Extracellular Lipase Enzyme Production by Seed-Borne Fungi under the Influence of Physical Factors

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Abstract

Present paper deals with the study of impact of physical factors on extracellular lipase activity of some dominant oilseed-borne fungi. In present investigation, seed-borne fungi were isolated from abnormal oilseeds by agar plate method. Of the 11 genera and 17 species of fungi isolated, 10 dominant fungi were selected to study their lipase enzyme activity. Various parameters such as temperature, pH, light and incubation period were studied in order to determine the optimum conditions for lipase production of ten dominant fungi. The lipase present in the broth was assayed by cup-plate method. It is revealed from the result that lipase activity of storage fungi was found to be optimum at temperature 20^0C, pH 6.5, incubation period of 20 days and in continuous light.

Keywords: Lipase activity, Physical factors, Oilseeds, Oilseed-borne fungi

1. Introduction

Seed is the plant part associated with either propagation of plant for its continuous existence or serves as food for human beings and it act as a catalyst in agricultural production. Several biological agents, mainly fungi damages oilseeds from the early stage of their formation on growing plants until their use and consumption.

Due to their high nutritive value seeds are prime target of attack of various microorganisms and insects. Among microorganisms fungi play a dominant role in infecting quality and longevity of seeds in storage (Christensen and Kaufman, 1969). Overall losses of grains due to diseases can vary from 10-25% annually throughout the world. In India we are losing an average 30 metric tons of food grains, 4 metric tons of oilseeds, 36 metric tons of sugarcane, 23 metric tons of fruits and vegetables. Oilseeds are rich in oil hence vigor of pathogenic fungi in the process of biodeterioration of seed may be related to their degree of lipase production (Chavan and Kakde, 2010). Lipases are the enzymes that catalyze the hydrolysis of fats and mono-and di-glycerides to free fatty acids and glycerol. Lipases (Triacylglycerol acylhydrolases) are found in animals, plants and microorganisms (Kamimura et al., 2001; Burkert et al., 2004). The production of lipases is influenced by many factors such as pH, temperature, carbon and nitrogen (George et al., 1999). All microorganisms have a characteristic optimal growth temperature at which they exhibit their highest growth and a range of temperatures they can tolerate (Cho et al., 2007). Many genera as *Penicillium, Rhizopus, Aspergillus* and *Fusarium* have been noted as producers of lipases with desirable properties. Little information on the factors and conditions that control lipase biosynthesis and secretion is available. Therefore, in present investigation an attempt was made to study the storage mycoflora which is associated with abnormal groundnut seeds and their degree of lipase production under the influence of cultural environment.

2. Materials and Methods

2.1 Isolation of mycoflora

In present research study, detection of seed mycoflora associated with the seed samples was done (ISTA, 1996). The groundnut seeds were further categorized according to their abnormalities like shrunkened, discolored and rotted seed to the fungi responsible for their abnormal nature. 10 seeds per pre-sterilized petri-plates were equispaced aseptically on autoclaved Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) media. The

plates were then allowed to incubate at room temperature. Detail observations of fungal characters were done under the binocular microscope and their identification was confirmed with standard literature (Ellies, 1971; Mukadam et al., 2006). Seventeen fungi were isolated from abnormal groundnut seeds from Tag-24 and SB-11 by Agar plate methods on Potato Dextrose Agar and Rose Bengal Agar medium.

2.2 Production of Lipase

Lipase activity was studied by growing the fungi in liquid medium at pH 5.6 containing oil-10ml, KNO3 -2.5g, KH_2PO_4- -1.0g, $MgSO_2$ – 0.5g and distilled water 1000ml. Treatments of different physical factors such as temperature, pH, light and incubation period were given to above basal medium. 25ml of the medium was poured in 100ml conical flasks and autoclaved at 15 lbs pressure for 30 minutes and then on cooling, the flasks were inoculated separately with 1.0ml spore suspension of the fungi. In case of temperature, pH and light, on 7th day, the flasks were harvested by filtering the contents through Whatman filter paper no.1. The filtrates were collected in pre-sterilized culture filtrate bottles and termed as crude lipase.

2.3 Assay Method (Cup-plate method)

Determination of lipase activity was done with the help of cup-plate method (Sierra, 1957). The medium contains Difco peptone-10g, NaCl-5g, $CaC1_2.2H_2O$-1.0g, Agar 20g and 10ml lipid substrate Serbitan mono laurate (Tween-20) (Pre-sterilized), distilled water- 1000ml was added to it. The pH of the medium was adjusted to 6.00. The medium was poured in each Petri plate. On solidifying the medium with the help of a cork borer (No.4) of 8mm diameter well was made in the centre of the plate and was filled with 0.1ml culture filtrate. The plates were incubated at 28^{o}C. After 24 hours, a clear circular zone was measured (mm) as lipase activity. (Fig.1)

2.4 Biostatic analysis

Data obtained was statistically analyzed for Critical difference (C.D.) by following Panse and Sukhatme (1978).

