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Fibrinolysis III. Nutritional and Environmental Requirements for Optimum Production of Fibrinolysin from Aspergillus (Aspergillin O). (25191)

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Many fungi are known to produce proteolytic substances as part of their metabolic activity and some of these substances are capable of fibrinolysis. Previously reported studies(1) on fibrinolytic activity of extracts from cultures of 260 fungi have singled out a strain of *Aspergillus oryzae* (B-1273) as the most promising producer of material with strong and relatively selective fibrinolytic activity. In continuing these studies, it soon became apparent that further progress would be greatly handicapped by the inability to obtain high yields of material. This paper describes observations on nutritional and environmental requirements of *Aspergillus oryzae* (B-1273) for increased production of fibrinolytic substance. For convenience, this material has been tentatively designated as "Aspergillin O" to distinguish it from the spore pigment, Aspergillin, previously isolated from *Aspergillus niger* (2). Properties and physiochemical characteristics of Aspergillin O are to be described.

Materials and methods. 1. *Culture media.* Czapek's solution agar slants and malt agar slants were prepared according to standard technics from commercial reagents (Difco). The liquid media consisted of following reagents: Sucrose 7.2 g, Dextrose 3.6 g, KH_2PO_4 13.69 g, KNO_3 2.0 g, Mg SO_4 1.23 g, in double distilled water to 1 liter. Two hundred ml of the media was transferred to 1 liter Roux culture flasks, then autoclaved 15 minutes at 15 lbs steam pressure (121°C). When needed, salts and vitamins were added to the liquid medium. Stock solutions of trace elements were prepared by dissolving 723 mg $FeSO_4$, 440 mg Zn SO_4, and 200 mg Mn SO_4 in 500 ml of double distilled H_2O. Sufficient volume of concentrated sulfuric acid was added to render solutions colorless and the solutions were then brought to 1 liter with double distilled H_2O. Two ml of each stock solution was added to the medium. Stock solutions of vitamins contained 10 mg of pyridoxine hydrochloride in 100 ml of 20% ethanol solution or 10 mg of thiamine hydrochloride in 100 ml of 20% ethanol solution in water. Other media were prepared in which the source of nitrogen was represented by NH_4NO_3, $NH_4H_2PO_4$, (NH_4) 2 HPO_4 and KNO_2 rather than KNO_3. These compounds were used in concentrations yielding a nitrogen equivalent to 2 g KNO_3/L. 2. *Fibrin plates.* Solutions of 350 mg % of bovine fibrinogen (Armour) and of bovine thrombin (Parke Davis) containing 30 NIH units/

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ml were prepared in phosphate buffer at pH 7. Nine ml of fibrinogen solution and 1 ml of thrombin solution were quickly mixed in test tube and poured into sterile Petri dish of 10 cm diameter. After clotting, the plates were heated at 85°C for 30 minutes and cooled before use. 3. *Growth of Fungi.* Parent cultures were transplanted to Czapek's solution agar slants, which served as stock cultures and incubated at 24° (± 2) C. Spores from stock tubes were transplanted to malt extract agar slants and cultured at same temperature to provide sufficient quantities of spores for inoculation of the liquid media. This was performed in a single transfer of spores from an agar slant by a sterile platinum loop (1 cm^2 area). Flasks were gently agitated to insure uniform distribution of the inoculum and cultures were incubated 5 days at room temperature in the dark or under different experimental conditions as desired. 4. *Extraction of fibrinolytic material.* The liquid medium was separated from the fungal mat after 5 days of incubation by filtration through filter paper (Whatman 40). The filtrate was passed through a column of IRA-400 resin in the Cl cycle. After addition of 1/10 volume of 1/10 N NaOH, the active material was precipitated by addition of 2 volumes of acetone A.G. The precipitate was resuspended in distilled water (2/5 volume of original filtrate) and any insoluble material was separated by filtration through paper. These represent preliminary steps for preparation of Aspergillin O. Full details are given elsewhere. Serial dilutions of the resuspended material from 1:1 to 1:8 were also prepared with distilled water. 5. *Weight of fungal mat.* This was determined on analytical balance after pressing the mat in paper toweling and drying at 80°C for 60 minutes or until constant weight was obtained. 6. *Assay of activity of material.* 0.05 ml of a 1:4 dilution of active material in water was placed on fibrin plates and these were incubated at 37°C for 6 hours. The 1:4 dilution was selected after preliminary work indicated that it would yield uniformly reproducible results, probably because of dilution of contaminating salts and other impurities. One unit of activity was considered equivalent to an area of 10 mm^2 of lysis. *All calculations were made on the basis of original volume of filtrate,* and in the experimental conditions, 0.05 ml of a 1:4 diluted material was equivalent to 1/32 ml of filtrate. The following formula was used for calculations of units of activity:

$$\text{units/ml filtrate} = \left(\frac{\text{lysis area, mm}^2}{10\ \text{mm}^2} \right) \times 32.$$

Results. (Fig. 1). The liquid medium appeared adequate for growth of the fungus, but, in the dilutions used, the material obtained had insignificant fibrinolytic activity. Table I indicates that addition of some trace

