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Extracellular Hydrolytic Enzymes Action of *Alternaria* Species under the Influence of Different Nutritional Sources

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Abstract

Influence of nutritional sources like carbohydrates, nitrogen, phosphorous and sulpher on amylase, lipase and protease action of six *Alternaria* species viz. *A. alternata, A. citri, A. crassa ,A. macrospora, A. dianthicola* and *A. tenuissima* were studied. It was reported that Carboxy methyl cellulose (CMC), ferrous sulphate, calcium sulphate, sodium sulphate, copper sulphate and sodium dihydrogen orthophosphate retarded the enzyme action of some *Alternaria* species.

1. Introduction

Seed is the basic and most vital input in crop production. About 90 % of world food crops are produced by using seeds. The seeds are also found to be responsible for transmission of diseases. More than 3000 diseases are known to be transmitted through seeds. This transmission takes place either in the field or in storage conditions. It is accepted that over all losses due to diseases can vary from 10 – 25 % annually through out the world. In India even it will take conservative estimates of around 15 % losses we are losing an average 30 metric tonnes of food grains, 4 metric tonnes of oilseed, 36 metric tonnes of cane, 23 metric tonnes of fruit and vegetables. The incidence of seed-borne pathogens mainly depends on climatic or physical conditions under which the seed crops are grown in the field. Similarly, storage conditions also enhance to develop various types of seed damages due to associated seed-borne pathogens such seeds show great loss in their chemical content. Neergaard (1977) reported several types of such abnormalities which mainly include seed abortions, sunken seeds, reduction in seed size, seed rots sclerotisation of seed, seed necrosis, loss in germinability, seed discolouration, toxification and other physiological disorders. Such seeds are considered poor in quality for seed industry and also for consumption. Fungi secrete hydrolytic enzymes and spoil the seed contents. Considering the fact attempt were made to study the impact of nutrional sources on hydrolytic enzyme production of *Alternaria* species.

2. Materials and Methods

Production of amylase

Production of amylase(s) was studied by growing the fungi in liquid medium containing 1%, KNO_3 0.25%, KH_2PO_4 0.1.% and $MgSO_4.7H_2O$ 0.05%, pH of the medium was adjusted at 5.5. twenty five ml of the medium was poured in 100ml conical flasks autoclaved and inoculated separately with 01 ml spore suspension of the fungi which were grown for 7 days on PDA slants. Unless otherwise stated, the flasks were incubated for 6 days at 25 ± 1°C with diurnal periodicity of light. On 7th day, the flasks were harvested by filtering the contents through Whatman filter No.1. The filtrates were collected in presterilized bottles and termed as crude enzyme preparation.

Assay method for amylase enzymes (Cup-plate method)

Determination of amylase activity was done with the help of cup-plate method which was adopted by Singh and Saxena (1982), where 20ml of starch agar assay medium (soluble starch – 10gm, Na_2HPO_4 – 2.84gm, NaCl – 0.35gm, Agar agar 20gm, distilled water 1000ml and pH 6.9) was poured in each petriplate. On solidification of the medium, a cavity (08 mm diameter) was made in the centre with the help of a cork borer (No.4) and was filled with 1ml culture filtrates (crude enzyme preparation) of the test fungi. The plates were incubated at 28°C for 24 hours, then they were flooded with Lugol's iodine solution as an indicator. A clear, non blue, circular zone obtained surrounding the central cavity; diameter of the zone was measured (mm) as the amylase activity zone. Similar

procedure followed for the control except pouring of culture filtrates in the central cavity instead of the activity enzyme.

Production of lipase

Lipase activity was studied by growing the fungi in liquid medium at pH5.6 containing oil-10ml, KNO_3 -2.5g, KH_2PO_4- -1.0g, $MgSO_2$ – 0.5g and distilled water 1000ml. Different sources of carbon, nitrogen, phosphorus and sulpher sources were added separately by replacing corresponding compounds in the above basal medium. 25ml of the medium was poured in 100ml conical flasks and autoclaved at 15 1bs pressure for 30 minutes, then on cooling the flasks were inoculated separately with 1.0ml spore suspension of the fungi which were incubated for 7 days at 25± 1°C with diurnal periodicity of light. On 7th day, the flasks were harvested by filtering the contents through Whatman filter paper no.1. The filtrates were collected in presterilized 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- 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°C. After 24 hours, a clear circular zone was measured (mm) as lipase activity.

Production of protease

Production of protease(s) was made by growing the fungi on liquid medium containing glucose 10g, gelatine 10g, dipotassium hydrogen phosphate 1.0g, $MgSO_4.7H_2O$ 500mg and distilled water 1000ml pH of the medium was adjusted at 5.5. Twenty five ml of medium was poured in 100ml Erlenmeyer conical flasks and autoclaved at 15lbs pressure for 20 minutes. The flasks on cooling were inoculated separately with 01 ml standard spore/mycelial suspension of test fungi prepared from 7 days old cultures grown on PDA slants. The flasks were incubated for 6 days at 25 ± 1°C with diurnal periodicity of light. On 7th day, the flasks were harvested by filtering the contents through Whatman's filter No.1. The filtrates were collected in the presterlised bottles and termed as crude enzyme preparation.

