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QUALITATIVE AND QUANTITATIVE PROTEASE ASSAY OF FUNGI ISOLATED FROM LEGUME SEEDS OF MARATHWADA REGION, INDIA

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ABSTRACT

Proteases are among the most commercially important enzymes used in biotechnology and industrial processes. Filamentous fungi are efficient producers of extracellular proteases due to their high secretion capacity and stability. The present study aimed to isolate fungi from protein-rich legume seeds collected from the Marathwada region and evaluate their protease production potential. Fifteen fungal species were isolated from seeds of chickpea, green gram, black gram, pigeon pea, and cowpea. Qualitative screening on skim milk agar revealed clear proteolytic zones, while quantitative protease activity was determined using the casein hydrolysis method. Among all isolates, *Aspergillus oryzae*, *Penicillium notatum*, and *Aspergillus clavatus* exhibited the highest protease activity across all seed substrates. The study demonstrates that legume seed-associated fungi represent promising candidates for industrial enzyme production.

Keywords: Protease, Fungi, Legume seeds, Skim milk agar, Enzyme assay, Marathwada

1. INTRODUCTION

Proteases (EC 3.4) catalyze the breakdown of proteins into peptides and amino acids and constitute nearly 60% of the global enzyme market (Gupta et al., 2002). They have extensive applications in detergents, leather processing, food fermentation, pharmaceuticals, diagnostics, and waste treatment (Rao et al., 1998; Sumantha et al., 2006).

Filamentous fungi are preferred producers of industrial enzymes because they secrete large quantities of extracellular proteins, grow rapidly on inexpensive substrates, and tolerate diverse environmental conditions (Pandey et al., 1999; Gupta and Ramnani, 2006). Several genera, including Aspergillus, Penicillium, Fusarium, and Rhizopus, are recognized as prolific protease producers.

Legume seeds are rich in storage proteins such as globulins and albumins (Duranti and Guerriero, 1993). These protein reserves create a favorable ecological niche for seed-associated fungi that produce extracellular proteases to utilize host nutrients (Strobel, 2003). Although studies exist on seed-borne fungi, limited attention has been given to their enzymatic potential, especially in the Marathwada region of Maharashtra.

Therefore, the present investigation was undertaken to isolate fungi from legume seeds and evaluate their protease activity using qualitative and quantitative methods.

2. MATERIALS AND METHODS

Sample Collection

Seeds of chickpea (*Cicer arietinum*), green gram (*Vigna radiata*), black gram (*Vigna mungo*), pigeon pea (*Cajanus cajan*), and cowpea (*Vigna unguiculata*) were collected from agricultural fields and local markets of the Marathwada region, Maharashtra, India.

Isolation and Identification of Fungi

Seeds were surface sterilized using 70% ethanol (1 min) followed by 1.5% sodium hypochlorite (3 min) and rinsed thrice with sterile distilled water (Schulz et al., 1993). Sterilized seeds were placed on Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 µg/mL) and incubated at 28 ± 2°C. Pure cultures were obtained by repeated subculturing. Identification was carried out based on colony morphology and microscopic features using standard manuals (Barnett and Hunter, 1998; Watanabe, 2002).

Qualitative Protease Assay

Fungal isolates were inoculated on skim milk agar plates and incubated at 28°C for 3–5 days. Clear zones around colonies indicated protease production (Casida, 1980).

Protease production was screened using skim milk agar medium. Fungal isolates were point inoculated and incubated at 28±2°C for 5–7 days. Clear zone formation around colonies indicated protease activity. Diameter of colony and zone of hydrolysis were measured.
The qualitative plate assay methodology was adopted following standard procedures described in Experiments in Microbiology, Plant Pathology and Biotechnology, Fungi and Food Spoilage, Food and Indoor Fungi, classical fungal taxonomy references such as The Fungi: An Advanced Treatise, and The Genus Fusarium.
The qualitative protease assay was performed following plate assay protocols described by Aneja (2012), Pitt & Hocking (2009), Samson et al. (2010), Cochrane (1958), and Booth (1971).

Quantitative Protease Assay

Protease activity was determined by the casein hydrolysis method (Kunitz, 1947; Lowry et al., 1951). Enzyme activity was expressed as U/mL (µmol tyrosine released per minute).

Protease activity was determined using the casein digestion method. Crude enzyme extract was incubated with casein substrate, and liberated tyrosine was measured spectrophotometrically using Folin–Ciocalteu reagent. Enzyme activity was expressed as Units per milliliter (U/mL), where one unit represents the amount of enzyme releasing 1 µg of tyrosine per minute under assay conditions.
The quantitative estimation method was originally described by M. L. Anson and later modified by McDonald & Chen (1965), Saran et al. (2007), Gupta et al. (2002), and Sandhya et al. (2005) for microbial protease studies.
Protease activity was quantified following the casein digestion method of Anson (1938) with modifications as described by McDonald & Chen (1965), Saran et al. (2007), Gupta et al. (2002), and Sandhya et al. (2005).

