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NOTES

Sulfur Reduction by the Extremely Thermophilic Archaebacterium *Pyrodictium occultum*

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The relationship between growth and biological sulfur reduction for the extremely thermophilic archaebacterium *Pyrodictium occultum* was studied over a temperature range of 98 to 105°C. The addition of yeast extract (0.2 g/liter) to the medium was found to increase hydrogen sulfide production significantly, especially at higher temperatures. Sulfide production in uninoculated controls with and without yeast extract was noticeable but substantially below the levels observed in samples containing the microorganism.

For microorganisms capable of growth at temperatures above 100°C, there appears to be an important relationship between thermophily and elemental sulfur. This relationship also is significant for other extreme thermophiles growing at slightly lower temperatures, as was demonstrated in recent studies focusing on the reduction of elemental sulfur at high temperatures under anoxic conditions (2). These studies showed significant liquid-phase sulfide production rates in abiotic samples, as well as in cultures of either a heterotrophic eubacterium (77°C and pH 7.5) (3) or a heterotrophic archaebacterium (88°C and pH 7.2) (1). Abiotic sulfide production rates were shown to increase with yeast extract addition (up to 1 g/liter), increasing pH, increasing temperature (above 80°C), or increasing amounts of elemental sulfur. Nonetheless, abiotic sulfide production rates were significantly below those found in the biotic samples.

Given the apparent biological significance of the sulfur chemistry of thermal waters from which a variety of extreme thermophiles have been isolated, the relationship between abiotic and biotic phenomena in these habitats merits further investigation. Along these lines, this study focused on sulfur reduction at elevated temperatures by the extremely thermophilic archaebacterium *Pyrodictium occultum*, first isolated by Stetter (4, 5). *P. occultum* is an obligate anaerobe which grows autotrophically (optimally, at 105°C and pH 5.0 to 7.0) and uses elemental sulfur apparently as an electron acceptor. Of interest was the relationship between growth under autotrophic conditions and sulfur reduction at temperatures of approximately 100°C.

P. occultum DSM2709 was cultured in 125-ml vials equipped with heavy-duty rubber stoppers and aluminum seals (Bellco Glass, Inc., Vineland, N.J.). Temperature baths (New Brunswick Scientific Co., Inc., New Brunswick, N.J.), modified for high-temperature operation and containing silicon oil (Dow Corning Corp., Midland, Mich.), were used for the growth-gas production experiments. Water obtained from the Chesapeake Bay was used for medium formulation; this water was both filter sterilized (0.2-μm-pore-size filter) and autoclaved. To 50 ml of the sterilized water were added 0.3 g of NaCl, 1.5 g of elemental sulfur, 1.1 ml of 0.2 N H_2SO_4 (for pH adjustment), and 0.5 ml of 0.01% (wt/vol) resazurin solution. When yeast extract (Difco Laboratories, Detroit, Mich.) was used, 0.2 g/liter was added. The vials containing the medium were preheated in the temperature bath to drive out oxygen and were sparged with an 80:20 H_2-CO_2 as mixture for approximately 4 min. Then 1.0 ml of a 2.5% (wt/vol) Na_2S aqueous solution was added to reduce any residual oxygen in the medium. The vials were sparged with the H_2-CO_2 gas mixture again until the solution turned clear. This procedure brought the medium to a pH of approximately 5.5 to 6.0. The pH was measured with a Beckman 45 pH meter (Beckman Instruments, Inc., Fullerton, Calif.) which has a combination electrode with a temperature range from −5 to 100°C. Finally, the vials were sealed and pressurized to approximately 4 bars (400 kPa absolute) with the H_2-CO_2 gas mixture. Samples were inoculated approximately 20 min after the vials were returned to the stationary temperature bath with a 2-day-old culture that had been prepared similarly. Periodically, samples were withdrawn from the vials by syringe.

Gas and liquid analyses for sulfide concentration were done with a flame photometric detector on a Varian 3700 gas chromatograph (Varian Associates, Sunnyvale, Calif.) interfaced to an Apple II+ microcomputer. For gas analysis, a Chromosil 310 packed column was used, while for liquid analysis, a Supelpak-S packed column was used; both were obtained from Supelco, Inc., Bellefonte, Pa. Cell counts were done by epifluorescence microscopy with acridine orange stain.

