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(U) Hydrogen/Sulfur Metabolism in the Hyperthermophilic Archaebacterium, Pyrodictium brockii

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The objective of this work is to understand the relationship between hydrogen uptake and sulfur reduction as it relates to the energetics of the hyperthermophilic archaebacterium, *Pyrodictium brockii*. In the first year of work, we have shown that *P. brockii* apparently uses a soluble sulfur species (i.e., a polysulfide) as a substrate for sulfur reduction. Also, we have established protocols for growing this organism in continuous culture under conditions such that molecular hydrogen, the energy substrate, is growth-limiting. It was also shown that several metals (chromium, nickel, molybdenum) were toxic at the 10 ppm level necessitating the use of an all glass system for cultivation. In the second year of work, we plan to estimate maintenance energies and growth efficiencies for *P. brockii* using an unstructured mathematical model for cell growth, substrate consumption and gaseous product formation. Also, we will investigate the relationship between growth, hydrogen and carbon dioxide uptake, and sulfide generation.

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DATE: January 22, 1990

PRINCIPAL INVESTIGATOR: Dr. Robert M. Kelly

CONTRACTOR: Department of Chemical Engineering, The Johns Hopkins University

CONTRACT TITLE: Hydrogen/Sulfur Autotrophy in the Hyperthermophilic Archaebacterium, *Pyrodictium brockii*

START DATE: January 19, 1989

RESEARCH OBJECTIVE: To investigate the bioenergetics of the chemolithoautotrophic hyperthermophile, *Pyrodictium brockii*, with particular emphasis on the metabolic roles of molecular hydrogen and sulfur.

PROGRESS (Year 1.0):

Fermentation/Metals toxicity

Our primary focus during this period of our work has been to develop an experimental system with which we can acquire the necessary data to investigate the energetics of hydrogen/sulfur autotrophy in *P. brockii*. This organism uses carbon dioxide as its carbon source and hydrogen as its energy source, making it a more tractable system for this study than hyperthermophilic heterotrophs for which sole carbon and energy sources have not been clearly established. Initial efforts to grow this bacterium in one of our fermentation systems used for hyperthermophile cultivation presented problems. This was somewhat surprising since we have routinely grown several other hyperthermophilic bacteria in this system. We have been able to grow *P. brockii* in smaller glass vessels. However, we had little success in our fermentation systems for reasons that turned out to be related to the leaching of toxic metals from the baffle cage, heating mantle, and other small metal parts. Along these lines, we have established that this organism is sensitive to several heavy metals, all of which are contained in the stainless steel in the fermentor. Growth was inhibited at 10 ppm for chromium, molybdenum and especially nickel while little or no inhibition was noted at 1 ppm levels of these metals and 10 ppm of iron, cobalt and manganese. We attempted to alleviate this problem by passivating the metal surfaces with nitric acid without any detectable improvement. Additionally, lowering the salt concentration of the medium from 25 g/l to 15 g/l and raising the pH from 5.5 to 6.0 did not result in satisfactory culture growth. The addition of chelator (EDTA 1mM) to the medium allowed the culture to grow to about 5×10^6 cells/ml which was as high as we have achieved in the steel system but significantly below the densities reached in glass ($2-4\times10^7$ cells/ml). Further characterization of the toxicity of metal ions and the effectiveness of chelators in preventing this toxicity is being pursued presently. However, we have switched to an all glass culturing system used in growing a variety of hyperthermophiles and extreme thermophiles in batch and continuous culture (see discussion below).

Screening for Sulfur Substrates

When considering the investigation of the energetics of the hydrogen/sulfur autotrophy of *P. brockii*, one objective was to find a soluble sulfur substrate. Up to now, elemental sulfur has been the nominal sulfur substrate but it is virtually insoluble at 98 °C in growth media. To estimate a Monod saturation parameter for this organism on sulfur (the first step in estimating maintenance requirements for this organism), a mass balance on sulfur must be done as a function of growth conditions. To this end, several organic di- and tri-sulfides were tested but it appeared that they were not stable under our growth conditions and were converted to elemental sulfur in the medium at an appreciable rate. The thiol, cysteine, did not appear to serve as a substrate. Neither did sulfate or sulfite. However, excellent growth and biotic H_2S production were obtained in the presence of thiosulfate.

