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Engineering Considerations for Growth of Bacteria at Temperatures Around 100°C

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ABSTRACT

Laboratory results from studies of three extremely thermophilic sulfur-dependent archaebacteria (*Pyrodictium occultum, Pyrodictium brockii,* and *Pyrococcus furiosus*) are used to illustrate both metabolic characteristics and engineering challenges associated with bacteria growing around 100°C. Low biomass yields, production of large amounts of hydrogen sulfide, and, in one instance, metabolic formation of a growth-inhibiting product are among the many characteristics that complicate the development of bioreactors and protocols for culturing these microorganisms. Engineering considerations related to the metabolic features of the sulfur-dependent archaebacteria are also discussed.

Index Entries: Extreme thermophiles; archaebacteria; sulfur respiration; gas metabolism; H_2S production.

INTRODUCTION

In recent years, a number of novel microorganisms, growing at temperatures near and above 100°C, have been isolated from submarine, volcanic environments. From a scientific perspective, intriguing questions about the existence of life at elevated temperatures and possible evolutionary implications have sparked considerable discussion. The related biotechnological opportunities created by these "super-thermophiles" (*1*) have not gone unnoticed; prospects of highly thermostable proteins,

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new genetic material, and novel biotransformations have attracted a considerable amount of attention to developments in this area.

As is often the case, the vast biotechnological potential associated with these high temperature microorganisms is tempered by a variety of technical obstacles. These range from very low biomass yields to strain instability to requirements for unusual culturing conditions (2). Whereas progress is continually being made in understanding the fundamental aspects of microbial growth at elevated temperatures, it is difficult to predict at what point biotechnological applications become feasible. As such, it is important to continue to attack the many technical problems from several angles, including an engineering perspective.

It is the purpose of this paper to illustrate some of the unique features associated with microorganisms growing at temperatures close to and above 100°C. Since, for the most part, bacteria found to grow at these elevated temperatures have been identified as belonging to the sulfur-dependent archaebacteria, data from laboratory growth experiments for three such microorganisms will be presented. These experiments will be used as a basis for the discussion of several engineering considerations associated with microbial growth at elevated temperatures.

THE MICROORGANISMS

The sulfur-dependent archaebacteria belong to a unique group of microorganisms distinct from both prokaryotes and eukaryotes (3). Whereas not all sulfur-dependent archaebacteria are capable of growth at extreme temperatures, most have optimal growth temperatures above 60°C. Three anaerobic strains isolated from shallow submarine hydrothermal vents by Stetter (4,5) have been shown to grow optimally at or above 100°C: *Pyrodictium occultum* (reported optimal growth temperature of 105°C), *Pyrodictium brockii* (reported optimal growth temperature of 105°C), and *Pyrococcus furiosus* (reported optimal growth temperature of 100°C). More information about each of these microorganisms is presented briefly in Table 1.

Work in our laboratory has focused on these three microorganisms for several reasons in addition to their elevated optimal growth temperatures. Each strain has some distinct characteristics. *P. occultum* is a strict chemolithotroph, depending on CO_2 for its carbon source. *P. brockii,* although similar in many ways to *P. occultum,* has been shown to be able to growth to higher cell densities in the presence of yeast extract (4), although it is not clear what role yeast extract plays in the growth of this bacterium. Both *Pyrodictium* species grow in H_2/CO_2 atmospheres and in the presence of elemental sulfur, usually under quiescent conditions. Experience to date shows that cell concentrations of approximately 10^7 cell/mL are obtained under seemingly optimal conditions.

P. furiosus, in contrast to the *Pyrodictium,* is a heterotroph. Although this bacterium can grow in the absence of elemental sulfur, higher

Table 1
Characteristics of the Microorganisms

	Pyrodictium brockii	*Pyrodictium occultum*	*Pyrococcus furiosus*
Shape	disc	disc	spherical
Size	.3–3.5 μm	slightly smaller than *P. brockii*	.8–2.5μm
G + C content	51.5–56.6 mol%	62 mol%	38 mol%
Temperature optimum	80–110°C, 105°C	80–110°C, 105°C	70–103°C, 100°C
pH Range optimum	5–7, 5.5	5–7, 5.5	5–9, 7
Energy/carbon sources	H_2, CO_2	H_2, CO_2	organic substrates
Know metabolic products	H_2S	H_2S	H_2, CO_2, and H_2S
Role of S°	e^- acceptor	e^- acceptor	detoxification for H_2 inhibition

growth yields are possible if sulfur is included in the media. Also, unlike the *Pyrodictium,* which use hydrogen and carbon dioxide as energy and carbon sources, respectively, *P. furiosus* produces both hydrogen and carbon dioxide as a consequence of growth. (The production of other exocellular materials has not been reported at this time.) In fact, the hydrogen produced by this bacterium is thought to inhibit growth. Fiala and Stetter (5) have suggested that the addition of elemental sulfur may help to reduce growth inhibition related to dissolved hydrogen through its biotic conversion to hydrogen sulfide.

As is the case for many thermophilic bacteria, it is not clear what factors are important in the cessation of growth at relatively low final cell densities. Also, Sturm et al. (2) have pointed out that often no real stationary phase exists for these extreme thermophiles, thus making cell harvesting for the purpose of protein recovery difficult. From a biotechnological perspective, low biomass yields, with all of its ramifications, represents a key obstacle.

To improve biomass yields, a better understanding of the growth characteristics and metabolic functions of the extremely thermophilic, sulfur-dependent archaebacteria is paramount. However, because these microorganisms grow at elevated temperatures, in the presence of elemental sulfur, under anaerobic and quiescent conditions, standard protocols that apply to the study of many other bacteria do not apply. Thus, in addition to the unusual characteristics associated with the sulfur-dependent archaebacteria, new approaches for their study and cultivation must be considered.

The purpose here is to illustrate through results obtained in our laboratory some of the characteristics of the sulfur-dependent archaebacteria growing in the vicinity of 100°C. Although these results and others indicate that progress is being made in working with these unusual microorganisms, they also suggest that there is still much to be done before related biotechnological applications can be considered.

METHODS AND MATERIALS

Culturing Protocols

The three strains used in this study were obtained from Deutsche Sammlung von Mikroorganismen, Federal Republic of Germany. Experiments were conducted using 125 mL glass culture vials which were sealed by heavy duty rubber stoppers and aluminum seals. Further details on experimental protocols can be found elsewhere (*6*). For the experiments reported here, several samples of water obtained from the Chesapeake Bay were used as the basis for growth media. This approach was used because of the low growth yields obtained with several defined media. In preparing the media, Chesapeake Bay water (CBW) was filtered through a 0.22 μm filter and autoclaved. It should be noted that the biomass yield obtained in a particular experiment was related to the sample batch of CBW used in the media. The batch of CBW used is noted where appropriate.

Pyrodictium species

To the media for *P. brockii*, yeast extract (2 g/L) was added prior to autoclaving the CBW, whereas for *P. occultum*, if yeast extract was used, 0.2 g/L was added after CBW was autoclaved. The point at which yeast extract was added to the media was subsequently found not to influence experimental results. To the autoclaved media, in either case, NaCl (6.4 g/L) and resazurin (.001 g/L) were added. Sulfuric acid (.2 N) was added to adjust the pH; the amount of acid added varied depending on the batch of sea water. Typical values were 11 mL/L for *P. occultum* and 25 mL/L for *P. brockii*. In all cases, the culture vial used for a growth experiment contained 50 mL of media and 1.5 gm of elemental sulfur.

