PRODUCTION OF PROTEOLYTIC ENZYME BY FUNGI ASSOCIATED WITH SOYBEAN SEEDS

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Abstract: Three dominant fungi viz. *Aspergillus niger, Rhizopus stolonifer* and *Fusarium oxysporum* were studied for protease synthesis. All these three fungi synthesized proteases in Czapek medium supplemented with casein hydrolysate and soybean seed meal. The enzymes synthesized in soybean seed meal medium were higher over casein hydrolysate supplemented medium. The enzyme production was affected by the pH and temperature. The optimum pH was found to be in the range of 5.5 and 40 °C was optimum temperature. The enzymes were partially purified and the molecular weight was determined by SDS PAGE. The molecular weight of the proteases synthesized by these fungi was in the renge of 63-66 kd.

Keywords: **Seed borne fungi, protease, *Aspergillus niger, Rhizopus stolinifer, Fusarium oxysporum***.

Introduction: The seed of soybean are rich in proteins and oil. The study on seed-borne fungi of *Glycine max* revealed association of 17 different species of fungi. This includes *Alternaria tenius*, *Aspergillus flavus*, *A. niger*, *A. tamarii*, *Chaetomium brassitiense*, *C. erectum*, *Cladosporium* sp., *Colletotrichum turncatum*, *Curvularia lunata*, *Fusarium moniliforme*, *F. semitectum*, *Helminthosporium* sp., *Macrophomina phaseolina*, *Monilia* sp., *Penicillium cyclopium*, *Phoma* sp., *Verticillium* sp. [1].

The storage of soybean (Glycine max) seed under tropical conditions can lead to deterioration that affects products taste and colour. In an earlier study designed to assess the effect of storage condition on seeds deterioration showed seed deterioration by fungi [2]. This deterioration of seed was attributed to the ability of fungi to produce proteases [3].

Fungal proteases have attracted the attention of researchers as fungi can grow on low cost substrates and secrete large quantities of enzymes into culture medium [4].

Different species of fungi have been studied for the synthesis of proteases, these includes genera of *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Humicola*, *Thermoascus*, *Thermomyces* etc. The physical and chemical parameters of protease from fungi have been widely reviewed [5].

The present work gives a comparative account of protease synthesis by *Aspergillus niger, Rhizopus stolinifer, Fusarium oxysporum.*

Material and Methods:

Isolation of fungi Fungi were isolated: from Glycine max: Linn seed Cv. JS 335, Cv. Prasad, Cv. Puja

Assessment of seed mycoflora: Three methods were used for isolation of externally and internally seed-borne fungi. Standard blotter test, Agar plating, Seed washates method [6].

Media: The following media were used in this study. i) Potato dextrose agar (PDA), ii) Glucose nitrate agar (GNA)iii) Czapek Dox's Agar (CZDA) and iv) Seed meal medium[7].

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.

Inoculum: Spore suspension was prepared by adding 10ml sterile water to a 8-day-old PDA slant culture and 5ml of this was used as inoculum in all experiments unless and otherwise stated.

Preparation of enzymes: Enzymes preparation medium was used for assessing synthesis of protease by seed fungi. Czapek medium supplemented with Seed meal/ casein hydrolysate instead of sucrose as carbon source was used for protease production. The seed meal medium was prepared from respective seed as described as liquid medium was used for production of protease. The method described earlier[3] was followed.

Measurement of Protease activity synthesized by seed fungi: 1% Casein dissolved in 0.1M phosphate buffer at pH 7 following method described earlier[3].

SDS - PAGE: SDS - PAGE was performed to check the purity and to determine molecular weight of enzymes by using standard protein marker. The protein bands were visualized on gel by staining it with coomassie brilliant blue.

Results: Proteolytic enzymes are synthesized by many species of bacteria and fungi, but the search for new micro organisms producing enzymes of higher specific activity and efficiency continues.

A series of experiments were undertaken to assess the ability of *Aspergillus niger*, *Rhizopus stolonifer* and *Fusarium moniliforme* to degrade protein present in the soybean seed. *Aspergillus niger*, *Rhizopus stolonifer* and *Fusarium moniliforme* was grown on soybean 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 general methods.

