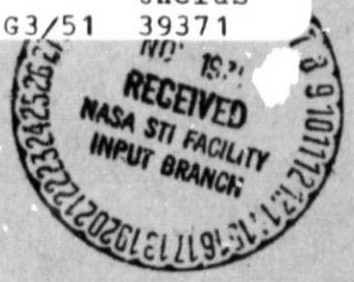
# THE PERFORMANCE AND CAPABILITIES OF TERRESTRIAL ORGANISMS IN EXTREME AND UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS



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SEMI-ANNUAL REPORT

# THE PERFORMANCE AND CAPABILITIES OF TERRESTRIAL ORGANISMS IN EXTREME AND UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS

Performance of Fungi in Exotic and Harsh Environments

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# INTRODUCTION

This report continues previous observations on the growth of *Penicillium* in saline and low temperature conditions, carrying the study to 15 months of incubation time.

It also includes two papers, one on *Penicillium* in saline and low temperature media, the other on geomycology. Both will appear in the transactions of the 18th Annual meeting of COSPAR. The geomycology paper is an extension of the study of supersaline media by replacing light metals (e.g. Na, K) with heavier cations. The context, however, is more ecological in the sense that it deals with the potential of fungi for modification of the surface geochemistry of the earth.

Further, the same group of fungi that here show outstanding adaptability to saline and low temperature environments exhibit the capacity for solubilizing and concentrating metals. These capabilities may well be related.

PROGRESS REPORT

## Performance Capabilities of *Penicillium notatum* in High Salt and Low Temperature Environments After 15 Months

There are organisms capable of growth in saturated NaCl from bacteria to brine shrimp, but the relative distribution of salt adaptability is not known. In nutrient media saturated with sodium chloride all known strains of *Penicillium notatum* cannot grow. This is also the case for many related species of *Penicillium* and the closely allied genus *Aspergillus*.

Our present strain of *Penicillium* (and most others as well) grows well in nutrient saturated with potassium or rubidium chloride (Table 1). Microcolonies appear at 24°C within 30 days in glucose-peptone-yeast (GPY) medium, although a somewhat longer lag period is required in Czapek nutrient containing nitrate as the sole N-source.

A major experiment combining extreme saline conditions with a range of temperatures and nutrient solutions was described previously after 6 months of incubation. Included were NaCl, Na-acetate, KCl, RbCl, $NH_4Cl$, $CaCl_2$ and two special salt mixtures of ecological interest. One of these, the Dead Sea mix is only a gross approximation of that body's composition consisting of a solution with saturating quantities of $Na_2SO_4$, NaCl and $MgSO_4$. The second,

Table 1. *Penicillium* Growth in Number of Cultures/5 Replicates Using Saturated Chloride Cations of Similar Size.

| | | | Temperature °C | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 24 | | | | 6 | | | | -6 | | | |
| | | | Months | | | | Months | | | | Months | | | |
| | | | 6 | 9 | 12 | 15 | 6 | 9 | 12 | 15 | 6 | 9 | 12 | 15 |
| Salt | Nutrient | Growth | | | | | | | | | | | | |
| KCl | Cz | Sub | 2 | 2 | — | — | 2 | 5 | 5 | 5 | 0 | 0 | 0 | 0 |
| | | Surf | 2 | 3 | 5 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Spor | 2 | 3 | 3 | 5 | 0 | 0 | 2 | 3 | 0 | 0 | 0 | 0 |
| | GPY | Sub | 1 | — | — | — | 4 | 4 | 4 | 3 | 1 | 2 | 3 | 5 |
| | | Surf | 4 | 5 | 5 | 5 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 0 |
| | | Spor | 4 | 5 | 5 | 5 | 1 | 1 | 2 | 3 | 0 | 0 | 0 | 0 |
| RbCl | Cz | Sub | 1 | — | — | — | 1 | 3 | 3 | 3 | 0 | 0 | 0 | 0 |
| | | Surf | 2 | 5 | 5 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Spor | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GPY | Sub | 1 | — | — | — | 1 | 3 | 3 | 3 | 1 | 2 | 2 | 4 |
| | | Surf | 2 | 5 | 5 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Spor | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $NH_4Cl$ | Cz | Sub | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Surf | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Spor | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GPY | Sub | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Surf | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Spor | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Sub = submerged; Surf = surface; Spor = sporulating

like Don Juan Pond in the U.S. Antarctic was a $CaCl_2$ brine but also contained small quantities of sulfate, chloride, potassium and sodium ions and iron.

