FACTORS INFLUENCING THE PRODUCTION OF EXTRACELLULAR ACID PROTEINASE BY *ASPERGILLUS FUMIGATUS*.* FRES IMI No. 215396

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Extracellular acid proteinases of many species of Aspergillus and Penicillium were comparatively studied. *Aspergillus fumigatus* was found to yield the highest enzyme activity. The influence of several cultural conditions viz. incubation period and temperature, pH of the medium, aeration and depth of the medium, on the production of extracellular acid proteinase by *A. fumigatus* was systematically investigated. The maximum concentration of the enzyme was obtained when the culture medium of pH 9.5 was incubated at 31°C for a period of 3 days. The important nutritional factors which affect the production of acid proteinase viz. the effects of different carbohydrates, different forms of organic and inorganic nitrogen, different metallic salts in the culture medium were critically investigated. Based on the results obtained, the composition of a medium for the maximum production of extracellular acid proteinase by *A. fumigatus* has been suggested.

In general proteinases of both molds and bacteria have wide applications in the fields of brewery,[1] textile,[2] dairy,[3] pharmaceutical[4] and leather industries.[5] In leather industry the enzymes are used mainly during the process of dehairing and bating of skins and hides. The chemical process of dehairing skins and hides using lime and sodium sulphide has not only the detrimental effect on the wool/hair, but also creates a major pollution problem in the forms of tannery effluents. The enzymic process, however, saves the quality of the valuable by-product, reduces the effluent as much as possible and avoids the use of toxic and sludge forming chemicals.

The acid proteinases, which have been purified and studied fully are of fungal origin and are extracellular in nature. The extracellular acid proteinases that have been studied so far are : (a) the trypsinogen activating enzymes *(Penicillopepsin)* from *Penicillium janthenellum*,[6] (b) Penicillocarboxypeptidase[7] from the same organism (c) trypsinogenkinase (Takadiastase) from *A. oryzae*,[8] (d) *Candida albicans* acid proteinase,[9] (e) *Paecilomyces varioti* acid proteinase,[10] (f) *Rhizopus chinensis* acid proteinase[11], aspergillopeptidase A from *A. saitoi*,[12] the milk clotting enzymes from *Endothia parasitica*,[13,14] and the mucor rennin, from *Mucor pusillus*[15,16] have also been reported. The acid proteinase from *Penicillium notatum*[17] has been isolated and purified. The acid proteinase from *Trametes sanguinea*[18] has been purified and

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crystallized. It is well known that acid, neutral or alkaline proteinases can be obtained from the culture medium of any strain of mold and the yield of enzyme depends on the various cultural conditions maintained for the growth of mold.

A good deal of work has been done on the factors influencing the production of proteolytic enzyme by molds and actinomycetes. Dion[19] studied the influence of several factors on the production of proteinases in some selected cultures. Maxwell[20] also studied the same in *A. oryzae*. Bose *et al.*[21] studied the influence of various cultural and nutritional conditions on the production of proteinase in *A. parasiticus*. All these investigations have been confined to either neutral or alkaline proteinases. However, very little work has been reported in the literature on the formation of acid proteinases in certain species of molds. In the present paper, a systematic investigation has, therefore, been directed towards elucidating the various nutritional factors and cultural conditions, which influence the production of acid proteinases, especially by *Aspergillus fumigatus*.

Materials and methods

About 40 strains of Aspergillus and Penicillium were obtained from the Centre for Advanced Studies in Botany, University of Madras and other Microbiology Departments. A few wild strains were also isolated from the soil. The chemicals and reagents used in this work are of analytical grade. The Bactopeptone and Agar powder were supplied by Difo Laboratories, U. S. A. The egg albumin flakes from E. Merck (Germany) and the Folin-Phenol reagent from Centron, Bombay.

Preparation of crude extract

The mold along with the culture medium of each flask was triturated in a mortar with acid washed sand, centrifuged and the supernatant was collected. The residue was triturated again with distilled water, centrifuged and the supernatant was mixed with the earlier extract. The total liquid collected was made upto a known volume and analysed for proteolytic activity.

Enzyme assay and enzyme unit

For estimating the proteolytic activity of the enzyme quantitatively, Anson's Colorimetric method[22] of estimating the liberated tyrosine during proteolysis as adopted by Yeshodha *et al.*,[23] was followed except that 2 ml aliquot of enzyme extract was added to 5 ml of 2.5 per cent egg albumin solution which was adjusted to pH 6.0, pH 2.5 or pH 2.1 as the case may be, and the mixture was allowed to be digested at 45°C for 30 miautes. After this period, the digestion mixture was analysed for the extent of hydrolysis by the usual method and the enzyme unit was calculated as the amount of enzyme that liberated one microgram of tyrosine from 2.5 per cent egg albumin under the normal experimental conditions.

