gus *Ustilago sphaerogena* is grown in a medium containing high levels of cobalt ions, large quantities of ferrichrome accumulate in the cells. The action of cobalt is completely reversed by zinc ions (see below). From the known effect of zinc on the cytochrome (*3*) and ferrichrome (*4*) content of *U. sphaerogena*, it appears that zinc ions are required for the utilization of iron by this organism.

Iron (as chloride) was added at a level of 16 mg/liter to a basal medium that was identical to that used previously (*5*), except that zinc was omitted and phosphorus was furnished as KH_2PO_4. The medium was dispensed in 50-ml quantities for sterilization [15 lb (6.8 kg), 15 minutes] and inoculated with 1 ml of a 2-day culture grown in the basal medium, and the cultures were incubated for 48 hours at 30°C with vigorous aeration by agitation. Total ferrichrome was extracted from the washed cells with 1*M* acetic acid and assayed as previously described (*6*). Cell mass was estimated by turbidity measurements and reference to a standard curve of dry weight versus absorbancy at 650 mμ.

The ferrichrome content of cells grown in the medium containing various concentrations of cobalt ions, and the effects of various concentrations of zinc on the accumulation of ferrichrome in the presence of cobalt ($3 \times 10^{-5}M$) are shown in Fig. 1. The dry weights of the cell mass in the cultures varied from 2 to 4 mg/ml. Neither manganese nor nickel ions at $3 \times 10^{-5}M$ concentration promoted the production of ferrichrome, nor did these ions reverse the effect of cobalt ion ($3 \times 10^{-5}M$). Since zinc does not inhibit the production of iron-binding compounds by iron-deficient cultures of *U. sphaerogena* (*5*), it seems likely that zinc is required for the utilization of iron. Further indirect evidence which supports the hypothesis that zinc deficiency, at least in some aspects, is essentially iron deficiency was the observation that diminished porphyrin synthesis occurs when cultures are grown in a medium low in zinc but containing normal amounts of iron. The speculation of Grimm and Allen (*3*) that depressed porphyrin synthesis occurs in zinc-deficient medium was proven correct by the extraction and measurement of porphyrins in the cells, and by the detection of a low specific activity of δ-aminolevulinic acid dehydrase in such zinc-deficient cells. Diminished porphyrin synthesis and low specific activity of δ-aminolevulinic acid dehydrase had been observed previously by Burnham (*7*) with iron-deficient cells of *U. sphaerogena*.

Ferrichrome is believed to act in microbial metabolism as a "coenzyme" for the transfer of iron. The present work suggests that the movement of iron from ferrichrome to the iron-containing enzymes and other iron-requiring sites within the cell involves the participation of zinc in a mechanism which can be blocked by cobalt.