The relationship between cell growth and sulfide production in gas phase for cultures with and without yeast extract can be seen in the results presented in Fig. 1 for temperatures of 98, 100, 103.5, and 105°C. Uninoculated controls were run for the 98 and 105°C cultures and resulted in sulfide levels significantly below those of the inoculated samples, as is evident in the linear curves of Fig. 2. The uninoculated control is more clearly shown in Fig. 3 for the 105°C culture, in which the addition of yeast extract (0.2 g/liter) caused a slight increase in the sulfide levels in gas phase; this was also true for the 98°C control.

Although only gas analyses were performed routinely for this set of experiments, one experiment which monitored dissolved sulfide concentration was conducted at 98°C for

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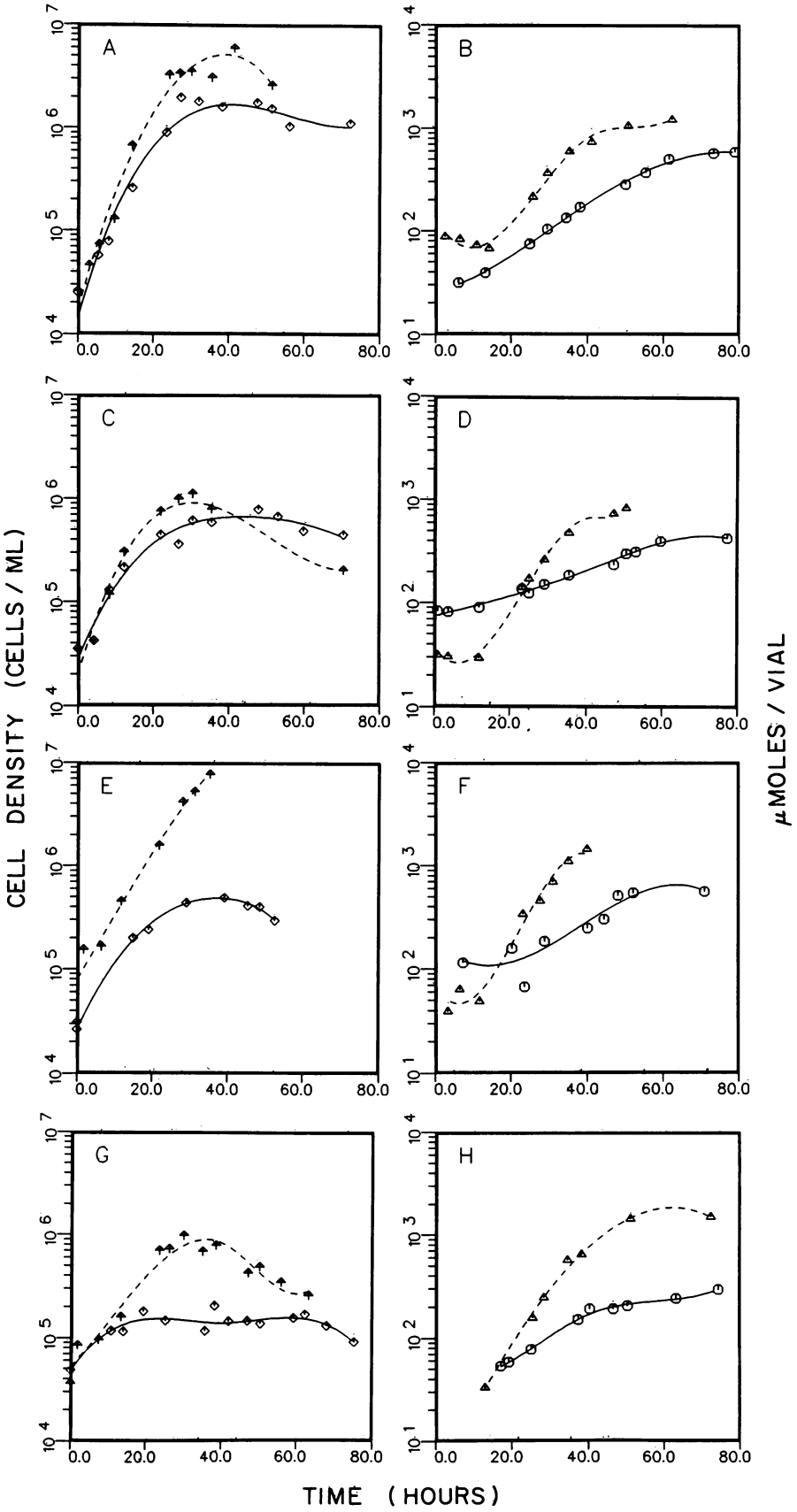

