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Mary M. Vinje
St. Ambrose College

James M. Vinje
Mast Development Company

Report on a Fungus Isolated From an Ozone Meter

MARY M. VINJE[1] AND JAMES M. VINJE[2]

Abstract. The fungus *Penicillium lilacinum* Thom was found to be responsible for the malfunction of ozone meters sent in from such widely divergent geographical locations as Iowa; Tennessee; Washington, D. C.; Maryland; Ottawa, Canada; and Hawaii. In 12 out of 15 isolations a pure culture of the fungus was obtained. The source of the fungus was traced to the rubber used in the assembly of the solution pump. The sensor solution used in the machine proved to be inhibitory but not fungicidal. Attempts at control included chemical additives of which methylparaben, scorbic acid, propylparaben, and sodium benzoate were all stimulants when used in the lower concentrations in conjunction with the sensor solution. The chemical which was most effective was o-Phenylphenol. The fungus showed unexpected tolerance for some chemicals.

In February 1961 a Mast Ozone Meter Model 724-1 was returned from the field as being inoperative. When it was determined that malfunction was not due to mechanical failure, it was suggested that some type of biological growth might be obstructing the orifices in the machine.

During an eighteen month period suspected components taken from malfunctioning equipment were investigated. The components included plastic tubing, rubber valves, diaphragms, and the sensor solution used in the meter. Units were received from the following geographical locations: Davenport, Iowa; Marshalltown, Iowa; Norris Dam, Tennessee; Washington, D.C.; Beltsville, Maryland; Ottawa, Ontario, Canada; and Hilo, Hawaii.

There were four phases to the problem:

1. The determination of the presence of a microorganism.
2. The identification of the microorganism isolated.
3. The determination of the source of the microorganism.
4. Investigation of possible control measures.

METHODS

Because the problem of "slime" control is a common one in the paper and pulp and the rubber industries, and is often concerned with fungi, it was anticipated that the current problem might be comparable. It was expected that mycological media would be productive of results in culture attempts. Such was the case for most of the samples, although in one instance the

[1]Professor of Biology, St. Ambrose College
[2]Mast Development Company, Davenport, Iowa

sample arrived in a dried condition and 168 trials were required before any growth was obtained. Had it not been that subsequent work confirmed that the fungus obtained was identical to that obtained from other later samples, a contaminant would have been suspected. Seven different media were tried but Sabouraud (maltose) agar was ultimately the choice for the isolation of the fungi. The data sheet used for routine recording of samples included date of arrival, geographic source, nature and gross appearance of the sample, and a history of the culture methods used in isolating the microorganisms.

Fifteen isolates were recovered from the components obtained from malfunctioning meters. Following isolation of the microorganisms identifications were made. Of the microorganisms recovered from the ozone meters, two were bacteria, one was a *Cephalosporium,* the others were all *Penicillia.* Because there was no evidence that the bacteria were pertinent to the problem no attempt was made to identify them. For the identification of the fungi, references by Raper and Thom (1) and by Gilman (2) were consulted. For the culture studies required to identify the fungi, petri dish cultures were made on the following media: Czapek solution agar, malt extract agar, corn meal agar, steep agar, and Sabouraud (maltose) agar. Three spot inoculations per plate were made. Micro-cultures were made on Shoemaker fungus microculture slides so that the organisms could be studied in situ. Drawings for confirmation of identification were made with a camera lucida, and all measurements were made with an ocular micrometer. When stained slides were required for comparative purposes, a 2% Phloxine stain was used. Since each of the isolates required the same analysis a "Descriptive Sheet for Identification of the Penicilla", as suggested by Raper and Thom (1) was used.

Identification

One of the most interesting features of this study was that an apparently pure culture of the same specific *Penicillium* was recovered from each of the malfunctioning meters. Because the specific *Penicillium* was recovered without difficulty even from the meter which yielded a *Cephalosporium* no further study was made of the latter microorganism.

The species of *Penicillium* recovered seemed to be most similar to *Penicillium lilacinum* Thom, as described by Raper and Thom (1). This identification was verified by Dr. J. J. Ellis of the Northern Regional Research Laboratory at Peoria, Ill.