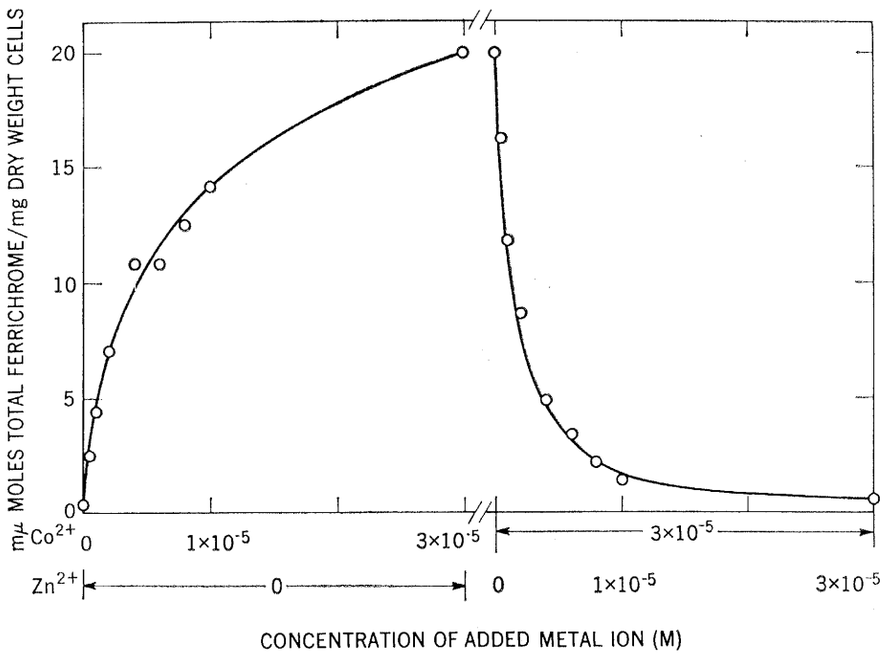

Fig. 1. Accumulation of ferrichrome by cells of *Ustilago sphaerogena* in a medium containing cobalt, and its counteraction by zinc.

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Laboratory Culturing of a Thermophilic Alga at High Temperature

Abstract. *Conditions for laboratory growth of the thermophilic alga* Cyanidium caldarium *at elevated temperatures have been developed. Growth characteristics of the organisms are described.*

Thermophilic algae that exist in hot springs and geysers are among the most resistant of all organisms to the deleterious effects of high temperature. It has been claimed that these microorganisms can grow at temperatures as high as 85°C in their native environment (*1–3*). However, growth above 70°C has not been reported for any thermophilic alga under laboratory conditions on a defined medium (*4*). Thermophiles appear to be a potential source of heat-stable enzymes. Our specific need for thermostable aminoacyl-sRNA synthetases with acidic *p*H optima (*5*) led us to investigate a species of algae that had been reported to grow at environmental temperatures as high as 82.5°C and to survive intermittent scaldings with boiling acidic water at 15- to 30-minute intervals (*3*). We now describe culture-growth characteristics of the thermophilic alga *Cyanidium caldarium* under laboratory conditions approximating its natural habitat of high temperatures and low *p*H.

Tilden first described this alga in 1896 and reported them growing at several locations in Yellowstone National Park (*6*). They have subsequently been found elsewhere, including the hot

springs of Sonoma, California (*2*), and the Sunda Islands of Indonesia (*7*). Wherever found, the waters are acidic; in fact, *Cyanidium* has been reported growing in 0.1*N* sulfuric acid (*8*).

Cyanidium caldarium has been variously classified as a blue-green alga (*9*), a green alga (*10*), a red alga (*11*), a coccoid cryptomonad (*12*), and even as a symbiont resulting from an association between a blue-green alga and a colorless chlorophyte (*13*) because it shares common features with all these forms. From its gross morphological characteristics, Geitler has regarded this organism as a transitional form, sufficiently unique to merit generic rank and classified *Cyanidium caldarium*, the only member of its genus, in a new family, the Cyanidiaceae (*14*).

Two sources of *Cyanidium* were used. One was a pure culture obtained from M. B. Allen. The other was isolated from a hot-spring sample withdrawn at Crater Hills in Yellowstone National Park. The two strains seem to be identical by all criteria of growth rate, nutrition, and morphology.

Cyanidium was isolated from the hot-spring water sample and freed of contaminants by repeated platings and transfers at 55°C on malt extract agar (Baltimore Biological Laboratories) supplemented with 1 percent Bactoagar (Difco) (which was acidified to *p*H 3.5 with H_2SO_4 after autoclaving). Bacterial contaminants were removed in an early transfer made to plates containing 0.3 mg of streptomycin sulfate per milliliter, incubated at 30°C. For final isolation, *Cyanidium* was transferred to the liquid inorganic culture medium described by Allen (*8*), but adjusted to *p*H 2 and incubated at 45°C with aeration and illumination. *Cyanidium* cultures were routinely maintained in a modified liquid culture medium described below. A *p*H of 2 was maintained and cultures were stored both at room temperature (22°C) and 55°C.

