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EFFECT OF CARBON, NITROGEN, SULPHUR, PHOSPHORUS, ANTIBIOTIC AND VITAMIN SOURCES ON HYDROLYTIC ENZYME PRODUCTION BY STORAGE FUNGI

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Abstract

In present investigation emphasis is given on to screen the lipolytic activity of storage fungi. Abnormal safflower seeds of PBNS-12 and Bhima varieties were collected from Marathwada region of Maharashtra state. Dominant fungi were isolated from abnormal oilseeds on Potato Dextrose Agar (PDA). Total twenty fungi were isolated. Out of that lipase enzyme activity of ten dominant fungi other than *Aspergillus sp.* was studied by using different nutritional sources like carbon, nitrogen, phosphorus, sulphur, antibiotic and vitamin sources. It was found that carbon sources like fructose and sucrose induces lipase activity while starch, lactose and carboxyl methyl cellulose (CMC) inhibits lipase activity. Nitrogen sources like casein and peptone which are organic forms stimulated maximum lipase enzyme production of storage fungi. Sulphur sources like calcium sulphate and ferrus sulphate reduced the lipase enzyme production by storage fungi while, phosphorus source like di-sodium hydrogen ortho-phosphate, ammonium phosphate and potassium di-hydrogen ortho-phosphate stimulated lipase enzyme production. Antibiotic like ampicillin, norfloxacin and tetracycline reduced the lipase production of storage fungi. Lipase activity of storage fungi was reduced in presence of vitamin source like riboflavin while, folic acid and vitamin C stimulated the lipase enzyme production.

Keywords: Abnormal safflower seeds, storage fungi, lipase enzyme activity, nutritional sources

Introduction

Safflower (*Carthamus tinctorus* L.) is an important oilseed crop in India. It is cultivated in both Rabi and Kharif seasons of Marathwada region of Maharashtra state. The seeds of the crops are used for extraction of oil that is used for consumption as well as for pharmaceutical purposes and the residue cake is used as cattle feed. Seed plays a vital role for the production of healthy crop. About 90 % of the world food crops are produced by using seeds. These seeds are also responsible for disease transmission. This takes place either in the field or in ill storage condition. Under hot and humid storage conditions, oilseeds frequently become invaded by storage fungi (Sharma, 1977; Mondal, et. al. 1981; Nandi, et. al. 1982). Fungi are the major cause of spoilage in stored grains and seeds in the technologically advanced countries, because insects and rodents are effectively controlled (Christensen and Kaufmann 1974). There is qualitative and quantitative reduction in oil production from the molds contaminated seeds. A major problem of agricultural production is loss of grain during and after harvest. Microorganisms, insects, and rodents contribute greatly to these postharvest losses. A commonly quoted estimate provided by the FAO for worldwide losses for all cereals, leguminous seeds, and oilseeds is 10% (Janicki and Green 1976). In the presence of seed-borne pathogens, several types of abnormalities occurred in the seed. Such seeds are rejected by seed industry and agriculture (Neergaard, 1973). When seeds are damaged in improper storage conditions or exposed to certain microorganisms, fungi secrets their biological weapon i.e. hydrolytic enzyme. Since oilseeds are rich in oil, lipid degradation reaction can occur. 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). Many genera as *Penicillium, Rhizopus, Aspergillus* and *Fusarium* produce lipases with desirable properties. Factors affecting microbial extracellular lipase production have been widely studied in bacteria (Lawrence et al., 1967; Mates and Sudakevitz 1973), moulds (Chander et al., 1980; Chopra and Chander, 1983) and yeasts (Ota et al., 1968). Few studies have been made with *Rhizopus oligoporus* to examine the control of lipase production (Smith and Alford, 1968). The production of lipase by several *Fusarium oxysporum* strains has been studied

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in terms of enzyme productions, protein properties and purifications (Hoshino et al., 1992; Maria de Mascena et al., 1999). Little information on the factors and conditions that impact on extracellular lipase biosynthesis and secretion is available. Therefore, in present investigation an attempt was made to study the storage mycoflora which is associated with abnormal safflower seeds and their degree of lipase production under the influence of different nutritional sources.