In addition to general salt and nutrient composition, a major variable was the presence or absence of K-ion as a supplement. The rationale for this was the preliminary finding that KCl but not NaCl regularly supported growth, even if slow. It was reasoned that the absence of growth in Na-salts was primarily a K-deficiency response, although Na-intoxication might well provide a secondary basis for response.

A complex nutrient (i.e., GPY containing pre-formed organic N) made little difference at 24°C at 6 months, but supports a doubled growth rate at 6°C. At -6°C only GPY permits any growth at all over the 6 month period. This we believe is the lowest constant temperature on record for the growth of filamentous fungi.

At all temperatures in KCl and RbCl microcolonies first appear as more or less spherical bodies in culture sediments. At 24°C they gradually enlarge but eventually "seed" surface colonies; at the lower temperatures the submerged colonies had not surfaced after 6 months.

Considering now, the overall 15-month picture, it is evident that only KCl and RbCl provide really effective media for extensive growth. Yet even here, Rb-ion fails to support sporulation, even after an incubation period of more than one year. Between 24° and

6°, growth per se is not highly temperature sensitive in KCL or RbCl media with either simple or complex nutrient media, whereas sporulation in KCL media becomes both temperature and nutrient dependent at 6°C. At -6° no sporulation was noted in any medium, but growth took place only in complex (organic N) nutrients.

In summary (Table 2) we can recognize growth-and sporulation-dependent conditions uniquely involving K-ion. In addition the requirement for K-ion (vs. Rb-ion) and the ability to convert nitrates to organic N serve as determinants of lower temperature limits.

In all other media (Table 3), growth was limited, and those salts in which it was observed have been denoted by "+". None of these conditions permitted sporulation.

In NaCl, growth occurs only with KCL supplementation at 24 and 6°C, but even the presence of K-ion could not offset an ambient -6°. Strikingly, K-ion failed to promote growth in Na-acetate medium at 24°, but did so at lower temperatures. Conceivably, there is a temperature-dependent toxic effect of acetate ion. One possibility is that acetate is converted to toxic levels of oxalate at 24°, and that conversion is suppressed or slowed at temperatures of 6° or less. The conversion of acetate to oxalte via glycolic and glyoxylic acids is known.

Ammonium-ion toxicity is also well known and may account for failure of K-ion to promote growth in $NH_4Cl$.

Table 2. A Summary of *Penicillium* Growth in Relation to N-Source Cations and Temperature

| Cation | Inorganic N | | Organic N | |
|---|---|---|---|---|
| | Growth | Sporulation | Growth | Sporulation |
| -K | negative | negative | negative | negative |
| +Rb | 6° | negative | -6° | negative |
| +K | 6° | 24/slow at 6° | -6° | 6° |

Table 3. Growth of *Penicillium* In Experimental Media of Varying Composition.