FIG. 1. Effect of yeast extract (YE) (0.2 g/liter) on the growth rate and gas-phase sulfide production of *P. occultum*. Sulfide present in samples was determined to be, and reported as, H_2S. Panels A, C, E, and G show growth curves (cells per milliliter versus hours) at 98, 100, 103.5, and 105°C, respectively. Panels B, D, F, and H show H_2S production (micromoles [of gas] versus hours) at 98, 100, 103.5, and 105°C, respectively. Cells per milliliter with (♦) and without (◇) YE; H_2S micromoles per vial with (▲) and without (▽) YE.

both inoculated and abiotic cases (Fig. 4). Approximately a 2-to-1 split of sulfide between gas and liquid phases was found at this temperature, which is what would be expected if Henry's law holds for this system. It was found that gas-phase sulfide level lagged liquid-phase level slightly, indicating that some degree of mass transfer limitation was present; this lag might be expected since no sparging or agitation took place during the experiments.

The results reported here demonstrate that there is a significant amount of sulfur biotransformation associated with the growth of *P. occultum* at all of the temperatures examined in this study. The gas production per cell (micromoles per cell) was estimated to be about a factor of 100 higher than that of the archaebacterium studied by Belkin et al. (2) (in each case, taking into account both gas and liquid phases). Our controls at 98 and 105°C, however, showed liquid-phase sulfide levels (micromolar per gram of S°) comparable to those measured at lower temperatures by Belkin et al. (2). Furthermore, the influence of yeast extract on sulfide production was pronounced and most evident at the higher growth temperatures. At 98 and 100°C, yeast extract had a much smaller effect on growth than it did on sulfide production, while at 103.5 and 105°C, yeast extract influenced growth more than at the lower temperatures and had a marked effect on sulfide production. Figure 2, which is plotted on a linear scale, illustrates the effect of yeast extract on sulfide production at 98 and 105°C. In all biotic cases, sulfide production was found to increase exponentially at a rate lower than the growth rate, and in general production lagged growth. This was also true for the dissolved sulfide levels monitored in the growth experiment at 98°C (Fig. 4). It is also interesting that sulfide production in inoculated samples continued after the onset of stationary phase.

The relative contributions of abiotic and biological phenomena in considering the effect of yeast extract on sulfide production are difficult to separate. Yeast extract may meet certain nutritional requirements, although the results at 98 and 100°C with and without yeast extract suggest that nutrition may be a minor consideration; these results are

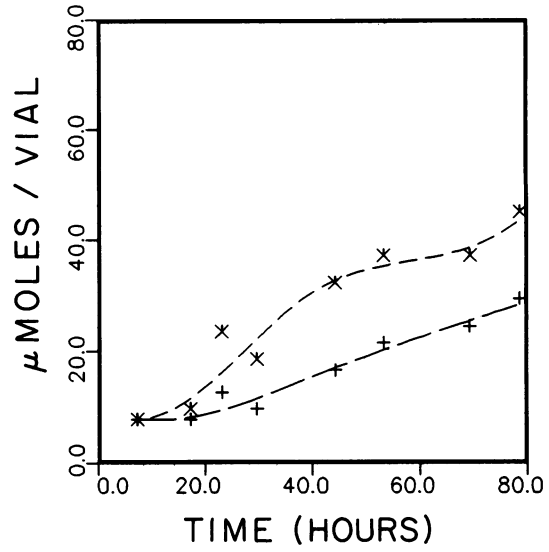

FIG. 3. Gas-phase sulfide production (micromoles per vial) for uninoculated controls at 105°C with (✱) and without (+) yeast extract (0.2 g/liter).