Materials and Methods

Collection of Oilseed samples

Oilseeds samples of safflower of two varieties viz. PBNS-12 and Bhima were collected from store houses of Parbhani district of Marathwada region of Maharashtra state. These seeds were then packed in pre-sterilized polythene bags.

Isolation of Seed mycoflora

Seeds were further categorized according to their abnormalities like shrunkened seeds, cracked seeds and undersized seeds, to know the fungi responsible for their abnormal nature. 10 seeds per pre-sterilized petriplates were equispaced asceptically on autoclaved Potato Dextrose Agar (PDA). Plates were then allowed to incubate at room temperature for seven days.

Identification and Preservation of Seed borne fungi

On seventh day of incubation the seeds were examined under stereoscopic microscope for the preliminary determination of fungal growth on them. Detail observation of fungal characters was done under the binocular microscope and their identification was confirmed with standard literature. Identification was done on the basis of size, shape, septation, and colour of conidia, presence or absence of chlamydospore. Pure culture of these fungi were made and maintained separately on Potato Dextrose Agar (PDA) slants (Ellies, 1971; Mukadam et al., 2006).

Production of Lipase

Lipase activity was studied by growing the fungi in liquid medium at pH 5.6 containing safflower oil-10ml, KNO3 -2.5g, KH_2PO_4- -1.0g, $MgSO_2$ – 0.5g and distilled water 1000ml. Treatments of different nutritional sources such as carbon, nitrogen, phosphorus, sulphur, antibiotics and vitamins 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 1.0 ml spore suspension of the fungi. 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.

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-1000 ml 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, well was made at the centre of each plate with the help of a cork borer (No.4) of 8mm diameter and well 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.

Results and Discussion

Seed mycoflora of two different varieties viz. PBNS-12 and Bhima of safflower cultivated in Marathwada region of Maharashtra state was isolated by using Potato Dextrose Agar (PDA) and the results are given in table 1. Four species of *Aspergillus* viz. *Aspergillus niger, Aspergillus flavus, Aspergillus ustus* and *Aspergillus terreus* were found to be associated on all the categories of safflower seeds of two varieties. on all the categories of safflower seeds of two varieties. PBNS-12 variety showed maximum seed-borne fungi. SH and CR categories showed maximum association of fungi. *Alternaria dianthicola* showed its quantitative dominance on PBNS-12 variety. *Rhizopus stolonifer* associated with SH and CR category of PBNS-12 variety. *Macrophomina phaseolina* showed its quantitative dominance only on PBNS-12 variety. *Fusarium oxysporum* occurred on CR category of PBNS-12 and US category of Bhima. CR category of Bhima showed association of *Fusarium equiseti, Penicillium chrysogenum, Penicillium notatum, Alternaria dianthicola, Trichoderma viridae* and *Curvularia lunata*. Only SH category of both the variety showed association of *Curvularia pellescens* (Table 1). Such type of work was earlier supported by several workers (ISTA, 1996; Agrawal, 1976; Chavan and Kakde, 2009a). Out of twenty 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 nutritional sources to lipase production.

Table 1: Abnormal safflower seed mycoflora on PDA

Fungi	Varieties					
	PBNS-12			Bhima		
	SH	US	CR	SH	US	CR
Aspergillus niger	20	10	-	-	20	10
Aspergillus flavus	-	-	10	20	-	10
Aspergillus terreus	-	-	10	-	-	-
Aspergillus ustus	-	-	-	10	10	-
Rhizopus stolonifer	30	-	10	20	-	-
Mucor sp.	20	-	10	-	-	-
Macrophomina phaseolina	20	10	30	-	-	-
Fusarium oxysporum	-	-	30	-	20	-
F. chlamydosporum	10	-	-	-	-	-
Fusariium roseum	-	-	10	-	-	-
Fusarium equiseti	10	-	30	-	-	10
Penicillium chrysogenum	-	10	-	20	-	20
Penicillium notatum	-	-	40	-	-	30
Alternaria dianthicola	40	-	10	-	-	10
Alternaria tennuisima	-	-	-	-	10	-
Trichoderma harzianum	-	-	10	-	-	-
Trichoderma viridae	10	-	10	-	-	20
Curvularia lunata	30	-	-	-	-	20
Curvularia pellescens	10	-	-	20	-	-