Table 1. Production of Protease on soybean meal medium

Age of culture filtrate (Days)	Protease activity (U/ml)		
	An	Rs	Fm
1	0.00	0.00	0.00
2	0.00	0.00	0.00
3	0.00	0.02	0.00
4	0.01	0.06	0.06
5	0.02	0.10	0.07
6	0.04	0.16	0.10
7	0.08	0.20	0.11
8	0.12	0.26	0.13
9	0.10	0.25	0.10
10	0.09	0.22	0.09

An-Aspergillus niger, **Rs**-Rhizopus stolonifer, **Fm**-Fusarium moniliforme

Aspergillus niger, *Rhizopus stolonifer* and *Fusarium moniliforme* synthesized protease in both the medium. 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 soybean seed cake medium (Table 1) followed by protease production medium (Table 2). The maximum amount of enzyme (0.12 U/ml, 0.26 U/ml and 0.13 U/ml) was secreted in 8 days in protease medium and thereafter decreased. In soybean seed cake medium the synthesis of enzymes (0.16 U/ml,0.34 U/ml and 0.15 U/ml) increased up to 9 days and thereafter it remained constant.

Table 2. Production of Protease on protease production medium:

Age of culture filtrate (Days)	Protease activity (U/ml)		
	An	Rs	Fm
1	0.00	0.00	0.00
2	0.00	0.00	0.00
3	0.01	0.01	0.00
4	0.02	0.06	0.03
5	0.05	0.10	0.07
6	0.09	0.16	0.12
7	0.12	0.21	0.13
8	0.13	0.26	0.14
9	0.16	0.34	0.15
10	0.15	0.32	0.12

An-Aspergillus niger, **Rs**-Rhizopus stolonifer, **Fm**-Fusarium moniliforme

The effect of temperature and pH on protease synthesis revealed that 40°C and 5.5 was optimum temperature (Table no. 4) and pH (Table 3) respectively.

Table 3. Effect of pH on production of Protease on soybean oil containing medium

pH	Protease activity (U/ml)		
	An	Rs	Fm
3.5	0.10	0.21	0.09
4.0	0.10	0.26	0.10
4.5	0.12	0.30	0.11
5.0	0.14	0.36	0.12
5.5	0.17	0.40	0.16
6.0	0.15	0.39	0.14
6.5	0.12	0.36	0.12
7.0	0.10	0.21	0.10
7.5	0.05	0.19	0.06
8.0	0.05	0.09	0.02
8.5	0.03	0.02	0.00
9.0	0.02	0.00	0.00

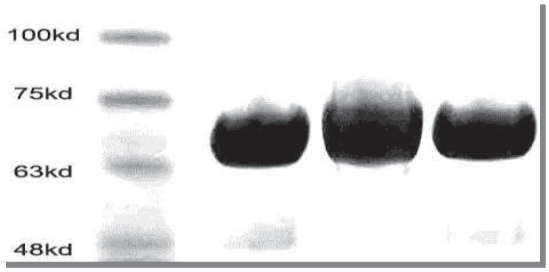
An-Aspergillus niger, **Rs**-Rhizopus stolonifer, **Fm**-Fusarium moniliforme

Table 4. Effect of Temperature on production of Protease on soybean seed meal medium

Temp (°C)	Protease activity (U/ml)		
	An	Rs	Fm
25	0.14	0.26	0.10
30	0.16	0.30	0.12
35	0.20	0.40	0.13
40	0.24	0.42	0.17
45	0.18	0.30	0.13
50	0.14	0.21	0.10
55	0.10	0.10	0.06

An-Aspergillus niger, **Rs**-Rhizopus stolonifer, **Fm**-Fusarium moniliforme

Sds-Page: The purified pectin lyase exhibited a single band on SDS-PAGE. When compare it with standard molecular weight markers, it showed molecular weight of ~66 kDa. A major proteasease excreted by *Aspergillus has* molecular mass of 63 kilodaltons (kDa)[9].



Discussion: The assessment of soybean seeds in storage for the quantities of total protein content

present in the seeds revealed that there was drastic reduction in total protein content. With increase in time of storage the quantity of total protein content decreased. The dominant fungi associated in all the cases were internal or endophytic fungi. This indicates there role in deteriorating total protein content and quality by secretion of proteases. The qualitative estimation for amino acids indicates the decrease in some amino acids with increase in time and accumulation of some amino acids with storage period [8].

The seed infestation by the common and dominant seed-borne fungi leads to deterioration of seed quality. The growth of the fungi associated with the seed can be attributed to the ability of the fungi to produce proteases. 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. [9].

It appears thus the fungi exhibit different trends of production of protease in relation to growth on different seeds. Amino acids acids released were utilized for growth by fungi. [10, 11].

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