Gas Requirements of Molds: The Importance of Dissolved Oxygen in the Medium for Germination and Growth of Several Molds

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IT HAS BEEN KNOWN for a long time that molds are aerobes and require oxygen for germination and growth. More recently it has been shown that these organisms have a wide range of oxygen tension that gives maximum growth (Brown, 1922; Golding, 1940; Miller and Golding, 1949). Good germination and growth were attained at extremely low concentrations of oxygen, provided carbon dioxide was not allowed to accumulate. The objective of this study was to investigate the importance to mold germination and growth of the solubility of oxygen in the medium. The prevention of food spoilage by molds is often affected by removal of oxygen but exactly how this is physiologically accomplished is not well understood.

Previous investigations. Golding (1945), working with four common molds, obtained results indicating ". . . that the inhibiting effect of the soluble carbon dioxide was, in general, the function of a straight line as the concentration of carbon dioxide increased." Miller and Golding (1949) demonstrated that a similar relationship also held for dissolved oxygen in the medium. A relationship was distinguished between the oxygen required by their test organisms and the amount of oxygen soluble in the medium. Thus, *Geotrichum candidum* was not significantly inhibited until the oxygen supply was below 0.30 volumes of oxygen at normal temperature and pressure soluble in 1,000 volumes of water. *Penicillium roqueforti* was inhibited significantly at 0.78 volumes of oxygen soluble in 1,000 volumes of water. These two organisms marked the lower and higher boundaries of oxygen-tension requirements of the test organisms.

Behavior of gases in solution. The solubility of gases in liquids follows definite laws or patterns, namely:

1. Henry's Law (Handbook of Chemistry and Physics, 1950): The volume of a slightly soluble gas that will dissolve in a liquid is proportional to the pressure of that gas over the liquid.
2. Gases are more soluble at low temperatures than at high temperatures.

3. Gases exhibit a change in solubility in salt and sugar solutions that is inversely proportional to the concentration of the solute, provided the gases are not also soluble in the solute.

Methods and Materials

Equipment. Cultural techniques and equipment used by Miller and Golding (1949) were also used in these studies.

Measurement of growth. The phases of mold growth against which planned environmental changes can be evaluated are many. The diameter of the giant colony, considered satisfactory in past gas requirement studies on molds, was used for this phase of the present study. All reported results are the average of at least five replicates.

Test organisms. The molds chosen for this investigation were *Aspergillus niger, A. Flavus, Geotrichum candidum, Penicillium notatum, P. roqueforti,* and *P. expansum.*

Results and Discussion

Loss of moisture. Loss of moisture from uninoculated and inoculated media during incubation at various temperatures is of considerable significance as the solubility of gases in the medium is intimately bound to the amount of moisture in the medium. It was found that whether inoculated or not inoculated the loss of moisture was far greater in control plates (averaged about 5.00 gms.) than in dextrose-enriched plates (averaged 0.50 gms.) and that this loss was at a maximum at the high temperatures. This is in accord with the laws covering the behavior of salts and sugars in solution. However, due to the methods of humidifying the continuously circulating atmosphere, in no instance was the moisture loss for the seven-day incubation period large enough to interfere with growth.

Growth on malt agar enriched with dextrose. Growth curves were drawn for all the test organisms based on growth at temperatures from 70° F to 95° F and on medium containing 0 to 60 gms. of added dextrose per 100 ml. of water. At optimum temperature of growth for the particular organism, concentrations of up to 20 gms. of dextrose per 100 ml. of water yielded giant colonies with the largest diameter. Above this concentration, colony diameter decreased as the concentration of dextrose was increased. It was here that the protective action of high concentrations of dextrose against thermal death of the molds was first noted.

Growth at reduced oxygen tension. When the atmospheric pressure over the inoculated plates was reduced to 74 mm. Hg., there resulted a partial oxygen pressure of 14.8 mm. Hg. as compared to the partial oxygen pressure of air of about 152 mm. Hg. Previous experiments by Golding (1945) have demonstrated that in air, low pressures or high pressures in themselves had slight effect on mold growth. In addition, many earlier investigators had pointed out the resistance of these microorganisms to very low or high pressures (Buchanan and Fulmer, 1928). Reductions of 10 per cent or more in the diameter of the giant colony on the enriched medium relative to the control colony on the basal medium when grown under the same environmental conditions were considered significant. Significant reductions in the diameter of the giant colony were obtained for every test organism. This would seem to indicate that the solubility of oxygen in the medium is essential for growth. When this solubility is retarded by high concentrations of dextrose and low pressures, fungal growth is also retarded.