Culturing vials were preheated in a high temperature bath (New Brunswick Scientific, New Brunswick, NJ) for one h. The vials were then removed from the bath and sparged with an 80:20 H_2/CO_2 gas mixture for 4 min. Following this, 1.0 mL of 2.5% w/v Na_2S was added to reduce the residual oxygen in the solution. The liquid in the vial was again sparged, for about 30 s, until the resazurin turned colorless, indicating the absence of oxygen. The vial was then sealed and returned to the temperature bath. After this procedure, the pH of the solution was approximately 5.5. The vials were inoculated one h after they had been returned to the bath. The inoculum for an experiment is from a culture vial prepared in a similar fashion and is about 48 h old. Subsequent to inoculation, the bottles are pressurized with 4 atm (absolute) of the H_2/CO_2 mixture.

For experiments involving different partial pressures of the nutrient gases (H_2 and CO_2), gas mixtures were blended in a mixing system described elsewhere (*7*). In these experiments, the gas mixture was used to sparge and pressurize the culturing vials.

Pyrococcus furiosus

For *P. furiosus,* yeast extract (1 g/L) and tryptone (5 g/L) were added to the CBW or artificial sea water (ASW), then autoclaved. (Note: ASW contains 1.3 gm/l $(NH_4)_2\ SO_4$, .28 gm/L KH_2PO_4, .25 gm/L $MgSO_4 \cdot 7\ H_2O$, .07 gm/L $CaCl_2 \cdot 2\ H_2O$ and has been used as a source of basal salts for the growth of another heterotrophic extreme thermophile *Sulfolobus acidocaldarius* (*8*).) Prior to inoculation, elemental sulfur (30 g/L) and resazurin (1.0 mg/L) were added to 50 mL of autoclaved media. The bottle containing the media was flushed with helium for five min followed by the addition of 1.0 mL 2.5% w/v Na_2S solution. An appropriate amount of 10 N sulfuric acid (typically 0.5 mL/L) was used to adjust the pH to around 7.0. The mixture was flushed for another 30 s until the solution turned colorless, suggesting that anaerobic conditions had been achieved. The bottle was then sealed and placed in the high temperature bath. The culture was inoculated after one h of preheating using a one-d-old inoculum from a culture vial that was prepared using the same procedure. Unlike the *Pyrodictium* species, the culture vial was not pressurized. As such, the experiment started with a gas phase containing about 1 atm of helium and water vapor.

Analytical Methods

Cell Enumeration

Epifluorescence microscopy (EFM) with acridine orange stain was used for cell enumeration. When sample dilution was necessary, distilled water and CBW was used for *Pyrodictium* and *P. furiosus,* respectively. A more detailed description of these procedures can be found in Sturm (*7*).

Gas Chromatography

Gas analyses for H_2S, H_2, and CO_2 were done using a Varian 3700 gas chromatograph (Varian Associates, Sunnyvale, CA). A flame photometric detector and a Chromosil 310 column (Supelco Inc., Bellefonte, PA) were used for hydrogen sulfide analysis, and a thermal conductivity detector and Carbosieve 100/120 column (Supelco Inc., Bellefonte, PA) were used for H_2 and CO_2 analyses. Peak areas were obtained using an Apple II^+ microcomputer interfaced to the gas chromatograph.

RESULTS

Hydrogen Sulfide Production by Pyrodictium

Because hydrogen-sulfur autotrophy characterizes the growth of the *Pyrodictium,* hydrogen sulfide is produced as a byproduct of metabolism (*4*). This can be seen quantitatively in Figs. 1 and 2, which show growth and sulfide production by *P. occultum* and *P. brockii* at 98°C. Stetter et al.

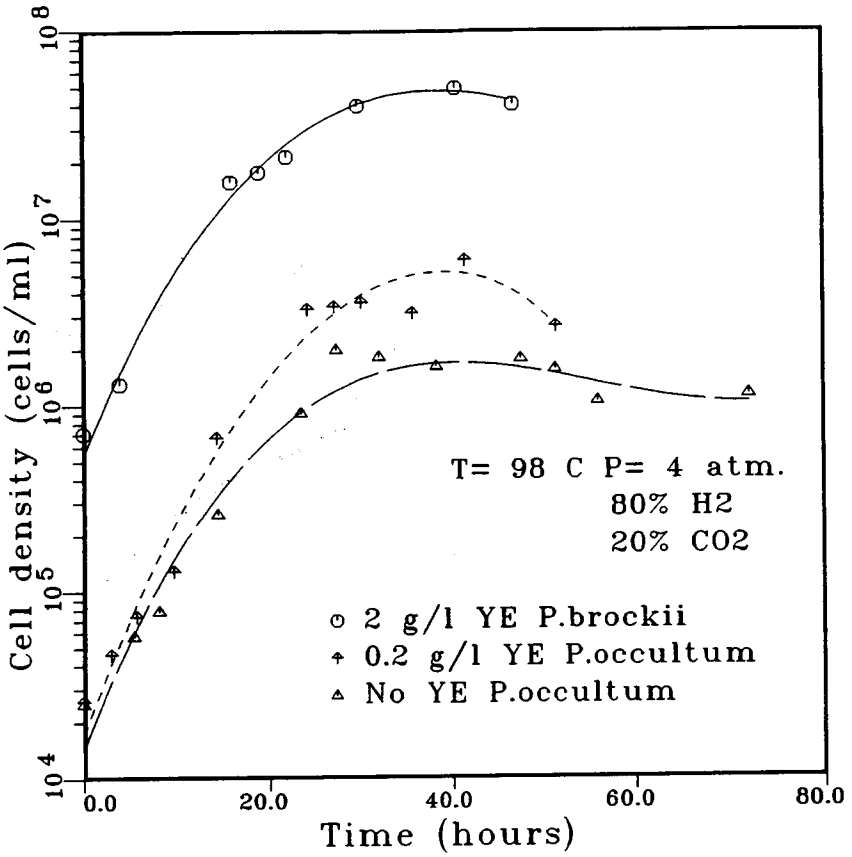

Fig. 1. Growth curves of *Pyrodictium occultum* and *Pyrodictium brockii* at varying yeast extract (YE) concentrations.

(4) have reported that the addition of yeast extract to the media does less to enhance *P. occultum* growth in comparison to the marked effect it has on the growth of *P. brockii*. This can be seen in Fig. 1, which shows the growth of both *Pyrodictium* species at approximately optimal levels of yeast extract. Note the lower growth for *P. occultum* when no yeast extract is added; this result is approximately the same for *P. brockii* when no yeast extract is added (*see* Fig. 3). Figure 3 also shows the effect of increasing yeast extract concentrations on the growth of *P. brockii*. Yeast extract levels above 2 g/L for *P. brockii* and .2 g/L for *P. occultum* do not improve growth rates or biomass yields in either case.

