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EFFECT OF GLUCOSE ON THE SURVIVAL OF *MUCOR PLUMBEUS* BON., AND *MUCOR RACEMOSUS* FRESEN., UNDER TEN ATMOSPHERS OF PURE OXYGEN

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Abstract

Studies have been made on the effect of the presence of glucose on survival and changes in catalase activity of *Mucor plumbeus* Bon, and *M. racemosus* Fresen., under 10 atmospheres of pure oxygen. The survival of these fungi were greatly reduced on glucose media under high pressures of oxygen and showed rapid drop in catalase activity. Investigations made to determine the effect of different pH values on the changes in catalase activity under high pressures of oxygen and the effect of pH on the changes in catalase activity and survival time suggested that glucose acts indirectly by decreasing the pH of the media which results in a decrease in catalase activity and consequently an early death of fungi on this substrate under high pressures of oxygen.

Introduction

It has been frequently suggested (Mann & Quastel, 1946; Dickens, 1946; Gerschman, Gilbert, Nye, Dwyer & Fenn, 1954) that generation of hydrogen peroxide is responsible for the toxic effects of increased oxygen tension on living organisms. These suggestions were further supported by Ahmed & Pritchard (1970) who showed a correlation between changes in catalase activity and survival of fungi under hyperbaric oxygen. They suggested that a high level of catalase activity enables the organisms to cope with higher rate of peroxide generation under high pressures of oxygen by breaking down the hydrogen peroxide which is toxic for living tissues.

In studies on the metabolic effect of hyperbaric oxygen on fungi (Ahmed, 1969) it became apparent that the ability of fungi to survive under prolonged treatment of hyperbaric oxygen (Caldwell, 1965; Robb, 1965) were markedly affected by nutritional factors, especially the carbon source. Ahmed & Pritchard (1970) have shown that the presence of glucose has a deleterious effect on the survival of fungi under hyperbaric oxygen. They suggested that the deleterious effect on survival and the inhibition of the induced increase in catalase activity could be due to a direct effect on induction process by 'catabolite repression' (Magasanik, 1961) or to an indirect effect on either activity or synthesis of catalase due to the large and rapid drop of pH which occurs on this substrate. But they provided no experimental evidence in support of either of these assumptions.

In the present paper investigations have been made to study the effect of pH values on the changes in catalase activity under high pressures of oxygen. The effect of pH on the changes in catalase activity and survival time under high pressures of oxygen in the presence of glucose has also been studied. The results obtained support the idea that glucose effect is probably due to low pH values.

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Materials and Methods

The work described in this paper was carried out on *Mucor racemosus* Fresen (CMI 35716) and *Mucor plumbeus* Bon. (CMI 10772).

Growth of fungus cultures:

Stationary cultures of fungi were grown on liquid glucose glutamate medium (Pritchard, 1965) at 25°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 growth the fungus pads were replaced on to a sterile solution of glucose. The fungus pads were first rinsed with sterile deionised water and then carefully floated on to the replacement medium.

High presure oxygen treatment:

The pressure vessels for maintaining the cultures in hyperbaric oxygen were a modification of the vessel described by Caldwell (1965). A pressure of 10 atmospheres of pure oxygen (hereafter abbreviated to HPO) were used for all the experiments described in this paper. The pressure was applied and released slowly to avoid the physical damage to the mycelium.

Viability of cultures following oxygen treatment was tested by subculturing the fungus on 2.5 per cent 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.

Catalase activity was determined by the method of Herbert (1954). First order velocity constants were calculated 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 slightly during treatment with 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 occured during treatment with HPO were closely related with 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.

Determination of keto-acid:

Keto-acids were determined by the method of Isherwood & Cruickshank (1954) as modified by Niavis (1956). This method is based on the chromatographic separation of 2-4 dinitrophenyl hydrazones and colorimetric estimation of the separated hydrazones.

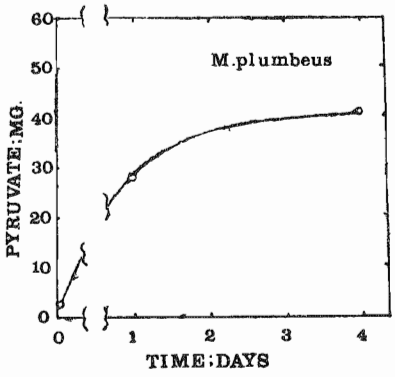

Fig. 1. Changes in the pyruvate content of the medium (3% glucose) of *M. plumbeus* cultures after a 4 day sojourn in 10 atm. of pure oxygen.

