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GERMINATION OF CONCENTRATED SUSPENSIONS OF SPORES FROM *ASPERGILLUS NIGER.*

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SUMMARY

Germination of condiospores from *A.niger* in very concentrated suspension was required to inoculate solid fermentation media, but a germination self-inhibitor was detected in spores. It was found that the inhibitory effect depended on the composition of the medium and was reduced when glucose was used as a carbon source. The effect of the self-inhibitor was eliminated by washing the spores and using glucose and a protein nitrogen source in the germination medium. By this method it was possible to increase about 100 times (10^6 to 10^8) spore concentration, keeping more than 90% germination.

INTRODUCTION

In the protein enrichment process of cassava by solid state fermentation (Raimbault et al., 1980) one third of the fermentation time corresponds to spore germination. The duration of the process would be greatly reduced, with possible economic benefits, if this phase could be performed separately in liquid suspension and pregerminated spores used to inoculate the solid substrates.

Such a procedure requires the ability to pregerminate spores in a very concentrated suspension. Thus the effect of spore concentration on germination was studied.

MATERIALS AND METHODS

* **Strain** *Aspergillus niger* No. 31 : (Raimbault et al., 1985). The strain was kept in conservation medium at 4°C and tranferred to a new culture every week .

* **Conservation medium**: cassava-agar (Raimbauult & Alazard, 1980).

* **Sporulation medium**: potato-dextrose-agar (PDA).

* **Germination medium**: Similar in composition to the solid state fermentation, except that cassava was substituted by starch (Barrios-González & Anaya, 1987) : soluble starch 10 g; $(NH_4)_2SO_4$ 0.5 g; urea 0.24 g; KH_2PO_4 0.97 g; distilled water 1 l. The pH was adjusted to 4 with H_3PO_4.

In some cases the starch was substituted by 10.5 g of glucose. Similary, the $(NH_4)_2SO_4$ and urea were substituted by 0.92 g of peptone and 0.92 g of casein peptone.

* **Inoculum preparation**: 250 ml Erlenmeyer flasks containing 50 ml of sterile sporulation medium were inoculated from a new working culture and incubated for one week at 30°C. After this time a spore suspension was prepared, filtered through double cheese cloth and its concentration determined microscopically, using a Neubauer chamber.

* **Spore washing**: Suspensions were centrifuged for 15 minutes at 5000 rpm, and the pellet resuspended in the required volume of sterile water (first wash).

* **Germination System**: 250 ml Erlenmeyer flasks containing 100 ml of germination medium were inoculated with the required volume of spore suspension to achieve the desired final spore concentration. The flasks were incubated at 35°C, with rotary agitation at 140 rpm. For sampling, flasks were hand agitated and 5 ml transferred into a test tube and a drop of tween 80 added. Before putting a sample into the Neubauer chamber, the tube was agitated vigorously with a Vortex for 2 minutes.

* **Dertermination of Germination rate**: In a Neubauer chamber, the number of germinated (already showing a germ tube) and ungerminated spores were counted.

RESULTS

Initially, the degree of germination obtained at different times was determined. The germination kinetics curve had a sigmoidal shape and a duration of between 8 and 12 hours, depending on the medium used. To study the effect of spore concentration on the rate of germination, the degree of germination after 12 hours was measured at different spore concentrations. Figure 1 shows the results obtained in an experiment using a germination medium with starch as a carbon source and ammonium sulphate and urea (ammonium) as a nitrogen source. It can be seen that at spore concentrations greater than 10^6spores/ml, the process is increasingly inhibited. The same figure shows that the inhibitory effect is lower in a medium with glucose (carbon source) and peptone (nitrogen source).

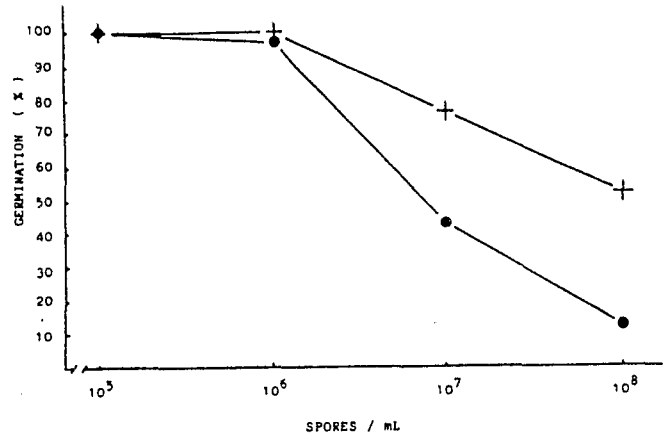

Figure 1. Effect of spore concentration on the degree of germination obtained ater 12 hours of incubation. Spores of A.niger (No. 31) were used to inoculate a medium with starch- amonium ($(NH_4)_2SO_4$-urea) (●) and a medium with glucose-peptone (+)

Since it was not known if this attenuation of the inhibition was due to the glucose or to the peptone, an experiment was performed comparing media containing: a) glucose and peptone, b) glucose and ammonium, c) starch and peptone , d) starch and ammonium.

