

Pak. J. Bot., 12 (1): 69- 76, 1980.

EFFECT OF DIFFERENT OXYGEN PRESSURES AND OF AGE ON CHANGES IN CATALASE AND PEROXIDASE ACTIVITIES OF *RHIZOPUS ORYZAE* UNDER HIGH PRESSURES OF OXYGEN

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Abstract

Changes in catalase activity of *Rhizopus oryzae* on ethanol medium under high pressures of oxygen varied with varying pressure. The optimum pressure for induced increase of catalase was found to be 4 atmospheres. This induced increase in catalase activity was affected by the age of fungus culture, 44 and 69 hour old cultures showed a decrease in enzyme activity under high pressures of oxygen, whereas an increase was observed in 24 hour old culture. Long term studies under optimum pressure and age revealed that the induced changes in catalase and peroxidase activities of *R. oryzae* are essentially similar under HPO, whereas alcohol dehydrogenase exhibits a decrease instead of an increase. These changes in enzyme activities in response to HPO are reversible on returning the treated samples to air.

Introduction

A previous study demonstrated that the presence of 1 per cent ethanol increased the survival time of *Mucor* sp. under 10 atmospheres of pure oxygen (Ahmed & Pritchard, 1970). This effect was correlated with the changes in catalase activity. In the presence of ethanol a big increase in the induced synthesis of catalase and peroxidase was observed. This induced activity may be due to the increased generation of hydrogen peroxide under high pressures of oxygen (HPO) which acts as an inducer for the activities of catalase and peroxidase (Ahmed, 1977a).

Preliminary studies with *Rhizopus oryzae* under similar conditions failed to give identical results as obtained for *Mucor* sp. This discrepancy in the behaviour of two filamentous fungi under identical conditions suggested that the optimum pressure requirement and age for the induced increase of catalase activity under HPO may vary from individual to individual. Observations of previous workers also supported this suggestion. Gerschman *et al* (1958) demonstrated that aminoethyl iso thiouranium (AET) protected mice at 2.9 and 2.5 atm, but was quite ineffective at 1 and 10 atm. Similarly cobalt increased the survival effect at high pressure. In the present investigation different oxygen pressures and age of the cultures were studied in order to get an optimum value for induced increase in catalase activity of *R. oryzae*. After determining these optimum values, long term studies were carried out to see the effect of the presence of 1 per cent ethanol on the activities of catalase, peroxidase and alcohol dehydrogenase. The results obtained are essentially similar to those of *Mucor* sp. (Ahmed & Pritchard, 1970).

Materials and Methods

Stationary cultures of *R. oryzae* Went and Geerl (CMI 40564) 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 three consecutive days. The medium was inoculated with 1 ml. of spore suspension. After 3-4 days the fungus pads were replaced on 25 ml of sterile solution of 1 per cent ethanol. The fungus pads were first rinsed with sterile deionized water and then carefully floated on the replacement medium.

The pressure vessels for maintaining the cultures in hyperbaric oxygen were a modification of the vessel described by Caldwell (1956). The pressure was applied and released slowly to avoid physical damage to the mycelium.

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 0.5 M phosphate buffer (pH 6.8) and homogenized in a MSE ultrasonic disintegrator, keeping the homogenate cool in an ice bath during treatment. The homogenate was centrifuged at 30,000xg for 10 minutes at 5°C . The supernatant was used for enzyme determination.

Catalase activity was determined by the method of Herbert (1954). First order velocity constants were calculated for each sample at two or three time intervals upto 60 seconds. Peroxidase activity is expressed as increase in absorbance at 340 nm per minute.

Alcohol dehydrogenase was determined by the method of Racker (1950). The reaction was followed spectrophotometrically at 340 nm and enzyme activity expressed as decrease in optical density units per minute. The activities of the enzymes have been expressed on fresh weight basis since the fresh weight often decreased slightly during treatment with high pressures of oxygen. In several early experiments protein determinations on the extract were made using the method of Lowry *et al* (1951) and it was found that the changes in fresh weight which occurred during treatment with HPO were closely correlated with changes in extractable protein.

