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EFFECT OF ETHANOL ON THE SURVIVAL AND CATALASE ACTIVITY OF SOME FILAMENTOUS FUNGI UNDER HIGH PRESSURES OF OXYGEN

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Abstract

Survival of replacement cultures of *Aspergillus niger*, *Mucor racemosus* *M. Plumbeus* and *Rhizopus oryzae* were markedly affected by the presence of one per cent ethanol under hyperbaric oxygen. This effect of ethanol on survival time was accompanied by an initial increase in catalase activity which was maintained for quite prolonged period of time. It is suggested that the protective effect of ethanol is probably due to its ability to stabilize catalase against inactivation by oxygen or hydrogen peroxide.

Introduction

The effect of high oxygen pressures on various living organisms have been under investigations for many years (Bert, 1878; Cleveland, 1925; Thaysen, 1934; Bean, 1945; McAllister, Stark, Norman & Ross, 1963; Caldwell, 1965; Gifford, 1968 Ahmed, 1969; Ahmed & Pritchard, 1970; Ahmed, 1973). Recent studies are mostly on the reaction of micro-organisms to treatment with hyperbaric oxygen. These studies have shown that micro-organisms are more resistant to hyperbaric oxygen than higher plants or animals (Pritchard & Hudson, 1967).

The ability of fungi to survive under prolonged treatment of hyperbaric oxygen (Caldwell, 1965; Robb, 1965; Pritchard & Hudson, 1967) were markedly affected by nutritional factors specially the carbon source (Ahmed, 1969). The presence of ethanol was found to have an enhancing effect on the survival of fungi under hyperbaric oxygen, whereas survival time decreases on glucose and other related compounds (Ahmed & Pritchard, 1970). This protective effect of ethanol against oxygen toxicity was probably due to the ability of ethanol to stabilize catalase against inactivation by oxygen or hydrogen peroxide.

In the present paper the effect of the presence of ethanol on survival time and catalase activity of *Rhizopus oryzae*, *Aspergillus niger*, *Mucor racemosus* and *Mucor plumbeus* under hyperbaric oxygen is reported. The results obtained further support the suggestion given by Ahmed & Pritchard (1970).

Materials and Methods

The work described in this paper was carried on *Mucor racemosus* Fresen (CMI 35716), *M. Plumbeus* Bon. (CMI 10772), *Aspergillus niger* Van Tiegh (E 10) and *Rhizopus oryzae* Went and Geerl (CMI 40564).

Growth of fungus cultures

Stationary cultures of fungi were grown on liquid glucose-glutamate medium (Pritchard, 1965) at $25 \pm 1^\circ\text{C}$. The medium was sterilized by steaming for 20 minutes on 3 consecutive days. The medium was inoculated with 1 ml. of spore suspension. After required period of growth the fungus pads were replaced on the sterile solution of 1 per cent ethanol and deionised water. The fungus pads were first rinsed with sterile deionised water and then carefully floated on to the replacement medium.

High pressure oxygen treatment

The pressure vessels for maintaining the cultures in hyperbaric oxygen were a modification of the vessels described by Caldwell (1965). A pressure of 10 atmospheres of pure oxygen was used in all the experiments described in this paper. The pressure was applied and released slowly in order to avoid the physical damage to the mycelium. Viability of cultures following oxygen treatment was tested by sub-culturing the fungus on 2.5 percent malt agar.

Determination of catalase activity

The mycelium was filtered and washed under suction, dried between filter papers, weighed to obtain the fresh weight and then frozen to -15°C for about 30 minutes. The frozen mycelium was thawed in 0.5M phosphate buffer (pH 6.8) and homogenised in a MSE disintegrator keeping the homogenate cool in an ice bath during treatment. The homogenate was centrifuged at 30,000 g for 10 minutes at 5°C and the supernatant was used for catalase assay.

The catalase activity was determined by the method described by Herbert (1954). First order velocity constants were determined for each sample at 2-3 time intervals upto 60 seconds. The activity of catalase is expressed on fresh weight basis. Since the fresh weight often decreases during treatment with high pressures of oxygen (HPO) this basis is not entirely satisfactory. However, in several early experiments protein determination on the extract were made using the method of Lowry, Roseborough Farr & Randall (1951) and it was found that changes in fresh weight that occurred during treatment with HPO were closely related with the changes in extractable protein. Consequently the use of fresh weight as a basis was adopted in later experiments. The pH of filtered medium was measured directly by using a glass electrode.