The supplemented liquid medium used for culturing *Cyanidium caldarium* consists of 0.001*M* KH_2PO_4, 0.001*M* $CaCl_2$, 0.001*M* $MgCl_2$, 0.015*M* $(NH_4)_2SO_4$, 0.5 percent D(+)-galactose, 0.05 percent malt extract, 0.3 parts of a trace-metals mixture per liter and sufficient 10*N* H_2SO_4 to bring the medium to the desired *p*H. The trace metals mixture contains in 300-ml, $Fe(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$, 4 g; H_3BO_3, 0.5 g; $MnSO_4 \cdot 2H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; $CuSO_4 \cdot 5H_2O$, 0.02 g; $(NH_4)_6Mo_4O_{24} \cdot 4H_2O$, 0.01 g; $VOSO_4 \cdot 2H_2O$, 0.01 g; $CoSO_4$, 0.01 g. The $(NH_4)_2SO_4$ must be added to a large volume of water to prevent precipitation once the $CaCl_2$ and $MgCl_2$ have been added. The medium was autoclaved after adjusting the *p*H with 10*N* H_2SO_4 at 2 atmospheres absolute pressure for 15 minutes.

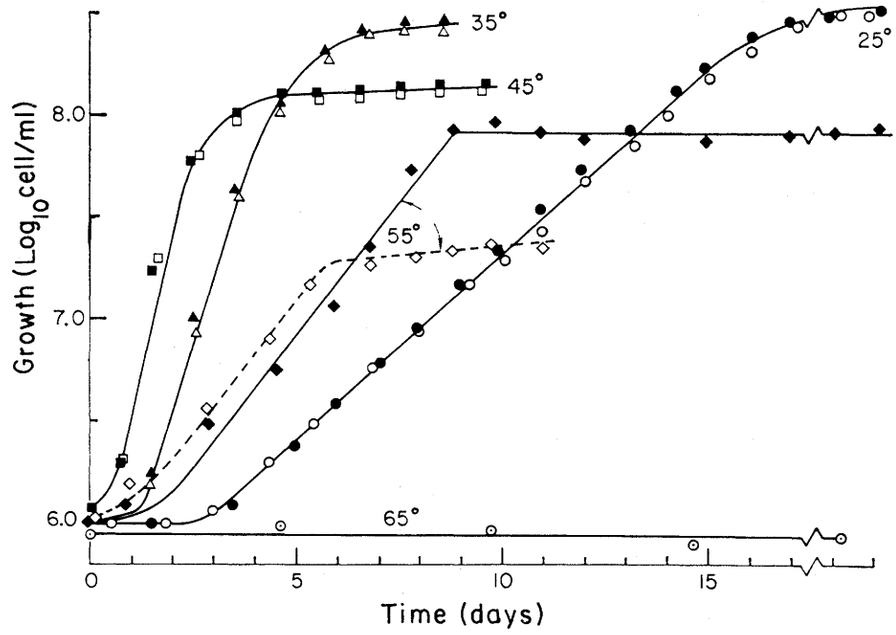

Fig. 1. Growth of *Cyanidium caldarium* as a function of temperature at *p*H 1 (open circles) and *p*H 2 (solid circles). Growth was followed in a Klett colorimeter (red filter No. 70) that had been calibrated against known numbers of *Cyanidium* cells.

This liquid medium differs from the usual algal medium, which is inorganic, in its content of galactose and malt extract. Without these supplements, growth at elevated temperatures and low *p*H was very limited. The use of galactose also eliminated the necessity of bubbling CO_2 through the medium. Moreover, it is difficult to maintain a sufficient CO_2 supply at the desired high temperatures and low *p*H because both these conditions greatly decrease CO_2 solubility. While cell yields improved with galactose alone, further supplementation with malt extract was required for logarithmic growth. A similar effect was not obtained with yeast extract, various protein hydrolyzates or mixed vitamin supplements. The beneficial effect of malt extract is consistent with the findings of Baker *et al.* that many strains of a thermophilic bacterial species behave as auxotrophs only when their temperature of growth is elevated (*15*). The medium described above afforded good growth up to 60°C in small culture flasks and in batch cultures (50-liter New Brunswick Scientific Co. fermacel, equipped with water-jacketed quartz illuminator), so long as moderate aeration and illumination were provided.

In the earlier experiments cultures in DeLong flasks were "read" in a Klett colorimeter. This method lacks some sensitivity because it does not discriminate between cell size and cell number. Later, direct cell counts were made with a Levy ultraplane hemacytometer.

