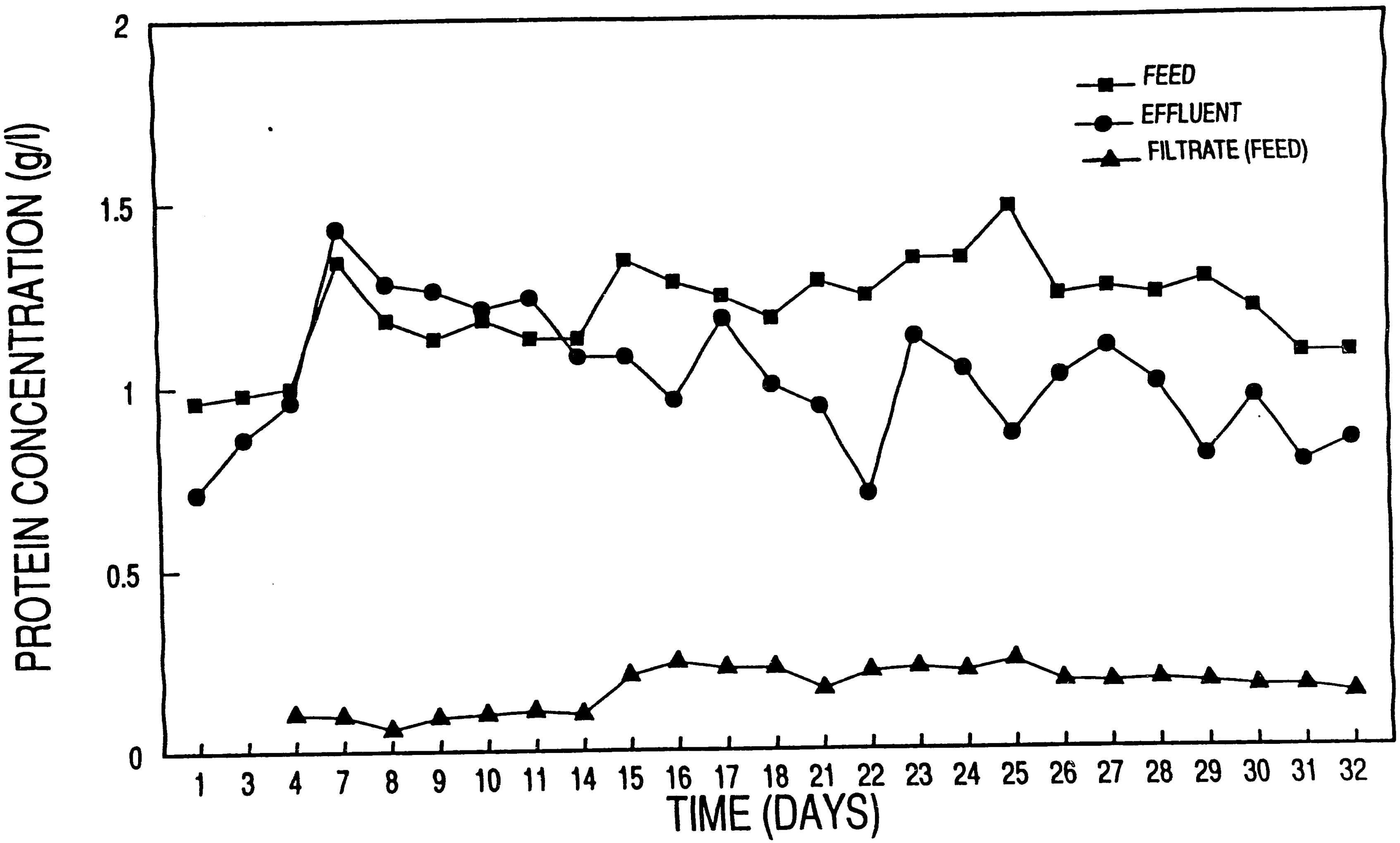


Figure 28a. Soluble protein concentration in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

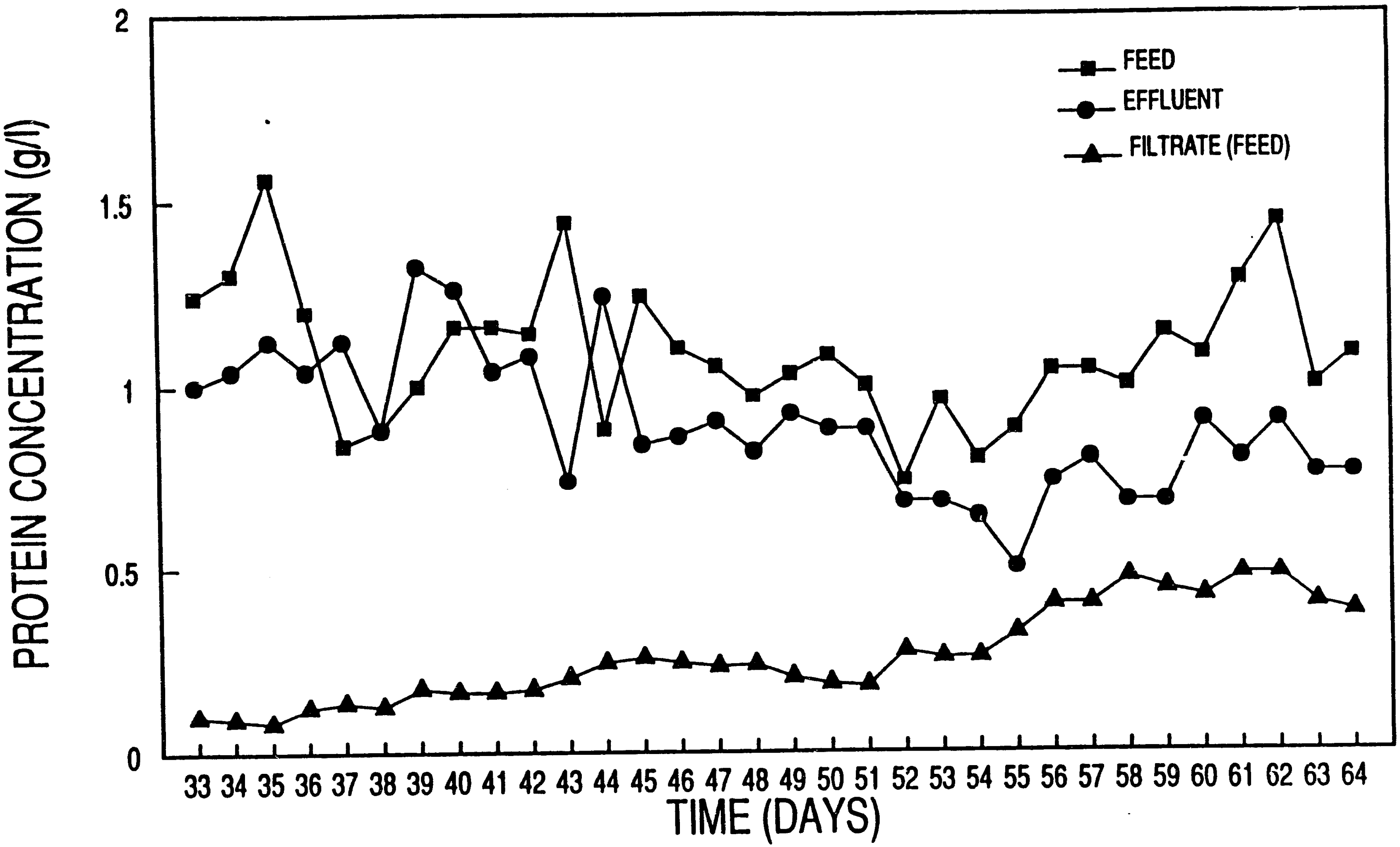


Figure 28b. Soluble protein concentration in the feed and effluent of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement.

effluent sulfate concentration. The latter would have been indicative of an increase in SRB activity.

5) Finally, the most important conclusion was that yeast extract could be eliminated from the feed if the feed is pretreated with heat under alkaline conditions.

After operating for 13 days on a pretreated sewage solids feed (autoclaved at 121°C at pH 12) with no yeast extract supplement, the biomass in the reactor remained well flocculated. Figure 29 gives settling curves for both feed and reactor effluent solids.

5.1.4 Start-up of a Continuous SO_2-Reducing Culture with a Pretreated Municipal Sludge Feed

The continuous culture described above was converted to SO_2-reducing conditions following operation for 13 days with a municipal sludge feed (pretreated by heating to 121°C for 30 min at pH 12) without yeast extract supplement.

Feed for the bioreactor was prepared as follows: 100 g of wet-packed municipal sludge was suspended in 1.0 L of the sulfate-free medium described in Table 20. The suspension was then adjusted to pH 12 with 10N NaOH and autoclaved (121°C) for 30 min. At the end of this time, the suspension was cooled to 30°C and the pH adjusted to 7.0 with 6N HCl. The feed reservoir was chilled with ice in an insulated container to slow subsequent microbial activity which might reduce the concentration of fermentable substrates in the feed. Feed was pumped to the fermenter (B. Braun Biostat M,1.5 L culture volume) by a B. Braun FE 211 piston pump at a rate of 12 mL/hr resulting in a dilution rate of 0.19 d^{-1}.

The SO_2-reducing culture was produced as follows:

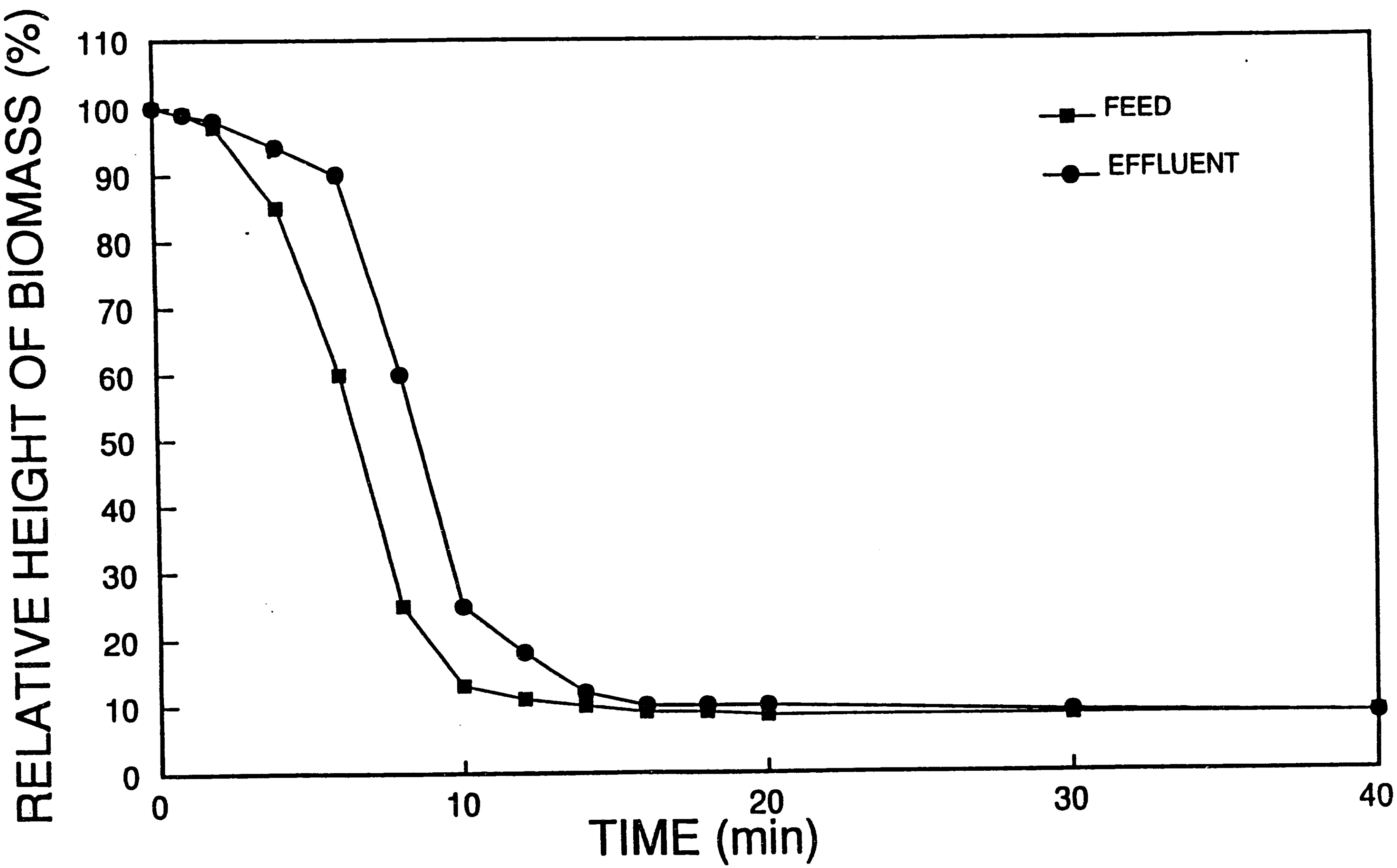


Figure 29. Settling properties of feed and effluent solids of a continuous SRB-bioreactor operating with a feed of heat and/or alkali treated municipal sewage sludge with and without yeast extract supplement. (Feed and effluent solids were diluted to 1.5 g/L for settling tests).

Biomass from the above referenced continuous culture was harvested by centrifugation at 4900g at 25°C and washed with sulfate-free minimal medium (Table 20). The biomass was then resuspended in the filtrate of a feed preparation (see above). This ensured that there was fermentable substrates in the culture during start-up.

Effluent from the reactor was continuously removed by means of a 1/4-in. stainless steel tube at the culture surface which withdrew mixed liquor from the reactor as the volume increased as feed was delivered. The effluent was withdrawn by means of a peristaltic pump.

The culture was maintained at pH 7.0 and 30°C. The agitation rate was 200 rpm. The culture received two gas feeds: 308 mL/min N_2 (to strip H_2S) and 12 mL/min of 1.0% SO_2, 5% CO_2, balance N_2. This corresponded to a molar flow rate of SO_2 of 0.295 mmoles/hr. During start-up, the H_2S concentration in the outlet gas and sulfite concentration in the aqueous phase of the bioreactor were monitored closely. If the culture could not use SO_2 as a terminal electron acceptor at all or if the molar SO_2 feed rate was in excess of the maximum specific activity of the biomass for SO_2 reduction then sulfite would accumulate in the bulk aqueous phase.

During this start-up period, some accumulation of sulfite (up to 80 μg/mL) was observed in the culture necessitating a reduction in the SO_2 feed rate. After two days of adjustments and monitoring, the bioreactor was stabilized with an SO_2 feed of 8.0 mL/min (1.0% SO_2, 5% CO_2, balance N_2) or 0.196 mmoles/hr SO_2. The sulfite concentration in the aqueous phase leveled off at 5 μg/mL.

Probably the most interesting observation during start-up was with regard to H_2S production. Some 24 hours after initiation of SO_2

feed, the H_2S concentration in the reactor outlet gas was about 6000 ppmv. This H_2S production was much too high to accounted for in terms of SO_2 reduction. Over the next 48 hours the H_2S concentration declined steadily until the concentration was 200-250 ppm where it remained for the duration of the experiment reported here. This extra H_2S production, over and above that produced by SO_2 reduction has been attributed to the metabolism by non-SRB heterotrophs of sulfur-containing substrates (probably S-containing amino acids) produced during heat/alkali treatment of sewage solids. These soluble substrates were present at very high concentrations during start-up.

The bioreactor was operated for 15 days under the conditions described above. After the non-SO_2 sources of H_2S were depleted, all of the inlet SO_2 was shown to be converted to H_2S with 1-2 s of gas-liquid contact time. Figures 30 and 31 show the total COD concentrations and the filtrate COD concentrations (minus solids) in the reactor feed and effluent during this start-up period. These figures show the utilization of about 50-60% of the soluble COD from the feed. Similarly Figure 32 shows that about 50% of the soluble protein in the feed was utilized in the reactor. This experiment definitely proved that pretreated municipal sewage sludge can serve as a carbon and energy source for SO_2-reducing cultures.

5.1.5 Long-Term Operation of a Continuous SO_2-Reducing Culture with a Pretreated Municipal Sludge Feed

By the end of the current reporting period the continuous SO_2-reducing culture described above had been operated for a total of over 110 days. Figures 35-37 document the feed and effluent MLSS, soluble COD, total protein and soluble protein during the entire course of

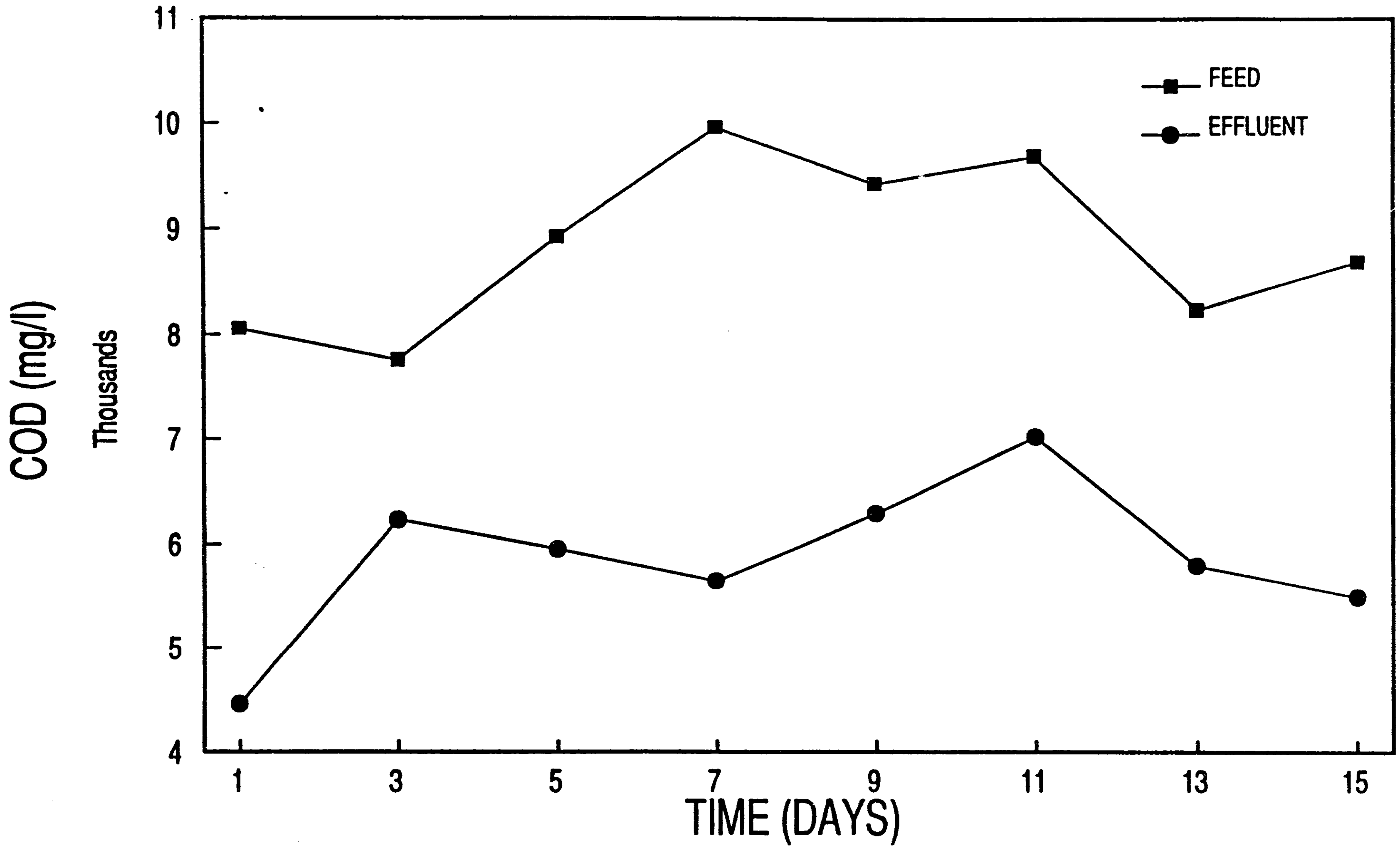


Figure 30. Total COD in the feed and effluent of an SO_2-reducing culture operating with a feed of pretreated municipal sewage sludge.

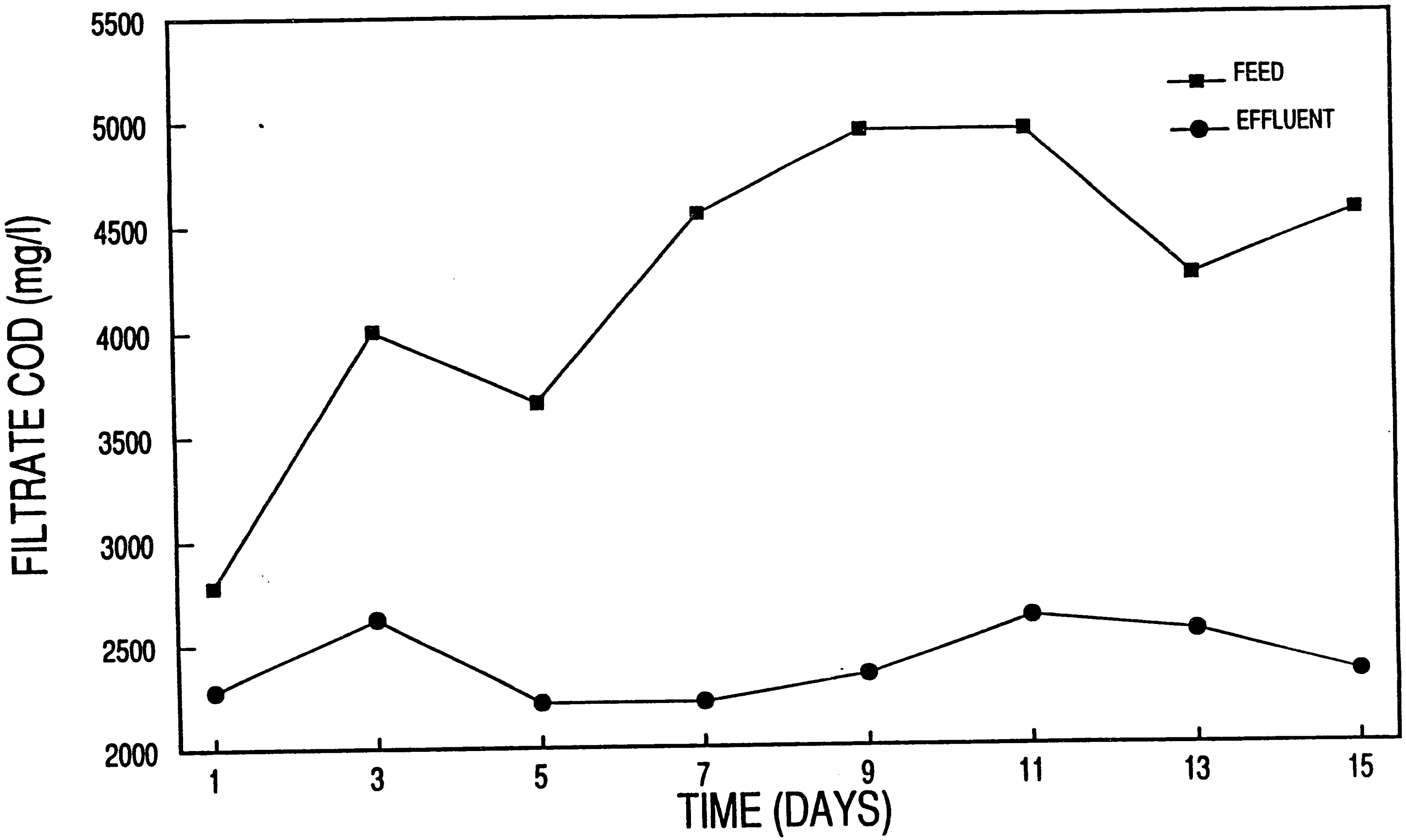


Figure 31. Filtrate or soluble COD in the feed and effluent of an SO_2-reducing culture operating with a feed of pretreated municipal sewage sludge.

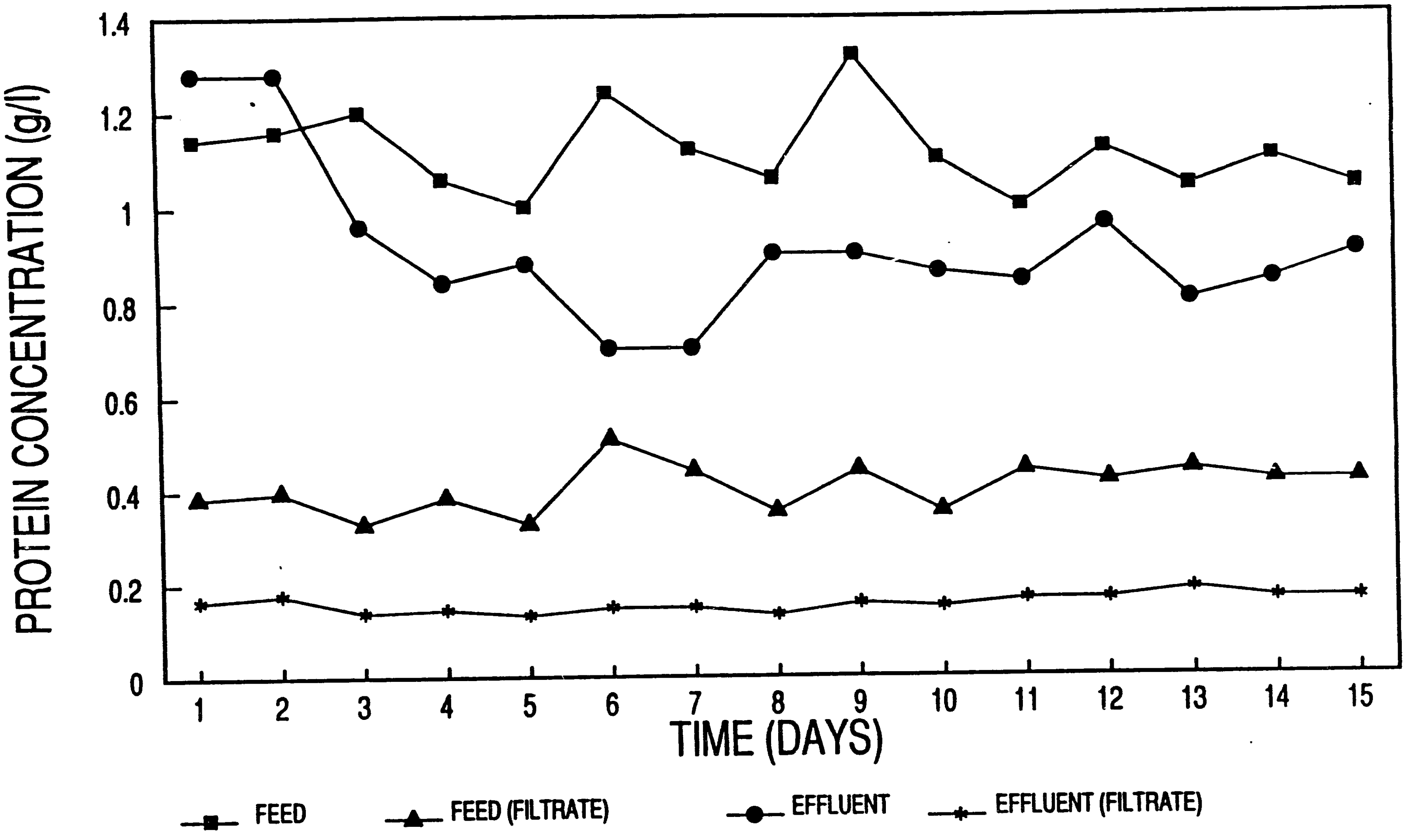


Figure 32. Protein concentrations in the feed and effluent of an SO_2-reducing culture operating with a feed of pretreated municipal sewage sludge.

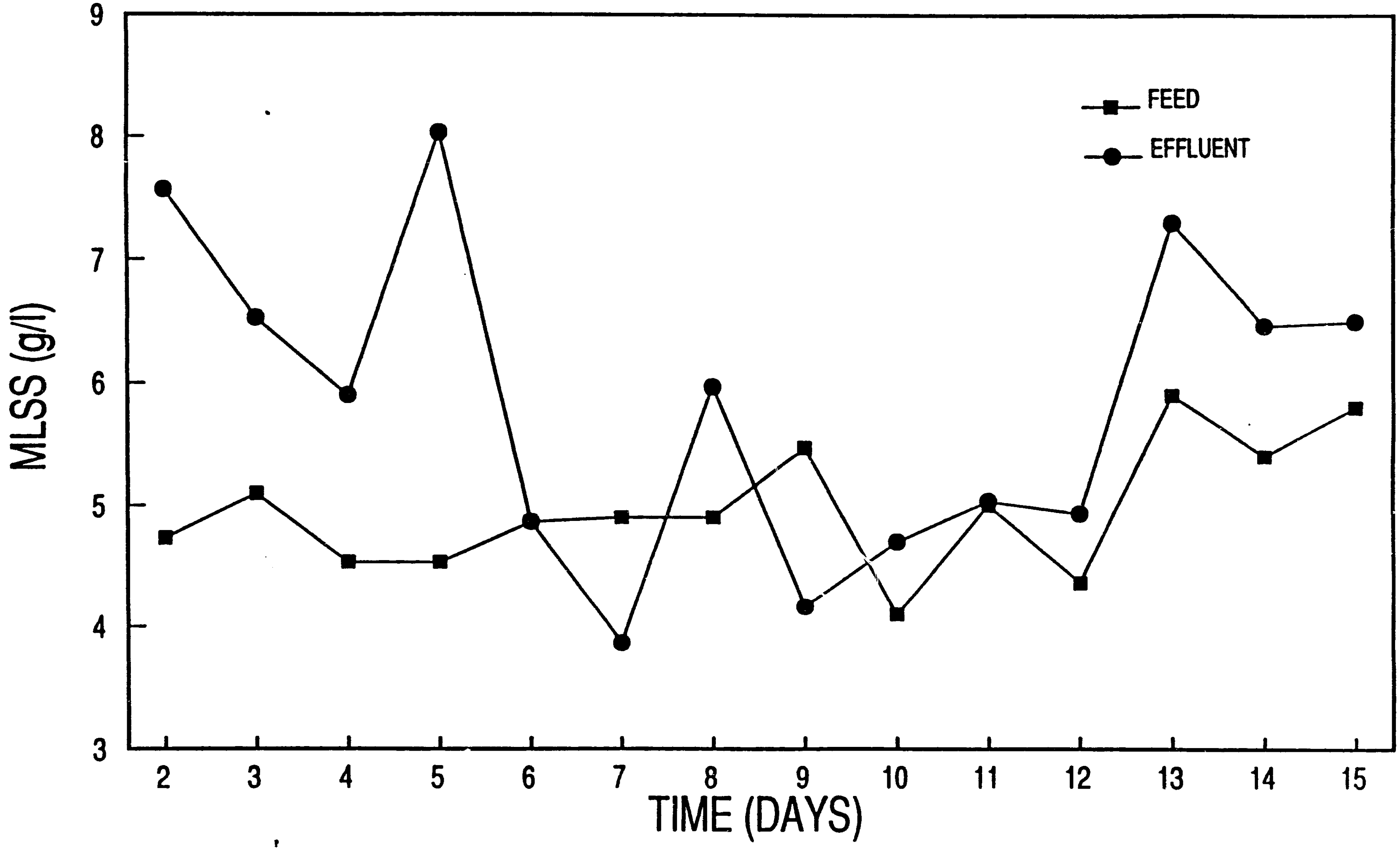


Figure 33. MLSS concentrations in the feed and effluent of an SO_2-reducing culture operating with a feed of pretreated municipal sewage sludge.

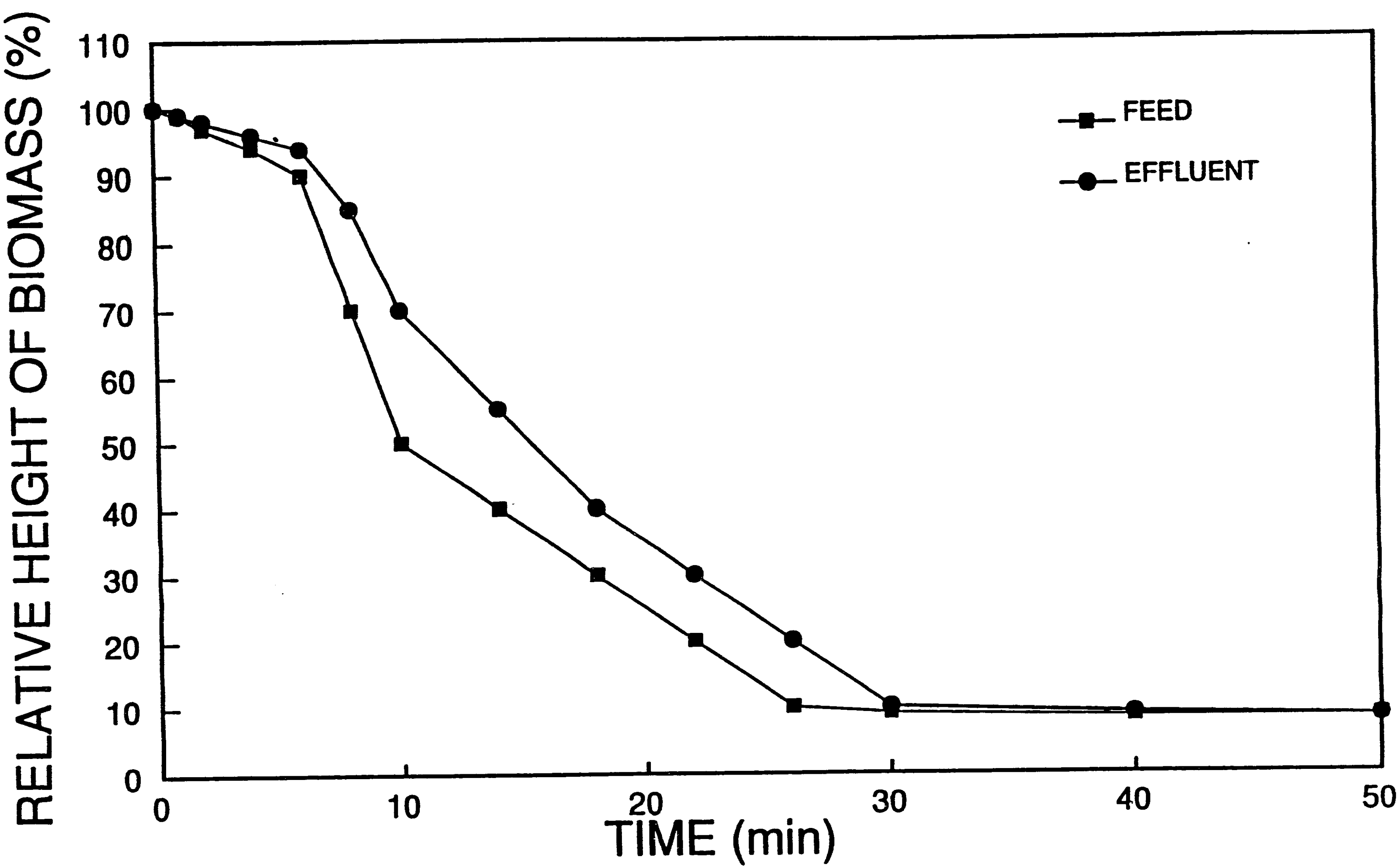


Figure 34. Settling properties of feed and effluent solids of an SO_2-reducing culture operating with a feed of pretreated municipal sewage sludge. (Feed and effluent solids were diluted to 1.5 g/L for settling tests.)

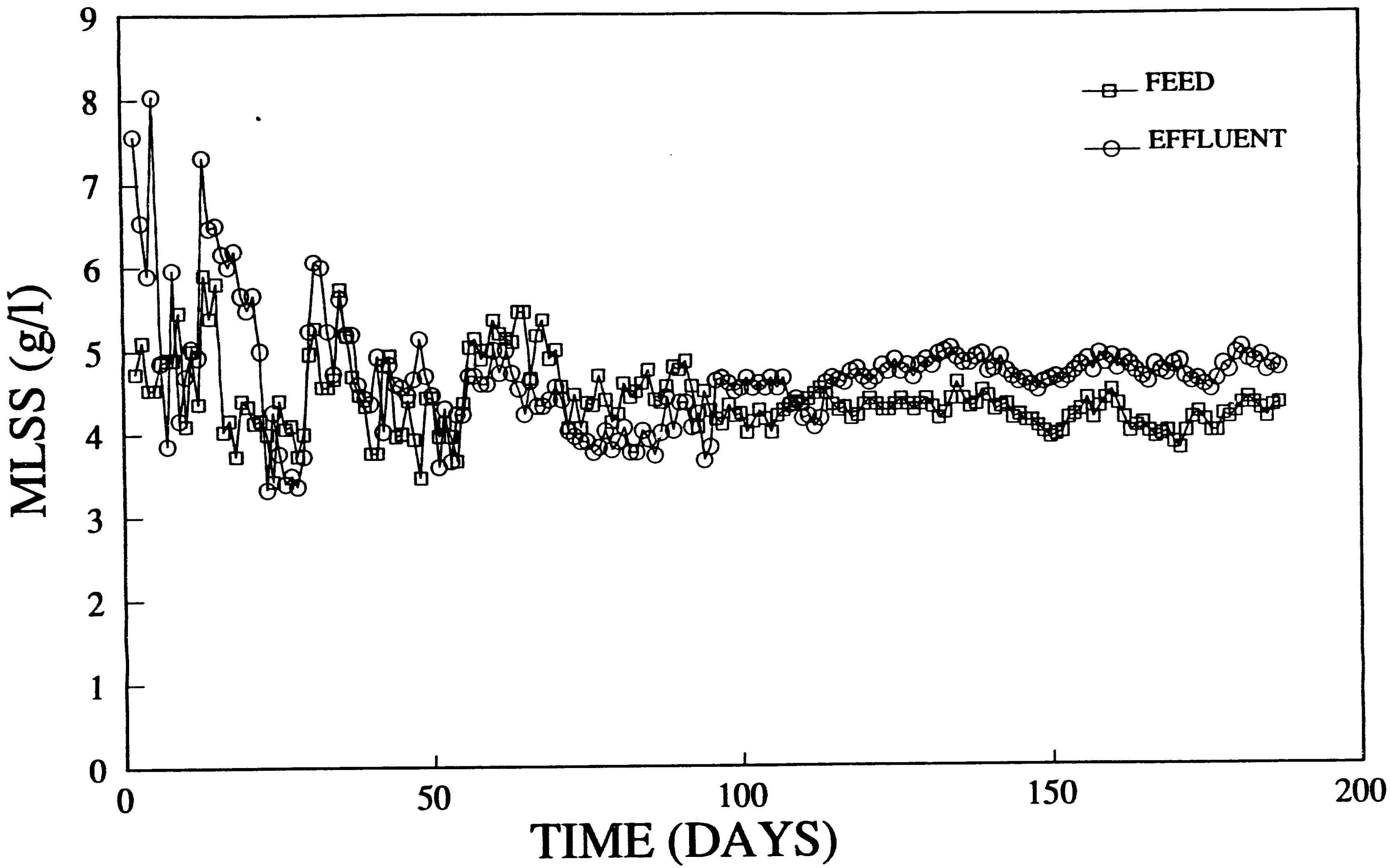


Figure 35. MLSS concentrations in the feed and effluent of a continuous SO_2-reducing culture operating with a feed on heat/alkali pretreated sewage sludge

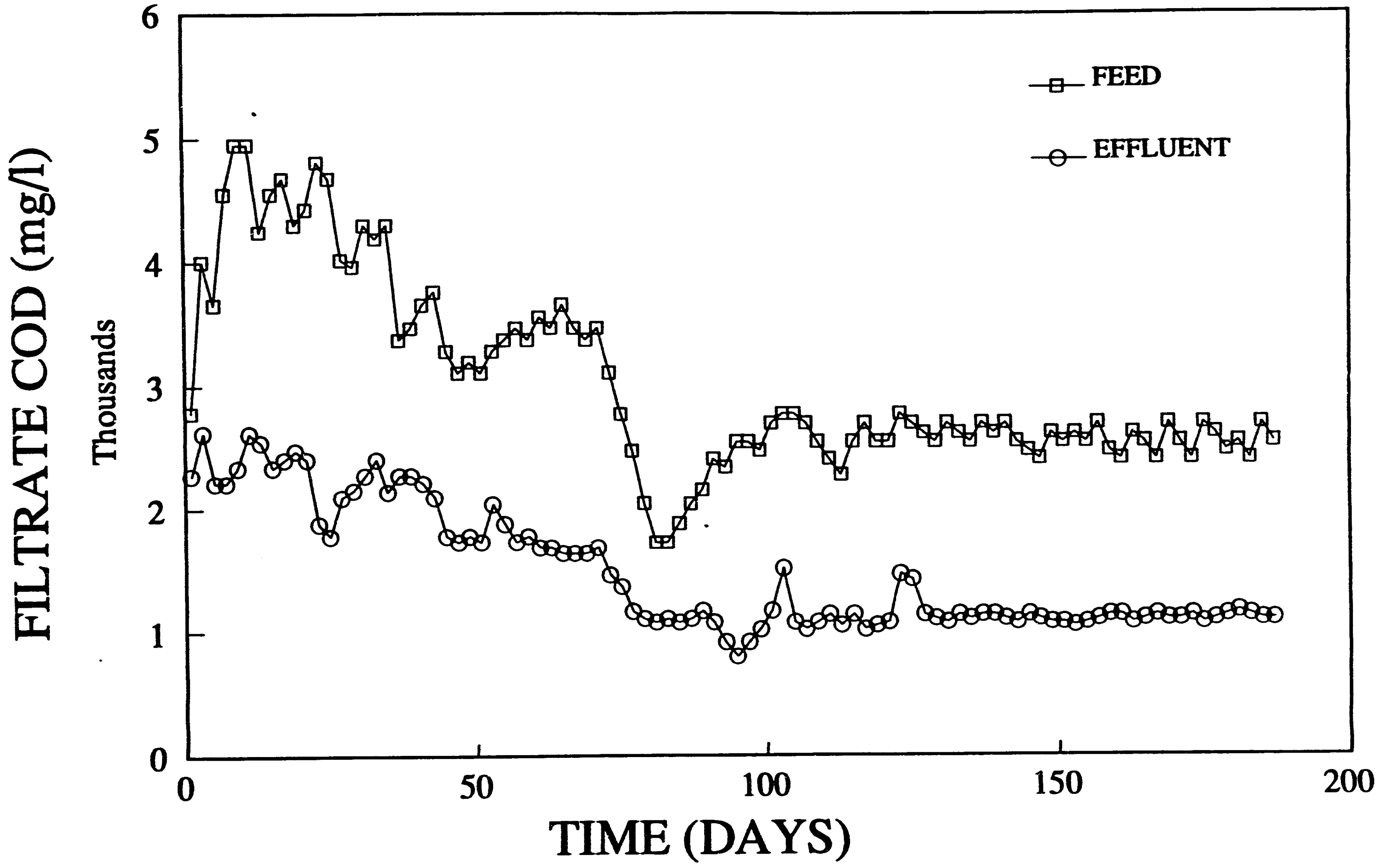


Figure 36. Filtrate or soluble COD concentrations in the feed and effluent of a continuous SO_2-reducing culture operating with a feed on heat/alkali pretreated sewage sludge.

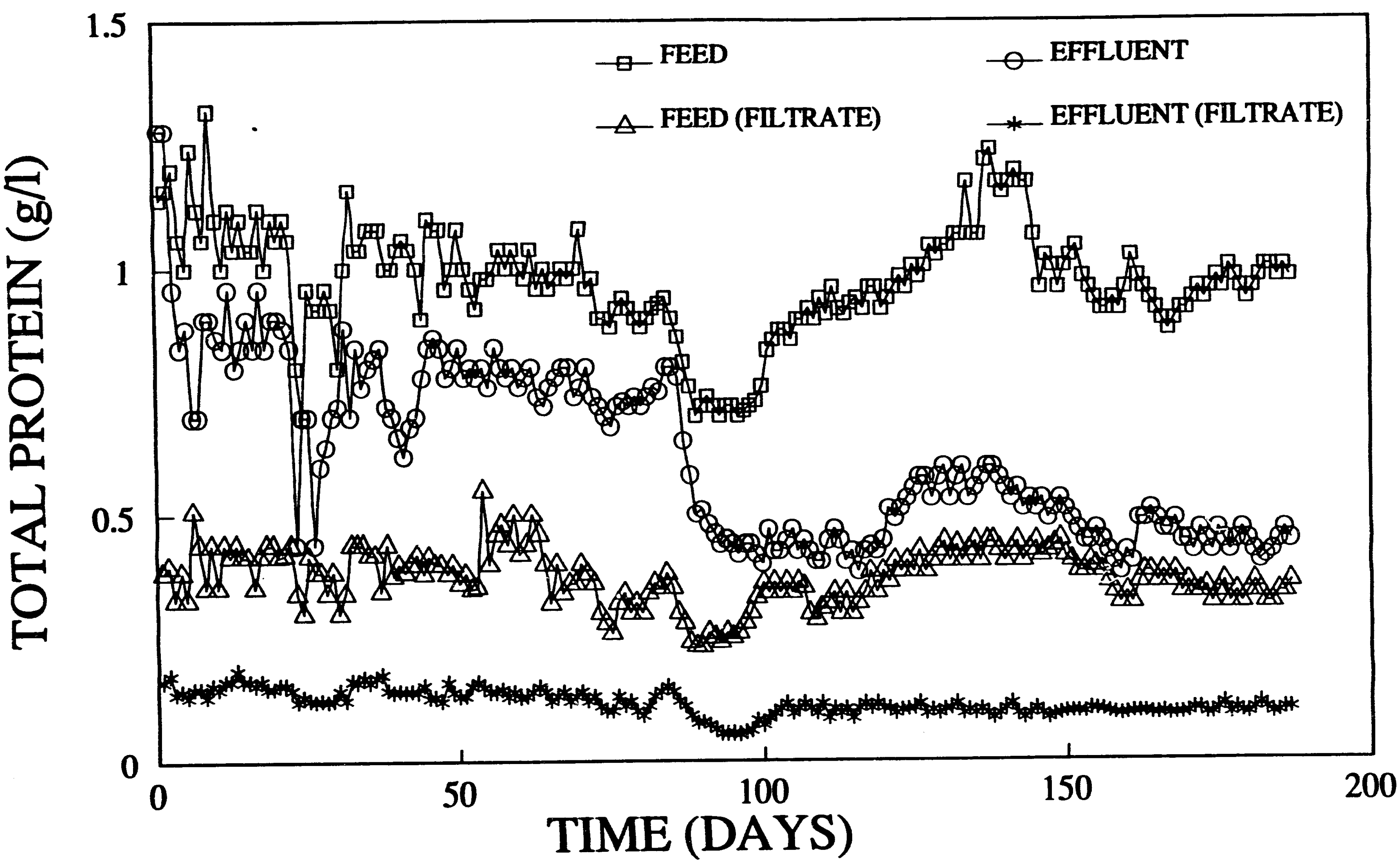


Figure 37. Protein concentrations in the feed and effluent of a continuous SO_2-reducing culture operating with a feed on heat/alkali pretreated sewage sludge.

operation of this reactor. Time zero corresponds to the initiation of SO_2 feed. The culture has continued to operate under the conditions described in Section 5.1.4 except for a decrease in the volumetric feed rate from 12 mL/hr to 8 mL/hr on day 59. The culture has operated at 8 mL/hr since then. As seen in Figures 35-34 the culture has been subject to variations in the feed composition with regard to soluble COD and total protein. Since the method of preparation of the feed has not changed during the course of the experiment, the variations have been attributed to variations in the sludge as obtained from the municipal sewage treatement system. Despite these variations the culture has been very stable with respect to SO_2 reduction. No upsets (as indicated by accumulation of sulfite in the culture medium) have been observed and as shown in Table 22 complete reduction of SO_2 to H_2S was observed. As seen in Figure 38 excellent settling properties of effluent solids have been maintained throughout the course of the experiment.

Since about 100 days the reactor has operated with about a 10% increase in MLSS from feed to effluent. Note, however, the roughly 50% decrease in total protein. During the last 60 days, there has been a 60% decreaes in the feed soluble COD compared to the effluent. As seen in Figure 37, there has been a similar decrease in the soluble protein comparing the feed to the effluent.

Feed and effluent filtrates were analyzed for total carbohydrates, carboxylic acids and lipids at about 130 days. The total feed and effluent were also analyzed for lipids. Total carbohydrates were determined by the Orcinol method with glucose as a standard. Carboxylic acids were determined by gas chromatography as

Table 22. Sulfur Balances in Continuous SO_2-Reducing Reactor Operated on Feed of Heat/Alkali Pretreated Sewage Sludge

Day	SO_2 Feed Rate (mmoles/hr)	H_2S Production Rate (mmoles/hr)	H_2S/SO_2
21	0.205	0.204	1.00
22	0.205	0.209	1.02
47	0.222	0.224	1.01
65	0.222	0.219	0.99
68	0.236	0.229	0.97
85	0.236	0.232	0.98

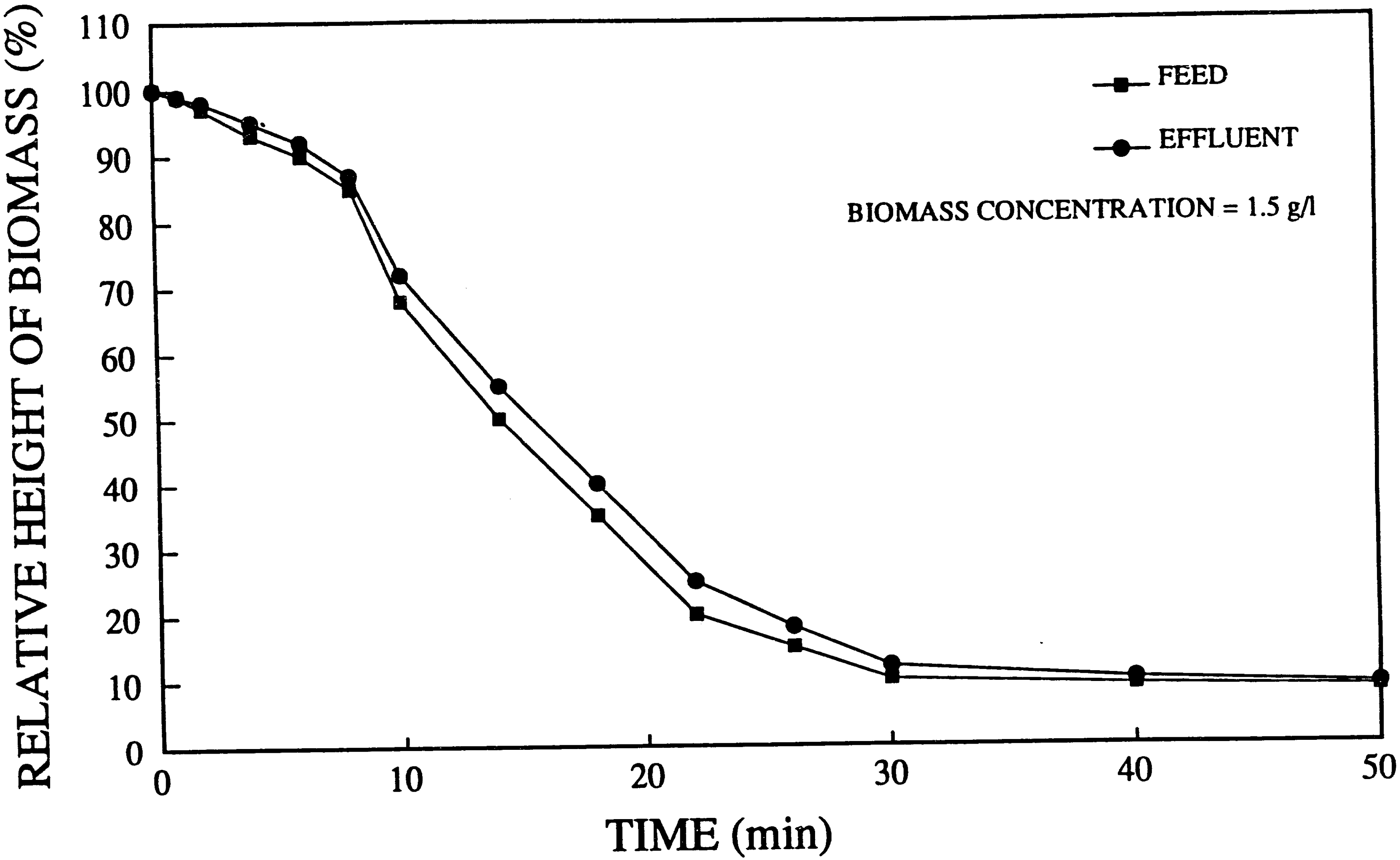


Figure 38. Settling properties of feed and effluent solids of an SO_2-reducing culture operated with a feed of pretreated municipal sewage sludge.

described in Table 19. Lipids were determined gravimetrically via two-stage extraction by diethyl ether. Results are shown in Table 23. As seen in Table 23, very little production of carboxylic acids was seen as has been noted previously. The data with regard to lipid analysis suggest under-utilization of the lipid fraction despite significant solubilization. The more efficient utilization of this fraction will be studied further.

Routine monitoring of H_2S in the outlet gas was done by gas chromatography as described in Table 17. However, more accurate chemical methods were also employed in which H_2S in the outlet gas was precipitated as ZnS and analyzed spectrophotometrically as follows: Reactor outlet gas was bubbled into 400 mL 0.1% zinc acetate for 2 hours. Two reagents were required for colorimetric analysis of the precipitated sulfide, DMPD reagent and ferric reagent. The DMPD reagent was prepared by dissolving 1.0 g of N,N-dimethyl-p-phenylenediamine sulfate, 1.0 g $Zn(CH_3COO)_2 \cdot 2H_2O$ and 50 mL of concentrated H_2SO_4 in distilled water and diluted to one liter. Ferric reagent was prepared by dissolving 5.0 g $FeCl_3 \cdot 6H_2O$ in 20 mL of distilled water. Suspensions of ZnS were analyzed by mixing 5.0 mL of the ZnS suspension (or a suitable dilution) with 4.9 mL DMPD reagent followed by immediate addition of 0.1 mL of ferric reagent. The absorbance at 660 nm was then read after at least 10 min incubation at room temperature. Sulfide standards were prepared by washing $Na_2S \cdot 9H_2O$ with distilled water, drying at room temperature and dissolving approximately 8 g in anoxic 0.01 N NaOH to a total volume of 1 L.

Table 23. Total Carbohydrates, Carboxylic Acids and Total and Filtrate Lipids in Feed and Effluent of Continuous SO_2-Reducing Culture

Analysis	Feed Filtrate	Feed Total	Effluent Filtrate	Effluent Total
Carbohydrates	0.98 g/L		0.53 g/L	
Carboxylic Acids:				
Acetic	73 mg/L		59 mg/L	
Propionic	2 mg/L		NT	
Isobutyric	ND		8 mg/L	
Butyric	ND		5 mg/L	
Isovaleric	ND		22 mg/L	
Lipids				
dried @ 60°C	0.24 g/L		0.18 g/L	
dried @ 40°C	0.27 g/L		0.22 g/L	
dried @ 60°C		0.38 g/L		0.27 g/L

ND = not detected

5.1.6 Operation of a Second Continuous SO_2 - Reducing Culture with a Pretreated Municipal Sludge Feed

The operation of the continuous SO_2-reducing culture described in Section 5.1.5 was interrupted for three weeks. During this time the process culture was stored at 4°C. The culture was restarted by harvesting the culture biomass by centrifugation and resuspending in the filtrate of a sample of pretreated municipal sludge feed in a manner similar to that used during the original start-up (see Section 5.1.4). A continuous feed of pretreated sewage sludge was started immediately and the culture operated under the conditios described in Section 5.1.5 at a volumetric feed rate of 8 mL/hr. Figures 39-41 document the feed and effluent MLSS, soluble COD, total protein and soluble protein during this reporting period. It is interesting to note that the performance of this reactor system duplicated that of the previous reactor (see Figures 35-37).

The culture exhibited excellent stability with respect to SO_2 reduction. No upsets (as indicated by the accumulation of sulfite in the culture medium) were observed. Complete reduction of SO_2 to H_2S was also observed. The effluent solids maintained excellent settling properties throughout the operation of this reactor.

Several variations were subsequently made in the feed (both SO_2 and sludge) to this second continuous SO_2-reducing culture. Figures 42 and 43 give the soluble and total protein and soluble COD concentrations in the feed and effluent during this part of this experiment. Time zero on these plots corresponds to day 70 in Figures 40 and 41. For the first 20 days of operation shown in Figures 42 and 43, the sludge feed was prepared as described above. The volumetric

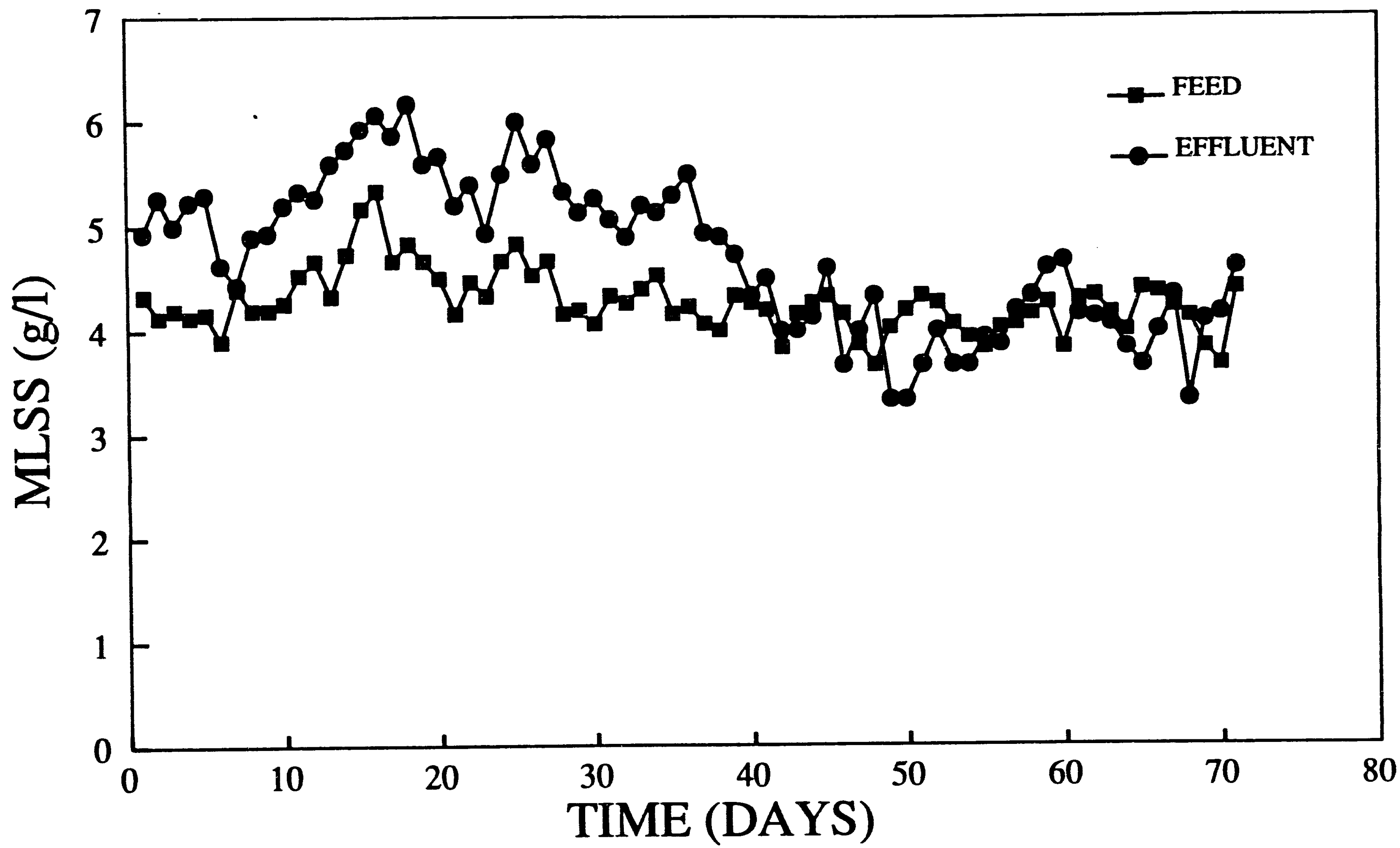


Figure 39. MLSS concentration in the feed and effluent of a second continuous SO_2-reducing culture operating with a feed of heat/alkali pretreated sludge.