OBSERVATIONS & RESULTS

Table 1 Qualitative protease assay of fungi isolated from pulses seeds

Sr. No.	Fungal species	Host seed	Colony colour	Clear zone (mm)
1	*Aspergillus niger*	Chickpea	Black	21.5 ± 1.2
2	*Aspergillus flavus*	Chickpea	Yellow-green	18.4 ± 0.9
3	*Fusarium oxysporum*	Chickpea	White-pink	16.3 ± 0.8
4	*Penicillium chrysogenum*	Green gram	Blue-green	19.6 ± 1.0

5	*Aspergillus terreus*	Green gram	Cinnamon brown	20.2 ± 1.1
6	*Rhizopus stolonifer*	Green gram	Grey-black	14.8 ± 0.7
7	*Fusarium solani*	Black gram	Cream	15.2 ± 0.8
8	*Aspergillus fumigatus*	Black gram	Smoky green	17.5 ± 0.9
9	*Penicillium expansum*	Black gram	Olive green	18.1 ± 1.0
10	*Aspergillus clavatus*	Pigeon pea	Pale green	22.4 ± 1.3
11	*Aspergillus oryzae*	Pigeon pea	Yellow	24.8 ± 1.4
12	*Fusarium moniliforme*	Pigeon pea	Pink	20.3 ± 1.1
13	*Aspergillus candidus*	Cowpea	White	21.2 ± 1.2
14	*Penicillium notatum*	Cowpea	Bluish green	23.1 ± 1.3
15	*Aspergillus versicolor*	Cowpea	Green	22.6 ± 1.2

The qualitative protease assay revealed significant variability among fungal isolates in their ability to produce extracellular proteases, as indicated by the diameter of the clear zone formed around colonies on casein-containing agar medium. The formation of a clear halo zone indicates proteolytic degradation of protein substrates, reflecting extracellular enzyme secretion efficiency.

Among the isolates, *Aspergillus oryzae* (24.8 ± 1.4 mm) exhibited the highest proteolytic activity, followed by *Penicillium notatum* (23.1 ± 1.3 mm), *Aspergillus versicolor* (22.6 ± 1.2 mm), and *Aspergillus clavatus* (22.4 ± 1.3 mm). These results suggest that species belonging to Aspergillus and Penicillium genera are potent producers of extracellular proteases.

Moderate protease activity was observed in *Aspergillus niger* (21.5 ± 1.2 mm), *Aspergillus candidus*, and *Fusarium moniliforme*, while comparatively lower activity was recorded in *Rhizopus stolonifer* (14.8 ± 0.7 mm) and *Fusarium solani*.

The dominance of *Aspergillus* species in protease production is consistent with earlier findings that members of this genus are efficient secretors of hydrolytic enzymes, including proteases, amylases, and cellulases (Gupta et al., 2002; Sandhya et al., 2005). The clear zone diameter correlates with enzyme diffusion capacity and substrate hydrolysis efficiency (Anson, 1938).

Seed-borne fungi such as *Fusarium* spp. and *Rhizopus* spp. showed comparatively lower halo formation, indicating limited proteolytic efficiency under in vitro conditions. However, these fungi are still important contributors to seed deterioration due to their ability to produce multiple degradative enzymes (Christensen & Kaufmann, 1969; Pitt & Hocking, 2009).

The variation in colony colour observed among isolates is characteristic of species-level morphological identification, supporting classical taxonomic descriptions (Watanabe, 2002; Samson et al., 2010).

Overall, the qualitative assay clearly demonstrates that pulse seeds harbor diverse protease-producing fungi, with Aspergillus and Penicillium species showing superior extracellular proteolytic potential.

Table 2. Protease activity (U/mL) of fungal species on different legume seeds

S.No.	Fungal species	Chickpea	Green gram	Black gram	Pigeon pea	Cowpea
1	*A. niger*	28.4	32.7	30.1	41.6	38.5
2	*A. flavus*	26.9	30.3	28.7	39.2	36.4
3	*F. oxysporum*	22.5	24.8	23.4	33.6	31.1
4	*P. chrysogenum*	25.6	34.1	29.6	40.8	37.9
5	*A. terreus*	27.4	36.2	31.4	43.5	39.6
6	*R. stolonifer*	18.2	22.6	20.4	29.7	27.5
7	*F. solani*	20.1	23.4	21.7	31.5	29.2

8	*A. fumigates*	23.8	28.7	26.4	36.9	34.2
9	*P. expansum*	24.9	29.3	27.8	38.1	35.4
10	*A. clavatus*	30.4	35.6	33.1	48.2	44.7
11	*A. oryzae*	32.1	38.9	35.4	52.6	48.3
12	*F. moniliforme*	26.3	31.7	29.2	41.3	37.6
13	*A. candidus*	29.7	34.5	32.0	45.1	41.8
14	*P. notatum*	31.5	37.4	34.6	49.3	46.2
15	*A. versicolor*	30.8	36.1	33.7	47.5	43.9

The quantitative protease assay demonstrated significant differences in enzyme production among fungal species and host substrates. Protease activity ranged from 18.2 U/mL to 52.6 U/mL, indicating substantial variability in metabolic efficiency.