It is not clear as to the role yeast extract plays in the growth of these microorganisms. We have made the observation that yeast extract addition helps to keep the elemental sulfur finely dispersed in the media, particularly at elevated temperatures (9). Since interaction between the bacteria and elemental sulfur is critical for growth, yeast extract addition possibly improves growth by increasing the available surface area. It also has been suggested that yeast extract may promote the interaction of bacteria with sulfur as a surface active agent (4). Experiments with both bac-

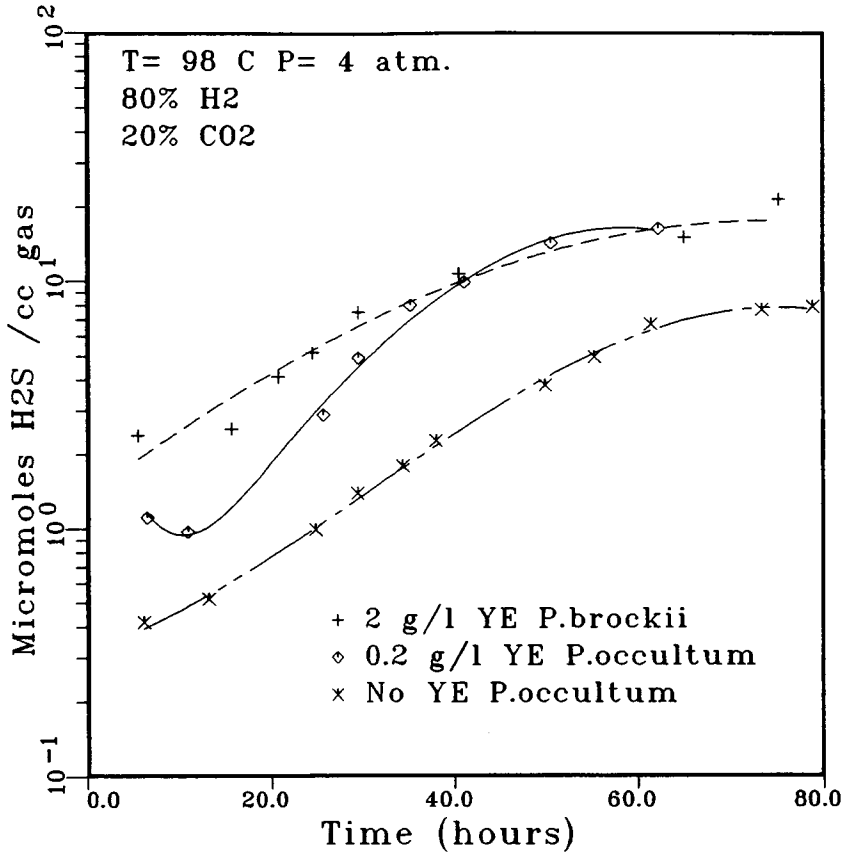

Fig. 2. Gas production by *Pyrodictium occultum* and *Pyrodictium brockii* at varying yeast extract (YE) concentrations.

teria have shown that no growth occurs in the absence of CO_2, indicating that yeast extract is probably not a carbon source. Of course, it is possible that yeast extract supplies some other nutritional requirement, but nothing has been found in this regard as yet. It is interesting to note that yeast extract has been shown to enhance biotic and abiotic sulfide production for other extreme thermophiles at lower temperatures (*10*).

The growth of both *P. occultum* and *P. brockii* is accompanied by the production of large amounts of sulfide. This is clear upon examination of Fig. 2. Parameswaran et al. (*9*) have shown previously the relationship between growth temperature and sulfide production for *P. occultum*. Sulfide production appeared to lag growth and increase at a rate slower than the growth rate. Reexamination of this same data provides some additional insight.

Figures 4 and 5 show the specific production of hydrogen sulfide for *P. occultum* at 98 and 105°C, respectively. Here, the specific production was determined by dividing the total hydrogen sulfide in the gas phase by the associated cell density. At 98°C, it appears that the specific pro-

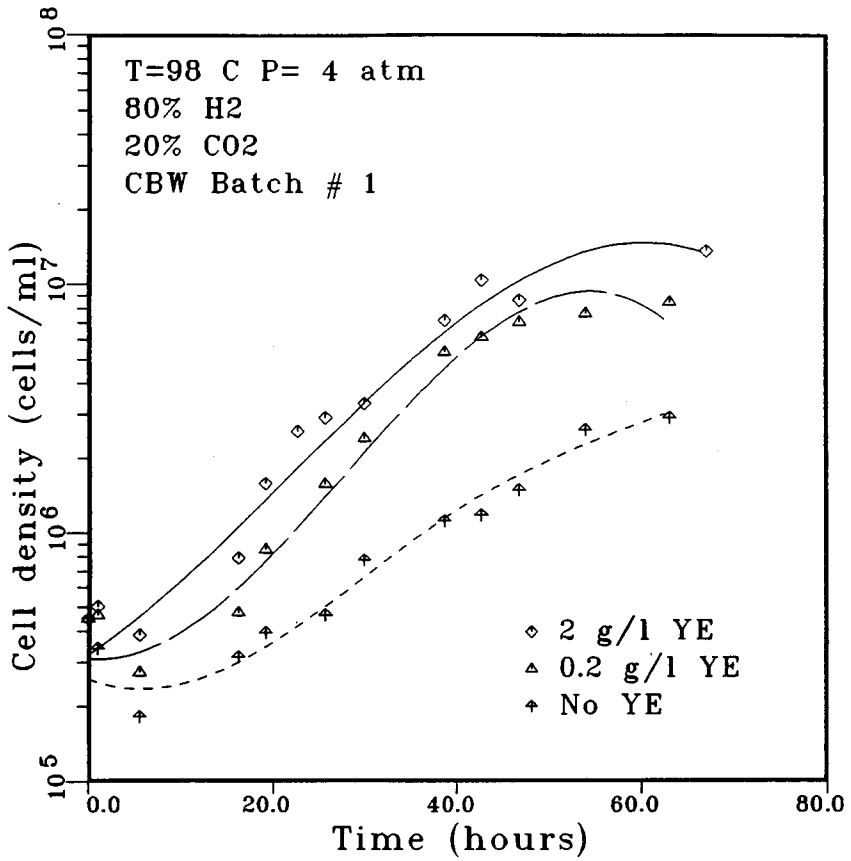

Fig. 3. Effect of yeast extract (YE) on the growth of *Pyrodictium brockii*.

duction is lowest during periods of rapid growth. This appears to be significant, since the concentration of hydrogen sulfide in the gas phase continues to increase during stationary phase even as the cell density declines. This does not appear to be the result of degassing since a similar relationship is seen at 98°C in the liquid phase (unpublished results). At 105°C, however, specific hydrogen sulfide production continues to increase throughout the experiment, even when the cells are in the exponential phase.

The significance of changes in specific hydrogen sulfide production during growth for this microorganism is not clear. Based on this preliminary information, it is interesting that the pattern seems to be different at 105°C than at 98°C. Whether the changes are related to strictly biotic phenomena or are the product of both biological and abiotic events is not certain. It also has been noted that there are significant changes in sulfur chemistry and structure as the temperature increases through 100°C (*10*). Nonetheless, changes that occur as the growth temperature increases should provide some insight into the basis for microbial growth at elevated temperatures.