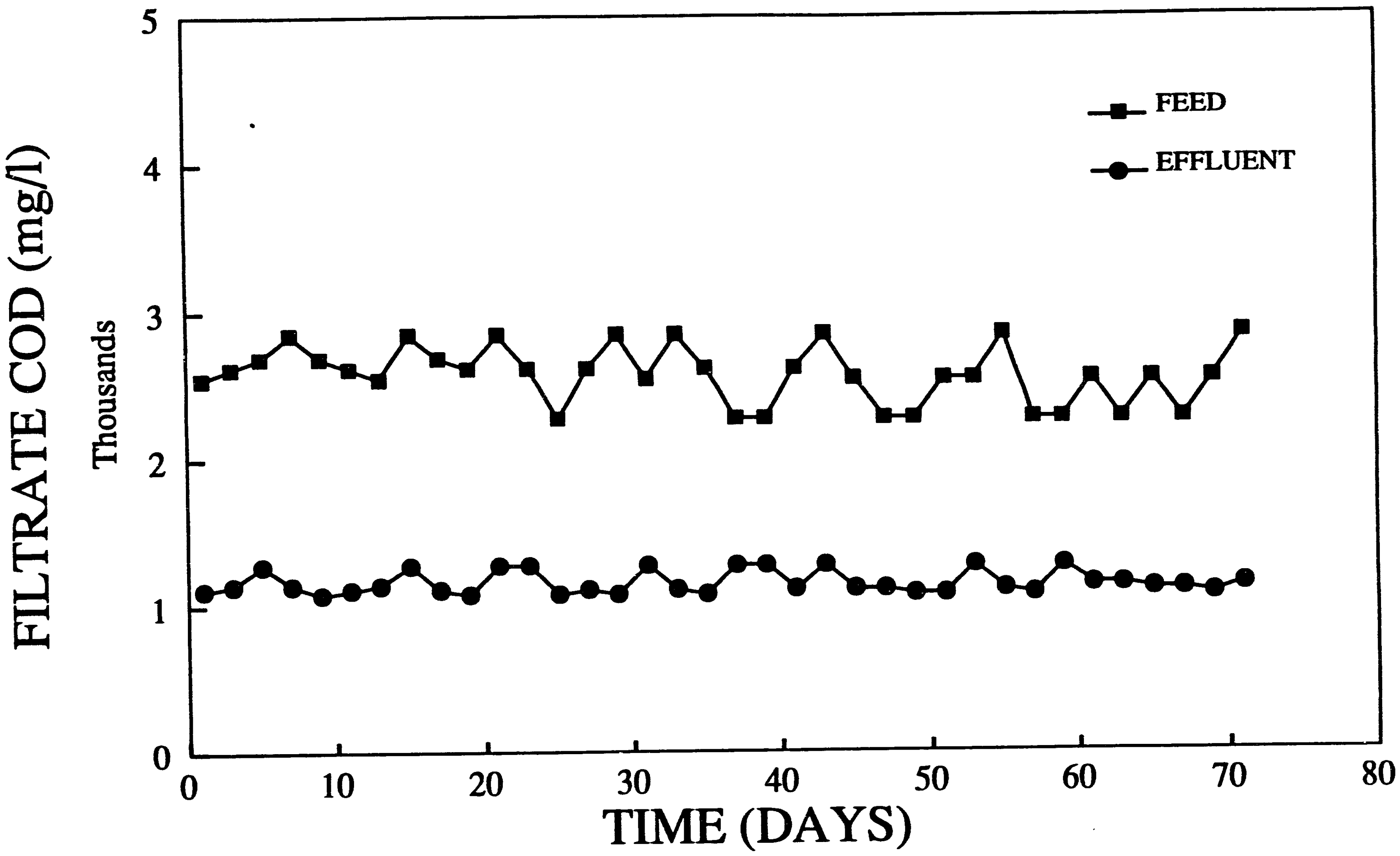


Figure 40. Filtrate or soluble COD in the feed and effluent of a second continuous SO_2-reducing culture operating with a feed of heat/alkali pretreated sludge.

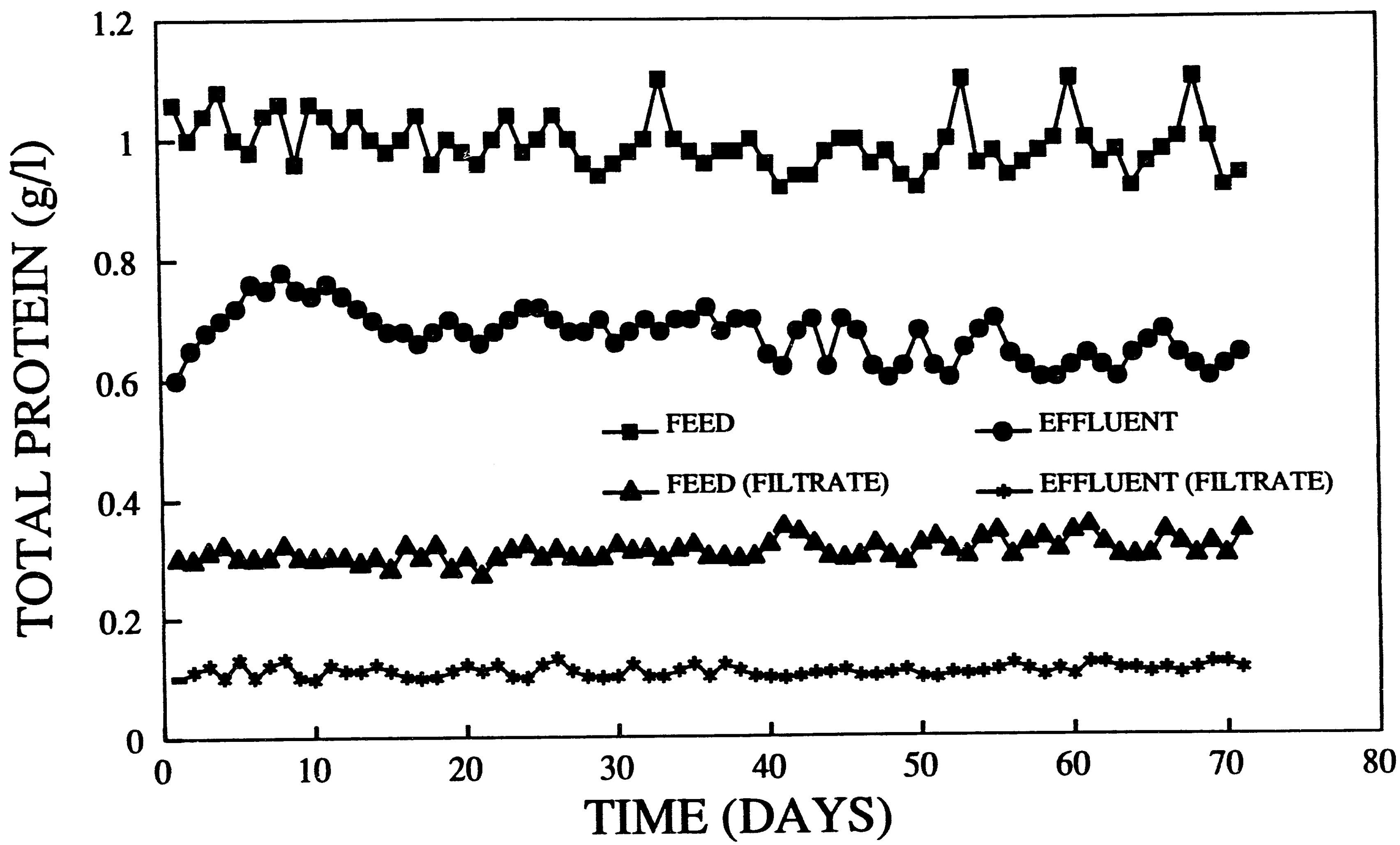


Figure 41. Protein concentrations in the feed and effluent of a second continuous SO_2-reducing culture operating with a feed of heat/alkali pretreated sludge.

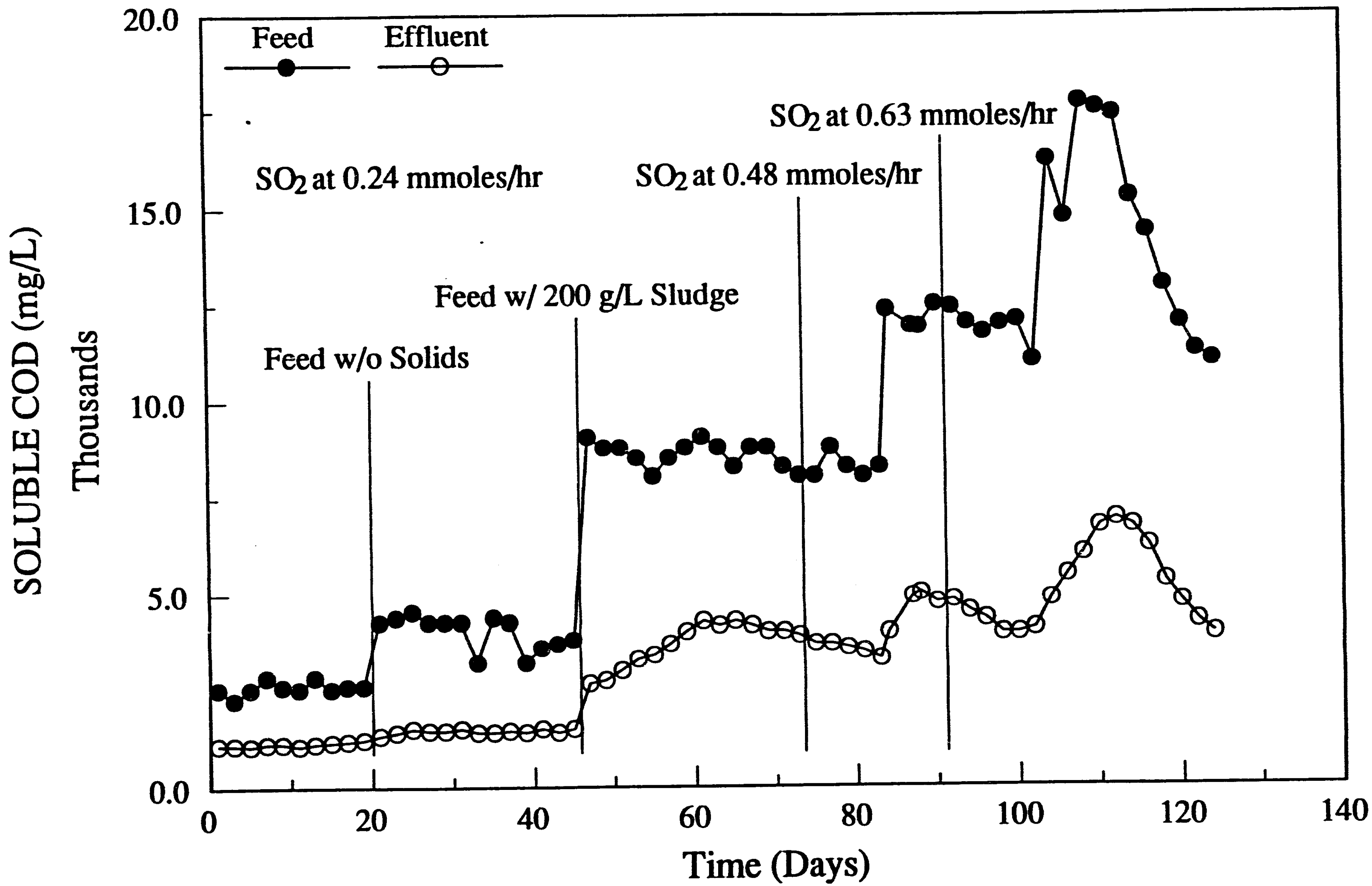


Figure 42. Soluble COD concentration in the feed and effluent of a second continuous SO_2-reducing culture operating with a feed of heat/alkali pretreated sewage sludge.

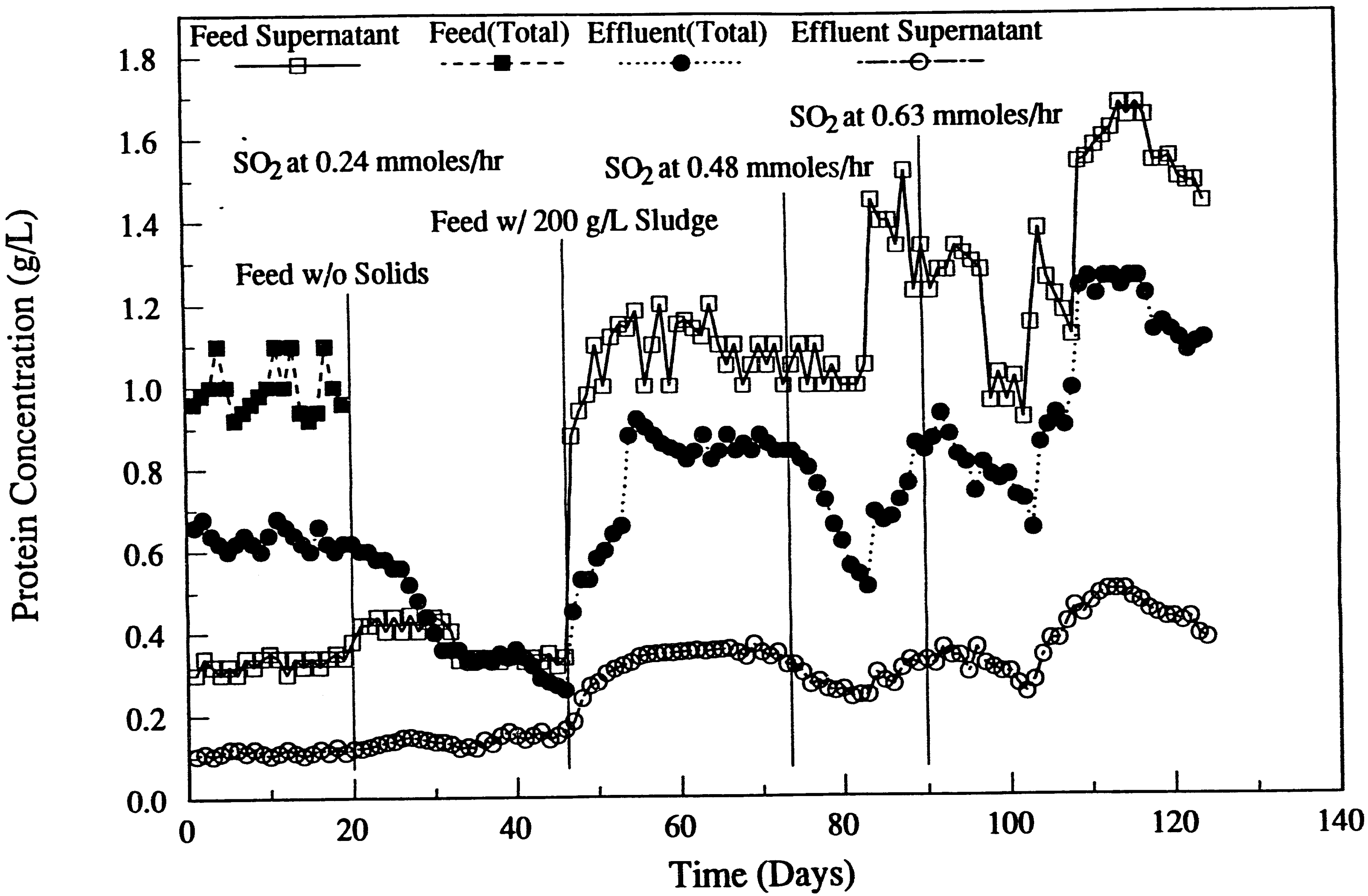


Figure 43. Protein concentrations in the feed and effluent of a second continuous SO_2-reducing culture operating with a feed of heat/alkali pretreated sewage sludge.

feed rate was 8.0 mL/hr. The SO_2 feed rate was 9.8 mL/min (1.0% SO_2, 5% CO_2, balance N_2) which corresponded to a molar SO_2 feed rate of 0.236 mmoles/hr. The culture also received a gas feed of 308 mL/min N_2. On day 20, the feed preparation was changed in that insoluble solids were removed from feed preparations by centrifugation at 5000 g and 25 C. After the culture reached steady state (Figure 43), the method of feed preparation was again changed in that 200 g of wet-packed sludge per liter was used in the feed preparation rather than 100 g/L. As seen in Figure 42, the soluble COD in the feed doubled. There was also a corresponding increase in the soluble COD in the effluent indicating increased availability of carbon and energy sources for the culture. This was a necessary prerequisite to increasing the SO_2 feed rate to prevent the culture from being limited by the carbon and energy source.

At 73 days the SO_2 feed rate was increased to 0.48 mmoles/hr. There was corresponding increase in H_2S production and after some time (Figure 44) a slight increase in the sulfite concentration in the liquid phase. This did not represent an upset condition which is characterized by runaway sulfite production. As seen in Figures 43 and 44, the increase in SO_2 feed rate also produced a decrease in the effluent soluble COD and soluble protein concentrations as expected. At about 82 days the soluble COD and soluble protein in the feed were seen to increase dramatically. This was not due to any change in the method of feed preparation but resulted from the variability of the municipal sludge. Another sharp increase was seen at about 105 days.

At 86 days the SO_2 feed rate was again increased to 0.63 mmoles/hr. There was another increase in the sulfite concentration in

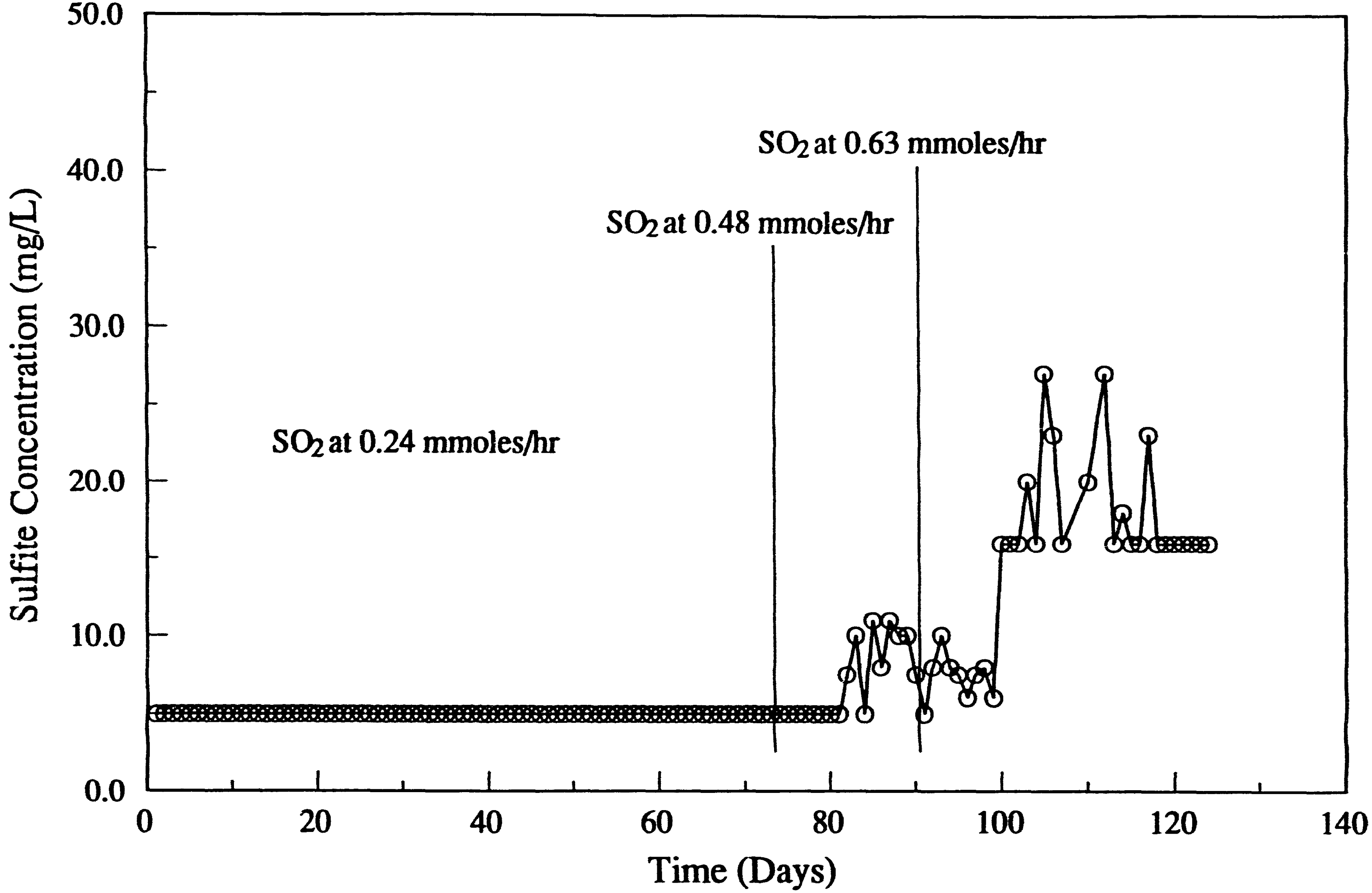


Figure 44. Sulfite concentration in the feed and effluent of a second continuous SO_2-reducing culture operating with a feed of heat/alkali pretreated sewage sludge.

the liquid phase (Figure 44); however, the concentration stabilized at about 15-17 mg/L. As the culture operates at this SO_2 feed rate the SRB concentration will slowly increase allowing further increases in SO_2 utilization. At present the maximum volumetric productivity observed is 0.44 mmoles SO_2 reduced /L-hr.

5.1.6.1 Improving Volumetric Productivity in a Continuous SO_2-Reducing Culture with Pretreated Municipal Sludge

During the operation of this second continuous SO_2-reducing culture efforts were initiated to improve the volumetric productivity (moles SO_2 reduced/unit volume of reactor). The reuse of effluent solids in formulation of the feed was considered as a means of improving the efficiency of utilization of carbon and energy sources and reducing waste streams. A suspension of effluent solids was subjected to heat/alkali treatment at pH 12, 121°C (autoclave) for 60 min. After cooling and neutralization the MLSS, soluble COD and soluble protein were determined. Results are shown in Table 24. As seen in this table very little solubilization of these solids took place. This is not surprising since this represents the second such treatment for a major fraction of these solids. As seen in Table 24, 46 mg of soluble COD was produced per g of MLSS treated. This compares to 290 mg/g for raw sewage sludge solids. Likewise heat/alkali treatment of effluent solids produced 0.044 g soluble protein per g of MLSS treated. This compares to 0.28 g/g for raw sewage sludge solids.

In a continuous bioreactor recycle or retention of the biomass allows the hydraulic and biomass retention times to be decoupled. Therefore, biomass recycle or retention is a key parameter in

Table 24. Heat/Alkali Treatment of a Suspension of Effluent Solids

Parameter	Before Treatment	After Treatment
MLSS (mg/L)	9550	8050
Filtrate COD (mg/L)	402	821
	388	844
Filtrate Protein (g/L)	0.02	0.44
	0.015	0.43

controlling volumetric productivity. However, before experiments in biomass recycle or retention can be initiated some means of enumerating key bacteria must be available. In the case of the continuous SO_2-reducing culture the process culture contains insoluble proteins and other bio-derived solids from the feed as well as the sulfate-reducing bacteria responsible for SO_2 reduction. Therefore, it would be difficult to determine SO_2-active biomass concentrations through protein determinations. Because of the solids in the process culture and the possibility of agglomeration of SRB and other culture solids, viable plate counts are impractical. We chose to use the most probable number (MPN) method (20). The MPN method employs serial dilutions of process cultures to inoculate a selective medium for SRB. From the dilutions showing growth of the SRB a statistical analysis is done to arrive at an estimate of the concentration of SRB in the original sample. This technique is frequently used to estimate bacterial counts in soils and sediments.

As previously noted the SO_2-reducing cultures contain a high concentration of settleable solids. However, the MLSS concentration in the culture is roughly the same as the MLSS concentration in the feed. With regard to the issue of biomass recycle or retention it is important to know how the SRB are distributed in the process culture between the solids and the liquor. A sample (100 mL) of reactor mixed liquor was diluted to a MLSS concentration of 1.5 g/L and the solids allowed settle under gravity. The supernatant (85 mL) and solids (15 mL) were then separated. A MPN count of SRB was then made in the supernatant using BTI-SRB medium from Bioindustrial Technologies, Inc. (Grafton, NY). The solids fraction was then diluted back to 100 mL and allow to settle again. This process was then repeated. After dilution back to the original volume of 100 mL, a MPN count of the SRB was obtained. The results of these counts are given in Table 25. As seen in Table 25, only 35% of the SRB in the process culture are associated with the solids. Therefore, recycle of a fraction of the effluent solids would be an inefficient way of recyling SO_2-active biomass. Future efforts in this regard will concentrate on 1) eliminating solids from the feed, 2) increasing the concentration of soluble COD and 3) addition of anaerobic floc-forming bacteria to the process culture. Increasing the concentration of soluble COD in the feed will increase the steady state concentration of SO_2-active biomass in the reactor. It is anticipated that the addition of anaerobic floc-forming bacteria (from an anaerobic digester) may flocculate the SO_2-reducing bacteria facilitating biomass recycle.

One last issue to address as we began this phase of the project is to estimate the specific activity of the SO_2-active biomass for SO_2 reduction. This can be accomplished now that we have a way of estimating the concentration of SRB using the MPN technique. A mixed liquor sample of the continuous SO_2-reducing process culture was obtained and a MPN count of SRB made. The result was 4.5×10^7 cells/mL. The SO_2 feed rate was the increased step-wise until sulfite began to accumulate in the culture medium indicating that the specific activity of the SO_2-active biomass had been exceeded. From this determination the maximum specific activity of the SO_2-active biomass was estimated to be 0.73 mmoles SO_2/hr - 10^{11} cells. This compares well with the specific activity of *D. desulfuricans* for SO_2 reduction in mixed cultures with glucose as a carbon and energy source (8).

Table 25. Results of Most Probable Number Count of *D. desulfuricans* in the Solids and Liquid Fractions of an SO_2-Reducing Culture Operating on a Feed of Pretreated Municipal Sewage Solids

Fraction	MPN (Cells/mL)	Percent of Total
Solids*	5.2×10^7	35
Liquid	1.0×10^8	65

*Based on original concentration of gravity settleable solids and corrected for contribution of accompanying liquid phase

5.1.7 Immobilization of *D. desulfuricans* by Co-Culture with Floc-Forming Anaerobes

As noted in Section 5.1.6.1, biomass recycle is a key parameter in controlling volumetric productivity in SO_2-reducing reactors. In practical terms, biomass recycle requires that the biomass be immobilized or flocculated to facilitate concentration of the biomass by gravity settling. The immobilization of *D. desulfuricans* by co-culture with the floc-forming anaerobes or sludge from an anaerobic digester has been investigated.

For the 10 days prior to the addition of solids from the anaerobic digester (described below), the continuous SO_2-reducing culture described in Section 5.1.6 was operated with a liquid feed of pretreated sewage sludge medium (minus solids) at 8 mL/hr and a gas feed (1.0% SO_2, 5% CO_2, balance N_2) of 26.1 mL/min or 0.63 mmoles/hr SO_2. The sulfite concentration in the culture medium was 8 ± 2 mg/L. Stoichiometric conversion of SO_2 to H_2S was observed. Effluent COD and protein determinations during this period are given in Figures 45 and 46.

Sludge was obtained from an anaerobic digester at a municipal waste treatment system in Tulsa, Oklahoma. Initially, sludge solids had a tendency to float due to settling of the solids. The supernatant liquid was decanted and discarded. Settled solids were then washed three times with two volumes of 20 mM phosphate buffer (pH 7.0). During each settling period, the mixed liquor was maintained under a nitrogen blanket. After the third washing, the solids were collected by centrifugation at 5000g for 15 min at 25°C and resuspended in 650 mL of pretreated sewage sludge medium (minus solids). This suspension was then mixed with 800 mL of the contents

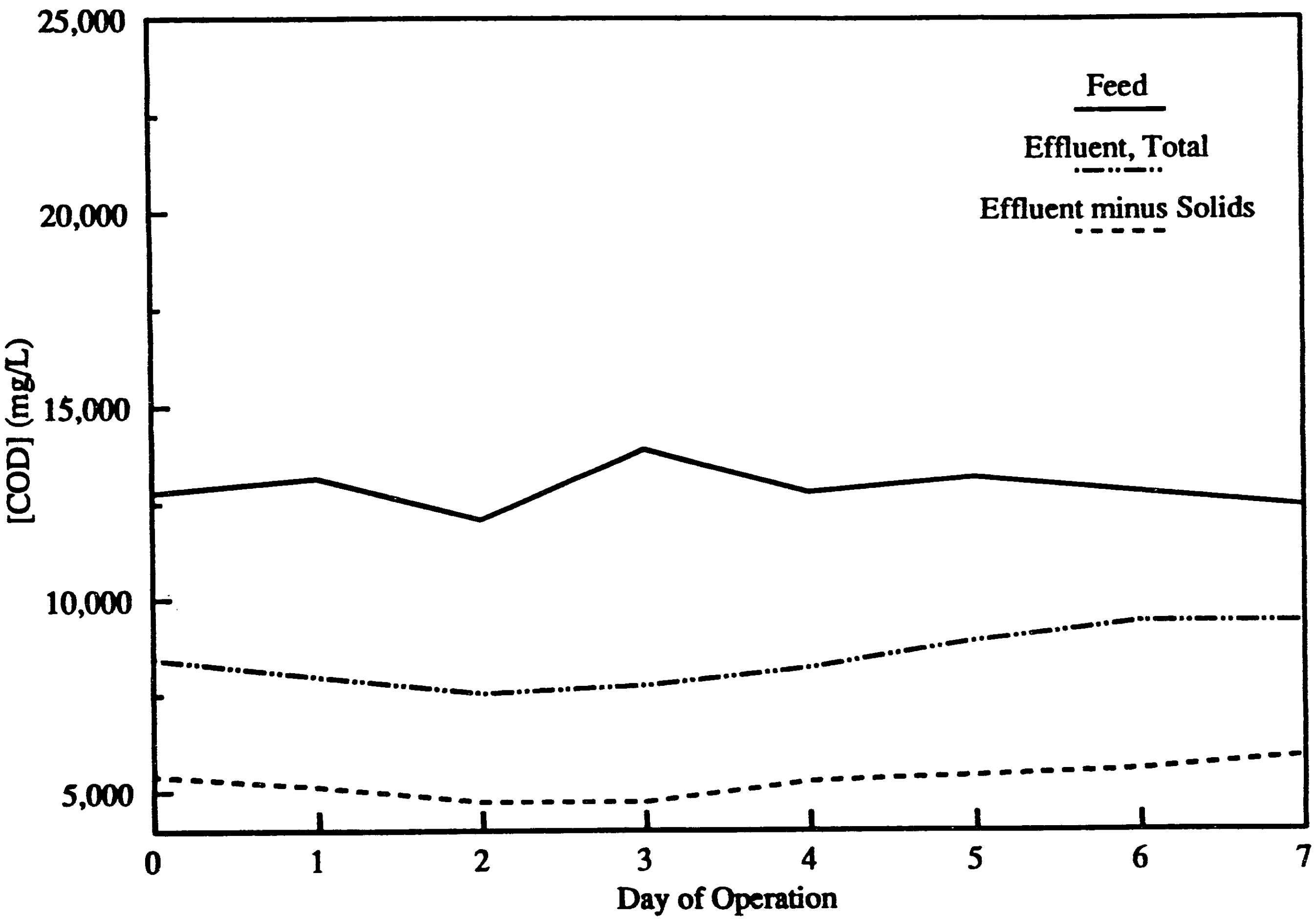


Figure 45. Total and soluble COD in the effluent of a continuous *D. desulfuricans* SO_2-reducing culture operating with a feed of pretreated sewage sludge medium. Time zero corresponds to one week prior to the addition of anaerobic digester solids.

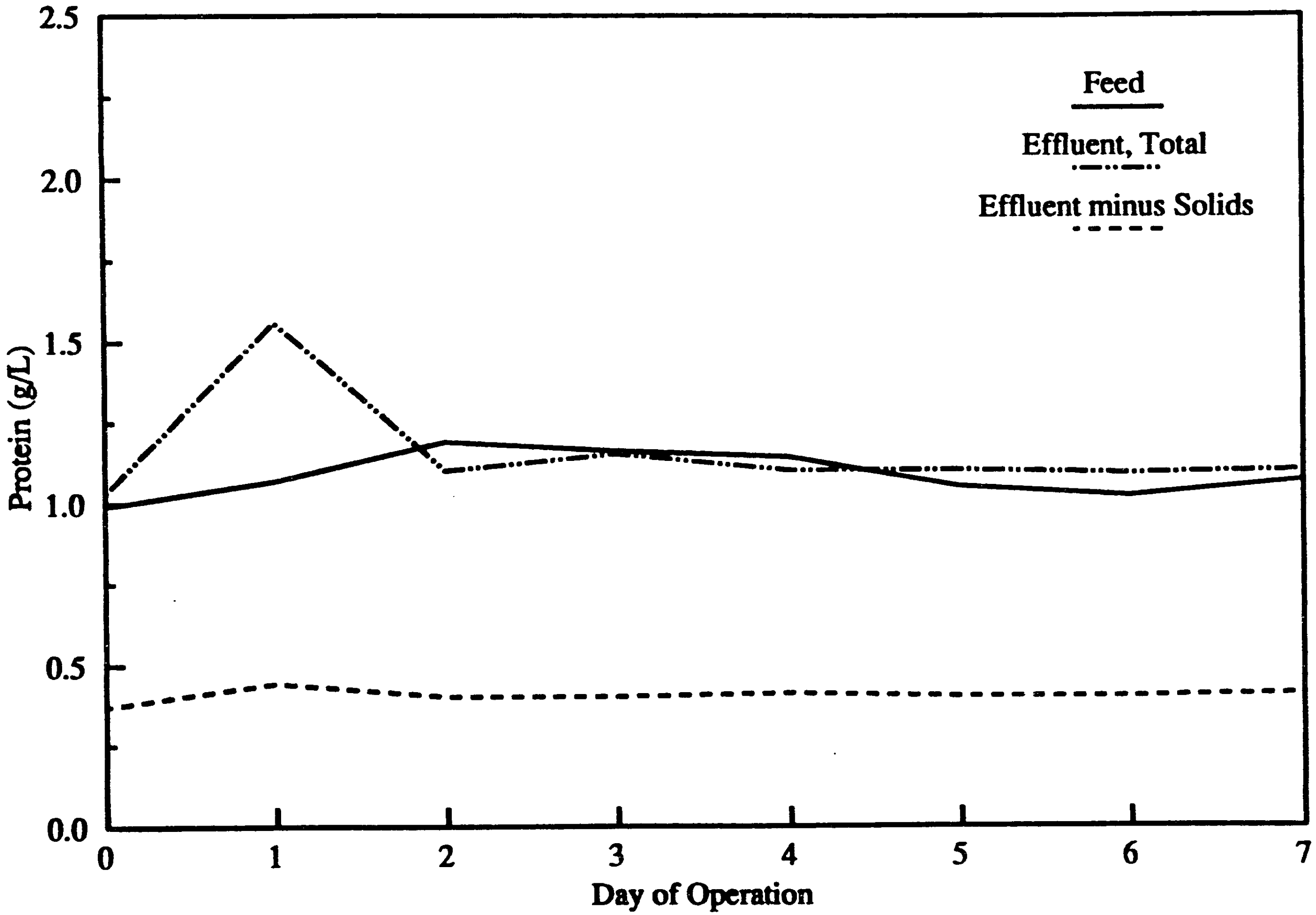


Figure 46. Total and soluble protein in the effluent of a continuous *D. desulfuricans* SO_2-reducing culture operating with a feed of pretreated sewage sludge medium. Time zero corresponds to one week prior to the addition of anaerobic digester solids.

of the SO_2-reducing culture described above in the B. Braun Biostat M fermenter. At this time, the SO_2 feed rate to the reactor was reduced to 10.4 mL/min (0.25 mmoles/her SO_2) and the agitation rate was reduced from 200 rpm to 150 rpm to minimize shear in the reactor. The pH continued to be maintained at 7.2 and the temperature at 30°C.

Following addition of anaerobic digester solids, the reactor was operated in a fed-batch mode. For the first 10 days of operation, the reactor received SO_2 feed for about 14 hrs/d only and turned off at night when no attendant was present. Subsequently, the reactor received SO_2 feed 24 hrs/d. About every 48 hrs, the gas feed and agitation were turned off and the reactor solids allowed to settle under gravity. The supernatant liquid was siphoned off, discarded and replaced with an equal volume of pretreated sewage sludge medium (minus solids). In this way, the culture was enriched with each fed batch cycle for *D. desulfuricans* which had become physically associated with the floc. The first fed batch feeding came 48 hrs after addition of anaerobic digester solids and initiatio of SO_2 feed. Settling of solids during the first feeding required about one hour to produce 500 mL of supernatant devoid of macroscopic solids. The supernatant, however, was still quite turbid. The dark brown/black color of the pretreated sewage sludge medium prevented actual turbidity measurements. After 3-4 cycles, solids settling during feeding became much faster requiring only 15-20 min to produce 700 mL of supernatant. The supernatant had also become much less turbid.

After three fed batch cycles, the SO_2 feed rate was gradually increased in increments over 52 days reaching a SO_2 molar feed rate of 0.57 mmoles/hr. Figures 47, 48 and 49 give the concentration of

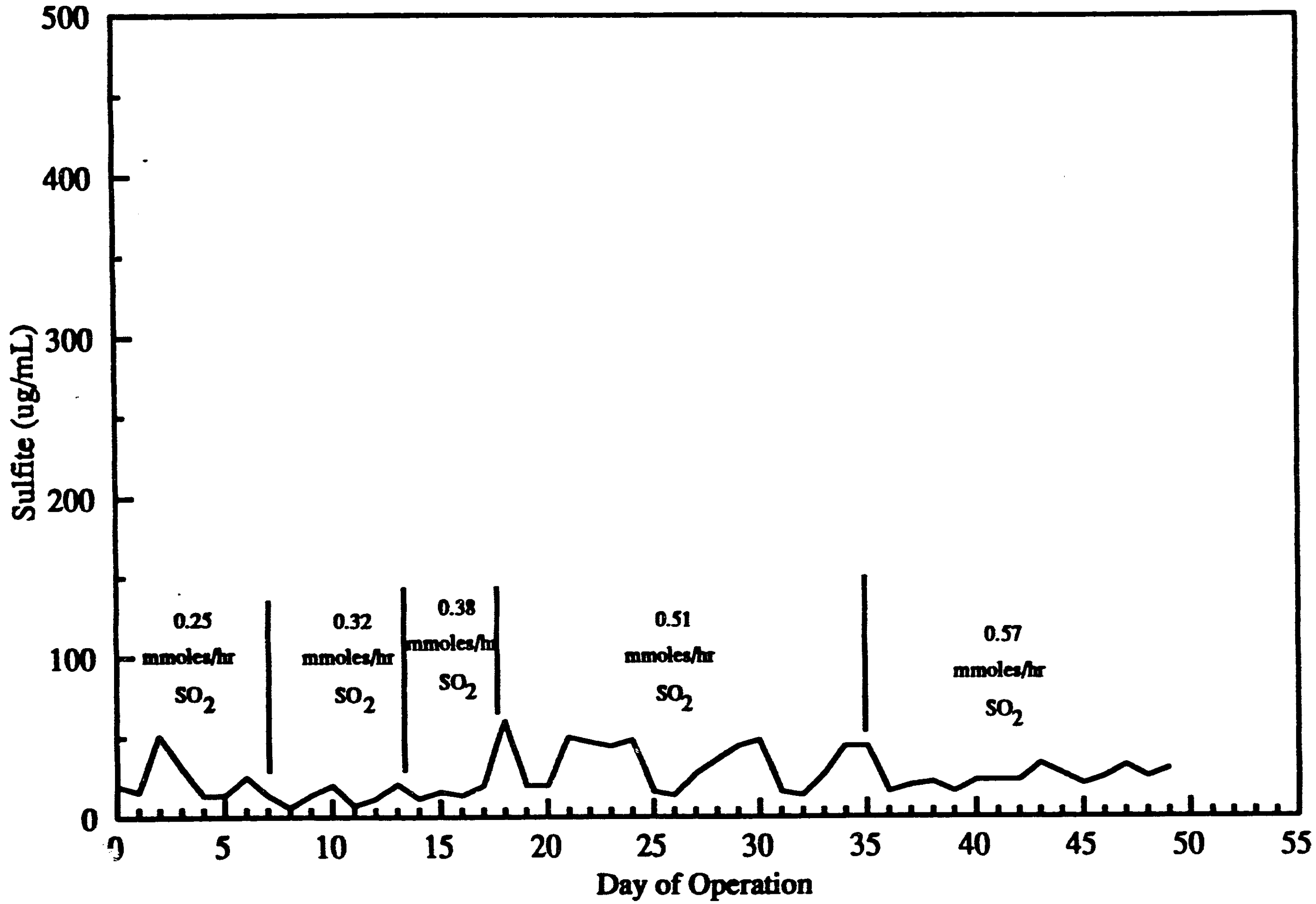


Figure 47. Sulfite concentration in a fed-batch *D. desulfuricans* SO_2-reducing culture in the presence of anaerobic digester solids. Time zero corresponds to the first addition of digester solids.

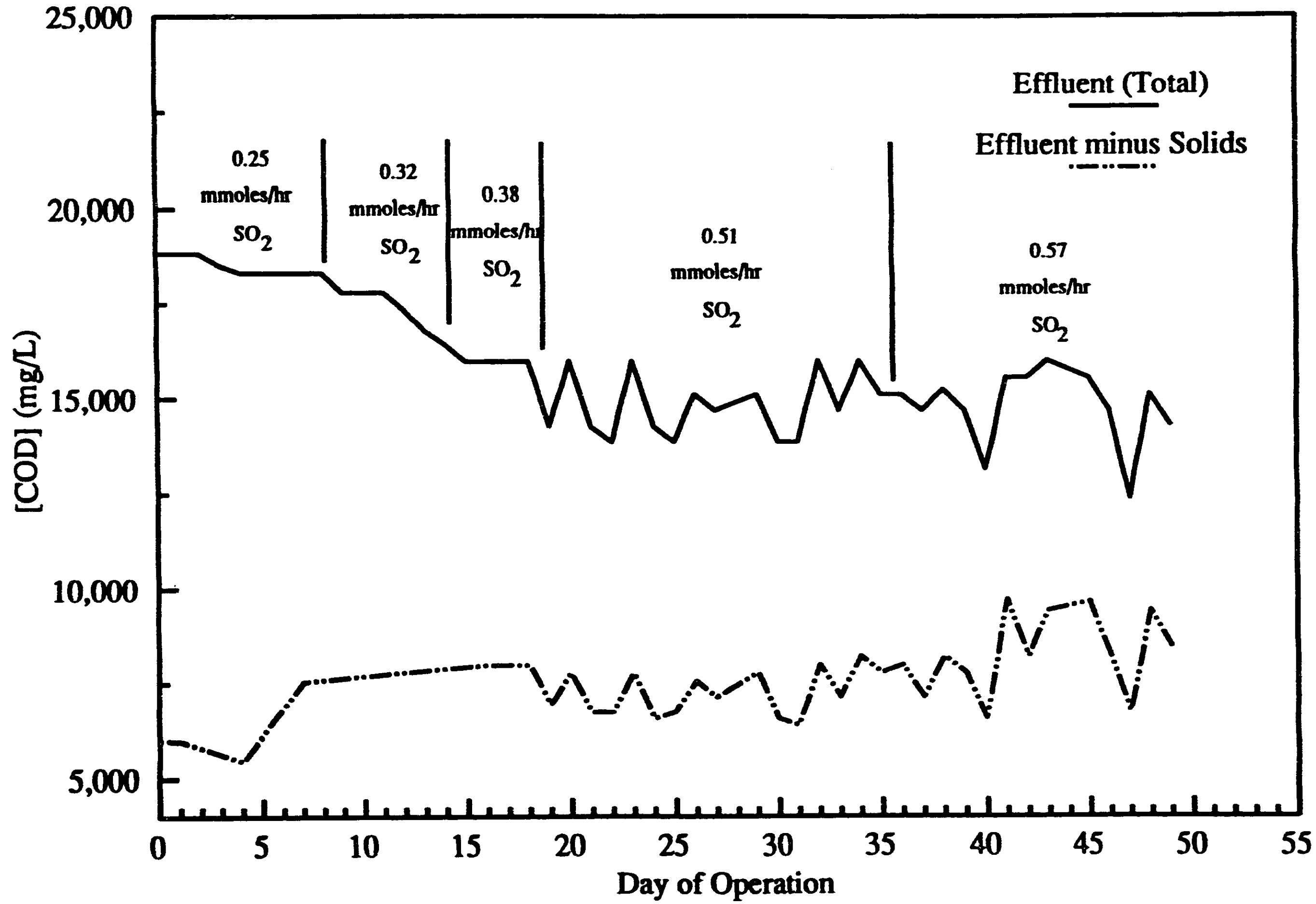


Figure 48. Total and soluble COD in a fed-batch *D. desulfuricans* SO_2-reducing culture in the presence of anaerobic digester solids. Time zero corresponds to the first addition of digester solids.

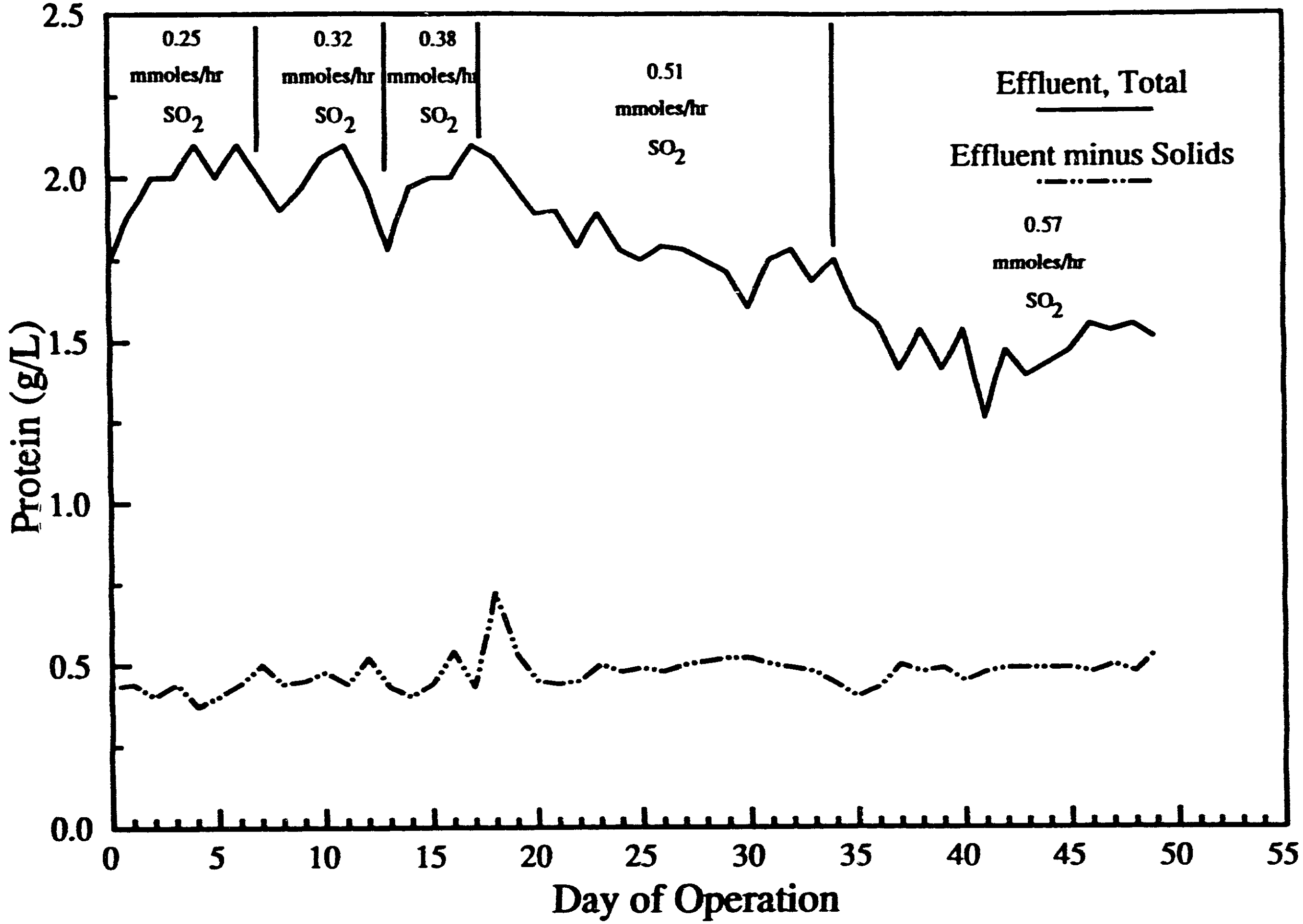


Figure 49. Total and soluble protein in a fed-batch *D. desulfuricans* SO_2-reducing culture in the presence of anaerobic digester solids. Time zero corresponds to the first addition of digester solids.

sulfite, total and soluble COD and total and soluble protein in the reactor effluent, respectively. As seen in Figure 47, sulfite in the culture medium remained under control during the course of the experiment indicating complete conversion of SO_2 to H_2S.

Once steady state operation of the reactor has been achieved at the highest SO_2 feed rate (Figures 48 and 49), the reactor was once again converted to continuous operaton with biomass recycle. A schematic diagram of the reactor system is given in Figure 50. The bioreactor or fermenter at start-up received a liquid feed of 8.0 mL/hr pretreated sewage sludge medium and a gas feed of 26.1 mL/min 1.0%, SO_2 5% CO_2, balance N_2. This corresponds to a SO_2 molar feed rate of 0.63 mmoles/hr. The effluent from the reactor was removed from the surface of the culture through a stainless steel tube positioned to maintain a constant culture volume of 1.45 L. The effluent was transferred by peristaltic pump to a gravity settler shown in detail in Figure 51. The head space of the settler was purged with N_2. Settled solids were recycled back to the reactor by peristaltic pump. The immediate goal of this experiment was to maximize the volumetric productivity of the reactor system for SO_2 reduction through biomass recycle. In this regard, the SO_2 feed rate was increased with 100% biomass recycle.

Effluent from the reactor was continuously removed by means of a 1/4-in stainless steel tube at the culture surface which withdrew mixed liquor from the reactor as the volume increased and feed was delivered. The effluent was withdrawn by means of a peristaltic pump at a rate of 6 mL/min and was transferred to a gravity settler as shown in Figure 50. The settled solids were then recycled back to the

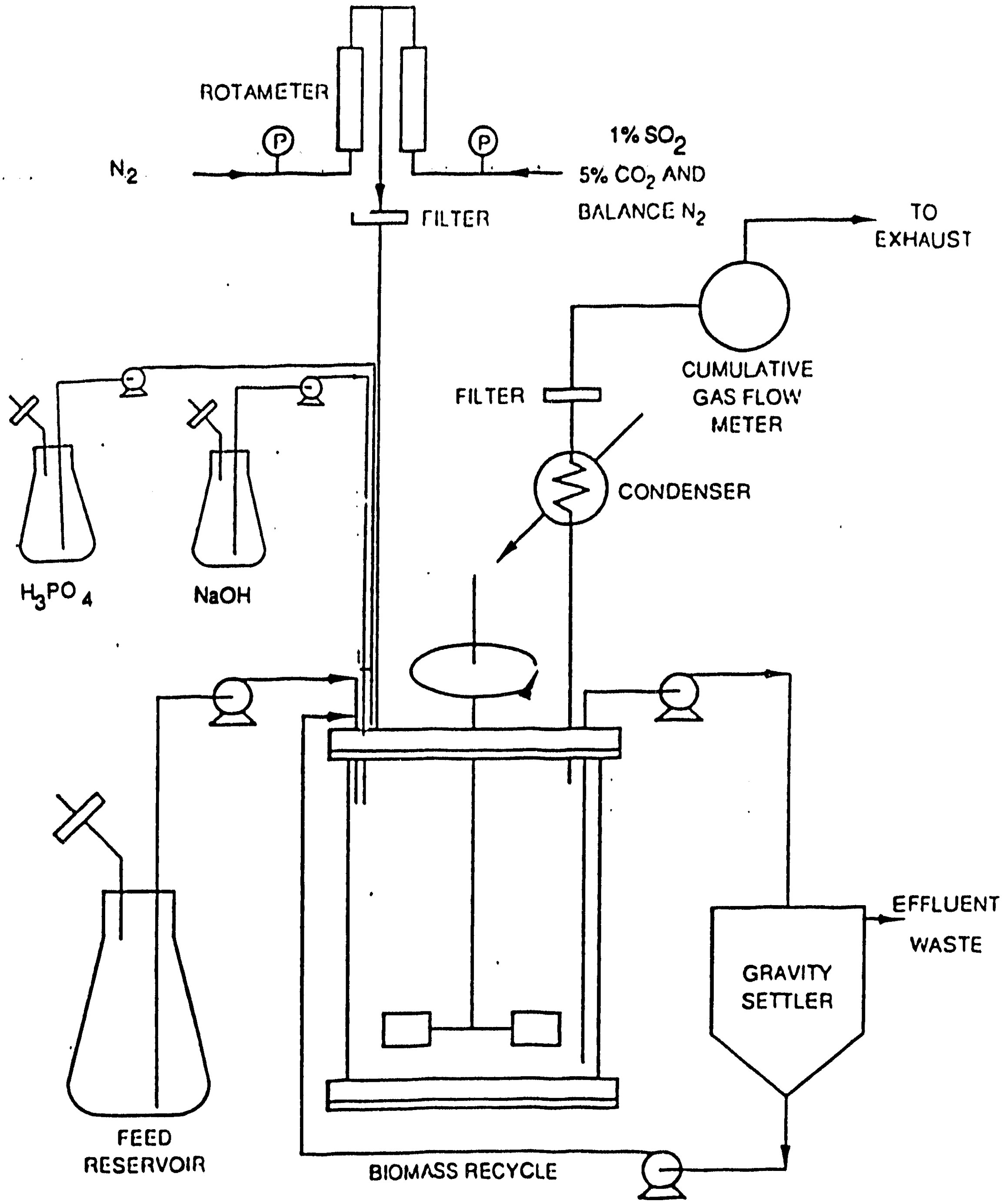


Figure 50. Schematic diagram of continuous *D. desulfuricans* SO_2-reducing reactor system with biomass recycle.

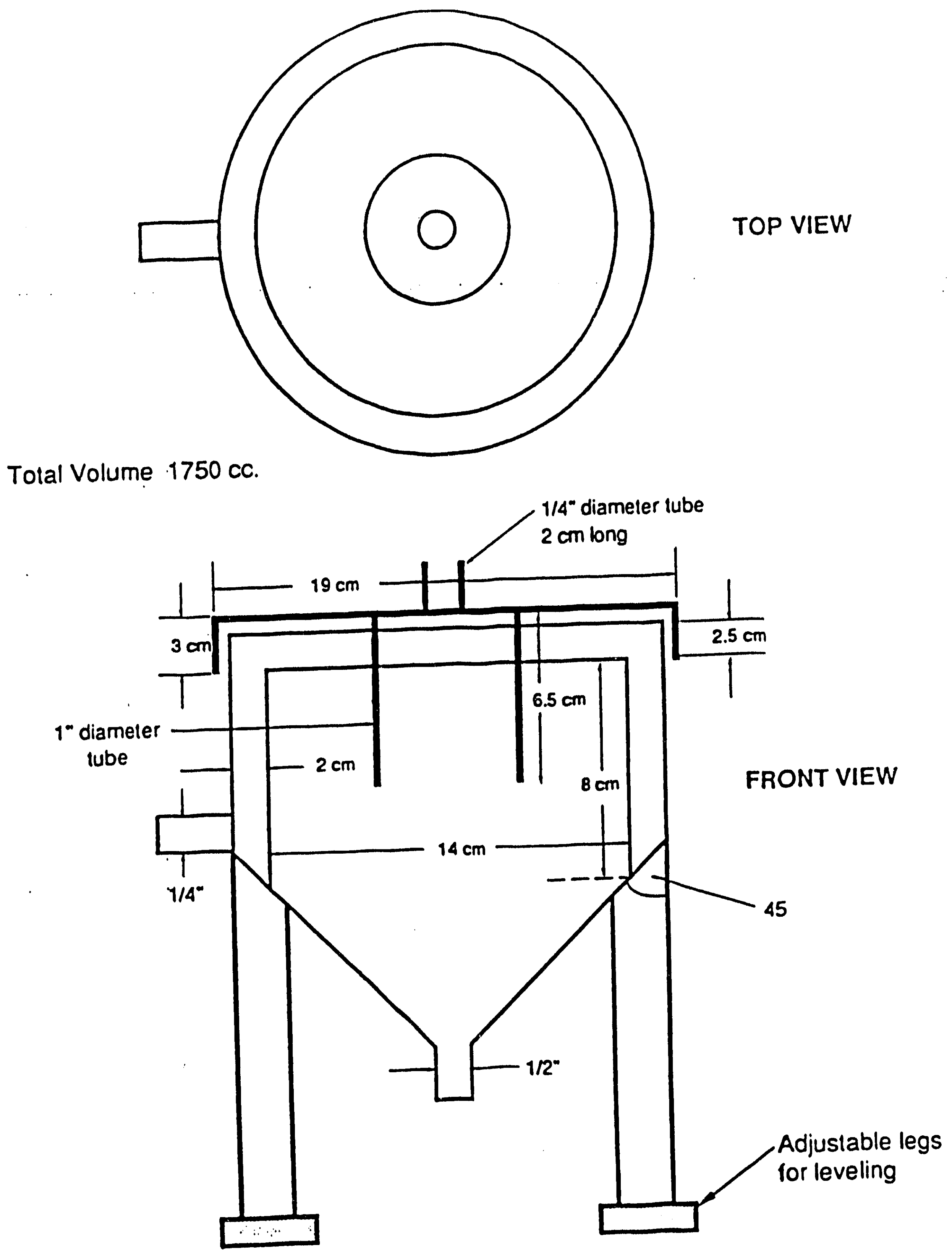


Figure 51. Details of construction of gravity settler used with continuous *D. desulfuricans* SO_2-reducing reactor system with biomass recycle.

reactor for 5 min out of every 30 min by means of a peristaltic pump at a rate of 6 mL/min. The agitation rate in the reactor was kept constant at 150 rpm. The heat/alkali treated sewage sludge was fed continuously at a rate of 8 mL/hr. The initial SO_2 feed rate was set at 0.64 mmoles/hr as described above. The reactor was operated at this feed rate for 6 days. The sulfite level in the reactor medium remained very low during this period; therefore, the SO_2 flow rate was gradually increased. Effluent samples were taken for MLSS analysis, protein, COD, and sulfite analyses.

The reactor was operated at SO_2 feed rates as high as 2.29 mmoles/hr with complete conversion of SO_2 to H_2S. The results of protein, COD and MLSS analyses are given in Figures 52-54. As shown in Figure 52, the total protein concentration in the total effluent was seen to increase since initiation of the experiment indicating the growth and accumulation of *D. desulfuricans* and other biomass as facilitated by recycle. The results of COD and MLSS analyses are shown in Figures 53 and 54. The sulfite results in Figure 55 showed no accumulation of sulfite in the reactor medium during the operating of the recycle reactor.

Sulfur balances were determined at two different SO_2 feed rates during this phase of operation as follows: The outlet gas was bubbled into 400 mL 0.5% zinc acetate solution for 1 hour to trap H_2S gas as ZnS. The ZnS suspension was then analyzed spectrophotometrically for sulfide as described earlier. The results of sulfur balance are given in Table 26. In these experiments, the ratio of H_2S produced to SO_2 consumed averaged 0.95 which in turn indicates the complete conversion of SO_2 to H_2S.