Among all isolates, *Aspergillus oryzae* showed the highest protease activity across all seeds, with maximum production recorded on pigeon pea (52.6 U/mL), followed by cowpea (48.3 U/mL). Similarly, *Penicillium notatum* (49.3 U/mL) and *Aspergillus clavatus* (48.2 U/mL) demonstrated high proteolytic activity on pigeon pea.

Protease production was consistently higher on pigeon pea and cowpea compared to chickpea and black gram. This variation may be attributed to differences in seed protein composition, substrate availability, and carbon–nitrogen ratio, which influence enzyme induction (Saran et al., 2007; Pandey et al., 2000).

Moderate enzyme production was observed in *Aspergillus terreus*, *Aspergillus versicolor*, and *Aspergillus candidus*, while comparatively lower activity was recorded in *Rhizopus stolonifer* and *Fusarium solani*.

The strong proteolytic performance of *Aspergillus* species aligns with previous studies indicating their commercial importance in enzyme biotechnology and food fermentation industries (Gupta et al., 2002; Sandhya et al., 2005). Seed deterioration by fungal proteases leads to degradation of storage proteins, resulting in reduced germination and seed vigor (Christensen & Kaufmann, 1969).

The data clearly indicate substrate-specific enzyme induction, with pigeon pea serving as the most favorable substrate for protease production among tested pulses.

DISCUSSION

The present investigation clearly demonstrates that legume seeds from the Marathwada region harbor a diverse population of protease-producing fungi. All fifteen isolates showed positive proteolytic activity on skim milk agar, confirming their ability to secrete extracellular proteases for protein degradation. Similar findings were reported by Gupta et al. (2002), who stated that seed-associated fungi utilize host protein reserves through secretion of hydrolytic enzymes.

Among the isolates, species of the genus Aspergillus dominated and exhibited the highest protease activities. *Aspergillus oryzae* showed maximum activity across all seed substrates (52.6 U/mL on pigeon pea; 48.3 U/mL on cowpea). This agrees with Pandey et al. (1999) and Gupta and Ramnani (2006), who identified *Aspergillus* species as superior producers of extracellular proteases for industrial use. The high enzyme yields may be attributed to the genetic ability of Aspergillus to secrete large quantities of hydrolytic enzymes and their rapid growth on protein-rich substrates.

The variation in protease activity among fungal species can be explained by differences in metabolic regulation, gene expression, substrate specificity, and environmental adaptation (Rao et al., 1998). Legume seeds differ in protein composition and concentration, which may influence enzyme induction. Pigeon pea and cowpea supported higher protease activity in most isolates, likely due to their higher globulin content (Duranti and Guerriero, 1993).

Species such as *Penicillium notatum* and *Aspergillus clavatus* also showed high enzyme activity, suggesting their potential for commercial exploitation. Penicillium species are known for producing stable proteases with wide pH tolerance (Sumantha et al., 2006). Moderate protease activity observed in Fusarium species and *Rhizopus stolonifer* indicates their ecological role in seed degradation rather than industrial efficiency.

The clear zone diameters on skim milk agar correlated well with quantitative protease values, supporting the reliability of SMA as a preliminary screening tool. Similar correlations were reported by Olajuyigbe and Fatokun (2017) and Katoch and Sharma (2007).

These findings highlight the biotechnological significance of seed-associated fungi as inexpensive and renewable sources of proteases. Further optimization of culture conditions, enzyme purification, molecular identification, and characterization of pH and temperature stability would enhance industrial applicability.

CONCLUSION

The present study confirms that legume seeds from the Marathwada region harbor diverse protease-producing fungi. Among the fifteen isolates, *Aspergillus oryzae*,*Penicillium notatum,* and *Aspergillus clavatus* exhibited the highest proteolytic activities. Pigeon pea and cowpea were found to be the most suitable substrates for enzyme production.

These fungi represent promising, eco-friendly sources of industrial proteases. With further optimization and characterization, they could be developed for applications in detergents, food processing, pharmaceuticals, leather industries, and agricultural waste management. The study also contributes to understanding the enzymatic potential of seed-associated fungi from semi-arid regions like Marathwada.

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