Assay method (Cup-plate method)

Determination of protease(s) activity was done with the help of cup plate method, adopted by Hislop *et.al.* (1982) and Rajamani (1990). A basal medium was prepared by adding 2 % (W/V) agar and one percent (W/V) gelatin. pH of the medium was adjusted at 5.6 with Mcllavaine's buffer. Then it was sterlized at 15lbs pressure for 15 minutes. About 15ml of the medium was poured in presterilized petriplates under aseptic conditions. On solidification 6mm diameter cups/cavities were made in the centre of each of the agar plate with a sterilized cork borer (No.4). The cups/cavities were filled carefully with about 0.5ml of culture filtrate (crude enzyme preparation). The plates were incubated at 25°C for 24 hours. Then the plates were flooded with 15 percent mercuric chloride in 7NHCl. After 10 minutes of standing, a clear transparent zone indicated the hydrolysis of gelatin by extra cellular proteolytic enzymes, whereas the rest of the region of the petriplates became opaque due to the coagulation of gelatin (protein) by mercuric chloride. Diameter of the clear zone was used as measure (mm) of protease activity, while non appearance of clear zone considered absence of protease(s) in the culture filtrates.

3. Results and Discussion

It is observed from the table 1 that all six species of *Alternaria* produced amylase. However CMC proved highly inhibitory for amylase production where as glucose and fructose stimulated the amylase activity in *Alternaria alternata, A. crassa, A. dianthicola* and *A. tenuissima.* Lipase production was found to be stimulated in the presence of disaccharides and polysaccharides in all the species of *Alternaria.* Protease activity was favored in the presence of fructose & sucrose as compared with glucose. However *Alternaria citri, A. crassa and A. dianthicola* retarded the activity in the presence of CMC.

Amylase production of A. *citri* totally inhibited by sodium nitrites whereas amylase action of most of the *Alternaria* species stimulated in presence of peptone, gelatin, casein, urea and sodium nitrate. It was observed that all the species of *Alternaria* stimulated lipase and protease production in the presence of different nitrogen sources (Table 2). It is observed from table 3 that amylase production of *Alternaria, A. crassa. A. macrospora* and *A. tenuissima* was totally inhibited in the presence of sodium

dihydrogen orthophosphate where as disodium hydrogenorthophosphate dihydrate and diammonium phosphate stimulated the amylase production in all species of *Alternaria*. All the sources of phosphorus stimulated lipase and protease production in all *Alternaria* species.

Amylase production of *A. tenuissima* inhibited in the presence of ferrous sulphate and sodium sulphate. Calcium sulphate inhibited lipase production of *A. crassa*, Lipase activity of *A. dianthicola* inhibited in the presence of ferrous sulphate, sodium sulphate and copper sulphate where as lipase production of *A. tenuissima* was inhibited in ferrous sulphate and zinc sulphate only. Protease activity of *Alternaria alternata, A. citri* and *A. teuissima* also inhibited in different sources sulphur (Table 4).

There are several reports that nitrogen and carbon sources behaves differently against lipase enzyme activity of oilseeds. Such type of work was earlier carried out by Sandikar. and Mukadam (1992), reported that stimulatory effect of different nitrogen sources for liipase production in seed-borne fungi. Rathod .(2007) observed that disaccharides and polysaccharides stimulates lipase enzyme activity and nitrogen sources as like Calcium nitrate, caesin, gelatin and peptone also increases lipase enzyme activity. Kesare (2009) found that nitrogen sources as like sodium nitrate, sodium nitrate, ammonium phosphate, ammonium sulphate, urea, gelatin and peptone inhibit lipase enzyme activity whereas, casein stimulates lipase enzyme activity of *Aspergillus glaucus, Fusarium roseum* and *Spicaria violecia* while sodium nitrate stimulates lipase enzyme activity of *Curvularia lunata.* Kakde and Chavan (2009) observed that fructose and sucrose stimulates lipase activity while lactose, carboxyl methyl cellulose and starch inhibited lipase activity. Sharma and Satyanarayana (1980) found that carbohydrate sources affects protease enzyme activity of *Helminthosporium, Curvularia* and *Alternaria* s*p.* Patil and Shastri (1982) reported that fructose and sucrose stimulated protease production in Alternaria alternata but glucose was found to no effect on protease activity.

It can be concluded that Carboxy methyl cellulose (CMC), ferrous sulphate, calcium sulphate, sodium sulphate, copper sulphate and sodium dihydrogen orthophosphate retarded the production of lipase, amylase and protease of some *Alternaria* species, such nature of inhibition of these may be useful to control the spoilage of seeds by fungi.