Source of the Fungus

When it was discovered that the same single species of *Penicillium* could be recovered from malfunctioning meters from

seven such widely divergent geographical areas, even the ubiquitous nature of the *Penicillia* did not seem adequate to account for the coincidence. This was especially true when it was noted that visual evidence of the contaminant, in the form of a slimy deposit on the walls of the plastic tubing and as shreds on the valves and diaphragm, was confined to the interior of the solution pump and its outlet tube. Since outside air does not pass through this portion of the equipment, this seemed to indicate that the probable common source for the fungus must be other than the place where the equipment was being used. It was therefore assumed that contamination must have preceded assembly of the meter. It seemed likely that either the air in the assembly plant or one of the solution pump components must have been contaminated with spores of the fungus. All these possibilities were investigated. Neither the air in the plant nor the new or stored plastic tubing used in assembly, nor the sensor solution itself, yielded *P. lilacinum.* But the rubber used for the diaphragms and valves proved very productive of fungi. When dilution plates were made from samples of the rubber used in assembly, three different species of *Penicillia* and two different species of bacteria were recovered. One of the fungi was the same *P. lilacinum* as had been recovered from malfunctioning units from the field. In an attempt to determine what would be the effect on the fungi of a long time exposure to the sensor solution, pieces of the rubber were placed in tubes of the Sensor Solution and left there for four months. This was an attempt to simulate field conditions where the rubber remains in the solution for long periods of time. It was interesting to note that at the end of the four month period the only microorganism recoverable by laboratory procedures was the *P. lilacinum.* This seems to indicate that the fungus is either antibiotic for those microorganisms which were originally found with the fungus on the rubber or that *P. lilacinum* has a greater tolerance for the chemicals used in the sensor solution than did the other microorganism with which it was associated.

Investigation of Control Measures *FOR PENICILLIUM LILACINUM*

The suggestion that the rubber be sterilized by autoclaving proved to be impractical from a manufacturing viewpoint. It was therefore decided to attempt control by chemical means.

We next considered that a chemical treatment of the components before assembly might prove valuable. Zinc chloride was the chemical chosen. The concentrations used were 5 percent, 10 percent, and 20 percent. A "10 Second Dip and Rinse" method and a "20 Minute Soak and Rinse" method were tried. One quarter inch squares of rubber and one quarter inch lengths

of plastic tubing, from assembly stock, were treated with the chemical, rinsed in distilled water and then dropped into test tubes containing either Czapek-Dox solution or the sensor solution as the available nutrient. Controls included untreated rubber and plastic tubing in Czapek-Dox solution and in the sensor solution. The rubber and plastic tubing remained in the respective solutions for four months. On the twelfth day, which marked the first appearance of visual growth in the experimental tubes, a transfer from all tubes which showed no such visual growth was made to Sabouraud agar in a petri dish, to determine the viability of any fungi which might be present. Subsequent viability checks were made at intervals for the duration of the experiment. On the last transfer the original pieces of rubber and plastic tubing were used.

The resulting effects of this type of chemical treatment on rubber were most disappointing. Although growth occured in the Czapek-Dox control tubes containing rubber samples within three days, and visible growth in experimental tubes did not occur for 12 days, this was but a temporary inhibition. If Czapek-Dox solution was used after the chemical treatment, only the 20 percent "20 Minute Soak and Rinse" method showed any inhibitory effect beyond the twelve day period. Even this apparent inhibition was found to be correlated with a particular sample of rubber which seemed to have less contamination than that found on other samples. When the sensor solution was used after the chemical treatment of rubber, there was no visible growth evident, and it was thought at first that inhibition was indicated. However, the sensor solution control itself showed no evidence of growth. Yet in every instance, experimental and control, the fungus was recoverable by plating out transfers onto Sabauraud agar (even after 130 days). It would appear that the sensor solution itself has inhibitory properties. Completely negative results were obtained from all tests involving plastic tubing, even the controls. Apparently either the plastic was not contaminated or microorganisms do not survive well on dry plastic in storage. Other experiments confirmed this impression.

These preliminary trials seemed to indicate that hope of control would depend upon the use of chemicals added to the sensor solution. Although the inhibition of the growth of microorganisms by mercurials offered attractive possibilities [this is the preference of the paper and pulp industry (3)], none of the mercurials were tried in this case because of the probability of ion exchange with the chemicals used in making up the sensor solution. This contained potassium bromide; potassium iodide; sodium phosphate, monobasic; and sodium phosphate dibasic.

