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Research Article

Comparative Production of Protease by seed borne *Aspergillus niger, A. flavusand A. terreus.*

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ABSTRACT
The synthesis ability of the *Aspergillus niger, A. flavus, and A. terreus.* In protease enzyme production which are cultured on protease production medium and pigeon pea seed cake medium. These fungi was able to produce in maximum amount in eight day old culture in protease production medium and there after decreased, pigeon pea seed cake medium the synthesis of enzyme increased up to ten day and there after it remained constant. The protease was active at P^H 5.5 and 40 ^{0}C. temperature.

Key words: Seed borne fungi**,** Protease, Pigeon pea seed cake

INTRODUCTION

Seed-borne plant pathogenic fungi cause losses in terms of seed quality and quantity in all pulse crops. These fungi also reduce the percentage of germination and storability of the seed. They are responsible for seed rot, seedling blight, root/stem rot diseases [1,2]. Seed deterioration due to various fungi is common feature that affects the germination and seedling emergence during the course of growth.

Thus seed deterioration due to these seed borne fungi is attributed to ability of production of hydrolytic enzyme. In pulses, the seed borne fungi produces protease which hydrolyses the stored protein in the seed [3]. Proteolytic enzymes are included in a subclassof the enzymes hydrolyses. These enzymes cause breakdown of proteins into smaller peptidesand amino acids by catalyzing the breakdown of peptide bonds.Proteolytic enzymes are the most important industrial enzymes, representing worldwide sales ofabout 60% of the total enzyme market.

They are high temperature resistant withhigh specific activities and superior physical andchemical characteristics which seem to be good forfuture biotechnological applications[4]

The deterioration of Pigeon pea seeds rich in protein was correlated with extracellular production of protease by seed borne fungi. *Aspergillus niger, A. flavus, A. tenuis A. fumigatus,A.alternata A. terreus* areassociated with pigeon pea are studied for protease production. This paper presented a detailed study

of protease produced by *Aspergillus niger, A. flavus,and A. terreus.*

MATERIALS AND METHODS

Isolation of seed borne fungi:

Pigeon pea Seeds were obtained from the Pulses Research Station (Marathwada Agricultural University, Badnapur, Jalna, Maharashtra), local farmers, warehouses, local dealers etc. The untreated seeds were stored at 22°C in cloth bags and used whenever needed. The seed-borne fungi of Pigeon pea were detected by agar plate and blotter test methods as recommended by International Seed Testing Association[5,6] (After isolation, pure cultures of individual fungi were obtained, each fungus was grown separately on PDA/GNA medium and its growth and sporulation were recorded. Comparing morphology of hyphae, conidiophores and conidia with standard manuals generic and specific names were determined.Cultures were incubated for 7-10 days at 27±2ºC in the laboratory and variation in temperature was recorded.

Seed meal medium

20gms of seeds were crushed to a homogenous paste in a little quantity of distilled water and then it was made up to 1000ml. This suspension was directly used as a medium for studies.

Protease production medium

Czapek medium-broth was added with casein hydrolysate instead of sucrose as carbon sources. The pH was adjusted to 6.0 by adding dilute HCl.

Preparation of enzymes

Czapek medium supplemented with casein hydrolysate and seed meal medium was prepared from respective seed as described as liquid medium was used for production of protease.

Fifty ml of the medium was poured in 250 ml flasks and was inoculated with 0.5 ml of spore suspension of the fungi. The flasks were incubated for 27±2°C for 10 days. Flasks were drawn after regular time interval and the contents were filtered through Whatman No. 1.

The culture filtrate was collected, centrifuged at 2000 rpm for 20 minutes to remove spore and suspended matter. The filtrate was dialyzed against running tap water for 24 hours. The preparation was used as enzyme sources during the study. Few drops of toluene was added to avoid contamination. The preparation was kept at 2-4°C in refrigerator.This Enzymes preparation medium was used for assessing synthesis of protease by seed borne *Aspergillus niger, A. flavus,and A. terreus.*

Measurement of Protease activity synthesized by seed fungi

Protease was determined using casein as substrate. Casein substrate was prepared by making 1% Casein in 0.1M phosphate buffer at pH 7 (as casein is sparing soluble in water. it is dissolved in a minimal quantity of 0.1 NaOH and the volume was raised to 100 ml with the buffer. the pH was adjust).