| Salt | Nutrients | Potassium Supplement | Temperature °C: 24 Months | | | | 6 Months | | | | -6 Months | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 6 | 9 | 12 | 15 | 6 | 9 | 12 | 15 | 6 | 9 | 12 | 15 |
| NaCl | Cz | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | + | + | + | + | + | 0 | 0 | + | + | 0 | 0 | 0 | 0 |
| | GPY | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | + | + | + | + | + | 0 | + | + | + | 0 | 0 | 0 | 0 |
| NaOAc | Cz | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | + | 0 | 0 | 0 | 0 | 0 | 0 | + | + | 0 | 0 | + | + |
| | GPY | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | + | 0 | 0 | 0 | 0 | 0 | 0 | + | + | 0 | 0 | + | + |
| $CaCl_2$ | Cz | - | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | + | + | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 |
| | GPY | - | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + |
| | | + | + | + | + | + | 0 | + | + | + | 0 | + | + | + |
| Dead Sea | Cz | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | + | + | + | + | + | 0 | + | + | + | 0 | + | + | + |
| | GPY | - | + | + | + | + | 0 | + | + | + | 0 | 0 | 0 | 0 |
| | | + | + | + | + | + | + | + | + | + | 0 | + | + | + |
| Don Juan Pond | Cz | - | + | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 |
| | | + | + | + | + | + | + | + | + | + | 0 | + | + | + |
| | GPY | - | + | + | + | + | 0 | + | + | + | 0 | 0 | 0 | + |
| | | + | + | + | + | + | 0 | + | + | + | 0 | + | + | + |

In $CaCl_2$ media, the organism responds as if in KCL (but slower) at 24°, but as if in NaCl at 6°. At -6°, However, it is not K-ion but organic N that determines growth. Possibly Ca-ion can replace K-ion in its less specific functions, thereby sparing whatever small quantities of K-ion may be present for specialized needs. At -6°, there may indeed be K-ion enough for the very limited growth observed but an absolute need for preformed organic N compounds. Note, however, that K-ion induces growth to appear sooner.

The two simulated natural media are more or less similar to one another and in a general way to $CaCl_2$ media. A specific difference of note is the need in Dead Sea salts for either K-ion or organic N and the shift to complete K-ion dependency at -6°.

These data again underscore the importance of K-ion and to some extent of organic N in saline media as the temperature falls.

These data also suggest that even the rather forbidding conditions simulated in the Don Juan Pond and Dead Sea media cannot eliminate eukaryotic life forms.

Although observations are planned on all of these cultures at least through 24 months in culture, and some may be continued on an indefinite schedule, the next phase of this study can now be initiated:

1) Using KCL and RbCl media, the growth of *Aspergillus* and *Penicillium* will be examined in more detail. Salt-free and saline cultures will be compared at low and high temperatures with respect to N-source metabolic poisons and related factors.

2) Production of phenolic metabolites will be measured as an index of environmental condition.

3) Search will continue for a NaCl-adapted strain.

## Geomycology

### Abstract

Fungi have long been known to have capabilities for reduction and alkylation of arsenate and selenate but their general capabilities for solubilizing and accumulating metallic substances have only been given serious attention with recent years.

Common members of the Aspergillaceae cultured on boron, copper, lead and other metals or oxides can solubilize and concentrate the elements or their compounds.

To account for bio-solubilization of the metals, we have set up a model study, incubating selected metals, e.g., mercury, in solutions of various metabolites including L-lysine and citric acid. Results of 100-300 days incubation showed that many metals can in fact be readily solubilized, and in some cases more effectively at pH 6-7 than at pH 1.5-2.

Established microbiological interactions upon or within the lithosphere include surface degradation of igneous rocks by lichens; oxidation and deposition of iron, manganese and sulfur by bacteria; and conversions of inorganic arsenic, selenium, tellurium and mercury to organic derivatives [1, 3-5, 8-10]. The utility of selective metal accumulation in higher plants has been recognized in geobotanical prospecting [2], but such capabilities have only recently been recognized in fungi, particularly members of the Aspergillaceae [6, 7].

Species of *Penicillium* and *Aspergillus* were grown in standard glucose-peptone media at 24-25°C. These media were either controls or supplemented with metals or metal oxides as specified below. In the cultures supplied B, Al, Bi, HgO and PbO, the elements were 100 to 150 mesh powders and the oxides, even finer, were sealed in dialysis bags floated in the medium to avoid errors because of adhesion to fungal filaments. The other elements were presented in thin sheet or foil form with shallow layers of medium so that contact could be made with the fungal mycelium. Cultures were inoculated with conidiospores and, in all results presented here, incubated for 30 days. Analyses for the metal were carried out both on filtered medium and washed mycelium using emmision spectography or atomic absorption spectrophotometry (Hg, Cd, Cu, Pb).