In order to eliminate any interference in results due to reduced pressures, similar experiments were conducted in which low partial pressures of oxygen were obtained by evacuating gas tanks down to low residual air content. The residual air was then diluted with oxygen-free nitrogen until normal atmospheric pressure was again obtained. If upon analysis the partial oxygen pressure was still too high, the gas was pumped through oxygen-absorbent solutions until the pressure was satisfactory. At sufficiently low oxygen pressures, a significant decrease in growth was obtained on the medium which contained dextrose in quantities calculated to yield giant colonies the approximate size of the basal medium colony. These results again indicated that high concentrations of dextrose decrease the solubility of atmospheric oxygen and consequently mold growth decreased as the oxygen tension became too low. This was observed best in the cases of *Penicillium expansum* and *Aspergillus flavus* (Fig. 1).

It was in the latter experimental series that the protective action of dextrose at above-optimum temperatures of growth for the particular organism was most apparent. This protection against thermal destruction occurs over a narrow range only, on either side of which there are a temperature and osmotic equilibrium equally destructive to the further activity of the fungus. The word, protective, may be deemed suitable for this reason. In some instances inoculated plates incubated at above-optimum temperatures for four to seven days were completely devoid of growth at the end of this period even under microscopic scrutiny. Such plates were held at optimum temperatures for as long as a week before discarding. If growth occurred at all, it appeared first in plates containing the medium enriched with dextrose while often the control (basal) medium remained barren of growth.

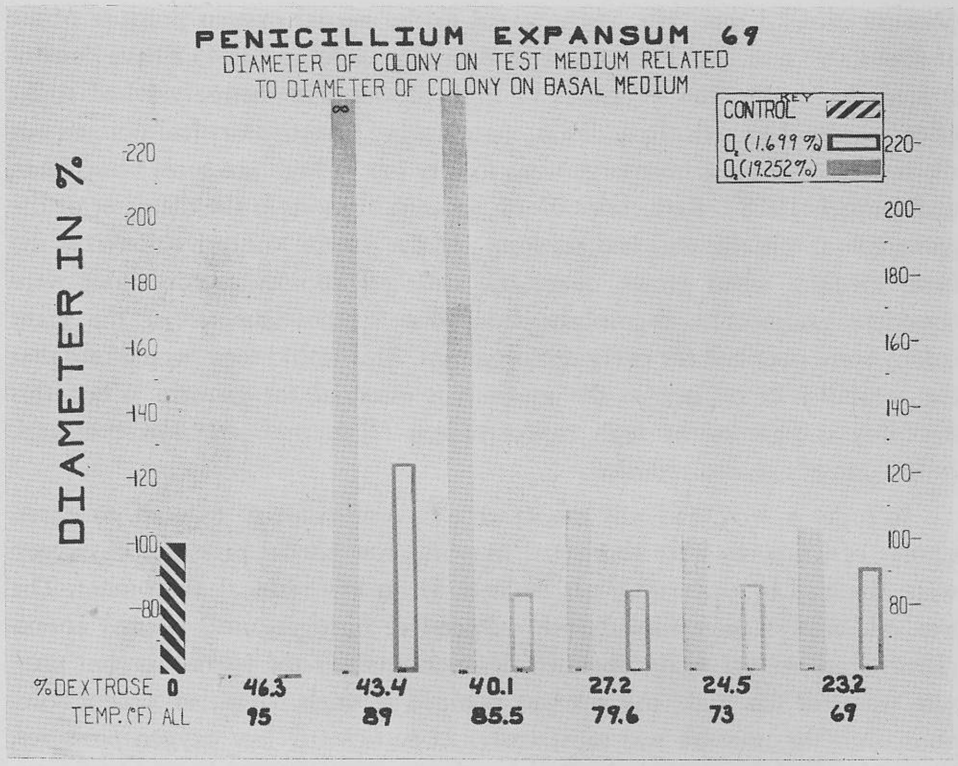

FIGURE 1.

Elimination of contact with oxygen of the atmosphere. Elimination of contact with atmospheric oxygen was accomplished by covering the site of inoculation with a large cover slip (24 x 50 mm.). The edges of the cover slip were sealed to the medium with melted petrolatum. This technique was used on basal medium and on dextrose-enriched basal medium. Incubation was at the optimum temperature of growth for the particular organism and at normal atmospheric oxygen pressure. All the test organisms were found to possess the ability to use dissolved oxygen for spore germination and growth when blocked off from atmospheric oxygen. That the dextrose served to retard the speed of solution and diffusion of oxygen in the medium was clearly evident. The basal medium with cover slips over the inoculation sites yielded macroscopic growth first (in 3 days) and at the termination of incubation the growth was much larger (breaking through the petrolatum seal) than the growth on dextrose-enriched medium.