Results

Effect of incubation period

In order to study and compare the influence of incubation period on the production of the enzyme of the mold strains, a number of conical flasks (100 ml) containing 15 ml of the basal liquid medium (4 parts glucose, 4 parts peptone and 100 parts water) which had been sterilized and cooled earlier, were inoculated with spore suspensions of 5 days old culture of the mold and incubated at room temperature (30-32°C) for different periods.

Everyday the mold along with the culture medium of each flask of each series was triturated in a mortar with acid washed sand in the same manner as described earlier.

The crude enzyme extract collected was analysed for proteolytic activity. The results obtained are presented in Fig. 1

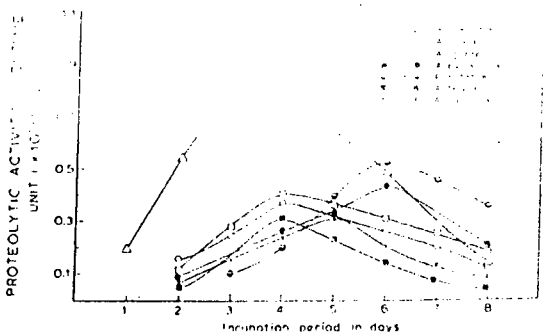

Fig. 1. Incubation period vs. proteolytic activity of the molds.

Of the seven mold strains compared for the proteolytic activity at pH 6.0, *A. fumigatus* was found to show the highest activity after 3 days of incubation. In order to find out, whether any other mold could show higher enzymic activity, if the estimation of enzymic activity has been carried out at highly acidic conditions, the experiment was repeated with *A. fumigatus* and *P. notatum*. The proteolytic activity for both the molds was estimated, by maintaining the pH of the substrate as 2.5. The results obtained are presented in Fig. 2.

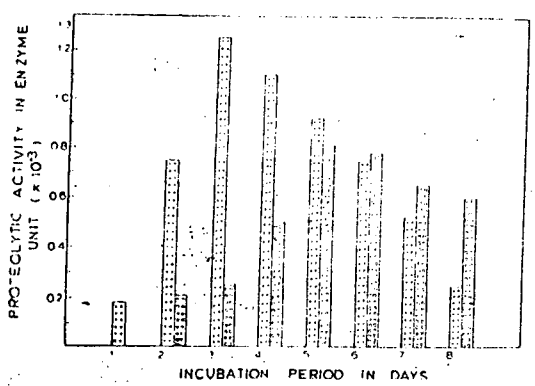

Fig. 2. Effect of incubation period on the production of acid-proteinase by *A. fumigatus* (xx) and *P. notatum* (°°)

Determination of extracellular proteinase elaborated by A. fumigatus

In order to estimate the extracellular acid proteinase produced by *A. fumigatus*, the mat and the culture medium were separated after 3 days of incubation, by passing through the Buchner funnel. The mat was triturated with acid washed sand and the extract was collected by centrifugation and made upto a known volume with distilled water. Both the enzyme extracts were analysed for the proteolytic activity separately. It was however observed that the maximum activity was found to be present in the culture medium alone and very insignificant activity was obtained in the mat formed by the mold. Hence, in all our future work, only the culture medium of *A. fumigatus* after 3 days of incubation was used as the crude enzyme extract.

Determination of the optimum pH and temperature for the proteolytic activity of the crude enzyme extract

In order to find out the effect of various pH values on the enzyme activity, the egg albumin solution (2.5%) was adjusted to different pH values with acid or alkali as the case may be. 5 ml aliquots of egg albumin

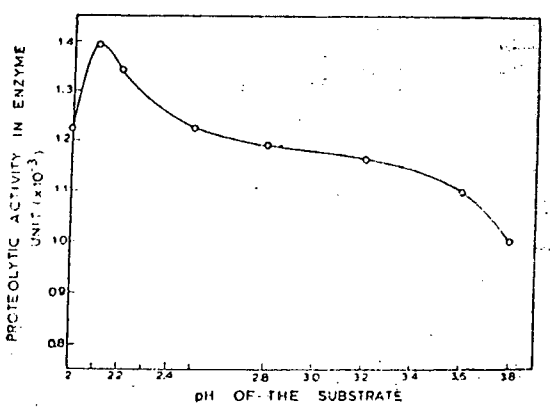

Fig. 3. Effect of pH on the crude enzyme activity of *A. fumigatus*.

solutions were mixed with 2 ml of enzyme extract in the 50 ml conical flasks and incubated at 45°C for 30 minutes. The proteolytic activity in each flask was determined as before. The results are shown in Fig. 3.