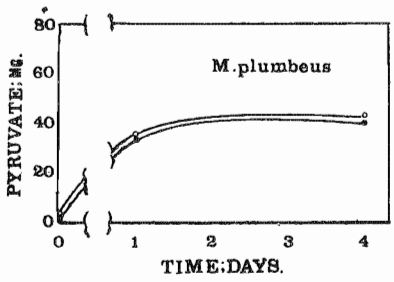

Fig. 2. Changes in pyruvate content of the medium during a 4 day sojourn in 10 atm. of pure oxygen on ●—● glucose+CaCo3 and o—o complete glucose glutamate medium.

All the keto-acid determinations were carried out on the medium only, because most of the amount accumulated is excreted into the medium and a very small amount remains in the mycelium (Pritchard, 1966).

Results

I. SURVIVAL AND GLUCOSE METABOLISM OF *M. plumbeus* under HPO.

A total of 14 cultures of *M. plumbeus* were grown on 25 ml. of complete glucose-glutamate medium in 100 ml. Erlenmyer flasks for four days at 25± 1°C. Thereafter they were replaced on 25 ml. of 3 per cent glucose under sterile conditions. After replacement two cultures were taken for initial readings and the remaining 12 cultures were allowed to remain in air for 24 hours. They were then subjected to 10 atmospheres of pure oxygen. The pressures were allowed to escape slowly on first and fourth days and studies were made for viability and dry weight of the mycelium, pyruvate and pH of the medium.

There was a large accumulation of pyruvate in the culture filtrates of HPO treated samples (Fig. 1). The fungus recovered after 24 hours treatment with HPO but there was no recovery after four days treatment which was quite contradictory to the previous report (Robb, 1965). On the fourth day the pH dropped from 5.0 to 2.4. Dry weight showed a gradual decrease. It was thought that the early death of the fungus might be due to either low pH value or to absence of mineral salts and nitrogen source in the medium. Therefore the same experiment was repeated again using 3 per cent glucose + calcium carbonate and complete glucose-glutamate medium as the replacement substrates. The experimental procedure was the same as described above. After 4 days growth 8 cultures were replaced on 25 ml of complete glucose glutmate medium. The remaining 8 cultures were replaced on 25 ml of glucose and 250 mg. of calcium carbonate was added to each of them.

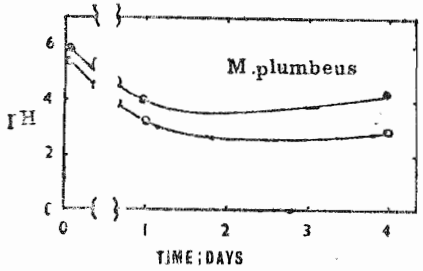

Fig. 3. Changes in pH value of the medium during a 4 day sojourn in 10 atm. of pure oxygen on ●—● glucoseCaCo3 and o—o complete glucose glutamate medium.

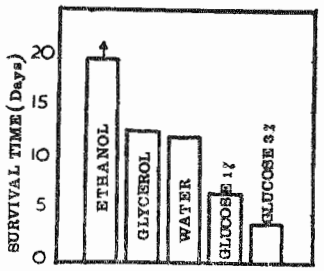

Fig. 4. Survival time of *M. plumbeus* on various substrate in 10 atm of pure oxygen.

Two cultures on 3 per cent glucose and calcium carbonate and two on glucose-glutamate medium were analysed after the one and four days treatment for viability, pH and pyruvate. On the fifth day cultures were tested for viability only. On complete glucose-glutamate medium no growth of the fungus occurred after four days treatment. On 3 per cent glucose + calcium carbonate however the clutures eventually grew after four days treatment with 10 atmospheres of pure oxygen, but there was no recovery after five days treatment. A large accumulation of pyruvate was observed on both the substrates (Fig. 2) and there was a drop in pH value (Fig. 3).

II. COMPARISON OF THE SURVIVAL OF *MUCOR SPP.* ON GLUCOSE MEDIA WITH THE SURVIVAL ON SOME OTHER SUBSTRATES UNDER HPO.

Cultures of *M. plumbeus* were grown on complete glucose-glutamate medium in 2 cm diameter boiling tubes for 4 days. Thereafter they were replaced on one per-cent glucose (V/V), three per cent glucose (V/V), one per cent ethanol (V/V), one per cent glycerol and deionised water. After 24 h period of adaptation to these media the cultures were subjected to 10 atmosphers of pure oxgyen. A total of nine pressure vessels were used each containing four replicates of each medium.