I(a). EFFECT OF ONE PER CENT ETHANOL AND DEIONISED WATER ON CATALASE ACTIVITY OF *M. plumbeus* UNDER HPO.

The cultures of *M. plumbeus* were grown in 100 ml Erlenmeyer flasks on 25ml of complete glucose-glutamate medium at a temperature of $25 \pm 1^\circ\text{C}$ for four days. Four day old cultures were replaced on 25ml of 1 per cent ethanol and water. After 24 hours in air the cultures were subjected to 10 atmospheres of HPO. Six pressure vessels were used each having two cultures of both the substrates. An equal number of cultures were left in air for control readings. Initial readings were taken on replacement media before subjecting to pressures.

Two control and two treated samples were analysed for catalase activity on each of six consecutive days (Fig. 1). Each reading in this figure represents an average of two readings. After 24 hours treatment a considerable increase in catalase activity to about five times the initial value was found in cultures replaced on 1 per

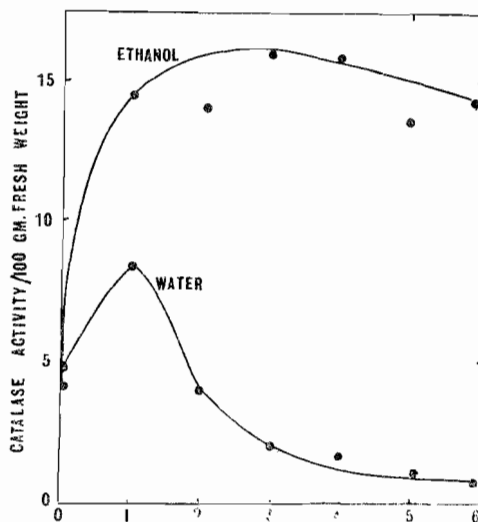


Fig. 1. Changes induced in the catalase activity of *Mucor plumbeus* during a 6 day sojourn in 10 atm. of pure oxygen on 1% ethanol and deionised water.

cent ethanol. On this substrate the induced increase in catalase activity was maintained at a high level upto 5 days and then started decreasing. On deionised water an initial increase in catalase activity was observed but it was less marked than that of ethanol and started decreasing after 24 hours treatment. The control samples showed no appreciable increase in catalase activity.

I(b). EFFECT OF ONE PER CENT ETHANOL AND DEIONISED WATER ON CATALASE ACTIVITY AND SURVIVAL TIME OF *M. racemosus* UNDER HPO.

Experiment I (a) was a short term experiment and therefore it was not possible to determine the exact survival time on ethanol and water. The present experiment was carried out for a longer period in order to determine the exact survival time.

Cultures of *M. racemosus* were grown as described in expt. Ia. On the fourth day two cultures were taken for initial readings. The rest of the cultures were replaced on ethanol and deionised water. After replacement the cultures were subjected to 10 atmospheres of pure oxygen. A total of six pressure vessels were used, each having two cultures on each of the two substrates. Initial readings were taken on the original growth medium before replacement.

The pressures were allowed to escape slowly on the 1st, 3rd, 6th, 9th, 12th and 16th day and cultures were analysed for catalase activity and survival time (Fig. 2). Each reading in this figure represents an average of two readings and arrows represent the death point of the fungus. Both the catalase activity and survival time was higher on ethanol than on deionised water. As in experiment Ia, the induced catalase activity on ethanol remained quite stationary upto five days then started decreasing slowly indicating that the presence of ethanol decreases the rate of fall of catalase activity. Another interesting feature which emerged from this experiment is that the death of the fungus occurred when some residual catalase activity was still present in the mycelium indicating that catalase at low level cannot protect tissues against oxygen poisoning.