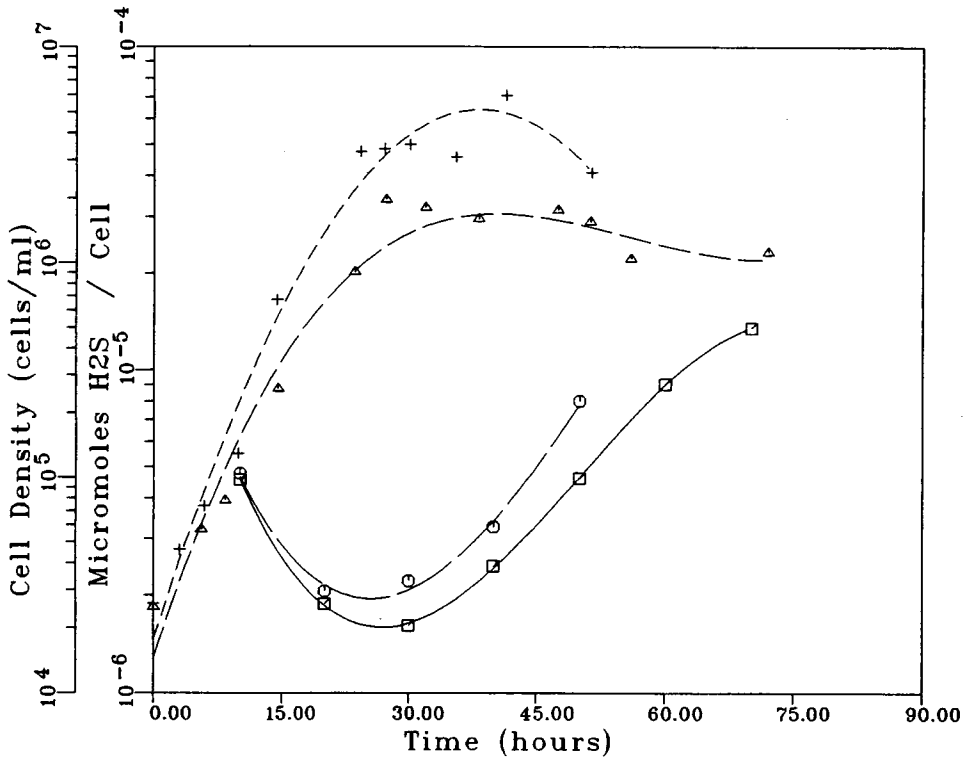

Fig. 4. Specific H_2S production for *Pyrodictium occultum* (98°C). The following legend applies: (+) cell density (with yeast extract); (○) specific H_2S production (with yeast extract); (□) specific H_2S production (without yeast extract); (△) cell density (without yeast extract).

Consumption/Production of Gases During the Growth of Pyrodictium brockii

One of the interesting characteristics of the growth of the two bacteria that presently comprise the genus *Pyrodictium* is the production and consumption of gases during growth. In the previous section, we have shown that large amounts of hydrogen sulfide are produced as these microorganisms grow. Here, we further explore gas phase phenomena.

Stetter et al. (4) proposed that the cultivation of the bacteria they isolated belonging to the *Pyrodictium* be done in the presence of a gas phase containing hydrogen and carbon dioxide. Since the proposed growth medium was similar to those used for the growth of methanogens, a gas phase mix of 80% hydrogen and 20% carbon dioxide was suggested. This, of course, represents the stoichiometric ratio for methane. No attempt was made to determine whether this ratio was optimal for the *Pyrodictium*.

For *P. brockii*, CO_2 and H_2 serve as carbon source and energy source, respectively. Although, as seen in Fig. 3, yeast extract promotes growth

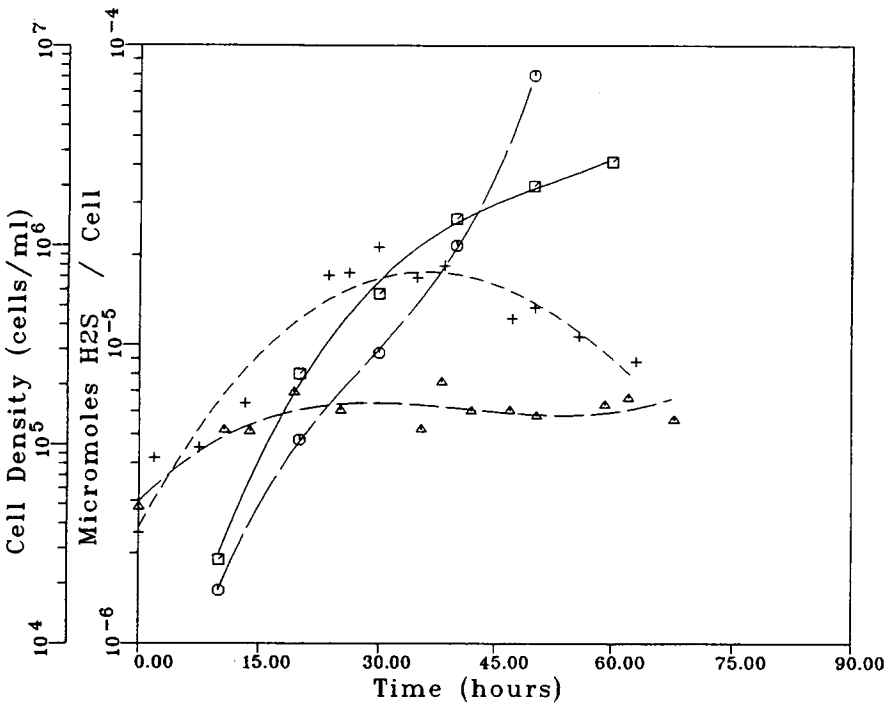

Fig. 5. Specific H_2S production for *Pyrodictium occultum* (105°C). The following legend applies: (+) cell density (with yeast extract); (○) specific H_2S production (with yeast extract); (□) specific H_2S production (without yeast extract); (△) cell density (without yeast extract).

for this bacterium, we have determined that it probably contributes little as either a carbon source or energy source; no growth is observed if either carbon dioxide or hydrogen are eliminated from the gas phase in the presence of yeast extract (*see* Table 2). It has not been determined whether there are conditions such that these gases are rate- and/or stoichiometrically-limiting.

Table 2
Effect of Lower Hydrogen Partial Pressures[a]

Percentage in gas phase		Max. cell density, cells/mL	Doubling time (hours)	H_2S production (μmole/vial)
H_2	CO_2			
100	0	no growth	—	70
80	20	4.4×10^7	2.9	2000
8	92	3.4×10^7	3.4	450
4	96	1.2×10^7	4.2	150
1	99	1.1×10^7	4.0	100
0	100	no growth	—	70

[a]System, vials; temperature, 98°C; total pressure, 4 bar; initial cell density, 5.8×10^5

To pursue this point, we have investigated the effect of hydrogen partial pressures on the growth of *P. brockii* at 98°C. First, we show in Fig. 6 that CO_2 concentrations at or above 8% (recall that each vial is pressurized to 4 atm, initially) have no effect on growth rate or final cell density. Figure 7 and Table 2 then show the effect of H_2 partial pressures on the growth of *P. brockii*. These data show that H_2 partial pressures can affect both the growth rate and cell yield.