Model experiments using aqueous solutions of selected metabolites were also set up using a variety of metals or compounds. Of these, trials with Cu, pyrite ($CuFeS_2$), Hg and cinnabar (HgS) are presented here. All analyses were carried out using atomic absorption. All experiments were duplicated and analyses run in duplicates or triplicate.

*Penicillium* was grown in the presence of elements representing four periodic groups and a range of redox and metallic properties (Table 1). In the case of B, the element and highly stable oxidized form $H_3BO_3$ were compared. The acid saturated medium (0.2M) constituted a stress condition in itself. In spite of its well-known refractory character, B was better solubilized and concentrated over 300-fold by the organism. Evidence was also obtained suggesting that one mode of B assimilation may take place via boranes synthesis.

The two concentration parameters of interest are the ratio, based upon wet weight : aqueous solution and percentage of total dry matter. Excluding $H_3BO_3$, the concentration ratios ranked, disregarding lesser difference:

B > Zn, Cu, Al > Cd, Bi.

On a dry matter basis, the ranking differed:

Cu > Al > Zn, B > Cd, Bi.

This suggests the complex nature of the interactions involving solubilization, uptake, intracellular deposition and cytotoxic limits.

Table 1. Element Accumulation in Cultures of Penicillium and Aspergillus.

| Organism | Element | Element Analysis (ppm) Medium[a] | Organism[b] | Ratio | % Dry wt. |
|---|---|---|---|---|---|
| Penicillium notatum | B° | << 10 | 3200 | << 320 | 1.6 |
| | $H_3BO_3$ | 1000 | 4000 | 4 | 2.0 |
| | Al° | ca.100 | 10,600 | 106 | 5.3 |
| | Zn° | 28 | 4600 | 164 | 2.3 |
| | Cd° | 12 | 1000 | 83 | 0.5 |
| | Bi° | ca.10 | 600 | 60 | 0.3 |
| | Cu° | 132 | 16,000 | 121 | 8.0 |
| Aspergillus clavatus | Hg° | 2 | 128 | 64 | 0.06 |
| | HgO | 234 | 2680 | 11 | 1.34 |
| | PbO | 32 | 1080 | 34 | 0.54 |

[a] In filtered culture medium after 30 days

[b] In washed mycelium, fresh wt. after 30 days

The uptake of Cu by *Penicillium* was evident prior to quantitative analysis in the distinct blue color of the mycelium and the massive precipitation in some cells of CuS upon exposure to ammonium sulfide (Figure 1). Etch marks on the Cu surface followed hyphal filament lines [7]. The actual Cu-content 8% or 1.4 mmoles $g^{-1}$ is extraordinary.

Experiments with *Aspergillus* showed that highly toxic Hg and Pb can also be rendered soluble and absorbed.

It was noted that the concentrations of some elements in the spent medium exceeded normal solubility levels. Thus, dissolved Cu at 132 ppm was *ca* 66-fold higher than in sterile nutrient of the same age and Hg at 2 ppm was 80-fold greater.

These enhanced concentrations in media after 30 days of culture suggested that fungal metabolites be considered a factor in metal solubilization. Accordingly, experiments were initiated as models using two elements and a native mineral derivative of each (Tables 2 and 3).

It is obvious from the calculated ratios solute : water that ammonium chloride and a variety of organic compounds enhance the solubility not only of the elements but also of their high refractory sulfides. Evidence for selectivity is provided by the differences in the efficacy of solutes toward Hg, HgS, Cu and $FeS_2$ respectively, and in the differential solubilization of Cu and Fe from pyrites.

Figure 1. Hyphal cells of Penicilliium notatum grown on copper after washing and exposure to ammonium sulfide. The dense black CuS is evident within some cells. Photographed at 950X.