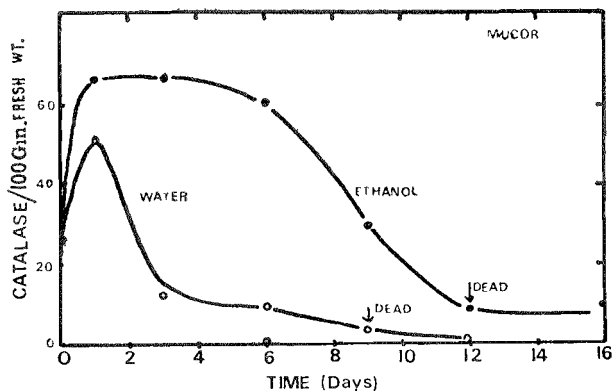


Fig. 2. Changes induced in the catalase activity of *Mucor racemosus* during a 12 day sojourn in 10 atm. of pure oxygen on 1% ethanol and water.

II. EFFECT OF ONE PER CENT ETHANOL AND DEIONISED WATER ON THE CHANGES OF CATALASE ACTIVITY AND SURVIVAL TIME OF *Aspergillus niger*.

Cultures of *Aspergillus niger* 4 day old were replaced on 25 ml. of one per cent ethanol and deionised water and subjected to 10 atmospheres of pure oxygen. Before subjecting to pressure one culture on each of the two substrates was taken for initial readings. Six pressure vessels were used each having two cultures on two different substrates. An equal number of cultures were left in air for control readings. The pressures were released slowly on the 1st, 3rd, 4th, 6th, 8th and 11th day and studies were made for catalase activity (Fig. 3.) Each reading in this figure represents an

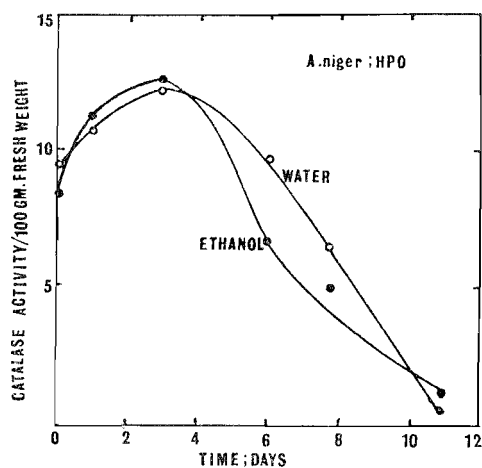


Fig. 3. Changes induced in the activity of *Aspergillus niger* during an 11 day sojourn in 10 atm. of pure oxygen on 1% ethanol and water.

average of two readings. After 24 hours treatment an increase in catalase activity was observed in cultures replaced on ethanol and water. The rise in catalase activity on ethanol and water continued for three days and then started decreasing very rapidly. After 11 days treatment the level of catalase activity was higher on ethanol than on deionised water. Although overall picture for the changes in catalase activity was more or less similar in *M. racemosus* and *A. niger* but increase on ethanol and water was less marked. The other difference between the two species was the rate of increase or decrease in the enzyme activity (compare Fig. 2 and Fig. 3). In this organism the induced increase in catalase activity was more or less similar on ethanol and water and after 4 days treatment it was higher on water than on ethanol. This discrepancy was found to be due to difference in pyruvate metabolism of *A. niger* and *M. racemosus* (Ahmed, 1969).

III. EFFECT OF ONE PER CENT ETHANOL AND DEIONISED WATER ON THE CHANGES IN THE CATALASE ACTIVITY AND SURVIVAL TIME OF *Rhizopus oryzae* IN 4 ATMOSPHERES OF PURE OXYGEN.

After a few preliminary experiments it was found that the optimum oxygen pressure for catalase induction in *Rhizopus oryzae* is four atmospheres. Therefore this particular experiment was carried-out under four atmospheres of oxygen instead of 10 atmospheres. Cultures of *R. oryzae* were grown in 100 ml. Erlenmeyer flasks having 25 ml. of glucose-glutamate medium for 24 hours in a controlled temperature chamber. Thereafter two culture flasks were taken for initial readings. The remaining cultures were replaced on 25 ml. of one per cent ethanol and deionised water and subjected to 4 atmospheres of pure oxygen. The pressures were allowed to escape slowly on the 1st, 4th 7th and 11th day. On each of these days two cultures on one per cent ethanol and two cultures on deionised water were analysed immediately for catalase activity, survival time and dry weight of all the fungus (Fig. 4). Each reading in this for catalase figure represents an average of two readings. After 24 hours a big increase in catalase activity was observed in the treated samples. But this increase was much higher on ethanol than on deionised water. The activity dropped to a very low value between first and fourth day of the treatment. With the longer exposures the decrease becomes very slow. Even after 11 days treatment considerable activity was still present in the mycelium and it was viable. But the activity came down to a very low value on deionised water and the death of all the fungus occurred after 11 days treatment.