Results (Figure 2) indicated that the attenuation is mainly due to glucose. To eliminate the inhibitory effect spores were

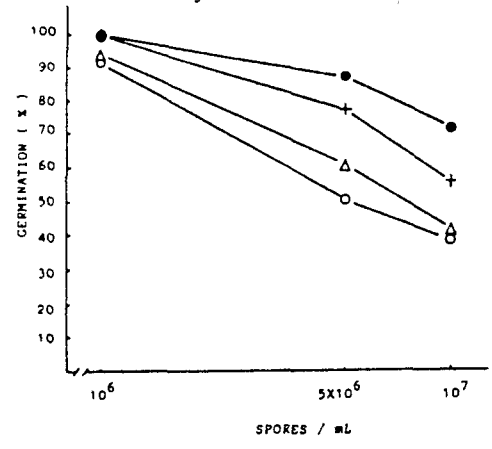

Figure 2. Influence of the medium composition on the inhibition of germination at high spore concentrations. Spores from A.niger (No.31) were incubated for 12 hours in media containing: glucose- peptone (●), glucose-amonium (+), starch-peptone (Δ) and starch-ammonium (0).

washed before germination in glucose-peptone medium. It was observed (Figure 3) that the inhibition was practically eliminated by this treatment ; the degree of germination obtained increased from 50% to 90% at 10^8. The experiment was repeated, but germination of the washed spores was tested in different media. Results showed that the increase in germination obtained by washing was: 29.5% in glucose-peptone, 26% in glucose-ammonium, 19% in starch-peptone and 2% in starch-ammonium.

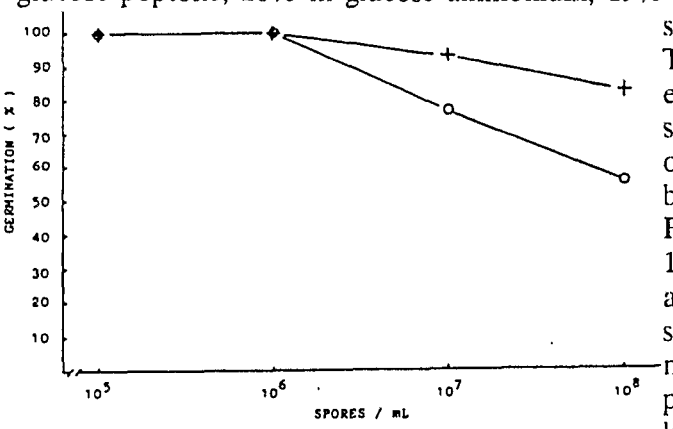

Figure 3. Elimination of the inhibitor of germination by washing the spores of A.niger (no.31) once. Washed (+) and non-washed spores (0) were incubated for 12 hours in medium with glucose-peptone.

The glucose - ammonium experiment was repeated with spores that had been washed once, twice and three times, but no difference was observed. Finally, a small quantity (10 and 15 ml) of the supernatant of a centrifuged 10^7 spores/ml suspension was added to a medium with glucose and peptone. It was seen that this liquid not only lowers the germination rate, but makes the kinetics erratic, the effect being proportional to the volume added.

DISCUSSION

A number of fungal spores in a wide range of taxonomic groups, contain compounds (called self-inhibitors) which prevent their germination (Macko et al., 1976). The spores of such fungi will not germinate until the inhibitor is leached away. The selective advantage of self-inhibitors is, probably, to prevent premature germination and rapid germination of all spores at the same time. In some cases, mainly among the rust fungi, the inhibitory compounds have been isolated and identified. (Van Etten et al., 1984).

An inhibitor has been reported in conidia of *A.niger* in conditions of spore crowding that affected only the initiation of spore growth (Anderson & Smith, 1972). The inhibitor appeared to be present at the time of spore formation and could be removed by washing (Krishnan et al., 1954).

In this work it was shown that the spores of *A.niger* (No. 31) also have an endogenous inhibitor of germination. This compound (or compounds) is water soluble and is responsable for the drastic decrease in germination rate when the spores concentration is above 10^6 spores/ml. It was also found that the intensity of the inhibition depended on the medium composition, particulary on the carbon source. When strarch is used, the inhibitory effect is more intense than when glucose is the carbon source. Surprisingly, it was found that this effect is only slightly affected by the nitrogen source (proteic or inorganic). But the use of starch and ammonium increases the inhibitory effect. In contrast, glucose and peptone were more favourable.

The inhibitory effect can be sensibly decreased by washing the spores, and this attenuation depends, again, on the composition of the medium. After glucose-peptone, the best germination of washed spores was obtained in glucose-ammonium medium. In this case, the washing treatment decreases the crowding effect, but a relatively small inhibition is still present.

It was also seen that the inhibitory compound remains in the supernatant of a concentrated spore suspensions. A small fraction of this supernatant added to a germination medium produced a decrese in germination rate and irregular shape kinetics, similar to ones described by Barrios-González & Anaya (1987) when old working cultures were used to produce spores, and germination was done in a starch-ammonium medium.

From a practical point of view, it can be concluded that there is an inhibition of *A.niger* spores germination when concentrated suspensions are used. The concentration of spores can be increased about 100 times (probably more, but was not tested), when spores are washed and the germination medium contains glucose and proteic nitrogen. In some applications, the use of proteic nitrogen for the germination stage is not economical. According to our results, if spores are washed and a medium with glucose and ammonium is used, the inhibitory effect is greatly diminished, and rapid, stable and predictable germination kinetics can be obtained.

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