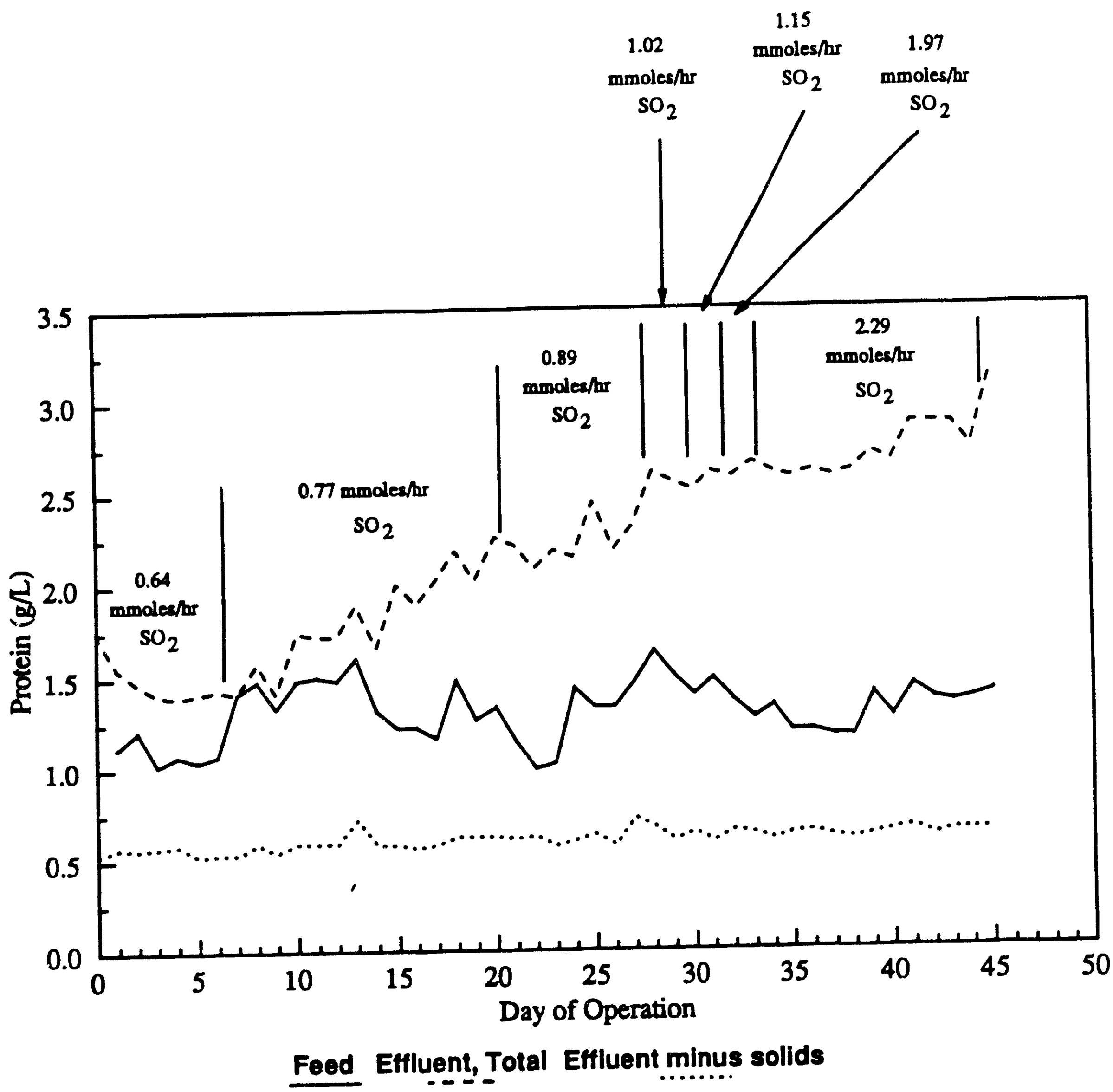


Figure 52. Soluble protein concentration in the feed and effluent of a continuous, immobilized *D. desulfuricans* SO_2-reducing culture operating with a feed of pretreated sewage sludge medium.

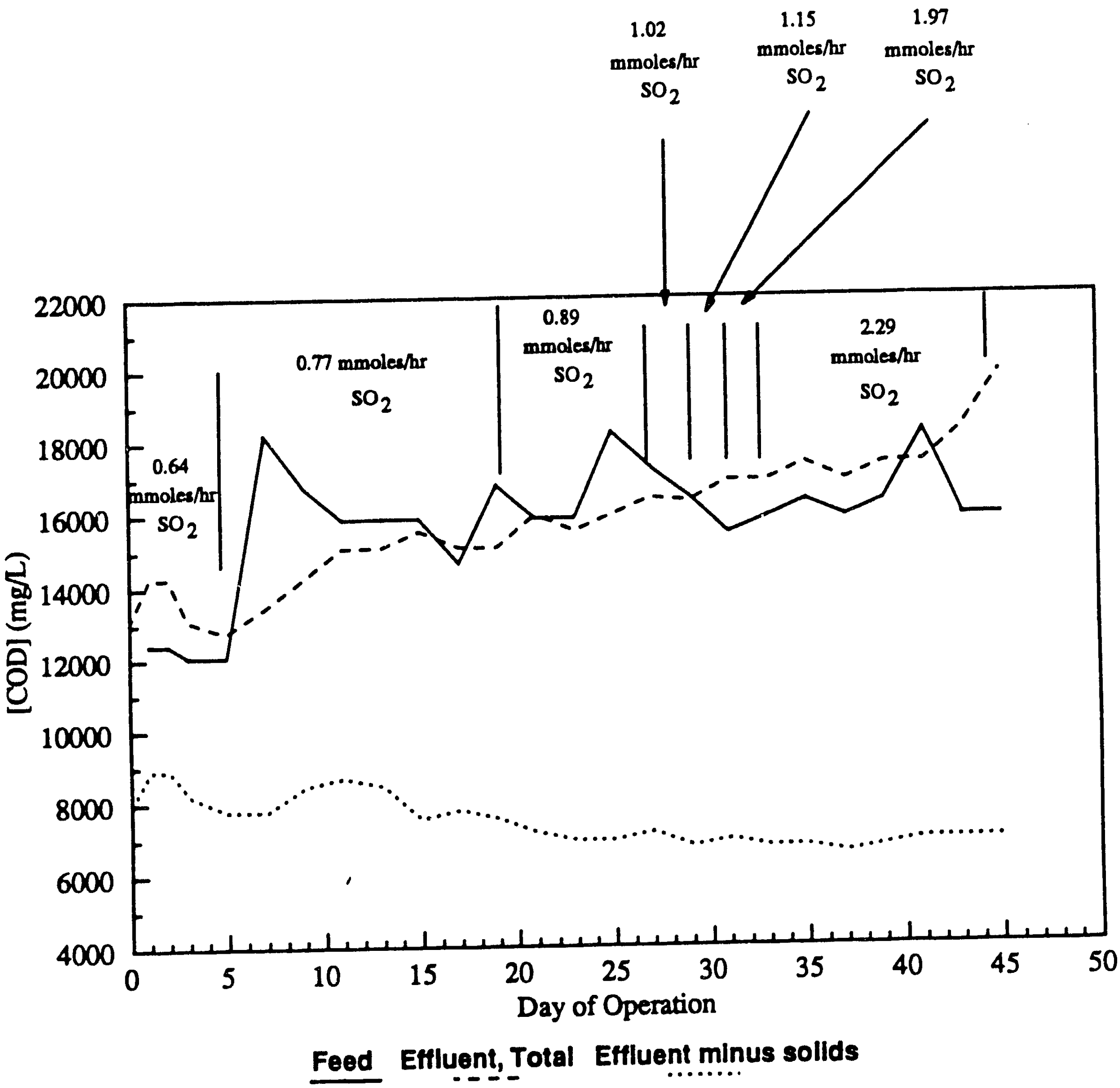


Figure 53. Total and soluble COD concentration in the feed and effluent of a continuous, immobilized *D. desulfuricans* SO_2-reducing culture operating with a feed of pretreated sewage sludge medium.

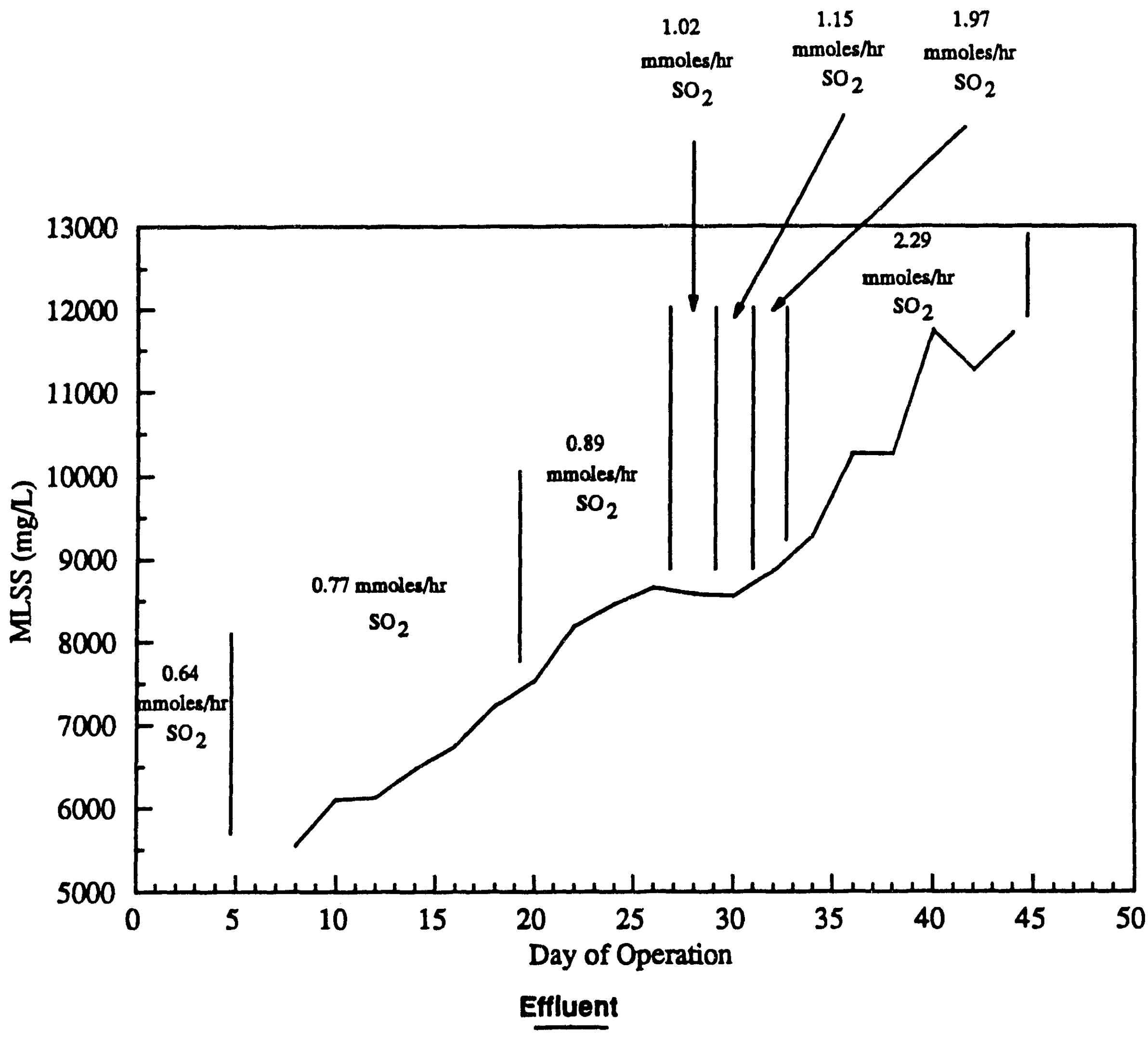


Figure 54. MLSS concentration in the effluent of a continuous, immobilized *D. desulfuricans* SO_2-reducing culture operating with a feed of pretreated sewage sludge medium.

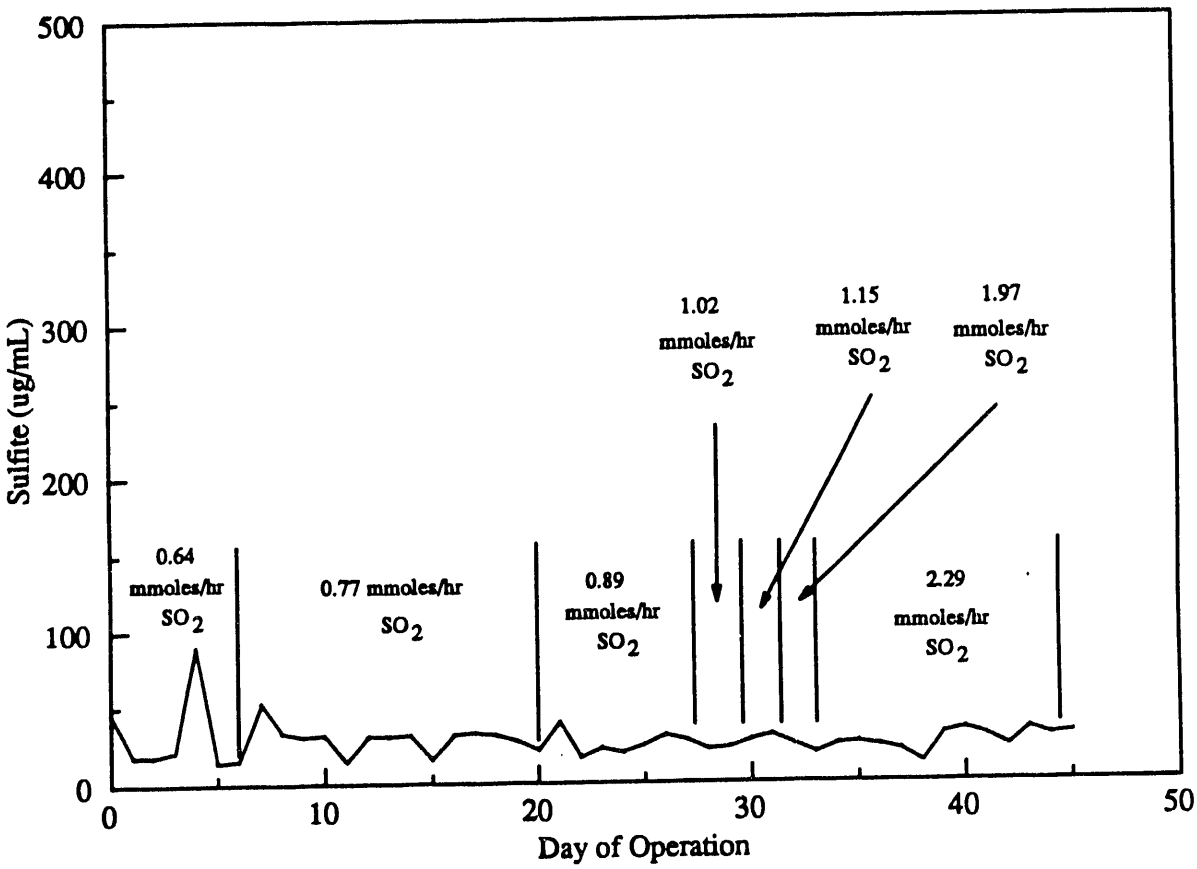


Figure 55. Sulfite concentration in the effluent of a continuous, immobilized *D. desulfuricans* SO_2-reducing culture operating with a feed of pretreated sewage sludge medium.

Table 26. Sulfur Balances in *D. desulfuricans* SO_2-Reducing Culture with Biomass Recycle

Experiment	mmoles SO_2 Consumed	mmoles H_2S Produced	H_2S/SO_2 (mole/mole)
1	1.97	1.89	0.96
2	2.29	2.15	0.94

5.1.8 Alternate Pre-Treatment Methodology for Sewage Sludge

An alternate treatment to replace alkali/heat treatment of sewage sludge was investigated.

In the anaerobic digestion process, the biological conversion of the organic matter is thought to occur in three steps as shown. The first step in the process involves the enzyme-mediated transformation (hydrolysis) of higher-molecular weight compounds into compounds suitable for direct use as a source of energy and cell carbon. The second step (acidogenesis) involves the bacterial conversion of the compounds resulting from the first step into identifiable lower-molecular weight intermediate compounds. The third step (methanogenesis) involves the bacterial conversion of the intermediate compounds into simpler end products, principally methane and carbon dioxide.

Chloroform inhibits the methanogenesis step. We have investigated the use of anaerobic digestion in the presence of chloroform as a means of producing fermentable substrates for SO_2-reducing cultures as follows. Two hundred grams of wet-packed biosolids from the DAF unit of a sewage treatment plant in Tulsa was suspended in 1 L of sulfate-free minimal medium (Table 20). The suspension was split into five identical 200-mL suspensions in 500 mL Erlenmeyer flasks. Chloroform was then added to four of these suspensions as follows:

Flask 1 - without $CHCl_3$

Flask 2 - 250 mg/L $CHCL_3$

Flask 3 - 500 mg/L $CHCl_3$

Flask 4 - 750 mg/L $CHCl_3$

Flask 5 - 1000 mg/L $CHCl_3$

The head space of all 5 flasks were purged with nitrogen and the flasks sealed with rubber stoppers. Samples were taken from each flask daily for first 8 days, at 12 days and after 22 days. Samples were centrifuged and the supernatants analyzed for soluble COD. The results are given in Figure 56. As seen in Figure 56, soluble COD levels increased by about 3000 mg/L in all the flasks except flask 1. These results suggests the inhibition of methanogenesis step in all 4 flasks containing chloroform. It was also observed that the solids in the flask 1 (w/o $CHCl_3$) were floating probably due to gas formation. Methane was found in the head space of flask 1 only. There was very little difference in soluble COD values among the flasks with different concentrations of $CHCl_3$ and also there was no significant increase in soluble COD level after 12 days.

Based on this experiment, we decided to incubate anaerobically the biosolids from the DAF unit suspended in the sulfate-free minimal medium in large volume with chloroform for about 10 days and use the supernatant as a feed to the *D. desulfuricans*
SO_2-reducing culture.

5.2 CO_2/H_2 as Carbon and Energy Sources in SO_2 - Reducing Cultures

5.2.1 SO_2 Reduction by *Desulfotomaculum orientis*

An investigation of the use of CO_2/H_2 as carbon and energy sources for SO_2-reducing cultures was initiated with a study of sulfate-reducing bacterium, *Desulfotomaculum orientis*. *D. orientis* has been shown to grow autotrophically on H_2 and CO_2 and

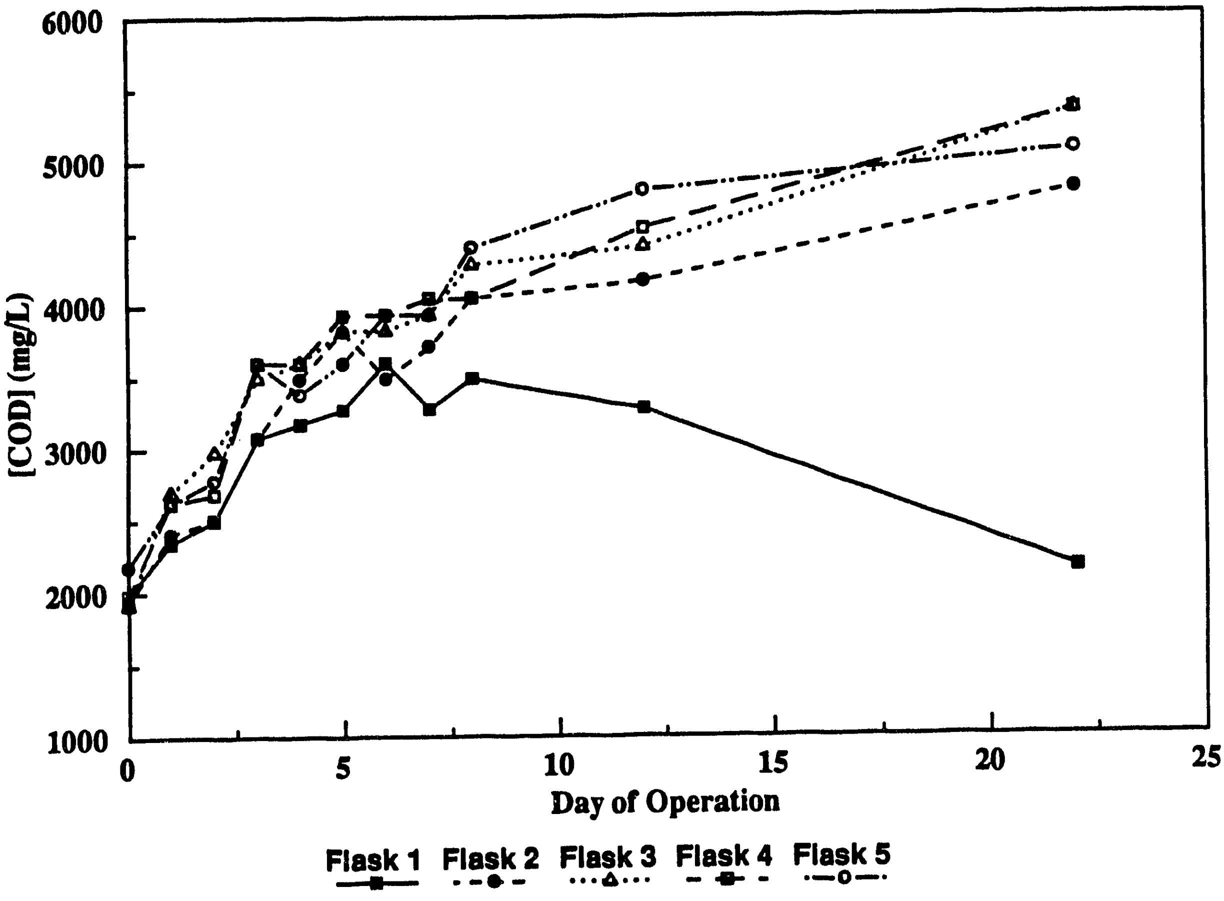


Figure 56. Soluble COD concentration during anaerobic digestion of municipal sewage sludge in the presence of chloroform.

heterotrophically on lactate, formate, methanol and ethanol with sulfate at the terminal electron acceptor (21).

D. orientis (ATCC 19365) was obtained from the American Type Culture Collection (Rockville, MD). Stocks were grown in an autotrophic medium (Table 27) in 100-mL septum bottles. Bottles contained 15 mL of medium and were gassed with $H_2/CO_2/N_2$ (5:5:90, v/v). When H_2 depletion was indicated bottles were regassed. Hydrogen in the bottle was determined by gas chromatography as described in Table 30. Some typical results are shown in Figure 57.

For SO_2-reducing experiments, *D.orientis* was grown septically in 1.5-L cultures in a B. Braun Biostat M fermenter at pH 7.3 and 30°C (agitation rate 200 rpm) in the autotrophic medium described in Table 31. Cultures were inoculated with fresh stocks (15 mL) with demonstrated H_2-utilizing capability.

The first of these cultures originally received two gas feeds: 10% H_2, 5% CO_2, balance N_2 and 5% CO_2, balance, N_2 at 50 mL/min and 150 mL/min, respectively. The original protein concentration in the reactor was 10 mg/L. (Protein was used as an indicator of biomass concentration). The protein concentration and the production of H_2S from sulfate remained quite low for six days. To increase the biomass growth rate 3.5 mL of 60% sodium lactate was added on the 7th day. Lactate addition was repeated on the 9th and 10th day. During this time, the H_2 feed was maintained in order to ensure that enzymes required for H_2 utilization remained induced. By the 10th day the biomass concentration had increased to about 86 mg/L. However, the concentration was flucuating due to some adherance of biomass to the

Table 27. Autotrophic Medium for *D. orientis*

Component	g/L
KH_2PO_4	0.3
NH_4Cl	0.5
$NaCl$	1.0
$MgSO_4$	1.0
Na_2SO_4	1.0
$CaCl_2 \cdot 2H_2O$	0.1
$NaHCO_3$	1.0
$Fe(NH_4)_2(SO_4)_2$	0.05
Trace Element Solution (Table 28)	1 mL
Wolfe's Vitamin Solution (Table 29)	1 mL

Table 28. Trace Element Solution for *D. orientis*

Component	Quantity/L
HCl(25%;7.7M)	10.0 mL
$FeCl_2 \cdot 4H_2O$	1.5 g
$ZnCl_2$	70.0 mg
$MnCl_2 \cdot 4H_2O$	100.0 mg
H_3BO_3	6.0 mg
$CoCl_2 \cdot 6H_2O$	190.0 mg
$CuCl_2 \cdot 2H_2O$	2.0 mg
$NiCl_2 \cdot 6H_2O$	24.0 mg
$Na_2MoO_4 \cdot 2H_2O$	36.0 mg

First dissolve $FeCl_2$ in the HCl, then dilute with water, add and dissolve the other salts, adjust pH to 6.0 with NaOH, finally adjust to 1.0 L with distilled water.

Table 29. Wolfe's Vitamin Solution

Component	mg/L
Biotin	2.0
Folic acid	2.0
Pyridoxine HCl	10.0
Thiamine HCl	5.0
Riboflavin	5.0
Nicotinic acid	5.0
Calcium pantothenate	5.0
Cyanocobalamine	0.10
p-Aminobenzoic acid	5.0
Thioctic acid	5.0

Table 30. Chromatographic Conditions for Analysis of H_2 in Reactor Outlet Gas

Instrument:	Hewlett Packard 5890
Column:	20 ft. x 1/8-in ID stainless steel 100/200 Haye Sep D
Carrier Gas & Flow Rate:	He , 30 mL/min
Oven Temperature:	40°C (2 min) then 24°C/min, 120°C max
Injection Oven & Detector Temperature:	100°C, 140°C
Detector:	Thermal Conductivity Detector

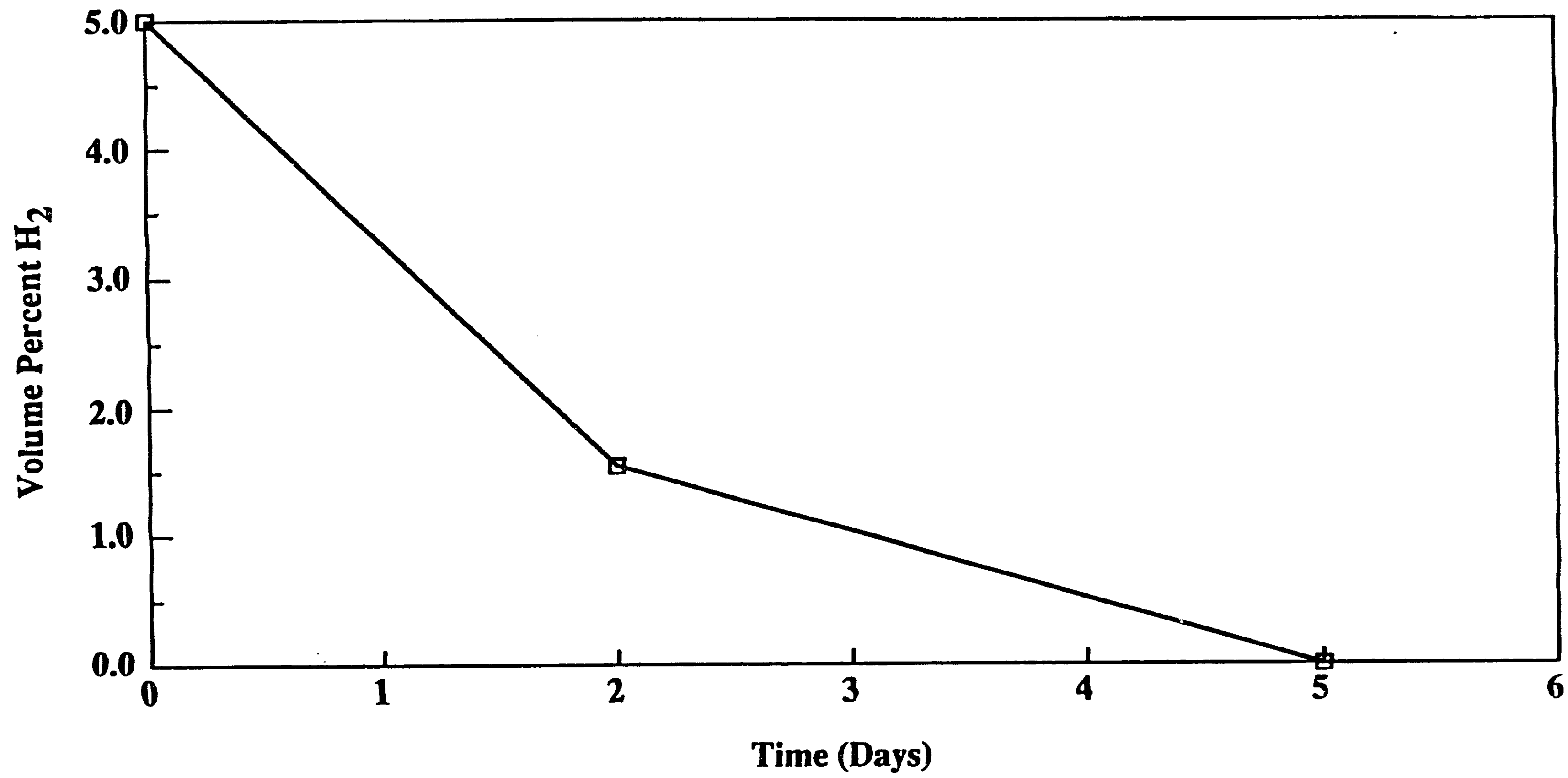


Figure 57. H_2 concentration in the gas space of a *D. orientis* stock culture.

Table 31. SO_2 Growth Medium for *D. orientis*

Component	g/L
KH_2PO_4	0.3
NH_4Cl	0.6
NaCl	1.0
$MgCl_2$	0.96
$CaCl_2 \cdot 2H_2O$	0.1
$NaHCO_3$	1.0
$FeCl_3$	0.02
Trace Element Solution (Table 28)	1 mL
Wolfe's Vitamin Solution (Table 29)	1 mL

reactor wall. The H_2S concentration in the outlet gas (approximately 200 mL/min) ranged from 500-1200 ppmv.

On the 11th day, the biomass was harvested by centrifugation at 4900g and 25°C, resuspended in autotrophic medium (Table 27) without lactate and transferred back to the fermenter. Gas feeds were restarted and the culture maintained at 30°C and pH 7.3. For the next 48 hours, the H_2S concentration in the outlet gas averaged about 150 ppmv. At this time, it was suspected that growth of *D. orientis* was mass transfer limited. To determine if this was the case, the H_2 mixture was replaced with 100% H_2. At this time, the gas feeds consisted of 60 mL/min H_2 and 150 mL/min 5% CO_2, balance N_2. The increase in H_2 partial pressure resulted in an increase in H_2S production giving an average H_2S concentration in the outlet gas of about 2000 ppmv. Therefore, growth of *D. orientis* had been clearly mass transfer limited and not limited by the intrinsic H_2 utilization rate of the biomass.

On day 27, the biomass was again harvested by centrifugation as described above and resuspended in modified autotrophic medium (Table 31). This medium is similar to that described in Table 27 with the exception that all sulfate had been removed. The suspension was then sparged with H_2 (60 mL/min) and 5% CO_2, balance N_2 (130 mL/min) for 2 hours to allow any residual sulfate to be consumed. At the end of this time, an additional gas feed 1.0% SO_2, 5% CO_2, and balance N_2 was introduced at a rate of 6.8 mL/min. This corresponds to a molar flow rate of 0.167 mmoles/hr SO_2. The culture was maintained under these conditions for an additional 15 days during which time the outlet gas was monitored for H_2S and the culture medium analyzed to demonstrate growth of *D. orientis* under these conditions.

With a molar flow rate of 0.167 mmoles/hr, the H_2S concentration in the outlet gas averaged 315 ppmv. The total outlet gas flow was 211 mL/min. Therefore, greater than 96% conversion of SO_2 to H_2S was demonstrated with 1-2 s of gas-liquid contact time. Sulfite concentrations in the bulk aqueous phase were relatively constant and averaged less than 10 mg/L indicating complete reduction of inlet SO_2 to H_2S. During this time, the biomass protein concentration in the culture medium was seen to increase as shown in Figure 58. Ammonium ion, a source of reduced nitrogen for the biomass, was also seen to decline as SO_2 was removed from the feed gas. To the best of our knowledge, this is the first time that growth of a sulfate-reducing bacterium on $H_2/CO_2/SO_2$ has been demonstrated.

These experiments were duplicated at higher biomass concentrations as follows. *D. orientis* working cultures (1.5 L) were prepared by growing the organism septically in a B. Braun Biostat M at pH 7.3 and 30°C in the autotrophic medium described in Table 27 supplemented with 5.9 mL of 60% sodium lactate. Cultures were inoculated with fresh stocks (15 mL) with demonstrated H_2-utilizing capability. Lactate was replenished as it was utilized (5.8 mL every 2 days) until the biomass total protein concentration was 0.2-0.3 g/L. At this time the biomass was harvested by centrifugation at 4900 x g and 25°C and resuspended in 1.5 L of fresh medium (Table 24 without sulfate) in the fermenter. After resuspension the fermenter received gas feeds of 140 mL/min 5% CO_2, balance N_2, 70 mL/min H_2 and 7.5-9.6 mL/min of 1% SO_2, 5% CO_2, balance N_2. The molar feed rate of SO_2 was

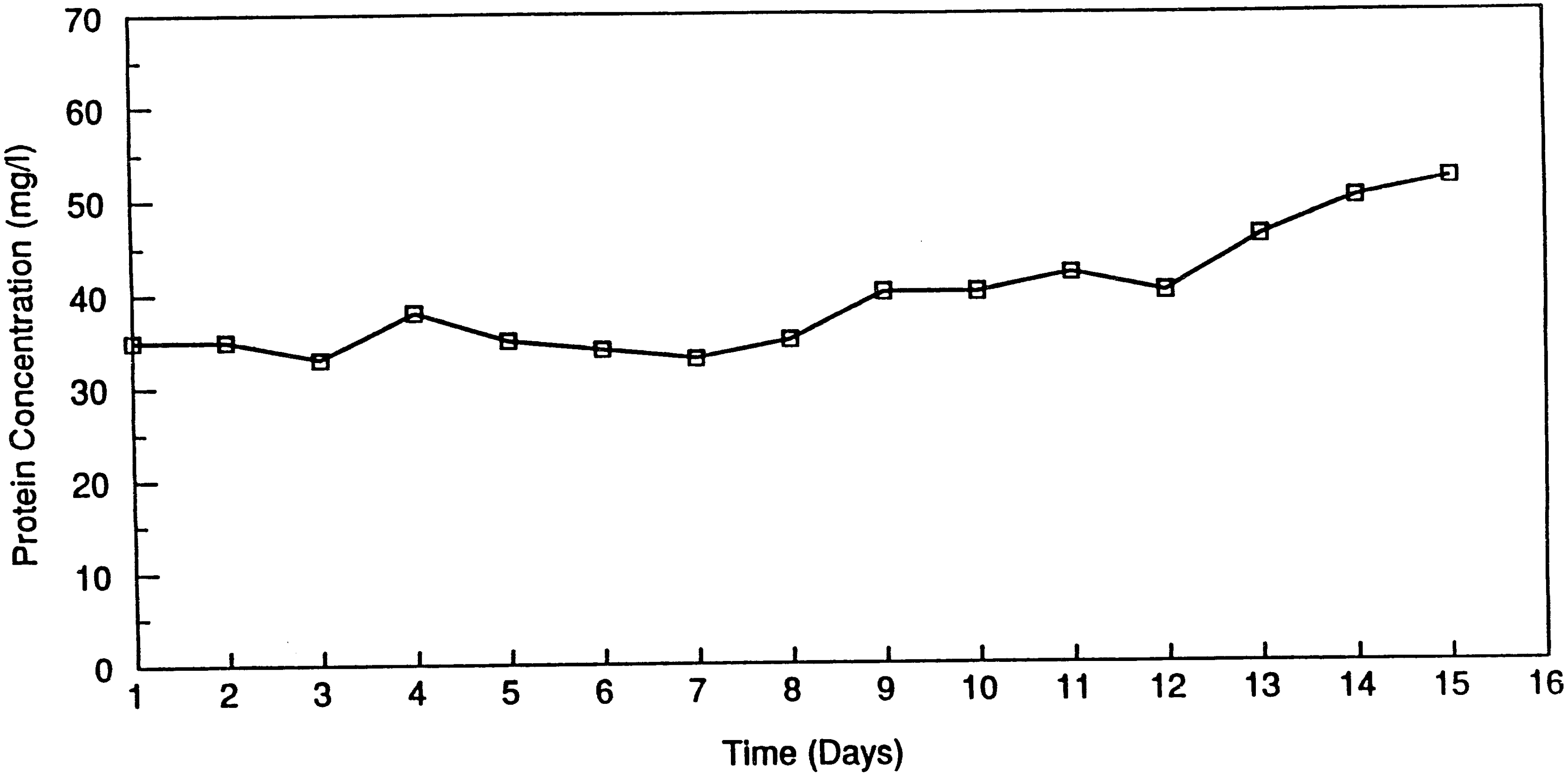


Figure 58. Biomass protein concentration in a batch SO_2-reducing culture of <u>D. orientis</u> operating with a CO_2/H_2 feed.

0.19-0.24 mmoles/hr. Cultures were maintained under these conditions at pH 7.3 and 30°C for 15-30 days during which time the outlet gas was monitored for H_2S and the culture medium analyzed to demonstrate growth of *D. orientis* under these conditions.

With a molar flow rate of 0.185 mmoles/hr, the H_2S concentration in the outlet gas (total flow rate 218 mL/min) averaged 340 ppmv. Table 32 shows the result of sulfur balances performed at various times during the course of three batch experiments. Complete conversion of SO_2 to H_2S is indicated. Sulfite concentrations in the bulk aqueous phase were relatively constant and average less than 5 mg/L. (Routine analysis of the outlet gas for H_2S was performed by gas chromatography as described in Table 17. However, outlet gases were also periodically analyzed by trapping of H_2S by precipitation as ZnS and subsequent spectrophotometric analysis as described in Section 5.1.5).

As SO_2 was removed from the feed gas and reduced to H_2S, the biomass protein in these reactors was seen to increase as shown in Figures 59 and 60. The data are somewhat erratic due to a tendency for the biomass to adhere to the walls of the vessel; however, a clear upward trend is evident indicating growth of the organism on $H_2/CO_2/SO_2$. Ammonium ion, a source of reduced nitrogen for the oragnism, was seen to decrease as SO_2 was removed from the feed gas (Figure 61).

Analysis of the culture medium from one of these batch experiments (B3) produced some rather surprising results. As seen in Figures 62 and 63, acetic acid and butyric acid were observed to accumulate to rather high concentrations as SO_2 was reduced to H_2S.

Table 32. Sulfur Balances in *D. orientis* SO_2-Reducing Batch Cultures

Experiment	SO_2 Feed Rate (mmoles/hr)	H_2S Production Rate (mmoles/hr)	H_2S/SO_2
B1	0.165	0.173	1.05
B2	0.237	0.236	1.00
		0.241	1.02
		0.236	1.00
B3	0.185	0.189	1.02
		0.182	0.98
		0.184	0.99

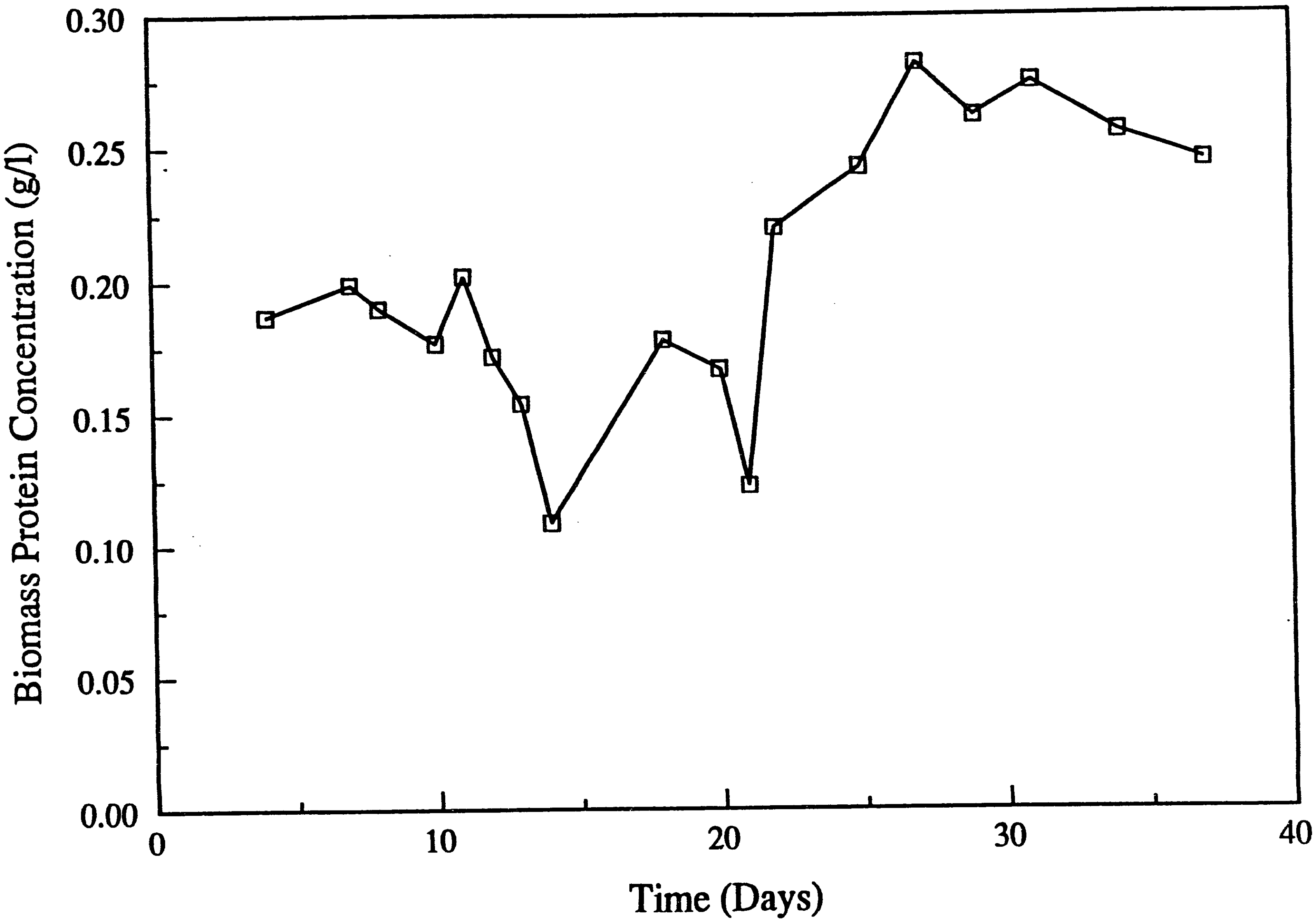


Figure 59. Biomass protein concentration in a batch SO_2-reducing culture (B2) of <u>D. orientis</u> operating with a CO_2/H_2 feed.

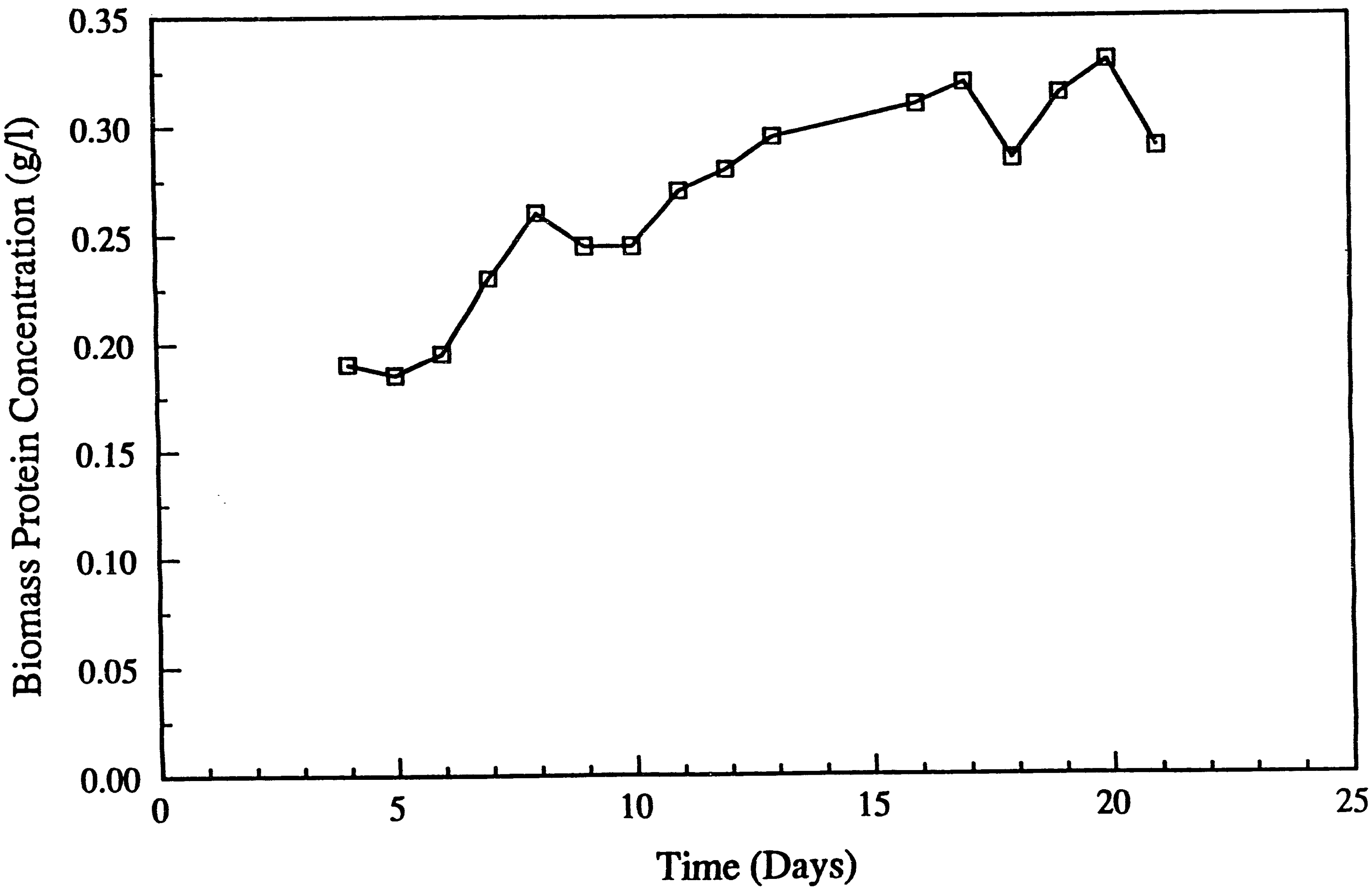


Figure 60. Biomass protein concentration in a batch SO_2-reducing culture (B3) of _D. orientis_ operating with a CO_2/H_2 feed.

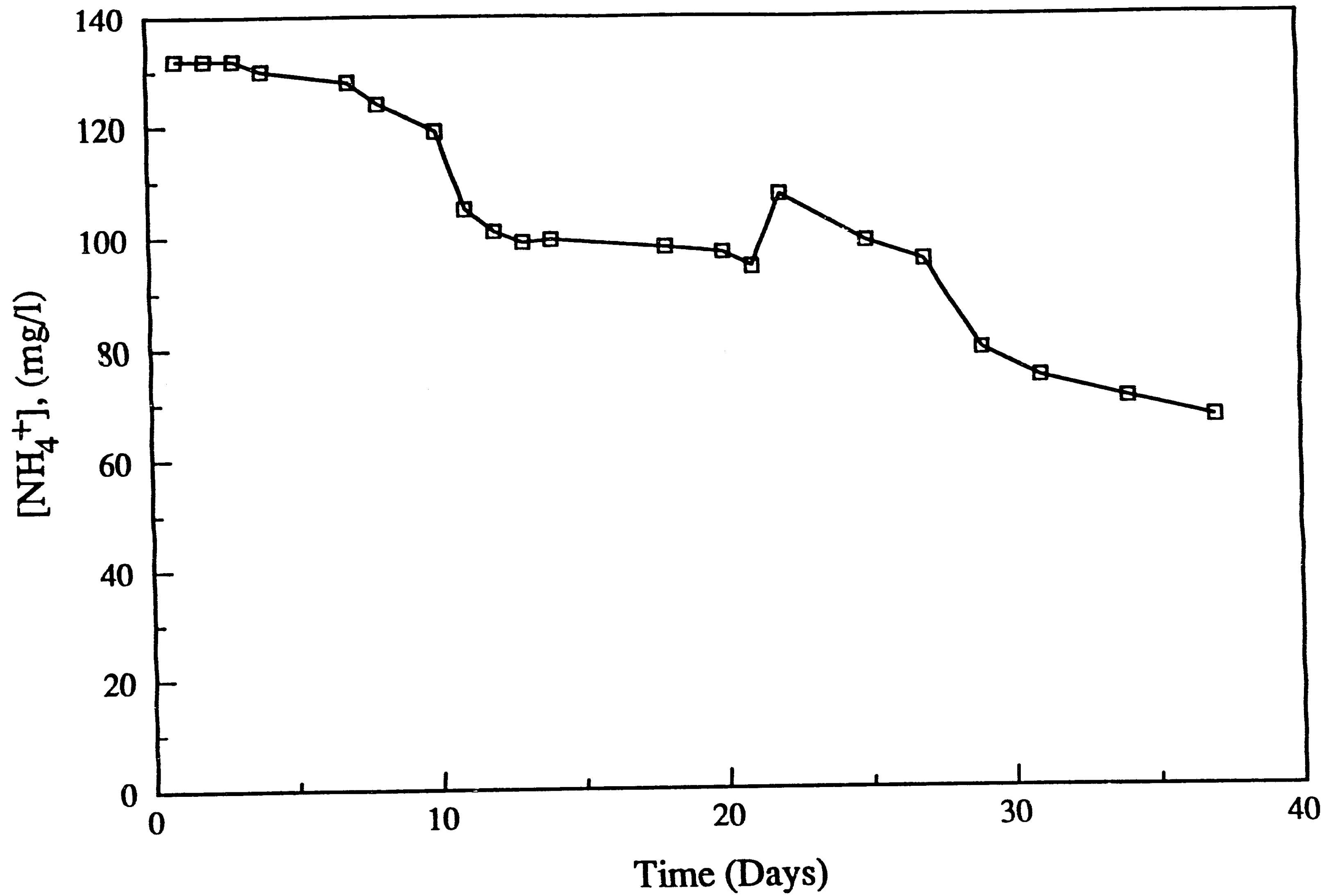


Figure 61. Ammonium in concentration in a batch SO_2-reducing culture (B3) of <u>D. orientis</u> operating with a CO_2/H_2 feed.

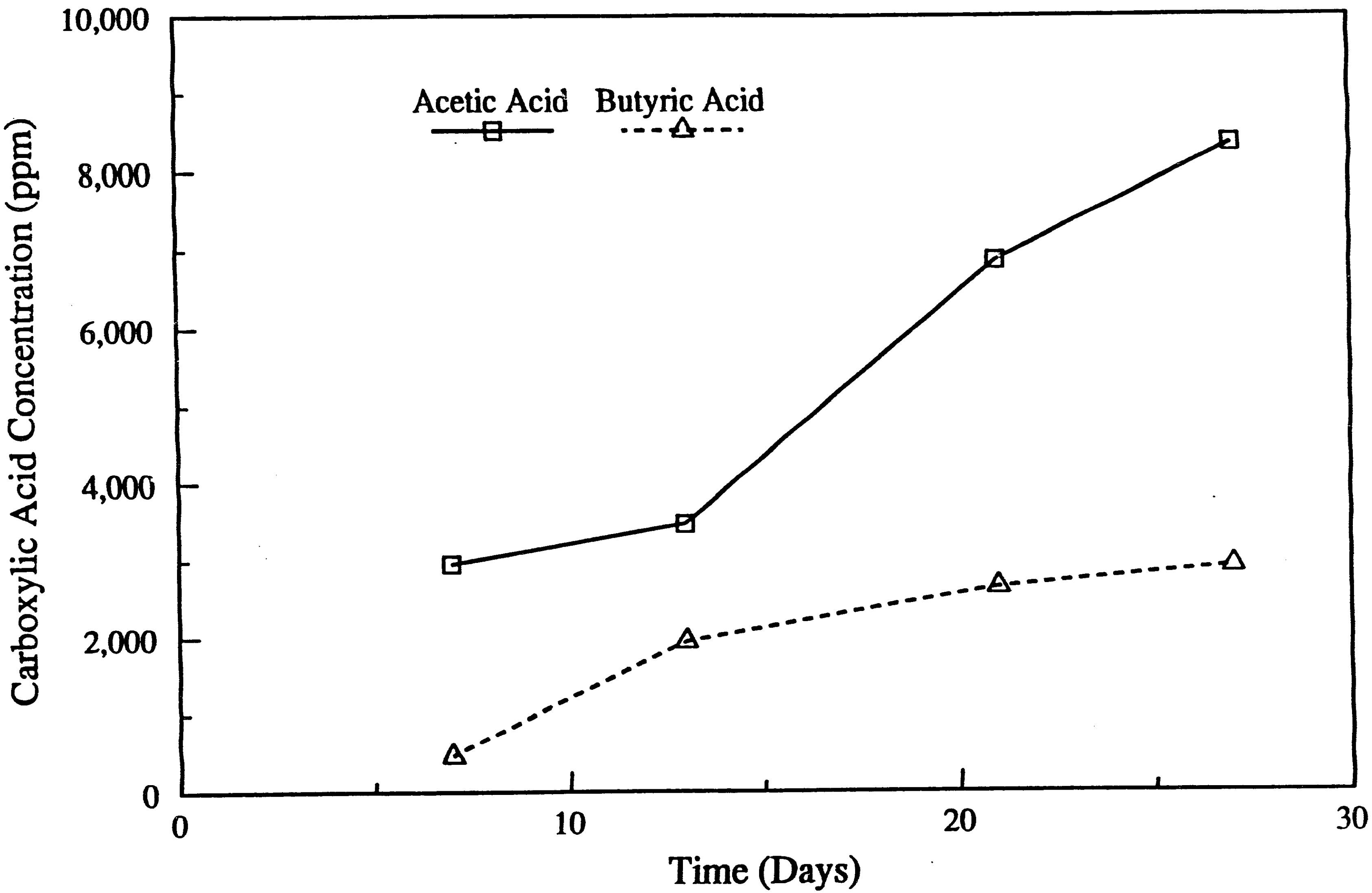


Figure 62. Acetic acid and butyric acid concentrations in a batch SO_2-reducing culture (B3) of D. orientis operating with a CO_2/H_2 feed.

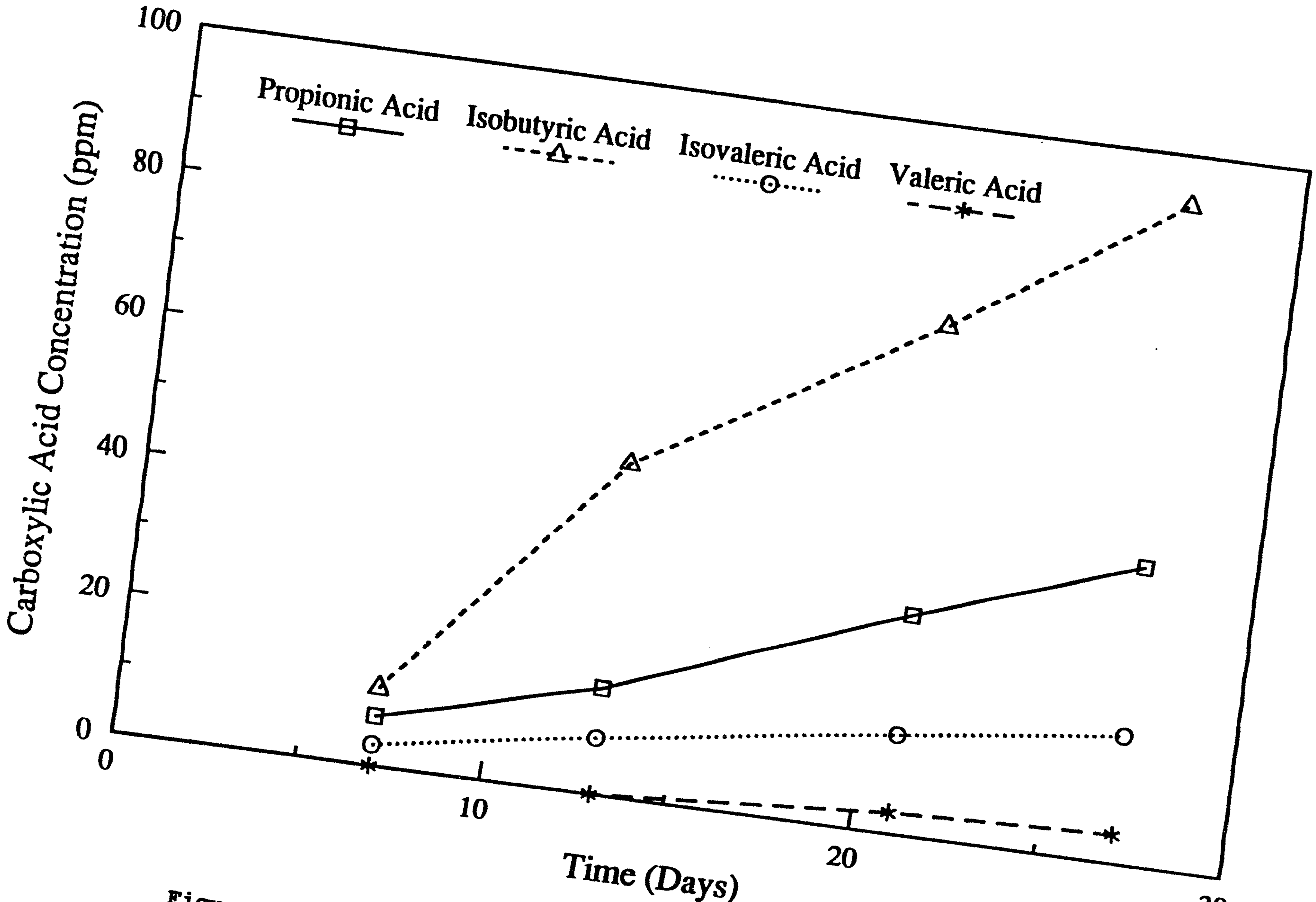


Figure 63. Concentrations of various carboxylic acids in a batch SO_2-reducing culture (B3) of <u>D. orientis</u> operating with a CO_2/H_2 feed.

Lesser amounts of propionic acid, isobutyric acid, isovaleric acid and valeric acid accumulated. These observations will be investigated further and if confirmed may represent a new method of producing carboxylic acids from H_2/CO_2. However, for SO_2-reducing applications it will be necessary to utilize these carboxylic acids to support SO_2 reduction in order to improve the efficiency of the process and reduce the effluent COD.

The equation for the purely chemical reduction of SO_2 by H_2 would be as follows:

$$3H_2 + SO_2 \rightarrow H_2S + 2H_2O$$

In D. orientis cultures operating on a feed of $H_2/CO_2/SO_2$, a H_2/SO_2 ratio of slightly higher than 3.0 would be expected since some H_2 oxidation would be required to reduce CO_2 for production of biomass. In the experiments reported here the molar ratio of H_2/SO_2 in the feed gas was on the order of 700-900. Therefore, it is difficult to determine the stoichiometry of the bioprocess with respect to H_2 utilization by analysis of the feed gas with any accuracy. GC analysis indicated a 4% loss of H_2 from the feed gas which is within experimental error for the analysis.