Results

Effect of different oxygen pressure on catalase activity

Cultures were grown in 100 ml Erlenmeyer flasks on 25 ml of complete glucose glutamate medium. After 4 days growth in air at $25 \pm 1^\circ\text{C}$, the cultures were transferred on 25 ml of 1 per cent ethanol (v/v) and subjected to 4, 6 and 8 atmospheres of HPO. After 24 hours treatment the cultures were analysed for catalase activity (Fig. 1.). Initial readings were taken on the original medium before replacement. Each reading represents an average of two readings. The cultures which were treated with 4 and 8 atm. of HPO showed a drop in catalase activity while the cultures treated with 6 atm. of HPO showed a very slight increase in catalase activity.

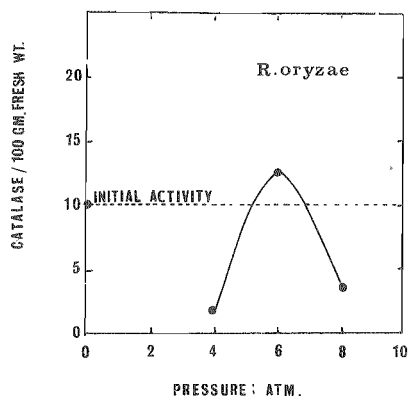


Fig. 1 Changes induced in the activity of catalase in 4 day old cultures of *R. oryzae* on an ethanol medium during a 24 hr sojourn in 4.6 and 8 atm of HPO

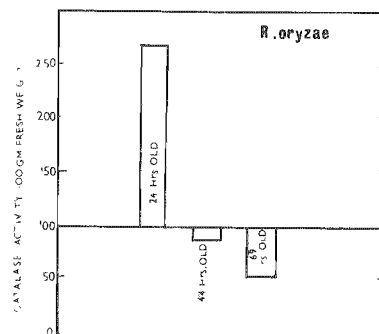


Fig. 2 Comparative changes in catalase activity of *R. oryzae* cultures of various ages during a 6 hr sojourn in 10 atm of pure oxygen

Effect of age of cultures on the changes in catalase activity

The culture vessels were kept in a controlled temperature chamber of $25 \pm 1^\circ\text{C}$. A total of four cultures were taken out every time after 24, 44 and 69 hours. Two of these were analysed for initial catalase activity. The remaining two cultures were replaced on 25 ml of 1 per cent ethanol and subjected to 10 atm of pure oxygen. After 6 hours treatment with HPO the cultures were analysed for catalase activity. The determination were made on duplicate samples.

The induced increase in catalase activity of *R. oryzae* decreased with the increasing age of the fungus (Fig. 2) After 6 hour treatment with 10 atm. of pure oxygen, an increase in catalase activity was observed in 24 hour old cultures whereas 44 hour old cultures showed a slight decrease in catalase activity. This decrease was more marked in 69 hour old cultures.

Effect of different oxygen pressures on the changes in catalase activity

The preceding two experiments suggested that both the age and the oxygen pressure can affect the change in catalase activity of *R. oryzae*. The optimum age for catalase was found to be 24 hours. In another experiment optimum pressure for the increase in catalase activity in 24 hours old culture of *R. oryzae* in HPO was determined.

The cultures were allowed to grow in air for 24 hours and then an initial pair of cultures was taken for analysis. The rest of the cultures were replaced on 1 per cent ethanol and subjected to 4.6 and 10 atm. of pure oxygen in three different pressure vessels. After 24 hours sojourn in pure oxygen, the cultures were analysed for catalase activity in terms of fresh weight (Fig. 3). Each reading represents an average of two read-

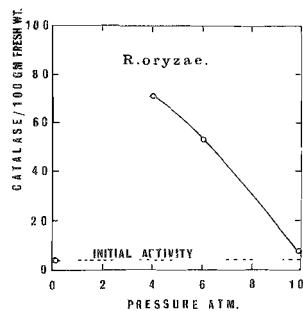


Fig. 3 Changes induced in the catalase activity of 24 hr old cultures of *R. oryzae* on an ethanol medium during 24 hr sojourn in 4.6 and 10 atm. of pure oxygen.