3. Results

3.1 Isolation of oilseed mycoflora

Seed mycoflora of two different varieties viz. Tag-24 and SB-11 of groundnut cultivated in Marathwada region of Maharashtra state was isolated by using Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) and the results are given in table 1. *Alternaria dianthicola* showed association with all categories of Tag-24 and on Sh category its quantitative dominance was observed. *Curvularia lunata* showed its quantitative dominance on Rot category of variety Tag-24 on PDA while, it occurred on all categories of SB-11 except Rot category. On PDA, Rot category of Tag-24 showed occurrence of *Curvularia pellescens.* Tag-24 on all its categories and both media showed maximum occurrence of *Fusarium oxysporum,* and *Fusarium equiseti* as compared to SB-11 variety. All categories of Tag-24 on PDA and all categories of SB-11 on RBA showed occurrence of *Macrophomina phaseolina. Rhizopus stolonifer* showed its quantitative dominance on Sh category of SB-11 on RBA. Quantitative dominance of *Penicillium notatum* was observed on all categories of Tag-24 variety. Dc category of SB-11 showed maximum occurrence of *Penicillium chrysogenum. Trichoderma viridae* showed its occurrence on both the varieties. Out of seventeen fungi isolated, *Alternaria dianthicola, Curvularia lunata, Curvularia pellescens, Fusarium oxysporum, Fusarium equiseti, Macrophomina phaseolina, Rhizopus stolonifer, Penicillium notatum, Penicillium chrysogenum* and *Trichoderma viridae* showed their quantitative dominance. Therefore, these ten fungi other than *Aspergillus* species were selected to study their lipase activity. All the ten dominant fungi were able to metabolize to varying grades of different types of physical factors to lipase production. Such type of work was earlier supported by several workers (Neergaard, 1973; Agrawal, 1976; Chavan and Kakde, 2009).

3.2 Effect of Temperature

The optimum temperature for the enzyme activity was determined, reactions being performed at various temperatures, from 10 to 60^{0}C. At 20^{0}C *Penicillium notatum* showed significant maximum lipase activity which was followed by *Fusarium equiseti*. *Penicillium chrysogenum* showed maximum lipase production at 30^{0}C as compared to other fungi. On the other hand *Curvularia lunata* and *Curvularia pellescens* did not show lipase activity at 10^{0} C while, at 30^{0}C lipase activity of *Fusarium oxysporum* was found to be increased (Table 2). Similar results were reported by Moataza et al. (2004). They found that *Fusarium oxysporum* produced maximum lipase at 30^{0}C.

3.3 Effect of pH

Alternaria dianthicola, Penicillium notatum, Penicillium chrysogenum, Fusarium oxysporum and *Fusarium equiseti* showed maximum lipase activity at pH range from 6.5 to 7.5. On the other hand, *Alternaria dianthicola,*

Curvularia lunata, Macrophomina phaseolina and *Trichoderma viridae* did not produce lipase enzyme at 8.5 pH (Table 3). This was also observed during the lipase production of *Fusarium oxysporum* (Moataza et al., 2004a).

3.4 Incubation Period

From Table 4 it is clear that as incubation period is increased, lipase enzyme production also increased. At 5th day of incubation period, *Alternaria dianthicola, Curvularia lunata, Curvularia pellescens* and *Fusarium equiseti* did not produce lipase enzyme. At incubation period 10 and 15 days, there is moderate lipase production. At 20th day of incubation period, *Fusarium oxysporum, Fusarium equiseti, Penicillium chrysogenum* and *Rhizopus stolonifer* showed maximum lipase enzyme production as compared to other fungi. *Penicillium notatum, Macrophomina phaseolina* and *Rhizopus stolonifer* were found to produce maximum lipase enzyme at 25th days of incubation period. It is interesting to note that at 10-15 days of incubation period *Penicillium notatum* and *Penicillium chrysogenum* showed same lipase action.

3.5 Light

Continuous light significantly favors maximum lipase production of fungi as compared to continuous dark. At Continuous light condition *Penicillium notatum* and *Penicillium chrysogenum* showed maximum lipase production as compared to other fungi, but it was reduced to some extent at alternate light and dark condition.