The pressures were released slowly on 2nd, 3rd, 5th, 7th, 9th, 10th, 13th, 16th, and 20th days and studies were made of survival of the mycelium and pH of the medium. The survival time of *M. plumbeus* was found to be different on different substrates (Fig. 4) under HPO. The cultures replaced on ethanol survived after 20 days treatment of HPO. The shortest survival time was observed in cultures replaced on glucose. On 3 per cent glucose, it was three days and on 1 per cent glucose it was seven days. Survival time of the cultures replaced on 1 per cent glycerol and deionised water was almost the same under HPO.

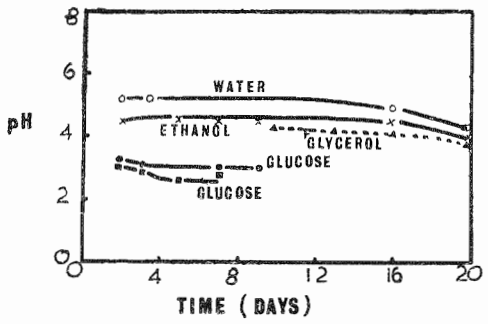

Fig. 5. Changes in pH of media of *M. plumbeus* culture during a 20 day sojourn of 10 atm. of pure oxygen.

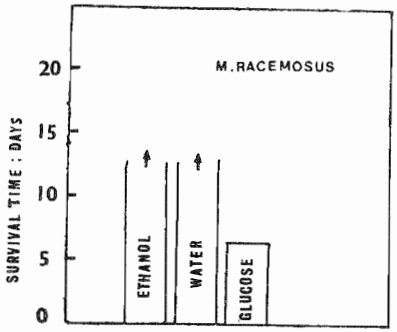

Fig. 6. Survival time on ethanol, glucose and water in 10 atm. of pure oxygen.

The pH dropped to a low value (Fig. 5) on 1 and 3 per cent glucose. Where ethanol and glycerol were used the pH were much higher than on glucose and remained more or less stationary up to 13 days and then decreased gradually.

To confirm the toxic effect of glucose, comparable studies were also made on *M. racemosus*. The cultures were replaced on one per cent ehtanol (V/V), one per cent glucose (V/V) and deionised water and subjected to 10 atmospheres of pure oxygen. The pressures were released slowly on the 4th, 7th, 10th and 13th days and studies were made for viability and pH on duplicate samples.

The results obtained were essentially similar to those of *M. plumbeus*. Shortest survival time of about seven days was observed on 1 per cent glucose (Fig. 6). The pH dropped to a very low value on glucose media while it remained quite high on deionised water and ethanol.

III. EFFECT OF GLUCOSE ON THE ACTIVITY OF CATALASE UNDER HPO AND COMPARISON OF ITS EFFECT WITH THE ACTIVITY ON SOME OTHER SUBSTRATES UNDER HPO.

The experiments described above suggests that glucose enhances and ethanol protects against oxygen toxicity. Pritchard & Hudson (1967) observed that HPO treatment induces a large increase in catalase activity in the fungi. They suggested that catalase may play an important role in determining the degree of oxygen toxicity. An experiment was therefore carried out to investigate the effect of glucose on catalase activity in order to find out some basis for the deleterious effect of glucose.

The experimental procedure was the same as described for previous experiments. Four days old cultures were replaced onto 1 per cent glucose (V/V), deionised water and 1 per cent ethanol and subjected immediately to HPO treatment. Samples were taken at 3—day interval over 15 days period for catalase activity and viability determinations. (Fig. 7). These results suggest that there exists a correlation between catalase activity and survival time under HPO. It would also suggest that glucose somehow decreases catalase activity, consequently an early death of the fungus occurs on this substrate.

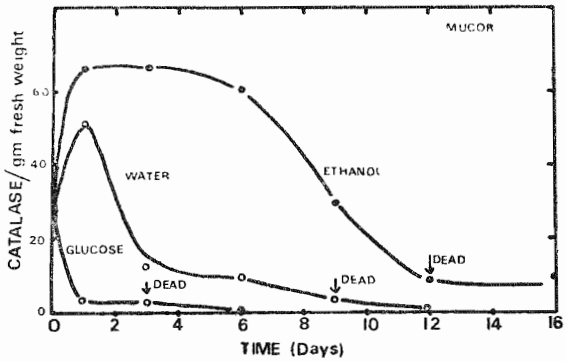

Fig. 7. Correlation between survival and changes in catalase activity of *M. racemosus* during a 12 day so journ in 10 atm. of pure oxygen.