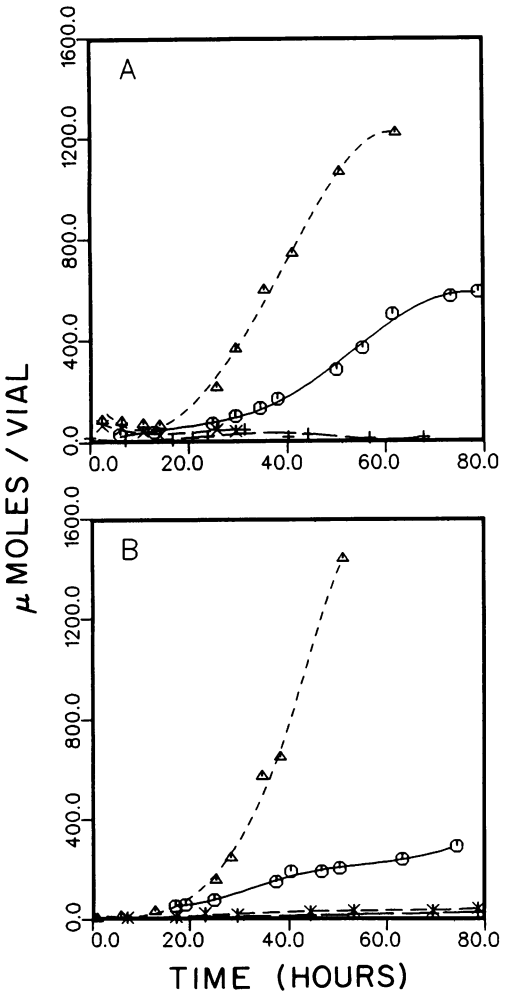

FIG. 2. Gas-phase sulfide production (micromoles per vial) for inoculated and uninoculated vials at 98°C (A) and 105°C (B) with and without yeast extract (YE). Inoculated vial with (△) and without (Ⓣ) YE; uninoculated control with (✱) and without (+) YE.

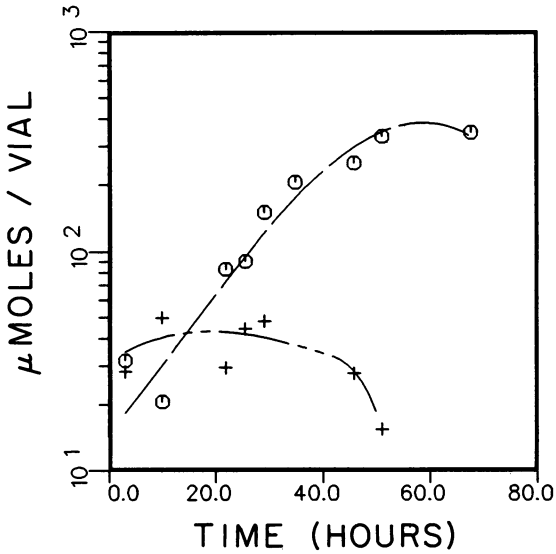

FIG. 4. Dissolved sulfide levels (micromoles per vial) for inoculated (Ⓣ) and uninoculated (+) samples at 98°C.

consistent with previous reports (5). In other experiments where larger amounts of yeast extract (up to 2 g/liter) were added to uninoculated samples (data not shown), it was clearly observed that the elemental sulfur remained as fine particles rather than agglomerating, as was the case when no yeast extract was added. This fact perhaps supports the suggestion by Stetter et al. (5) that yeast extract serves as a wetting agent promoting a higher surface area of sulfur per unit volume of culture. It may be speculated that yeast extract helps to increase the surface area available for cell-sulfur interaction, which may be more important at temperatures above 100°C, given the results of these experiments.

The results reported here reinforce the suggestion made by Belkin et al. (2) as to the importance of abiotic phenomena in considering biological activity in marine and terrestrial hot springs. Clearly, more work is needed to determine the nature of cell-sulfur interactions and the role that yeast extract or other additives play in systems like this one at elevated temperatures.

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