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Production of enzymes by test fungi isolated from stored mustard seeds.

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ABSTRACT

Brown mustard (*Brassica juncea* cv Pusabold) is one of the oilseed crop commonly grown in cooler region of India and widely distributed. Pure and clean seeds stored in cotton bag and proceed for the incidence and isolation of seed mycoflora in every month of a year storage period. Total 28 fungi were isolated from mustard seeds but frequently occurring dominant fungi (test fungi) throughout the year were *Alternaria alternata*, *Aspergillus flavus*. *A. fumigatus, A. niger*, *Curvularia lunata*, *Fusarium moniliforme* and *Penicillium oxalicum*. In the present study, attempts have been made for the production of extra cellular lipase, protease and amylase enzymes by test fungi from mustard seeds. The test fungi were grown in sterilized czapek's broth liquid medium and incubated for 7, 14 and 21 days at 28 ±1^{0}C. After completion of every incubation period the contents were filtered and centrifuged. The filtrates were used as crude enzyme extract for the respective enzyme assay. In the present investigation, it is found that species of *Aspergillus* found higher lipase production than other test fungi. *Aspergillus flavus, Aspergillus niger* and *Penicillium oxalicum* gives higher ability to produce protease enzymes while *Aspergillus flavus* had been recorded as higher ability to produce amylase than other fungi. In the enzyme assay, it is seen that the enzyme activity depends on incubation of test fungal filtrates.

Key words: Mustard, Enzyme assay, Test fungi, Lipase, Protease, Amylase

INTRODUCTION

Mustard is one of the important traditional oilseed crop grown in north India. The seed coat colour is brown to dark brown. The oil content is 40% in seeds and oil containing 46% erucic acid and 140 to 150 µ mol. glucosinolate in defatted meal. Microorganisms exhibit a variety of metabolic activities, which are of immense economic importance. They are also better known to destroy food stuff in stored plant materials particularly in food grains. Microorganisms have a role during the process of seed deterioration and have been considered helpful to their invasion and colonization. Microorganisms use enzymes to process specific biological molecules during metabolism and growth. The fungi are known to produce many enzymes extracellularly. These

enzymes degrade their respective substrate available in the seeds due to their association during storage, which lowers the nutritional values. Oilseeds mustard have high percentage of oil content, proteins & also carbohydrates, which are very important in human diet. So it was found necessary to study the ability of the 'test fungi' to produce lipases, proteases and amylases.

MATERIALS AND METHODS

Brassica juncea (L.) Czern, & Coss. cv. Pusabold commonly known as traditional mustard selected for experimental study. Isolation of seed mycoflora was done in every month of one year of storage period by both blotter as well as agar plate method as recommended by ISTA (1966). After collecting the data of percentage of fungal incidence, most frequent or dominant fungi were selected as, 'test fungi'. The test fungi are *Alternaria alternata*, *Aspergillus flavus*. *A. fumigatus, A. niger*, *Curvularia lunata*, *Fusarium moniliforme* and *Penicillium oxalicum*,

Preparation of crude enzyme extract for lipase: Production of lipase was studied by growing the 'test fungi' on liquid medium at pH 5.6 containing oil 10 g, KNO_3 2.5 g KH_2PO_4 l g, $MgSO_4$ 0.5 g and distilled water 1000 ml. Twenty five ml of the medium was poured in 150 ml of conical flasks and autoclaved at 15 lb pressure for 30 min then on cooling the flask were inoculated separately with 1 ml of spore suspension of the ' test fungi'. The flasks were incubated for 7, 14 and 21 days at 25 ± 1 ^{0}C with diurnal periodicity of light. After incubation period the flasks were harvested by filtering the content through whatman filter paper no.1. The filtrates were collected in pre-sterilized culture filtrates bottles and termed as crude lipase.

Assay method for Lipase:
Determination of lipase activity was done with the help of cup-plate method, which was adopted by Sierira (1957). On solidifying the medium, with the help of cork borer (No. 4) cavity (8 mm diameter) was made in the center and was filled with 0.1 ml culture filtrate of each 'test fungi'. The plates were incubated at 28±1^{0}C. After 24 hours, a clear circular zone was found surrounding to the central cavity. Diameter of the zone was measured (mm) as the lipase activity control was kept by pouring sterile growth medium.

Preparation of crude enzyme extract for protease and amylase:
The test fungi were grown in sterilized czapek's broth and incubated for 7,15 and 21 days at 28 ±1^{0}C. After incubation period the contents were filtered through whatman filter paper No. 1 and then centrifuged at 5000 rpm for 10 minutes. The filtrates were used as crude enzyme extract.