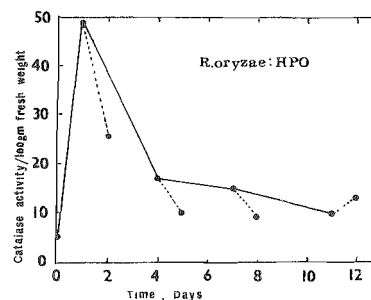


Fig. 4 Changes induced in the activity of catalase in 24 hr old cultures of *R. oryzae* during an 11 day sojourn in 4 atm. of pure oxygen. The broken line represents the activity of the culture which were analyzed 24 hr after the release of pressures.

ings. An increase in catalase activity was observed at 4 atm. of HPO, then it started decreasing and came down to a very low value at 10 atm. Although maximum increase in catalase activity was observed at 4 atm. but even at 6 atm. the activity was fairly high. These results indicate that optimum age for inducing an increase in catalase activity of *R. oryzae* was 24 hours and optimum pressure was 4 atm.

Changes in the activity of catalase, peroxidase and alcohol dehydrogenase in air after HPO treatment

After determining an age and a pressure which gives maximum increase in catalase activity of *R. oryzae* a long term experiment was carried out to see how far the changes in enzyme activity were similar to those of *Mucor* sp. (Ahmed & Pritchard, 1970). The cultures were allowed to grow on complete glucose glutamate medium in air for 24 hours in a controlled temperature chamber of $25 \pm 1^\circ\text{C}$. There after 2 culture flasks were taken for initial readings. The rest of the cultures were replaced on 25 ml of 1 per cent ethanol. A total of 16 cultures were subjected to 4 atm. of HPO in four different pressure vessels each containing four culture flasks. The remaining 8 cultures were left in air for control readings.

The pressures were allowed to escape slowly after 1, 4, 7 and 11 days. At these intervals two samples were analysed immediately for fresh weight and activity of catalase, peroxidase and alcohol dehydrogenase and the other two samples were kept in air for a further 24 hours before analysis in order to obtain some information on the reversibility of enzyme changes (Fig. 4-7). Samples maintained in air throughout were also analysed. Each reading represents an average of two readings. After 24 hours a very big increase in catalase activity was observed in treated samples (Fig. 4) which dropped to a quite low value between 1 and 4 day of treatment. With longer exposure the rate of decrease became very slow and even after 11 days treatment considerable activity was still present in the mycelium. On returning the treated samples to air for a further 24 hours the catalase

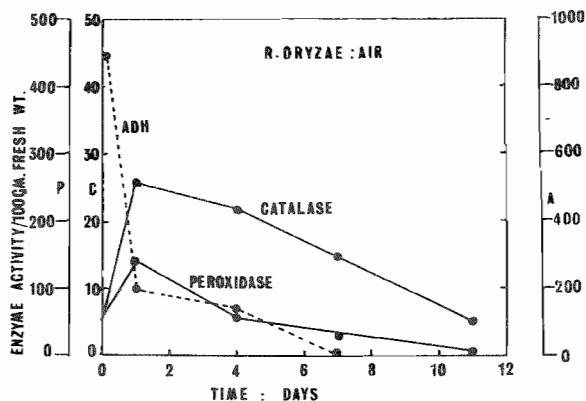


Fig. 5. Changes in the activities of catalase, peroxidase and alcohol dehydrogenase of *R. oryzae* during an 11 day sojourn in air on an ethanol medium.

activity decreased markedly in the case of one day sample. Even after 4 and 7 days treatment when the catalase activity decreased to a much lower value in oxygen it decreased further when returned to air. After 11 days treatment however, samples returned to air showed an increase in catalase activity. Cultures maintained throughout in the air, an increase in activity was observed after 24 hours of replacement, then it started decreasing gradually and came down to a very low value after 11 days (Fig. 5).