Table 2. Effect of Metabolites on the Solubility of Mercury and Cinnabar (HgS).

| Solute (100 mM) | pH | Hg in Solution (100 days) | | | |
|---|---|---|---|---|---|
| | | Hg° | | HgS | |
| | | μM | solute / water | μM | solute / water |
| Water | 5.5 | 0.12 | — | 0.01 | — |
| Ammonium chloride | 7.0 | 20 | 167 | 0.35 | 350 |
| D-glucoseamine | 5.5 | 29 | 242 | 0.42 | 42 |
| L-lysine | 6.5 | 28 | 233 | 1.56 | 156 |
| L-asparagine | 7.0 | 118 | 983 | — | — |
| α-ketoglutaric | 1.5 | 24 | 200 | — | — |
| Citric acid | 1.5 | 24 | 200 | 0.60 | 60 |

Table 3. Effect of Metabolites on the Solubility of Copper and Pyrites ($CuFeS_2$).

| Solute (100 mM) | pH | Cu in Solution (300 days) Cu° (μM) | Pyrite (μM) |
|---|---|---|---|
| Water | 5.5 | 20 | 8.0 (180)[a] |
| $NH_4Cl$ | 7.0 | 3000 | 15.8 (666) |
| D-glucoseamine | 5.5 | 2000 | 15.8 (1260) |
| L-Lysine | 6.5 | 1120 | 17.6 (810) |
| Citric acid | 1.5 | 650 | 14.4 (378) |

[a](180) denotes μM Fe in solution containing 8 μM Cu, etc.

On the basis of the biological and chemical data presented here, we suggest that more attention be given to fungi as participants in or initiators of transformations of primary rock surfaces and as factors in biogeochemical cycling of inorganic substances.

## References


1. Alexander, M. 1961. "Introduction to Soil Microbiology". John Wiley & Sons, New York.

2. Cannon, H. 1960. Botanical prospecting for ore deposits. Science 132: 591.

3. Challenger, F. 1955. Biological methylation. Quart. Rev. 9: 755.

4. Jensen, S. and A. Jernelöv. 1969. Biological methylation of mercury in aquatic organisms. Nature 223: 753.

5. Puckett, K. J., E. Nieboer, M. J. Gorynski and D. H. S. Richardson. 1973. The uptake of metal ions by lichens: a modified ion-exchange process. New Phytol. 72: 329.

6. Roberts, K. and S. M. Siegel. 1967. Experimental microbiology of saturated salt solutions and other harsh environments III. Growth of salt-tolerant *Penicillium notatum* in boron-rich media. Plant Physiol. 42: 1215-1218.

7. Seaward, M. R. D. 1973. Lichen ecology of the Scunthorpe heathlands. I. Mineral accumulation. Lichenologist 5: 423.

8. Siegel, S. M., 1973. Solubilization and accumulation of copper from elementary surfaces by *Penicillium notatum*. Environmental Biology and Medicine 2: 19-22.

9. Williams, M. E. and E. D. Rudolph. 1974. The role of lichens and associated fungi in the chemical weathering of rock. Mycologia LXVI: 648-660.

# Performance of Fungi in Low Temperature and Hypersaline Environments


## Abstract

During the past ten years, we have observed a broad array of stress capabilities in common fungi including ability to grow in aqueous ammonia and other alkaline solutions; in acids; in the presence of heavy metals; and in various salt media at low temperature.

This report is concerned primarily with (a) the performance of Aspergillaceae in a variety of saturated salts; (b) distinctive roles for $K^+$ and $Rb^+$-ions; and (c) the lowest temperatures at which growth in nutrient brines has been observed, namely 267°K in as little as 14 days. We also describe a novel solid medium based upon gelatin, glycerol and water in which fungal cultures growing at 248°K can be directly examined under oil-immersion magnification.

The performance capabilities of the Fungi show that tolerance or adaptability to harsh and extreme physical-chemical environments cannot be considered a unique feature of prokaryotic life forms.

Salt flats, brine pools and other natural hypersaline environments have long been recognized as real ecological niches harboring a range of biota from pseudomonad bacteria and green algae to specialized crustaceans. A notable omission in this ecological record is the fungi, although the group is known to include marine forms.