SH- Shrunkened; US- Undersized; CR- Cracked

Effect of Carbon sources

It was found that carbon sources like fructose (monosaccharides) and sucrose (disaccharides) induced lipase activity while lactose (disaccharides), starch and carboxyl methyl cellulose (CMC) (polysaccharides) inhibited lipase activity. *Alternaria dianthicola, Curvularia lunata, Fusarium oxysporum* and *Penicillium chrysogenum* produced maximum lipase enzyme in presence of carbon sources as compared to other storage fungi (Table 2) (Fig. 1).

Fig. 1 Production and assay of lipase enzyme

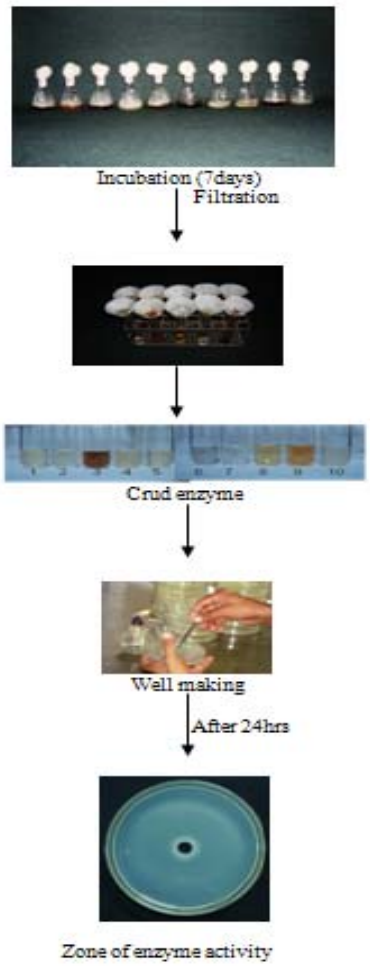
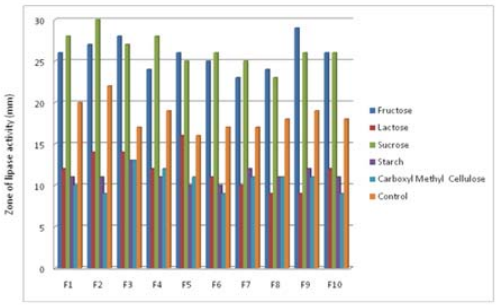


Table 2: Effect of Carbon sources on lipase enzyme activity of fungi from abnormal oilseeds

Sr no	Carbon Sources	Fungi									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	Fructose	26	27	28	24	26	25	23	24	29	26
2	Lactose	12	14	14	12	16	11	10	09	09	12
3	Sucrose	28	30	27	28	25	26	25	23	26	26
4	Starch	11	11	13	11	10	10	12	11	12	11
5	Carboxyl Methyl Cellulose	10	09	13	12	11	09	11	11	11	09
6	Control	20	22	17	19	16	17	17	18	19	18
	C.D. (at 0.05)	8.3	9.1	7.3	7.5	7.1	8.0	6.7	6.9	8.8	7.9

Graph. 1 Effect of carbon sources on lipase enzyme activity of fungi from abnormal oilseeds