In order to firmly establish that SO_2 reduction in these cultures was occurring at the expense of the H_2 oxidation, two experiments were conducted in batch SO_2-reducing cultures in which H_2 feed was turned off and the results observed. If reducing equivalents required for SO_2 reduction came from H_2 oxidation, the cessation of H_2 feed would produce a reduction in the outlet H_2S concentration and an accumulation of sulfite in the culture medium. As shown in Figure 64 and 65 this was indeed the case. Before the H_2 feed was turned off

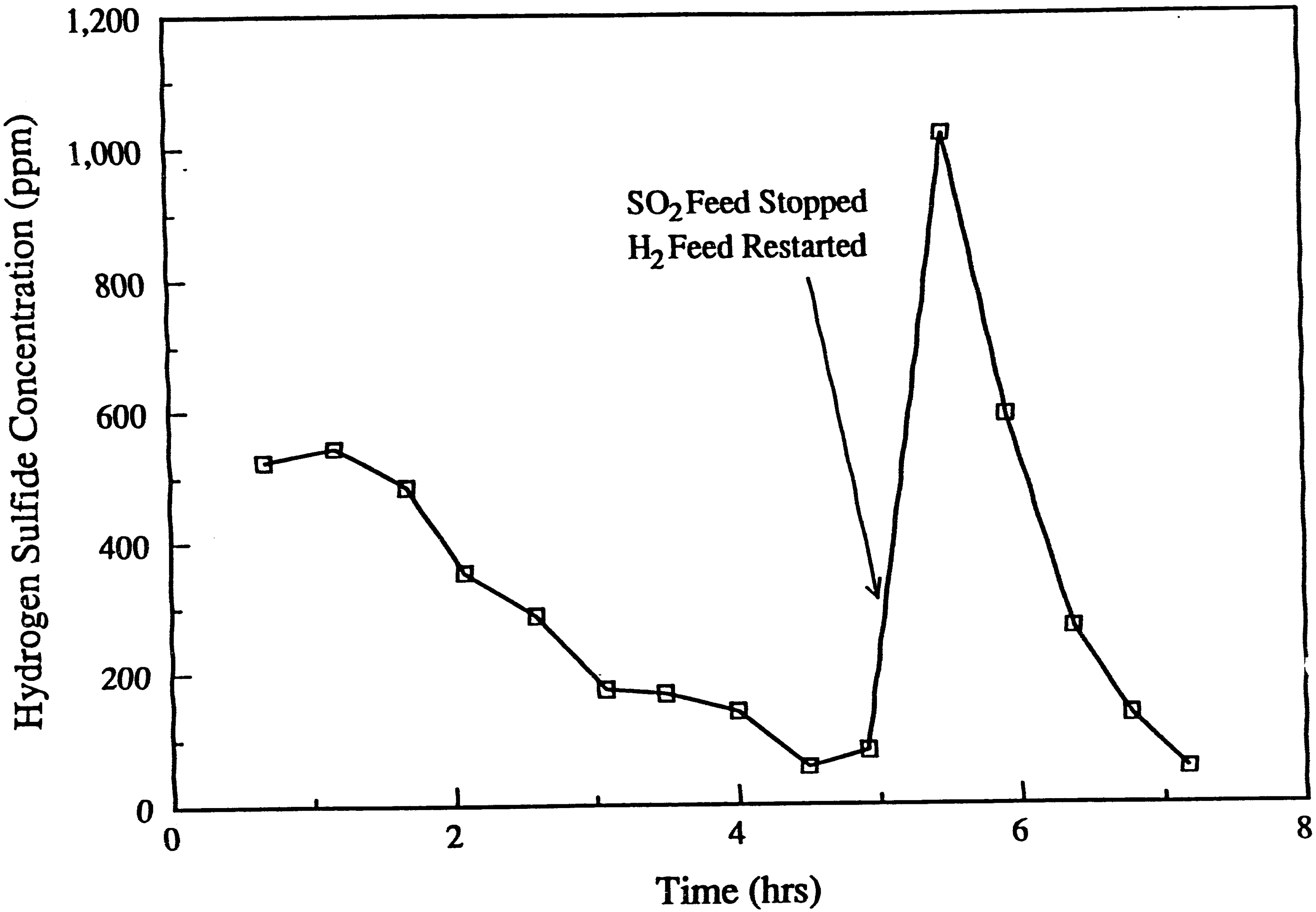


Figure 64. Outlet gas H_2S concentration in a batch SO_2-reducing culture of <u>D. orientis</u> following cessation of H_2 feed.

the total gas feed rate in these experiments was 220 mL/min consisting of 9.6 mL/min of 1% SO_2, 5% CO_2, balance N_2; 71 mL/min H_2; and 140 mL/min of 5% CO_2, balance N_2. The H_2S concentration in the outlet gas was about 340 ppm. When the H_2 feed was turned off the H_2S concentration in the outlet gas increased to 500 ppm due the decrease in total gas flow. As seen in Figure 64 the H_2S concentration then decreased dramatically over the next four hours. At the same time sulfite was observed to accumulate in the culture medium (Figure 65). Five hours after the H_2 feed was turned off, the SO_2 feed was turned off and the H_2 feed restarted. Consequently, the sulfite concentration returned to very low levels within 30 min and there was a corresponding transient surge in H_2S production. After another 3.5 hrs the SO_2 feed was restarted at the original feed rate. The H_2S concentration in the outlet gas then returned to about 340 ppm representing stoichiometric reduction of SO_2 to H_2S.

As noted above, rather high concentrations of acetic acid and butyric acid were observed in cultures of *D. orientis* operating on a feed of H_2, CO_2 and SO_2. Additional experiments were conducted to better document the role of carboxylic acids in these cultures for two reasons. First, as noted previously, these cultures may represent a new method of producing carboxylic acids from H_2/CO_2. Secondly, these carboxylic acids represent the under-utilization of electron donors in the cultures. Either the accumulation of these carboxylic acids must be inhibited or additional species of SRB capable of utilization of carboxylic acids (and SO_2 reduction) must be added to the culture.

For these experiments, working cultures (1.5 L) of *D. orientis* were prepared by growing the organism septically on lactate as

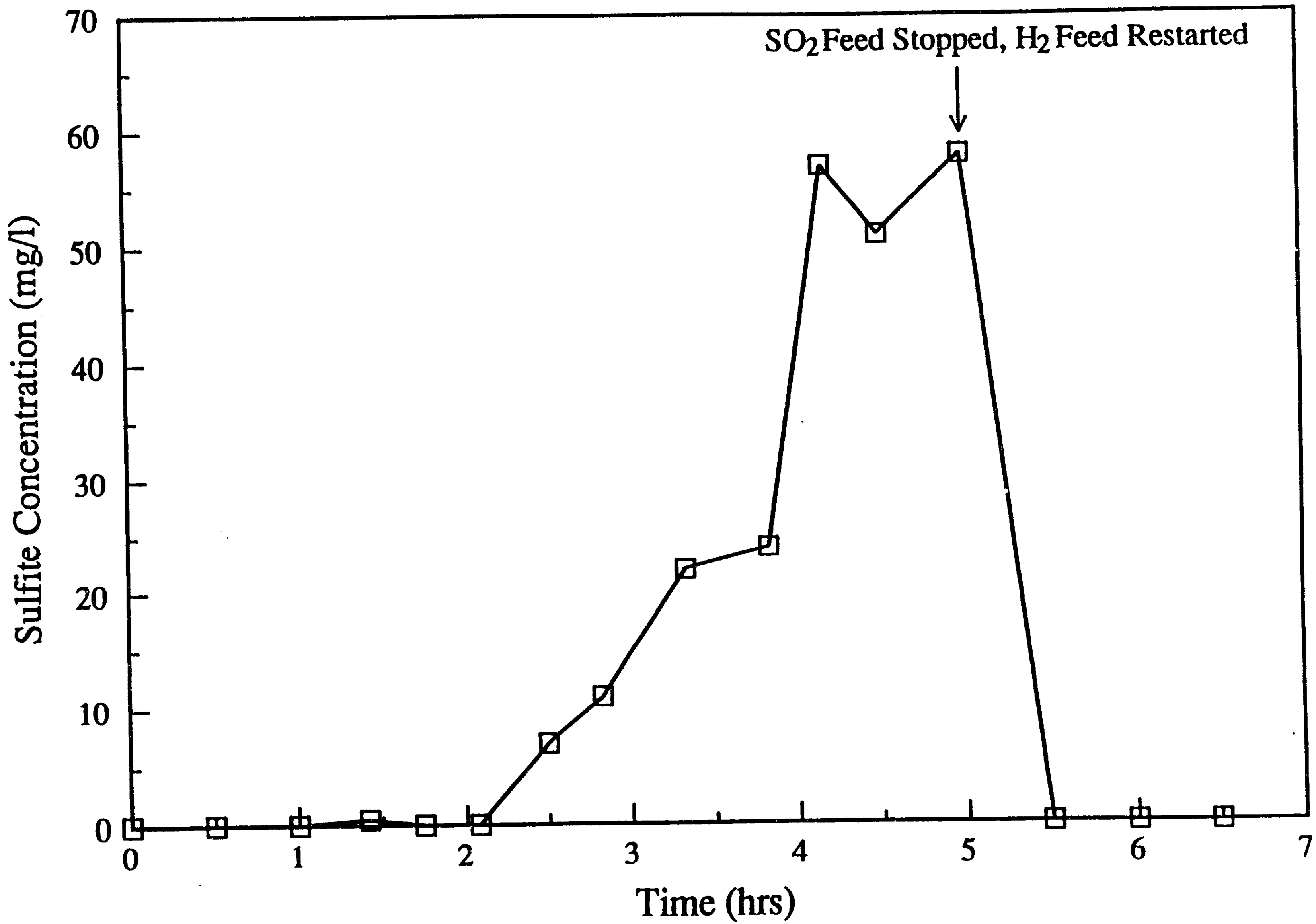


Figure 65. Sulfite concentration in a batch SO_2-reducing culture of <u>D. orientis</u> following cessation of H_2 feed.

described above followed by transfer to an autotrophic medium (Table 31). After resuspension, the fermenter received gas feeds of 140 mL/min of 5% CO_2, balance N_2; 70 mL/min H_2; and 7.6 mL/min 1.0% SO_2, 5% CO_2, balance N_2. The molar feed rate of SO_2 was 0.185 mmoles/hr.

Two experiments were conducted using these operating conditions. In the first experiment the culture was operated for 145 hrs on the SO_2 feed with accumulation of acetic acid and butyric acid in the culture medium as seen in Figure 66. At this time, the SO_2 feed was terminated; however, gas feeds of 140 mL/min of 5% CO_2, balance N_2 and 70 mL/min H_2 were continued. As shown in Figure 66, the production of acetic acid and butyric acid essentially leveled off indicating that production of carboxylic acids was linked to SO_2 reduction by *D. orientis*. Figure 67 shows the biomass protein concentration in the reactor during the experiment. Some continued growth of biomass is indicated after the SO_2 feed was stopped.

In a second similar experiment, the SO_2 feed was stopped (while CO_2/N_2 and H_2 feeds were maintained) at a lower accumulated acetic acid concentration (about 5000 ppm). For about 50 hrs following the cessation of SO_2 feed the acetic acid concentration continued to increase at roughly the same rate (Figure 68). However, after 50 hrs the acetic acid concentration remained relatively constant for the next 120 hrs of operation without a SO_2 feed. As shown in Figure 68, the butyric acid accumulation ceased almost immediately after the SO_2 feed was stopped. After 175 hrs of operation without the SO_2 feed, the SO_2 feed was restarted at the original rate (0.185 mmoles/hr). As shown in Figure 68, acetic acid and butyric acid again began to accumulate in the reactor medium. Figure 69 shows the biomass protein

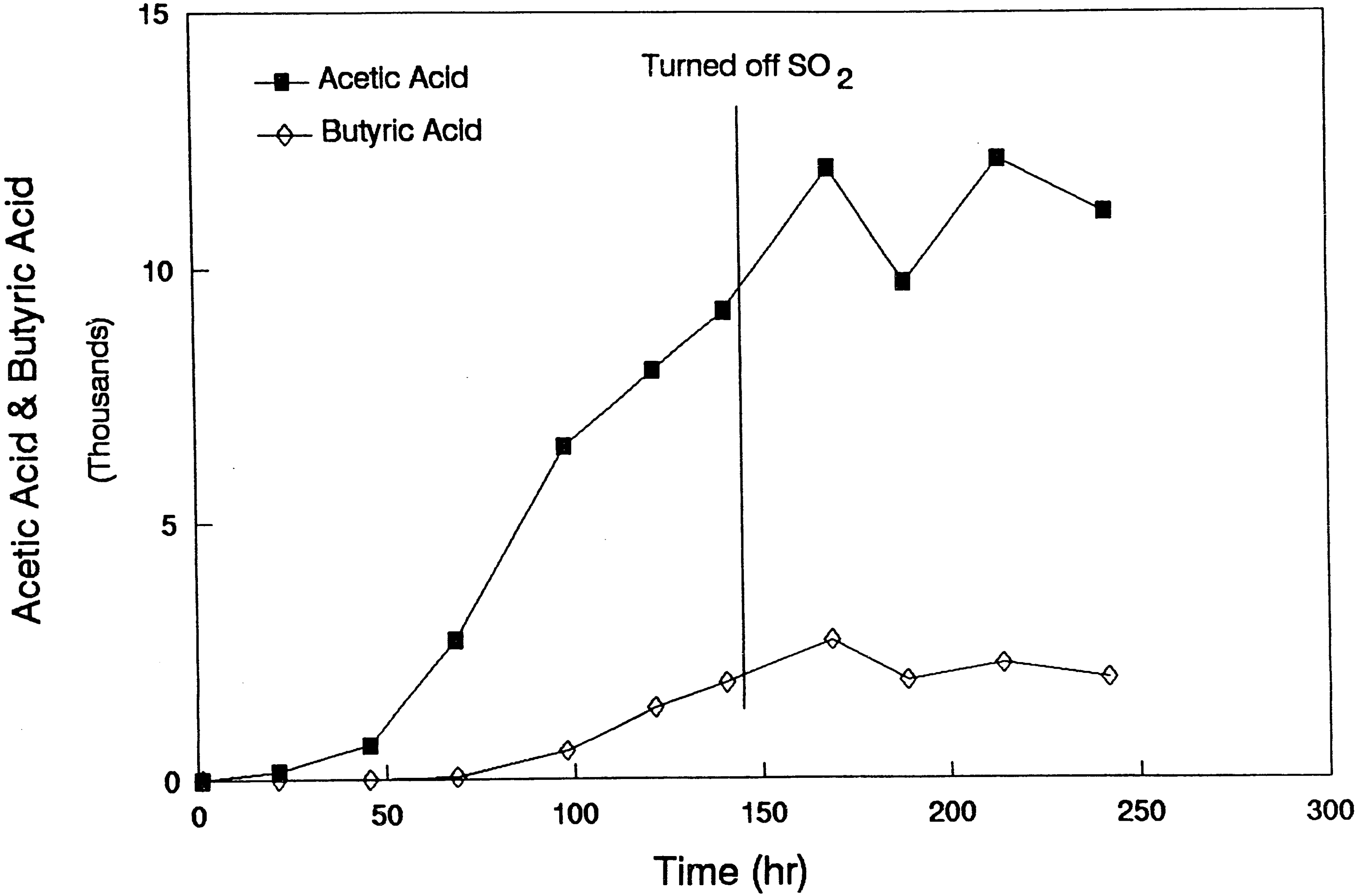


Figure 66. Accumulation of acetic acid and butyric acid in a culture of *D. orientis* with and without SO_2 feed (E1).

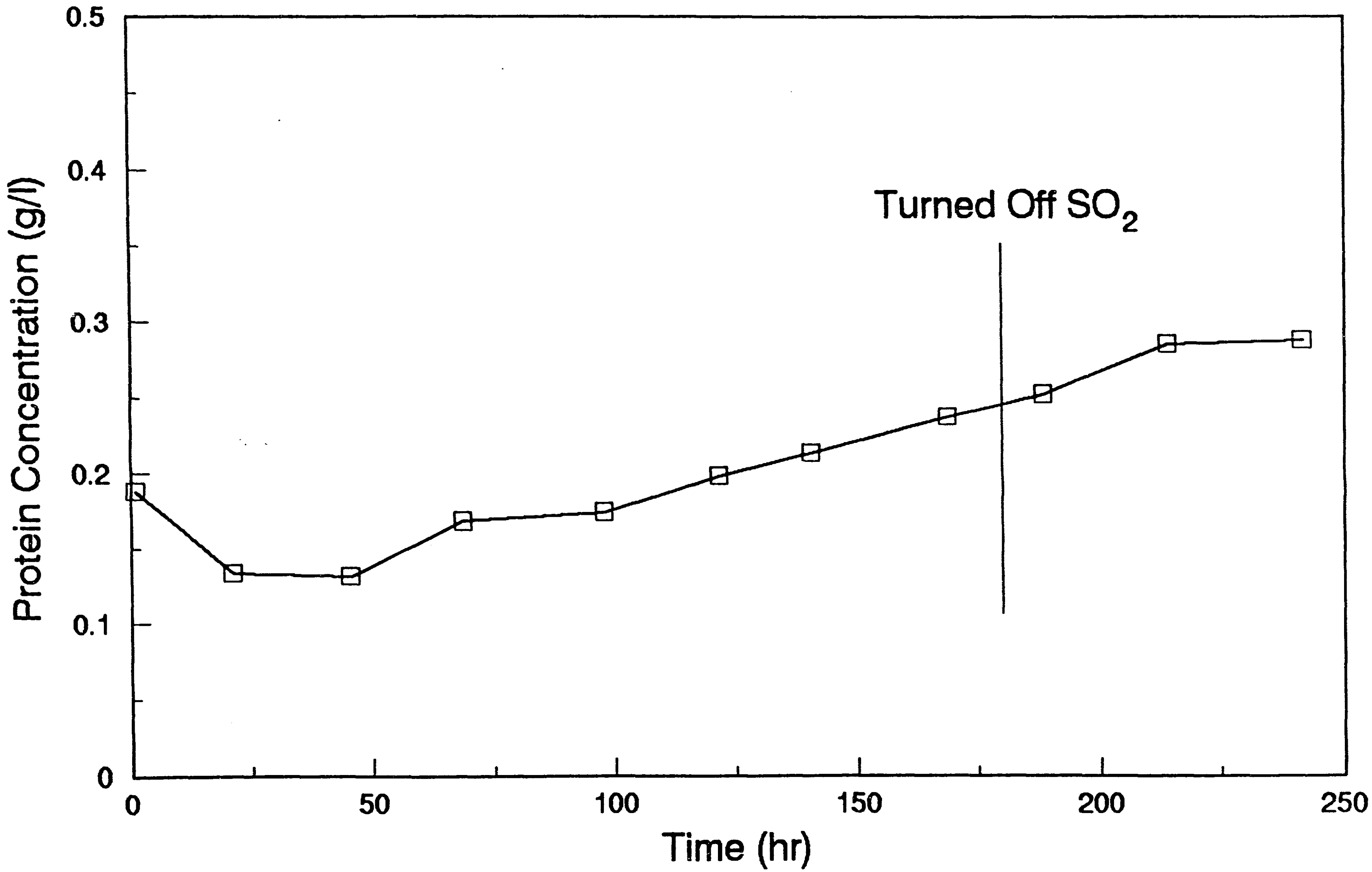


Figure 67. Biomass protein concentration in a *D. orientis* culture with and without SO_2 feed (E1).

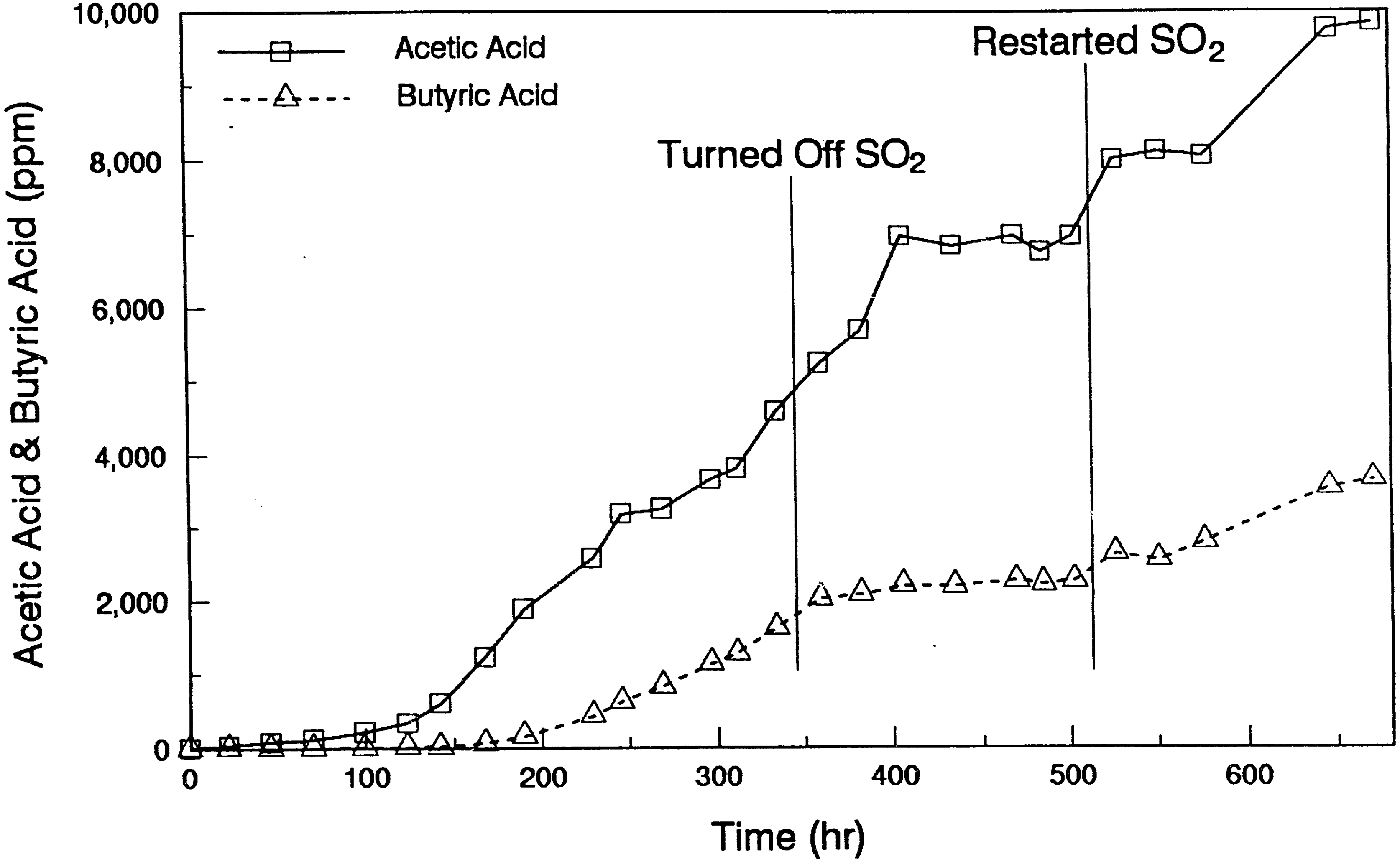


Figure 68. Accumulation of acetic acid and butyric acid in a culture of *D. orientis* with and without SO_2 feed (E2).

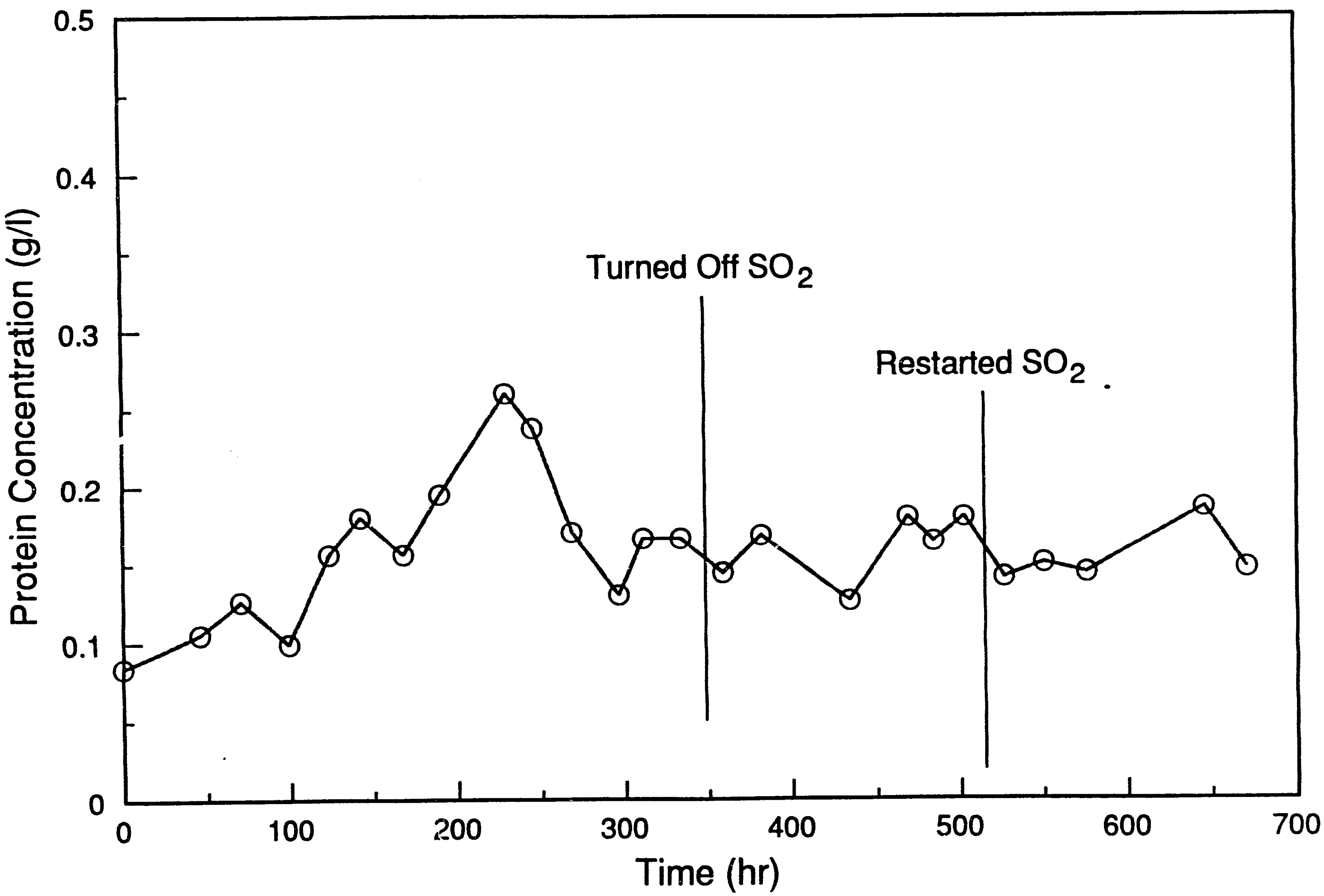


Figure 69. Biomass protein concentration in a *D. orientis* culture with and without SO_2 feed (E2).

concentration in the culture during the course of the experiment. At present, we have no explanation for the decrease in biomass protein prior to the cessation of SO_2 feed. However, as shown in Figure 69, the biomass protein concentration was relatively constant from the time the SO_2 feed was stopped until the end of the experiment. As shown in Figure 70, the ammonium ion (source of reduced nitrogen) concentration was seen to decline while SO_2 was available to the culture but essentially leveled off when the SO_2 feed was stopped.

It was our intention when initiating the further experiments described below to create mixed cultures of *D. orientis* and SRB capable of utilizing carboxylic acids as carbon and energy sources. The goal was to prevent the accumulation of carboxylic acids in the culture medium and improve the utilization of electron donors for SO_2 reduction. However, as detailed below the carboxylic acids never again appeared in any appreciable concentration.

Working cultures (1.5 L) of *D. orientis* were prepared by growing the organism septically on lactate as described above followed by transfer to an autotrophic medium (Table 31). After resuspension the fermenter received gas feeds of 140 mL/min of 5% CO_2, balance N_2; 70 mL/min H_2; and 6.8 mL/min 1.0% SO_2, 5% CO_2, balance N_2. The molar feed rate of SO_2 was 0.166 mmoles/hr.

The culture was maintained on lactate for eight days. At the end of this time the outlet gas typically contained over 1000 ppmv H_2S (150 mL 5% CO_2 in N_2 as inlet). Following transfer to the autotrophic medium the culture was operated on an SO_2 feed for an additional 15 days. Complete reduction of SO_2 to H_2S was observed. However, no carboxylic acids were observed.

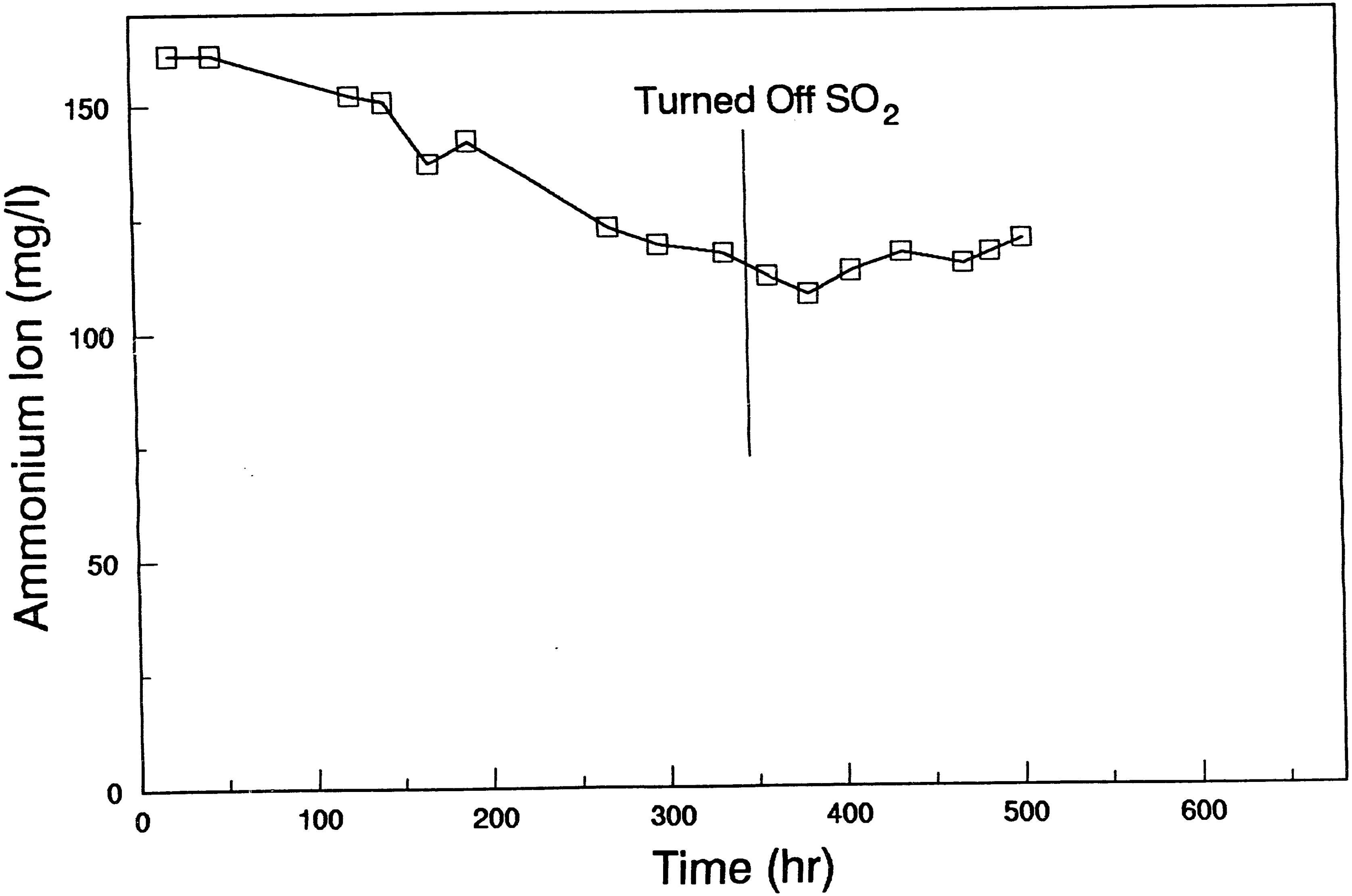


Figure 70. Ammonium ion concentration in a *D. orientis* culture with and without SO_2 feed (E2).

After 15 days on an SO_2 feed (Phase I of growth on SO_2), the culture biomass was harvested by centrifugation and resuspended in fresh autotrophic medium with lactate as the electron donor and sulfate as the electron acceptor. The culture was maintained with daily additions of lactate (as previously described) for seven days. at the end of this time the culture biomass was harvested and again transferred to the autotrophic medium described in Table 31 (without sulfate). Gas feeds of $H_2/CO_2/SO_2$ were initiated as described above (Phase II of growth on SO_2). The culture was maintained under these conditions for another 19 days. The culture operated normally with respect to SO_2 reduction to H_2S. However, no carboxylic acids were found until the 17th day. For the last three days of operation carboxylic acids were found at the following concentrations: acetic acid, 15-30 mg/L; propionic acid, 12-16 mg/L; isobutyric acid, 9 mg/L; and butyric acid, 17-18 mg/L. These are the same carboxylic acids observed in previous experiments but the concentrations observed in this experiment are orders of magnitude lower.

Figure 71 shows the biomass protein in the culture medium during growth on SO_2 as a terminal electron acceptor. Very little accumulation of biomass was observed. However, these observations must be viewed with some caution since *D. orientis* biomass has a tendency to adhere to the walls of the fermenter.

In a similar experiment the culture was developed in lactate/sulfate for nine days and then transferred to fresh autotrophic medium without sulfate and established on $H_2/CO_2/SO_2$ as described above. Complete reduction of SO_2 to H_2S was again observed. However, after 12 days of operation on a feed of $H_2/CO_2/SO_2$

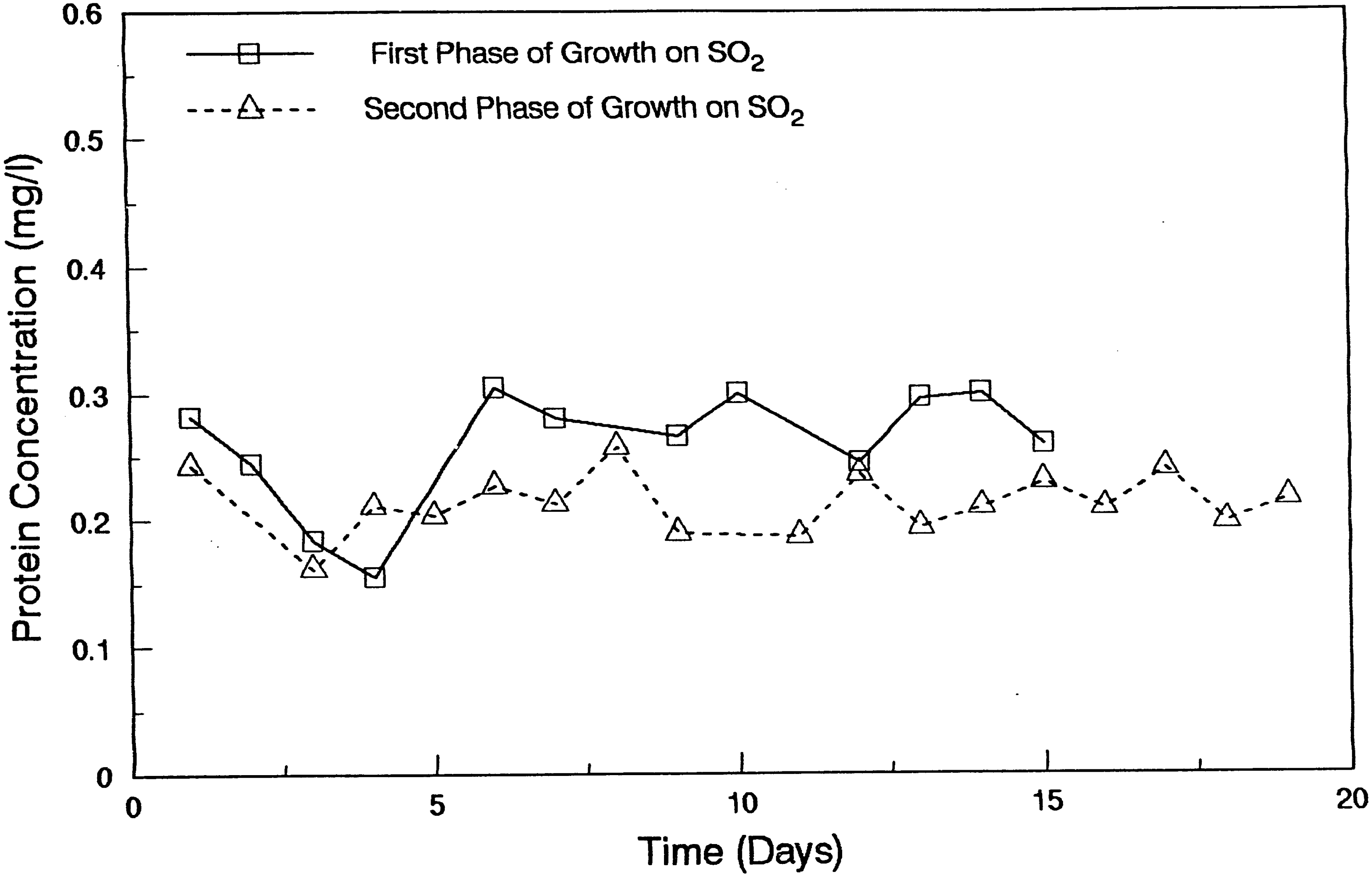


Figure 71. Protein concentration in the first of two SO_2-reducing cultures of *D. orientis* in which no carboxylic acids formation was observed.

no carboxylic acids have been observed. Figure 72 shows the biomass protein concentration in this culture. A slow increase in the biomass protein was observed.

At this point in time these observations are without explanation. These experiments described early in this section clearly indicate that when carboxylic acids are found in significant quantities their accumulation is linked to SO_2 reduction. One possible explanation is that those cultures were contaminated with an acetogen which produced carboxylic acids from CO_2. However, the linkage to SO_2 reduction is hard to explain. In addition, it is hard to accept that two cultures started from different stocks of the inoculum (*D. orientis*) both became contaminated with an acetogen but two subsequent cultures operated in the same lab in the same manner did not.

Two additional experiments were conducted to verify the sulfur balance in the absence of carboxylic acid formation and to estimate the specific activity of the organism for SO_2 reduction.

In the first experiment *D. orientis* was first grown on lactate and sulfate as described in Section 5.2 before resuspension in fresh medium (Table 31) and initiation of H_2 and SO_2 gas feeds. After resuspension gas feeds consisted of 140 mL/min of 5% CO_2, balance nitrogen; 8.1 mL/min 1.0% SO_2, 5% CO_2, balance N_2; and 74.1 mL/min H_2. The culture was maintained on $SO_2/CO_2/H_2$ feed for eleven days following initiation of SO_2 feed. No carboxylic acids were seen to acccumulate during this time beyond trace quantities (<10 mg/L). Table 33 gives the results of a series of sulfur balances performed during this time. Hydrogen sulfide was determined by precipitating H_2S from the outlet gas as ZnS followed by analysis by the methylene blue method.

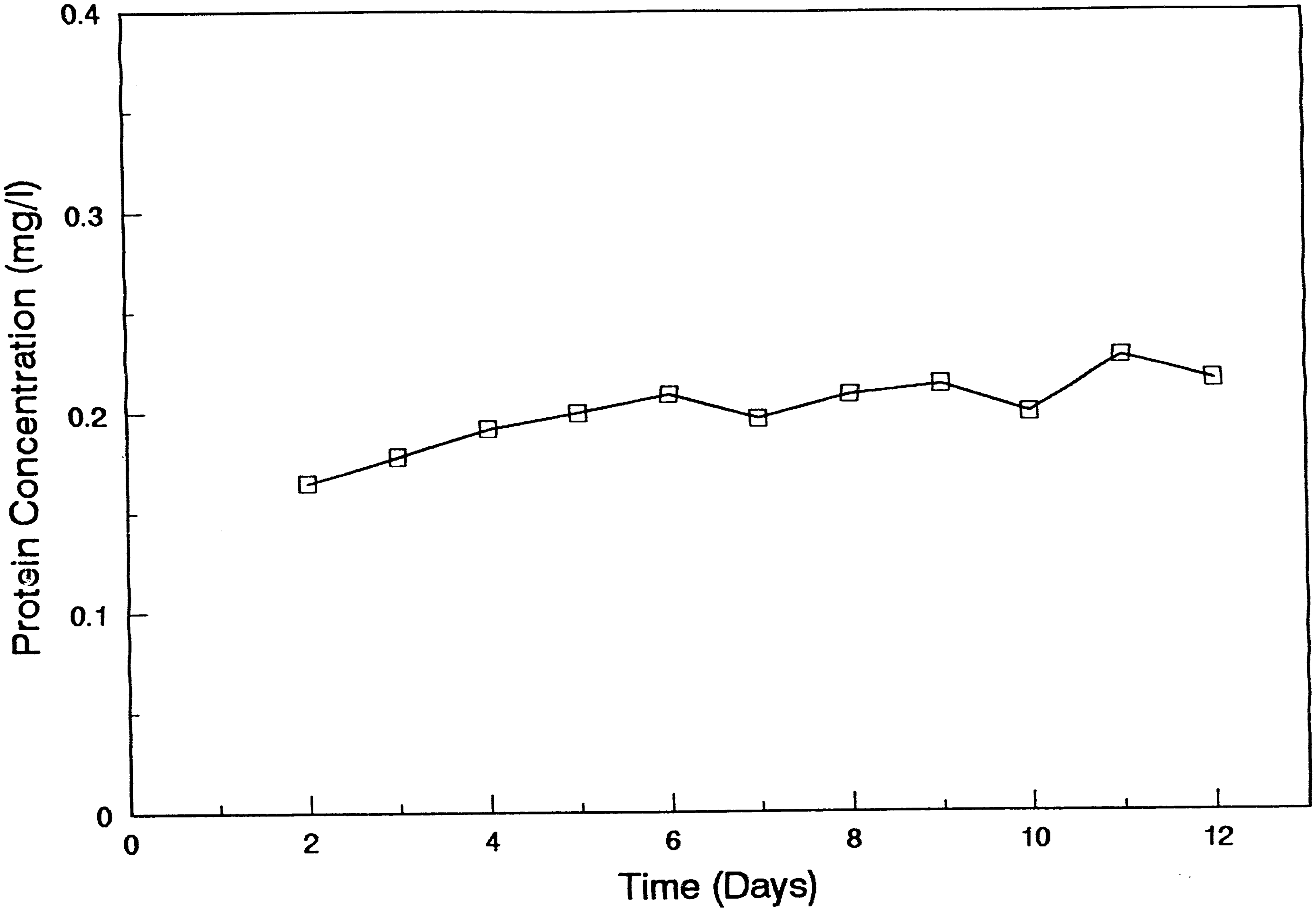


Figure 72. Protein concentration in the second of two SO_2-reducing cultures of *D. orientis* in which no carboxylic acids formation was observed.

Table 33. Sulfur Balances in a Batch Culture of *D. orientis* Receiving an SO_2 Feed

Sample #	SO_2 Feed Rate (mmoles/hr)	H_2S Production Rate (mmoles/hr)	H_2S/SO_2
1	0.200	0.194	0.97
2		0.209	1.05
3		0.192	0.96
4		0.179	0.90
5		0.189	0.95
6		0.186	0.93
7		0.181	0.91
8		0.182	0.91
9		0.188	0.94
10		0.174	0.87
		Avg.	0.94

Esentially 100% reduction of SO_2 to H_2S was observed. The liquid phase sulfite concentration was always less than 5 mg/L.

A second similar experiment produced the results shown in Table 34. Again essentially no carboxylic acids were observed. Following the establishment of the sulfur balance the SO_2 feed rate was increased stepwise until runaway sulfite production was observed indicating that the specific activity of the biomass for SO_2 reduction had been exceeded. The upset could not be reversed by increasing the H_2 feed rate. Only a reduction in the SO_2 feed rate could result in a decrease in the liquid phase sulfite concentration. The maximum SO_2 feed rate achieved was about 39 mL/min (1.0% SO_2) or 0.96 mmoles SO_2/hr. The total biomass protein in the reactor at that time was 148 mg. Therefore, the specific activity was 6.5 mmoles SO_2/hr - g of total biomass protein. Since this was a septic culture all of the protein cannot be attributed to *D. orientis*.

The stoichiometry of microbial reduction of SO_2 with H_2 as electron donor was again investigated to determine the H_2/SO_2 ratio. As noted above, the purely chemical reduction of SO_2 by H_2 would be described by the equation below:

$$3H_2 + SO_2 \rightarrow H_2S + 2H_2O$$

In *D. orientis* cultures operating on a feed of $H_2/CO_2/SO_2$, a H_2/SO_2 ratio of slightly higher than 3.0 would be expected since some of H_2 oxidation would be required to reduce CO_2 for production of biomass.

In experiments reported previously in this section, the H_2/SO_2 molar ratio in the feed gas was on the order of 700-900 since pure H_2 was used as the H_2 source. Therefore, if was difficult to determine

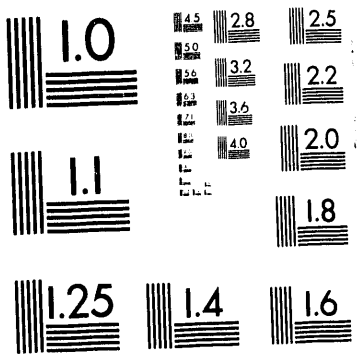

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

Table 34. Sulfur Balances in a Batch Culture of *D. orientis* Receiving an SO_2 Feed

Sample #	SO_2 Feed Rate (mmoles/hr)	H_2S Production Rate (mmoles/hr)	H_2S/SO_2
1	0.206	0.202	0.98
2	0.206	0.211	1.02
3	0.191	0.176	0.92
4		0.184	0.96
5		0.187	0.98
6		0.185	0.97
7		0.211	1.11
8		0.177	0.93
9		0.184	0.96
10		0.180	0.94
11		0.173	0.91
		Avg.	0.97

the stoichiometry of the bioprocess with respect to H_2 utilization by the analyses of the feed and effluent gas streams with any accuracy.

A gas mixture containing 10.0% H_2, 5% CO_2, balance N_2 (v:v:v) was obtained to use as a source of H_2. With a lower concentration of H_2 in the feed gas mixture, changes in the H_2 concentration resulting from contact with the process culture could be more accurately determined.

An SO_2-reducing culture of *D. orientis* received the gas feeds indicated in Table 35. The H_2 consumed in the process culture was determined by GC analysis (Table 36) of the total feed gas and effluent gas streams. Hydrogen and SO_2 consumed during two duplicate experiments are also given in Table 35, along with the H_2/SO_2 ratio observed. The average ratio was 3.28, very close to that predicted for the purely reaction. The extra H_2 oxidation produced reducing equivalents for reduction of CO_2 for biomass production.

5.2.2 Gas Recycle in D. orientis SO_2-Reducing Culture

Gas recycle has been employed in a *D. orientis* culture operating with a feed of $H_2/CO_2/SO_2$ to improve the percent conversion of H_2 in the feed gas. Prior to the initiation of gas recycle, the gas feeds to the reactor were as follows: 1) 29.3 mL/min 10% H_2, 5% CO_2, balance N_2; 2) 73.1 mL/min 5% CO_2 in N_2; and 3) 9.3 mL/min 1.0% SO_2, 5% CO_2, balance N_2 (0.228 mmoles SO_2/hr). Hydrogen consumed in the culture was determined by GC analysis (Table 36). The H_2/SO_2 ratio was 3.28 (Table 35).

After 14 days of operation on a once through H_2 feed, recycle of the exhaust gas was initiated. The feed gases to the reactor were intially changed to 1) 11.6 mL/min of 10% H_2, 5% CO_2, balance N_2, 2)

Table 35. Stoichiometry of H_2 Consumption in an SO_2-Reducing Culture of *D. orientis*

	Gas Flowrates (mL/min)					
Trial #	10% H_2, 5% CO_2 bal N_2	5% CO_2 in N_2	1.0% SO_2, 5% CO_2 bal N_2	H_2 Consumed mmole/hr	SO_2 Consumed (mmole/hr)	H_2/SO_2
1	29.3	73.1	9.36	0.770	0.230	3.35
2	29.3	73.1	9.14	0.719	0.224	3.21

Table 36. Gas Chromatographic Conditions for Analysis of H_2 in Feed and Effluent Gas Streams in an SO_2-Reducing Culture of *D. orientis*

Instrument:	Hewlett Packard - HP 5980
Detector:	Thermal Conductivity
Carrier:	Nitrogen at 30 mL/min
Temperatures:	

Column - 50 C for 2 min, 24 C/min to 120 C final

Injector - 110 C

Detector - 140 C

64.7 mL/min N_2 and 3) 9.3 mL/min 1.0% SO_2, 5% CO_2, balance N_2. Subsequent changes in gas feeds are given in Table 37. Exhaust gas was withdrawn from the gas outlet by peristaltic pump at 44.3 mL/min and sent first to a vapor/liquid separator then to a caustic scrubber to remove H_2S formed from SO_2 reduction. Following H_2S scrubbing, the gas flowed back to the reactor and was bubbled into the culture through a second sparger. The reactor operated under recycle conditions for 20 days.

At each feed condition described in Table 37, the H_2 utilization rate was determined and the H_2/SO_2 ratios calculated. As seen in Table 37, the H_2/SO_2 observed during recycle experiments were lower that predicted from purely chemical reduction of SO_2 by H_2 and lower that those observed in a *D. orientis* SO_2-reducing culture without gas recycle (Section 5.2.1). As indicated in Table 37, carboxylic acids, especially acetic acid, was observed in the culture medium during gas recycle. It is not know whether there is a relationship between these carboxylic acids and the low H_2/CO_2 ratios.

5.2.3 <u>Sulfate Production from SO_2 by *D. orientis*</u>

Using 10% H_2, 5% CO_2, balance N_2 as a source of H_2 with gas recycle resulted in H_2/SO_2 ratios of 0.9-1.5 (Table 37) as opposed to the expected ratio of approximately 3.0. These observations were further investigated. A new batch culture of *D. orientis* was developed on lactate and switched to a $H_2/CO_2/SO_2$ gas feed as. The initial gas feed to this culture consisted of 30.5 mL/min 10% H_2, 5% CO_2, balance N_2; 9.3 mL/min 1% SO_2, 5% CO_2, balance N_2; and 80.5 mL/min N_2. After 10 days, the feed gas was changed to 15.9 mL/min 10% H_2, 5% CO_2, balance N_2; 11.8 mL/min 1% SO_2, 5% CO_2, balance N_2 and 80.5

Table 37. Stoichiometry of H_2 Consumption in an SO_2-Reducing Culture of *D. orientis* with Gas Recycle

Day	Gas Feed Rates (mL/min) 10% H_2	N_2	1.0% SO_2	H_2 Used mmole/hr	SO_2 Feed (mmole/hr)	H_2/SO_2	Acetic Acid mg/L
0	11.6	64.7	9.3	0.318	0.228	1.40	
7	6.1	44.8	11.8	0.321	0.290	1.11	50
8	6.1	44.8	11.8	0.333	0.290	1.15	134
9	6.1	44.8	9.3	0.260	0.228	1.14	142
10	11.6	64.7	9.3	0.350	0.228	1.54	104
13	11.6	64.7	9.3	0.347	0.228	1.52	75
15	11.6	64.7	9.3	0.324	0.228	1.42	92
16	11.6	64.7	9.3	0.321	0.228	1.41	
17	11.6	64.7	9.3	0.350	0.228	1.53	
20	11.6	64.7	9.3	0.266	0.228	1.17	
21	11.6	64.7	9.3	0.209	0.228	0.92	

mL/min N_2. This feed condition was maintained for an additional 21 days. As shown in Table 38, the H_2/SO_2 ratio observed with both gas feeds was 0.79-2.31. However, the H_2/SO_2 ratio was generally higher at the higher overall percent H_2 in the feed gas although significantly lower than the H_2/SO_2 ratio of 3.28 observed in a previous experiment with virtually the same gas feed.

On the 13th day of operation, this culture (over 2000 mg/L) was found in the culture medium. This sulfate had accumulated over the previous 12 days of operation in a $H_2/CO_2/SO_2$ feed and continued to accumulate while this feed was maintained. (Figure 73)

Analysis of the outlet gas for H_2S showed less than stoichiometric production of H_2S based on the SO_2 feed rate (Table 38). However, during this time, the $(SO_4^{-2} + H_2S)/SO_2$ ratio average 1.02. Therefore, SO_2 was both reduced to H_2S and oxidized to sulfate. This explains the depressed H_2/SO_2 ratios; less than stoichiometric utilization of H_2 was observed because only a fraction of the SO_2 was reduced to H_2S. The remainder was oxidized to sulfate.

On day 32 of this experiment, the H_2 feed was eliminated. The reactor continued to receive a gas feed of 11.8 mL/min 1% SO_2, 5% CO_2, balance N_2 and 80.5 mL/min N_2. During this time, H_2S continued to be produced but at a slower rate (Table 38). The rate of sulfate accumulation increased and the $(SO_4^{-2} + H_2S)/SO_2$ ratio average 0.92. A feed of 15.9 mL/min 10% H_2,5% CO_2, balance N_2 was reinitiated on the 38th day. As seen in Table 38, the rate of sulfate accumulation decreased and H_2S production increased in response to the renewed aavailability of H_2.

Table 38. Stoichiometry of the Oxidation/Reduction of SO_2 in a D. *orientis* Culture with a $H_2/CO_2/SO_2$ Feed (EI).

Day	H_2 consumed (mmole/h)	SO_2 in (mmole/h)	SO_4^{-2} (mmole/h)	H_2S (mmole/h)	H_2/SO_2	H_2/H_2S
4	0.424	0.228			1.86	
5	0.527	0.228			2.31	
11	0.406	0.290			1.40	
12	0.382	0.290			1.32	
18	0.344	0.290	0.183		1.19	
19	0.230	0.290	0.150		0.79	
21		0.290	0.174	0.136		
25		0.290	0.132	0.129		
26	0.310	0.290	0.167	0.148	1.07	2.09
32	0	0.290	0.195	0.060		
33	0	0.290	0.235	0.051		
37	0	0.290	0.202	0.052		
39	0.355	0.290	0.151	0.109	1.22	3.25

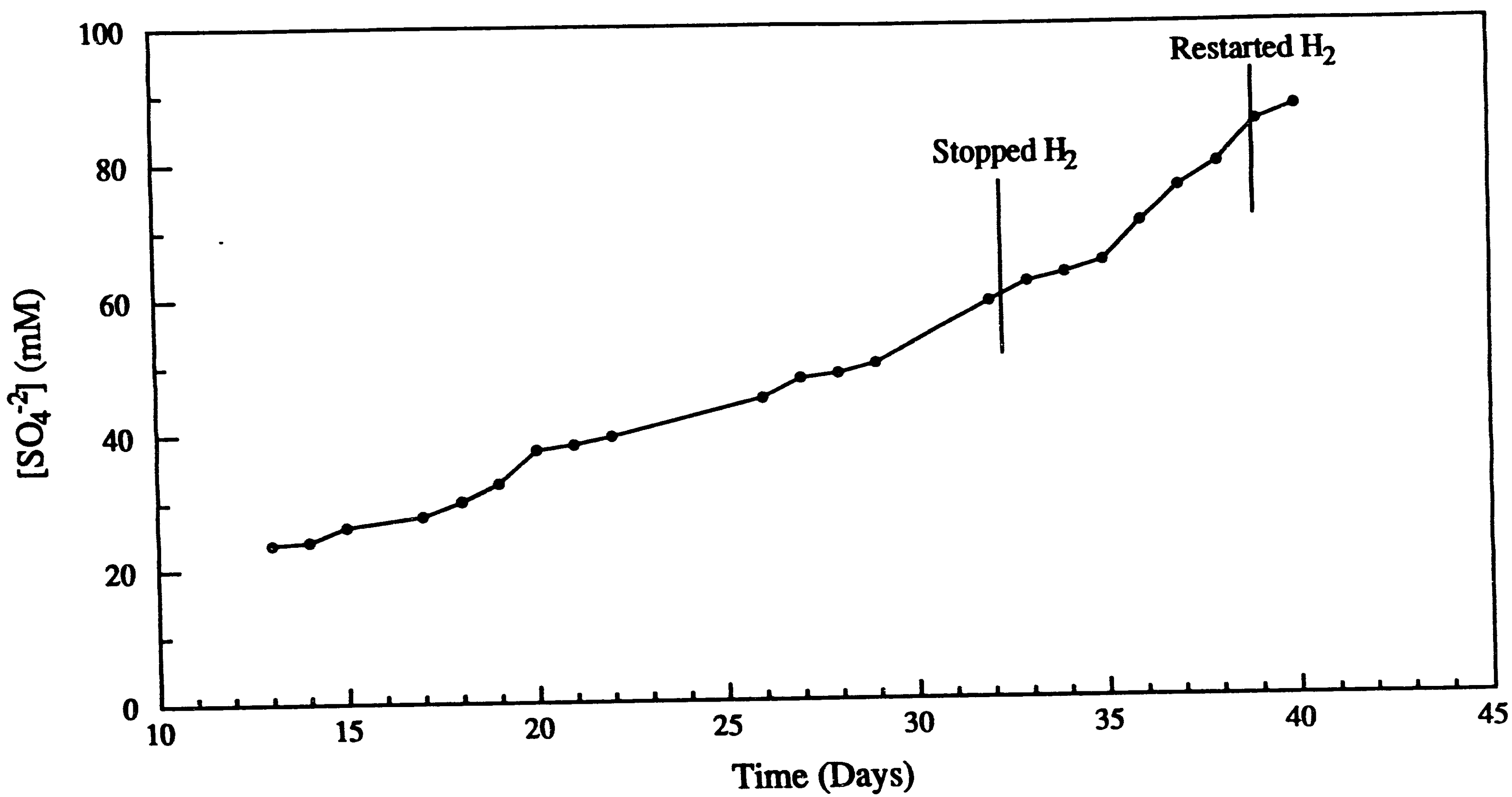


Figure 73. Sulfate concentration in a *D. orientis* culture receiving a $CO_2/H_2/SO_2$ feed. Refer to Table 45.

At the conclusion of this experiment, biomass was harvested by centrifugation and resuspended in fresh autotrophic medium. The culture then again received a gas feed of 15.9 mL/min 10% H_2, 5% CO_2, balance N_2; 11.8 mL/min 1% SO_2, 5% CO_2, balance N_2; and 80.5 mL/min N_2. As seen in Figure 74, sulfate was again seen to accumulate although at somewhat lower rates (Table 39) than in the previous experiment. The average $(H_2S + SO_4^{-2})/SO_2$ ratio observed was 0.98; therefore, all SO_2 was again either oxidized to sulfate or reduced to H_2S.

The H_2/H_2S ratio average 3.2. The chemical reduction of SO_2 by H_2 would be given by

$$3H_2 + SO_2 \rightarrow H_2S + 2H_2O$$

Therefore, that fraction of the SO_2 feed reduced to H_2S, was apparently reduced at the expense of H_2 oxidation only in this experiment. This may not have been the case in the previous experiment where the H_2/H_2S ratios were < 3.0. The electron acceptor for SO_2 oxidation to sulfate is unknown. If the electron acceptor is SO_2, three moles of SO_2 would be oxidized for every mole of SO_2 reduced according to the equation below:

$$4H_2O + 4SO_2 \rightarrow 3SO_4^{-2} + H_2S + 6H^+$$

Production of H_2S from this reaction would result in a H_2/H_2S ratio of < 3.0. This hypothesis is supported by the observation that alkali addition was required to maintain the pH at 7.3 when sulfate was observed to accumulate.

On the 21st day of this experiment, the gas feed was changed to the following: 32.4 mL/min H_2; 9.3 mL/min 1% SO_2, 5% CO_2, balance N_2; and 91.0 mL/min 5% CO_2, balance N_2. As shown in Figure 74, the

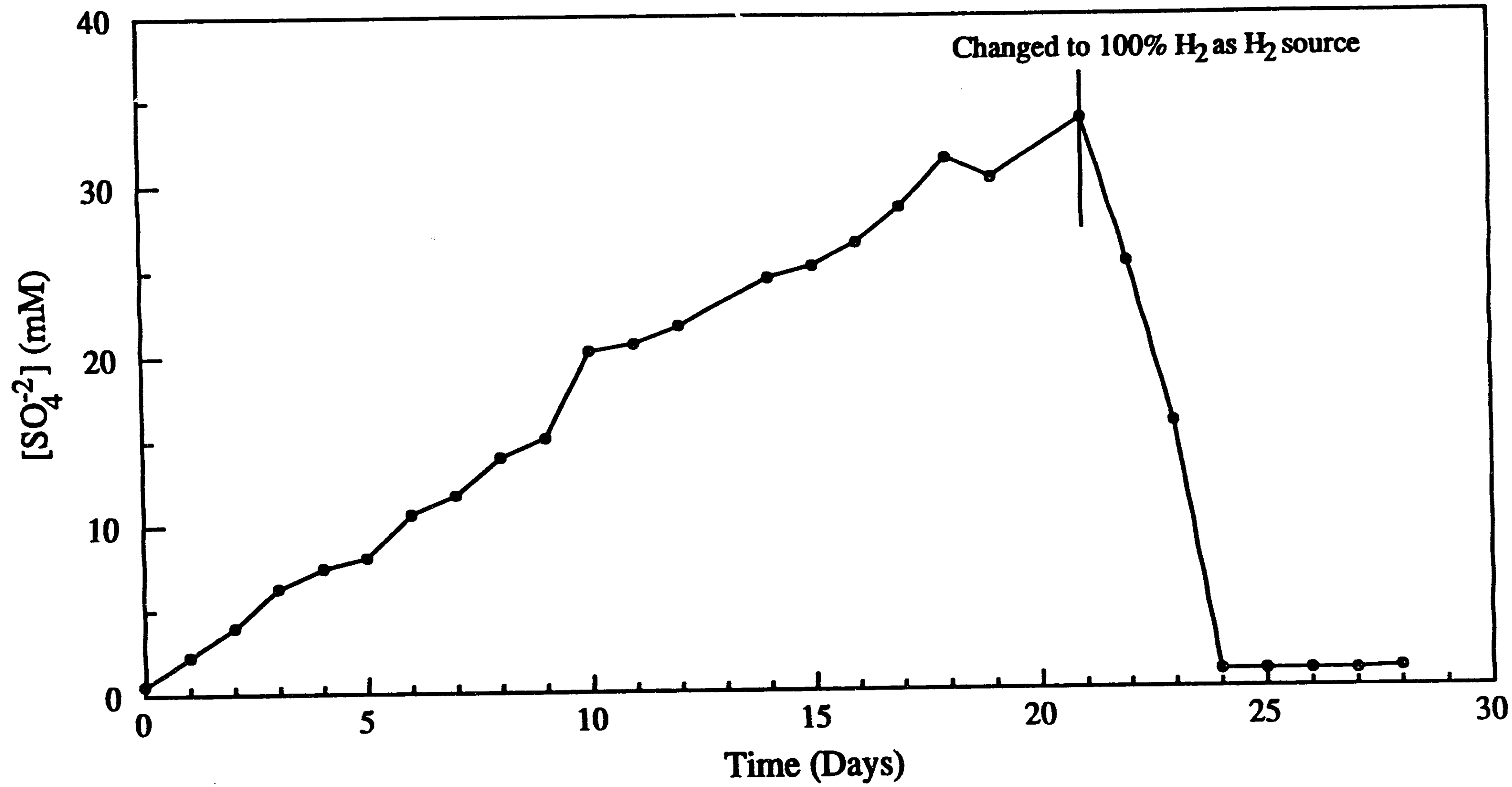


Figure 74. Sulfate concentration in a *D. orientis* culture receiving a $CO_2/H_2/SO_2$ feed. Refer to Table 46.