Because fungi are highly versatile metabolically and important in the recycling of organic matter in eco-systems generally, we have undertaken an extended study of their stress capabilities over the past ten years [1-5]. In addition to growth in ammonia, alkalies and acids and their ability to solubilize and accumulate metals and metalloid elements, the fungi have displayed salt tolerances comparable with a superior to those of common halophiles without the benefit of evolutionary or laboratory selection or conditioning. The ability to grow in the presence of hypertonic salt solutions also opened prospects for their use in cryobiologic investigations, that is at temperatures lower than the freezing point of ordinary aqueous nutrient media.

Most of these studies of fungi in severe or exotic environments have been concerned with the Aspergillaceae, a widespread terrestrial group without marine affinity and commonly observed on decaying vegetable foodstuffs as saprophytes, but also known as occasional animal pathogens (e.g. "Aspergillosis"). The standard culture medium contains 1% each of glucose and peptone with 0.5% yeast extract and ranges in pH from 6.0 to 6.5. Such media are modified for experimental purposes by saturation with salts as desired with respect to depression of the freezing point. All cultures were set up in quintuplicate.

In early studies (cited above) it was found that *Penicillium notatum* and other forms could be cultured on freeze-thaw diurnal

cycles, for example 12 hours each at 293°K and 243°K without marked reduction in growth rates provided they were incubated in media saturated with acetate chlorides or other salts of K, Mg or Ca. The cyclic temperature regimes left open, however, the possibility that growth took place only during the warm phase. It did show that a fungus can grow while being exposed to severe cold shock repeatedly over 30 or 60 consecutive days. Comparable capabilities were found in various species of *Penicillium* and the closely allied genus *Aspergillus*.

Subsequently, the growth and development of *Penicillium notatum* was examined over a more modest range of continuous temperatures, 266-297°K (Table 1). The time required for spore germination to begin; for the appearance of 1 mm colonies; and for spore differentiation were noted. Saturated KCl slowed development substantially at 297°K, but in saturated NaCl no development at all was seen during a period of 120 days. When, however, the NaCl medium was supplemented with 0.5M KCl or RbCl, the performance was nearly the same as that seen in KCl at saturation. It is of interest to note that $Rb^+$ with an ionic radius of 1.47 Å is closer to $K^+$ (1.33 Å) than the latter is to $Na^+$ (0.97 Å) in charge-size relations. Neither $Li^+$ (0.68 Å) nor $Cs^+$ (1.67 Å) would replace $K^+$ or $Rb^+$. Thus it seems clear that the non-performance of the organism in NaCl is an expression of $Na^+$ imbalance and induced $K^+$ requirement of unusual magnitude. With reduction in temper-

Table 1. Time-Temperature Relations in the Development of *Penicillium notatum* in Salt-Saturated Glucose-Peptone Medium.

| Temperature °K | Stage | Days Required for Stage in Chloride Medium[a] | | | | |
|---|---|---|---|---|---|---|
| | | None | $K^+$ | $Na^+$ | $Na^+ + K^+$ | $Na^+ + Rb^+$ |
| 297 | Germination | <1 | 2 | >120 | 2 | 3 |
| | Macrocolony[b] | 2 | 34 | — | 35 | 35 |
| | Sporulation | 3 | 62 | — | 67 | 68 |
| 279 | Germination | 18 | 18 | >120 | 24 | 26 |
| | Macrocolony | 30 | 78 | — | 87 | 88 |
| | Sporulation | 67 | 100 | — | 110 | 113 |
| 266 | Germination | >120 | 24 | >120 | 30 | 32 |
| | Macrocolony | — | 88 | — | 104 | 109 |
| | Sporulation | — | >120 | — | >120 | >120 |

[a] "$Na^+ + K^+$" and "$Na^+ + Rb^+$" indicate 0.5M $K^+$ or $Rb^+$ added to 3.5M NaCl.