Penicillium expansum showed the maximal lipase activity at initial pH 5.5-6.0 and temperature 26 °C (Dazhang Daiand Liming Xia, 2008). Licia et. al., (2006) tested the effect of pH on the enzyme activity of *Aspergillus niger* at pH range of 2.0–8.0, temperature range of 4-55^{0}C and incubation period range of 1-6 days. They found that *Aspergillus niger* showed maximum lipase activity at pH 6.5, temperature at 37^{0}C and incubation period at 4th day. Pimente, et. al. (1997) found that, the optimum temperature for lipase activity for *Penicillium citrinum* was 37^{0}C, pH 2.4 and 5 days of incubation period. Vaidehi and Jagdamba (1984) recorded maximum lipase activity of *Aspergillus niger* and *A.flavus* at 6th day and pH 6 and *Fusarium oxysporum* and *Rhizoctonia solani* showed maximum lipase activity at 8th day and pH 8. The maximum lipase

activity was obtained on the 5th day of incubation at 20 °C. Lipase from *P. chrysogenum* showed the greatest activity at pH 7.9–8.1 and 45 °C (Ferrer *et al*., 2000). Garcı´a-Lepe et al. (1997) tested filamentous fungi like *Fusarium oxysporum, F. equiseti*, *F. moniliforme, F. solani, Rhizopus stolonifer, Penicillium oxalicum* and *Trichoderma harzianum* at 3·5, 6·5 and 9·2 pH. They found that at pH 6.5 and incubation period 4 hr and at 25°C, these fungi produced maximum lipase enzyme. Ruiz et al. (2001) studied the optimum pH for *P. candidum* lipase activity in a range from pH 5 to 11. The enzyme was found to be most active at pH 9.0 and showed its stability in the pH range from 4 to 6 when incubated at 37°C for 30 min.

4. Discussion

Potato Dextrose Agar is favourable for fungal growth, as it showed maximum occurrence of fungi than Rose Bengal Agar (RBA). The results presented in this work i.e. low temperature range from 5-10^{0}C, minimum storage period 5 days and low pH 3.5 and 4.5 suggest that by manipulating the physical factors the lipase production can be minimize which ultimately reduce the loss of groundnut. Knowledge gained through such studies may be applied to control the seed-borne diseases by changing the environmental conditions to the disadvantage of the pathogen.

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Table 1. Associated mycoflora of groundnut on PDA and RBA

Fungi	Varieties											
	Tag-24						SB-11					
	PDA			RBA			PDA			RBA		
	DC	Sh	Rot	DC	Sh	Rot	DC	Sh	Rot	DC	Sh	Rot
Alternaria dianthicola	30	10	-	20	40	30	30	-	-	20	20	30
Alternaria tennuisima	10	-	-	-	-	-	-	20	-	-	-	-
Aspergillus niger	30	-	10	-	-	60	40	-	40	-	20	-
Aspergillus flavus	-	-	20	10	-	-	-	30	-	50	-	10
Aspergillus fumigatus	10	-	20	30	-	-	20	10	20	30	-	60
Curvularia lunata	20	-	30	20	-	-	20	20	-	20	-	-
Curvularia pellescens	-	-	50	-	40	-	10	-	30	20	-	20
Colletotrichum sp.	-	10	-	50	-	-	20	-	-	30	-	-
Fusarium oxysporum	10	30	20	20	70	50	-	20	-	-	10	30
Fusarium equiseti	10	20	20	-	20	80	-	-	-	20	20	30
Macrophomina phaseolina	50	40	40	20	-	-	20	50	-	40	40	40
Rhizopus stolonifer	-	10	30	20	-	30	20	30	-	-	50	30
Penicillium notatum	20	30	-	20	-	20	-	-	50	-	-	-
Penicillium chrysogenum	30	-	-	40	-	20	10	-	-	50	-	30
Verticillium sp.	-	-	-	-	-	-	10	-	-	30	-	-
Helminthosporum sp.	10	-	-	-	-	-	10	-	-	-	-	-
Trichoderma viridae	-	20	10	-	-	-	30	20	-	-	10	-

Dc: Discolored; Sh: shrunkened; Rot: Rotted

Table 2. Effect of temperature on lipase enzyme production

Temperature (^{0}C)	Fungi									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
10	09	-	-	10	11	12	13	12	11	09
20	16	14	15	16	18	13	16	19	17	12
30	18	17	19	19	20	17	18	20	22	15
40	16	15	13	17	15	19	20	19	21	17
50	12	13	13	10	11	16	22	16	17	15
60	09	12	10	-	09	11	14	12	11	09
C.D. at 0.05	3.5	5.9	6.1	6.7	4.1	2.9	3.3	6.0	4.6	4.0