Because of the low *p*H of stock cultures, inoculation was routinely carried out with a known number of cells washed with distilled water. This procedure did not adversely affect the cells. Inoculated cultures were agitated at 150 rev/min in a New Brunswick (model G-77) gyrotatory shaker at the desired temperature ± 0.5°C. Continuous illumination was provided by two 14-watt fluorescent warm-white bulbs located 15 cm from the surface of the medium. The *p*H was measured before the beginning and after termination of growth. Growth rates were compared in terms of the mean generation time (T_g), which is defined as the time required for the population of cells to double during the phase of logarithmic growth. Temperature, *p*H, and the influence of the temperature at which the cell inoculum had been grown were studied.

The curves in Fig. 1 describe the dependence of growth rate on temperature at *p*H 1 and *p*H 2 from stationary-cell inocula raised at *p*H 2 at tempera-

tures close to the experimental temperatures. While there is a definite temperature effect, there is no distinction between growth at *p*H 1 and 2 below 55°C. At that temperature, however, there was nearly a threefold diminution in final cell density at *p*H 1 compared with that at *p*H 2, even though T_g was unaffected. Furthermore, at *p*H 1 the cells tended to remain unicellular, became slightly larger and pale yellow in color, and tended to settle out of the medium. Those cells growing at *p*H 2 remained green, but they aggregated, forming colonies (up to 100 cells) that remained in suspension.

The growth rate at *p*H 2 (Table 1) on an enriched medium reaches a maximum close to 45°C, similar to that found by Allen with the inorganic medium (*8*). No such maximum was observed for limiting cell density, however. Instead, the cell yields decreased with increasing temperature in essentially linear fashion, with no cell yield near 65°C. In fact, at 65°C no measurable cell division occurs even over an extended period of time (Fig. 1). After 18 days at 65°C, lowering the temperature to 45°C did not bring about resumption of growth. Microscopic examination revealed no apparent morphological changes after such prolonged incubation, nor was there any lysis of cells. When stationary phase cells grown at 45°C were transferred to fresh medium and incubated at 65°C for 24 hours or less, although they bleached rapidly, they were still able to resume growth (after a lag of 3 days) once returned to fresh medium at 45°C. Normal pigmentation returned to these cells after 5 days. By contrast, when stationary cells at 45°C were not transferred to a fresh medium they began lysing after bleaching rapidly. This suggests that maintenance of cell viability at 65°C might require a continuing supply of essential nutrients.

Investigation of the dependence of growth at 45°C on *p*H from *p*H 1 to 6 was limited by the inability of the medium to buffer the acid produced by the growing cells. Consequently, cultures not titrated during growth eventually approached *p*H 2 regardless of their initial *p*H, and the amount of growth similarly approached that observed at *p*H 2. When such cultures were adjusted to the intended *p*H's during the log phase of growth, the growth rate diminished with increasing *p*H, so that above *p*H 5 further growth did not take place.

Table 1. Growth of *Cyanidium caldarium* at *p*H 2.0. All cultures were inoculated with that number of cells which resulted in an initial density of 10^6 cells per milliliter.

Growth temperature of:		Mean generation time (hr)	Yield (10^8 cells/ml)
Culture (°C)	Inoculum (°C)		
25	25	41	3.5
35	25	12	2.8
45	35	8	1.4
55	55	26	0.8
65	55	∞	< 0.01

Figure 2 depicts the effects of abruptly changing the incubation temperature of cells that had been acclimatized to some other temperature. Cells in the mid-log phase were grown at 25° or 55°C and used as inocula for cell growth at 25°C (Fig. 2*a*). It is evident that cells not acclimatized to 25°C exhibit a significant lag before commencing growth. By contrast, T_g and final cell density are unaffected by the previous history of the inocula.