[b] Colony diameter $\geq$ 1mm.

ature, but still above freezing, the difference in developmental rates with and without salts is far smaller. That is, even a modest reduction in temperature affects rates more profoundly in the absence of salts at saturating levels. Finally, at 266°K, the salt-free cultures are frozen and fail to develop in 120 days, whereas development through the macrocolony phase is almost as rapid as it was at a 13° higher temperature level and above the usual freezing point. The most important difference observed was the failure at the lower temperature of sporogenesis, at least within the present time limits.

One of the more striking results of this and other studies was the apparent reduction in temperature effects in saturated salt media. This effect is supported by another series of studies using mycelial growth rate data (weight basis) to calculate Arrhenius heats of activation with and without salts (Table 2). It is evident that whatever saturating salt is used, the activation energy for growth is always appreciably lower than in salt-free media.

Thus, the presence of high salt concentrations does in fact reduce the temperature dependency of the growth process in *Penicillium*.

Exploratory studies indicated that a constant 266°K was far from the lower limit for these fungi, however the technical problems in the observation and separation of individual small

Table 2. Salt-Induced Lowering of Activation Energy for Growth Based upon Biomass of *Penicillium notatum* in Glucose-Peptone Medium.

| Saturating Salt | Temperature Range °K | log Increase in Biomass | $E_a$ (kcal/mole)[a] |
|---|---|---|---|
| None | 277-295 | 0.793[b] | 16.5±2.1 |
| | 265-277 | — | — |
| KCl | 277-295 | 0.293 | 6.1±0.6 |
| | 265-277 | 0.167 | 4.7±0.5 |
| KOAc | 277-295 | 0.270 | 5.6±0.7 |
| | 265-277 | 0.147 | 4.1±0.5 |
| $Mg(OAc)_2$ | 277-295 | 0.267 | 5.5±0.6 |
| | 265-277 | 0.149 | 4.2±0.5 |

[a]Arrhenius Heat of Activation ("Activation Energy") ± Standard Error.

[b]After 10 days incubation without salts and 60 days with salts.

colonies in a slurry of medium and fine salt crystals are considerable. On the other hand, agar media which provide a good surface, are destroyed by freezing or by the addition of many salts. An alternative medium was devised by the dispersion of gelatin in 40-60% (v/v) aqueous glycerol, together with the usual proportions of glucose, peptone and yeast extract. When 20-40g of gelatin are introduced rapidly into 100 ml of the glycerol-water solution, the resulting mixture remains fluid enough to pour for 5-10 minutes. When solidified in plates, this gel medium has the translucency and resiliency of 1-2% agar gels and is completely stable at temperatures of 248°K or less. Gel surfaces were easily scanned at oil-immersion magnification by first placing a cover slip over the area to be examined followed by the usual immersion oil.

These cryogels represent severely dessicating environments and do not in general support microorganismal growth even at room temperatures. When inoculated with suspensions of *Aspergillus niger* spores and incubated at 248°K, the following germination responses were noted: at 30 days, 0 per 1000; at 60 days, 0.8; at 120 days, 1.3; and at 180 days, 2.5. These experiments and others concerned with the lower thermal limits of fungi are still in progress. They already show however the importance of including eukaryotic organisms in any consideration of the environmental capabilities of terrestrial life.

## References

1. Roberts, K. and S. M. Siegel. 1967. Experimental microbiology of saturated salt solutions III. growth of salt-tolerant *Penicillium notatum* in boron-rich media. Plant Physiol. 42: 1215-1218.

2. Siegel, S. M. 1967. Elements of space biology. Adv. in Space Sci. and Technology 9: 1-100.

3. Siegel, S. M. and C. Giumarro. 1965. Survival and growth of terrestrial microorganisms in ammonia-rich atmospheres. Icarus 4: 37-40.

4. Siegel, S. M., T. Speitel and R. Stoecker. 1969. Life in earth extreme environments: a study of cryobiotic potentialities. Cryobiology 6: 160-181.

5. Siegel, S. M. 1971. Experimental biology of extreme environments and its significance for space bioscience - 3. Spaceflight 13: 183-186.