Table 1 Effect of carbohydrates on enzyme production of *Alternaria* species

Species of *Alternaria*	Carbohydrates					
	Glucose ©	Fructose	Maltose	Sucrose	CMC	Starch
	Amylase Production					
A. alternata	15	22	18	11	10	12
A. citri	12	14	13	15	10	13
A. crassa	17	20	19	19	14	12
A. dianthicola	13	20	21	16	11	12
A. macrospora	13	12	10	18	10	12 ↓
A. tenuissima	16	22	14	12	10	14 ↓
	Lipase Production					
A. alternata	18	22	24	26	25	20
A. citri	21	24	17	21	20	15
A. crassa	18	20	21	25	21	22
A. dianthicola	28	25	20	29	30	24
A. macrospora	14	27	20	23	17	21

A. tenuissima	21	24	27	27	26	20
Protease Production						
A. alternata	18	22	24	26	25	20
A. citri	21	24	17	21	15	20
A. crassa	22	20	18	16	20	19
A. dianthicola	18	17	15	24	20	17
A. macrospora	20	12	20	21	25	22
A. tenuissima	20	22	17	19	21	20

Activity zone in mm

Table 2 Effect of nitrogen sources on enzyme production of *Alternaria* species

Species of *Alternaria*	Sources of nitrogen							
	KNO3 Control (C)	Sod Nitrate	Calcium Nitrate	Sod Nitrate	Urea	Casein	Gelatine	Peptone
Amylase Production								
A. alternata	10	14	14	18	17	17	18	18
A. citri	10	-	12	15	15	12	13	13
A. crassa	12	13	05	16	11	15	12	16
A. dianthicola	11	13	10	17	15	20	17	20
A. macrospora	12	17	10	18	11	14	15	15
A. tenuissima	08	15	15	17	14	15	15	16
Lipase Production								
A. alternata	21	15	20	17	20	24	20	17
A. citri	20	20	24	20	19	18	20	29
A. crassa	17	20	18	22	25	21	18	20
A. dianthicola	15	17	20	19	21	25	22	31
A. macrospora	18	17	20	18	15	27	24	20
A. tenuissima	20	28	21	25	24	25	25	29
Protease Production								
A. alternata	16	18	17	20	20	15	19	19
A. citri	20	14	15	18	17	20	24	20
A. crassa	18	19	18	14	17	19	20	15
A. dianthicola	20	17	17	13	20	16	22	18
A. macrospora	21	23	24	20	19	14	14	20
A. tenuissima	17	20	17	13	20	22	24	21

Activity zone in mm

Table 3 Effect of phosphorus sources on enzyme production of *Alternaria* species

Species of *Alternaria*	Sources of phosphorus			
	Potassium di Hydrogen Orthophosphate (c)	Di.sod. Hydrogen Orthopho. Dihydrate.	Sod.dihydrogen Orthosphosph Ate.	Diammonium Phosphate.
	Amylase Production			
A. alternata	10	11	12	12
A. citri	10	09	10	13
A. crassa	08	08	-	14
A. dianthicola	14	15	10	10
A. macrospora	13	12	-	12
A. tenuissima	13	11	-	15
	Lipase Production			
A. alternata	25	27	23	28
A. citri	25	28	26	29
A. crassa	25	29	27	26
A. dianthicola	28	25	30	25
A. macrospora	25	27	23	25
A. tenuissima	25	23	33	29
	Protease Production			
A. alternata	18	18	19	18
A. citri	21	28	20	19
A. crassa	20	21	23	24
A. dianthicola	24	26	21	18
A. macrospora	26	23	21	20
A. tenuissima	20	20	20	19

Activity zone in mm

Table 4 Effect of sulphure sources on enzyme production of *Alternaria* species

Species of *Alternaria*	Sources of sulphure					
	Magnecium Sulphate (c)	Ferrous Sulphate	Sodium sulphate	Calcium sulphate	Zinc sulphate	Copper sulphate
	Amylase Production					
A. alternata	08	12	12	10	10	13
A. citri	12	13	14	15	15	15
A. crassa	08	10	17	12	08	18
A. thinla	10	11	14	09	10	12
A. macrospora	07	12	15	14	14	14

A. tenuissima	11	10	10	12	12	11
Lipase Production						
A. alternata	20	29	27	23	25	21
A. citri	18	24	29	25	25	20
A. crassa	19	20	22	18	23	25
A. dianthicola	22	20	20	25	21	20
A. macrospora	25	27	25	28	28	22
A. tenuissima	24	20	28	25	20	25
Protease Production						
A. alternata	24	18	28	19	19	24
A. citri	23	20	20	20	18	18
A. crassa	23	28	25	29	26	20
A. dianthicola	20	27	20	24	22	25
A. macrospora	30	32	24	28	20	24
A. tenuissima	25	18	19	18	20	15

Activity zone in mm

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