For the determination of the optimum temperature for enzyme activity, 5 ml portions of egg albumin solution, which was adjusted to pH 2.1 were mixed with 2 ml portions of enzyme extract in 50-ml conical flasks and incubated at different temperatures for 30 minutes. After the period the proteolytic activity in each flask was determined as before and the results are presented in Fig. 4.

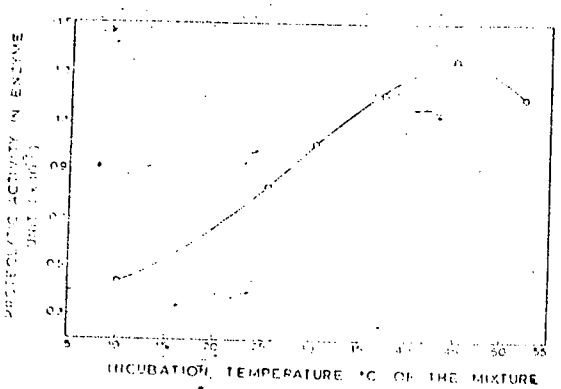

Fig. 4. Effect of temperature on the crude enzyme activity of *A. fumigatus*.

Effect of pH of the medium

To determine the influence of pH of the culture medium on the production of acid proteinases, 15 ml portions of sterile medium having different pH values were taken in several conical flasks (100 ml) and inoculated with the spore suspension of 5 days old culture slant of *A. fumigatus*. The flasks were incubated at room temperature (30-32°C) for 3 days. The culture filtrate was collected from each flask and analysed for the proteolytic activity. The results are presented graphically in Fig. 5.

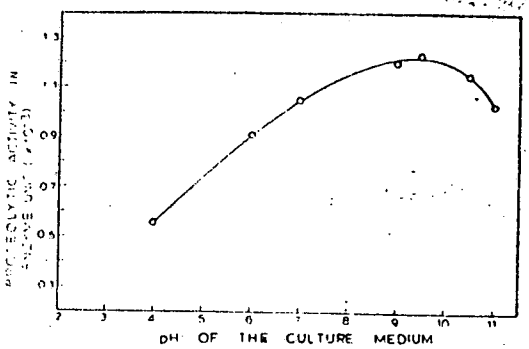

Fig. 5. pH of the culture medium vs. proteolytic activity of *A. fumigatus*.

Effect of temperature of incubation

15 ml portions of sterile culture medium which had been adjusted to pH 9.5 was taken in several sterile conical flasks, inoculated with *A. fumigatus* and the flasks were incubated at different temperatures for 3 days. At the end of the period, the proteolytic activity of the culture filtrate in each flask was determined. The temperature of incubation *vs* proteinase activity curve is presented in Fig. 6.

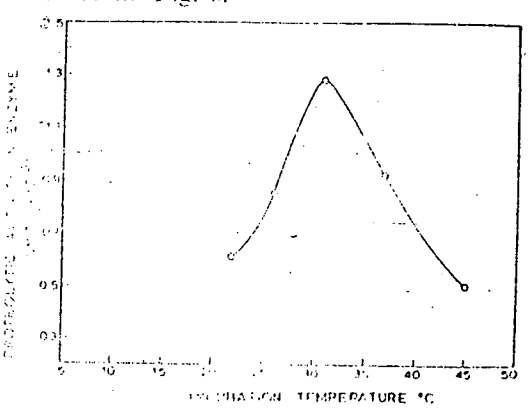

Fig. 6 Incubation temperature vs. proteolytic activity of *A. fumigatus*.

Effect of aeration and depth of the culture medium

To determine the influence of the depth of culture medium on the production of extra-

cellular acid proteinases, varying volumes of the sterile medium (pH 9.5) were put in several sterile conical flasks (250 ml) and were inoculated with the five days old culture of *A. fumigatus*. After the incubation for 3 days at 31°C, the proteolytic activity of the culture filtrate in each flask was determined. The results are presented in Table 1. 20 ml portion of culture medium in 250 ml conical flask was found to be sufficient for the maximum production of the proteinase.