Table 39. Stoichiometry of the Oxidation/Reduction of SO_2 in a *D. orientis* Culture with a $H_2/CO_2/SO_2$ Feed (EII).

Day	H_2 consumed (mmole/h)	SO_2 in (mmole/h)	SO_4^{-2} (mmole/h)	H_2S (mmole/h)	H_2/SO_2	H_2/H_2S
1		0.228	0.141			
2		0.290	0.104			
3		0.290	0.116			
5		0.290	0.045			
6	0.608	0.290	0.156	0.187	3.25	2.10
7	0.791	0.290	0.070	0.238	3.32	2.73
8	0.538	0.290	0.130	0.179	3.01	1.86
9		0.290	0.069	0.193		
11		0.290	0.030			
12		0.290	0.071	0.216		
14		0.290	0.075	0.189		
15		0.290	0.032	0.206		
16		0.290	0.075	0.906		
17		0.290	0.127			
18		0.290	0.146			
22		0.185	-0.557			
23		0.228	-0.545	0.522		
24		0.228	-0.673			
25		0.228	0			
26		0.228	0			
27		0.290	0			
28		0.290	0	0.287		
33		0.290	0	0.306		
35		0.290	0	0.284		

sulfate concentration in the culture medium immediately began to decline. After three days under this feed condition, the sulfate concentration was less than 1 mM.

At the time of this writing, we are continuing to operate this reactor to further study the oxidation/reduction of SO_2 to sulfate and H_2S. This mode of sulfur metabolism seems to be triggered by H_2 starvation and may indicate that H_2 can be eliminated as an energy source.

5.3 Microbial Reduction of Nitric Oxide by Denitrifying Bacteria

5.3.1 *Paracoccus denitrificans*

A survey of denitrifying bacteria for the capability of using NO as a terminal electron acceptor (with reduction to N_2) was initiated with a study of *Paracoccus denitrificans*.

P. denitrificans (ATCC 13543) was originally grown anaerobically in a succinate minimal medium (Table 40) in Marubishi MD 300 fermenter (culture volume 2L) at pH 7.0 and 30°C. When the optical density (520 nm) reached about 0.6, the biomass was harvested aseptically by centrifugation at 4900g and 25°C and resuspended in the same medium without nitrate and transferred back to the fermenter. At this time, a gas feed of 5000 ppmv NO, 5% CO_2, balance N_2 was initiated at 30 mL/min. The initial agitation rate was 150 rpm. As seen in Figure 75, removal of NO from the feed gas was observed which could be increased by increasing the agitation rate. Ultimately NO was undetectable in the outlet gas by chromophoric Gas Tech analyzer tubes. Accompanying the removal of NO was an increase in optical density of the culture (Figure 76) and a decrease in the ammonium ion

Table 40. Minimal Medium for *Paracoccus denitrificans*

Component	Quantity/L
KNO_3	5.0 g
KH_2PO_4	2.0 g
$NaHCO_3$	1.0 g
NH_4Cl	0.5 g
$MgSO_4$	0.8 g
Succinic acid, disodium salt hexahydrate	10.0 g
Trace element solution (Table 41)	2.0 mL

Table 41. Trace Element Solution for *Paracoccus denitrificans*

Component	g/L
EDTA	50.0
$ZnSO_4$	3.0
$CaCl_2$	6.8
$MgCl_2$	7.9
$FeSO_4$	7.0
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.15
$CuSO_4$	2.1
$CoCl_2$	1.6

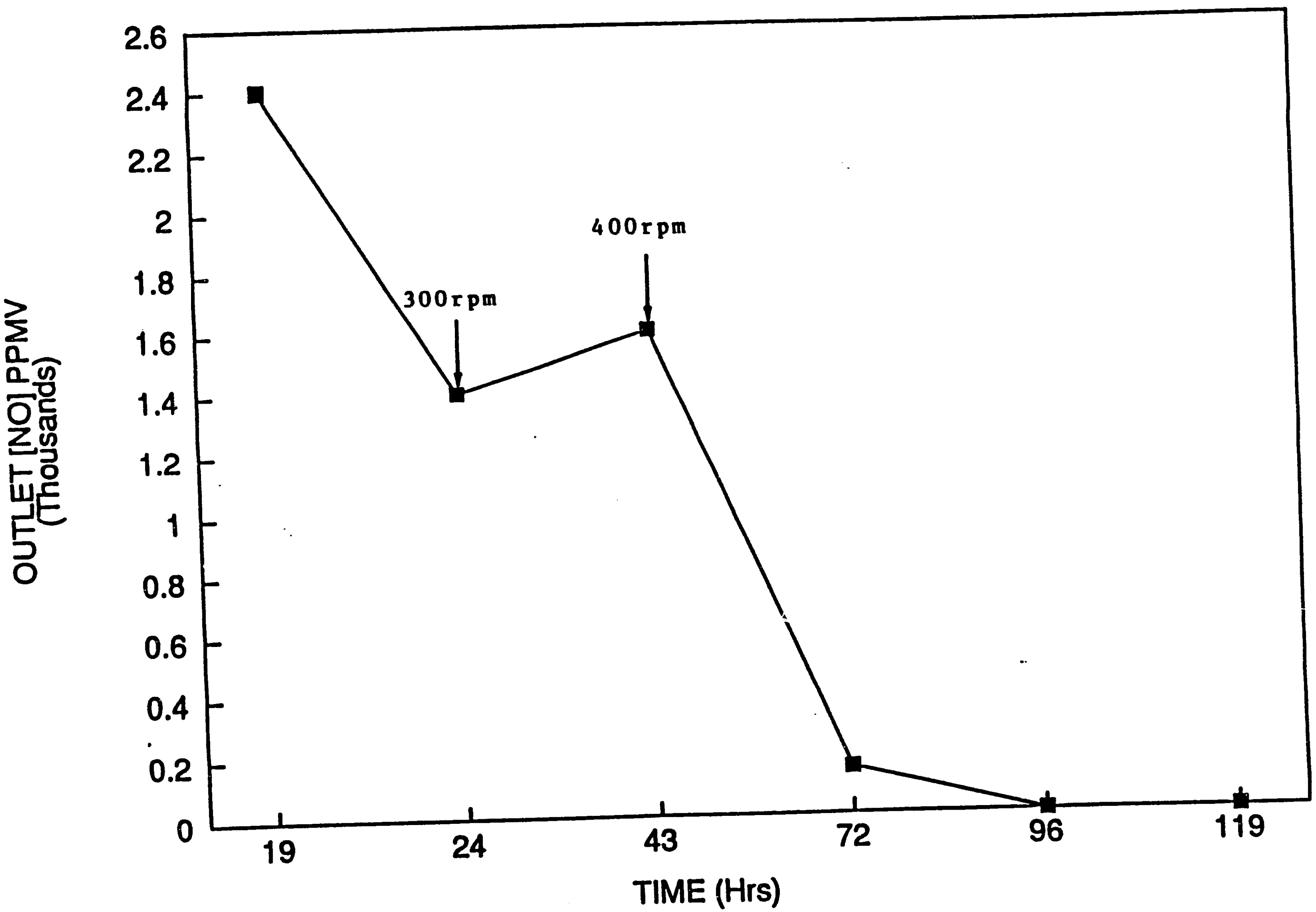


Figure 75. NO concentration in the outlet gas of a culture (2L) of <u>Paracoccus denitrificans</u> receiving a feed of 30 mL/min 5000 ppmv NO.

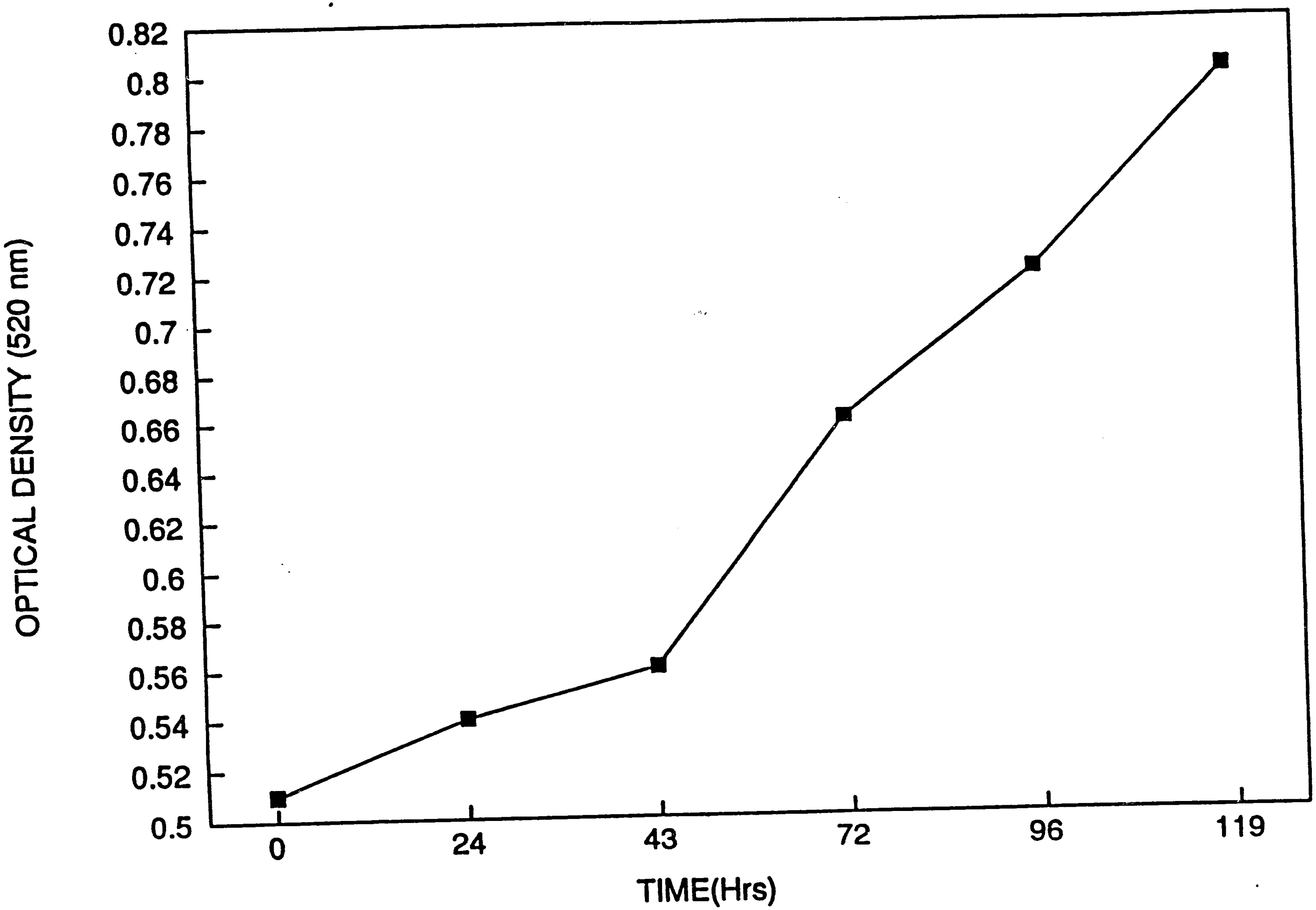


Figure 76. Optical density (520 nm) of a culture (2L) of *Paracoccus denitrificans* receiving a feed of 30 mL/min 5000 ppmv NO.

concentration (Figure 77) which served as a source of reduced nitrogen.

Following these preliminary experiments, a more comprehensive study of the reduction of NO by *Paracoccus denitrificans* has been undertaken as follows. *P. denitrificans* was grown anaerobically in succinate minimal medium (Table 40) in a Marubishi MD300 fermenter (culture volume 2L) at pH 7.0 and 30°C. When the optical density (520 nm) reached about 0.6, the cells were harvested aseptically by centrifugation at 4900 x g and 25° and resuspended in the same medium without nitrate and transferred back to the fermenter. At this time a gas feed of 0.5% NO, 5% CO_2 and balance N_2 was initiated at 30 mL/min. The agitation rate was 450 rpm.

Nitric oxide in the feed gas and outlet gas was determined by two methods. Routine measurements were obtained by using chromophoric analyzer tubes (GasTec, Yokohama, Japan). More accurate analyses were done by gas chromatography as described in Table 42.

Complete removal of NO from the feed gas was observed. As NO was removed from the feed gas, succinate (Figure 78) and ammonium ion (Figure 79) were depleted from the medium. As seen in Figure 80 there was a corresponding increase in the optical density of the culture medium. These data indicate that *P. denitrificans* was growing on succinate as a carbon and energy source and NO as a terminal electron acceptor.

In another experiment, *P. denitrificans* was again grown in succinate minimal medium as described in Section 5.3 with nitrate as a terminal electron acceptor. When the optical density (520 mm) reached about 0.6, the cells were harvested aseptically by centrifugation and

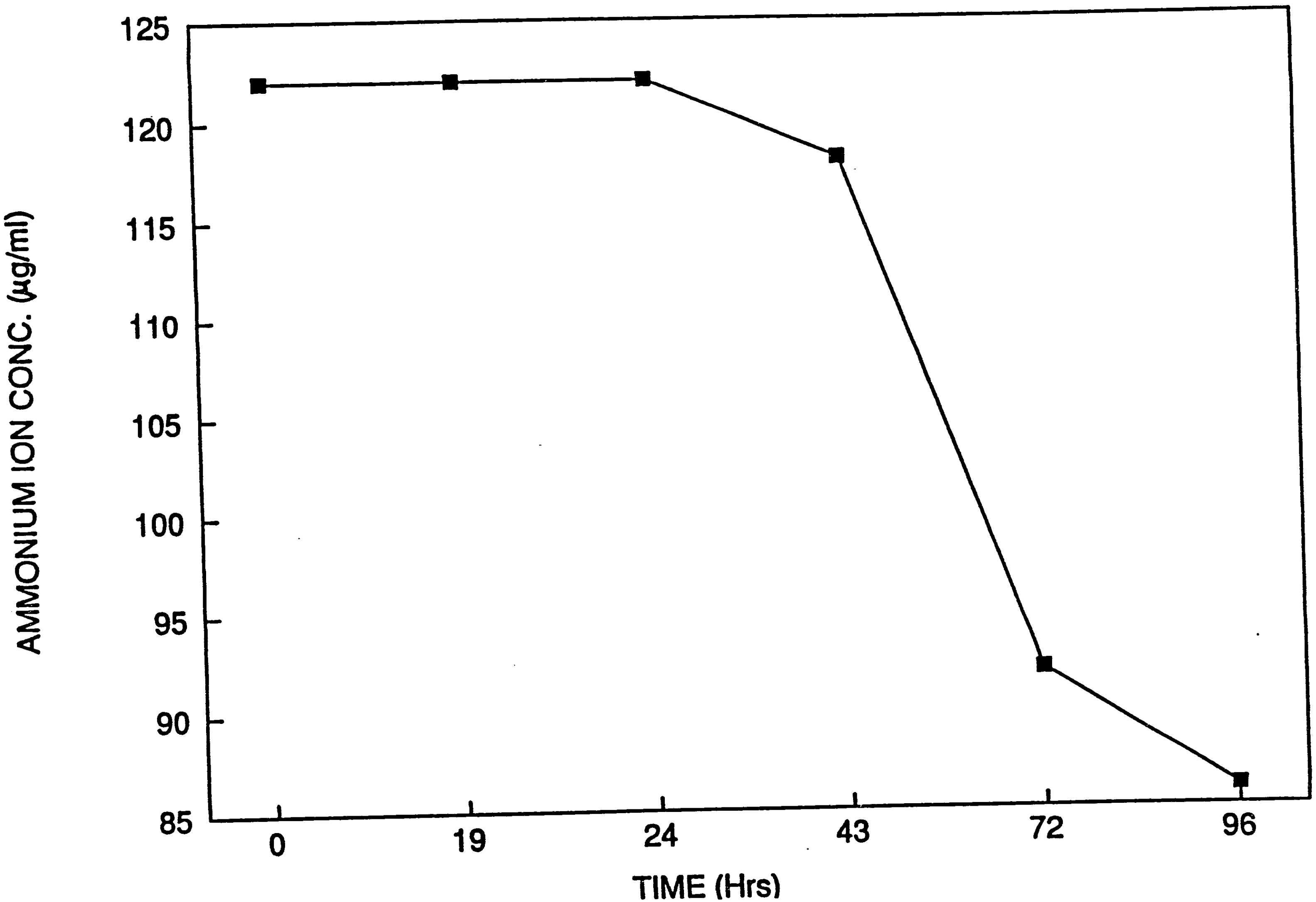


Figure 77. Ammonium ion concentration in a culture (2L) of *Paracoccus denitrificans* receiving a feed of 30 mL/min 5000 ppmv NO.

Table 42. Chromatographic Conditions for Analysis of NO in Reactor Outlet Gas

Instrument:	Hewlett Packard 5890
Column:	30 ft. x 1/8-in. ID stainless steel Haye Sep DB 100/120 mesh
Carrier Gas & Flow Rate:	He , 30 mL/min
Oven Temperature:	25°C
Injector Oven Temperature:	25°C
Detector Oven Temperature:	140°C
Detector:	Thermal Conductivity Detector

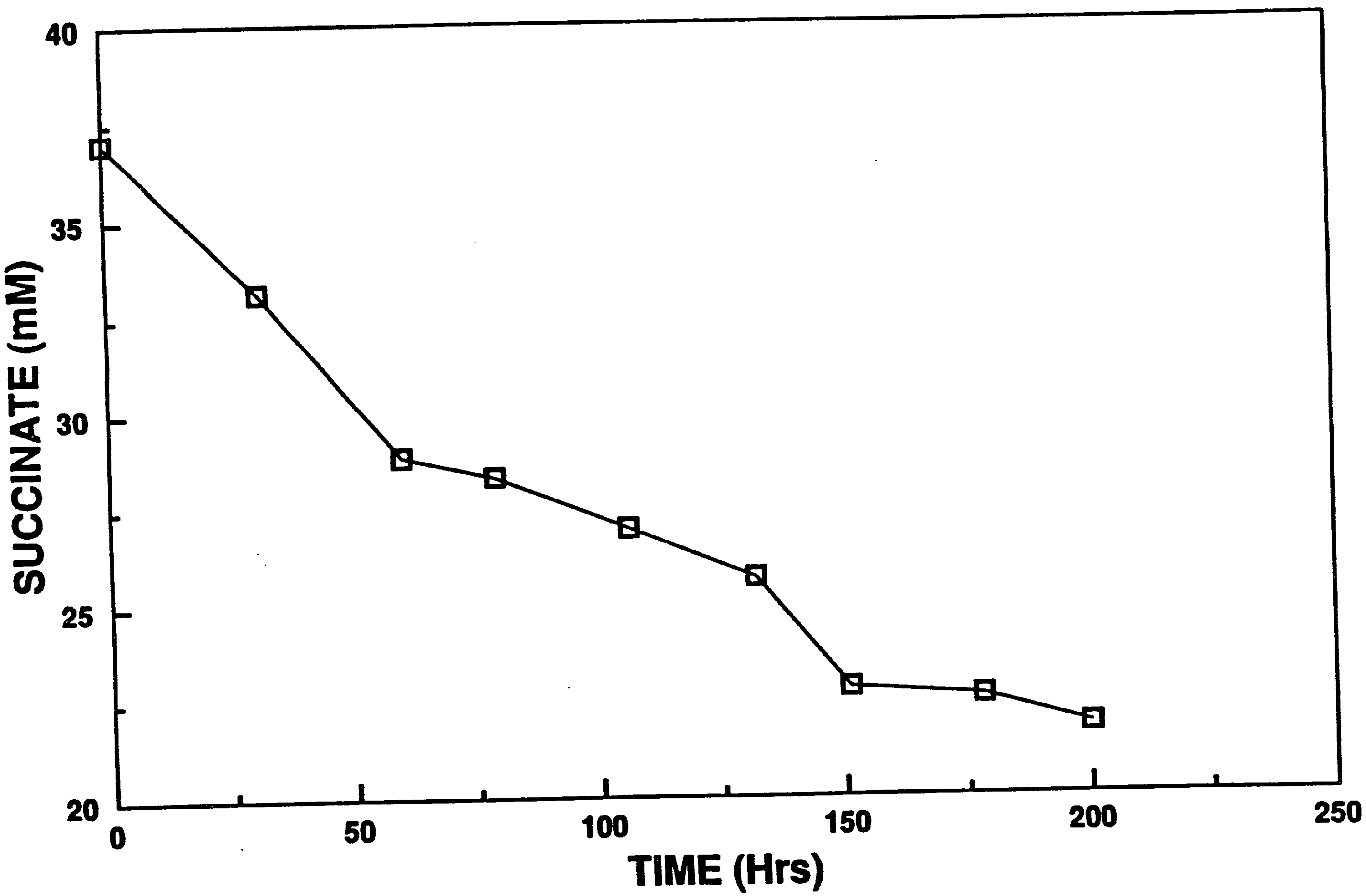


Figure 78. Succinic acid concentration in a *P. denitrificans* batch culture operating with a NO feed.

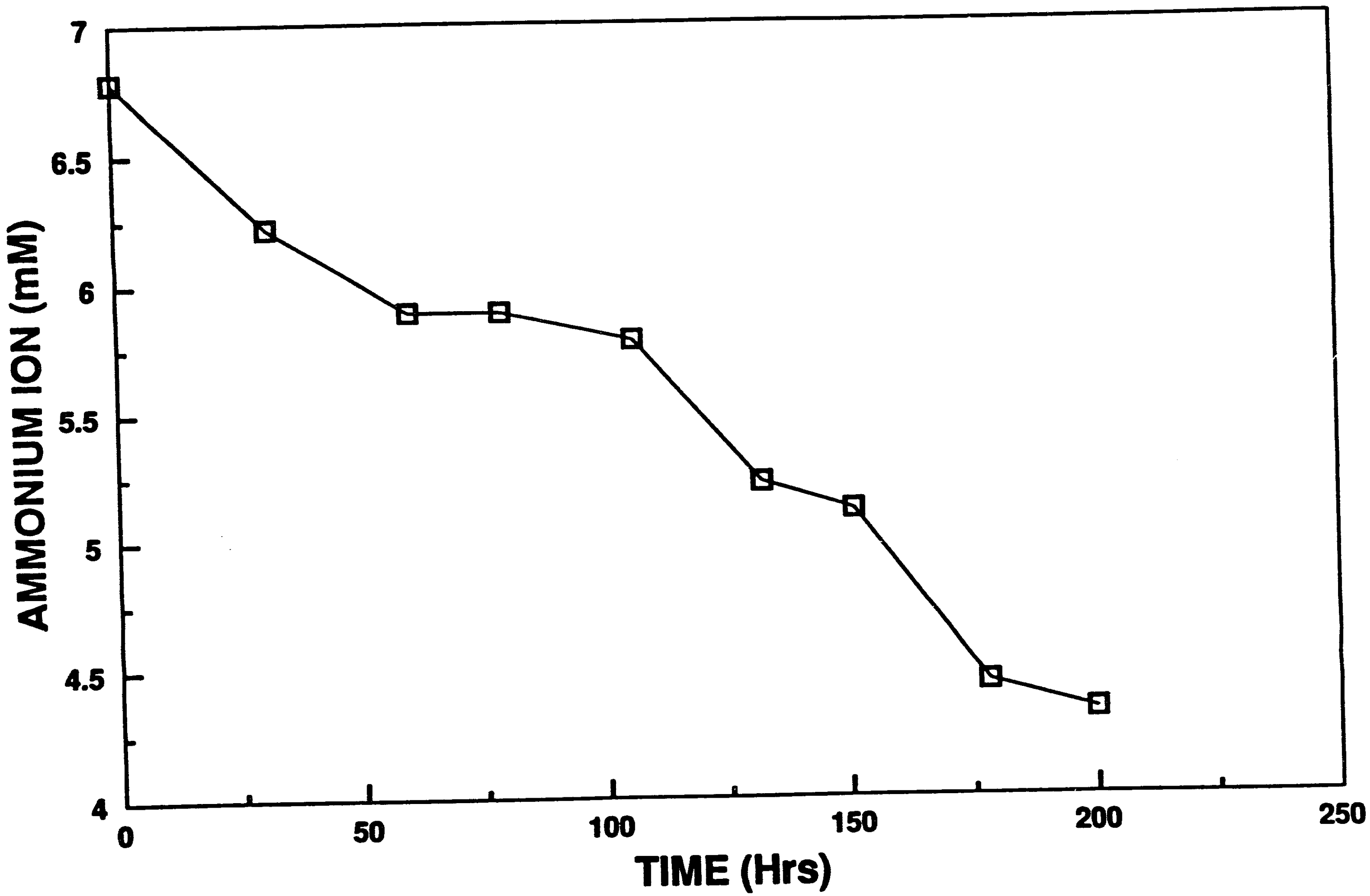


Figure 79. Ammonium ion concentration in a *P. denitrificans* batch culture operating with a NO feed.

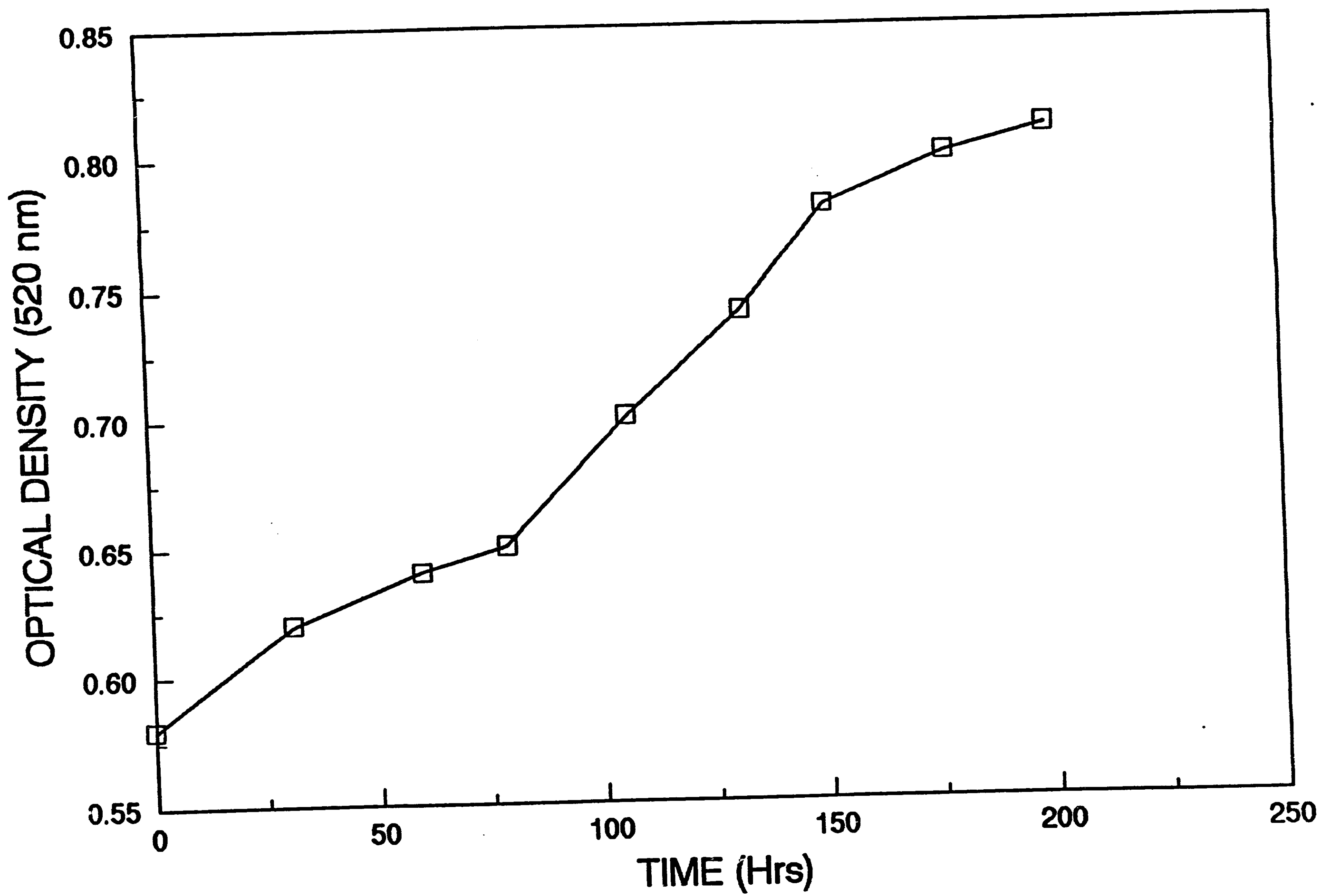


Figure 80. Optical density (520 nm) of a *P. denitrificans* batch culture operating with a NO feed.

resuspended in the same medium without nitrate and transferred back to the fermenter. At this time, a gas feed of 0.5% NO, 5% CO_2 and balance N_2 was initiated at 30 mL/min.

Similar results (Figures 81-84) were obtained compared to those described above. Complete removal of NO from the feed gas was observed. A more careful analysis of the culture medium allowed the stoichiometry to be determined. During the course of the experiment 33 mmoles of NO was reduced accompanied by the utilization of 52.6 mmoles of succinate and 11.2 mmoles of ammonium ion and the production of approximately 253 mg of biomass. *P. denitrificans* cells were difficult to break by sonication for protein determination. Therefore, biomass concentrations have been estimated based on an optical density vs biomass concentration correlation developed for another organism of similar size (*Thiobacillus denitrificans*). This estimation procedure cannot account for the contribution of storage granules (see below).

The purely chemical oxidation of succinate by NO would require 7 moles of NO per mole of succinate oxidized to CO_2. The NO/succinate ratio observed in this experiment was 0.63. In other words, the NO utilized can account for the complete oxidation of only 4.7 mmoles of succinate. The metabolic fate of the remaining succinate is not clear. Some succinate carbon was incorporated into biomass. Additional succinate carbon was likely converted into poly-β-hydroxybutyrate which is a common storage polymer in this organism. Under electron acceptor-limiting conditions the organism would be expected to accumulate storage polymers. Other possible pathways for succinate carbon include fermentation although no smaller molecular

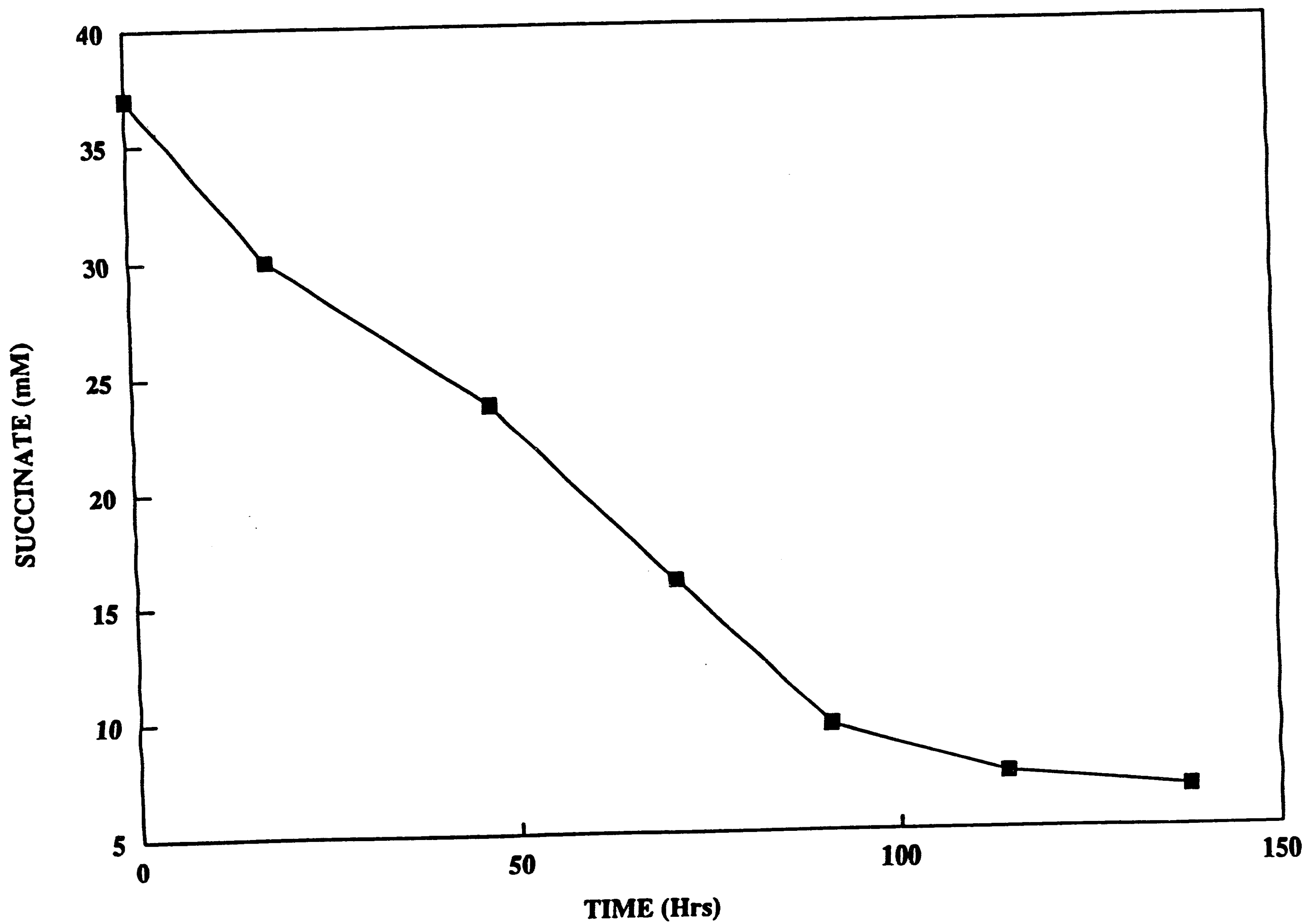


Figure 81. Succinic acid concentration in a *P. denitrificans* batch culture operating with a NO feed.

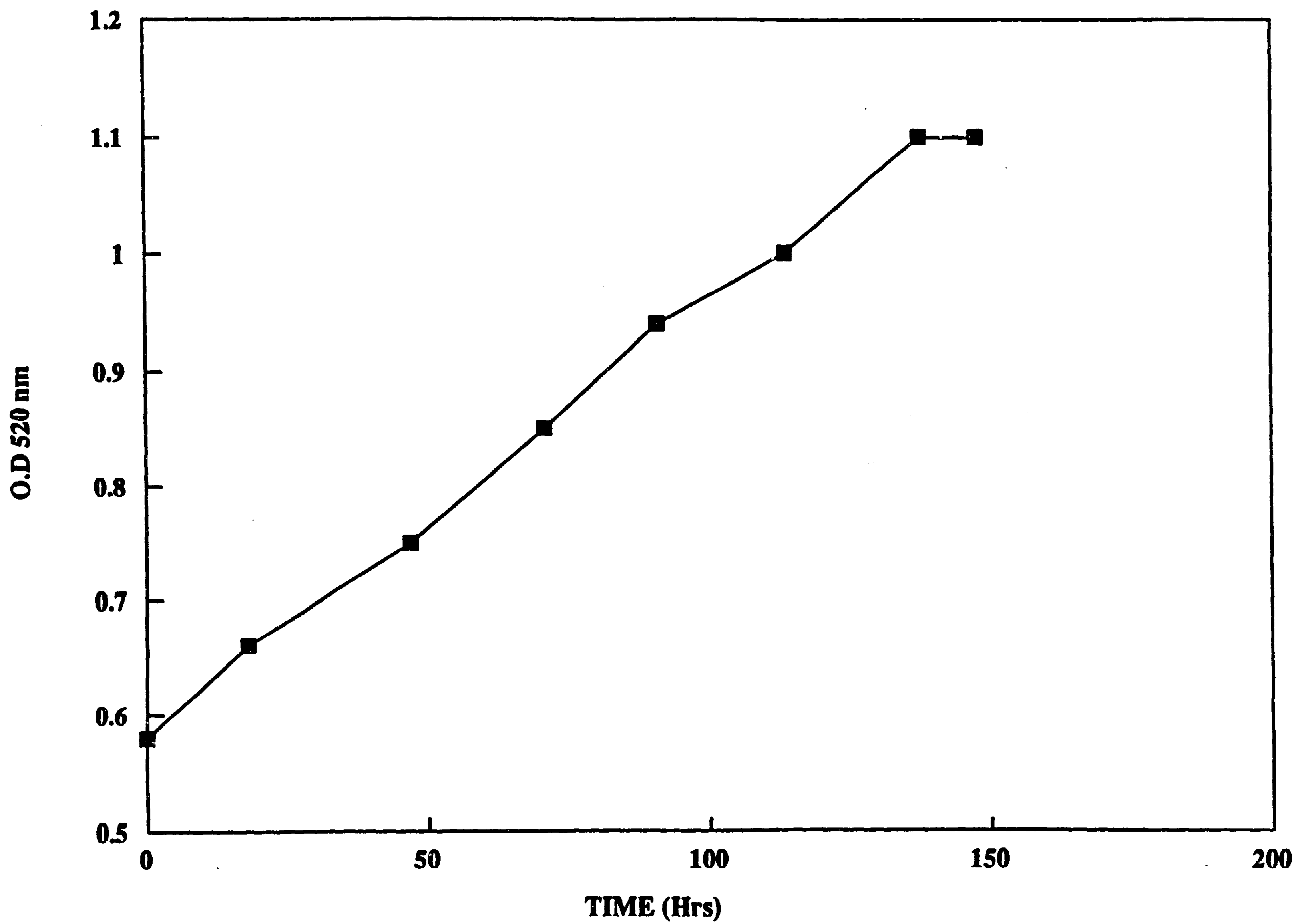


Figure 82. Optical density (520 nm) of a *P. denitrificans* batch culture operating with a NO feed.

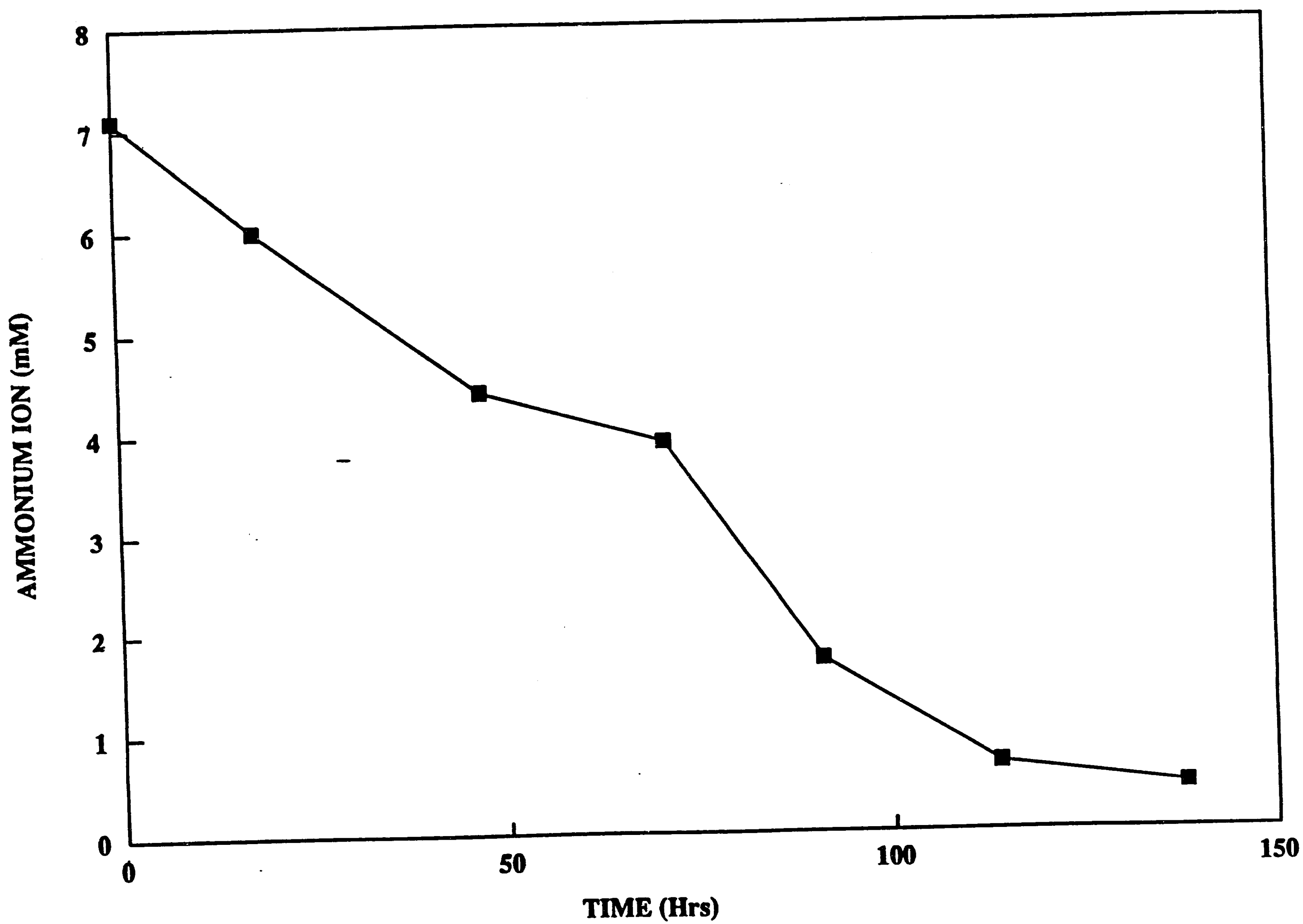


Figure 83. Ammonium ion concentration in a *P. denitrificans* batch culture operating with a NO feed.

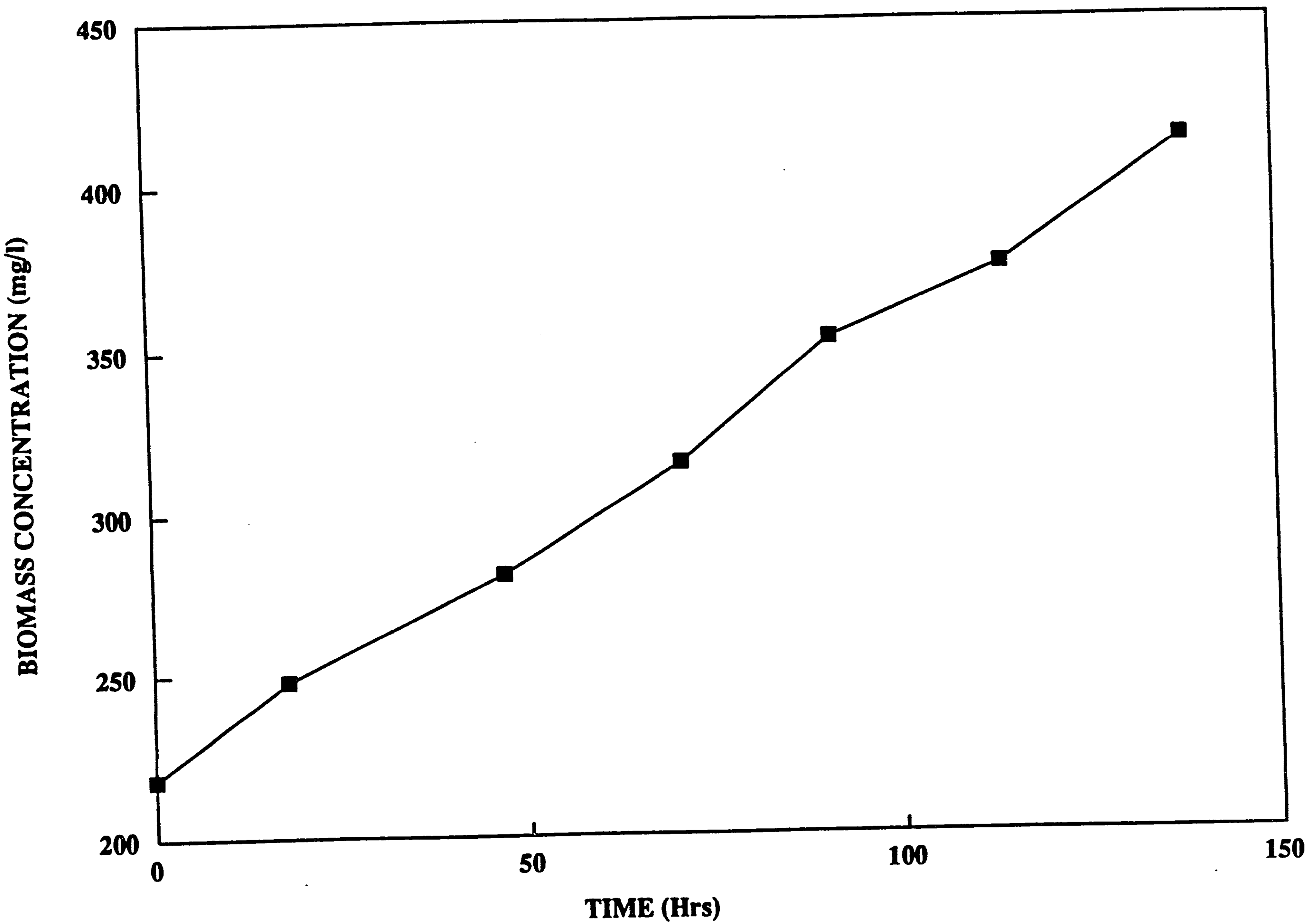


Figure 84. Biomass concentration in a *P. denitrificans* batch culture operating with a NO feed.

weight carboxylic acids (possible end products) were detected. The utilization of succinate for growth by this organism under electron acceptor-limiting conditions may result in loss of the more oxidized carbons as CO_2.

A similar series of experiments were conducted to determine if *P. denitrificans* could utilize heat and alkali pretreated sewage sludge as a carbon and energy source with NO as a terminal electron acceptor. *P. denitrificans* was first grown on succinate in the minimal medium (Table 40) as described above. Cells were then harvested by centrifugation and resuspended in medium prepared as follows: 100 g of wet-packed sludge was suspended in 1L of the medium described in Table 35 without succinate or nitrate. The pH was adjusted to 12 with 10N NaOH and the suspension autoclaved at 121°C for 30 min. The cooled suspension was adjusted to pH 7.0 with 6N HCl, diluted to 1.5 L with additional medium and transferred to the fermenter.

At this time a gas feed consisting of 0.5% NO, 5% CO_2 and balance N_2 was initiated at 30 mL/min. The agitation rate was 450 rpm. The pH and temperature were maintained at 7.0 and 30°C, respectively. Nitric oxide in the feed and outlet gases was determined as described above.

Complete removal of NO from the feed gas was observed. As NO was removed from the feed gas there was a corresponding decrease in the concentration of soluble COD (Figure 85). These data indicate that *P. denitrificans* was utilizing biomolecules solubilized from the sewage sludge as sources of carbon and energy and NO as a terminal electron acceptor. The ammonium ion concentration was seen to increase (Figure 86) as NO was removed from the feed gas. This has

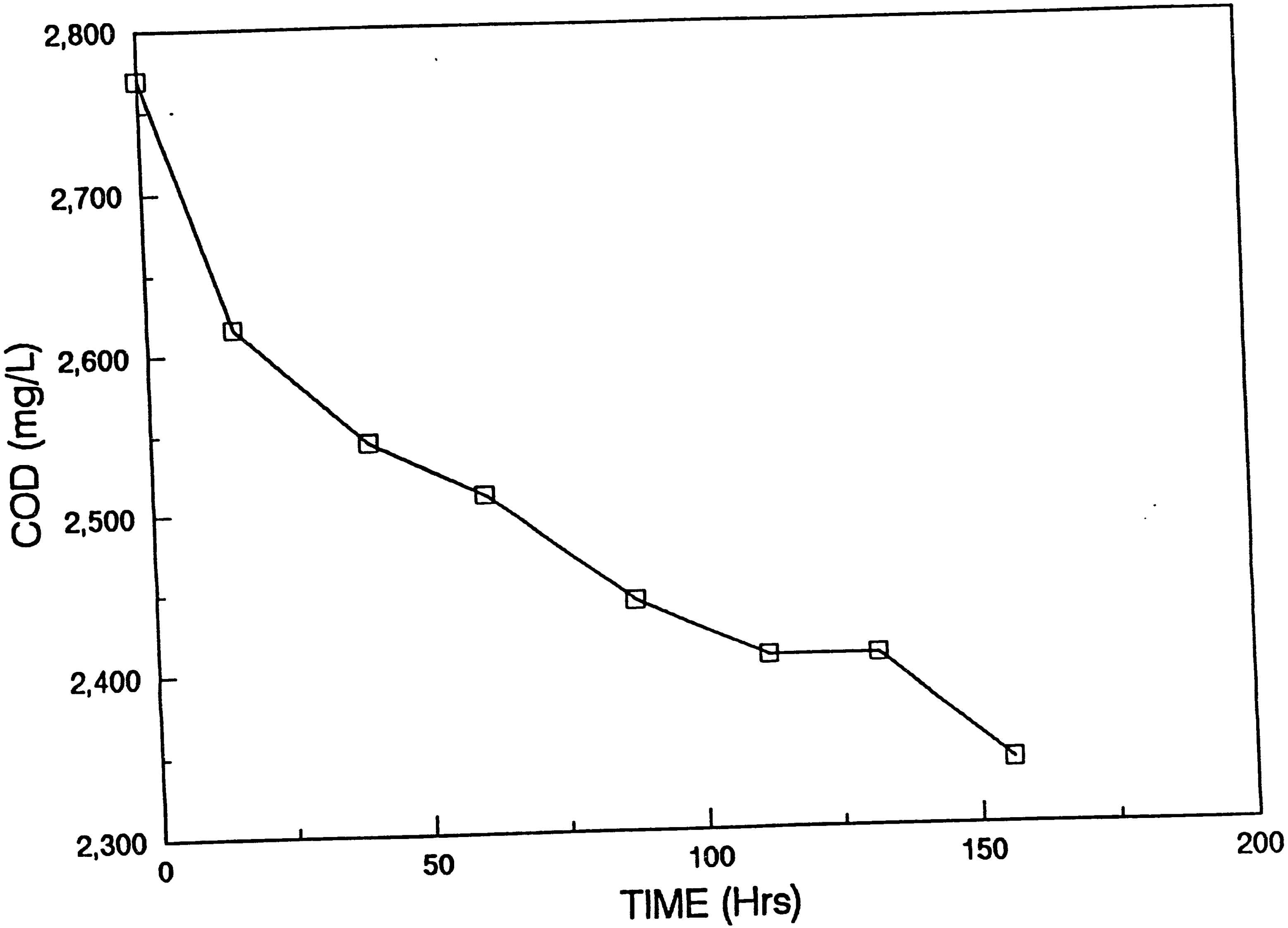


Figure 85. Soluble COD in a _P. denitrificans_ batch culture using pretreated sewage sludge as a carbon and energy source with a NO feed.

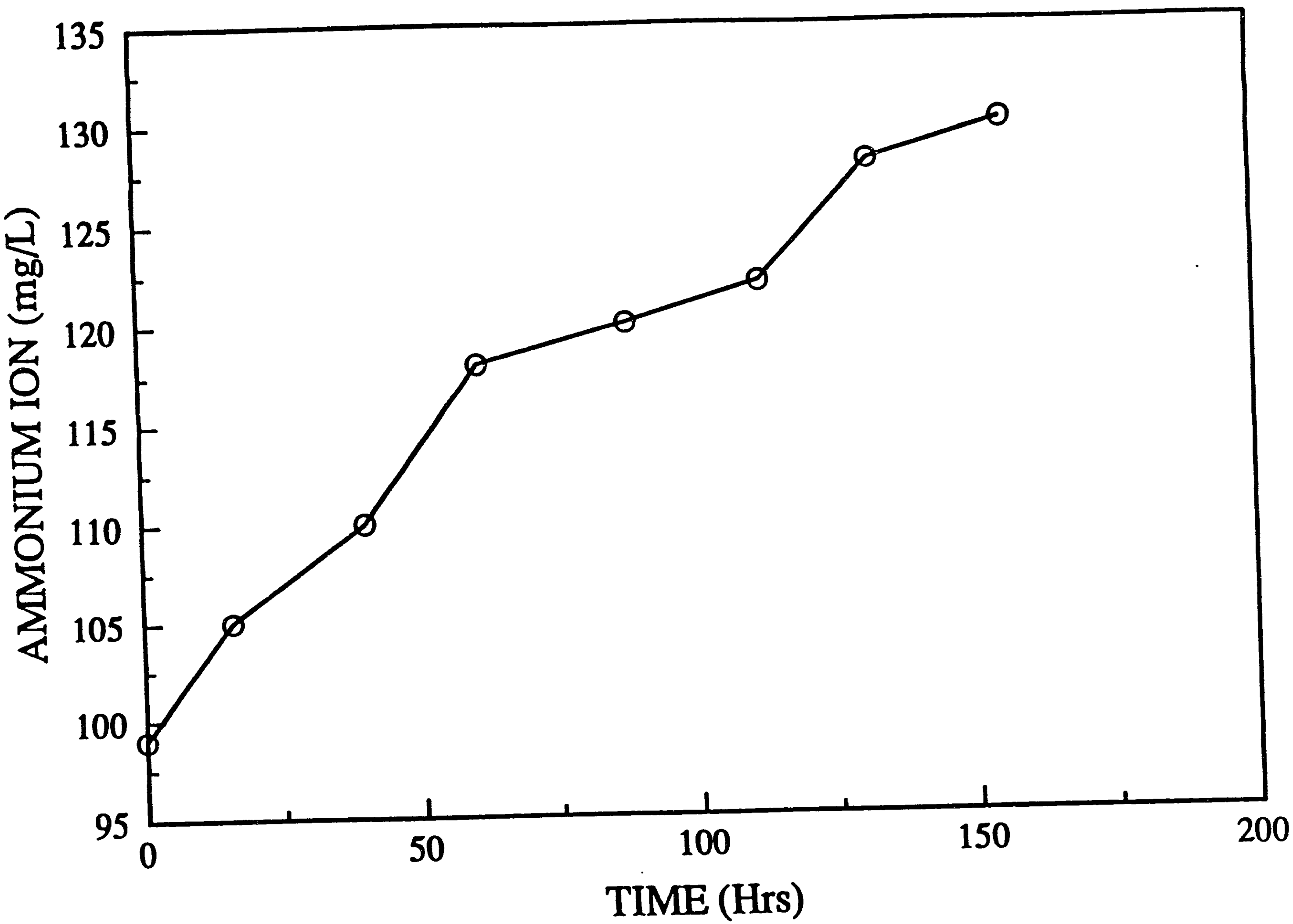


Figure 86. Ammonium ion concentration in a _P. denitrificans_ batch culture using pretreated sewage sludge as a carbon and energy source with a NO feed.

preliminarily been attributed to the liberation of NH_4^+ during metabolism of N-containing compounds from the sewage sludge.

In order to demonstrate clearly that the utilization of soluble COD in these cultures was directly linked to the utilization of NO as a terminal electron acceptor, two additional types of experiments were conducted. In the first type, *P. denitrificans* cultures were grown on a pretreated sewage sludge and a NO gas feed as described above. When utilization of soluble COD in the culture medium was clearly established the NO gas feed was stopped. As seen in Figure 87, when NO was no longer available as a terminal electron acceptor, the soluble COD concentration remained stable. When the NO feed was restarted about 72 hrs later the soluble COD concentration again began to decline.

In a second type of experiment, nitrate (3 g/L KNO_3) was added to a *P. denitrificans* culture growing on pretreated sewage sludge and NO. If NO were acting as a terminal electron acceptor in these cultures nitrate should suppress the utilization of NO. Within 72 hrs of the addition of KNO_3, NO was seen to breakthrough in the outlet gas at concentrations of about 1000 ppm. Further addition of pretreated sewage sludge did not reverse the NO breakthrough.

One additional observation is worthy of note. *P. denitrificans* cultures growing on pretreated sewage sludge were occasionally subject to NO breakthrough even when soluble COD was still available in the medium. However, addition of more pretreated sludge resulted once again in completed NO removal. These observations indicate that *P. denitrificans* could use only certain components of the soluble COD as carbon and energy sources.

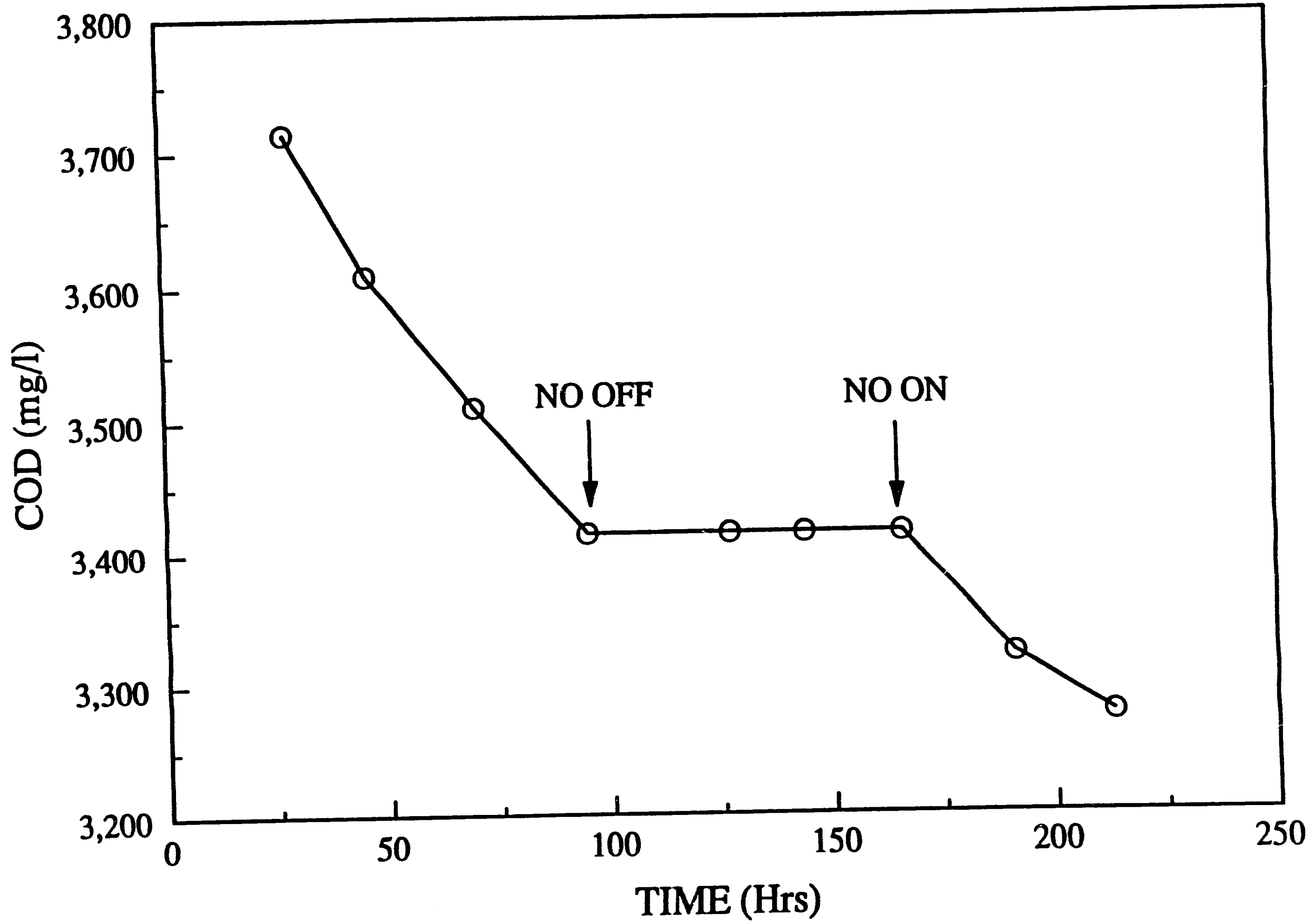


Figure 87. Soluble COD in a P. denitrificans batch culture using pretreated sewage sludge as a carbon and energy source with an intermittent NO feed.

Because of the unexpectedly low NO/succinate stoichiometric ratio observed when *P. denitrificans* was grown on succinate as a carbon and energy source and nitric oxide as a terminal electron acceptor, that experiment was repeated during the current reporting period. *P. denitrificans* was again grown in succinate minimal medium (Table 35) with nitrate as a terminal electron acceptor. When the OD (520 mm) reached about 0.6, the cells were harvested aseptically by centrifugation and resuspended in the same medium without nitrate and transferred back to the fermenter. At this time, a gas feed of 0.5% NO, 5% CO_2 and balance nitrogen was initiated at 30 mL/min.

