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STUDIES ON PROTEOLYTIC ENZYME
Part I. Characteristics of Protease Synthesis by *Penicillium expansum*

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The effect of environmental parameters on the growth and the protease synthesis by *Penicillium expansum* was investigated. The maximum production was achieved at 35°C at initial pH 6.0 after 48 hours incubation when 1 % Millet husk was used as a carbon and energy source. The addition of carbon and nitrogen sources to mineral Millet husk medium tested, gelatin, peptone, and sodium nitrate promoted protease synthesis whereas glucose inhibited protease formation.

Key words: Penicillium expansum, Millet husk, Protease enzyme.

INTRODUCTION

Studies of the regulation of enzyme biosynthesis in bacteria and other microorganisms will lead to economic production of many enzymes, some of which are employed for industrial purposes. Enzymes used in bulk are often impure and contain only a small percentage of the required enzyme. Such enzymes are less expenses, because of ready availability and small labour cost involved in their purification [1].

The present study was carried out to explore the possibilities to use agricultural waste (Millet husk) as a carbon and energy source for producting a protease enzyme by *Penicillium expansum* under submerged culture. The production of extracellular protease is a common feature among fungi, while microbial proteases have been isolated from *A.oryzae* [2,3], *A.saitoi* [4], *P.variol* [5], *L.mexicana* [6], *Butyl bacteria* [7], *and B.subtilis* [8]. Although the literature dealing with proteases of fungal origin is abundant, there is no work on protease enzymes of the genus *Penicillium expansum.* The fungus has been reported to produce amylase [9], pectinase, protopectinase [10], β-glucosidase [11] and invertase [12].

MATERIALS AND METHODS

Strain. Penicillium expansum strain CMI 39761 was used which was obtained from the Department of Microbiology, University of Strathclyde Glasgow. The stock culture was maintained at 27°C on agar stants, containing 2 % bactoagar, 1 % peptone and 2 % dextrose slopes.

Basal medium. Basal medium was used for the growth of *Penicillium expansum* as reported by Burrel *et al* [13] without altering chemical composition, containing following reagents (per 1 litre of solution): glucose, 10g; $(NH_4)_2SO_4$, 2.5g; fumaric acid, 2.0g; $KH_2PO_4.2H_2O$, 1.0g; $MgSO_4.7H_2O$, 0.5g; $(NH_4)Fe(SO_4)_2.12H_2O$, 0.2mg; $ZnSO_4.7H_2O$, 0.2mg; $MnSO_4.H_2O$, 0.1mg and thiamine hydrochloride, 0.1mg. The pH of the medium was adjusted to 6.0.

Inoculum. A spore suspension of the fungus was prepared by adding sterile distilled water to stock culture to get 50 x 10^6 spores/ml.

Cultivation condition. Fifty ml of basal medium with and without glucose supplemented with 0.5g of Millet husk fine powder (40 mesh) was taken in 250 ml conical flask plugged with cotton wool and autoclaved at $15 lb/in^2$ for 20 minutes. These sterilized media cooled at room temperature were inoculated with 0.5 ml of the inoculum containing 50 x 10^6 spores/ml. These flasks were incubated in an incubator (Gallenkamp) at 27°C. The flasks were shaken twice a day. The culture broth was separated from mycelium after an interval of 24 hours incubation period by filtration through What man No:1 filter paper.

Determination of protein. Protein content of the broth was determined by the method of Lowry *et al* [14], with bovine serum albumin as a standard.

Determination of protease activity. Protease activity in the culture broth was determined by the method of Penner and Aston [15] in combination with that of lowery *et al* [14].

To 1.0 ml of culuture broth, 3.0 ml of phosphate buffer (pH 7.6) and 1.0 ml of casein solution (0.1 % pH7.6) were added and incubated at 35°C for one hour.

To 2.0 ml of the above reaction mixture, 2.0 ml of 15 % trichloroacetic acid was added and the precipitate was removed by filtration through Whatman No: 1 filter paper.