Zone of Enzyme Activity is expressed in mm

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Table 3. Effect of pH on lipase enzyme production

pH	Fungi									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
3.5	09	11	10	13	15	16	16	14	12	09
4.5	10	09	12	14	16	15	15	16	18	10
5.5	13	13	14	16	15	16	16	19	18	13
6.5	14	15	14	18	29	17	19	20	22	17
7.5	17	19	18	20	21	19	17	21	20	19
8.5	-	-	-	11	09	-	10	14	16	-
C.D. at 0.05	5.7	6.3	6.0	3.2	6.6	6.5	2.8	3.0	3.3	6.4

Zone of Enzyme Activity is expressed in mm

Table 4. Effect of Incubation Period on lipase enzyme production

Incubation Period	Fungi									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
5	-	-	-		-	12	11	10	11	09
10	12	11	10	12	19	13	15	17	17	12
15	18	13	15	19	20	17	18	20	20	15
20	19	15	17	22	23	19	20	19	21	17
25	17	18	19	18	20	23	22	24	20	20
C.D. at 0.05	4.0	7.5	8.3	7.3	10.7	4.9	4.7	5.6	6.0	4.6

Zone of Enzyme Activity is expressed in mm

Table 5. Effect of Light on lipase enzyme production

Physical factor	Fungi									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Continuous Dark	15	14	13	16	17	15	19	20	21	15
Continuous Light	13	15	16	18	19	17	18	21	22	19
Alternate dark and light	14	16	15	17	15	16	18	19	19	16
C.D. at 0.05	2.0	2.0	3.1	2.0	4.0	2.0	1.1	2.0	3.0	4.2

Zone of Enzyme Activity is expressed in mm

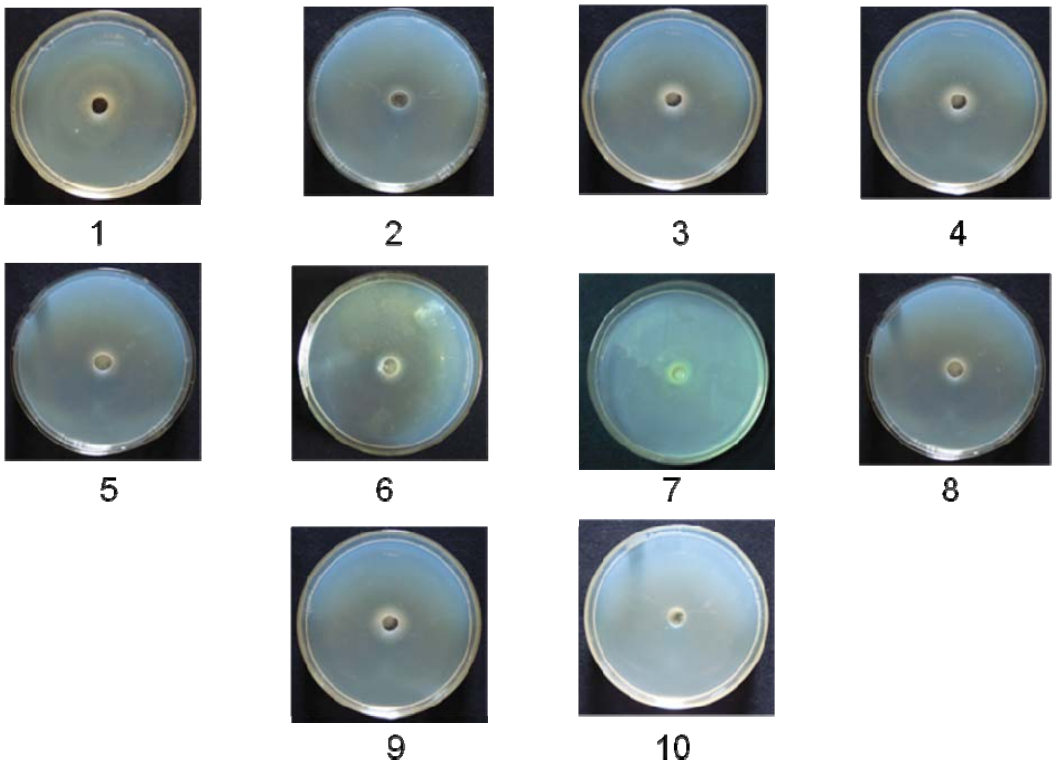

Figure 1. Effect of temperature on lipase enzyme production; Plates Showing the Zone (mm) of Lipase enzyme activity

F1. *Alternaria dianthicola*
F2. *Curvularia lunata*
F3. *Curvularia pellescens*
F4. *Fusarium oxysporum*
F5. *Fusarium equiseti*
F6. *Macrophomina phaseolina*
F7. *Rhizopus stolonifer*
F8. *Penicillium notatum*
F9. *Penicillium chrysogenum*
F10.*Trichoderma viridae*

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