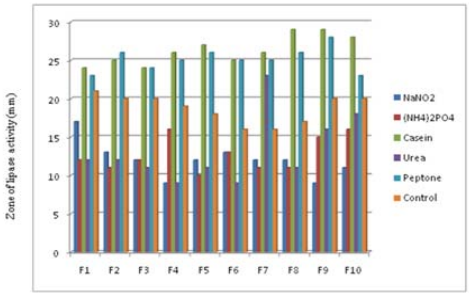
Effect of Nitrogen sources

Nitrogen sources like nitrate, nitrite, amide, ammonium, and protein showed its effect on lipase enzyme of fungi. Casein and peptone which are organic forms stimulated maximum lipase enzyme production of storage fungi. Both the species of *Penicillium* viz. *Penicillium notatum* and *Penicillium chrysogenum* showed maximum extracellular lipase activity in presence of casein and Peptone. Urea which is an amide form and ammonium phosphate which is an ammonium form hampered the extracellular lipase enzyme production of seed-borne fungi (Table 3).

Table 3: Effect of Nitrogen sources on lipase enzyme activity of fungi from abnormal oilseeds

Sr no	Nitrogen Sources	Fungi									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	$NaNO_2$	17	13	12	09	12	13	12	12	09	11
2	$(NH_4)_2PO_4$	12	11	12	16	10	13	11	11	15	16
3	Casein	24	25	24	26	27	25	26	29	29	28
4	Urea	12	12	11	09	11	09	23	11	16	18
5	Peptone	23	26	24	25	26	25	25	26	28	23
6	Control	21	20	20	19	18	16	16	17	20	20
	C.D. (at 0.05)	5.6	7.0	6.5	7.8	8.0	7.0	7.0	8.1	8.2	6.1

Graph.2 Effect of nitrogen sources on lipase enzyme activity of fungi from abnormal oilseeds



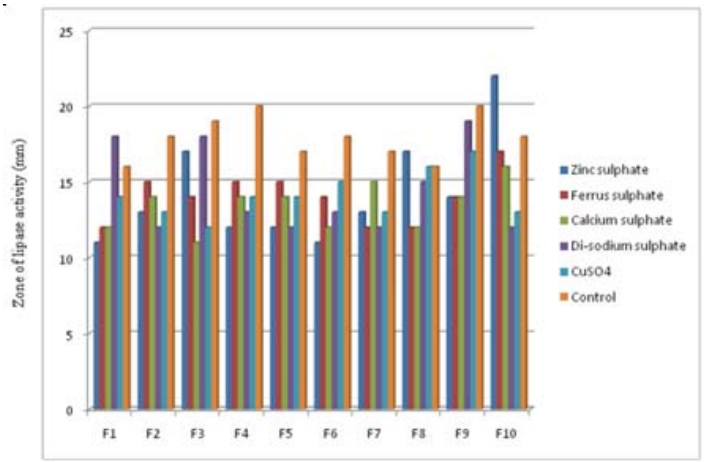
Effect of Sulphur sources

In case of sulphur sources; Calcium sulphate and di-sodium sulphate stimulated maximum lipse activity. *Alternaria dianthicola* in presence of di-sodium sulphate showed maximum extracellular lipase production. Lipase activity of *Penicillium chrysogenum* was hampered in zinc sulphate, ferrus sulphate and calcium sulphate as compared to control. Lipase activity of *Fusarium oxysporum, Fusarium equiseti* and *Macrophomina phaseolina* was low in sulphur source like zinc sulphate and ferrus sulphate. Lipase activity of *Trichoderma viridae* was stimulated in presence of sulphur source like ferrus sulphate (Table 4).

Table 4: Effect of Sulphur sources on lipase enzyme activity of fungi from abnormal oilseeds

Sr no	Sulphur Sources	Fungi									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	Zinc sulphate	11	13	17	12	12	11	13	17	14	22
2	Ferrus sulphate	12	15	14	15	15	14	12	12	14	17
3	Calcium sulphate	12	14	11	14	14	12	15	12	14	16
4	Di-sodium sulphate	18	12	18	13	12	13	12	15	19	12
5	$CuSO_4$	14	13	12	14	14	15	13	16	17	13
6	Control	16	18	19	20	17	18	17	16	20	18
	C.D. (at 0.05)	3.4	2.2	3.4	2.9	4.8	2.6	2.0	4.4	7.0	3.7