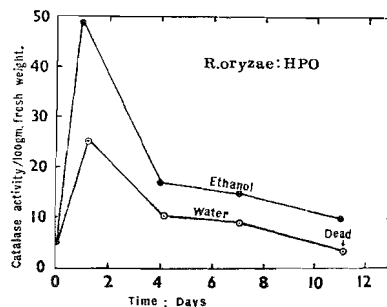


Fig. 4. Changes induced in the catalase activity of *Rhizopus oryzae* during an 11 day sojourn in 10 atm. of pure oxygen on 1% ethanol and water.

Discussion

The results obtained were essentially similar in all the three fungi tested except a little discrepancy in the case of *Aspergillus niger* which was found to be due to the difference in pyruvate metabolism of *Aspergillus niger* and *Mucor racemosus* (Ahmed, 1969). Under hyperbaric oxygen a big increase in catalase activity occurred both in the presence of ethanol and water in all the three fungi tested. This observation is consistent with the notion that some inducer is generated under hyperbaric oxygen which causes the induced synthesis of catalase (Pritchard & Hudson, 1967). But there were some basic difference in response to HPO on these two substrates. 1. The survival period under HPO was longer in the presence of ethanol than on water (Figs. 2 and 4) suggesting some protective effect of ethanol against oxygen toxicity. 2. The induced catalase activity was apparently higher on ethanol than on water. 3. On ethanol the high level of induced activity was maintained for a longer period than of water except in *A. niger*.

As suggested by Pritchard & Hudson (1967); Ahmed (1969); and Ahmad & Pritchard (1970) the increase in catalase activity may be due to slow formation of hydrogen peroxide under hyperbaric oxygen resulting in an induced synthesis of this enzyme. The rapid drop of enzyme activity on water may be explained by assuming that hydrogen peroxide generated under HPO soon attains a toxic level and causes a drop of catalase activity. High concentration of hydrogen peroxide is known to inactivate catalase. But this assumption does not explain the maintenance of high level of catalase activity on ethanol for a much longer period. The fact that on ethanol after an initial increase, the catalase activity remained more or less stationary for a long period suggests either that no further synthesis of enzyme takes place during this period or that a new steady state between synthesis and breakdown is reached. Two possibilities for the action of ethanol may be (1) that it affects the enzyme directly by causing alteration in existing enzyme molecules or (2) that it affects the balance between synthesis and breakdown.

The direct effect of ethanol on enzyme molecule is unlikely since the increase in catalase activity occurs even in the absence of ethanol. A more probable explanation for 'the ethanol effect' is that the presence of ethanol somehow maintains a balance between enzyme synthesis and breakdown, maintaining a constant level of enzyme for a much longer period than on water. In fact there are reports in early literature stating that the presence of ethanol may reduce the inactivation rate of catalase by oxygen (Marks, 1936). It is interesting to note that even extremely low concentration of ethanol may reduce the inactivation rate of catalase. Marks (1936) reported that the addition of 0.004M ethanol reduces the inactivation rate of catalase by atmospheric oxygen to more than half compared to control value and the addition of 0.25M ethanol (approximately 1%) completely inhibits the inactivation rate. More or less similar results were obtained by Golzow & Jankowsky (1927). Thus the fact that the presence of ethanol reduces the breakdown of catalase by molecular oxygen is fairly well established and there are good reasons to believe that the same mechanism may be operating in the present case.

If oxygen toxicity is due to slow accumulation of hydrogen peroxide (Mann & Quastel, 1946) the maintenance of catalase activity at a high level will certainly reduce the toxic effect of oxygen and that is why the survival period under HPO was longer on ethanol than on water. Thus the assumption that 'the ethanol effect' is due to the effect of ethanol on enzyme breakdown might explain both the manifestations observed in the presence of ethanol i.e. (1) maintenance of catalase activity at a high level and (2) protection against oxygen toxicity.

Acknowledgements

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