A list of fifty different chemicals with fungistatic or fungicidal

properties was then submitted to the chemist at Mast Developmen Company for consideration as to their compatibility with the sensor solution used in the ozone meter. Seven of these chemicals were chosen to be tried in different concentrations for their effect upon the fungus which had been isolated from the malfunctioning equipment. The chemicals chosen were methylparaben, scorbic acid, o-Phenylphenol, propylparaben, sodium benzoate, Dowicide-B (85% sodium trichlorophenate), and "Caroline Preservative" (proprietary).

Several series of experiments were conducted, the first using percentages based on the manufacturer's recommendations for the fungistatic properties of the chemicals being tried. Subsequent trials increased or decreased the percentages as indicated by the results obtained. Seven cultures were prepared for each percentage of the chemical being investigated. In all cases the procedure was the same. From a pure culture of the fungus *P. lilacinum* in a petri dish, a small piece of inoculum was removed from the margin of a giant colony and added to the test tube containing a nutrient plus the chemical. To insure that the amount of inoculum was comparable in all cases a small "cookie cutter" with a diameter of three millimeters was used as the transfer needle. The seven cultures included: sensor solution as used in the ozone meter; Czapek-Dox solution plus a small sample of sterile rubber; Czapek-Dox solution plus a sample of sterile plastic tubing; and Czapek-Dox solution only. On the assumption that the fungus might require a solid for anchorage, Czapek agar in one case and Sabouraud agar in another was supplied in the bottom of the test tube with the liquid nutrient above it. The controls included Czapek-Dox solution only plus the fungus inoculum; Czapek-Dox solution only plus the fungus and a sterile sample of rubber; and the sensor solution only plus the fungus inoculum.

The experiment lasted seven months. Observations were made daily but recorded at intervals only. In all cases the recording dates chosen coincided with the first appearance of the growth of the fungus in at least one of the cultures. Twenty six days after the inoculation date transfers were made from those test tubes which gave no visual evidence of growth to petri dishes containing Sabouraud agar. This was to determine whether or not the fungus might still be alive. Similar viability checks were made from time to time. The last such transfer, made 173 days after the inoculation date in one series of trials, used the original inoculum as the transfer material.

Very often there was no visual evidence of growth in the test tube containing the chemical additive, but a transfer from such a tube to a nutrient agar soon produced a luxurient mycelium,

indicating that the fungus had survived without difficulty. One of the most interesting features of the experiment was the confirmation that the sensor solution itself was inhibitory but not fungicidal. In the control cultures, which contained the sensor solution as the only available nutrient material, there was never any visual evidence of the growth of the fungus. Yet, when transfers were made to a nutrient agar, the fungus always grew very well. It was also noted that, although the sensor solution itself seemed to be an inhibitor, when the lower concentrations of some of the chemicals were used as additives, these acted as a stimulant for *P. lilacinum* and the fungus grew well even in the sensor solution.

Only after extensive experimentation was it realized that all of the chemicals except sodium benzoate were effective in ranges lower than those recommended by the manufacturer. Therefore, in the interests of brevity, the individual tables which recorded only the absence of growth have been omitted in favor of a summary which indicates the range investigated, and, when determined, the limits of effectiveness. Evidence of incompatibility between the chemical and the sensor solution or an adverse effect on the rubber or plastic tubing was considered a valid reason for abandoning further experimentation on that particular chemical.

METHYLPARABEN: (0.05% – 0.20%)
0.05% - Not only ineffective as an antifungal agent but a stimulant when added to the sensor solution.
0.10% - Fungistatic in 33% of the trials but not fungicidal; a stimulant when added to the sensor solution.
0.20% - Fungistatic in 100% of the trials but fungicidal in only 90% of the trials. Apparently represents the edge of tolerance of this fungus for this toxicant.
SCORBIC ACID (0.1% – 2.0%)
0.10% - Not only ineffective as an antifungal agent but a stimulant when added to the sensor solution.
0.50% - Fungistatic in 80% of the trials but in no case fungicidal.
1.00% - Fungistatic and fungicidal for *P. lilacinum*.
O-PHENYLPHENOL (0.025% – 1.0%)
0.025% - Fungistatic but not fungicidal
0.050% - Fungistatic and fungicidal
PROPYLPARABEN (0.01% – 0.05%)
0.01% - Not only ineffective as an antifungal agent but a stimulant when added to the sensor solution.
0.025% - Not only ineffective as an antifungal agent but a stimulant when added to a sensor solution.
0.050% - Fungistatic in 80% of the trials; but in no case fungicidal.
SODIUM BENZOATE (0.10% – 1.50%)
0.10% - Neither fungistatic nor fungicidal
0.20% - Not only ineffective as an antifungal agent but a stimulant when added to the sensor solution.
0.30% - Same
0.40% - Same
0.50% - Neither fungistatic nor fungicidal but no longer a stimulant when added to the sensor solution.
1.00% - Same
1.50% - Same. Abandoned because concentration becomes impractical.