Further experiments were then conducted to establish the effect of hydrogen partial pressure on cell yield and H_2S production at 98°C. These experiments were run using 20% CO_2, varying concentrations of H_2, with the balance being helium. Figure 8 shows the effect of H_2 partial pressures for this set of experiments, and Fig. 9 shows that H_2 S production increases with increasing partial pressures of H_2. Note that H_2S production is approximately linear with increasing H_2 partial pressure. How-

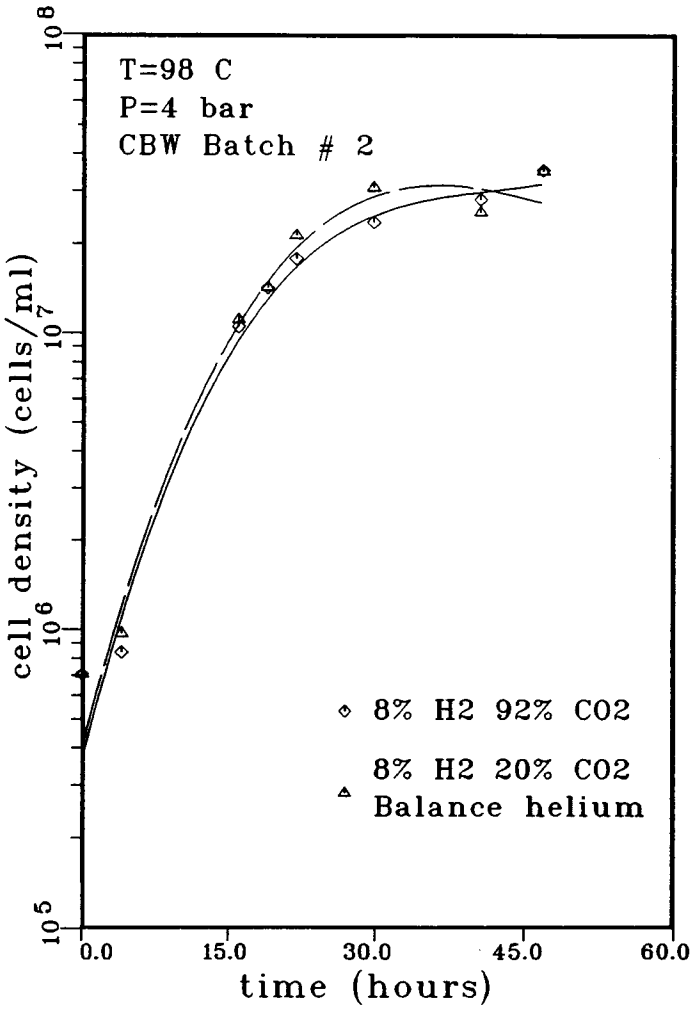

Fig. 6. Effect of CO_2 partial pressure on the growth of *Pyrodictium brockii*.

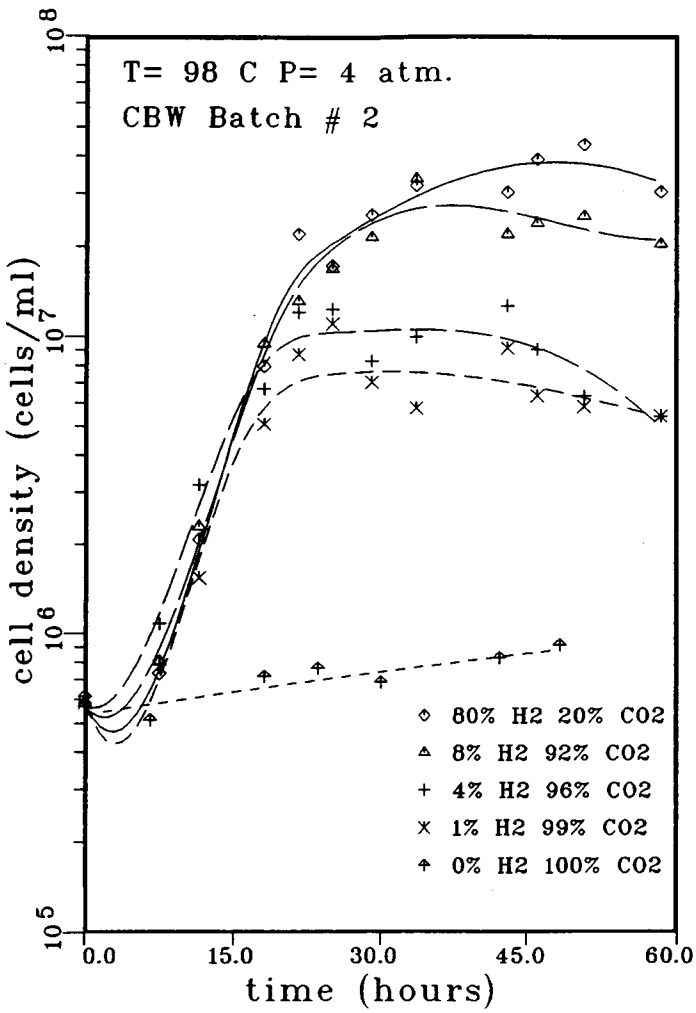

Fig. 7. Effect of H_2 partial pressure on the growth of *Pyrodictium brockii*.

ever, cell yields follow a highly nonlinear pattern with increasing H_2 partial pressure. This can also be seen in the experiments shown in Table 2 where the increase in H_2S production does not follow changes in cell yield. For example, for a 80-fold reduction in H_2 partial pressure (from 80% to 1%), only a four-fold reduction in cell density is noted. In addition, for this case, H_2S production decreased by a factor of 20. These results suggest that sulfide reduction is not always associated with growth. In fact, elevated hydrogen partial pressures have been found to be inhibitory to *P. brockii* (unpublished results) and so it is possible that sulfide production may be a mechanism to offset inhibition caused by excess dissolved H_2.

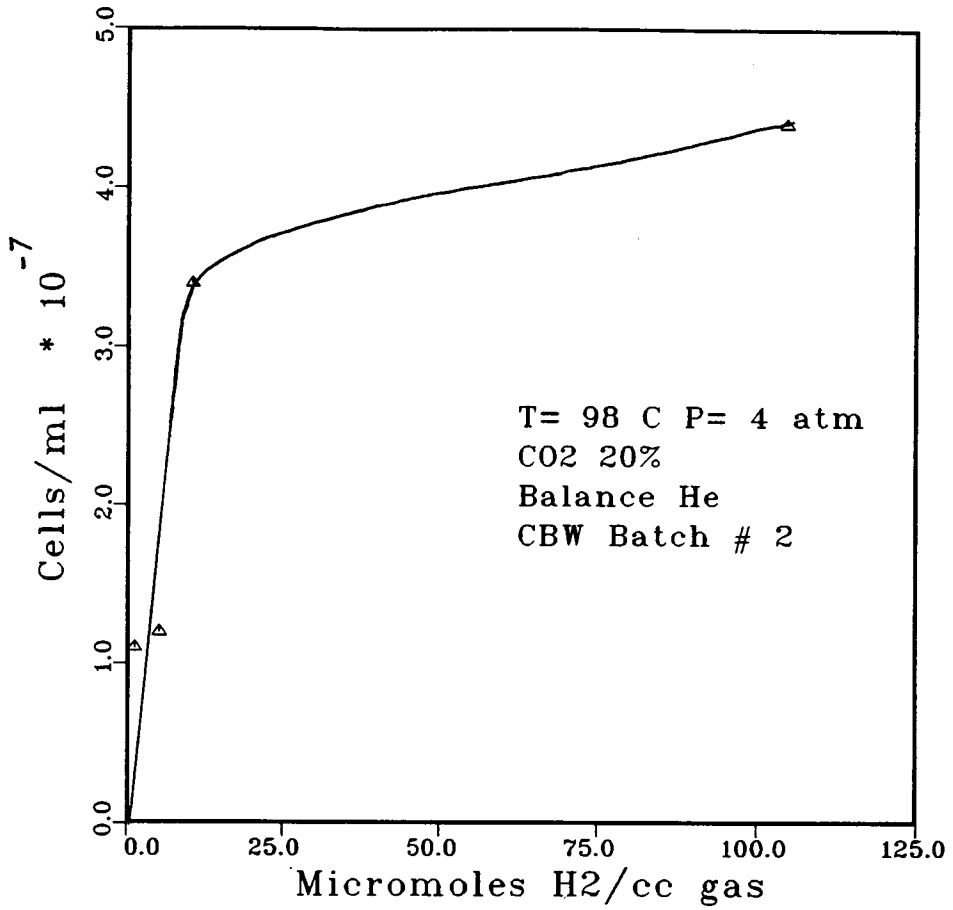

Fig. 8. Maximum cell density of *Pyrodictium brockii* as a function of H_2 concentration.