In complementary experiments where the temperature was abruptly increased, a similar effect was observed (Fig. 2*b*). Thus, the inocula grown at 25°C now showed the lag when used to inoculate a medium at 55°C. Again, T_g and final cell densities were unaffected. The cells acclimatized and raised at 25°C

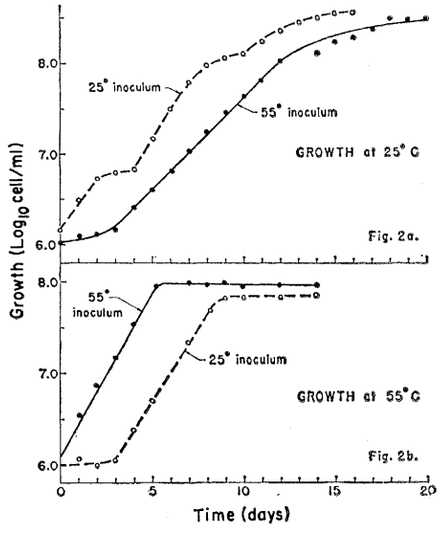

Fig. 2. (*a*) Growth of Cyanidium *caldarium* at *p*H 2.0 and 25°C. (These experiments were monitored by direct cell counting.) ●——● inoculated with mid-log phase cells raised at 55°C; ○——○ inoculated with cells in the mid-log phase of growth, raised at 25°C. (*b*) Growth of *Cyanidium caldarium* at *p*H 2.0 and 55°C. ●——● inoculated with mid-log phase cells raised at 55°C; ○——○ inoculated with mid-log phase cells raised at 25°C.

exhibit an unusual growth pattern characterized by occasional lags. This may be due to heterogeneity in the stage of endospore formation in inocula raised at 25°C where growth is very slow. Such a growth pattern has not been observed at higher temperatures. Furthermore such an anomalous growth pattern was not observed in the growth experiments described in Fig. 1, presumably because growth there was followed with the Klett colorimeter.

Our work indicates that reports of *Cyanidium* growth at temperatures greater than 80°C must be interpreted as an ability to survive periodic exposure to such high temperatures. It seems unlikely that *Cyanidium* can undergo cell division at temperatures much greater than 60°C, particularly under the conditions of minimum nutrition prevalent in its natural habitat. Indeed, all reports of growth of thermophiles in excess of 70°C require quantitative validation. Kempner has shown that many species of hot-springs algae are unable to incorporate P^{32} into their nucleic acids at environmental temperatures greater than 73°C (*16*). Experiments in this laboratory have indicated survival of *Cyanidium* after exposure for many hours to temperatures 10° to 20°C higher than the maximum temperature of growth, 60°C. This is in contrast to the findings of others that the upper temperature limits for growth in some algal and bacterial thermophiles are very close to the temperatures lethal for these cells (*17–19*).

Our previous work has indicated unusual resilience towards heat for certain biosynthetic enzymes extracted from *Cyanidium* (*5*). These enzymes, capable of functioning at 37°C, showed relatively little loss of activity when assayed at 37°C after brief exposures to temperature in excess of 90°C, or to exposure for longer periods of time at somewhat lower temperatures. However, these enzymes were not functional at temperatures near 90°C or for that matter at temperatures much in excess of the cell growth temperatures. Thus, the activities observed at 37°C must represent a renaturing effect.

These observations are consistent with the notion that *Cyanidium* can adapt to its environment by being able to multiply over a wide range of moderate temperatures. While cell division ceases whenever unfavorable environmental temperatures occur, the enzymes retain the capacity to function at moderate temperatures because of the unusual resistance of the *Cyanidium* proteins to

the deleterious effects of high temperatures.

Our work has indicated that *Cyanidium* is an obligate acidophile. Earlier reports of growth above *p*H 5 may well be due to the capacity of the organism to acidify the culture medium rapidly, or else the method of inoculation may have introduced acid into the medium (*8, 20*).

The lag periods induced in growth by abrupt temperature changes have also been observed for thermophilic bacteria (*21*), as well as for some mesophilic organisms (*22*). Such lags may be due to adaptive processes involving temperature-sensitive control mechanisms.

From our studies further work with *Cyanidium caldarium* is more feasible. There are indications that this organism offers an opportunity to study the mechanism of accommodation of cells to elevated temperatures, while at the same time serving as a source of heat-stable enzymes.