DOWICIDE–B (0.10% – 1.0%)
0.10% - Fungistatic and fungicidal
CAROLINA PRESERVATIVE (1.0% – 3.0%)
1.00% - Fungistatic and fungicidal.

As a matter of academic interest, one other experiment was conducted. This concerned an inquiry as to whether or not the addition of ethylene glycol to a bromide sensor solution would have any effect on the specific fungus being investigated. For this experiment a series of eleven test tubes were prepared. Five of the test tubes contained the bromide sensor solution plus the fungus inoculum. Two of these five tubes also contained a nutrient, Czapek-Dox or Sabouraud solution. Another five test tubes were identical to the first five except that 6 percent ethylene glycol had also been added. The eleventh test tube was the control and consisted of Czapek-Dox solution plus the fungus inoculum. This experiment lasted ten months. During that time any test tubes which failed to show visible evidence of growth were checked for viability of the fungus by making transfers to Sabouraud agar. Such viability checks were made monthly for the duration of the experiment.

The results of this experiment were extremely interesting and illustrated well the amazing chemical tolerance of *P. lilacinum*. At no time during the ten month period was there any visible growth of the fungus in any test tube which contained either the bromide sensor solution alone or the bromide sensor solution with the additive ethylene glycol. But when transfers were made from these test tubes to Sabouraud agar, the fungus immediately plated out, thus indicating that the solutions had been inhibitory but not fungicidal. The only apparent effect of having added ethylene glycol was that the growth was less luxurient in all transfers made from a solution containing this chemical. In all test tubes where a nutrient had been added to the bromide sensor solution, irrespective of the presence or absence of ethylene glycol, the fungus grew well within two days. Because of the long period of time involved in the experiment, the bromide sensor solution underwent evaporation to such a degree that, at the termination of the experiment, all that was left in the test tubes was either a collection of very fine crystals or one large cubic crystal with the fungus inoculum in the center. When such a crystal was broken open and the fungus inoculum transferred to Sabouraud agar, growth of the fungus occured.

Conclusions

1. *Penicillium lilacinum* Thom was the fungus responsible for the malfunction of the ozone meter.

2. The source of *P. lilacinum* was the rubber used in the assembly of valves and diaphragms in the solution pump.

3. *P. lilacinum* was not the only fungus which was encountered in assembly materials, but it was the only survivor in the experiments.

4. The fungus showed unexpected tolerance for some chemicals.

5. The sensor solution used in the ozone meter had inhibitory properties but was not fungicidal for *P. lilacinum.*

6. The lower concentrations of methylparaben, scorbic acid, propylparaben, and sodium benzoate were all stimulants for *P. lilacinum* when used in conjunction with the sensor solution.

7. O-Phenylphenol was the most effective of the chemicals invesigated.

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The Plasmodium of the Myxomycete Cribraria Violacea[1]

SISTER MARY ANNUNCIATA MCMANUS, R.S.M.[2]

Abstract. Cribraria violacea is a minute myxomycete which could be expected to possess a protoplasmodium similar to the plasmodia of other minute species such as *Clastoderma debaryanum, Echinostelium minutum,* and *Licea parasitica.* Observations reported here show that it differs from protoplasmodia previously described in forming a small network with a fanlike area at the advancing front, and in showing protoplasmic streaming which did not reverse its direction of flow during two 40-minute periods of continuous observation.

The classic picture of "the" myxomycete plasmodium has recently undergone several modifications. It is now known that at least two types of plasmodia exist among the myxomycetes which do not conform to the usual description. Textbooks and sources of general information usually describe the type which has been designated the "phaneroplasmodium" by Alexopoulos (1). This plasmodium is readily visible to the naked eye, be-

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[2]Department of Biology, Mount Mercy College, Cedar Rapids, Iowa.