Growth Characteristics of Pyrococcus furiosus

Media Development

Unlike *P. brockii* and *P. occultum*, *P. furiosus* is a heterotroph although it was isolated by Fiala and Stetter (*5*) from the same location. It is different from the *Pyrodictium* in that it requires no gas phase nutrients and will produce gases in addition to H_2S. Since this is the fastest growing bacterium growing at temperatures at or above 100°C and has been shown to grow to higher cell density, it is, perhaps, the best candidate for obtaining reasonable quantities of particular proteins. Hence, efforts to better understand its growth and metabolism to improve biomass yields are appropriate. Preliminary work along these lines will be presented.

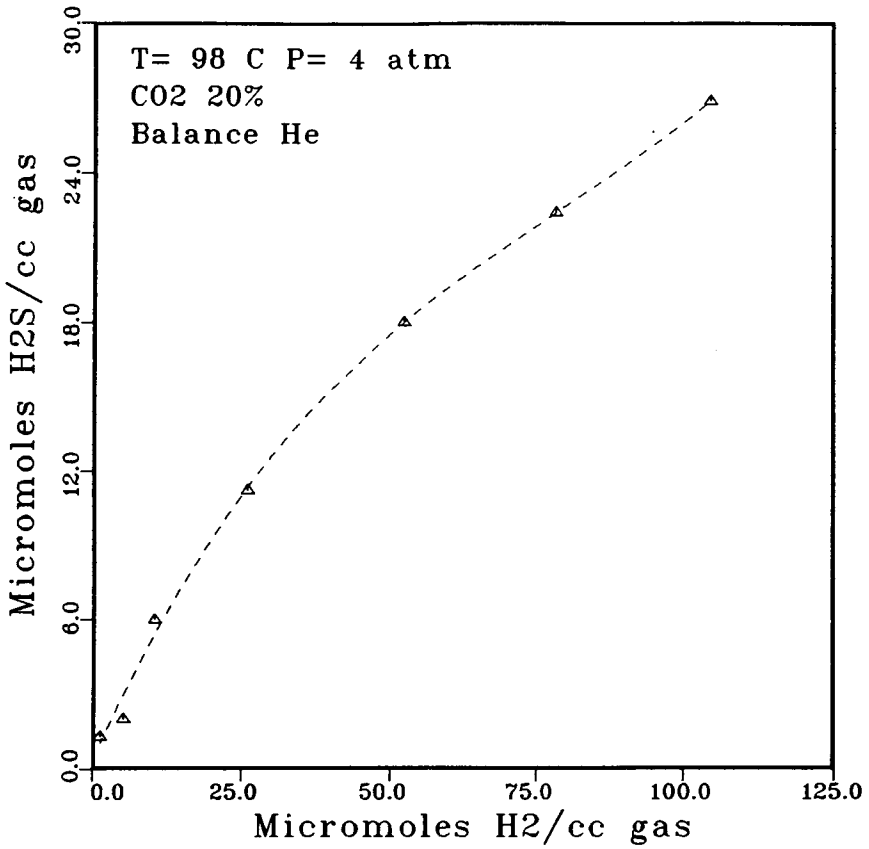

Fig. 9. H_2S production by *Pyrodictium brockii* as a function of H_2 concentration.

A series of experiments were conducted seeking to improve media composition (*see* Table 3). A media containing .1% yeast extract, .5% tryptone, and 3% elemental sulfur added to CBW was found to provide

Table 3
Effects of Different Media on Growth of *Pyrococcus furiosus*

Media Composition[a]	Final cell density, cells/mL
.5% YE[b] in CBW[c]	8.7×10^7
.1% YE, .5% tryptone, .5% peptone in CBW	2.0×10^8
.1% YE, .5% peptone in CBW	2.0×10^8
.1% YE, .5% tryptone in CBW	2.0×10^8
1.0% NaCl, .1% YE, .5% tryptone in ASW[d]	1.0×10^8

[a]All media contain 30 gm/L of elemental sulfur.
[b]YE, yeast extract.
[c]CBW, Chesapeake Bay water.
[d]ASW, artificial sea water.

cell yield of 2×10^8 cell/mL. This compares favorably to reported results (5) in which a more complicated undefined media was used for cultivation: We noted a doubling time of two hours and cell yield of 2×10^8 cell/mL at 98°C compared to literature reports of a doubling time of 37 min and cell yield of 3×10^8 cell/mL at 100°C. Upon examining Table 3, about the same final cell density (2×10^8) was observed when peptone was used in place of tryptone and a significantly lower cell yield was obtained if either peptone or tryptone was left out of the media. Tryptone was used in all subsequent experiments because it was observed, with its

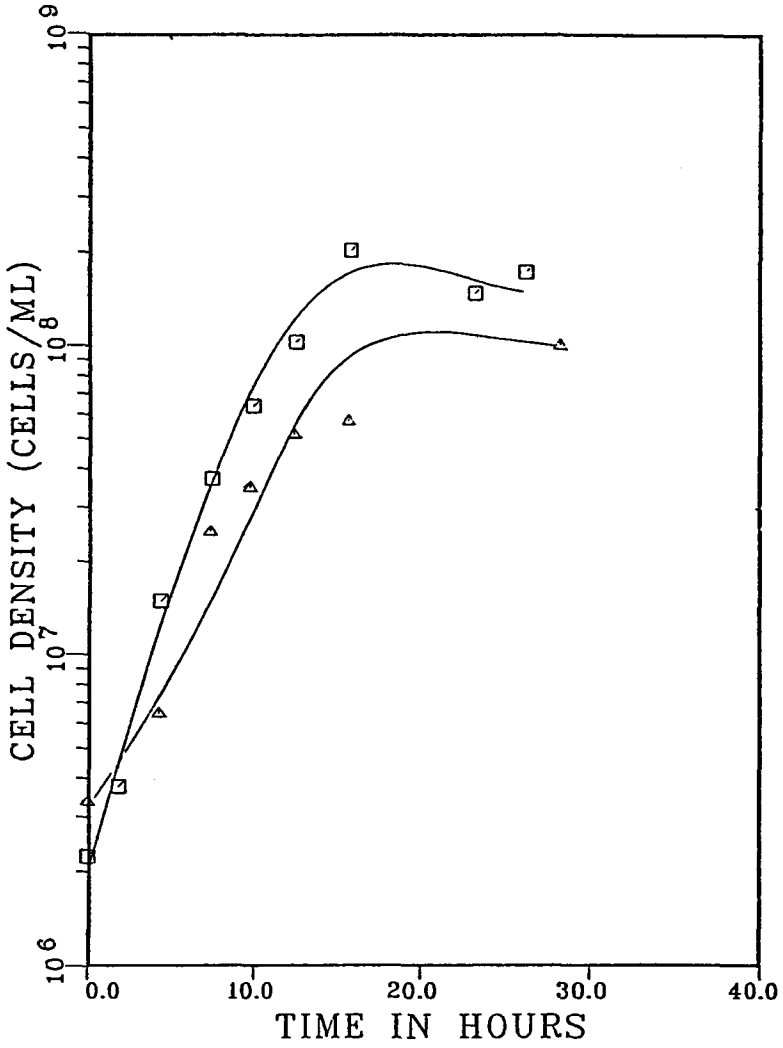

Fig. 10. Growth of *Pyrococcus furiosus* on Chesapeake Bay water (□), t_d = 2.3 h; and artificial sea water (△), t_d = 3.0 h. In both cases, 1.5 gm/50 mL of S° is added and growth occurs under quiescent conditions.

use, that a more uniform cell morphology resulted upon microscopic examination and the cell fluorescence was stronger when examined by EFM. Our previous work with other extreme thermophiles have suggested that these factors are indicative of a healthy culture (*6*).