Assay method for Protease: -
Determination of protease activity was done with the help of cup plate method suggested by Hankin and Stakis (1975). On solidification, a cavity was made with the help of cork borer in the center. Then 0.1 ml of crude enzyme extract was poured in the cavity and plates were incubated for 24 hours at 28 ± 1^{0}C. The plates were flooded by saturated solution of ammonium sulphate. After 30 min, a clear zone appeared around the cavity. Diameter of the zone was measured in mm and was considered proportional to the activity of protease enzyme while non-appearance of zone was considered as absence of protease. Control was kept by using sterile growth medium instead of crude enzyme extract.

Assay method for Amylase –
Determination of amylase was done with the help of cup plate method, which was adopted by Singh and Saxena (1982). On solidification of medium, a cavity (8 mm dia.) was made in the center with the help of cork borer and filled with 0.1 ml of crude enzyme extract of the test fungi. The plates were incubated at 28 ± 1^0 C for 24 hours and then were flooded with lugol's iodine solution as an indicator. A clear non-blue circular zone was obtained surrounding the central cavity. Diameter of the zone was measured (mm) as the amylase activity zone. Control was kept by pouring sterile growth medium. Diameter of the zone was directly proportional to the activity.

RESULT AND DISCUSSION

The fungi have capability to produce extra cellular enzymes and the experimental data on enzyme assay of lipase, protease and amylase is tabulated.

Enzyme assay for Lipase production:
The lipase activity by different test fungi presented in Table 1. Culture plates inoculated with sterile czepak's broth medium found clear zone with 8 mm in diameter. *Aspergillus flavus* and *Aspergillus niger* exhibited higher

enzyme production than *Alternaria alternata, Curvularia lunata, Fusarium moniliforme* and *Penicillium oxalicum.* Among these test fungi *A.niger* showing highest lipase activity with increase in incubation period of fungal filtrate.

Enzyme assay for Protease production:

Table 1 shows capability of extra cellular protease production for different incubation period. *Aspergillus flavus, Aspergillus niger* and *Penicillium oxalicum* shows higher ability to produce protease enzyme than *Alternaria alternata, Aspergillus fumigatus, Curvularia lunata* and *Fusarium moniliforme.*

Enzyme assay for Amylase production

The ability to produce amylase enzyme was presented in Table 1. All three species of *Aspergillus* showed higher enzyme secretion of amylase enzyme than remaining test fungi that is *Alternaria alternata, Curvularia lunata, Fusarium moniliforme* and *Penicillium oxalicum.*

Table 1: Activity of Lipase, Protease and Amylase enzymes

S. N.	Name of Organism	Lipase activity			Protease activity			Amylase activity		
		Incubation period (days)			Incubation period (days)			Incubation period (days)		
		7	14	21	7	14	21	7	14	21
1	Control	8*	8	8	8*	8	8	8*	8	8
2	*Alternaria alternata*	8	10	12	8	8	10	8	10	12
3	*Aspergillus flavus*	14	16	20	10	14	18	10	16	26
4	*A. fumigatus*	14	14	20	8	10	14	10	14	18
5	*A. niger*	16	20	25	9	14	17	14	18	20
6	*Curvularia lunata*	8	16	18	8	9	12	8	8	10
7	*Fusarium moniliforme*	12	16	18	8	8	10	8	10	12
8	*Penicillium oxalicum*	11	14	18	8	14	16	10	13	15

* Diameter of clear zone (including cavity - 8 mm) in mm

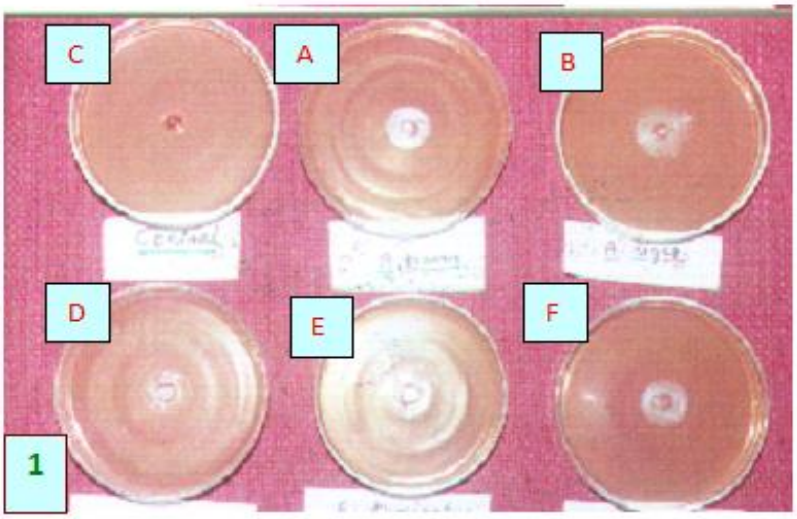
In the present investigation, species of *Aspergillus* found higher lipase production than *Alternaria alternata, Curvularia lunata* and *Fusarium moniliforme. Aspergillus niger* shows higher lipase activity with older fungal filtrate. While *Aspergillus flavus, Aspergillus niger* and *Penicillium oxalicum* gives higher ability to produce protease enzymes than the other test fungi studied. *Aspergillus flavus* have recorded as higher ability to produce amylase than other fungi, which were studied in present investigation. In enzyme assay, it is seen that the enzyme activity depends on incubation of test fungal filtrates (Table 1).