In order to study the effects of aeration on the production of the proteinase, 20 ml portions of the sterile culture medium of pH 9.5 were taken in 250 ml conical flasks and inoculated as before. One flask was tightly stoppered with cotton plug, another was evacuated and sealed and the third one was connected with one outlet of Seitz-filter. The outlet of Seitz-filter was connected with a water suction pump, for the continuous bubbling of the sterile air. The results obtained are presented in Table 1.

TABLE 1

Effect of aeration and depth of culture medium on the production of extracellular acid proteinase by *A. fumigatus*

Volume of the medium (ml)	*Proteolytic activity (enzyme unit)*
10	1100
15	1120
20	1356
30	860
45	600
60	375
Normal	1338
Aerated	620
Vacuum	150

Effect of different carbohydrates in the medium

In this case, the composition of the medium used was the same as before, except that glucose was replaced by other sugars or starch. As the solution of the starch would be affected by heat sterilization, 4 g. of soluble starch powder and 100 ml of 4% peptone solution were separately autoclaved and mixed under aseptic conditions. The results obtained are given in Table 2.

TABLE 2

Effect of different carbohydrates in the culture medium on the production of acid proteinase by *A. fumigatus*

Carbohydrate used (percent)		*Proteolytic activity (enzyme unit)*
Sucrose	4.0	800
Glucose	4.0	1336
Soluble starch	4.0	416
Maltose	4.0	1410
Lactose	4.0	100
Fructose	4.0	1120
Glucose + Fructose	2.0 + 2.0	1185

Effect of different nitrogen sources in the medium

In order to study the influence of different forms of organic nitrogen on the acid proteinase production by *A. fumigatus*, a number of culture media prepared replacing peptone with either a protein or with an amino acid were used. To determine the effect of inorganic source of nitrogen, instead of using a protein or an amino acid, certain nitrogen containing inorganic salts were used. The concentration of the amino acid or an inorganic salt in the medium was selected as to provide approximately the same level of nitrogen as would be derived from 1% concentration of a protein. As the solution of casein would be affected by heat steriliza-

tion, casein powder was separately autoclaved and then mixed with the sterile sugar solution under aseptic condition. Since egg albumin would be denatured by autoclaving and hence would be insoluble, the sterile medium with this protein was prepared by washing with 70% ethyl alcohol and then dissolving in sterile sugar solution. Aliquots of the medium thus prepared were tested for sterility and were found to be microbiologically sterile. The ammonium salt solution and the sugar solution were autoclaved separately and then mixed under aseptic condition. The results obtained are presented in Table 3.

TABLE 3

Effect of organic and inorganic sources of nitrogen in the medium on the production of acid proteinase by *A. fumigatus*.

Nitrogen source and concentration (%)		*Proteolytic activity (enzyme unit)*
Proteins :		
Egg albumin	(2 . 0)	685
Soluble casein	(2 . 0)	468
Peptone	(2 . 0)	1005
Gelatin	(2 . 0)	102
Amino acids :		
Glycine	(0 . 85)	322
Valine	(1 . 33)	358
Leucine	(1 . 49)	727
Methionine	(1 . 7)	714
Arginine*	(0 . 5)	179
Histidine*	(0 . 59)	428
Lysine	(0 . 84)	286
Aspartic acid	(1 . 5)	179
Cysteine*	(1 . 0)	No growth
Tryptophan	(1 . 14)	506
Inorganic nitrogen sources :		
Ammonium chloride	(0 . 6)	325
Ammonium sulphate	(0 . 76)	286
Ammonium nitrate	(0 . 92)	215
Sodium nitrate	(0 . 96)	452
Urea	(0 . 4)	No growth

* as a mono hydrochloride.

Effects of minerals in the medium

Lastly, the influence of a number of minerals in the medium on the production of acid proteinases by *A. fumigatus* was studied. The medium used was composed of 4% peptone, 4% glucose and suitable concentration of a metallic salt in 100% distilled water. The results obtained are presented in Table 4.