Some effort has been directed at establishing a defined medium. For example, ASW with its salinity adjusted with 1% NaCl was used in place of CBW. It resulted in a lower cell yield and longer doubling time when compared to results with CBW-based media formulation (*see* Fig. 10 and Table 3). This result suggests that certain components in CBW significantly affect the growth rate and biomass yield for this microorganism. An effort is underway to determine which component(s) may be responsible for these differences.

Gas Production by P. furiosus

In the absence of elemental sulfur, Fig. 11 shows that the cell density starts to decay when hydrogen levels exceed 1.6 μmol (gas phase)/mL culture (approximately .02 atm H_2). Fiala and Stetter (*5*) have suggested that hydrogen is inhibitory to growth for this microorganism and that sulfur addition to the media offsets this inhibition. To further explore the potentially inhibitory effect of H_2, an experiment was run with one atm of H_2 initially in the gas phase with no sulfur added to the media. As seen in Fig. 12, no growth was observed.

Hydrogen sulfide is produced by *P. furiosus* when elemental sulfur is added to the media and higher cell yields are subsequently obtained (*see* Fig. 13). There are several possible explanations for this phenomenon. It may be that elemental sulfur reacts with the hydrogen that is produced by this microorganism as a metabolic product to form H_2S through a biotic mechanism which, at the same time, reduces the inhibitory effect associated with H_2. Another possibility is that H_2 production is repressed in the presence of elemental sulfur and H_2S is produced through an indirect mechanism. In any case, the metabolic relationships between H_2, CO_2, H_2S as these compounds are produced by this microorganism must be better understood to improve cell yields.

Engineering Considerations

The results presented here illustrate several interesting features of microbial systems at temperatures around 100°C. These range from the consumption/production of several gases that have reduced solubilities at elevated temperatures to the simultaneous necessity in most cases of a solid substrate, i.e., elemental sulfur. There continues to be a steady stream of new discoveries of microorganisms at these elevated temperatures which undoubtedly will include many different bases for growth such as methanogenesis. For these novel microorganisms to be useful in a biotechnological context, not only must their growth and metabolism

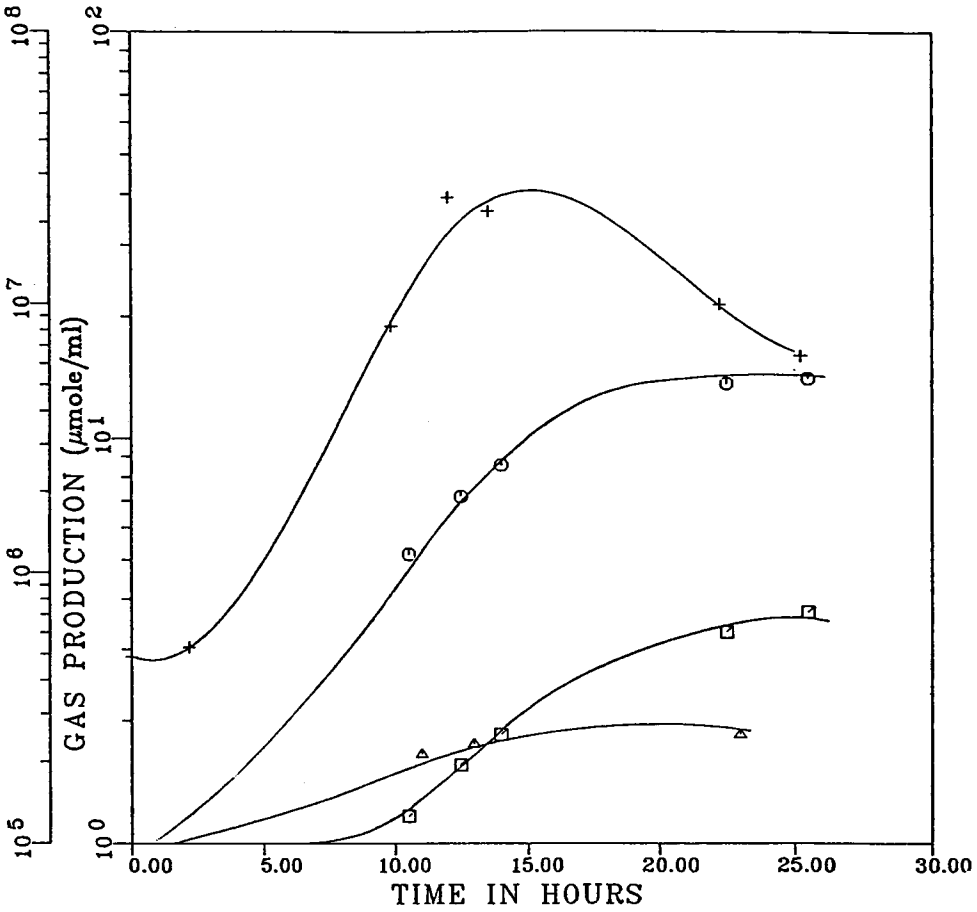

Fig. 11. Gas production by *Pyrococcus furiosus* in the absence of elemental sulfur. The following legend applies: (+) cell growth; (○) CO_2 production; (□) H_2 production; (△) CO_2 produced from media in uninoculated control. No H_2 production was observed in the uninoculated control. All data for gas production are reported as μmol gas produced in the gas phase per mL of liquid volume.

be better characterized but new bioreactor concepts and processing protocols must also be developed.

For the sulfur-dependent archaebacteria described here, there are still many bioprocessing challenges to be addressed. Although these microorganisms are sensitive to shear (*4*), it is not clear how this sensitivity must be dealt with on a larger scale. Also it has yet to be determined whether low levels of mixing will lead to gas–liquid mass transfer limitations, especially in conjunction with reduced gas solubilities at elevated temperatures. The influence of temperature on shear sensitivity will likely be important as the fluidity of membranous material will increase with temperature.