The next step was to determine if indeed the thiosulfate was being cleaved by *P. brockii* with the sulfane sulfur being reduced to sulfide and the sulfite sulfur being released as sulfite or sulfate. Thiosulfate was consumed as determined by colorimetric assay and H_2S was produced as determined both by gas chromatography (TCD) and colorimetric assay. However, no significant increase in either sulfite or sulfate could be detected in cultures grown on thiosulfate as determined by enzymatic assay and barium precipitation, respectively. The situation is complicated in that the interconversion of these compounds in aqueous solutions under our growth conditions may be possible. Thorough abiotic controls have not yet been performed. In addition, the disproportionation of thiosulfate to sulfur and sulfite in acidic solution is also possible. Therefore, it is not yet possible to pin-point the actual substrate or substrates and products with regard to sulfur. Using elemental sulfur contained in a low molecular weight cut-off dialysis membrane, it was found that cell contact with elemental sulfur for reduction to sulfide was unnecessary. This result points to the existence of a soluble sulfur substrate being released from elemental sulfur in the presence of a growing culture. The soluble compounds are probably polysulfidic which are released in aqueous media in the presence of a nucleophile such as sulfide or bisulfide ions. Polysulfides are not very stable at pH's below 8.5 but we were able to detect them in *P. brockii* cultures using a colorimetric assay.

Another impetus for wanting to model the growth, substrate consumption and product formation for *P. brockii* is to determine a maintenance coefficient. For this one needs a yield coefficient for growth on the energy substrate which is being limited. We would like to do this for hydrogen and sulfur as well as for the carbon source, carbon dioxide. In the case of sulfur, it would be technically difficult to run a sulfur-limited chemostat by delivering a solid slurry of elemental sulfur. Even though the situation with thiosulfate appears to be complicated, it still represents a way to control the rate of delivery of sulfur to a chemostat and should allow us to do the necessary experiments to calculate a sulfur yield coefficient. In the meantime we will continue to investigate any chemical equilibria involving our sulfur substrate which may be important under our growth conditions.

Continuous Culture Experiments

Due to the lack of success in growing *P. brockii* in the stainless steel fermentation system, we have switched to a glass culture vessel using free-standing temperature and pH meters/controllers as well as a dissolved hydrogen probe. The mass spectrometer is utilized for on-line analysis of the effluent gas. In early experiments with this system using elemental sulfur as the substrate, added periodically to the flask, we obtained some baseline data for cell density and hydrogen sulfide production versus dilution rate and hydrogen delivery rate (see Figures 1 and 2, and Table 1). We cannot state unequivocally that sulfur was not limiting in these experiments and therefore we will switch to thiosulfate or some colloidal suspension of sulfur for any future runs. However, we were able to show that hydrogen can become limiting in this system, apparently due to the mass transfer limitations. Thus, we will be able to establish conditions under which an energy substrate is limiting to investigate maintenance and growth efficiencies for this bacterium.

Using the mass spectrometer and colorimetric assay, it is possible to quantify total sulfide production. The same can be achieved for hydrogen with the mass spectrometer and hydrogen probe. With this system we will be able to limit either substrate and measure the sulfide produced. One of our first goals will be to close the mass balance on the conversion of hydrogen and sulfur to hydrogen sulfide and at the same time investigate the apparent uncoupling of hydrogen sulfide production from growth. If we are successful in obtaining the requisite data we will apply an unstructured maintenance model as discussed above and examine the relationship of maintenance energy to temperature and compare the maintenance requirement of *P. brockii* to mesophiles and other thermophiles.

WORK PLAN (Year 2.0):

Using the continuous culture system described above, we will explore issues of maintenance requirements and growth efficiencies for *P. brockii* under a variety of culturing conditions. This will involve developing a mathematical model relating growth to substrate consumption and product formation. Continuing our collaboration with Professor Robert Maier of the Department of Biology at Hopkins, we will investigate the relationship between hydrogen uptake and sulfide generation by this organism. Starting with information gained from our studies of the heterotrophic hyperthermophile, *Pyrococcus furiosus*, we will attempt to identify the enzyme or enzymes involved in the sulfur reduction.