Results are shown in Figures 88-90. Complete removal of NO from the feed gas was observed. However, the rate of utilization of suiccinic acid in this experiment was much lower than previously observed. During the course of the experiment 32.3 mmoles of NO was reduced accompanied by the utilization of 5.0 mmoles of succinic acid. The NO/succinate ratio was, therefore, 6.5. This is much closer to the NO/succinate ratio of 7.0 predicted from the purely chemical oxidation of succinate (to CO_2 and H_2O). The difference can be attributed to diversion of succinate reducing equivalents for biosynthesis rather than growth. The NH_4^+/NO stoichiometric ratio observed in this experiment was 0.023 which is more than ten times less NH_4^+ utilization than previously observed. Lastly, the reduction of 32.3 mmoles of NO was accompanied by the production of 99.3 mg of biomass protein (by Bradford with a bovine serum albumin standard). If it is assumed that *P. denitrificans* is about 50% protein by weight, this corresponds to about 200 mg of biomass. This is comparable (on the low side) to that observed in previous experiments. However,

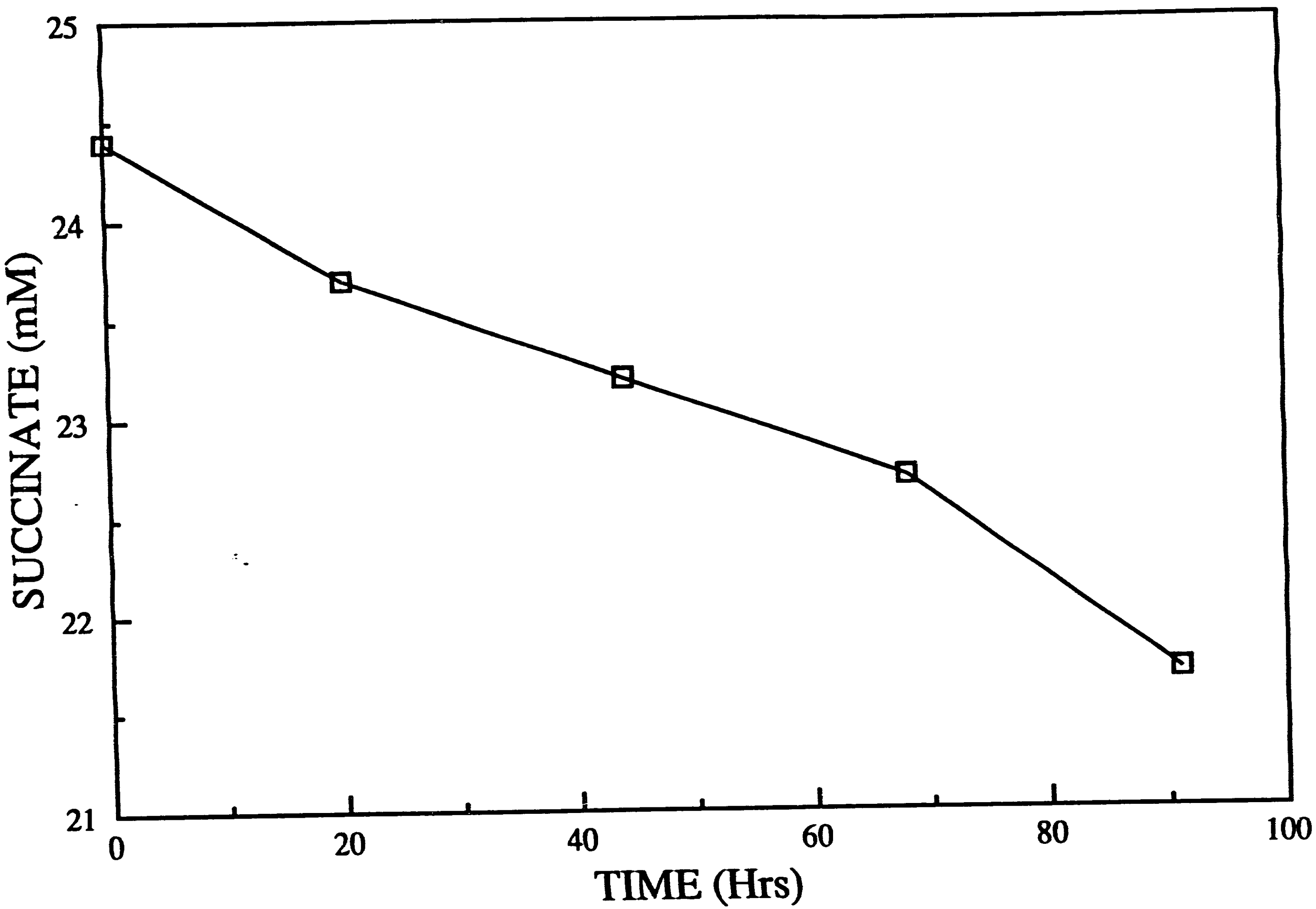


Figure 88. Succinic acid concentration in a *P. denitrificans* culture receiving a NO feed (30 mL/min of 0.5%).

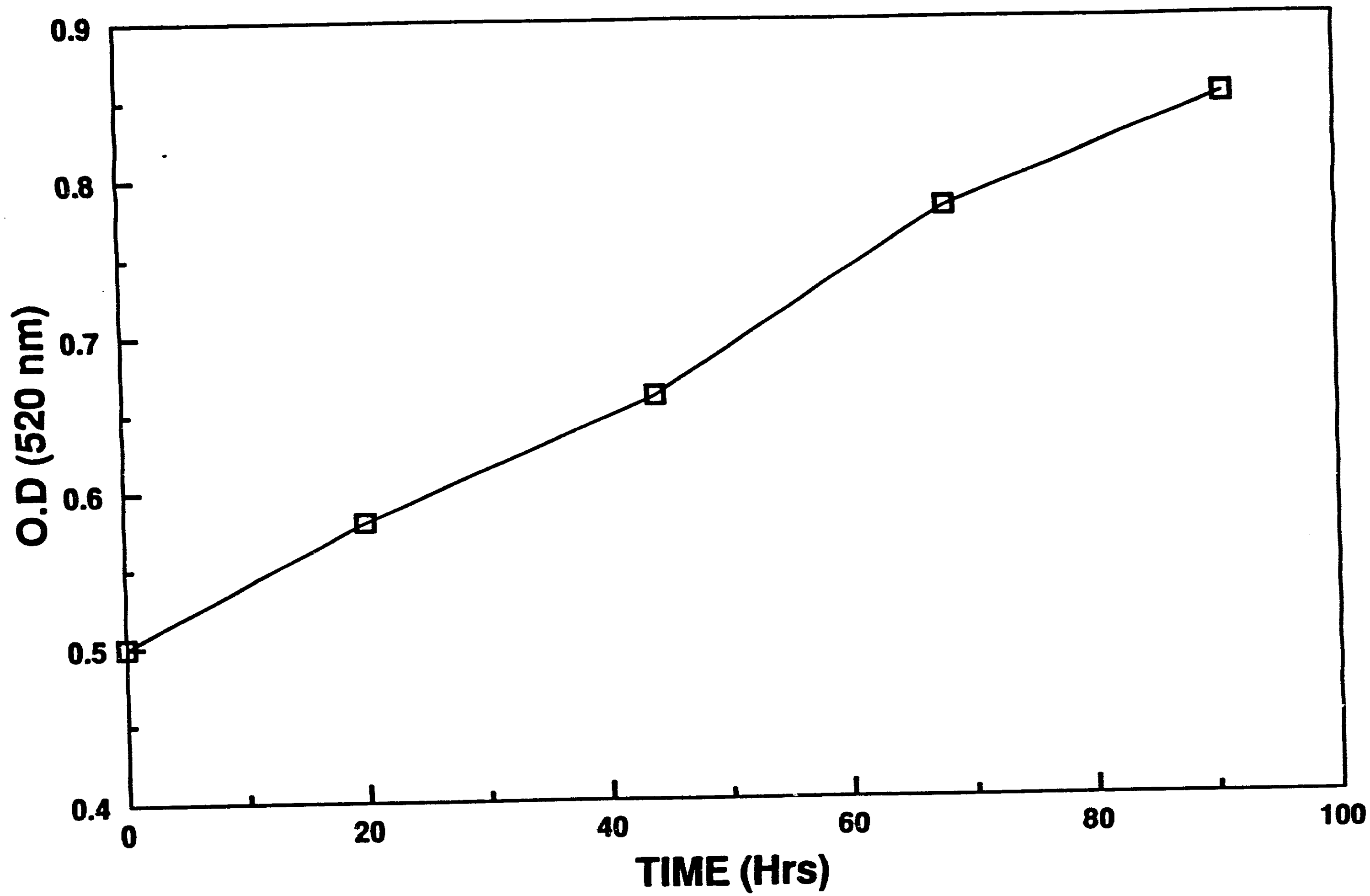


Figure 89. Optical density in a *P. denitrificans* culture receiving a NO feed (30mL/min of 0.5%).

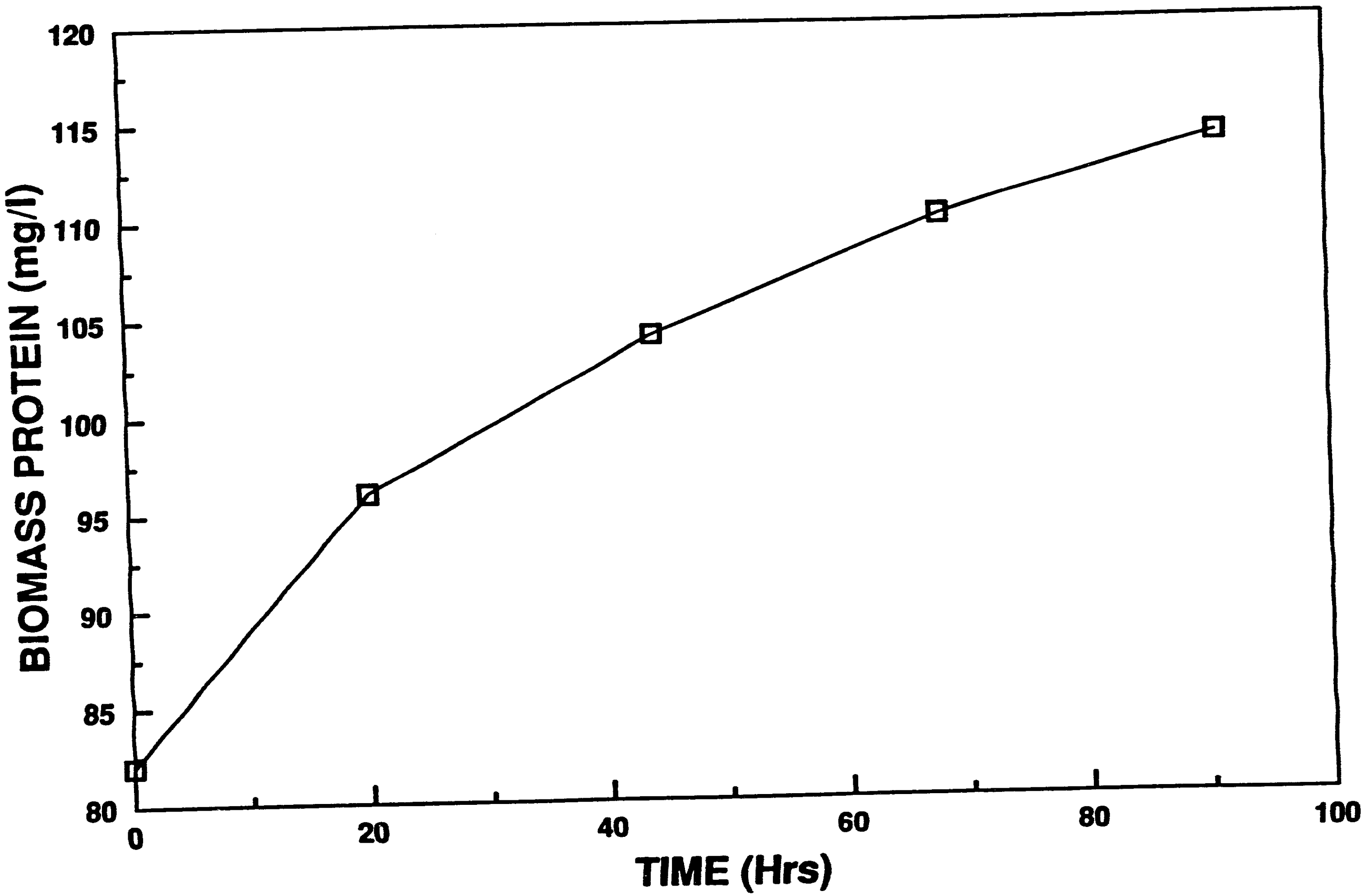


Figure 90. Biomass protein concentration in a *P. denitrificans* culture receiving a NO feed (30mL/min of 0.5%).

previous experiments used a turbidity/biomass relationship developed for another organism.

In summary, the stoichiometry observed in this experiment appears to be in line with what would be predicted based on a comparison with a purely chemical oxidation of succinic acid by NO. Previously observed low NO/succinate ratios may have resulted from contamination. However, *P. denitrificans* is a Gram-negative coccus. Most common laboratory contaminates are also Gram-negative but are short rods and easily distinguishable from coccus forms. Reference was made above to the production of poly-β-hydroxybutyrate as a storage polymer in this organism. The production (or lack of production of) storage granules would be expected to have a significant impact on the succinic acid utilization rate.

At the conclusion of the experiment described above, the culture was diluted with fresh succinic acid medium to a final biomass protein concentration of 48 mg/L. Two liters of this suspension was transfered back to the fermenter and the NO feed reinitiated. Over the next four hours the NO volumetric feed rate was increased stepwise in an effort to exceed the specific activity of the biomass for NO reduction. Results are shown in Figure 91. As shown in Figure 91 specific feed rates as high as 24.6 mmoles NO/g-biomass failed to produce significant breakthrough (beyond mass transfer limitations) of NO. Following 17 hrs of operation at a NO feed rate of 200 mL/min (2.36 mmoles/hr), the feed rate was lowered to 140 mL/min. The outlet NO concentration decreased to zero and remained so for 72 hrs of operation at this flow rate. During this time, the succinate, ammonium ion and biomass protein concentrations were monitored.

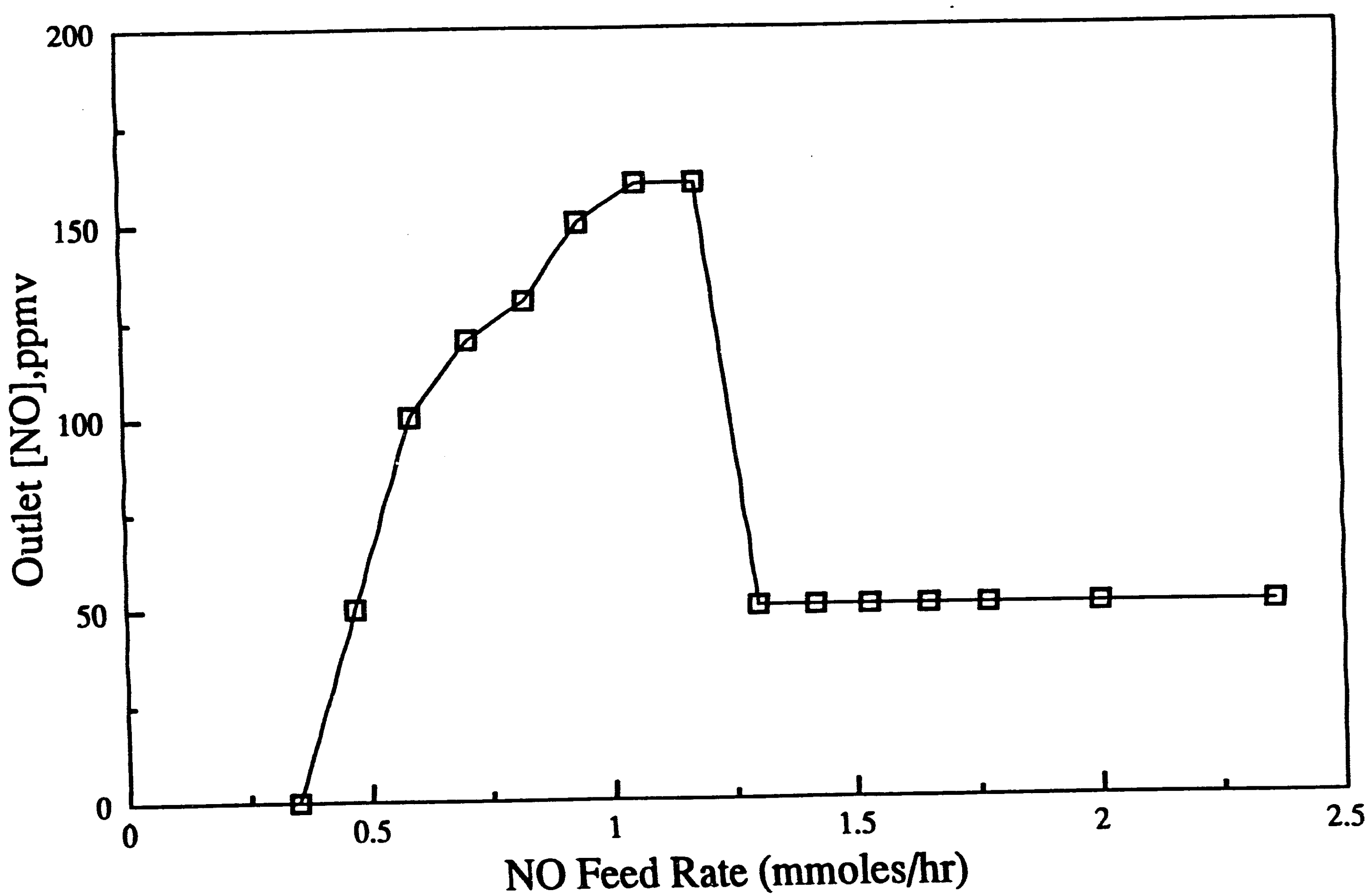


Figure 91. Outlet NO concentration from a 2 L *P. denitrificans* culture (on succinic acid) with increasing NO feed rate. Data collected over two hrs at a biomass protein concentration of 48 mg/L.

Results are shown in Figures 92-94. The reduction of 119 mmoles of NO was accompanied by the oxidation of 22.1 mmoles succinate and the utilization of 5.0 mmoles of NH_4^+. A total of 280 mg of biomass protein was produced. The corresponding stoichiometric ratios are:

$$\frac{NO}{Suc} = 5.4 \text{ mole/mole}$$

$$\frac{NH_4^+}{NO} = 0.042 \text{ mole/mole}$$

$$\frac{\text{Biomass protein}}{NO} = 2.35 \text{ g/mole}$$

5.3.2 *Pseudomonas denitrificans*

Ps. denitrificans (ATCC 13867) was grown anaerobically in a yeast extract, mineral salts medium (Table 43) in a Marubishi MD300 fermenter (culture volume 2L) at pH 7.0 and 30°C. When the optical density reached about 0.25, the cells were harvested aseptically by centrifugation at 4900 x g and 25°C and resuspended in the same medium (without nitrate) and transferred back to the fermenter. At this time, a gas feed of nitrogen was initiated (30 mL/min) and maintained for 40 hrs. The agitation rate was 450 rpm. At the end of this time the N_2 feed was replaced with a gas feed of 0.5% NO, 5% CO_2 and balance N_2 at 30 mL/min.

As seen in Figure 95 the optical density of the culture remained constant while the culture received the N_2 feed indicating that no growth was taking place without a terminal electron acceptor. When the NO feed was initiated the optical density began to increase (Figure 95) and the culture COD (Figure 96) began to decrease indicating growth of the organism on yeast extract components as

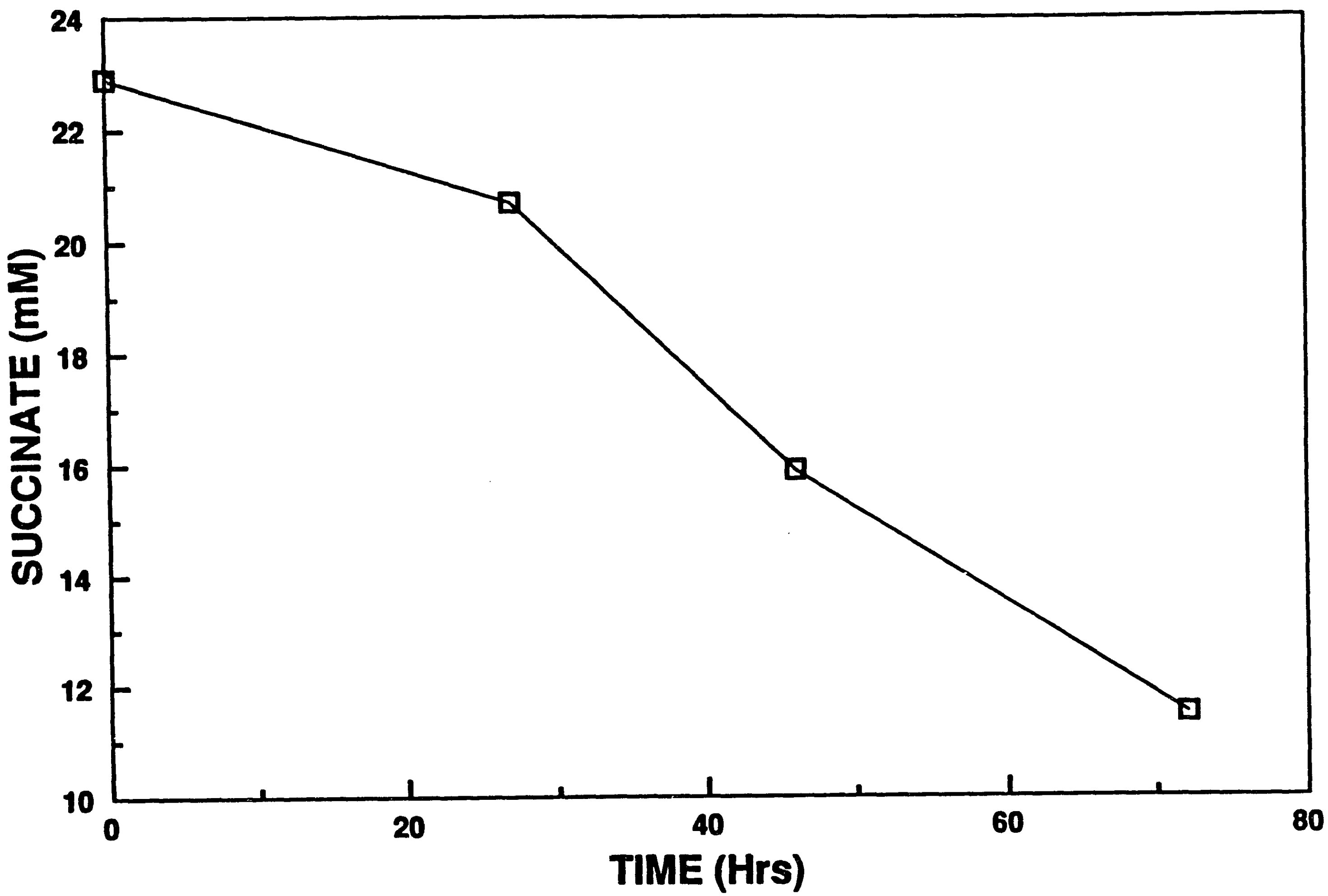


Figure 92. Succinic acid concentration in a *P. denitrificans* culture receiving a NO feed (140 mL/min of 0.5%).

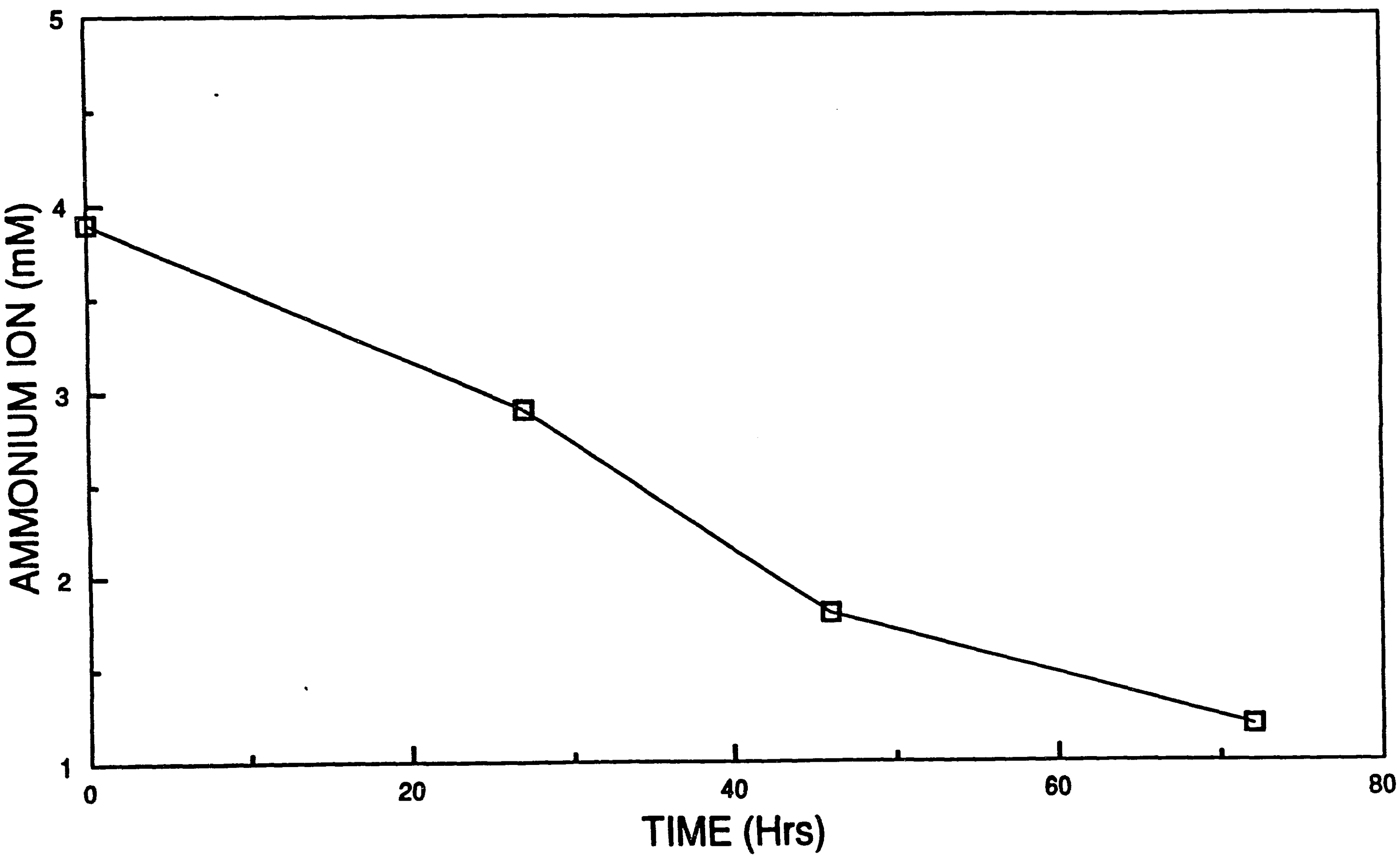


Figure 93. Ammonium ion concentration in a *P. denitrificans* culture receiving a NO feed (140 mL/min of 0.5%).

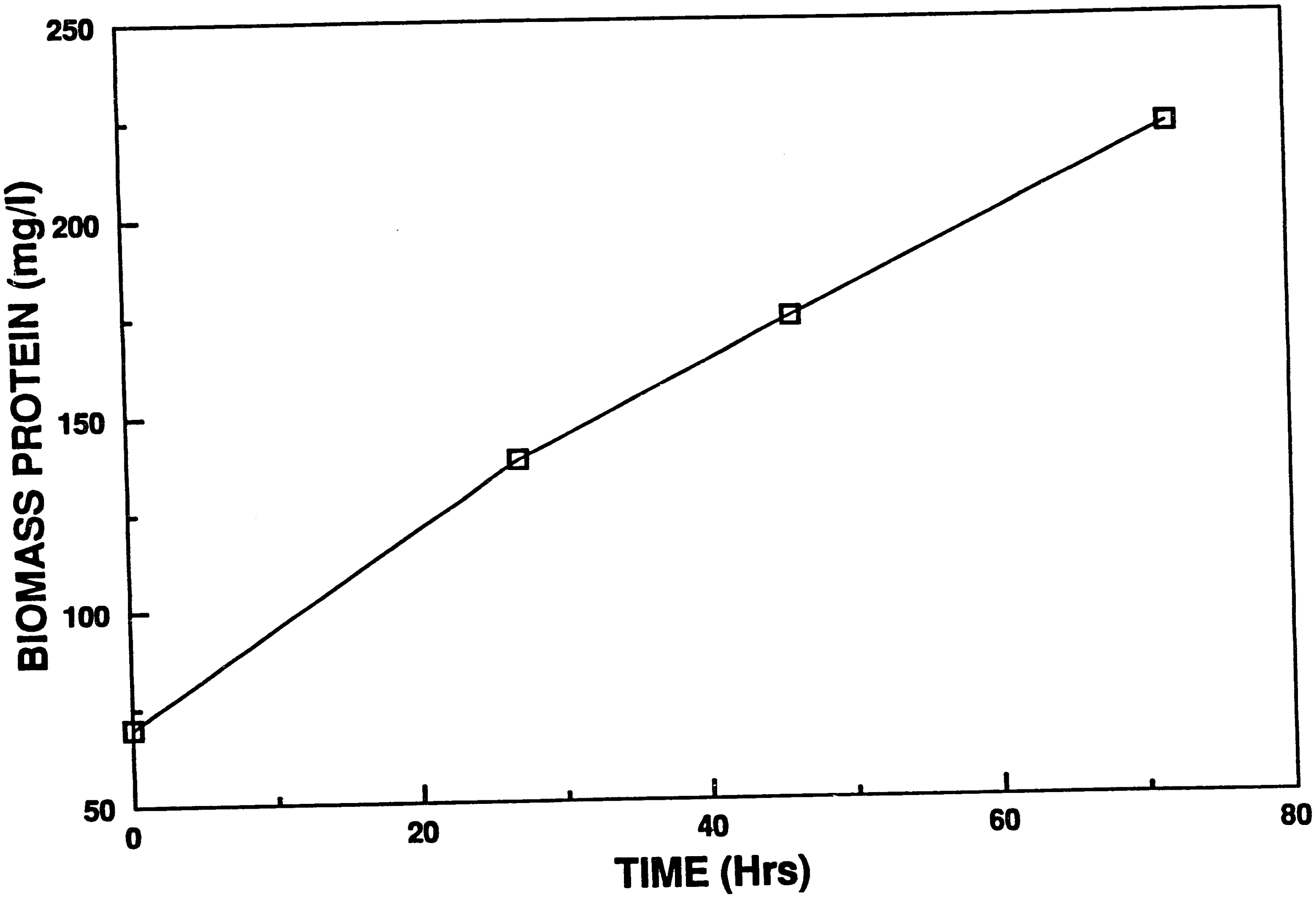


Figure 94. Biomass protein concentration in a *P. denitrificans* culture receiving a NO feed (140 mL/min of 0.5%).

Table 43. Yeast Extract/Mineral Salts Medium for *Pseudomonas denitrificans*

Component	quantity per liter
KNO_3	5.0 g
KH_2PO_4	2.0 g
$NaHCO_3$	1.0 g
NH_4Cl	0.5 g
$MgSO_4$	0.8 g
Yeast extract	3.0 g
Trace element solution (Table 14)	2.0 mL

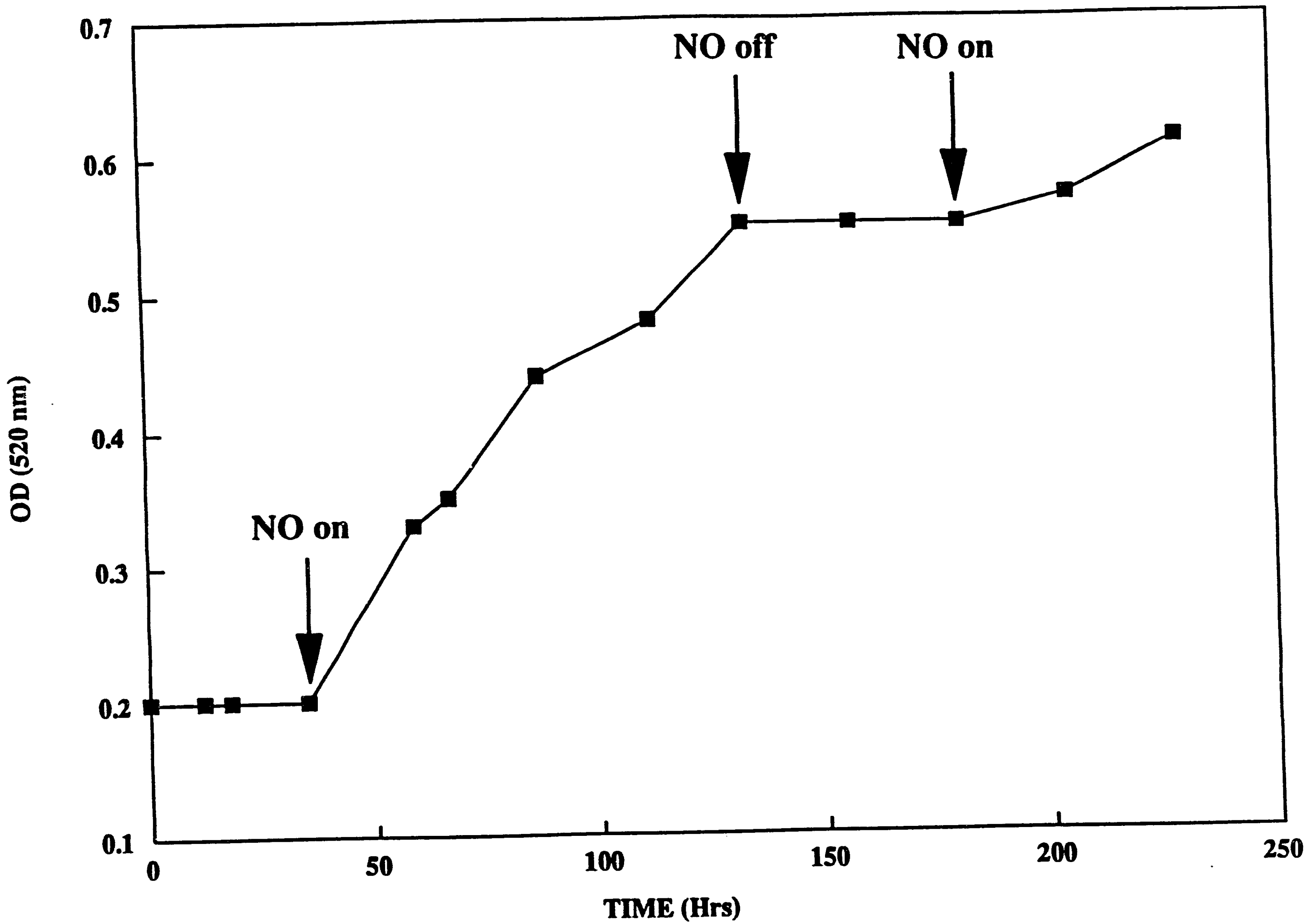


Figure 95. Optical density in a culture of *Pseudomonas denitrificans* receiving a gas feed of NO.

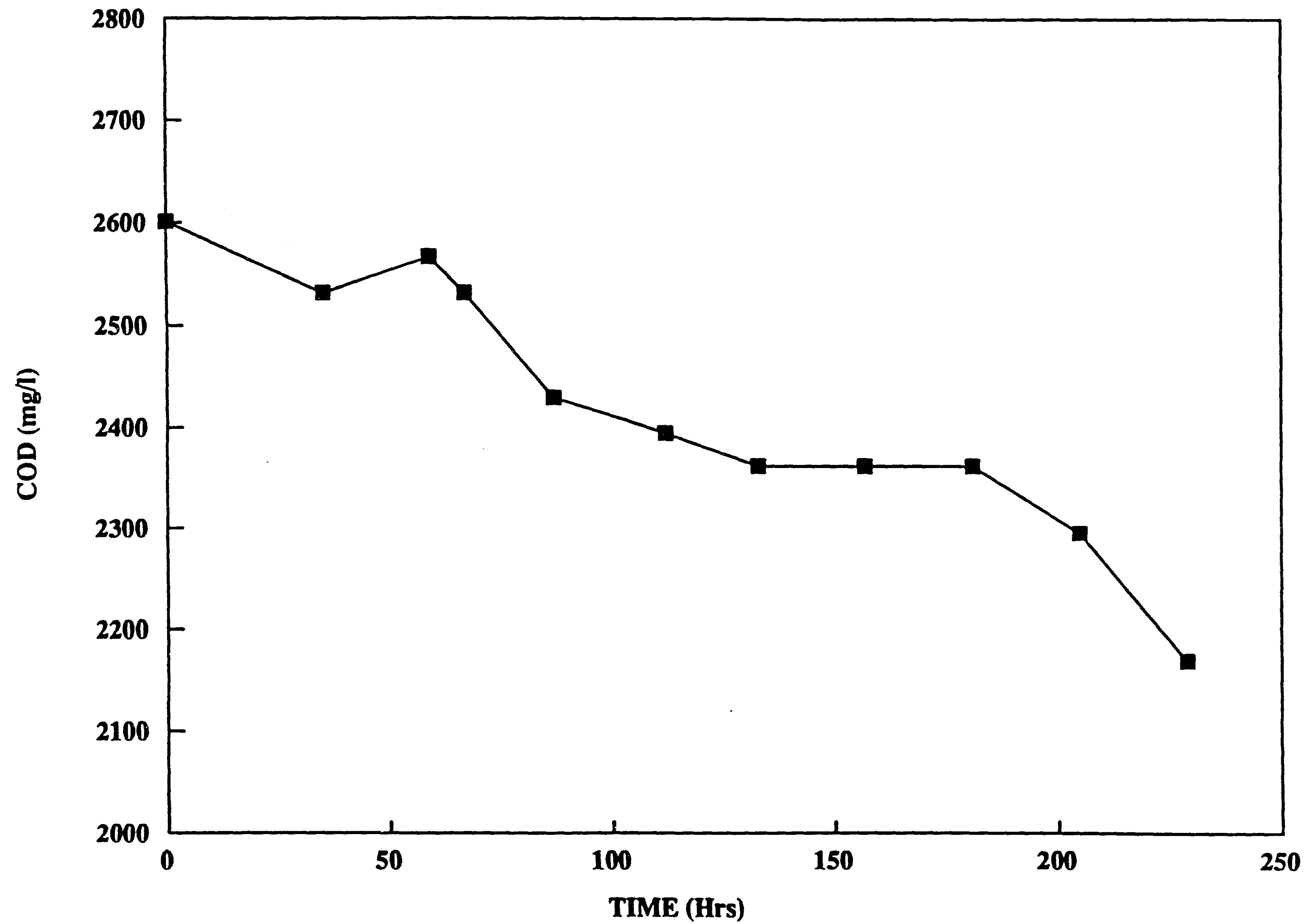


Figure 96. COD concentration in a culture of *Pseudomonas denitrificans* receiving a gas feed of NO (ref Figure 67).

carbon sources and NO as a terminal electron aceptor. Complete removal of NO from the feed gas was observed.

After about 100 hrs of operation on the NO feed the NO feed gas was again replaced with N_2. The result as shown in Figures 95 and 96 was the optical density and COD concentration in the culture leveled off as growth stopped. When the NO feed was reinitiated after about 48 hrs, growth resumed with further increase in optical density and decrease in COD. Figure 97 shows the total biomass protein in the culture during the course of the experiment. As seen in Figure 97, the organism grew only when NO was available as a terminal electron acceptor.

Ps. denitrificans was also grown on heat/alkali pretreated sewage sludge as a carbon and energy source and NO as a terminal electron acceptor. *Ps. denitrificans* was first grown on yeast extract medium as described to an optical density of about 0.8. Cells were then harvested by centrifugation as described previously and resuspended in a pretreated sludge medium. The sludge medium was prepared as follows: 100 g wet-packed sludge was suspended in 1L of the medium described in Table 43 without nitrate or yeast extract. The pH was adjusted to 12 with 10N NaOH and the suspension autoclaved at 121°C for 30 min. The cooled suspension was adjusted to pH 7.0 with 6N HCl and diluted to 2L with additional mineral salts medium.

The inoculated heat/alkali pretreated sludge medium was transferred to a Marubishi MD 300 fermenter. The temperature was brought to 30°C and the pH controlled at 7.0. The agitation rate was 450 rpm. At this time a gas feed consisting of 0.5% NO, 5% CO_2 and balance N_2 was initiated at 30 mL/min.

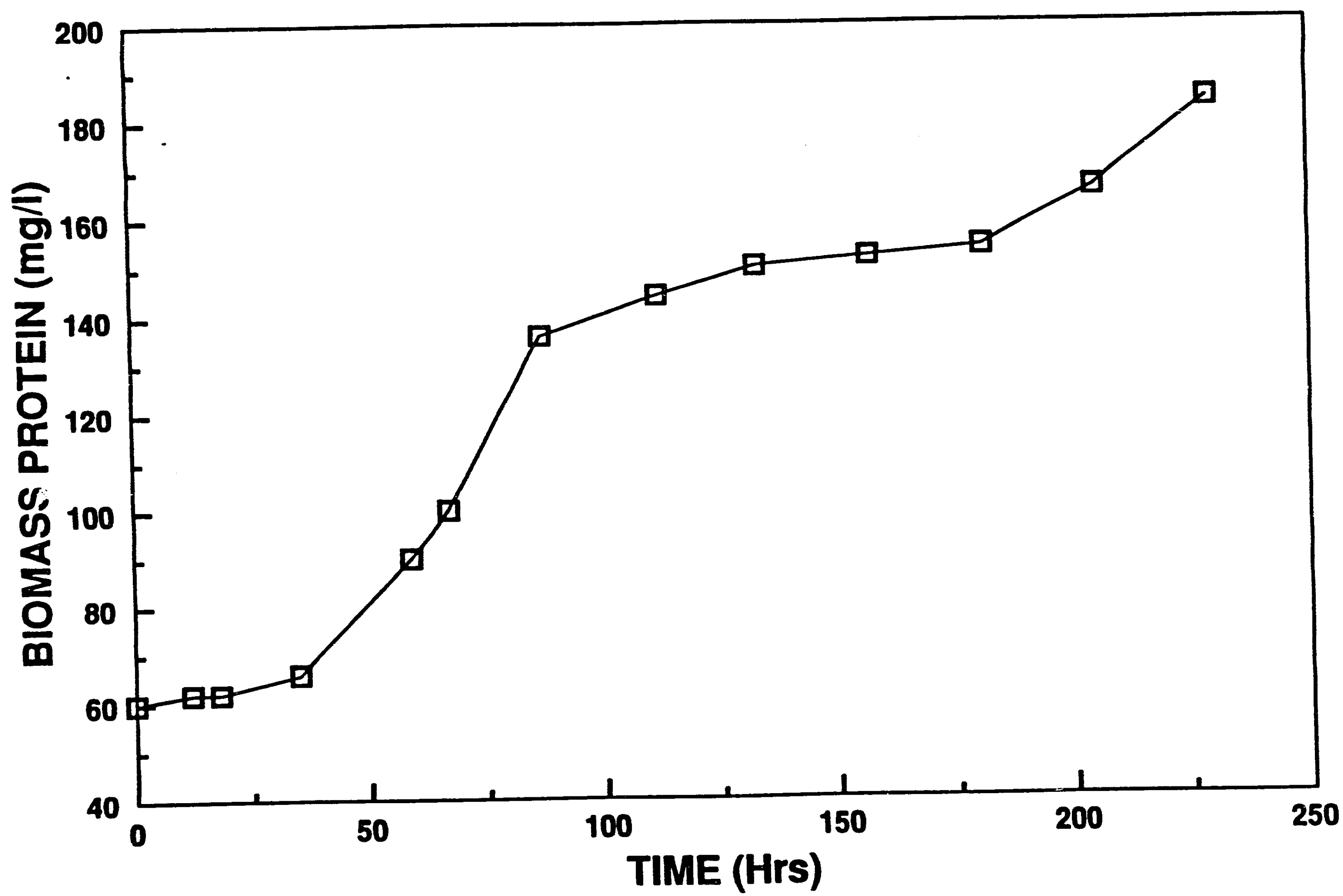


Figure 97. Biomass protein concentration in a culture of *Ps. dentitrficans* in yeast extract medium receiving a NO feed (see companion Figures 63 & 64).

Complete removal of NO from the feed gas was observed. As NO was removed from the feed gas there was a corresponding decrease in the concentration of soluble COD (Figure 98). After about 110 hrs of operation the NO feed was turned off. As shown in Figure 98 the soluble COD concentration remain constant during this time. When the NO feed was restarted the soluble COD again began to decline. Figure 99 shows the ammonium concentration in the reactor medium while receiving the NO feed. Figure 99 indicates that the NH_4^+ concentration increased as NO was removed from the feed gas. This type of behavior was also noted when *P. denitrificans* was grown in a similar medium. The increase in NH_4^+ concentration probably corresponds to the conversion of organic-N to NH_4^+ during oxidation of nitrogen-containing biomolecules liberated from the sludge by heat/alkali treatment. Note that the increase in NH_4^+ concentration stopped when the NO feed was turned off and the soluble COD concentration remained constant. The concentration of biomass protein in the reactor medium during the course of the experiment is shown in Figure 100. These protein determinations reflect both the *Ps. denitrificans* biomass and insoluble proteins from the pretreated sludge. However, an upward trend is evident.

These data indicate that *Ps. denitrificans* was utilizing biomolecules solubilized from the sewage sludge as sources of carbon and energy and NO as a terminal electron acceptor.

At the conclusion of this experiment the culture was diluted with mineral salts medium to a protein concentration of 200 mg/L. This dilution (2L) was then transferred back to the fermenter and the NO feed reinitiated. Over the next six hours the NO feed rate was

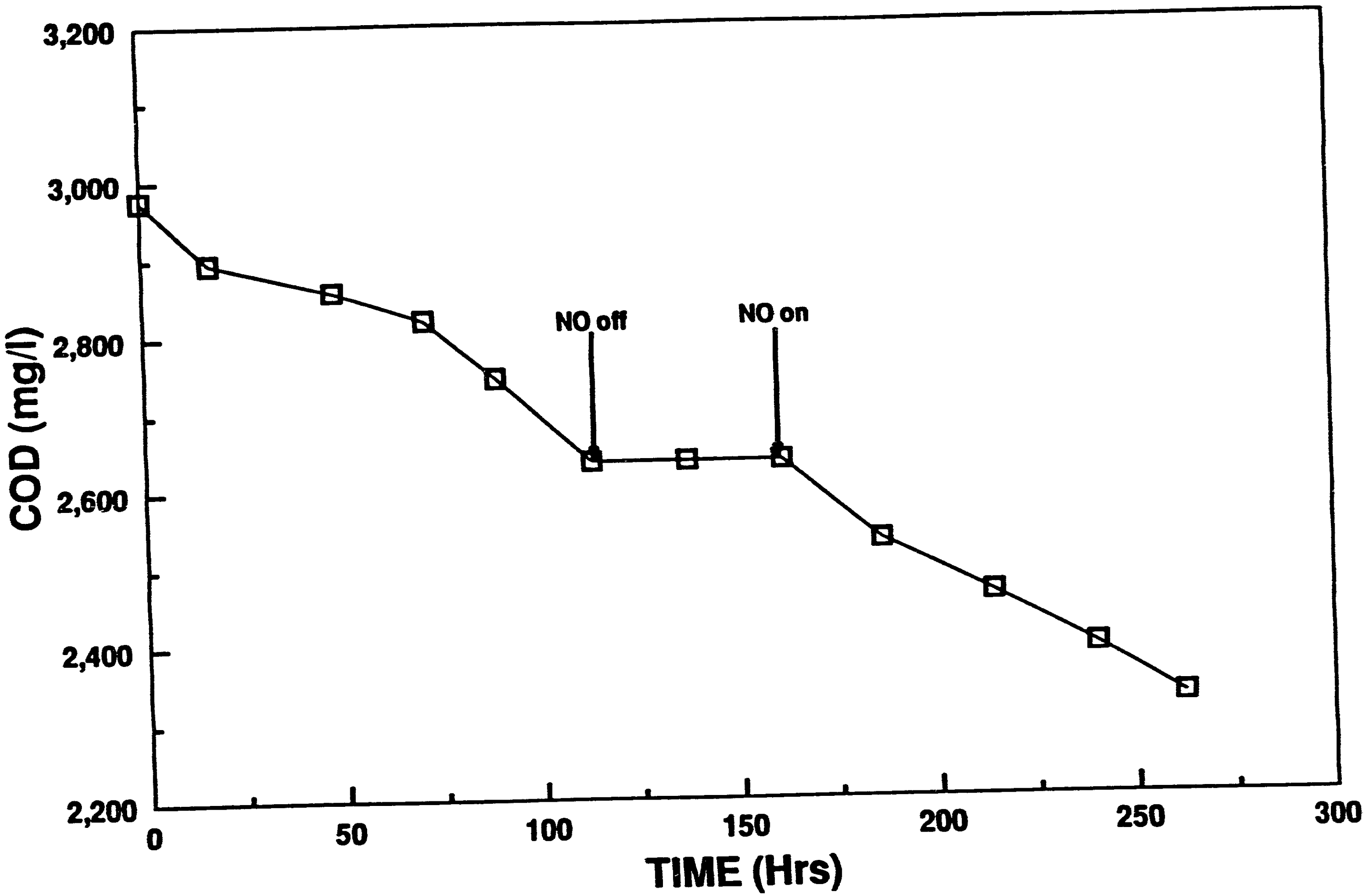


Figure 98. Soluble COD concentration in a *Ps. denitrificans* culture growing on heat/alkali pretreated sewage sludge as a carbon and energy source and NO as a terminal electron acceptor.

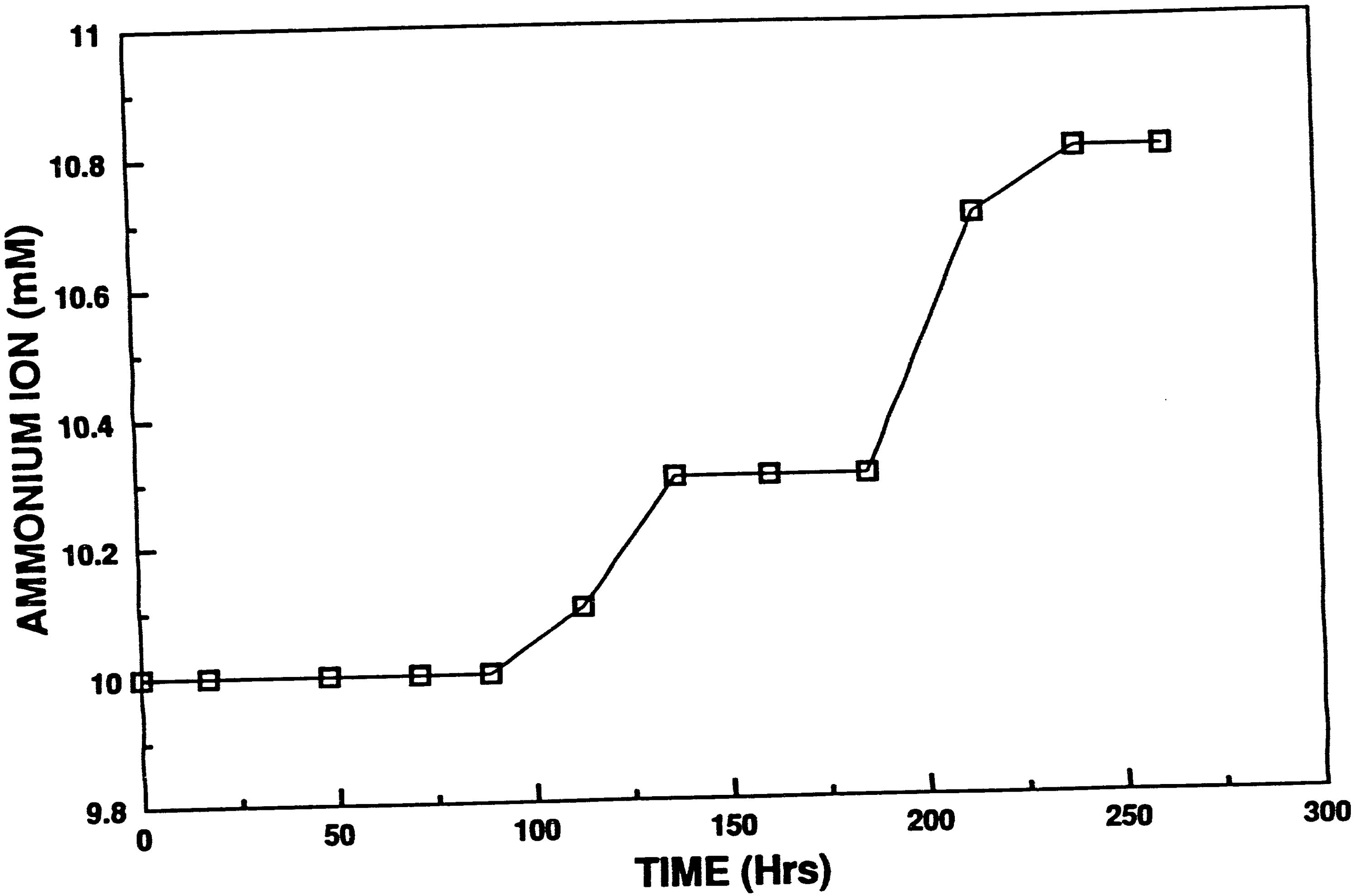


Figure 99. Ammonium ion concentration in a *Ps. denitrificans* culture growing on heat/alkali pretreated sewage sludge as a carbon and energy source and NO as a terminal electron acceptor.

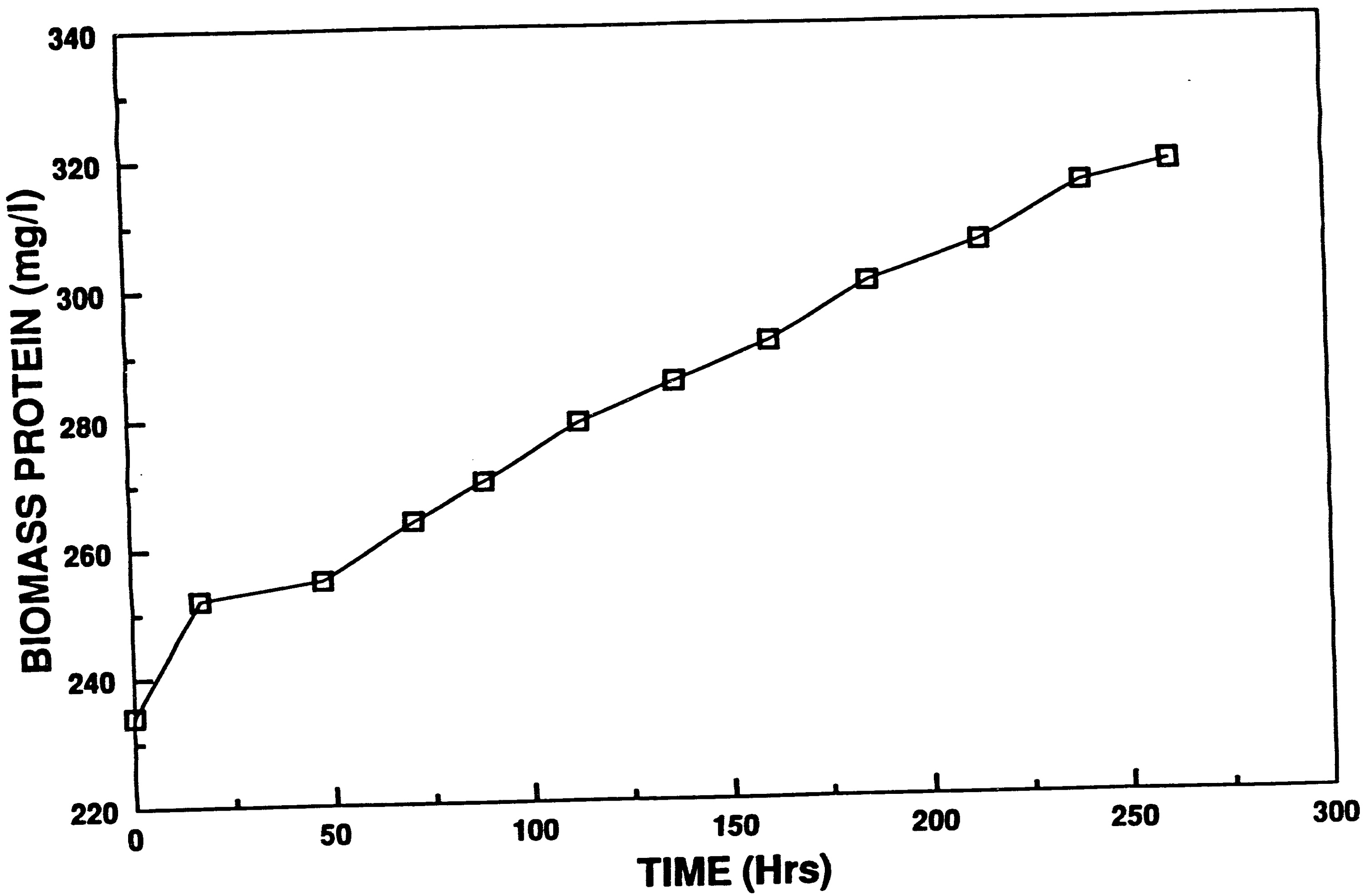


Figure 100. Biomass protein concentration in a *Ps. denitrificans* culture growing on heat/alkali pretreated sewage sludge as a carbon and energy source and NO as a terminal electron acceptor.

increased stepwise in an effort to exceed the specific activity of the biomass for NO reduction. Results are shown in Figure 101. As seen in Figure 101 each increase in the NO feed rate produced a corresponding increase in the outlet NO concentration. On first glance it appears that the specific activity of the biomass was exceeded at a NO feed rate of 0.35-0.47 mmole/hr. The behavior is curious in that the rate of NO utilization by the biomass also increases as the NO feed rate increases. For example if the specific activity of the biomass were actually exceeded at a molar NO feed rate of 0.35 mmoles/hr then at a molar feed rate of 2.12 mmoles/hr the outlet NO concentration would have been over 4000 ppm.

5.3.3 *Alcaligens denitrificans*

A. denitrificans (ATCC 31040) was grown anaerobically in a yeast extract, mineral salts medium (Table 43) in a Marubishi MD 300 fermenter (culture volume 2L) at pH 7.0 and 30 C. When the optical density (520 nm) reached about 0.6, the cells were harvested aseptically by centrifugation at 5000 g and 25 C and resuspended in the same medium (without nitrate) and transferred back to the fermenter. At this time, a gas feed of 0.50% NO, 5% CO_2 , balance N_2 was initiated (30 mL/min) and maintained for about 115 hrs. At this time, the NO feed was replaced with N_2. At a total elapsed time of about 215 hrs the NO feed was reinitiated. (The culture remained on a N_2 feed for about 100 hrs.)