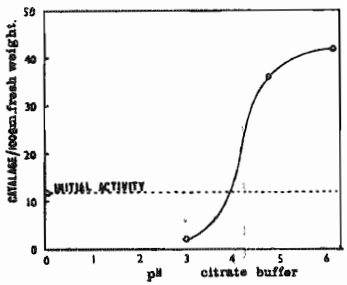

Fig. 8. Changes induced in the catalase activity of *M. racemosus* at various pH values duringa 24 hrs. sojourn in 10 atm. of pure oxygen in 0.1M citrate buffer.

IV. EFFECT OF pH CHANGES ON CATALASE ACTIVITY OF *M. racemosus* UNDER HPO.

Cultures of *M. racemosus* were grown for four days as described in previous experiments. Two flasks were then taken for initial readings. The remaining cultures were replaced on 0.1M citrate buffer pH 3.0, 4.8 and 6.2. The cultures were then subjected to 10 atmospheres of HPO in a pressure vessel containing two flasks of each of the three different pH values used. After a period of 24 hours in pure oxygen, culture flasks were analysed tor catalase activity.

The results obtained are shown in (Fig. 8). Each reading in this figure represents an average of two readings. After 24 hours treatment a marked drop in catalase activity, was observed on pH 3 buffer (the usual pH value obtained on glucose media after 24 hours treatment) but quite big increase in catalase activity was observed at pH 4.8 & 6.2. These observations indicate that low pH values are deterimental for the induced increase in catalase activity.

Effect of the presence of citrate buffer & CaCO3 was also determined on the changes in catalase activity of *M. racemosus* under HPO on glucose medium. After four days growth, two culture flasks were taken for initial readings and 6 cultures were replaced on 3 per cent glucose in deionised water and 150 mg. of CaCO3 was added to each culture flask. The remaining 6 cultures were replaced on 3 per cent glucose in 0.1M citrate buffer pH 6.2. After replacement the cultures were subjected to HPO.

The pressures were released slowly on the first, second and third dsys and determinations were made of catalase activity and pH. After 24 hours treatment the pH values of glucose + CaCO3 was 3.4 while on glucose + buffer the pH values were considerably higher (5.5). The decrease in catalase activity on glucose + CaCO3 was very high while the decrease in catalase activity on glucose in buffer solution was much less (Fig. 9). After two days treatment the catalase activity on glucose + CaCO3 was very low and no activity was observed after three days treatment. The ultimate pH value on this substrate was 3.0. However, the rate of decrease on glucose + citrate buffer was very slow and even after three days treatment a relatively high catalase activity was observed. The ultimate pH value on this substrate was 4.9. Two main conclusions can be drawn from the result of this experiment.

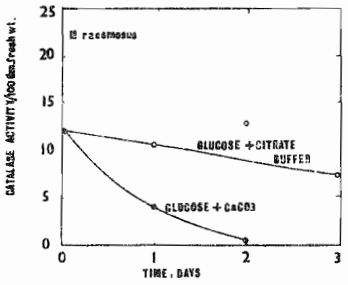

Fig. 9. Changes induced in the catalase activity of *M. racemosus* on 3% glucose in 0.1M citrate buffer (pH) 6.2) and 3% glucose + CaCo30

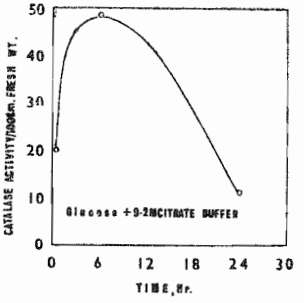

Fig. 10. Changes induced in the activity of *M. racemosus* on 3% glucose in 0.2M citrate buffer (pH 6.2) during a 24 hrs. sojourn in 10 atm. of pure oxygen.

1. The glucose effect can be altered by increasing the pH values.

2. CaCO3 is not an effective buffering agent for glucose under HPO.

In the experiment described above 0.1M citrate buffer decreased the fall of catalase activity on a glucose medium under HPO. On the basis of this observation it was assumed that a higher strength of citrate buffer may even permit an increase in catalase activity in the presence of glucose. To investigate the validity of this assumption, same experiment was repeated using 0.2M citrate buffer.

Culture were allowed to grow from spore inoculum for four days. Thereafter two cultures were taken for initial readings and four cultures were replaced on glucose 3 per cent in 0.2M citrate buffer pH 6.2. The cultures were subjected to ten atmospheres of HPO.