The data obtained for the activity of peroxidase were essentially similar to those for catalase (Fig. 6). In the treated samples a big rise in the peroxidase activity was observed after 24 hours treatment, which was very similar to the rise observed for catalase. As with catalase, a sudden drop in enzyme activity was observed between the first and the fourth day and then the decrease became quite gradual. On returning treated samples to air for a further 24 hours the peroxidase level decreased markedly in the case of 1 day

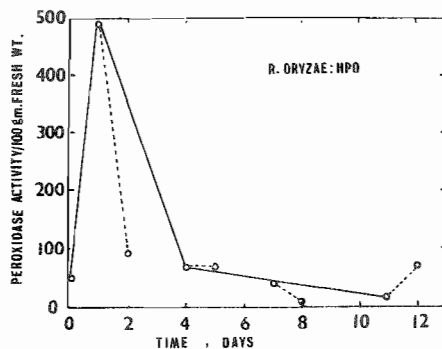


Fig. 6. Changes induced in the activity of peroxidase in 24 hr old cultures of *R. oryzae* during an 11 day sojourn in 4 atm. of pure oxygen. The broken line represents the peroxidase activity of the cultures which were analysed 24 hr after the release of pressure.

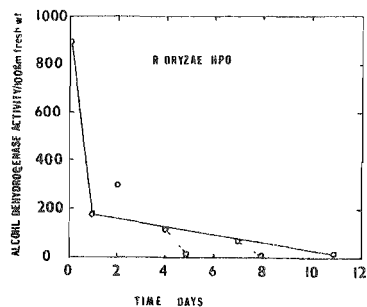


Fig. 7. Changes induced in the activity of alcohol dehydrogenase in 24 hr old cultures of *R. oryzae* during an 11 day sojourn in 4 atm. of pure oxygen. The broken line represents the alcohol dehydrogenase activity of the cultures which were analyzed 24 hr after the release of pressure.

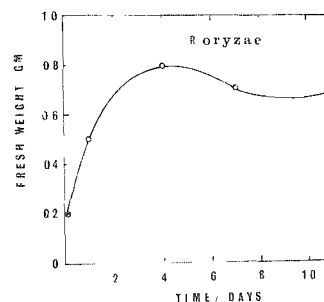


Fig. 8. Changes in the fresh weight in 24 hr old cultures of *R. oryzae* in 4 atm. of pure oxygen.

sample. Even after 4 and 7 days when peroxidase activity had decreased to a much lower value in oxygen, it decreased further when returned to air, but as in the case of catalase after 11 days treatment cultures after return to air, showed an increase in peroxidase activity. In the air control after a slight rise after 24 hours (Fig. 5) on ethanol medium the peroxidase activity started decreasing and came down to a very low value after 11 days treatment. The changes in catalase and in peroxidase activities were not so similar in the air control samples as in the treated samples.

The initial dehydrogenase activity was quite high in 24 hours cultures of *R. oryzae*, but 24 hours after replacement both in the treated samples and the control samples a big drop in the activity of alcohol dehydrogenase was observed (Fig. 7) and after 7 days treatment no activity could be detected in control samples. On returning the treated samples to air for a further 24 hours the alcohol dehydrogenase activity increased slightly in 24 hours treated samples, but when the 4 and 7 days samples were returned to air the alcohol dehydrogenase activity could not be detected. A considerable increase in fresh weight was observed in treated samples up to 4 days (Fig. 8), then it started decreasing gradually. The viability of the fungus was tested throughout the experimental period and even after 11 days treatment, recovery occurred.