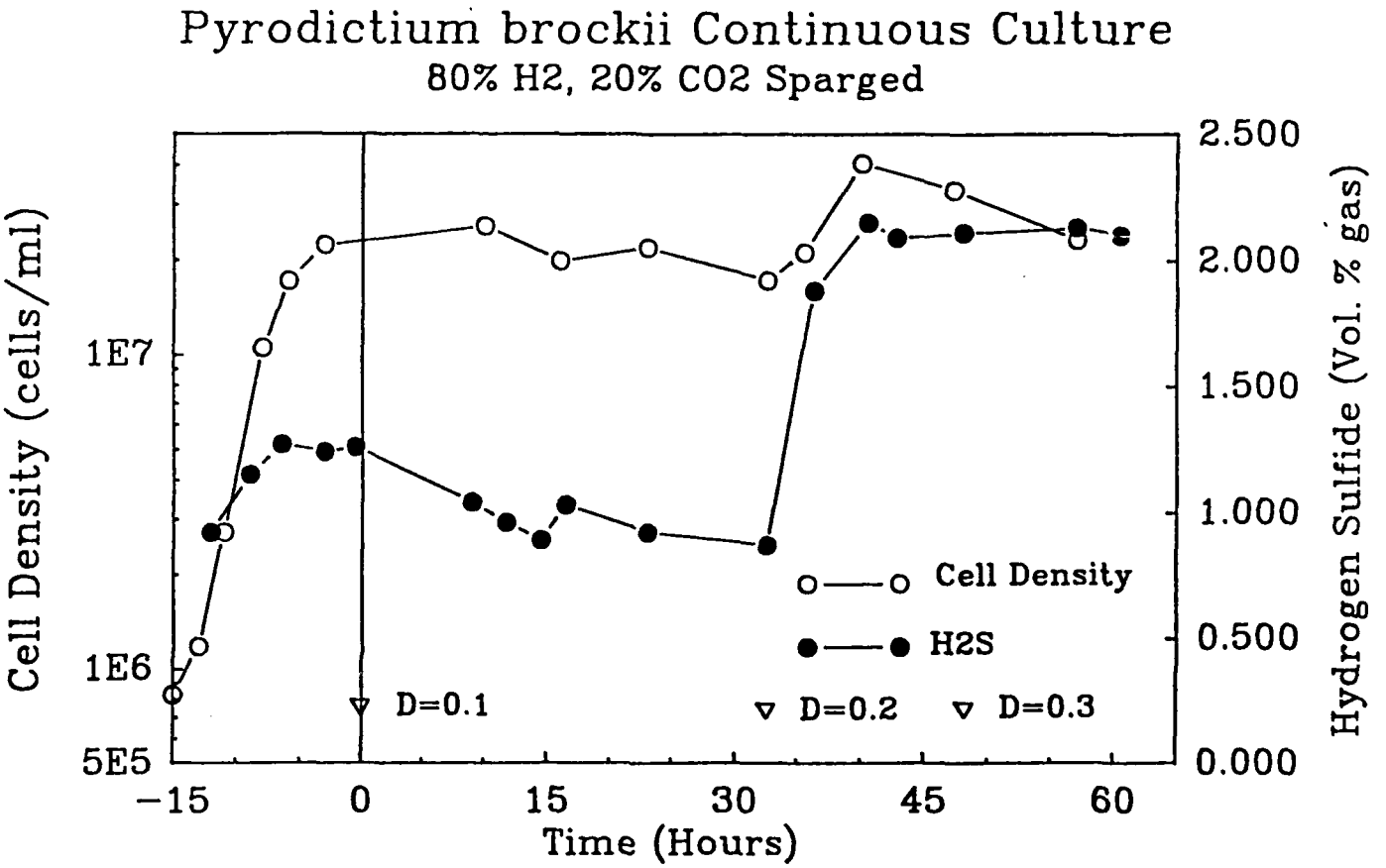

Fig. 1 Cell density and concentration of H_2S in the effluent gas during a continuous culture are plotted. The medium was artificial sea water supplemented with 2 g/l yeast extract and trace elements, culture volume was 1 liter. The temperature was 98 °C. The feed gas 80% H_2, 20% CO_2 and was sparged at a rate of 100 cc/min or 0.1 vvm. The batch portion of the experiment is represented by the time before zero, the dilution rate changes are designated by the inverted triangles and are in reciprocal hours. Elemental sulfur was added periodically. The plot illustrates a range of dilution rates over which the culture density is relatively stable but the production of hydrogen sulfide shows a sharp increase. Whether the discontinuity is due primarily to the discrete additions of elemental sulfur or not is uncertain.