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Feedback Inhibition of Glycerol Kinase, a Catabolic Enzyme in Escherichia coli

Abstract. *Fructose-1,6-diphosphate is a feedback inhibitor of the catabolic enzyme, glycerol kinase, in* Escherichia coli. *A mutant was isolated which produced a desensitized enzyme. Glucose was no longer as effective in preventing the utilization of exogenous glycerol by cells which synthesized constitutively such an altered enzyme, even though the usual degree of catabolite repression still operated.*

Feedback inhibition plays a part in the regulation of biosynthetic pathways in bacteria (*1*). A similar type of control has also been discovered in an anaplerotic (or replenishing) pathway (*2*). We now describe a case of feedback inhibition in a catabolic pathway encountered in our study of glycerol dissimilation by *Escherichia coli*.

The utilization of glycerol by cells of *E. coli* is obligatorily dependent upon a specific kinase (*3*). This kinase for glycerol not only mediates the first reaction in the dissimilatory pathway, but it also is responsible for the trapping of the substrate which is freely diffusible across the cell membrane (*4*). Utilization of glycerol can be greatly reduced by the metabolism of glucose, which exerts strong catabolite repression on the glycerol kinase even in cells producing this enzyme constitutively (*5*). During the course of screening for mutants in which the formation of glycerol kinase was resistant to glucose repression, using the ability of a colony to incorporate C^{14}-glycerol in the presence of glucose as a criterion (*6*) we uncovered an anomalous variant (strain 43). This mutant was still as sensitive to the repression as its parent, strain 7, but was able to incorporate glycerol while growing on glucose. This unexpected characteristic suggested that there is another type of control mechanism in addition to catabolite repression and that relief from either of these two controls is sufficient to permit simultaneous utilization of glycerol and glucose.

Results from a study of suspensions of whole cells indicated that the additional control was effected through a kinetic feedback mechanism. Cells of the parental constitutive strain, growing in the logarithmic phase on glucose, were promptly collected and suspended in basal medium. When glycerol was added immediately to such a cell suspension together with phenazine methosulfate and a tetrazolium dye, only a slight stimulation of the rate of reduction of the dye occurred (Fig. 1*A*). However, if the cells were first fasted for 30 minutes at 25°C, there was a rapid reaction when glycerol and the electron

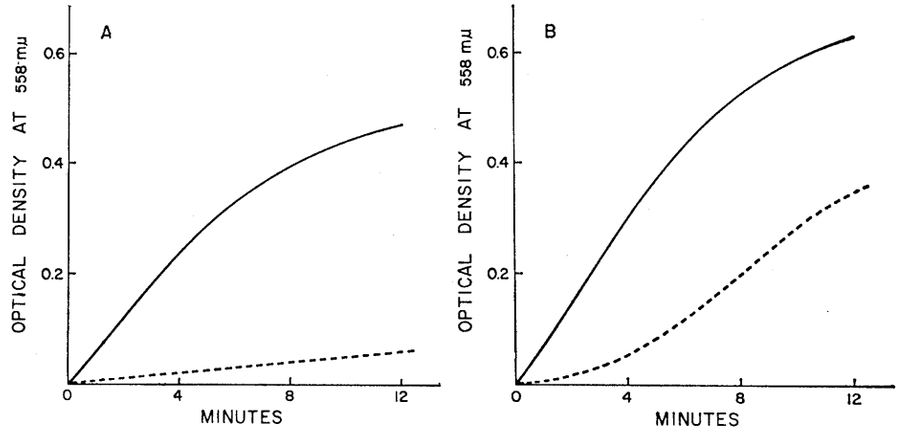

Fig. 1. The cells were grown on 0.2 percent glucose in 25-ml cultures incubated in 300-ml erlenmeyer flasks. When the culture density reached 100 Klett units (420 mμ filter), the cells were collected by centrifugation, washed twice with cold basal inorganic medium, and resuspended in 0.5 ml of the same solution at 0°C. One portion of cells (------) was tested immediately for the glycerol-dependent reduction of 3(4,5-dimethylthiazolyl-1,2)2,5-diphenyltetrazolium bromide in the presence of phenazine methosulfate (*3, 15*). Another portion was first incubated for 30 minutes at 25°C before exposure to glycerol and the hydrogen acceptors (——). The reduction of the tetrazolium was monitored in a Gilford Model 2000 spectrophotometer with automatic recording device. The curves were corrected for the blank in which glycerol was omitted. *A*, Parent strain; *B*, mutant strain.