Discussion

The increase in catalase and peroxidase activities on ethanol medium under HPO was consistent with the notion that some inducer is generated which causes induced synthesis of these enzymes. Ahmed (1977a) suggested that the rate of generation of hydrogen peroxide is accelerated under HPO and after short exposure the peroxide acts as an inducer for catalase and peroxidase, but after long exposure the peroxide accumulates to a toxic level and consequently the enzyme activity drops. The observations presented in this paper further support this suggestion. The effect of ethanol on catalase and peroxidase activities have been reported by Ahmed (1977b).

Studies on the reversibility of enzyme changes are in favour of the idea of induced synthesis of catalase and peroxidase. The activities of these two enzymes in the treated cultures (Figs. 4 and 6) came down to their normal air control values 24 hours after return to air. In cultures exposed to oxygen for up to 7 days, the catalase and peroxidase activities were higher in cultures which were analysed for enzyme activity just after releasing the pressures than in the cultures which were analysed 24 hours after releasing the pressure. But after 11 days exposure the activities of these enzymes were higher in cultures which were allowed to remain in air for 24 hours than in cultures analysed for enzyme activity just after releasing the pressures. This observation supports the view that the observed increase in catalase and peroxidase activities under HPO was due to the formation of some inducer (H_2O_2) and not due to the changes in enzyme molecules themselves. The decrease in the activities of these enzymes after return to air may be due to the rapid break down of H_2O_2 by catalase resulting in its complete disappearance. Since the rate of generation of H_2O_2 in air is very much less than in HPO, the induced synthesis of enzyme and the enzyme activities come down to their normal air control values. However, after long exposures the amount of H_2O_2 may attain such a high level that it becomes toxic for enzyme activity. This explains the observation that it becomes toxic for enzyme activity. This explains the observation that catalase and peroxidase activities after long exposures to HPO (11 days) were higher in cultures which were left in air for 24 hours before analysis than in cultures which were analysed immediately after the release of pressure, because in this case the enzyme activity is inhibited by high concentrations of H_2O_2 under HPO. After return to air H_2O_2 disappears and the enzyme activity attains its normal value.

A decrease in catalase activity of *R. oryzae* was observed when the cultures were 44 and 69 hours old, whereas 24 hours old cultures showed an increase in catalase activity (Fig. 2). It is difficult to define the physiological state in a filamentous fungus, but since *R. oryzae* is a very rapidly growing fungus, it may be assumed that in 44 and 69 hour cultures much of the mycelium was in a stationary phase and thus physiologically old. In 24 hour old cultures the mycelium is young and therefore much more active. The ability of an organism to synthesise an enzyme will obviously vary with the age depending upon its metabolic activity. The observation that catalase induction varies with the age of the fungus, further supports the view that increase in catalase was due to its synthesis rather than the changes in the activity of the existing molecules.

The changes in catalase activity were found to vary with the varying pressures and there was an optimum pressure for its activity (Fig. 3). These results suggest that biological variability of oxygen toxicity is rather large. One of the possible reasons for this variability may be that different amount of H_2O_2 is formed under different pressures depending upon the peroxide generating system of an organism. It has been suggested by many previous workers (Stadie, Riggs & Haugaard, 1944; Bean, 1945; Caldwell, 1963; Caldwell, 1964; Caldwell, 1965) that oxygen sensitivity varies from species to species. This variation may also be due to the differences in the peroxigenic system of various species. Elaboration of this point needs further investigations on peroxigenic systems of test organisms.

The observation that there is a high increase in the activities of the catalase and peroxidase (Fig. 4 and 6) which are maintained for several days further supports the suggestion given by Pritchard & Hudson (1967) and Ahmed & Pritchard (1970), that resistance of micro-organisms to H₂O₂ toxicity (Caldwell, 1963; Mc Allister *et al* 1963; Robb, 1965) is due to induced increase in catalase activity.

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