Initially it was thought that fungi could not utilize lipids as an energy source since the changes of crude lipid in culture media were so small. The increased in the lipolytic activity results into loss in the oil content (Agrawal, 1965 and Prasad et al, 1983). Christensen (1974) has stated that fat in seeds are readily broken down by lipase into fatty acids and glycerol.

McGee and Christensen (1970) also found similar results. Gupta *et al* (1986) reported that formation of free fatty acids and activity of enzymes like lipase and peroxidase were correlated with storability of the seeds of mustard, soybean and maize when they stored under high RH. Roberts *et al* (1987) studied the extra cellular lipase production by fungi from sunflower seed. Farag *et al* (1985) have studied the effect of fungal lipases on different classes of lipids.

Microbial proteases facilitate the invasion of seed tissue (Pernollet, 1978), which leads to destruction of the tissue. Autolysis by host enzymes then begins and seed proteins are hydrolyzed to peptides and free amino acids (Cherry, 1983). In peanuts, invasion results in the decomposition of protein to low molecular weight components, the depletion of some enzymes, and intensification of others and also the production of new enzyme symptoms (Mall *et al*, 1986). The production of amylase by fungi was found to be increased with increase in the concentration of starch in the medium (Chapman *et al*, 1975). Dhake (1995) reported maximum amylase production by *Aspergillus flavus* followed by *Aspergillus niger, Fusarium moniliforme, Trichoderma viride* and *Alternaria alternata.*

PLATE 1: Extracellular enzymes secreted by fungi in 21 days of incubation.

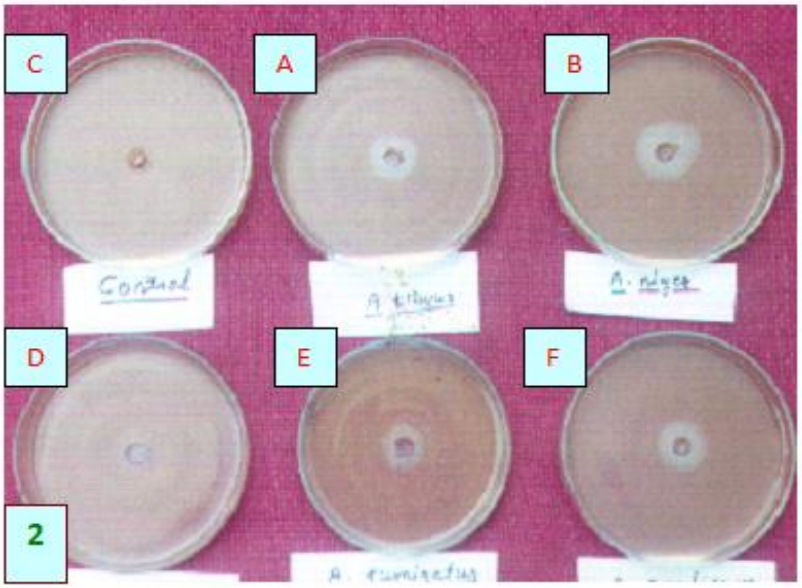


1. Assay for lipase.

C. Control
A. *A. flavus*
B. *A. niger*
D. *Fusarium moniliformae*
E. *A. fumigatus*
F. *P. oxalicum*

2. Assay for protease.

C. Control
A. *A. flavus*
B. *A. niger*
D. *Fusarium moniliformae*
E. *A. fumigatus*
F. *P. oxalicum*



3. Assay for amylase.

C. Control
A. *A. flavus*

CONCLUSION

From the above enzyme assay, it was clear that different test fungi produced all three enzymes i.e. lipase, protease and amylase and the secretion of the respective enzymes increases with increase in the incubation period of test fungi (plate 1).

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