Use of the oxidation-reduction indicator dye, resazurin. This experimental study dealt with the feasibility of using resazurin to demonstrate visibly the solution of oxygen in the medium. The final concentration of resazurin was in all instances approximately .00001 gm. per ml. The penetration of oxygen into the medium, indicated by the oxidation of the colorless dihydroresorufin to the pink resorufin, was measured in mm. from the bottom of the meniscus. When the concentration of dextrose was placed on a per cent basis in all cases, it was observed that the penetration of oxygen into the medium was inversely proportional to the concentration of dextrose. However, the water present in the medium markedly influenced the oxygen penetration up to a concentration of 50 per cent dextrose. The penetration of oxygen averaged slightly higher at 75° F than at 95° F. This is in conformity with the commonly observed gas solubility-temperature relation. A small amount of a reducing substance in the medium is necessary to cause complete reduction of the resazurin to the colorless form, dihydroresorufin, thus allowing a sharp differentiation as the oxygen penetrates into the medium (Fig. 2).

Similar experiments replacing dextrose with glycerol, ethylene glycol, and sucrose have borne out the observation by Buchanan and Fulmer (1928) that, "The lowering of solubility is generally independent of the nature of the

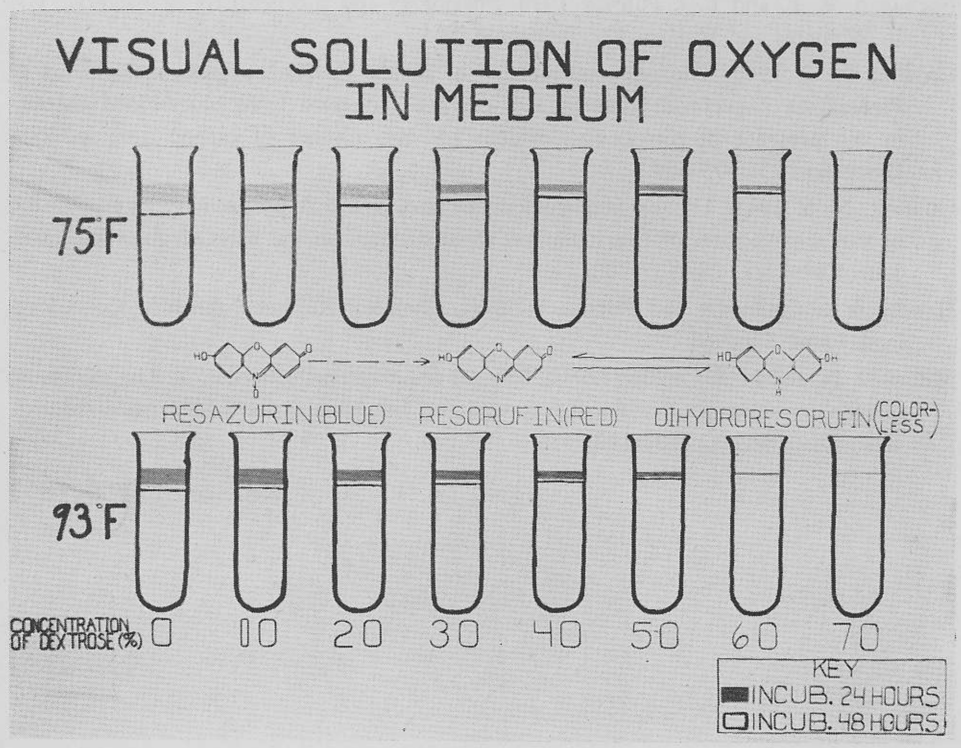

FIGURE 2.

gas and is a function of the nature of the solute." The rate and degree of solution of oxygen were directly proportional to the concentration of those solutes which were not themselves solvents for oxygen.

Summary

This study has suggested that the strains of *Aspergillus niger, A. flavus, Geotrichum candidum, Penicillium notatum, P. roqueforti,* and *P. expansum,* used as test organisms can utilize oxygen dissolved in the medium for germination and growth by absorption through the submerged hyphae. Moreover, there seems to be indicated, although incompletely demonstrated, that the dissolved oxygen may be the most important if not sole source of oxygen for these aerobic organisms. The use of resazurin as a visible indicator of the rate and degree of oxygen solution in the solid medium used in this study has been found to be a useful tool.

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