The cultures were analysed for catalase activity after 24 hours of treatment. After 6 hours treatment with HPO an increase in catalase activity was observed, but after 24 hours treatment a slight decrease in catalase activity below the original activity was observed (Fig. 10). The ultimate pH value of the substrate was 5.6. These results illustrate that rapid drop in catalase activity in the presence of glucose observed in previous experiments was probably due to low pH value and not due to any specific effect of glucose.

The increase in the catalase activity of *M. racemosus* on the glucose medium in the presence of 0.2M citrate buffer pH 6.2 under HPO suggested the idea of studying the survival time of this fungus on a glucose medium in the presence of 0.2M citrate buffer pH 6.2 under HPO.

Fully grown fungus pads were replaced on 25 ml. of three per cent glucose in 0.2M citrate buffer pH 6.2 and subjected to ten atmospheres of pure oxygen. The pressure were released on the 2nd, 4th, 8th, and 11th days and the studies were made for the viability pH and colony diameter of subcultures of the fungus on duplicate samples.

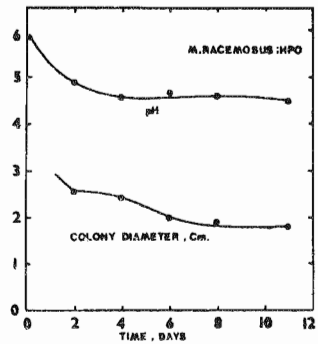

Fig. 11. a. Changes in pH values of the medium of *M. racemosus* cultures during 11 day sojourn in 10 atm. of pure oxygen on 3% glucose in 0.2M citrate buffer.

b. Colony diameter of subcultures taken from treated samples of M. racemosus 24 hrs. after inoculation on agar plates.

The fungus recovered even after 11 days treatment of HPO . After this period the experiment was discontinued. After an initial drop the pH remained fairly high throughout the experimental period (Fig. 11). The colony diameter of subcultures of treated cultures 24 hours after inoculation showed a slight decrease with the increasing period of exposure indicating a probable time lag in recovery (Fig. 11).

Discussion

Presence of glucose decreased the survival time of *M. racemosus* and *M. plumbeus* under HPO, suggesting that its presence somehow enhances oxygen toxicity. Its presence also resulted in decrease in the activity of catalase.

The present studies have given an indication that pyruvate metabolism of all the fungi tested is inhibited under HPO and almost all the pyruvic acid accumulated is excreted into the medium, making the solution very acidic (pH 3.5-2.5). Such similar observations have been made by Pritchard & Hudson (1967). Chance (1952) reported that catalase activity is pH insensitive over the range 5-9 but below pH 4 its activity towards H_2O_2 diminshes. A similar report came from Molland (1947) for bacterial catalase. He reported that the activity of bacterial catalase is completely inhibited below pH 3.5 but is fairly stable between 5.5—8.5. These results suggested that decrease in catalase activity in the presence of glucose may be due to the indirect effect of glucose on pH values.

A series of experiments carried out confirm the hpothesis that "the glucose effect" on catalase was due to the low pH value attained on this substrate under HPO. Catalase activity was greatly affected by pH under HPO. The catalase activity of *M. racemosus* after 24 hours treatment of HPO increased significantly at pH 6.2 & 4.2. The catalase activity at pH 3 (the usual pH observed on glucose medium) came down to a very low value. The degree of decrease in activity on pH 3 buffer was approximately equal to the decrease observed on glucose after 24 hours treatment with HPO. These observations strongly support the assumption that the decrease in catalase activity in the presence of glucose is a consequence of the pH decrease.

Studies with buffered glucose further supported this idea. When glucose solution was made up in deionised water and no buffering agent was added to it the catalase activity of *M. racemosus* decreased to a very low value after 24 hours treatment with HPO, when glucose solution was made up in 0.1M citrate buffer (pH after 24 hours 5.5) the rate of decrease was much slower and when 0.2M citrate buffer was used instead of 0.1M an actual increase in catalase activity was observed after 6 hours treatment with HPO. The results of these experiments would indicate that glucose effect on catalase can be neutralised by keeping the pH values fairly high.

Some further data in support of this hypothesis came from the studies of survival time of *M. racemosus* using 3 per cent glucose in 0.2M citrate buffer pH 6.2 as the replacement medium. The usual survival time of *Mucor spp.*, was found to be 4 days on 3 per cent glucose but on 3 per cent buffered glucose the fungus recovered even after 11 days treatment with HPO. By maintaining the pH above a certain level both the effects manifested by glucose *i. e.*, decrease in catalase activity and early death of the fungus under HPO can be altered. The glucose effect is due largely to a drop in pH value as a consequence of the inhibition of pyruvate metabolism under HPO.

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