Graph.3 Effect of sulphur sources on lipase enzyme activity of fungi from abnormal oilseeds



Effect of Phosphorus source

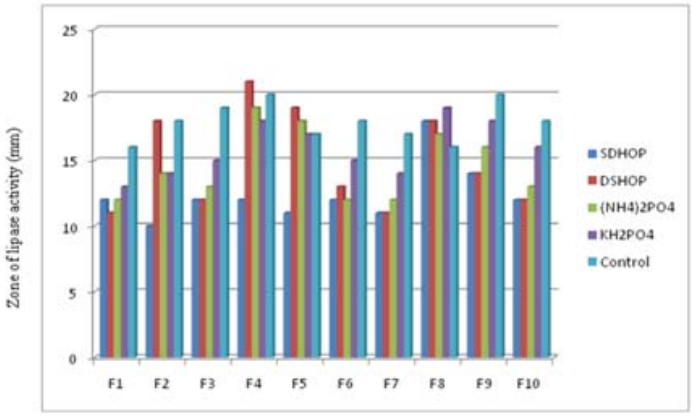
In case of phosphorus sources, *Fusarium oxysporum* and *Fusarium equiseti* produced more extracellular lipase in presence of di-sodium hydrogen ortho-phosphate, ammonium phosphate and potassium di-hydrogen ortho-phosphate. Sodium di-hydrogen ortho-phosphate and di-sodium hydrogen ortho-phosphate reduced the lipase activity of *Curvularia lunata, Rhizopus stolonifer, Penicillium chrysogenum* and *Penicillium notatum* (Table 5).

Table 5: Effect of Phosphorus sources on lipase enzyme activity of fungi from abnormal oilseeds

Sr no	Phosphorus Sources	Fungi									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	SDHOP	12	10	12	12	11	12	11	18	14	12
2	DSHOP	11	18	12	21	19	13	11	18	14	12
3	$(NH4)_2PO_4$	12	14	13	19	18	12	12	17	16	13
4	KH_2PO_4	13	14	15	18	17	15	14	19	18	16
5	Control	16	18	19	20	17	18	17	16	20	18
	C.D. (at 0.05)	2.5	4.0	3.5	4.2	3.7	3.0	3.0	3.0	3.1	3.2

SDHOP- Sodium di-hydrogen ortho-phosphate; DSHOP- Di-sodium hydrogen ortho-phosphate

Graph. 4 Effect of phosphorus sources on lipase enzyme activity of fungi from abnormal oilseeds



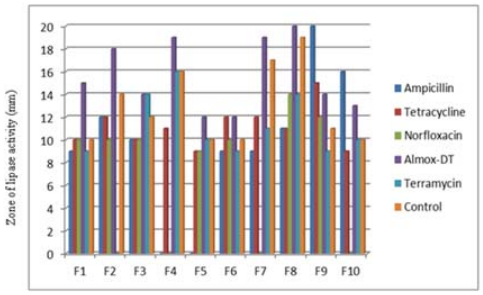
Effect of Antibiotic sources

Alternaria dianthicola showed minimum lipase activity in presence of Ampicillin and terramycin. ampicillin, norfloxacin and tetracycline reduced the lipase production of storage fungi. Lipase activity of *Fusarium oxysporum* was completely hampered due to ampicillin and norfloxacin while, almox-DT stimulated the lipase production of *Fusarium oxysporum.* Norfloxacin inhibited the lipase activity of *Rhizopus stolonifer* and *Trichoderma viridae. Curvularia lunata* produced the maximum lipase enzyme in presence of almox-DT while, lipase activity was completely hampered due to terramycin (Table 6).