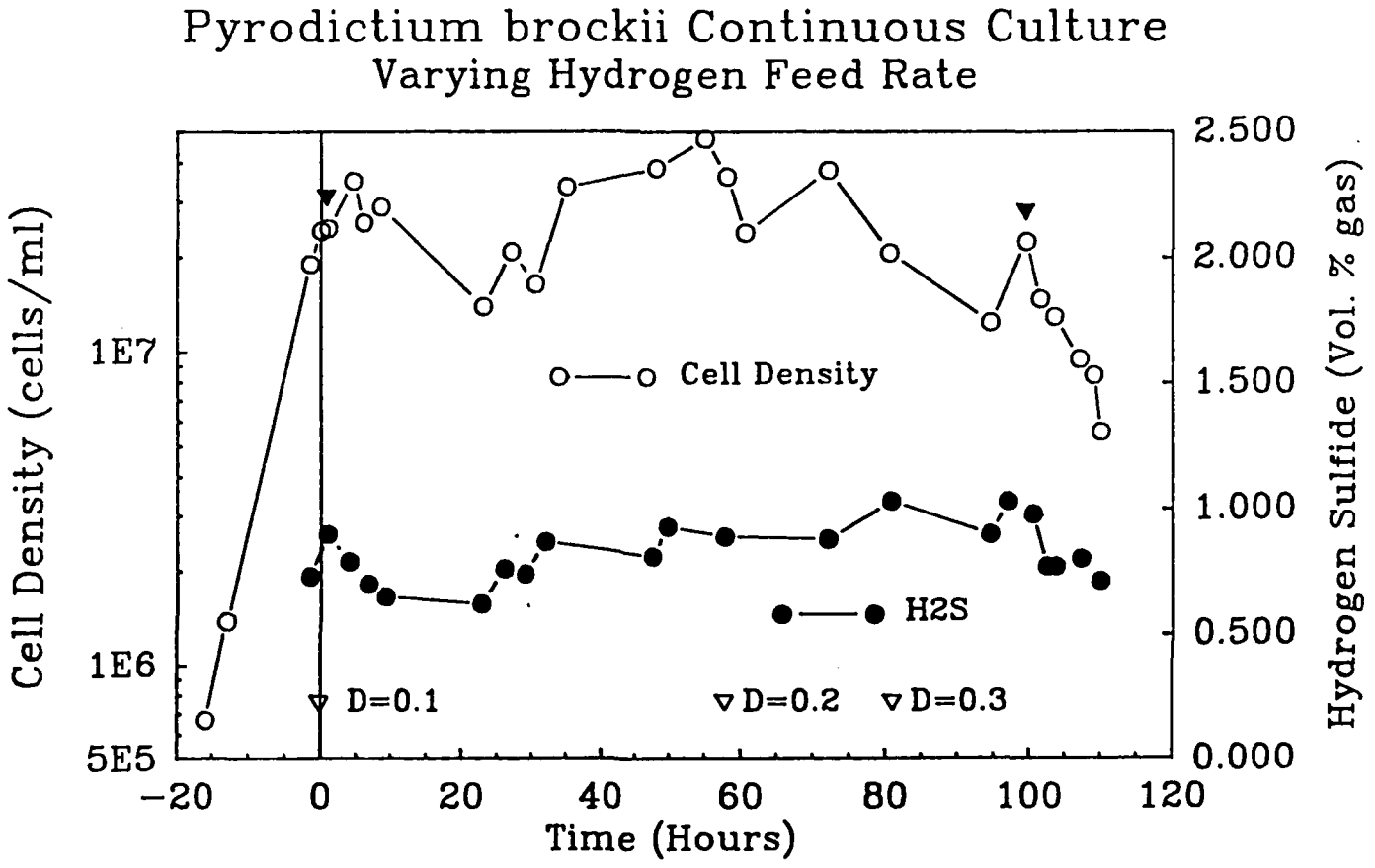

Fig. 2 The same variables are plotted for a second continuous culture experiment run under identical conditions except that the feed rate of H_2 was varied by diluting with N_2. The culture was grown up in batch mode with the same feed gas as the first run, 80/20, H_2/CO_2. Shortly after the start of continuous operation the feed mix was changed to 40/40/20 $N_2/H_2/CO_2$ and the total sparge rate kept the same, 100 cc/min. Note that the cell densities are essentially the same as in the first run but the H_2S production rates are lower for the most part. Note also that at about 100 hours the feed rate for H_2 was lowered again to the composition 60/20/20 $N_2/H_2/CO_2$ with the same volumetric rate, 100 cc/min. At this feed rate the culture was unable to maintain its cell density at the dilution rate of 0.3 h^{-1} even though there was significant H_2 (~ 15 %) in the effluent gas, indicating a gas/liquid mass transfer limitation of an autotrophic culture.

Metabolic Parameters

Hydrogen-Sulfur Activities at Different Growth Rates and Hydrogen Feed Rates				
Dilution Rate	Specific Hydrogenase Activity (nmol H_2 / μg protein · min.)		Specific Hydrogen Sulfide Production (mol$\times 10^{15}$ / cell · min)	
D ($Hour^{-1}$)	80% H_2	40% H_2	80% H_2	40% H_2
0.1	7.05	2.68	1.83	0.92
0.2	3.77	1.63	2.48	1.55
0.3	4.04	5.23	4.06	2.17
all cases: 20% CO_2, balance N_2 for 40% H_2 case				

Table 1 No induction of hydrogenase activity is noted at either feed rate over the range of growth rate investigated. But significant uncoupling in the production of hydrogen sulfide is apparent in the specific production rates. Up to twice as much H_2S was produced per cell at the higher hydrogen feed rate. Note also that specific H_2S production increased with growth rate for both H_2 feed rates.