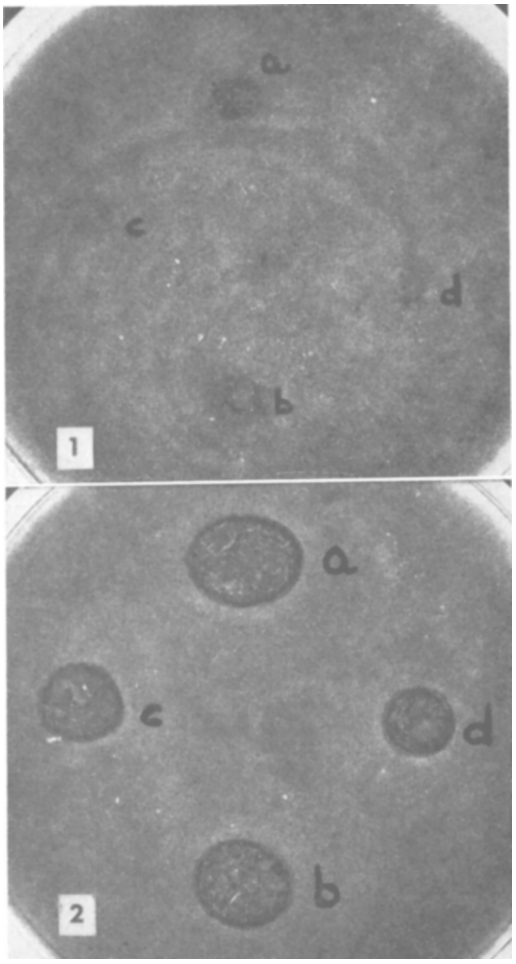

FIG. 1. Lysis of fibrin plates by preparations of Aspergillin O from different cultures. (1) Material from fungi grown in ordinary medium; (2) material from fungi grown in media enriched with trace elements; a, b, c, and d represent yield from .12, .06, .03, .015 ml of original filtrate.

TABLE I. Effects of Trace Minerals and Vitamins on Rate of Growth and Production of Fibrinolytic Material by *Aspergillus oryzae* (B-1273).

Additions	pH Initial	pH 5th day	Growth mycelium, dry wt (mg)	Activity, units/ml filtrate
None	4.3	4.8	56	0
Zn^{++}	"	4.9	286	58
Fe^{++}	4.2	4.7	66	0
Mn^{++}	4.3	"	88	0
Zn^{++}, Fe^{++}	4.2	5.9	787	500
Zn^{++}, Mn^{++}	"	5.4	446	0
Fe^{++}, Mn^{++}	4.3	4.6	74	0
Zn^{++}, Mn^{++}, Fe^{++}	4.2	5.7	753	552
Zn^{++}, Mn^{++}, Fe^{++} Vit. B_1	4.4	5.9	739	552
Zn^{++}, Mn^{++}, Fe^{++} Vit. B_6	4.0	"	842	550
Zn^{++}, Mn^{++}, Fe^{++} Vit. B_1 and B_6	4.2	"	808	554

minerals to the medium had the effect of increasing growth and fibrinolytic activity, while producing a shifting of pH (measured by direct reading on Beckman's) toward the alkaline side. Considerable enhancement of activity was obtained with addition of Zn^{++}; Zn^{++} and Fe^{++}; and Zn^{++}, Fe^{++} and Mn^{++} to the medium. Oddly enough, addition of combinations of Zn^{++} and Mn^{++} or of Fe^{++} and Mn^{++} to the medium did not enhance fibrinolytic activity, although the first combination increased the growth appreciably. The combination of Zn^{++} and Fe^{++} appeared primarily responsible for the increased yield of active substance, which was further enhanced by the addition of Mn^{++}. Addition of vitamins did not appear to be important, since their presence in the medium without trace minerals did not increase growth or activity.

Substitution of NH_4 salts for NO_3 salts to the medium enhanced the growth of the mycelium but did not influence the yield of Aspergillin O. This, in fact, dropped to zero. Thus, although a close relationship usually existed between these 2 variables, abundant growth is not necessarily a criterion for predicting the yield of Aspergillin O by the fungus. It should be noted, however, that the pH was shifted to a very acid value and this change might have destroyed any active material produced. Addition of KNO_2 in lieu of KNO_3 failed to induce growth, production of Aspergillin O or pH changes in the medium. Environmental conditions were relatively insignificant. Exposure of cultures to sunlight inhibited both growth and activity. Culturing at 37°C apparently reduced amount of growth, but did not affect the production of Aspergillin O. Further studies have indicated that *Aspergillus oryzae* (B-1273) seems unable to utilize lactose but will produce material optimally in the presence of either sucrose, maltose, dextrose, galactose or fructose. It has also been observed that the C/N ratio of the medium as used is optimal for growth and production of Aspergillin O. Any increase in carbon (carbohydrate) concentration resulted in decrease in pH and loss of fibrinolytic activity in the filtrates.

Discussion. The results of these studies indicate the possibility of greatly increasing the yield of fibrinolytic material from *Aspergillus oryzae* (B-1273) by addition of trace minerals to the culture medium. Whether the nutritional requirements described here are specific for production of Aspergillin O or are generic requirements for optimal metabolic activity of the fungus is being investigated.

Summary. A method is described for obtaining higher yields of fibrinolytic material (Aspergillin O) from cultures of *Aspergillus oryzae* (B-1273) by addition of trace minerals (zinc, iron and manganese) to the liquid medium, incubated at room temperature and in darkness.

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