In an 1.0 ml aliquot of the filtrate, 4.0 ml of 0.5 N sodium hydroxide and 1.0 ml of Folin phenol reagent (1 : 1) were added. The total volume was then made up to 10.0 ml by adding 4.0 ml double distilled water. A blue colour developed was determined after 5 minutes at 625 mu.

One unit of the protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine under the standard assay conditions described above.

RESULTS AND DISCUSSION

Many factors that affect the synthesis and release of extra-cellular enzymes into the medium have been discussed in detail by Davies [16], and Kundu and Das [17]. There is no definite relationship between time of growth and amount of enzymes released. The yield of enzyme varies with the organism employed, the species of enzyme as well as with the composition of the medium and conditions of growth. Most enzymes, however, were produced during cell growth with small variation. The yield of enzyme usually reached to the maximum when the growth ceased. In this study the production of protease reached its highest level after 48 hours. Fig. 1 shows that further incubation decreased the production of protease rapidly. Thus, in subsequent experiments the cultures were incubated for 48 hours for enzyme production.

Fig. 2 shows the effect of temperature on protease synthesis by *Penicillium expansum.* The incubation was made at different temperatures from 25 – 50°C for 96 hours. The protease synthesis was maximum at 35°C and it dropped abruptly.

The yield of protease by *Penicillium expansum* strain CMI 39761 was dependent on pH of the medium. pH was adjusted to 4.0 – 5.5 with acetate buffer and to 6.0 – 8.0 with phosphate buffer. The maximum synthesis of protease was observed at pH value of about 6.0 (Fig. 3).

The effect of Millet husk fine powder (40 mesh) and glucose in the concentration ranging from 0.5 to 2.5g per 100 ml as a carbon source was shown in Fig. 4. However, when glucose was sterilized separately and added aseptically before inoculation, the synthesis of enzyme reached the maximum at 1.5 % glucose and then it declined. On the other hand, in case of Millet husk the synthesis of enzyme was found to increase by increasing its concentrations. The utilization of glucose as a carbon source gives rise to good growth with decrease in protease formation. These observations were consistant with the observations reported for the enzyme synthesis by other organism [18,3]. The carbon source which appeared to be poorly utilized energy source for growth, synthesized much larger amount of enzyme than the carbon source used for energy purpose [19]. Such observations were also found

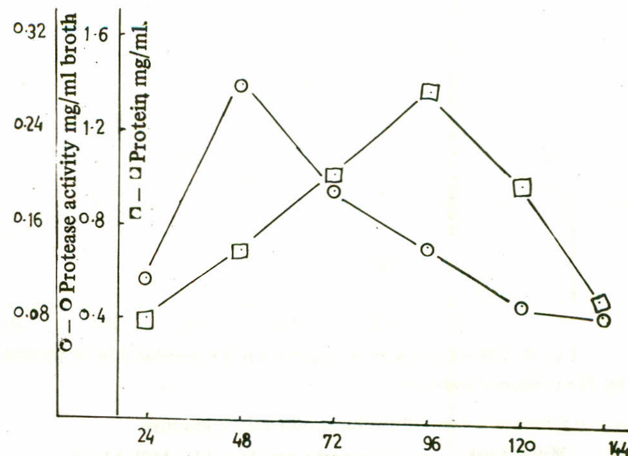

Fig. 1. Effect of time period on the rate of protease and protein synthesis by *Penicillium expansum* strain CMI 39761.

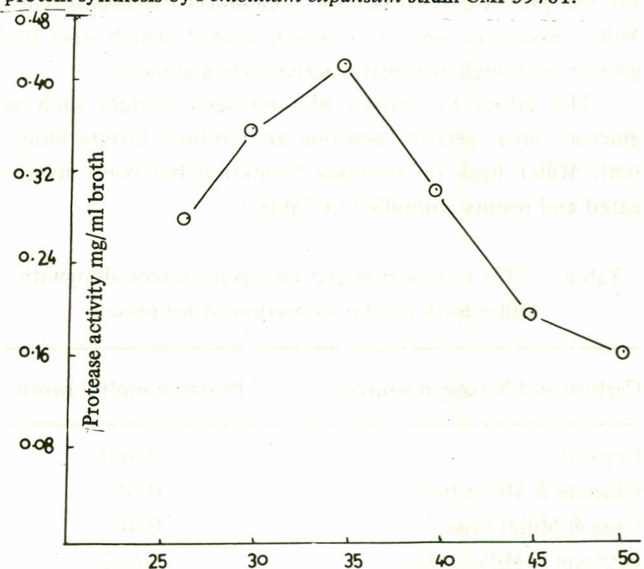

Fig. 2. Effect of temperature on the rate of protease production by *Penicillium expansum* CMI 39761.