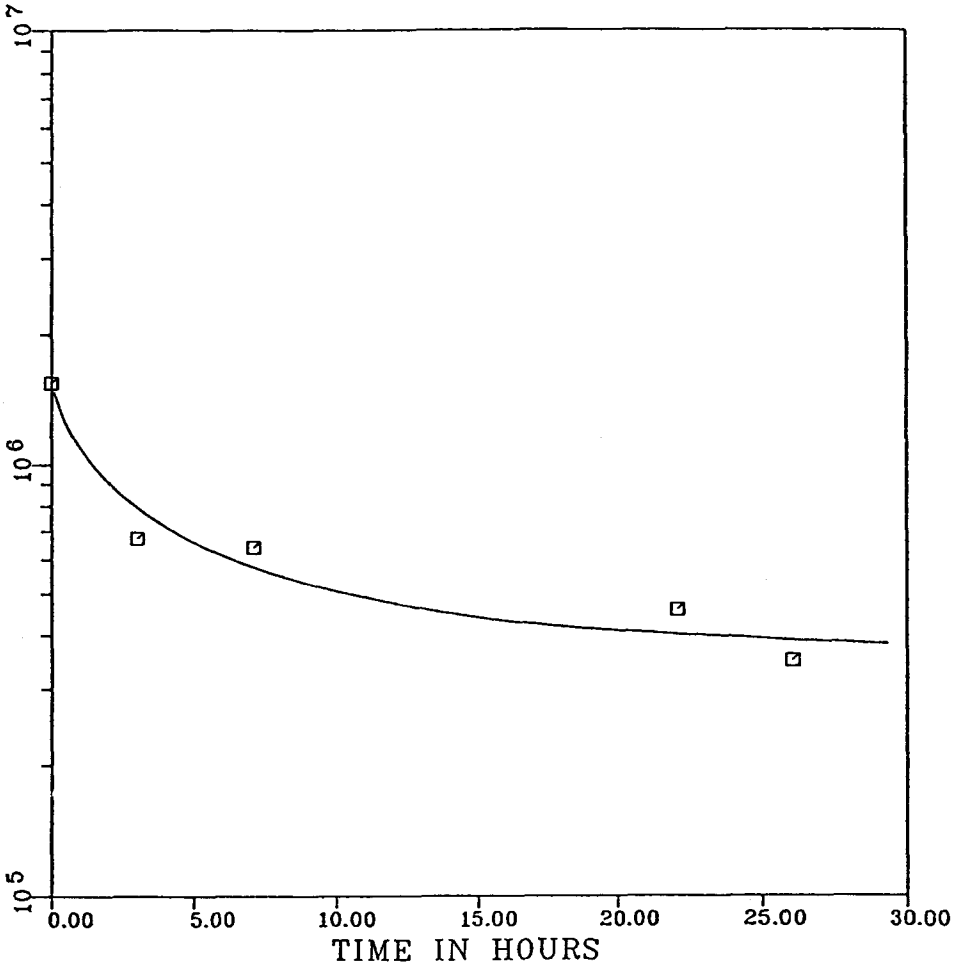

Fig. 12. Inhibitory effect of hydrogen (1 atm) on the growth of *Pyrococcus furiosus*.

It appears that high biomass yields will be associated with the production of significant amounts of hydrogen sulfide for the three bacteria studied. This, of course, may lead to serious materials problems as a result of high corrosion potentials which are exacerbated at high temperature. While other microorganisms are known to produce hydrogen sulfide as they grow (e.g., anaerobic digestion), few do so at such high temperatures and at such high rates. For *P. brockii*, limiting H_2 partial pressure was found to reduce H_2S production but this approach must be balanced against lower cell yields. It is less clear how to address this problem for *P. furiosus*, which grows better in the presence of elemental sulfur. It appears that *P. occultum* produces less sulfide during periods of rapid growth; this may be a key to minimizing sulfide production.

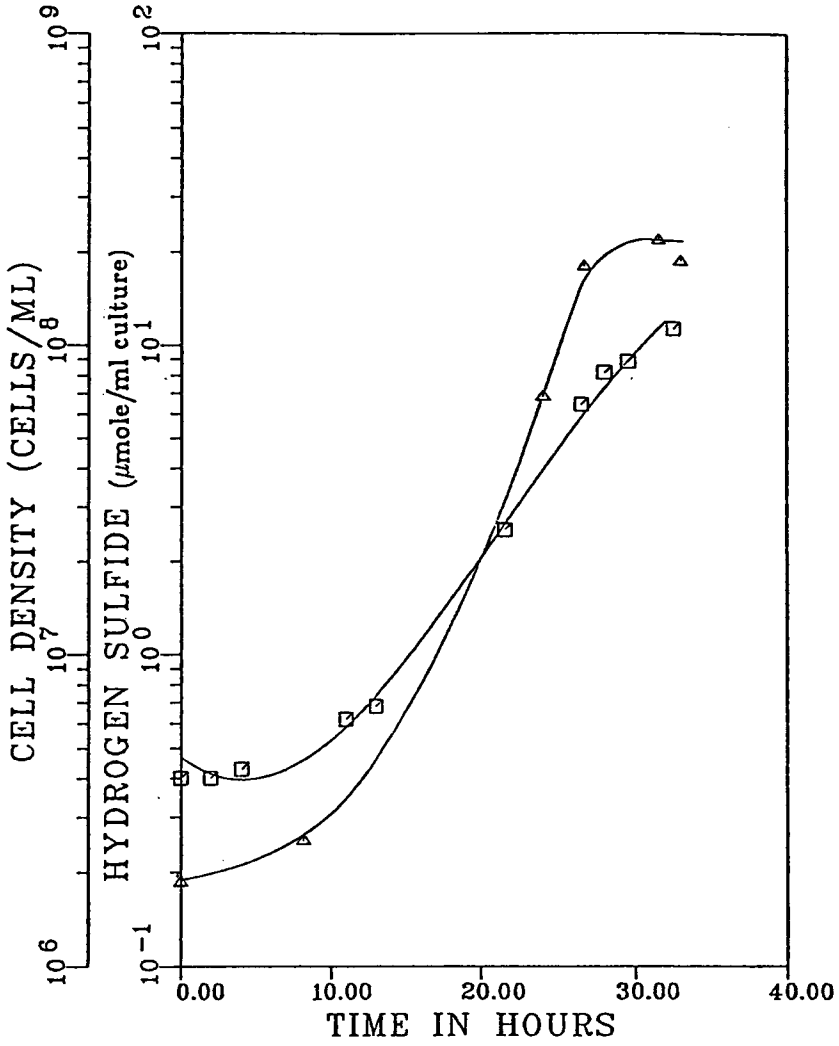

Fig. 13. Production of hydrogen sulfide by *Pyrococcus furiosus* growing in the presence of elemental sulfur (1.5 g S°/50 mL). The following legend applies: (□) Hydrogen sulfide formed in the gas phase per mL of culture; (△) Cells/mL.

There are other technical areas that must be better studied if proteins and other biomolecules are to be isolated from these bacteria. It is not known how particular proteins from these microorganisms might stand up to lower temperature recovery operations and whether particular proteins are stable in the exocellular environment. Also, the nature of the growth curves shown here suggests that a prolonged stationary phase does not exist. This may require a very well characterized protocol for biomass generation that includes harvesting at a precise time for batch systems. Operation under chemostatic conditions is also a possibility,

but dealing with a heterogeneous feed will require defining both solids and liquid dilution rates.

The type of bioreactor to be used for these systems is not clear. Although some modification of a conventional fermentor might be effective, gaslift systems or membrane biorectors should also be considered. Another feature that may be important is the capability to operate at elevated pressures to simulate conditions for the microorganisms from deep sea environments.

In any case, there are a multitude of important questions that must be answered in regard to the cultivation of the "super-thermophiles." These technical problems are especially suited to the interaction of scientists and engineers. Given the biotechnological opportunities that could arise, microbial activity at elevated temperatures warrants more vigorous study.

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