Ten ml of casein solution was taken into a test tube. 1ml of 0.1 M phosphate buffer, pH 7 and 5 ml of enzyme preparation was added. The contents were mixed thoroughly and the mixture was incubated at 30^0 C in a water bath. Boiled enzyme at zero time served as control. Aliquots of 1 ml were withdrawn at various intervals and the reaction was stopped by the addition of ninhydrin reagent. The amino acid content of the solutionwas estimated from a standard curve prepared from aspartic acid or glutamic acid standards. The enzyme activity was determined as the amount of amino acids released/unit time/g of protein. One unit of enzyme was defined as the amino acid released / unit time / gm of protein.

RESULTS

A series of experiments were undertaken to assess the ability of *Aspergillus niger*, *A. flavus* and *A. terreus* to degrade protein present in the Pigeon pea seeds. *Aspergillus niger, A. flavus* and *A. terreus* were grown on Pigeonpea seed cake medium as well as on protease production medium. 8th day old culture filtrate was used as crude enzyme source.

Protease enzyme activity was assayed following method given in material and methods. *Aspergillus niger*, *A. flavus* and *A. terreus* synthesized protease in both the media. The synthesis increased with increase in time of incubation in both the media. However the amount of enzymes varied in both the media, maximum enzymes were secreted in Pigeon pea

seed cake medium followed by protease production medium (Table No.1) The maximum amount of enzyme (0.15 U/ml, 0.30 U/ml and 0.16 U/ml) was secreted in 8 days in protease medium and thereafter decreased. In Pigeonpea seed cake medium the synthesis of enzymes (0.20 U/ml, 0.33 U/ml and 0.12 U/ml) increased up to 10 days and thereafter it remained constant.

The effect of temperature and pH on protease synthesis revealed that 40°C and 5.5 was optimum temperature (Table No.2) and pH (Table 45) respectively.

Table No. 1 Production of Protease by *Aspergillus niger, A. flavus and A. terreus* on protease production medium and seed cake medium.

Age of culture filtrate	Protease activity (U/ml)					
	Protease production medium			Seed cake production medium		
	A.niger	A. *flavus*	A.terreus	A.niger	A .*flavus*	A.terreus
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.02	0.00	0.01	0.01	0.00
4	0.01	0.04	0.05	0.02	0.05	0.03
5	0.02	0.09	0.09	0.05	0.09	0.07
6	0.05	0.15	0.11	0.07	0.15	0.10
7	0.09	0.21	0.13	0.11	0.20	0.12
8	0.15	0.30	0.16	0.15	0.27	0.15
9	0.11	0.22	0.10	0.17	0.31	0.14
10	0.07	0.20	0.09	0.20	0.33	0.12

Table No.2 Effect of pH on production of Protease by *Aspergillus niger*, *A. flavus* and *A. terreus*on Pigeonpea seed cake medium

pH	Protease activity (U/ml)		
	A.niger	*A. flavus*	*A.terreus*
3.5	0.07	0.15	0.07
4.0	0.09	0.19	0.10
4.5	0.10	0.26	0.13
5.0	0.15	0.32	0.15
5.5	0.17	0.38	0.18
6.0	0.13	0.37	0.16
6.5	0.11	0.30	0.11
7.0	0.10	0.22	0.09
7.5	0.07	0.15	0.05
8.0	0.05	0.07	0.01
8.5	0.03	0.01	0.00
9.0	0.02	0.00	0.00
9.5	0.00	0.00	0.00
10.0	0.00	0.00	0.00

Table No.3 Effect of temperature on production of Protease by *Aspergillus niger, A. flavus* and *A. terreus* on Pigeonpea seed cake medium

Temp. (°C)	Protease activity (U/ml)		
	A.niger	A. *flavus*	A.terreus
20	0.10	0.17	0.07
25	0.18	0.23	0.09
30	0.21	0.27	0.11
35	0.29	0.35	0.14
40	0.31	0.37	0.20
45	0.22	0.21	0.12
50	0.10	0.15	0.06
55	0.03	0.00	0.00
60	0.00	0.00	0.00
65	0.00	0.00	0.00

This indicates the proteolytic abilities of the dominant fungi associated with the respective seeds. The study of the dominant fungi for synthesis of proteases revealed that they were ardent producers of proteases. So the deterioration of proteins can be attributed to the proteolytic ability of fungi. Similarly the decrease in total protein content indicates their utilization as substrate by these fungi. In this present work it is clear that *Aspergillus niger, A. flavus,and A. terreus*. Produces proteasenecessary to degrade proteins which is stored in pigeon pea seeds.

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