The results of this experiment was shown in Figures 102-105. As seen in Figure 102, the soluble COD (yeast extract components) declined only when NO was available as a terminal electron acceptor. As indicated in Figures 103 and 104, there was essentially no

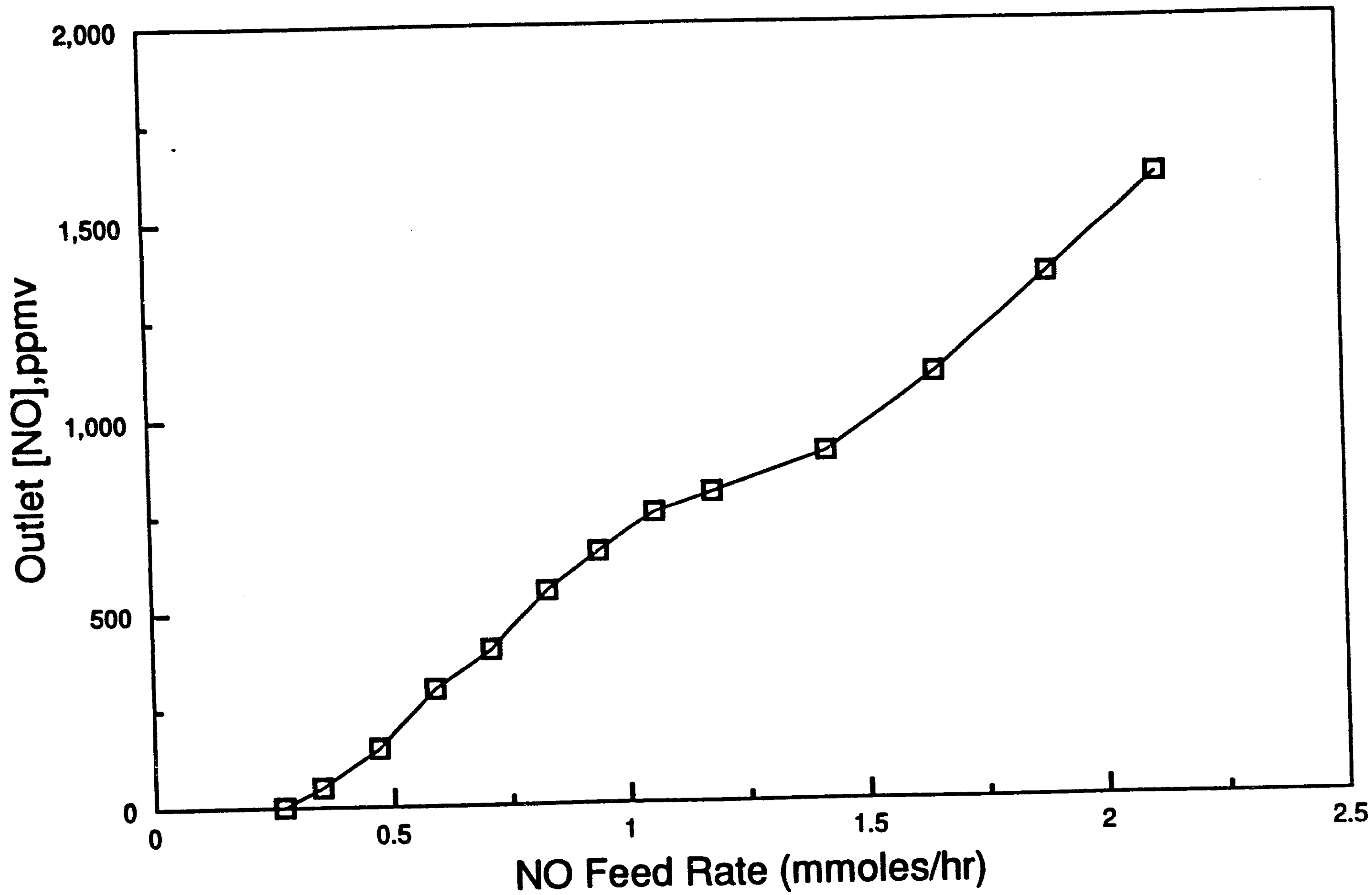


Figure 101. Outlet NO concentration from a 2 L *Ps. denitrificans* culture (on pretreated sewage sludge) with increasing NO feed rate. Data collected over six hrs at a biomass protein concentration of 200 mg/L.

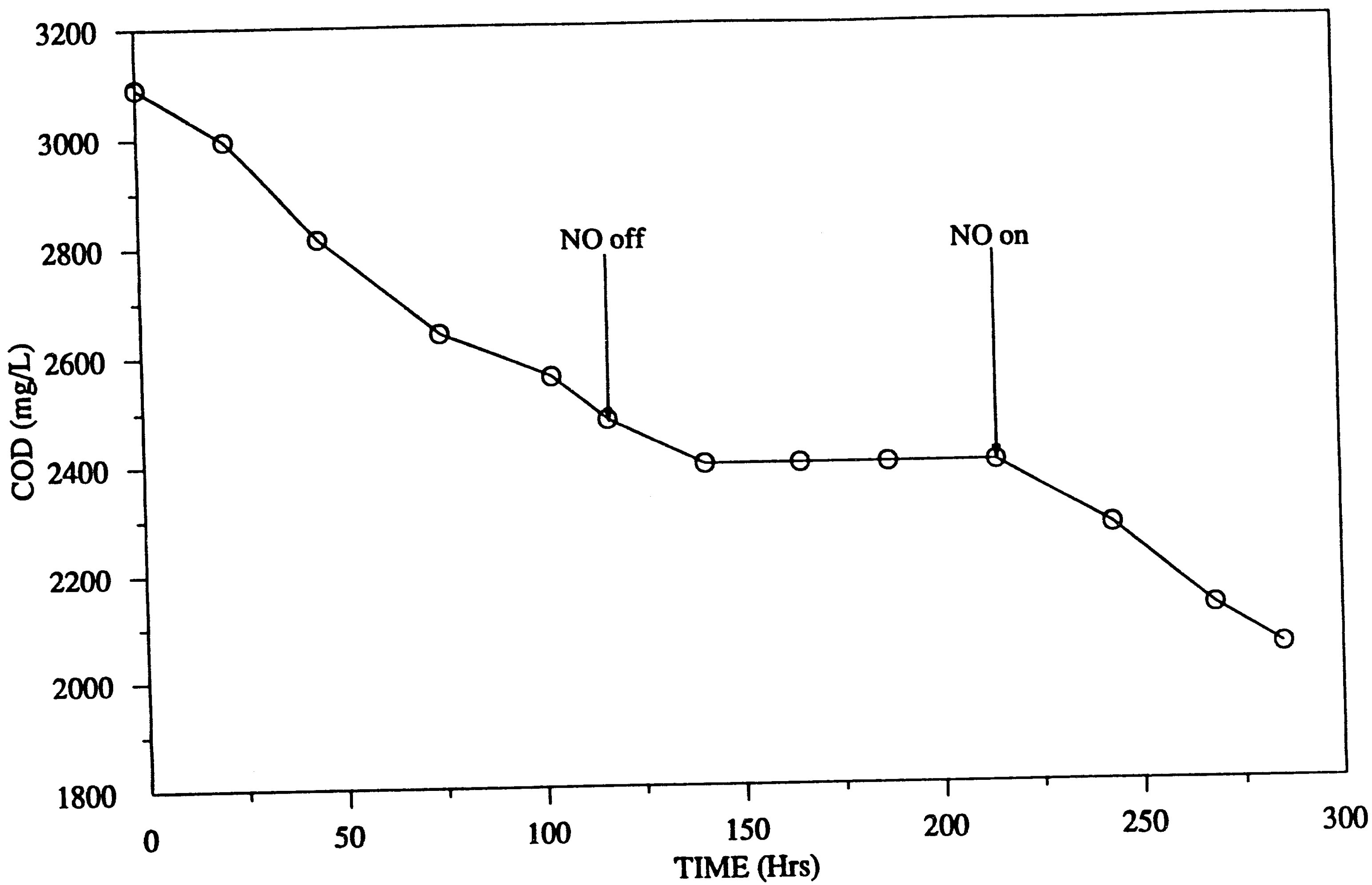


Figure 102. Soluble COD concentration in an *A. denitrificans* culture growing on yeast extract as a carbon and energy source with an intermittant NO feed.

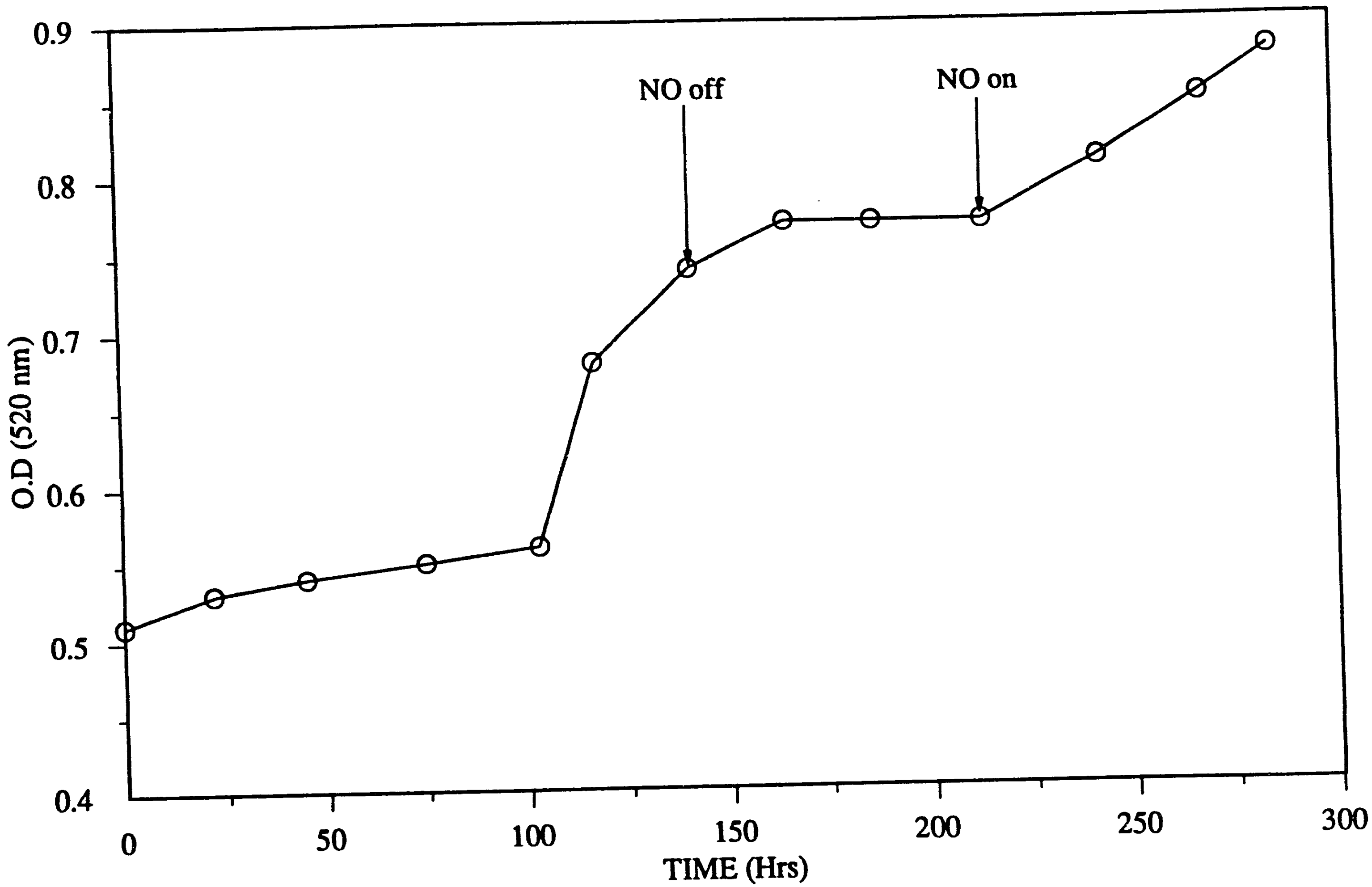


Figure 103. Optical density (520 nm) in an *A. denitrificans* culture growing on yeast extract as a carbon and energy source with an intermittant NO feed.

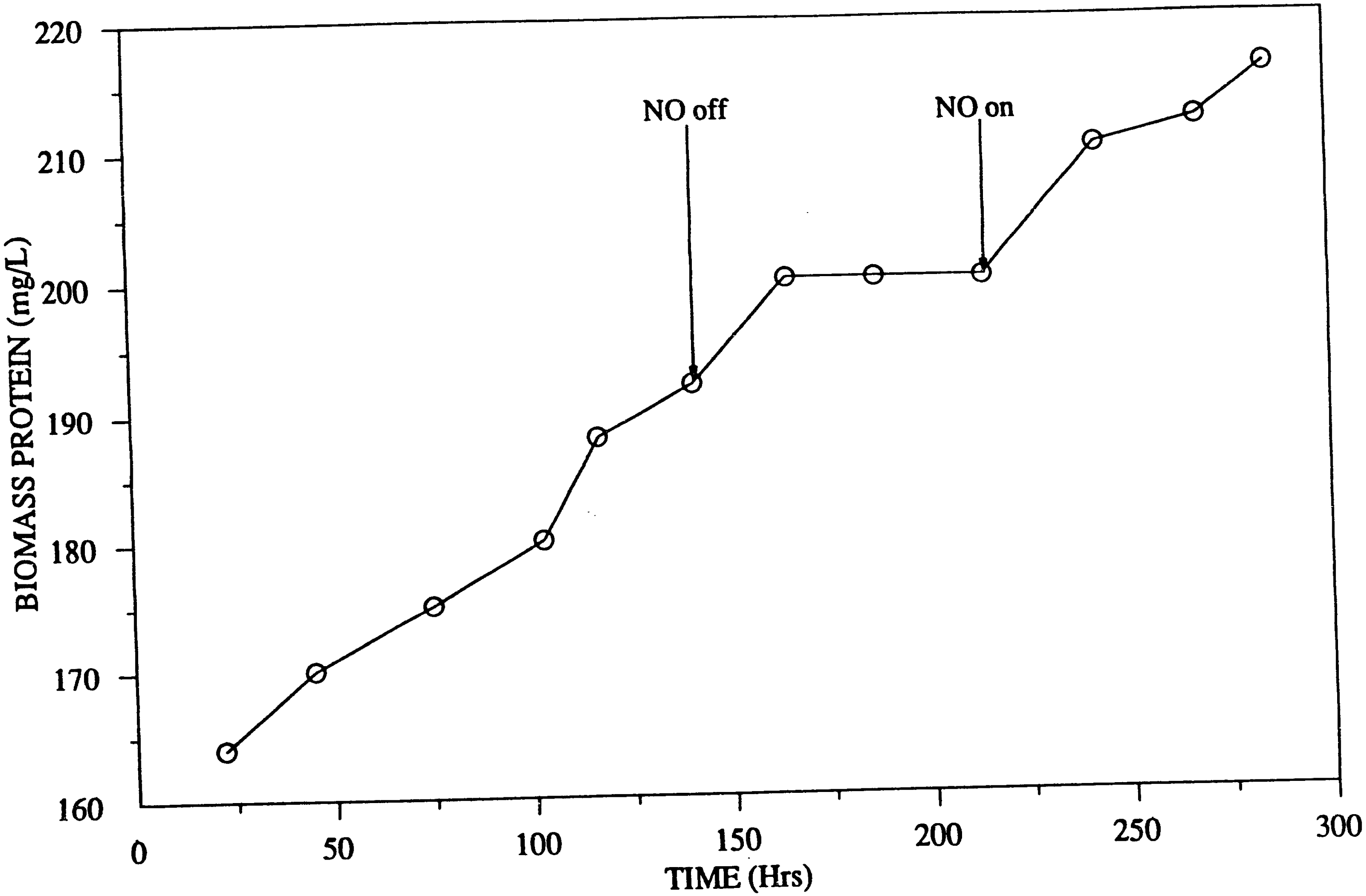


Figure 104. Biomass protein concentration in an *A. denitrificans* culture growing on yeast extract as a carbon and energy source with an intermittant NO feed.

accumulation of biomass in the absence of NO. Growth of the organism on yeast extract as a carbon and energy source and NO as a terminal electron acceptor is clearly indicated. As seen in Figure 105, the ammonia nitrogen concentration increased with time while NO was available. This has been seen before with complex carbon and energy sources which contain nitrogen.

A similar series of experiments were conducted to determine if *A. denitrificans* could utilize heat-and alkali-pretreated sewage sludge as a carbon and energy source with NO as a terminal electron acceptor. *A. denitrificans* was first grown on yeast extract in the minimal medium (Table 43) as described above. Cells were then harvested by centrifugation and resuspended in medium prepared as follows: 100 g of wet-packed sludge was suspended in 1L of the medium described in Table 43 without yeast extract or nitrate. The pH was adjusted to 12 with 10N NaOH and the suspension autoclaved at 121 C for 30 min. The cooled suspension was adjusted to pH 7.0 with 6N HCl, diluted to 1.5 L with additional medium and transferred to the fermenter.

At this time, a gas feed consisting of 0.50% NO, 5% CO_2 and balance N_2 was initiated at 30 mL/min. The agitation rate was 450 rpm. The pH and temperature were maintained at 7.0 and 30 C, respectively. Nitric oxide in the feed and outlet gases was determined as described above.

Complete removal of NO from the feed gas was observed. As NO was removed from the feed gas there was a corresponding decrease in the concentration of soluble COD (Figure 106). These data indicate that *A.denitrificans* was utilizing biomolecules solubilized from the sewage sludge as sources of carbon and energy and NO as a terminal electron

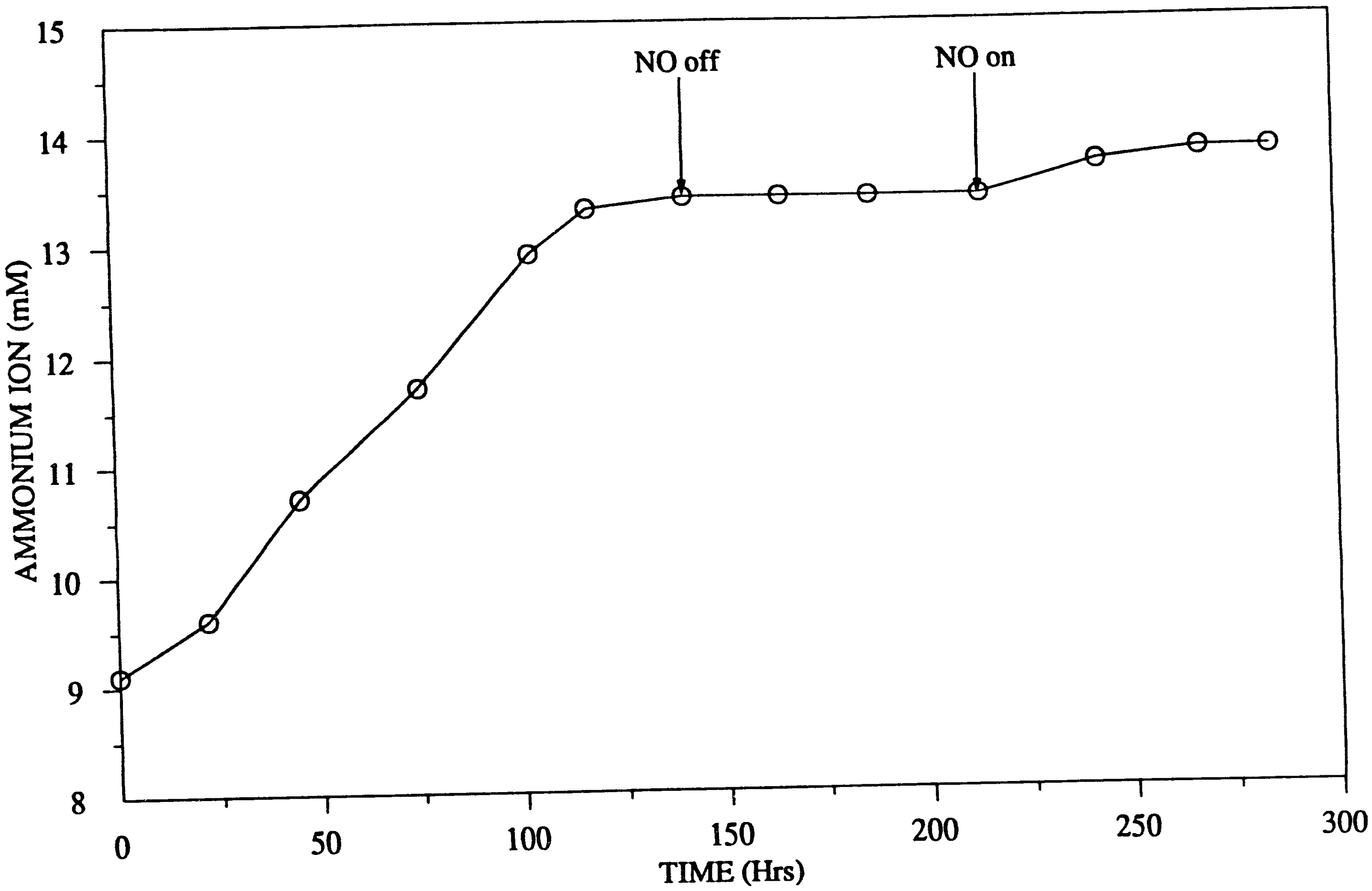


Figure 105. Ammonium ion concentration in an *A. denitrificans* culture growing on yeast extract as a carbon and energy source with an intermittant NO feed.

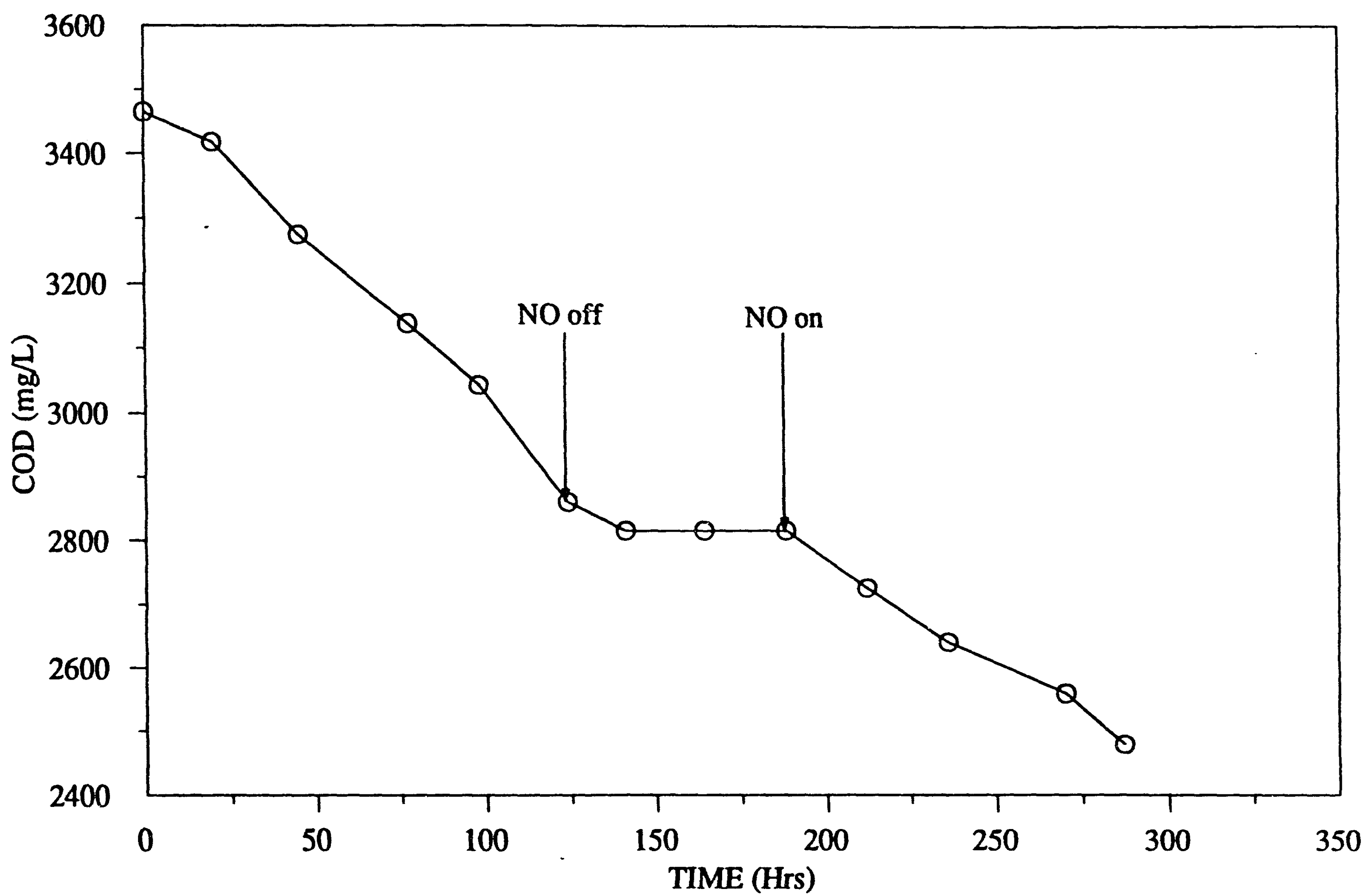


Figure 106. COD concentration in an *A. denitrificans* culture growing on pretreated sewage sludge as a carbon and energy source with an intermittant NO feed.

acceptor. When utilization of soluble COD in the culture medium was clearly established the NO gas feed was replaced by pure N_2. As seen in Figure 106, when NO was no longer available as a terminal electron acceptor, the soluble COD concentration remained stable. When the NO feed was restarted about 72 hrs later the soluble COD concentration again began to decline. The results of a duplicate experiment are shown in Figure 107.

5.3.4 Specific Activity of Denitrifying Bacteria for NO Reduction

The specific activity of *Ps. denitrificans*, *P. denitrificans*, *A. denitrificans* and *T. denitrificans* for NO reduction have been compared under similar experimental conditions. In each case the organism was grown anoxically in a Marubishi MD 300 fermenter under optimum growth conditions in a yeast extract/mineral salts medium (*A. denitrificans*, *Ps. denitrificans*, *Ps. denitrificans*) or thiosulfate mineral medium (*T. denitrificans*) to an optical density of about 0.8. In each case nitrate served as the terminal electron acceptor. Cells were then harvested by centrifugation at 5000 g and 25 C and resuspended in the same media without nitrate. Cell suspensions (25.0 mL) were then transferred to 125 mL serum bottles and gassed with 0.50% NO, 5% CO_2, balance N_2. Bottles were then shaken in an environmental shaker at 30 C. The gas volume was sampled periodically and analyzed for NO by gas chromatography as previously described. Controls containing 25 mL of medium without nitrate or cells were treated and analyzed in an identical manner. At the end of each experiment, each suspension containing cells was analyzed for total biomass protein.

As seen in Figures 108-111, NO disappeared from the gas space of each septum bottle containing a denitrifying bacterium. There was no

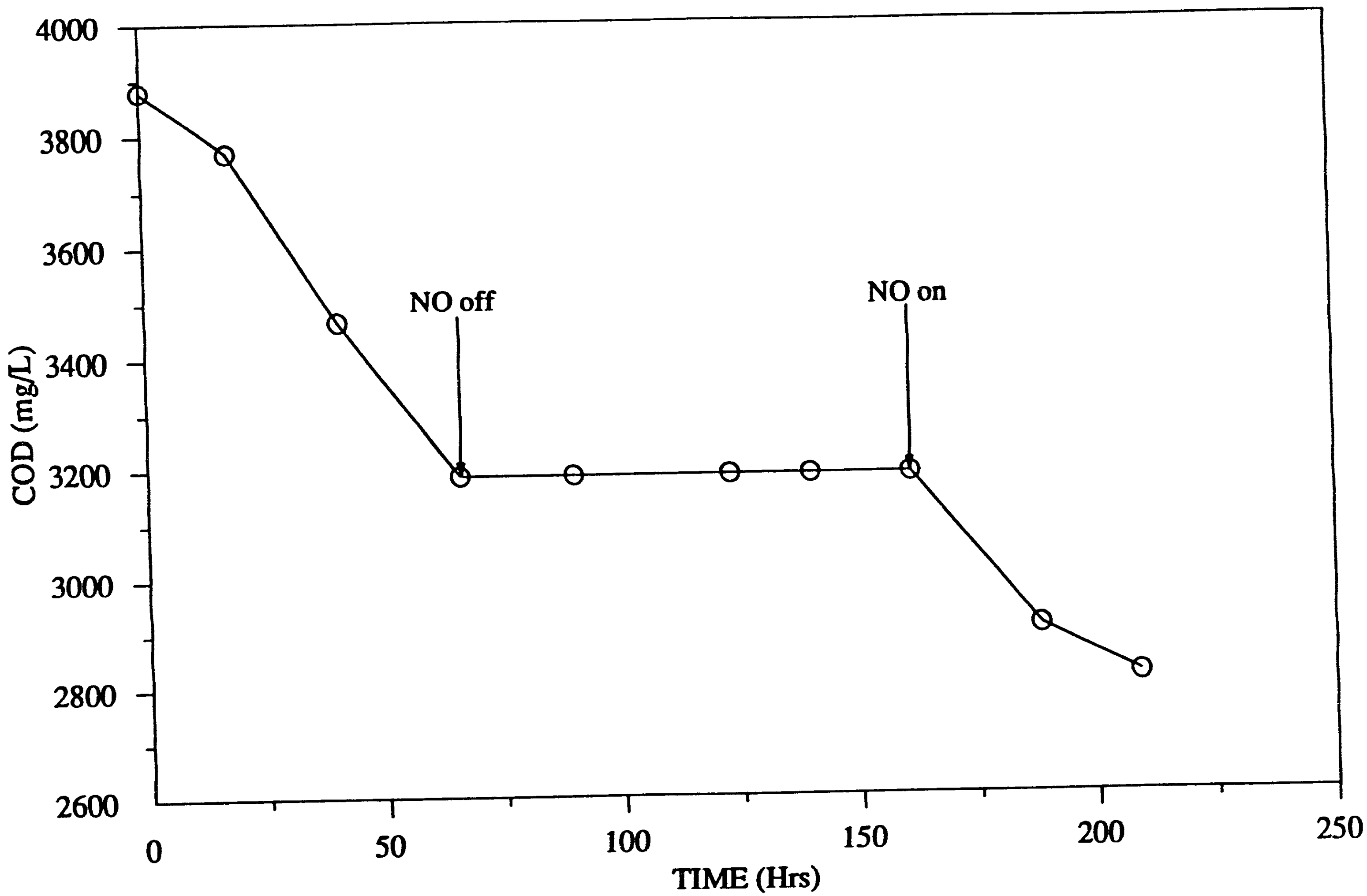


Figure 107. COD concentration in a second *A. denitrificans culture* growing on pretreated sewage sludge as a carbon and energy source with an intermittant NO feed.

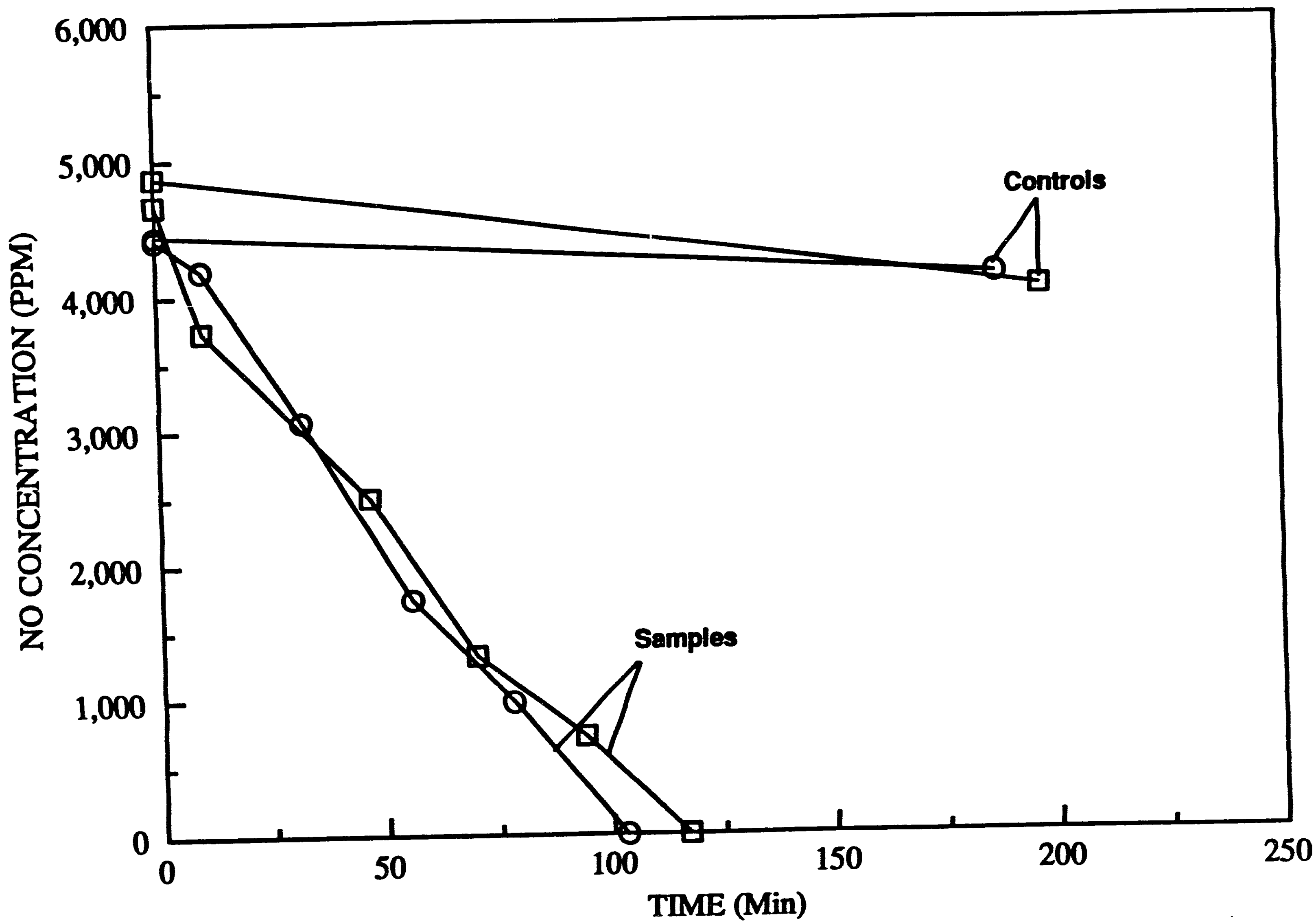


Figure 108. Uptake of nitric oxide by a suspension of *A. denitrificans* in yeast extract/ mineral salts medium without nitrate.

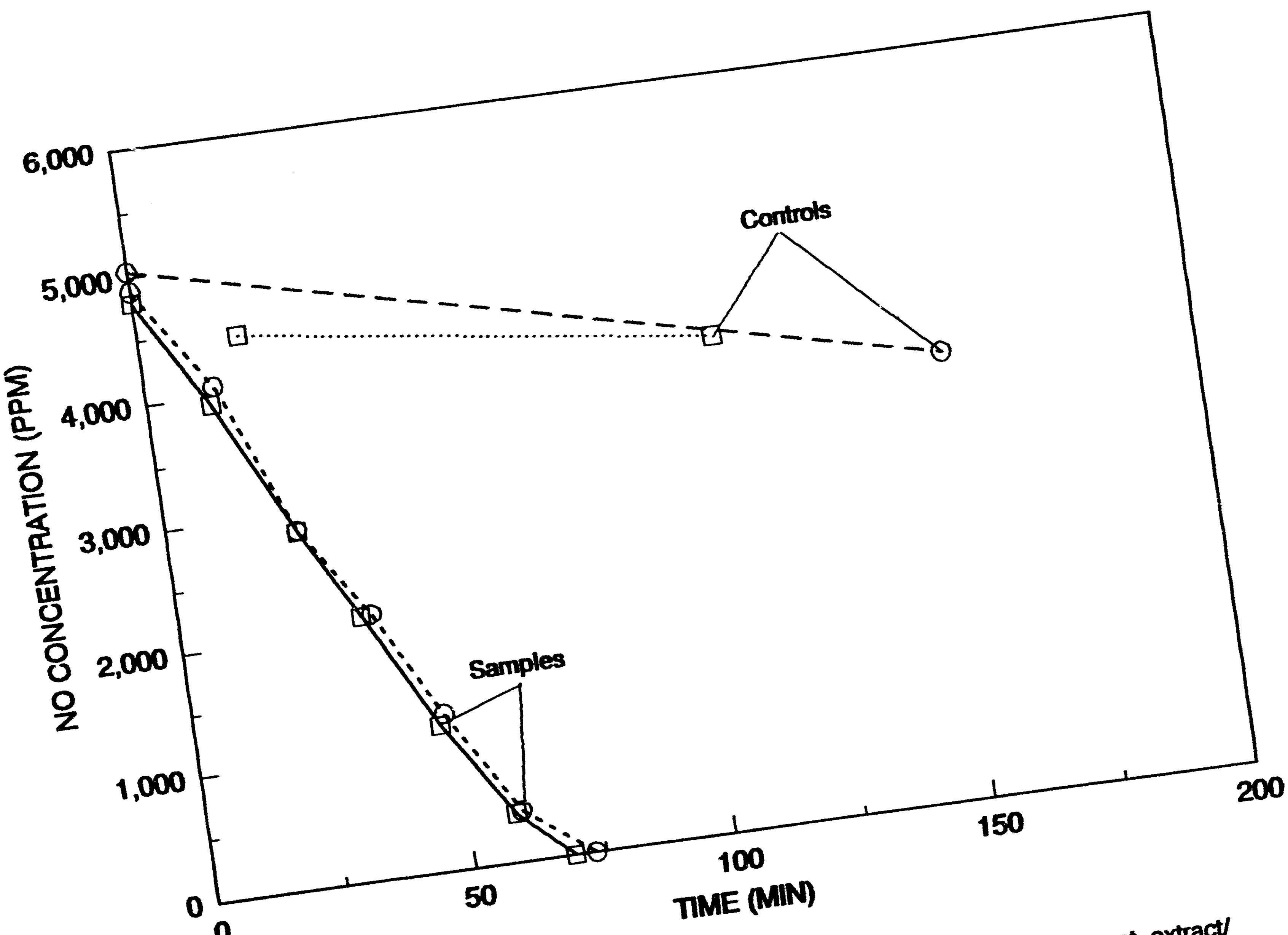


Figure 109. Uptake of nitric oxide by a suspension of *P. denitrificans* in yeast extract/ mineral salts medium without nitrate.

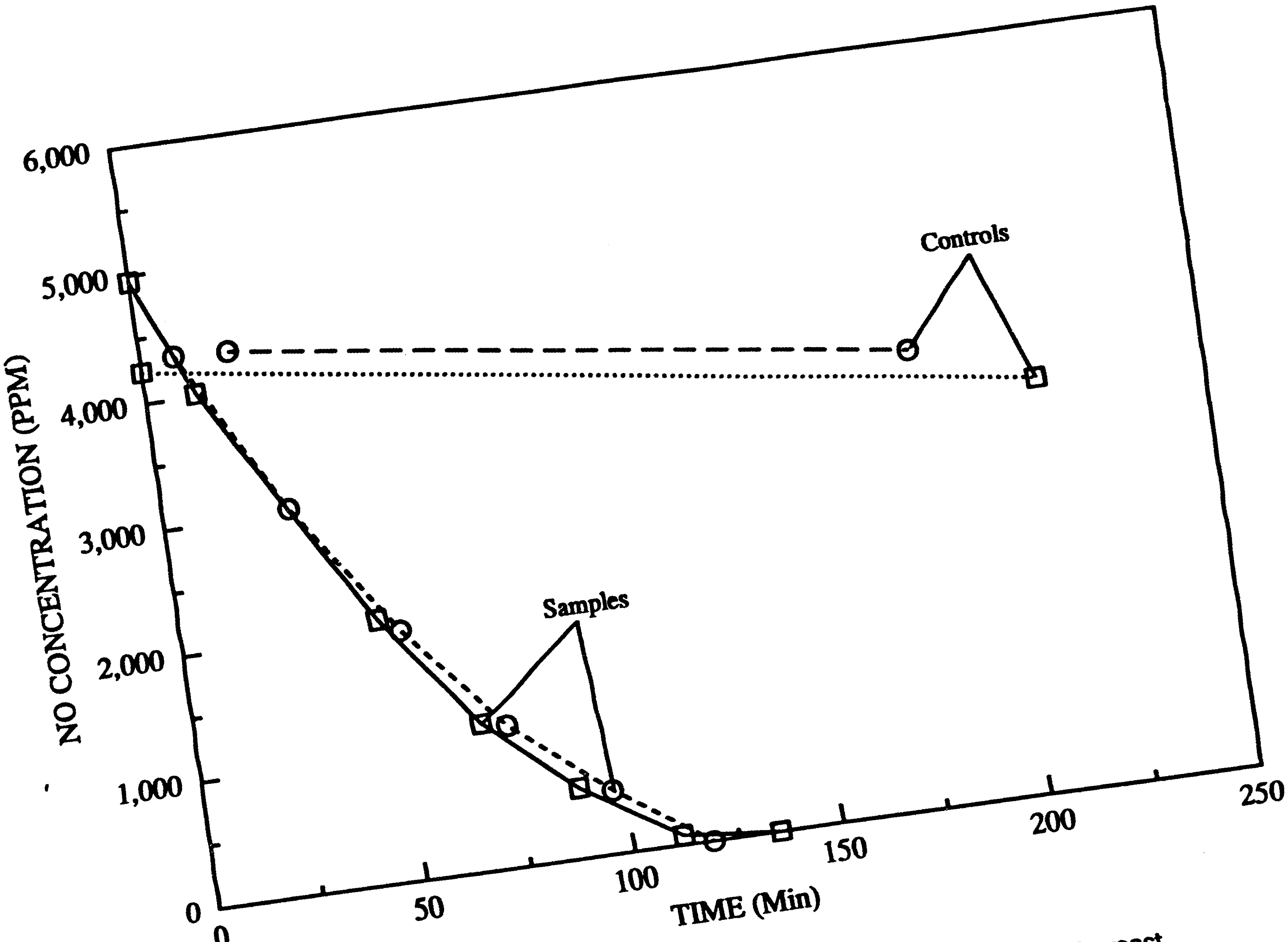


Figure 110. Uptake of nitric oxide by a suspension of *Ps. denitrificans* in yeast extract/mineral salts medium without nitrate.

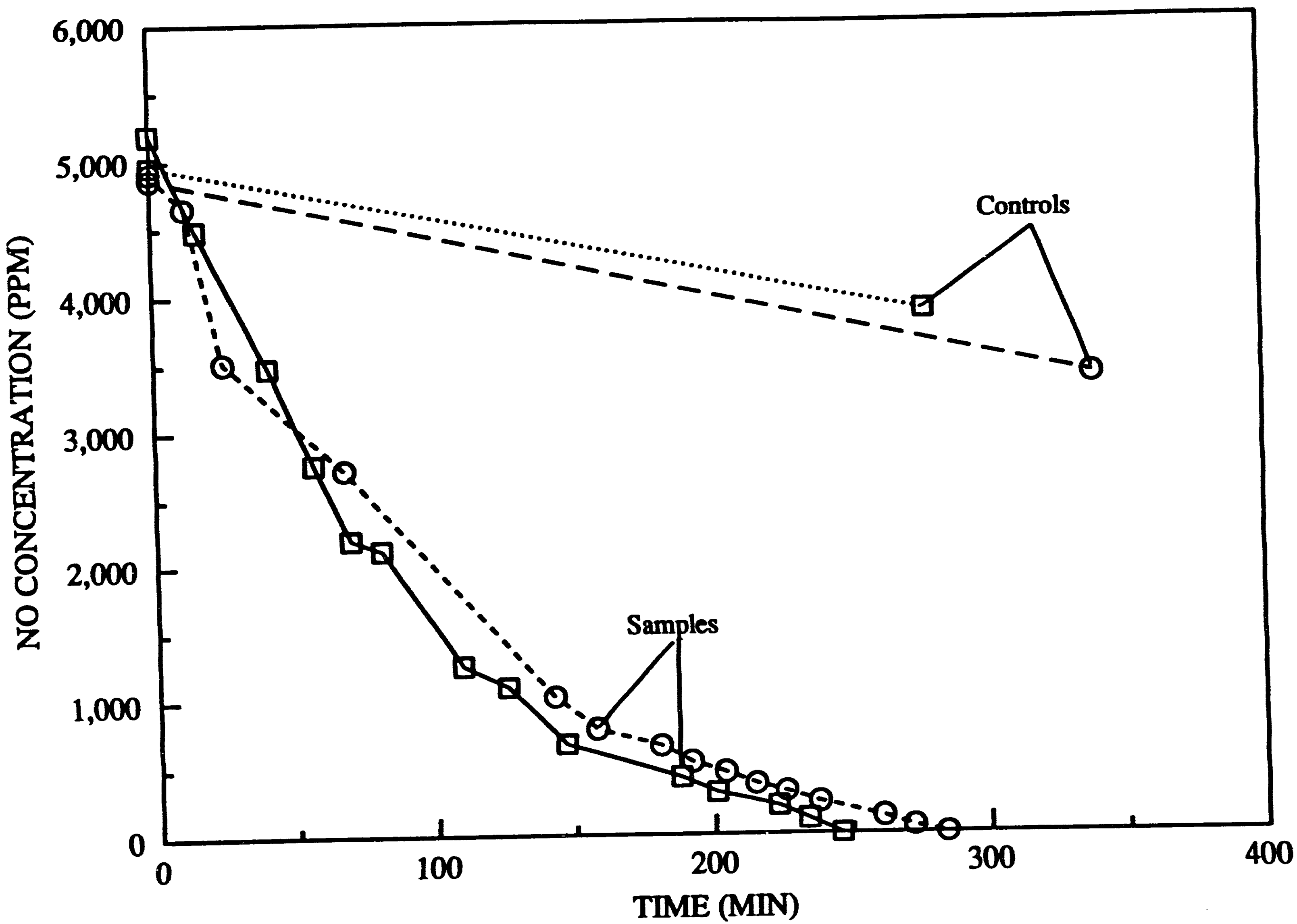


Figure 111. Uptake of nitric oxide by a suspension of *T. denitrificans* in a thiosulfate mineral salts medium without nitrate.

significant decrease in the NO concentration in control bottles. Nitric oxide depletion rates were determined relative to conrols (Table 44). From these depletion rates and the biomass protein concentrations, the specific activity of each organism for NO reduction was calculated (Table 44). *P. denitrificans* gave the highest NO depletion rates per unit weight of biomass protein under these conditions. The specific activity for NO reduction by *P. denitrificans* in serum bottle (1.15 mmoles/hr - g giomass protein) was lower than the specific activity for NO reduction by *P. denitrificans* in stirred tank reactor (24.6 mmoles/hr - g biomass protein). This pattern is indicative of NO inhibition and po r mass transfer (gas - liquid) to *P. denitrificans* in the serum bottle.

5.3.5 *Thiophaera pantotropha*

T. pantotropha (ATCC 35512) was grown anaerobically in a yeast extract/mineral salts medium (Table 45) in a Marubishi MD300 fermenter (culture volume 2L) at pH 8.0-8.2 and 30°C. When this optical density (520 nm) reached 0.6, the cells were harvested aseptically by centrifugation at 5000g and 25C and resuspended in the same medium (without nitrate) and transferred back to the fermenter. At this time, a gas feed of 0.50% NO, 5% CO_2, balance N_2 was initiated (30 mL/min). After about 20 hrs, the NO feed was replaced with N_2 at 30 mL/min. After another 95 hrs of operation, the N_2 was replaced with the 0.5% NO feed, again at 30 mL/min. The agitation rate was 450 rpm.

When NO feed was initiated, the optical density (Figure 112) began to increase while the culture soluble COD (Figure 113) began to decrease indicating growth of the organism on yeast extract components as carbon sources and NO as a terminal electron acceptor. Complete

Table 44. Specific Activity of Denitrifying Bacteria for NO Reduction

Organism	Biomass Protein (mg)	NO depletion rate (mmoles/hr)	Specific Activity (mmoles NO/hr-g protein)
A. denitrificans	14.2	7.92×10^{-3}	0.56
	14.1	9.84×10^{-3}	0.70
P. denitrificans	13.0	1.52×10^{-2}	1.17
	12.3	1.39×10^{-2}	1.13
Ps. denitrificans	12.5	1.27×10^{-2}	1.02
	12.5	1.13×10^{-2}	0.90
T. denitrificans	10.6	8.82×10^{-3}	0.83
	9.6	6.54×10^{-3}	0.68

Initial gas phase NO = 0.20 mM

Table 45. Yeast Extract/Minimal Salts Medium for *Thiosphaera pantotropha*

Component	g/L*
Na_2HPO_4	4.2
KH_2PO_4	1.5
NH_4Cl	0.3
$MgSO_4 \cdot 7H_2O$	0.1
KNO_3	5.0
Yeast Extract	3.0
Trace Element Solution (Table 14)	2.0 mL

pH 8.0-8.2

*unless otherwise indicated

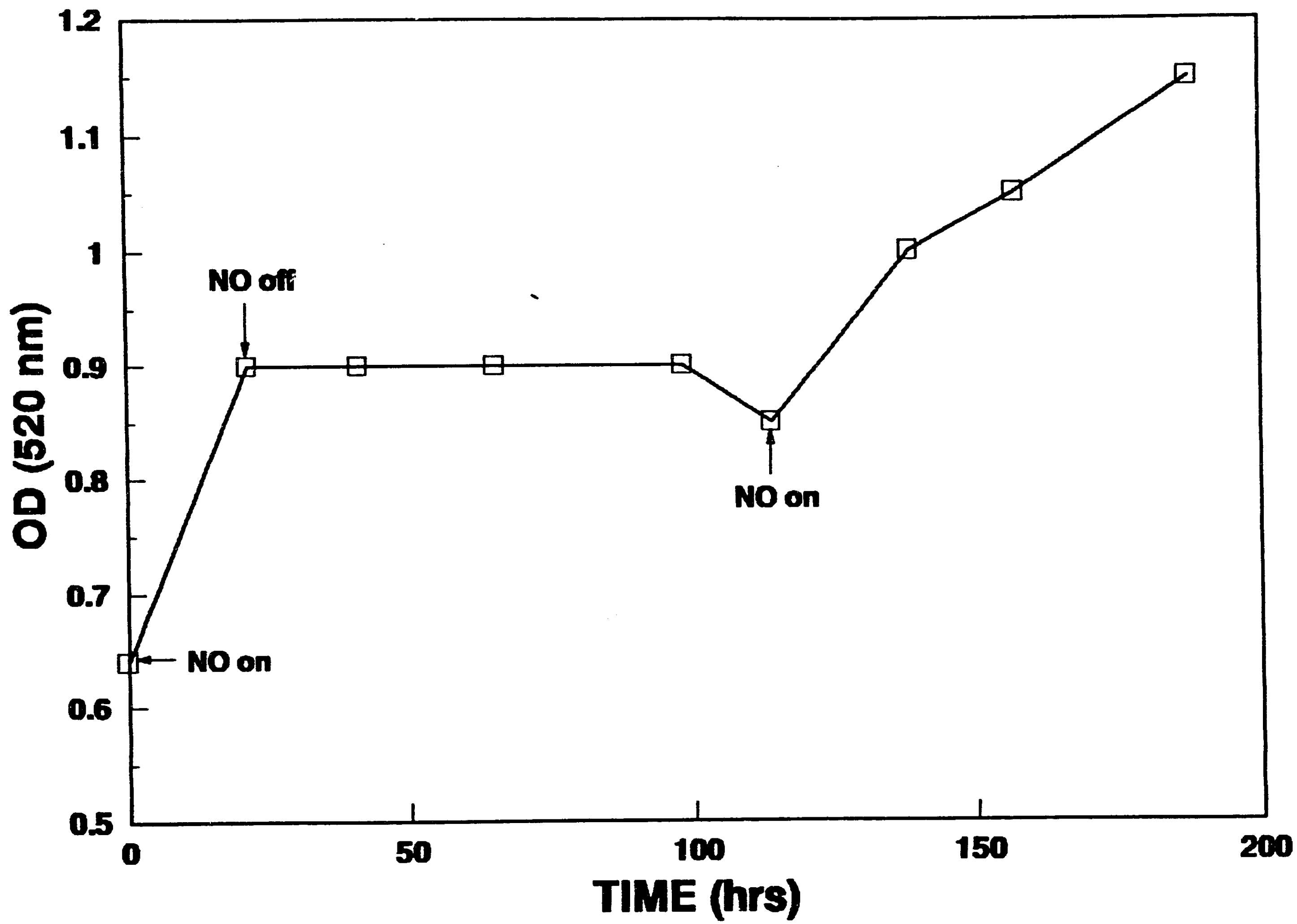


Figure 112. Optical density (520 nm) in a *T. pantotropha* culture growing on yeast extract with an intermittent NO feed.

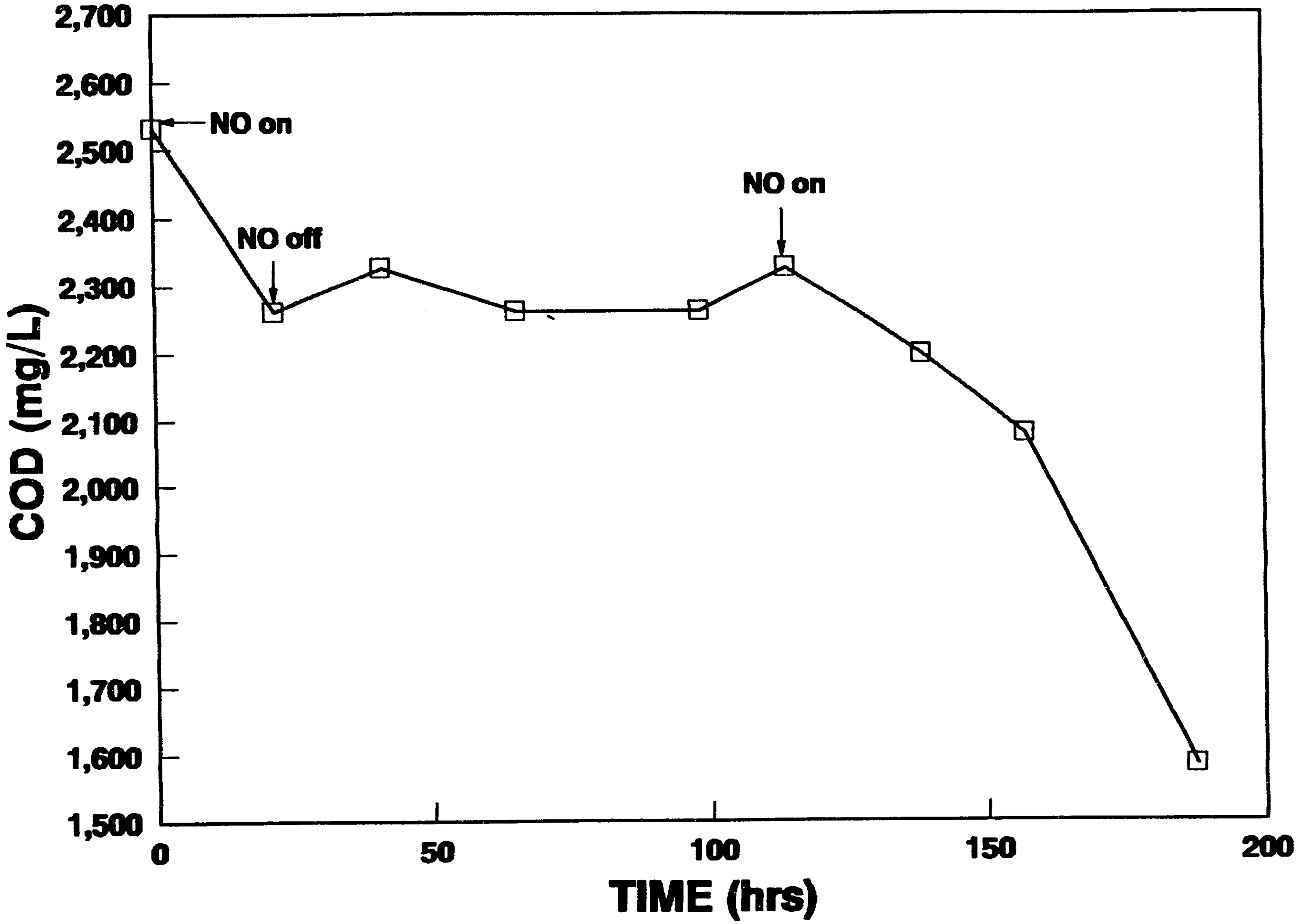


Figure 113. Soluble COD in a *T. pantotropha* culture growing on yeast extract with an intermittent NO feed.

removal of NO from the feed gas was observed. When the NO feed was replaced with N_2, both the OD and COD leveled off as seen in Figures 112 and 113. However, when the NO feed was restarted, the OD again began to increase and the soluble COD decrease. Figure 107 shows the NH_4^+ concentration during the course of this experiment. The NH_4^+ concentration was seen to increase with time while NO was available but remain constant when NO was replaced by N_2. Generating NH_4^+ corresponds to use of N-containing components of the yeast extract as carbon sources.

T. pantotropha was also grown on heat/alkali pretreated sewage sludge and NO. *T. pantotropha* was first grown on yeast extract in the minimal medium (Table 45) as described above to an optical density (520 nm) of 0.6. Cells were then harvested by centrifugation as described above and the supernatant was discarded. The cells were washed with the same medium but without nitrate, then resuspended in sewage sludge medium which was prepared as follows: 100 grams of wet-packed activated sludge was suspended in 1 L of the medium described in Table 43 without yeast extract or nitrate. The pH of this suspension was adjusted to 12 with 10 N NaOH and the suspension was autoclaved at 121 C for 30 min. The cooled suspension was adjusted to pH 7.0 with 6 N HCl. This suspension was diluted to 2 L with additional medium and transferred to the fermenter. At this time, a gas feed consisting of 0.5% NO, 5.0% CO_2 and balance N_2 was introduced at a feed rate of 30 mL/min. The agitation rate was maintained at 450 rpm. The pH and temperature were maintained at 8.0-8.2 and 30 C, respectively. The fermenter was sparged with NO feed for about 24 hours and then replaced with N_2 for about 60 hours to see if there was any growth of *T. pantotropha* in the absence of NO. After 60 hours, the NO feed was again sparged to the fermenter at a rate of 30 mL/min.

When heat/alkali pretreated sewage sludge was used as the carbon and energy source in a batch culture of *T. pantotropha* complete removal of NO was again observed. As NO was removed from the feed gas, there was corresponding decrease in the concentration in the soluble COD (Figure 115). When NO was replaced by N_2, the soluble COD remained constant. These data indicate that *T. pantotropha* was utilizing biomolecules from sewage sludge as sources of carbon and energy and NO as a terminal electron acceptor.

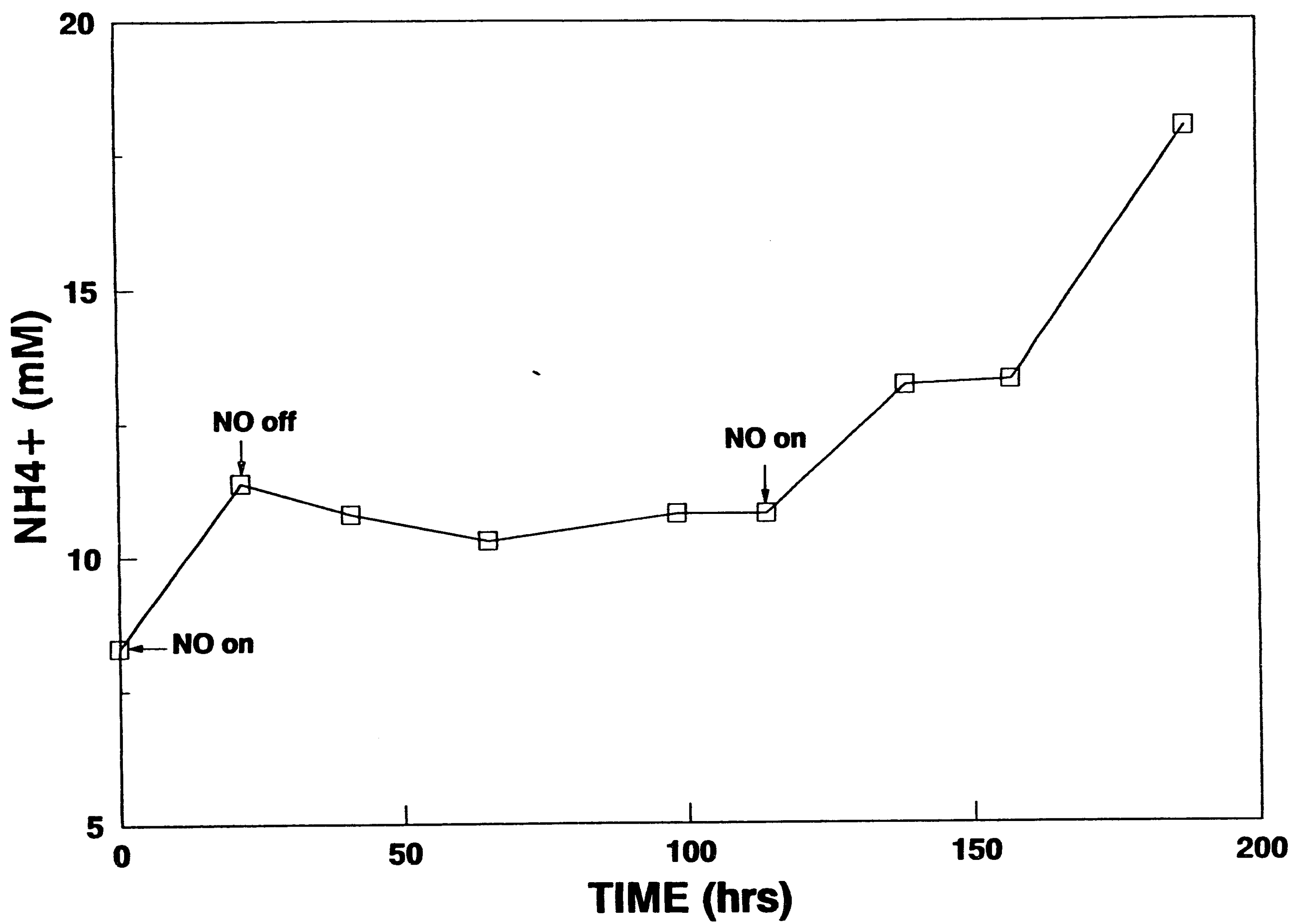


Figure 114. Ammonium ion concentration in a *T. pantotropha* culture growing on yeast extract with an intermittent NO feed.