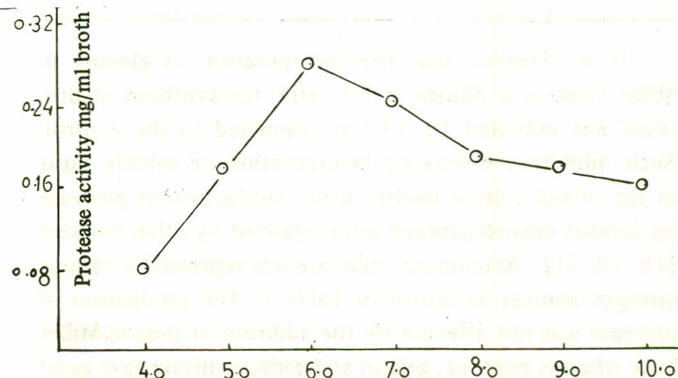

Fig. 3. Effect of pH on the rate of protease production by *Penicillium expansum* CMI 39761.

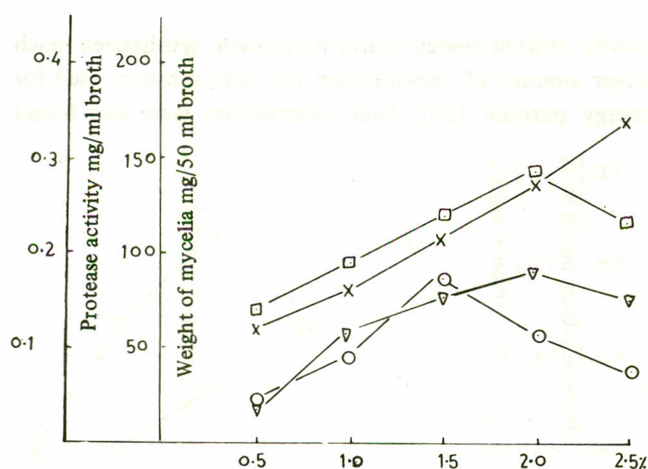

Fig. 4. Effect of carbon sources on the production of protease by *Penicillium expansum.*

o – o Glucose x – x mycelia produced by glucose.

□ – □ Millet husk v – v mucelia produced by Millet husk.

for the protease synthesis by *Penicillium expansum* when Millet husk was used as a carbon source, which gave less growth with high enzymatic activity than glucose.

The effect of carbon and nitrogen sources such as glucose, urea, gelatin, peptone and sodium nitrate alongwith Millet husk on protease formation has been investigated and results compilled in Table 1.

Table 1. Effect of carbon and nitrogen sources alongwith Millet husk on the formation of protease.

Carbon and Nitrogen sources	Protease mg/ml broth
Control	0.464
Glucose & Millet husk	0.352
Urea & Millet husk	0.462
Peptone & Millet husk	0.632
Gelatin & Millet husk	0.528
Sodium nitrate & Millet husk	0.472

It is observed that the incorporation of glucose in Millet husk as a diluent in 1:1 ratio, the synthesis of protease was inhibited by 15% as compared to the control. Such inhibitory effects by incorporation of soluble sugar in the mixed culture media, in the produciton of protease by various microorganisms were reported by other workers [18, 20, 21]. Ammonium sulphate was replaced by various nitrogen sources as shown in Table 1. The production of protease was not affected by the addition of urea in Millet husk whereas peptone, gelatin and sodium nitrate gave good response to stimulated 20%, 15% and 7.5% enzyme production .respectively. The stimulatory effect of peptone, gelatin and sodium nitrate in protease production was shown by other werkers [22, 23, 24].

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