TABLE 4

Effect of metallic salts in the medium on the production of acid proteinase by *A. fumigatus*

Metallic salt concentration (%)		*Proteolytic activity (Enzyme unit)*
Basal medium (without metallic salt)		1158
A Calcium sulphate	(0 . 01)	1262
B Potassium dihydrogen-phosphate	(0 . 01)	1208
Potassium chloride	(0 . 05)	1180
C Sodium sulphate	(0 . 05)	1290
D Magnesium sulphate	(0 . 05)	1210
Nickel chloride	(0 . 05)	1050
E Manganese sulphate	(0 . 005)	1275
F Zinc sulphate	(0 . 005)	1175
A + B + C + D + E + F		1450

Discussion

From Fig. 1, it is quite clear that different molds would need different periods of incubation, for the maximum production of the acid proteinase. The proteinase production by *A. fumigatus* was maximum after three days of incubation, whereas *A. Japonicus* showed the maximum activity after an incubation period of five days. *A. oryzae*, *A. flavus* and *A. tereus* produced their maximum production of acid proteinase in about four days. *A. parasiticus* and *P. notatum* showed the maximum after six days. It can be seen from Fig. 2 that *A. fumigatus* produced

maximum acid proteinase after three days of incubation, compared to that produced by *P. notatum* when enzyme activity was estimated at pH 2.5.

A. fumigatus was, therefore, selected for all subsequent studies. It was also observed that the maximum production of acid proteinase by *A. fumigatus* was found in the culture medium as extracellular enzyme. From Figs. 3 and 4, it is evident that the maximum activity of the crude enzyme extract was found when the enzyme estimation was carried out at pH 2.1 and 45°C temperature for a period 30 minutes.

Fig. 5 shows that the formation of acid proteinase by *A. fumigatus* was possible within the pH range of 4 to 11 – the maximum pH for the production of acid proteinase being in the neighbourhood of 9.5. It was therefore essential to adjust the pH of the culture medium at about 9.5 for the maximum production of proteinases. In the majority of the experiments carried out in the present investigation, the pH of the media after the growth of *A. fumigatus* for 3 days was found to be within the range 4.5 to 5.0.

From Fig. 6, it appears that the acid proteinase formation by *A. fumigatus* after an incubation period of three days was maximum at 31°C.

Table 1 shows that there appears to be a lot of variations in the production of acid proteinase, when the volume of the culture medium was altered. There was a gradual increase of proteolytic activity, when the volume of the culture medium was maintained from 10-20 ml. However, the activity in the flasks having 30 ml medium was found to be lower than those obtained in 10 to 20 ml. This decrease in enzyme activity might be due to the use of a thick layer of culture medium with less surface area. From the experiment on aeration it can be seen that *A. fumigatus* needed a normal amount of oxygen for its growth and maximum production of acid proteinase. Excess sterile oxygen when bubbled through the culture medium, considerable reduction in proteinase production was observed. The flask which was evacuated and sealed, might have some dissolved oxygen in the medium.

Among the different carbohydrates (Table 2) which were used in the culture media, maltose gave the highest and lactose the lowest activity. It appears that these carbohydrates when arranged in the descending order on the production of extracellular acid proteinases were: maltose, glucose, fructose, sucrose, starch and lactose. It can also be observed from the Table that *A. fumigatus* can assimilate glucose and fructose individually in a much better manner than sucrose, which on hydrolysis produces glucose and fructose.

It is evident from Table 3 that the production of the acid proteinase by inorganic nitrogen was very much less than that by organic nitrogen in the medium. The presence of urea in the medium was found to inhibit the growth of the mold. Among the different forms of organic nitrogen sources, the peptone was the best one for the maximum production of acid proteinase and the gelatin was the least. Among the several amino acids, the cysteine was found to inhibit the growth of the mold completely. In case of amino acids like arginine, aspartic acid and lysine the production of the enzyme was very poor. The proteinase production was observed moderately in certain amino acids viz. glycine, valine leucine, methionine, histidine and tryptophan.

Table 4 shows that most of the metallic salts tested had the beneficial influence on the formation of acid proteinase. The

addition of 0.1% calcium sulphate or potassium dihydrogen phosphate in the medium was found to increase the proteinase production. 0.05% of sodium sulphate and potassium chloride were beneficial for the production of the enzyme. Nickel chloride in the medium reduces the enzyme activity to an extent. Among all the salts tested sodium sulphate was found to give the highest proteolytic activity.

The final medium selected for the maximum production of acid proteinase by *A. fumigatus* therefore contained 4 g. of maltose, 4 g. of peptone, 0.1 g. of calcium sulphate, 0.1 g. of potassium dihydrogen phosphate, 0.05 g. of of magnesium sulphate and 0.05 g. of sodium sulphate, 0.005 g. each of zinc sulphate and manganese sulphate/100 ml distilled water. The presence of all salts in that particular concentration was found to enhance the proteolytic activity to a much greater extent, than any of these salts.

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