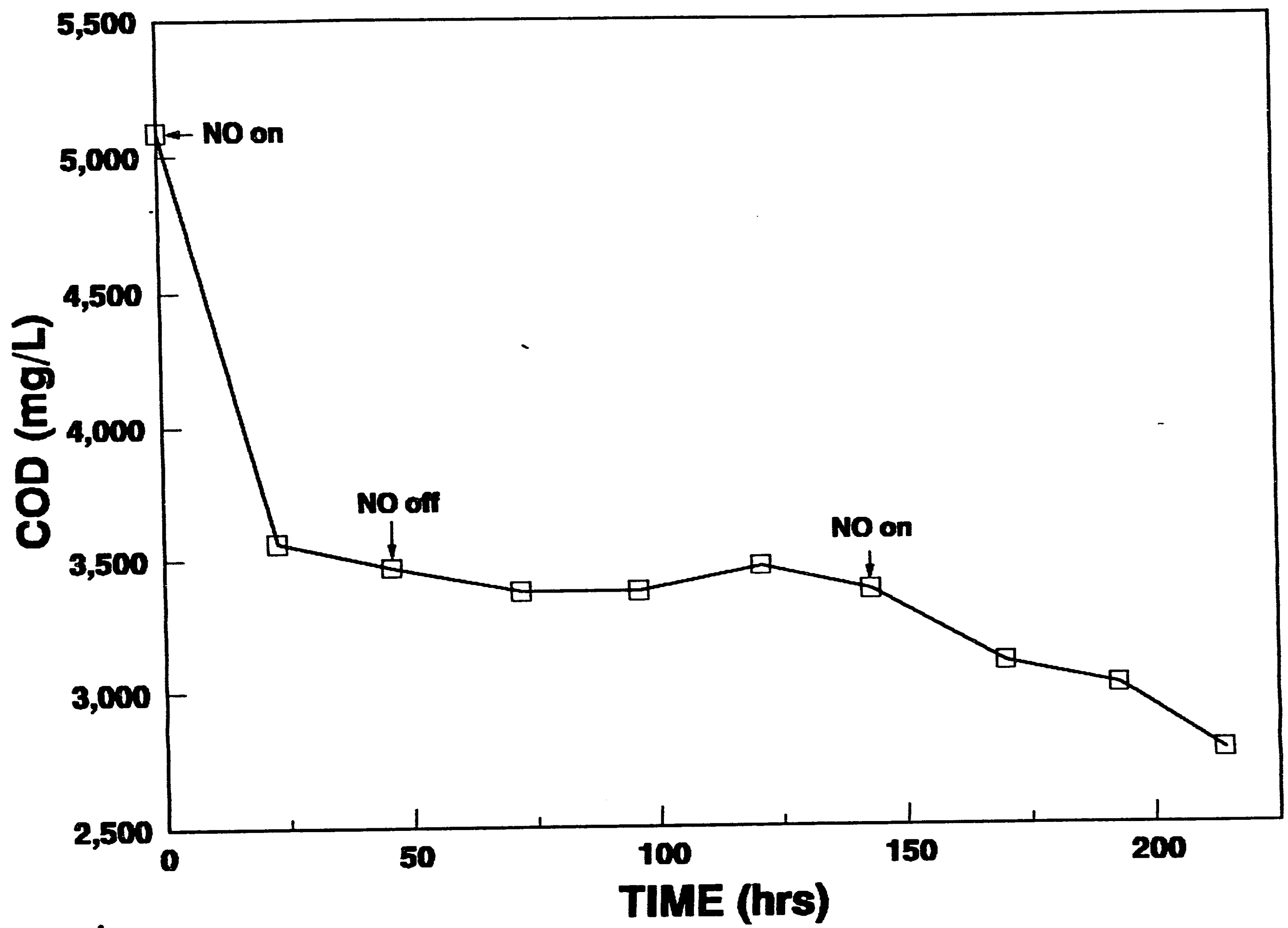


Figure 115. Soluble COD in a *T. pantotropha* culture growing on heat/alkali pretreated sewage sludge with an intermittent No feed.

6.0 WORK COMPLETED DURING THE CURRENT REPORTING PERIOD

6.1 Anaerobically Digested Sewage Solids as Carbon and Energy Sources for SO_2-Reducing Cultures of *D. desulfuricans*

6.1.1 Feed Preparation

About 3 kg of wet-packed municipal sewage solids collected from the DAF unit of a Tulsa sewage treatment plant was suspended in 13 L of sulfate-free minimal medium in a 5-gal glass bottle. The gas space was sparged with N_2, 4.4 mL $CHCl_3$ was added and the bottle sealed. This gave a $CHCl_3$ concentration of about 400 ppm. The bottle was incubated at room temperature and sampled periodically for soluble COD. These results are shown in Figure 116. Prior to use as a feedstock the mixed liquor was allowed to settle overnight and the supernatant siphoned off. This supernatant served as the feed.

6.1.2 Use of Anaerobically Digestsed Sewage Solids as a Carbon and Energy Source for SO_2-Reducing Batch Cultures of *D. desulfuricans*

About 2 g of wet-packed solids from the settler of the continuous *D. desulfuricans* reactor system with biomass recycle described in Section 5.1.7 were suspended in 1.5 L of feed preparation described above (6.1.1) in a Marubishi MD 300 fermenter. The reactor was operated at 30 C, pH 7.0, and 150 rpm with gas feeds of 400 mL/min N_2 and 10 mL/min 1.0% SO_2 (0.24 mmoles/hr). Within two hours of initiation of SO_2 feed, sulfite accumulated in the culture medium (113 mg/L). At this time, the SO_2 feed was terminated and the culture maintained with a N_2 feed overnight. The next day the sulfite concentration was < 3 mg/L and the SO_2 feed was reinitiated at 0.24 mmoles/hr. Again sulfite accumulated rapidly in the culture medium. These results

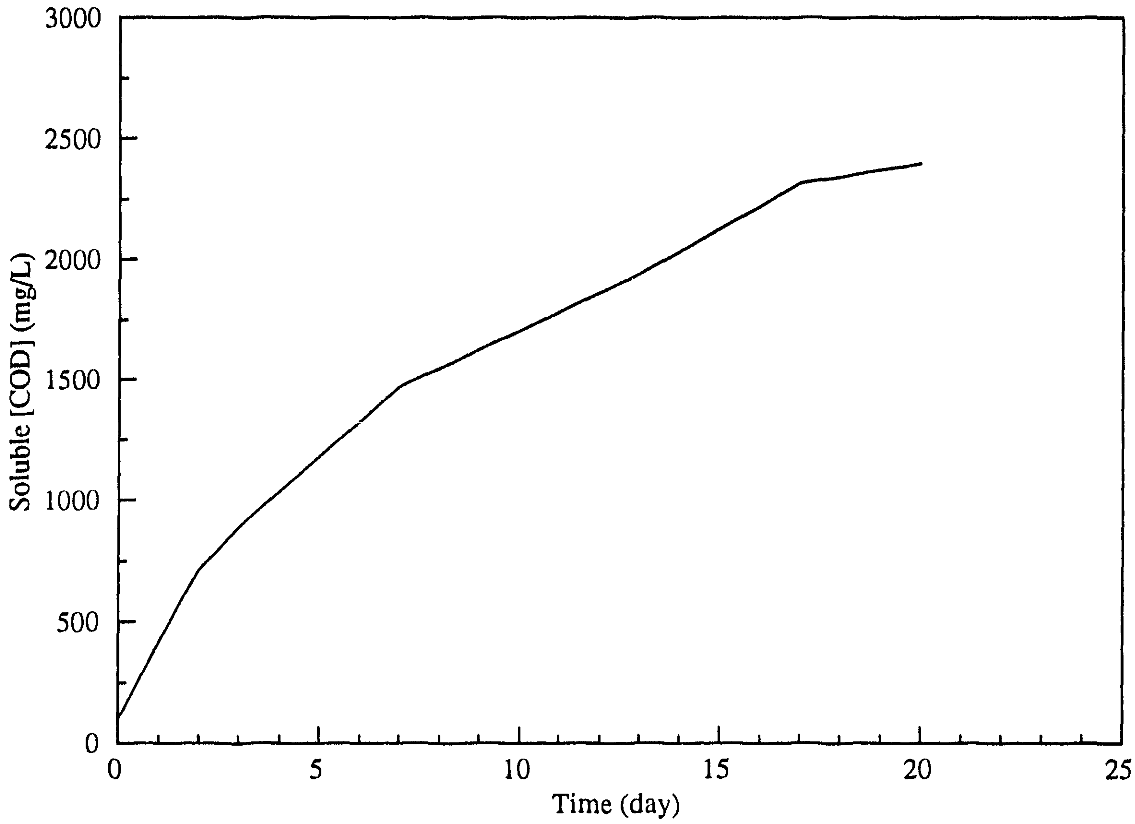


Figure 116. Accumulation of soluble COD during anaerobic digestion of municipal sewage sludge solids in the presence of 400 ppm chloroform.

indicated that 1) the feedstock did not contain compounds that could be used as carbon and energy sources by the culture, 2) the inoculum did not contain sufficient SRB to reduce the SO_2, or 3) the inoculum did not contain sufficient numbers of non-SRB heterotrophs to allow the feedstock to be used as a carbon and energy source.

The reactor medium was centrifuged to recover biosolids and resuspended in 1.5 L of sulfate-free medium containing 1 g/L yeast extract. This suspension was returned to the fermenter which was operated as before at 150 rpm, 30 C and pH 7.0. When the suspension had reached temperature, an SO_2 feed was initiated at 10 mL/min 1.0% SO_2 (0.24 mmoles/hr). Within 3 hrs sulfite was detected in the medium (100 mg/L). The SO_2 feed was turned off overnight and reinitiated the next morning with the same results. Since yeast extract is readily used by mixed cultures of *D. desulfuricans* and fermentative heterotrophs as a carbon and energy source, this experiment suggests that 1) the settler solids used to inoculate this batch reactor did not contain sufficient *D. desulfuricans* to reduce 0.24 mmoles/hr SO_2 or 2) the settler solids did not contain sufficient fermentative non-SRB heterotrophs to ferment the yeast extract components and make substrates available to *D. desulfuricans*.

At this point, effluent (500 mL) from the gravity settler (Section 5.1.7) was centrifuged at 5000 g at 25 C to collect cells to small to be collected in the settler. These cells were suspended in a small amount of buffer and added to the Marubishi fermenter. When the SO_2 feed was reinitiated little or no sulfite was observed in the medium at SO_2 feed rates of up to 0.48 mmoles/hr. Evidently, a significant fraction of the *D. desulfuricans* cells in the effluent of the continuous reactor described in Section 5.1.7 are not associated with the gravity-settleable solids.

After operating at these conditions for two days, the reactor biosolids were recovered by centrifugation at 5000 g and 25 C and resuspended in 1.5 L of the anaerobically digested sewage solids feed (Section 6.1.1) and transferred back to the Marubishi fermenter (30 C, pH 7.0, 150 rpm). A SO_2 feed of 10 mL/min 1.0% SO_2 (0.24 mmoles/hr) was then initiated. The culture medium was analyzed periodically for sulfite and soluble COD. Complete conversion of SO_2 to H_2S was observed with sulfite concentrations less than 5 mg/L at SO_2 feed rates as high as 1.1 mmole/hr. The COD concentrations are shown in Figures 117. Sulfite did not accumulate in the culture medium until the soluble COD dropped to about 500 ppm. However, clearly the anaerobically digested sewage solids medium contains usable carbon and energy sources for SO_2-reducing mixed cultures of *D. desulfuricans* and fermentative heterotrophs when suitable numbers of both types of organisms are present.

Additional experiments of this type with various media were conducted to determine the relative importance of the settler solids and cells obtained from the settler effluent as components of an inoculum (and recycle stream in a continuous system). These results are summarized in Table 46. Although both settler solids and effluent solids alone could in some cases support SO_2

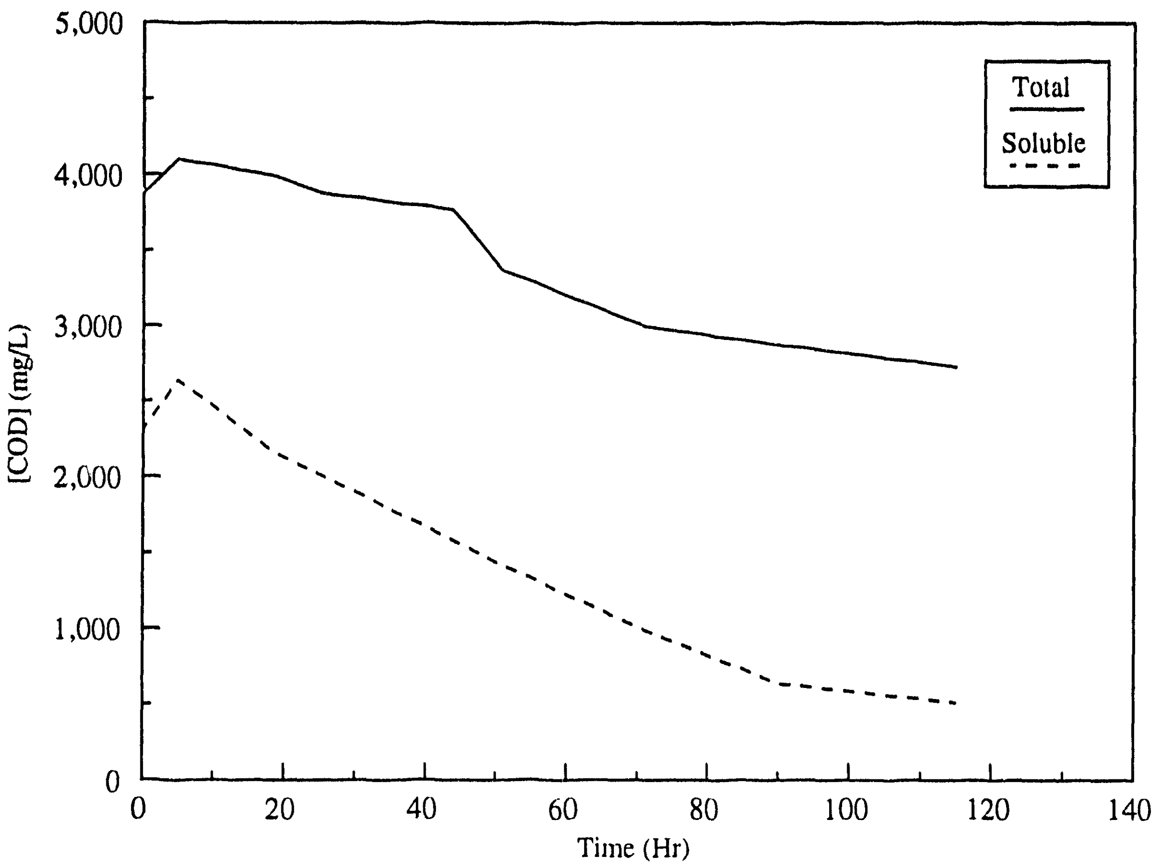


Figure 117. Utilization of soluble COD in a batch culture of *D. orientis* in anaerobically digested municipal sewage solids medium with an SO_2 feed.

Table 46. Performance of Settler Solids or Effluent Solids As Inocula of Support SO_2 Reduction

Medium	Inoculum	mmoles/hr SO_2 Feedrate	SO_2 Reduction
AD-MSS (Batch 1)	2g settler solids	0.24	-
	2g settler solids+ effluent solids (0.5 L)	1.1	+
SSM-YE	2g settler solids	0.24	-
	2g settler solids + effluent solids (0.5 L)	0.48	+
H/A-MSS	effluent solids (0.96 L)	0.24	-
	effluent solids (0.96 L) + 2g settler solids	0.48	+
	2g settler solids	0.48	+
AD-MSS (Batch 2)	2g settler solids	0.48	+
	effluent solids (1.2 L)	0.24	+
		0.48	-
	2g settler solids + effluent solids (1.7)	0.88	+

AD-MSS = anaerobically digested municipal sewage solids medium

SSM-YE = sulfate-free minimal medium with 1g/L yeast extract

H/A-MSS = heat/alkali pretreated municipal sewage sludge medium

Settler solids = solids collected by gravity settling from settler of continuous SO_2-reducing system (Section 5.1.7), wet-weight

Effluent solids = solids collected by centrifugation of effluent from settler at 5000g and 25 C. number in parenthesis indicates volume of effluent centrifuged.

reduction, best performance was observed with both an inocula. We suspect that the settler solids are rich in non-SRB heterotrophs and the effluent solids rich in SRB.

6.1.3 Use of Anaerobically Digested Sewage Solids as a Carbon and Energy Source for a Continuous SO_2-Reducing Culture of *D. desulfuricans*

As noted above, it was demonstrated that anaerobically digest sewage solids medium prepared as described in Section 6.1.1 could support SO_2 reduction in mixed batch cultures of *D. desulfuricans*. In the current reporting period, we have replaced the heat/alkali pretreated sewage sludge medium with the new anaerobically digested sewage sludge (AD-MSS) medium in the continuous SO_2-reducing culture of *D. desulfuricans* with biomass recycle (Section 5.1.7).

Two days prior to the introduction of the AD-MSS feed, the SO_2 feed rate in the reactor was reduced to 1.4 mmoles/hr. The AD-MSS feed was introduced at a rate of 30 mL/hr. This is nearly 4 times higher than previous rate because the COD concentration in the AD-MSS feed is 4 times lower than in the heat/alkali pretreated sewage sludge medium. At this time, the recyling rate was also increased to 90 mL/hr (from 60 mL/hr). Initially, there was no sulfite accumulation in the reactor; however, the protein concentration, COD and MLSS started decreasing (Figures 118-120). After 2 days, the sulfite concentration in the reactor medium (Figure 121) increased sharply. At this time, the SO_2 feed rate was reduced to 0.98 mmoles/hr. The reactor then operated for another 13 days without further significant sulfite accumulation. However, the decrease in protein, COD and MLSS continued. After

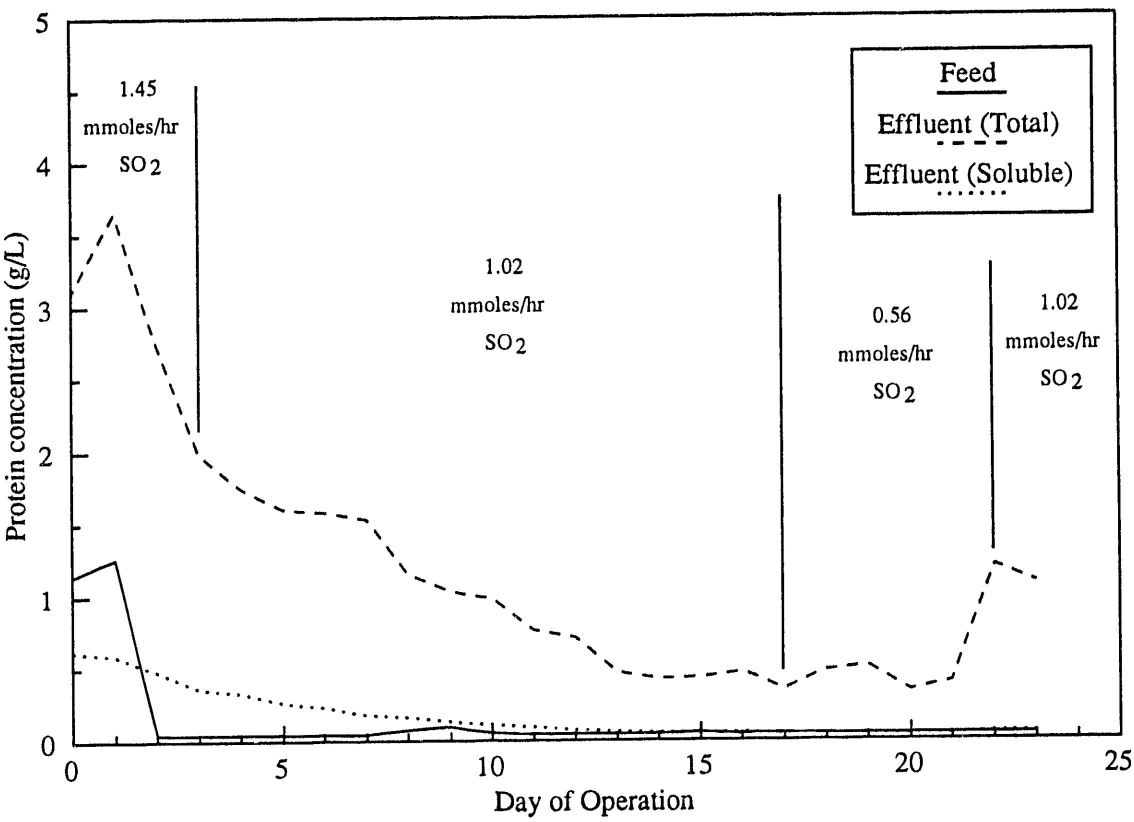


Figure 118. Protein concentration in the effluent of a continuous SO_2-reducing culture of *D. desulfuricans* (with biomass recycle) receiving a feed of anaerobically digested municipal sewage sludge medium.

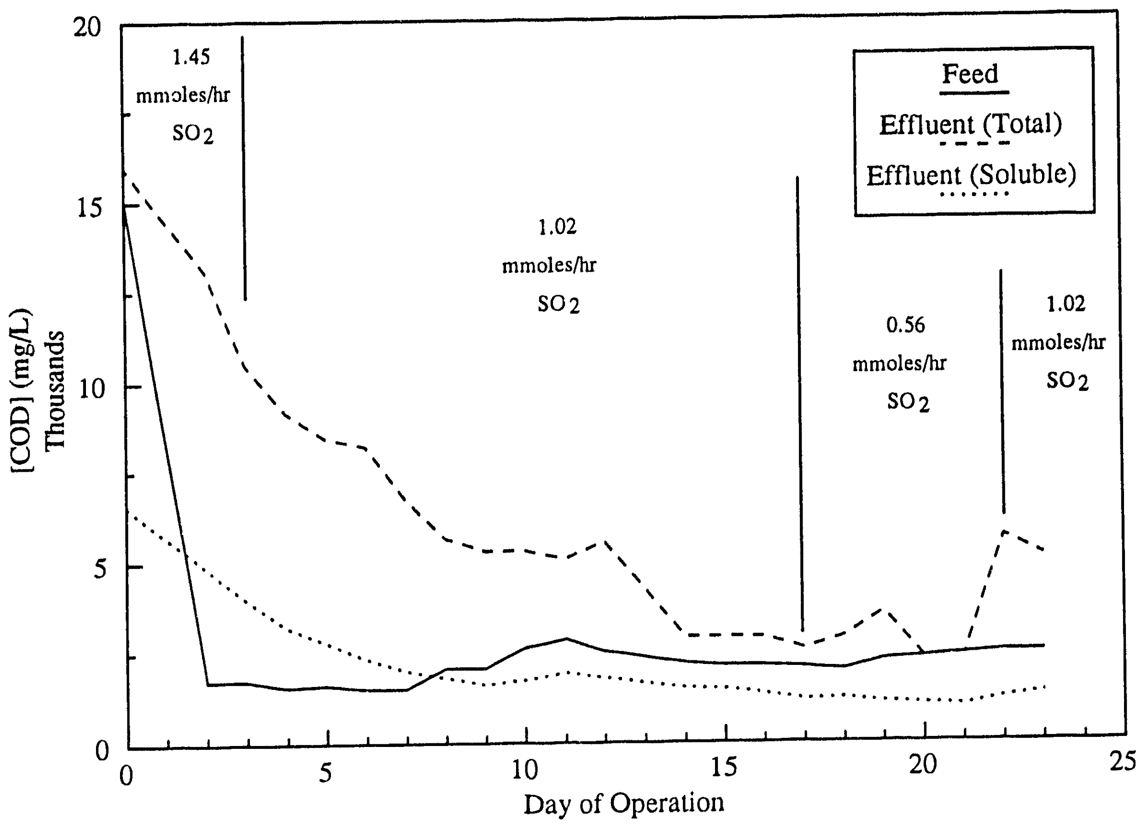


Figure 119. COD concentration in the effluent of a continuous SO_2-reducing culture of *D. desulfuricans* (with biomass recycle) receiving a feed of anaerobically digested municipal sewage sludge medium.

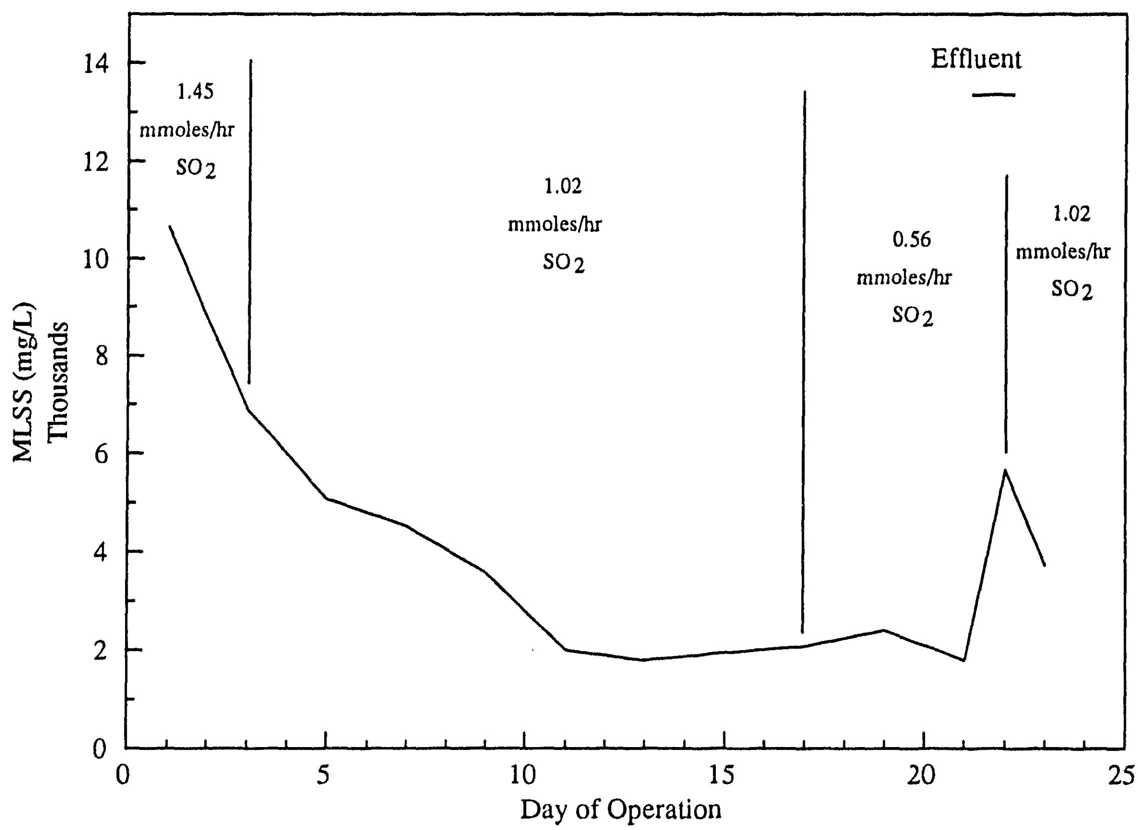


Figure 120. MLSS concentration in the effluent of a continuous SO_2-reducing culture of *D. desulfuricans* (with biomass recycle) receiving a feed of anaerobically digested municipal sewage sludge medium.

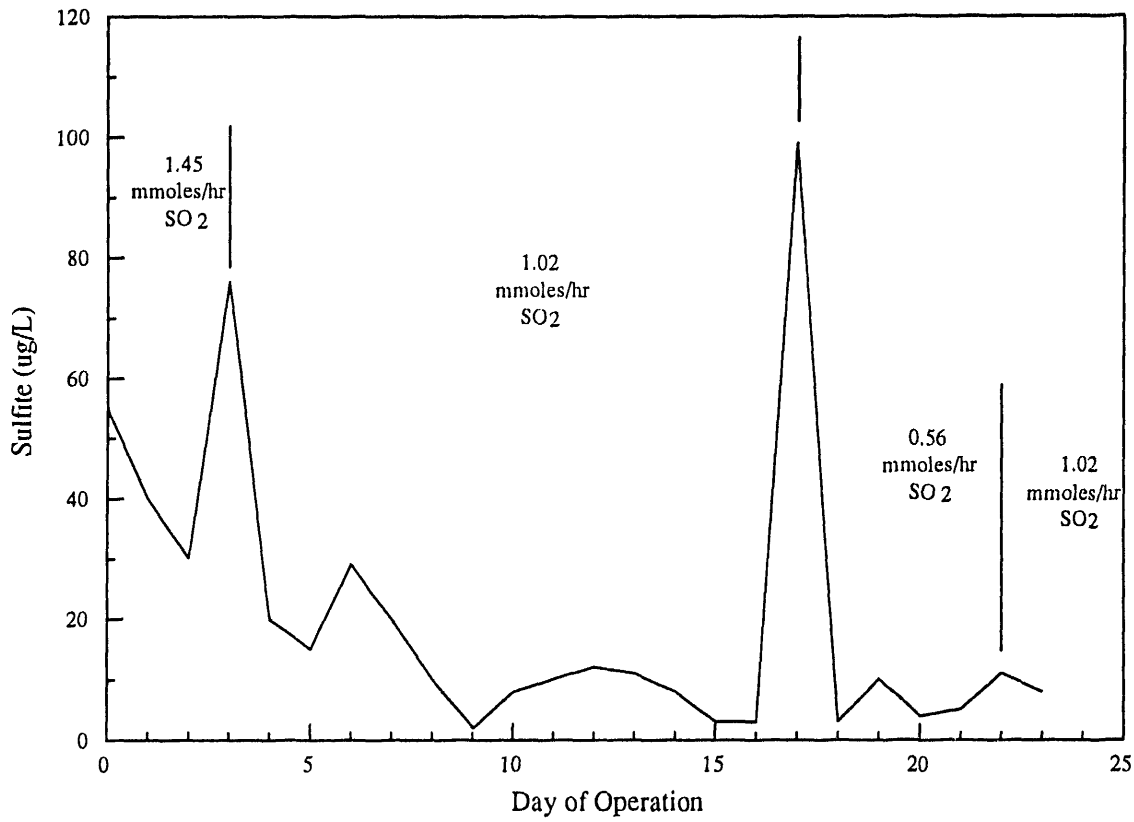


Figure 121. Sulfite concentration in the effluent of a continuous SO_2-reducing culture of *D. desulfuricans* (with biomass recycle) receiving a feed of anaerobically digested municipal sewage sludge medium.

13 days at an SO_2 feed rate of 0.98 mmoles/hr, the sulfite concentration again increased sharply. The SO_2 feed rate was then reduced again to 0.55 mmoles/hr. At this time, the feed and recycle streams were turned off and the reactor allowed to operate batch-wise for 48 hrs. No sulfite accumulation was observed during this time indicating that the previous upset condition resulted from insufficient SO_2-reducing biomass in the reactor as opposed to a nutrient limitation.

The considerable decrease in MLSS in the reactor with the change in feed and feed rate has resulted from poor recovery of solids in ther settler. This seems to be due to loss of good settling characteristics of solids that were previously recovered with heat/alkali pretreated sewage solids feed and less residence time in the settler. The latter is especially important with regard to the poorly settling fraction which may contain the majority of the SRB's. In any regard, the loss of biosolids from the system has led to major reductions in volumetric productivity of the system in terms of SO_2 reduced per unit volume per unit time.

In order to correct this situation, we have begun to collect the settler effluent, harvest biosolids daily and return then to the reactor. This was begun on day 21 following initiation of the AD-MSS feed.

6.2 Use of SO_2 as Both Energy Source and Terminal Electron Acceptor by *D. orientis*

In the current reporting period, we have investigated the elimination of H_2 as an energy source in SO_2-reducing cultures. In these cultures SO_2 would serve as an energy source with oxidation to sulfate and also a terminal electron acceptor with reduction to H_2S.

A new batch culture of *D. orientis* was developed on lactate/sulfate and switched to a $H_2/CO_2/SO_2$ gas feed as described in Section 5.2.3. The initial gas feed to this culture was 58 mL/min H_2; 11.8 mL/min SO_2, 5% CO_2, balance N_2; and 91.2 mL/min 5% CO_2 balance N_2. During this time, complete reduction of SO_2 to H_2S was observed with no production of sulfate or accumulation of sulfite in the culture medium (Figure 122). On the eighth day following initiation of $H_2/CO_2/SO_2$ feed, the source of H_2 was changed to 10% H_2, 5% CO_2 balance N_2. The feed rate of this gas was subsequently reduced and finally eliminated as indicated in the following outline of observations and operational changes:

Day 1-7 - 58 mL/min 100% H_2; 11.8 mL/min 1% SO_2, 5% CO_2 bal N_2; and 91.2 mL/min 5% CO_2 bal N_2.

Day 8 - Changed 100% H_2 to 15.9 mL/min 10% H_2, 5% CO_2 bal, and 5% CO_2, bal N_2; to 100% N_2 (at 80.5 mL/min). SO_2 feed rate unchanged.

Day 15 - Decreased H_2 flowrate to 9.7 mL/min.

Day 17 - Decreased H_2 flowrate to 5.8 mL/min.

Day 23 - Turned off H_2. SO_3^{-2} gradually accumulated to 90 mg/L. Turned on H_2 at 5.8 mL/min overnight (SO_2 @ 11.8 mL/min)

Day 24 - SO_3^{-2} was 103 mg/L in the am
Turned off SO_2 for 4 hrs and turned it back on at 11.8 mL/min, SO_3^{-2} began to accumulate again. Decreased SO_2 to 7.6 mL/min.

Day 27 - No sulfite, increased SO_2 to 9.3 mL/min.

Day 28 - Turned off H_2 for 2 days. SO_3^{-2} gradually accumulated to 111 mg/L.

Day 31-Day 36 - Decreased SO_2 to 7.6 mL/min, SO_3^{-2} still accumulated.

Day 37-Day 41 - Added lactic acid (7g of 60% sodium lactate per day, no H_2 feed).

Day 42 - Resuspended cells in fresh autotrophic medium. Reinitiated feed of 5.8 mL/min 10% H_2, 5% CO_2, bal N_2; 9.3 mL/min 1% SO_2, 5% CO_2, bal N_2; and 80.5 mL/min N_2.

Day 48 - Turned off H_2. Other gas feeds unchanged.

Day 62 - SO_3^{-2} began to accumulate, turned off SO_2.

Day 63 - Centrifuged, resuspended cells in fresh medium
Started the reactor at 7.6 mL/min SO_2 and 80.5 mL/min N_2, SO_3^{-2} began to accumulate immediately. SO_2 was then decreased to 3.8 mL/min, but SO_3^{-2} increased to 97.5 mg/L. Turned off SO_2 and turned on 10% H_2 at 3.1 mL/min.

Day 64 - Turned off H_2, SO_3^{-2} went up to 78 mg/L and then dropped to 4 mg/L in the afternoon.

Day 65 - 15 mg/L SO_3^{-2}

Day 66 - SO_3^{-2} was averaged 55 mg/L during the day. Decreased SO_2 to 5.9 mL/min.

Day 71 - SO_3^{-2} began to accumulate again. Turned off SO_2.

As seen in Figure 122, sulfate production was again stimulated by low H_2 partial pressure in the feed gas, i.e. H_2 starvation. However, repeated attempts from Day 23-37 to eliminate H_2 entirely resulted in accumulation of sulfite in the culture medium. During this time, there was a dramatic decrease in the biomass protein concentration (Figure 123) indicating a high death rate in the culture during H_2 starvation. Figure 124

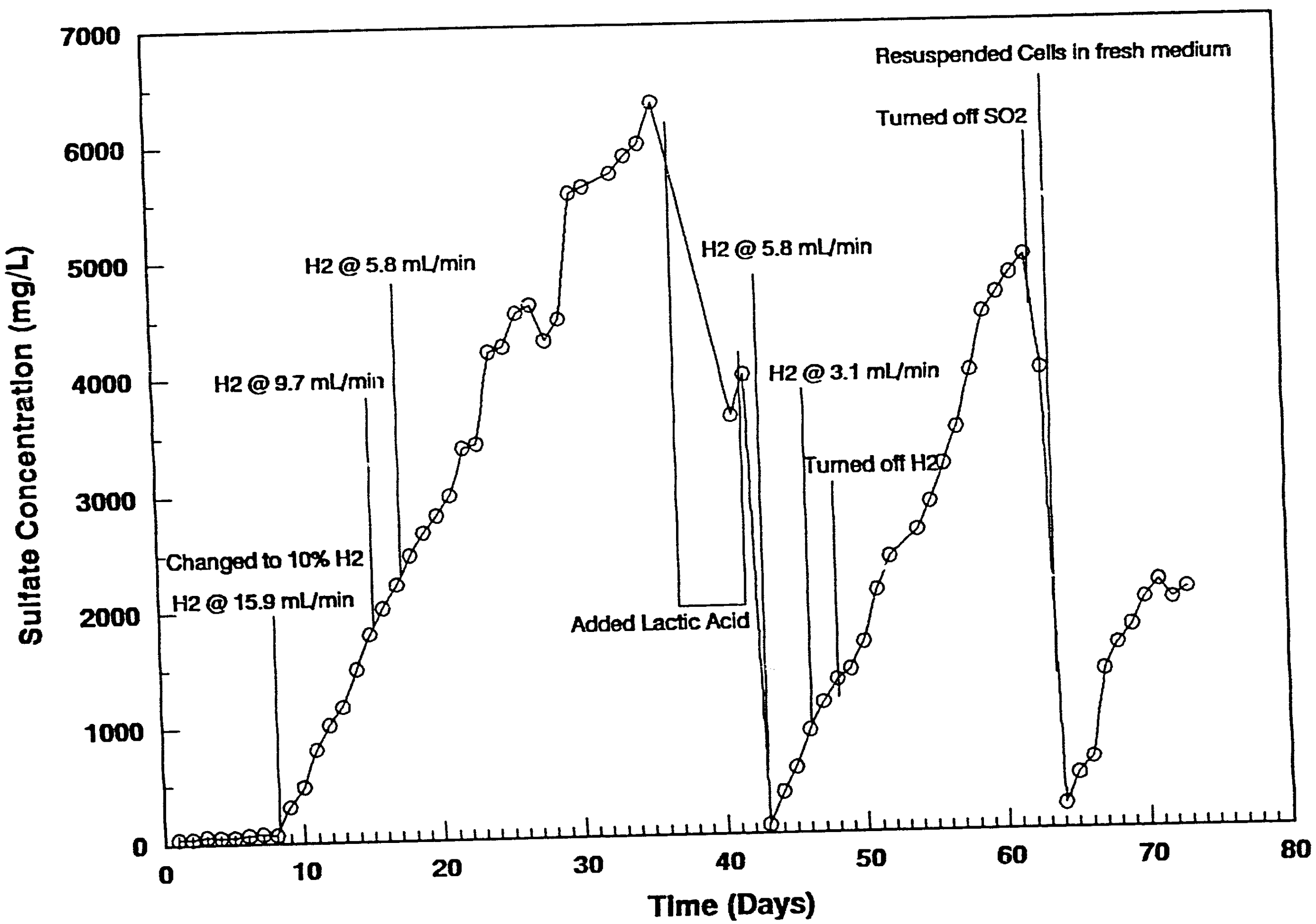


Figure 122. Sulfate concentration in a batch *D. orientis* culture receiving a gas feed of SO_2/CO_2 and intermittently H_2 under H_2 starvation conditions.

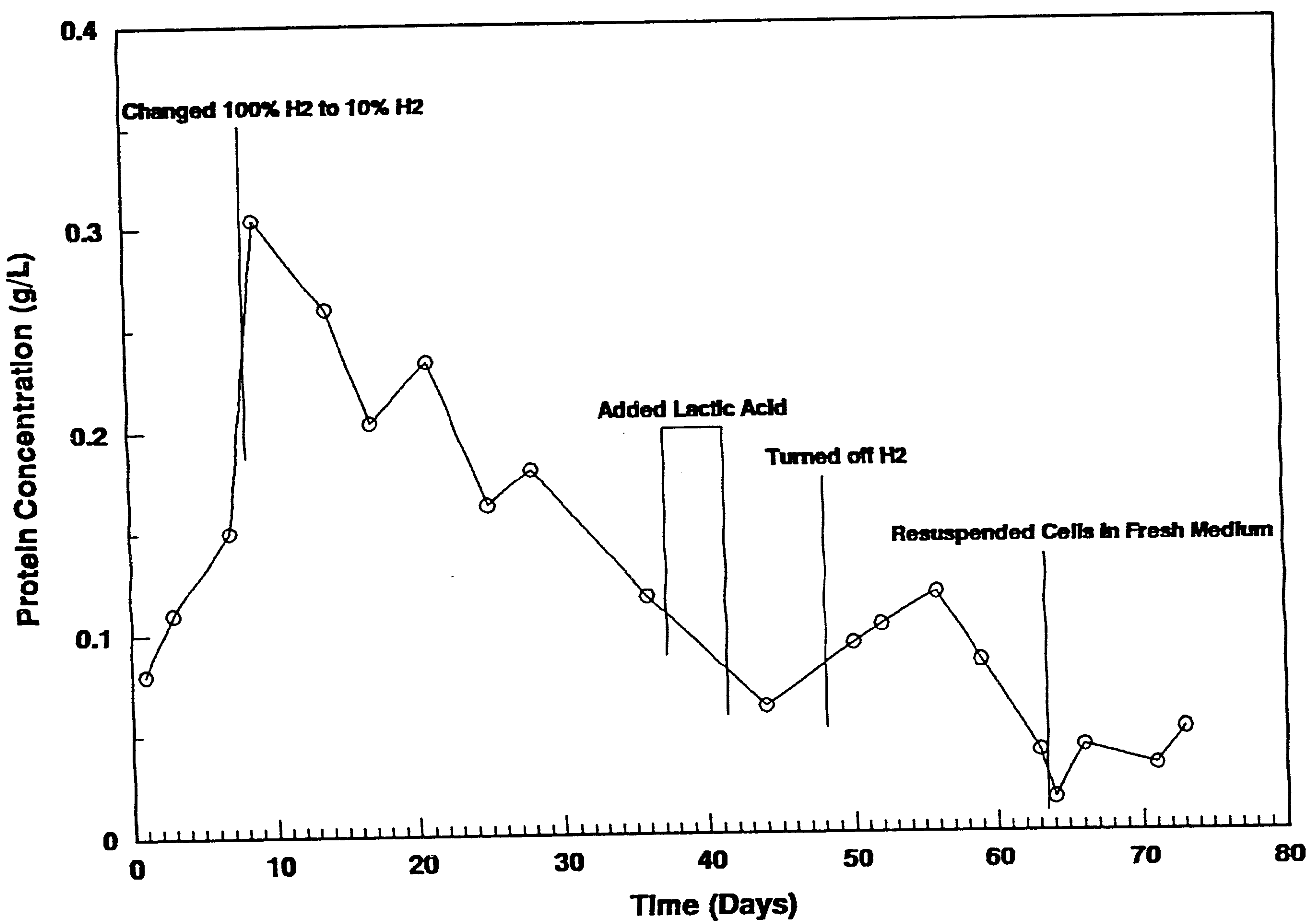


Figure 123. Protein concentration in a batch *D. orientis* culture receiving a gas feed of SO_2/CO_2 and intermittently H_2 under H_2 starvation conditions.

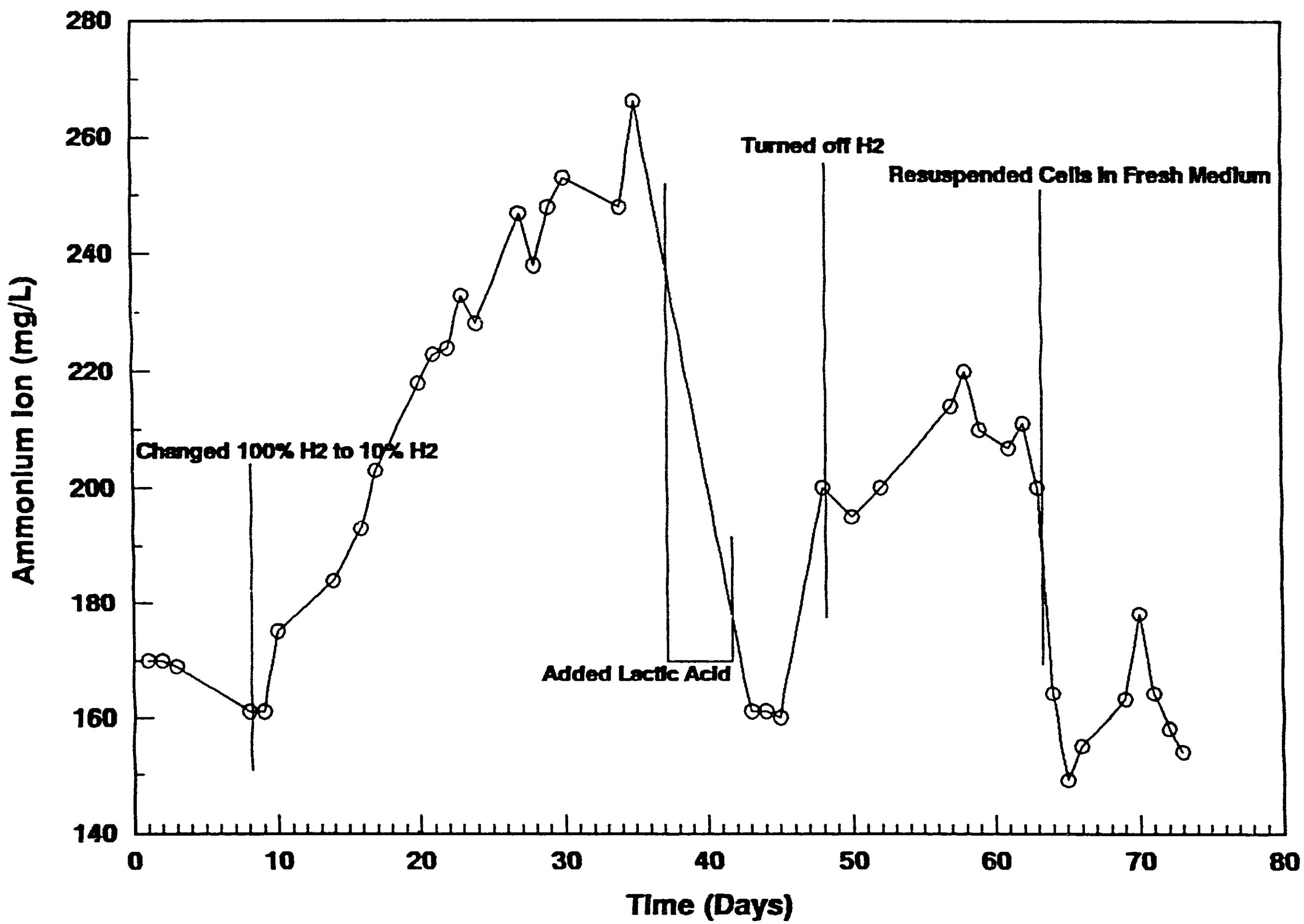


Figure 124. NH_4^+ concentration in a batch *D. orientis* culture receiving a gas feed of SO_2/CO_2 and intermittently H_2 under H_2 starvation conditions.

shows that there was a corresponding increase in the ammonium ion concentration. This may have resulted in lysis of *D. orientis* and metabolism of *D. orientis* protein by mixed heterotrophs in the culture or simply endogenous metabolism of macromolecules by *D. orientis* under these energy starvation conditions.

Sulfate also accumulated during the 20 days (in two periods) that the culture operated without H_2 feed. Table 47 gives sulfur balances with low partial pressure H_2 feed and without H_2. As shown in Table 47, while sulfate was accumulating, the (H_2S + SO_4^{-2})/SO_2 ratio averaged 0.95 indicating that essentially all of the SO_2 was either oxidized to sulfate or reduced to H_2S. Table 47 also shows that production of sulfate relative to H_2S increased with decreasing availability of H_2. This is not surprising since the loss of H_2 as an energy source would be expected to result in greater utilization of SO_2 as an energy source.

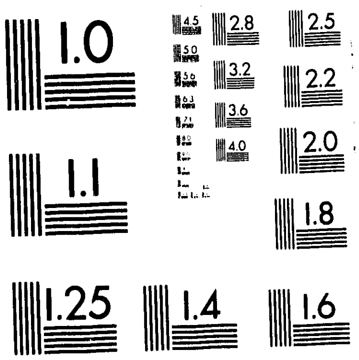
The stoichiometry of sulfate production relative to H_2S production is detailed in Table 48. Also shown in Table 48 is the rate of production of reducing equivalents from oxidation of SO_2 to sulfate compared to the rate of consumption of reducing equivalents from reduction of SO_2 to H_2S. As shown in Table 48, there was consistently an excess of reducing equivalents produced by SO_2 oxidation suggesting that when only SO_2 was available as an energy source some other species in addition to SO_2 also acted as an electron acceptor. In the first period of operation without H_2 feed (Day 48-62), sulfite began to accumulate at the end of this time. Replenishment of the medium resulted in a

Table 47. Sulfur Balance in D. orientis Batch Culture with SO_2 Feed Under Conditions of H_2 Starvation

Day	10% H_2 (mL/min)	SO_2 in (mmol/hr)	H_2S Feed (mmol/hr)	SO_4^{-2} produced (mmol/hr)	$(H_2S/SO_4^{-2})/SO_2$
9	15.9	0.290	0.129	0.144	0.94
10	15.9	0.290	0.120	0.134	0.88
14	15.9	0.290	0.141	0.171	1.08
16	9.7	0.290	0.119	0.126	0.85
22	5.8	0.290	0.105	0.182	0.99
45	5.8	0.228	0.0485	0.172	0.97
48	3.1	0.228	0.0474	0.162	0.92
50	0.0	0.228	0.0267	0.201	1.00
51	0.0	0.228	0.0207	0.209	1.01
52	0.0	0.228	0.0230	0.177	0.88
69	0.0	0.146	0.0239	0.110	0.92
70	0.0	0.146	0.0236	0.108	0.90

Table 48. Stoichiometry of SO_2 Reduction and Oxidation in *D. Orientis* Batch Culture UnderConditions of H_2 Starvation

Day	10% H_2 (mL/min)	SO_4^{-2}/H_2S	Reducing eq. prod (mmol/hr)	Reducing eq. consumed (mmol/hr)
9	15.9	1.12		
10	15.9	1.12		
14	15.9	1.21		
16	9.7	1.06		
22	5.8	1.73		
45	5.8	4.20		
48	3.1	3.41		
50	0	7.52	0.402	0.160
51	0	10.1	0.418	0.124
52	0	7.69	0.354	0.138
69	0	4.61	0.220	0.143
70	0	4.57	0.216	0.142


1.0
2.8
2.5
3.2
2.2
3.6
2.0
4.0
1.1
1.8
1.25
1.4
1.6

second period of operation without H_2 feed (Day 64-71) without significant sulfite accumulation. However, SO_2 feed rates had to be reduced. Replenishment of the medium may have provided needed electron acceptors.

We are continuing to use this culture to study the stoichiometry of SO_2 oxidation and reduction by *D. orientis*.

6.3 Porphyrin-catalyzed Reduction of Nitric Oxide

Porphyrins are naturally occurring biomolecules containing tetrapyrolle ring structures which are metal chelating and may coordinate with a single metal cation (Figure 125). In nature, porphyrins are found complexed with a metal cation such as Fe^{+2} in hemoglobin or Mg^{+2} in chlorophyll. In hemoglobin, the chelated or bound metal ion is directly involved in the bonding of oxygen. In chlorophyll, the Mg^{+2}-porphyrin complex is involved in the adsorption of light in photosynthesis. Porphyrins are also found in the active sites of enzymes involved in oxidation/reduction reactions such as enzymes involved in the reduction of oxides of nitrogen in denitrifying bacteria.

In the current reporting period, we have investigated the porphyrin-catalyzed reduction of NO. Porphyrin-metal complexes offer considerable advantages over the use of viable microorganisms. The use of a viable microorganism requires the maintenance of conditions conducive to viability and growth of the organisms; i.e., nutrients, temperature, pH, oxygen concentration, etc. Porphyrin-metal complexes in general are much less sensitive to environmental conditions than are viable microorganisms or even in vitro enzymes. Porphyrin-metal complexes, for example, are tolerant of temperatures which denature all known enzymes exhibiting melting points (without decomposition) as high as 200-300°C.

The role for cobalt-centered hematoporphyrin in the reduction of nitric oxide (NO) was investigated. The reactions were monitored directly using Fourier Transform Infrared

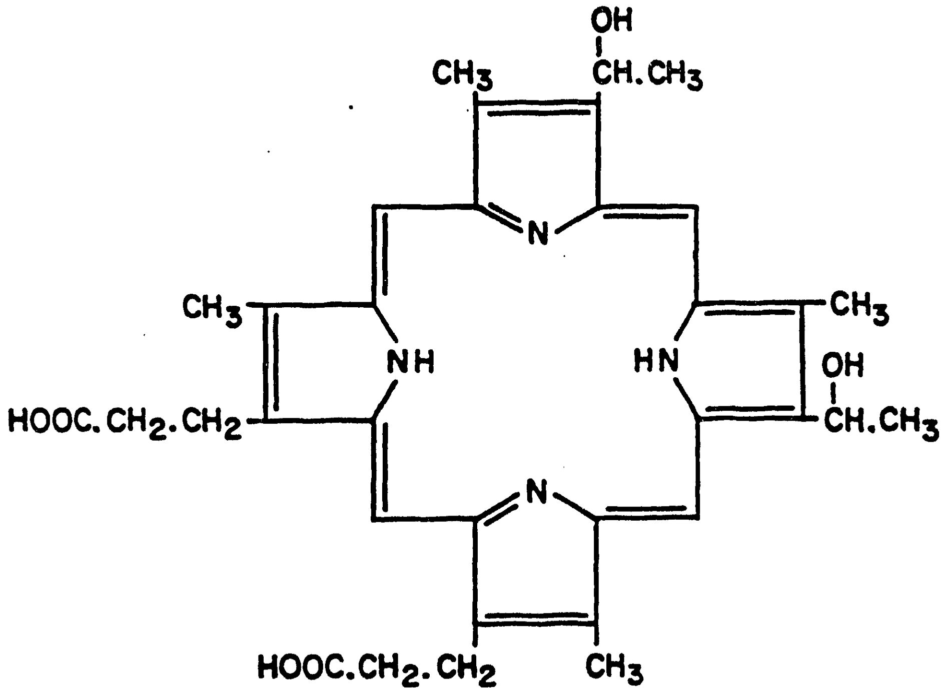


Figure 125. Hematoporphyrin

Spectroscopy (FTIR) in which the vapor phase spectra were recorded directly above aqueous solutions containing the hematoporphyrin. This procedure allowed for continuous, quantitative monitoring for NO reduction as well as for the production of other gaseous products which are infrared active.

Aqueous solutions of Co^{+3}-hematoporphyrin (2,4 hydoxyethyl-6,7 proprionic acid-1,3,5,8 tetramethyl porphyrin) catalyzed the reduction of NO in the presence of dithiothreitol (DTT) reducing agent. Anaerobic solutions containing 0.9 μmole Co^{+3}-hematoporphyrin and 0.5 mmoles of DTT were prepared directly in a 10 cm pathlength gas cell containing CaF_2 windows (total volume 30 cm^3, total solution volume 4 ml). FTIR of the vapor phase above the aqueous solution were obtained directly upon initiation of the reaction by the addition of 0.12 mmoles of NO. No attempt was made to facilitate mass transfer between the gas/solution interface. Under anaerobic conditions, the NO vibrational stretch bands between 1800 cm^{-1} and 1900 cm^{-1} could be observed following subtraction of water vapor bands. The NO concentration exhibited a steady decrease in concentration which was correlated to the production of nitrous oxide (N_2O) with its distinct vibrational stretch bands near 2200 cm^{-1}. The half-life for NO elimination under these conditions with limitations in mass transfer and DTT reducing power was on the order of two hours.

A similar experiment was conducted in which the reaction was followed by gas chromatography. To a 125 mL serum bottle was added 5 mL of a reaction mixture containing 0.36 mM Co^{+3}-centered hematoporphyrin and 53 mM DTT in 100 mM Tris buffer (pH 9.0).

The gas space was flushed with 10% NO in He and the serum bottle incubated at 30 C in a rotary shaker. Controls contained all components except the porphyrin. The gas phases were monitored periodically for NO and gaseous reaction products. Results are shown in Figure 126. Nitrous oxide was observed as one reaction product but no N_2 was detected.

The system is currently under further investigation.

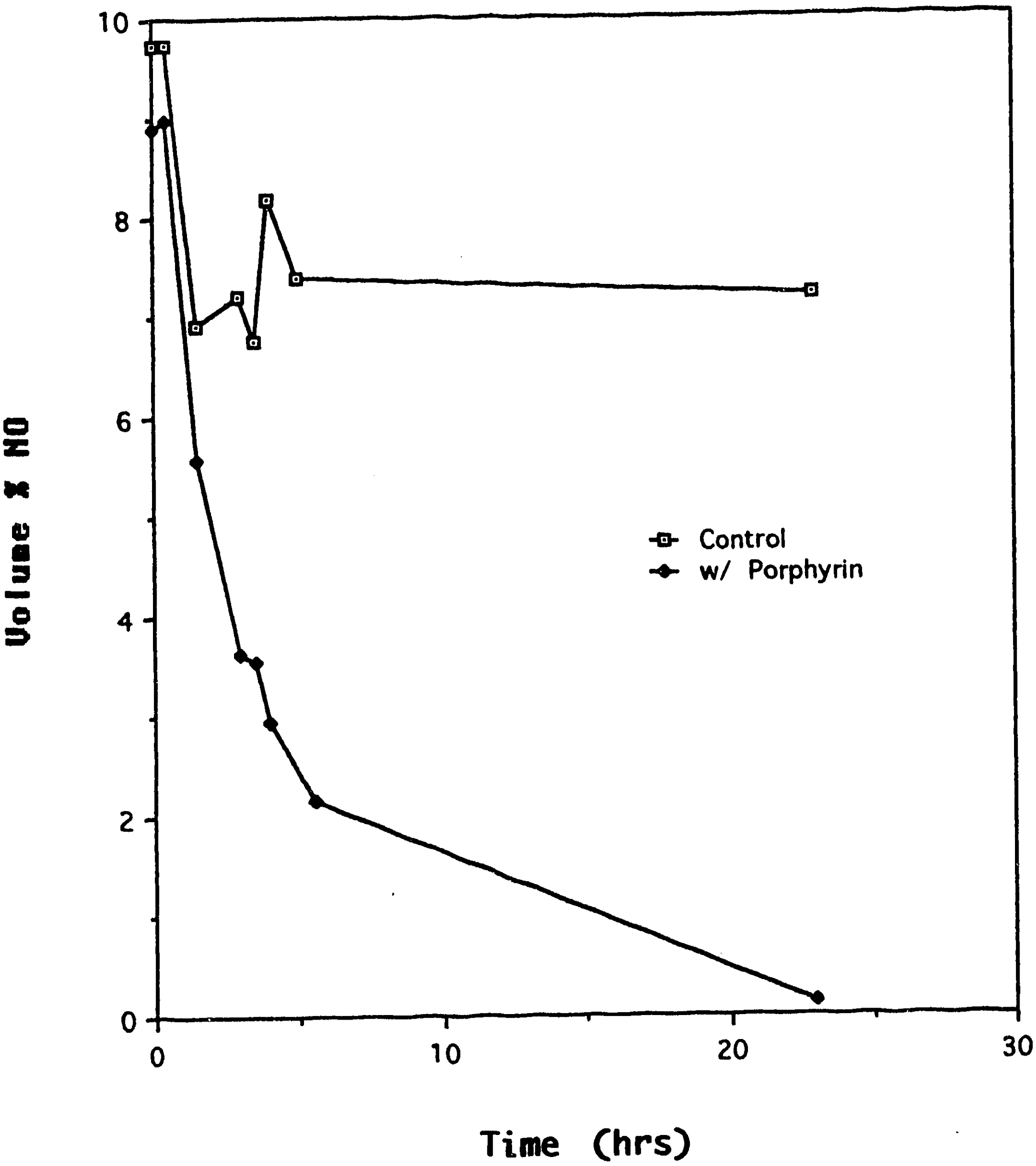


Figure 126. Reduction of nitric oxide (NO) by dithiothreitol catalyzed by Co^{+3}-centered hematoporphyrin.

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