Table 6: Effect of Antibiotics sources on lipase enzyme activity of fungi from abnormal oilseeds

Sr no	Antibiotics	Fungi									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	Ampicillin	09	12	10	-	-	09	09	11	20	16
2	Tetracycline	10	12	10	11	09	12	12	11	15	09
3	Norfloxacin	10	10	10	-	09	10	-	14	12	-
4	Almox-DT	15	18	14	19	12	12	19	20	14	13
5	Terramycin	09	-	14	16	10	09	11	14	09	10
6	Control	10	14	12	16	10	10	17	19	11	10
	C.D. (at 0.05)	5.6	6.2	2.0	8.7	4.3	1.6	6.9	4.0	3.9	5.7

Graph. 5 Effect of antibiotic sources on lipase enzyme activity of fungi from abnormal oilseeds



Effect of Vitamin sources

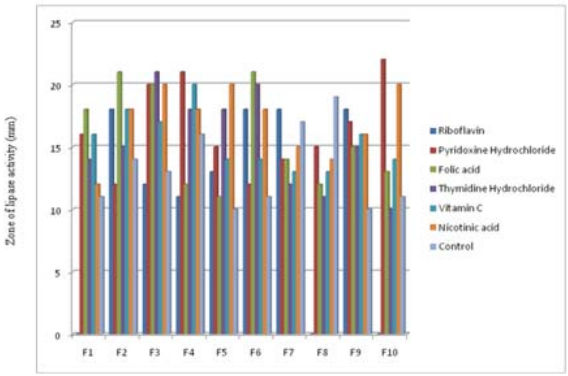
Riboflavin completely hampered lipase activity of *Alternaria dianthicola, Penicillium notatum* and *Trichoderma viridae* while folic acid stimulated the lipase production of *Curvularia lunata, Alternaria dianthicola* and *Macrophomina phaseolina*. *Penicillium notatum* and *Trichoderma viridae* did not produce the lipase enzyme in presence of riboflavin. Pyridoxine hydrochloride stimulated maximum lipase production in *Fusarium oxysporum* and *Trichoderma viridae. Curvularia pellescens* and *Macrophomina phaseolina* produced the maximum lipase due to thymidine hydrochloride as compared to other storage fungi. *Fusarium oxysporum* and *Curvularia lunata* produced maximum lipase in presence of vitamin C. Nicotinic acid stimulated maximum lipase in *Curvularia pellescens, Fusarium equiseti* and *Trichoderma viridae* (Table-7).

Table 7: Effect of Vitamins sources on lipase enzyme activity of fungi from abnormal oilseeds

Sr no	Vitamins	Fungi									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	Riboflavin	-	18	12	11	13	18	18	-	18	-
2	Pyridoxine Hydrochloride	16	12	20	21	15	12	14	15	17	22
3	Folic acid	18	21	20	12	11	21	14	12	15	13
4	Thymidine Hydrochloride	14	15	21	18	18	20	12	11	15	10
5	Vitamin C	16	18	17	20	14	14	13	13	16	14
6	Nicotinic acid	12	18	20	18	20	18	15	14	16	20
7	Control	11	14	13	16	10	11	17	19	10	11
	C.D. (at 0.05)	5.5	2.8	3.4	3.5	3.3	3.6	1.9	5.3	2.3	6.6

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*

Graph. 6 Effect of vitamin sources on lipase enzyme activity of fungi from abnormal oilseeds



Chavan and Kakde, (2009b) studied the lipase enzyme activity of storage fungi under the influence of carbon and nitrogen sources. They found that carbon sources as like fructose and sucrose induces lipase activity while starch, lactose and carboxyl methyl cellulose (CMC) inhibits lipase activity. Nitrogen sources as like nitrate, nitrite, amide, ammonium, and protein affect in different ways on lipase enzyme of fungi. Ely (1988) observed that carbohydrates were good source of growth of *Rhizopus oligoporus* but low lipase production was obtained. Kakde et. al., (2009) reported the impact of nutritional factors on lipase enzyme production.The fungi may survive by production of lipase on the surface of seed or inside the seed in the off season. Therefore, the nutrients which are unfavorable for lipase production may help in control of seed-borne diseases. Considering this situation, it was thought worthwhile to investigate the effect of different nutritional factors on degree of lipase production. Such nature of inhibition in different sources may be recommended for the production of different systematic fungicides which will be helpful to control field fungi as well as storage fungi.

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