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Semi-Solid Agar Medium for Detection of Fungal Enzymes

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The fungal plant pathogens were isolated from different host plants grown at different locations in Egypt. *Alternaria alternata, Alternaria solani, Curvularia lunata, Fusarium solani, Fusarium oxysporum, Macrophomina phaseolina, Pyrenochaeta lycopersici, Rhizoctonia solani, Stemphylium botryosum, Trichoderma viride* and *Thielaviopsis basicola* were recovered. Polygalacturonase (PG), chitinase (CH), cellulase (Cx) were determined using a suggested modified plate method with very low agar content (5gm/l). All fungal isolates produced remarkable activity of PG, CH and produced less cellulase (81.8% frequency) at 28°C. Six isolates (54.5%) were scored active PG producers namely *T. viride, Th. basicola, F. oxysporum, P. lycopersici, M. phaseolina, S. botryosum,* four isolates (36.4%) were moderate in this regard, *R. solani* (9.1%) was scored non producer at 21°C. Similar variation in Cx for the activity of three fungal isolates (27.3%) was recognized as active producers for *T. viride, Th. basicola, F. oxysporum* and three isolates (27.3%) were scored as moderate producers for *P. lycopersici, F. solani, A. solani.* on the other hand, five isolates, (45.5%) were found to be non-producers in this regard, *A. alternata, R. solani, M. phaseolina, S. botryosum* and *C. lunata* at 21°C. Moreover, two fungal isolates (18.2%) were highly active producers of chitinase (CH) activity for *F. oxysporum* and *F. solani.* Three isolates (27.3%) failed to produce chitinase (CH) under the conditions of the experiment for *A. alternata, R. solani, M. phaseolina,* though six isolates (54.5%) were found to be moderate chitinase (CH) producers by *A. solani, C. lunata, P. lycopersici, S. botryosum, T. viride* and *T. basicola.* Seven fungal isolates (63.6%) reacted positively at higher temperature (28°C) and higher production of (PG), (CH) and (Cx). The highest temperature tends to increase qualitatively the enzyme activity, while lower temperature decreased such effect. Four isolates, *F. oxysporum, Th. basicola, T. viride,* and *P. lycopersici* were scored active PG, CH and Cx at two different temperatures, 21 and 28°C, at four days incubation**.** It is worth noting that the semi solid agar medium (5 gm. agar/l) is being favorable for accurate detection of PG, Cx, and CH. Further trial with the modified semi-solid agar medium for evaluation of other enzymes involved in pathogenicity are needed.

Keywords: Bromo cresol purple, chitinase, cellulase, N-acetyl-glucosamine, semi-solid agar, PG, Cx, CH, relative enzyme.

Plant pathogenic organisms are able to produce a wide range of cell-wall-degrading enzymes (Amit *et al*., 2014). The pectinases (a group of pectinolytic enzymes) are the first enzymes considered by most fungal pathogens when attacking plant cell walls, followed by hemicellulases and cellulases (Vallejo Herrera *et al*., 2004). Pectinase is produced by a large number of microorganisms including bacteria, actinomycetes, yeasts, and filamentous fungi (Gomes *et al.,* 2009).

A positive correlation has been established between the production of pectinolytic enzymes, virulence and disease symptoms in several path systems (Kikot *et al*., 2009). Gawade *et al*., (2017) reported that pathogenic fungi are producing large quantities of PG in culture and in inoculated tissue is being correlated with their virulence.

Many plant pathogenic organisms are capable of degrading cellulose by producing a cellulase complex which involves the synergistic action of three main enzymatic complexes, endoglucanase, exoglucanase that releases either glucose or cellobiose, and β-1,4-glucosidase that hydrolyzes cellobiose and cellodextrins to glucose (Okunowo *et al*., 2010). Fungi belonging to genera such as *Trichoderma, Penicillium, Aspergillus, Myrothecium, Fusarium* and *Chaetomium* species etc., produce cellulases under suitable conditions (Sherief *et al*, 2010).

Zhang *et al*. (2014) reported that Cx degrades cellulose to cellobiose and is being correlated with virulence of pathogens (Zhou *et al*., 2016). The activity has been observed in culture and in diseased tissue inoculated with pathogens such as *Colletotrichum acutatum* (Fernando *et al*., 2001) and *Fusarium sulphureum* (Yang *et al*., 2012). *Thanatephorus cucumeris* (Zhao *et al*., 2014).

Chitin is one of the most abundant nitrogenous carbohydrate of ecosystem (Schickler *et al*., 1998). It is composed of β-(1-4) linked N-acetyl-D-glucosamine units. Chitinases are chitin-degrading enzymes that hydrolyze the β-1, 4-glycosidic bonds between the N-acetyl glucosamine residues of chitin and are widely distributed in nature (Kitamura and Kamei, 2003). They are well known producers of chitinolytic enzymes and used commercially as a source of these components. Additional interest in these enzymes is stimulated by the fact that chitinolytic strains of *Trichoderma* are among the most effective agents of biological control of plant diseases (Karlsson *et al*., 2010). Using natural substrates or derived from natural one, the detection of enzyme activity relies on chemical redox reaction (Ferrari *et al*., 2014). Fungal chitinase have also been observed to play a key role in the nutrition, morphogenesis, and developmental processes in fungi (Sharma *et al*., 2018).

The optimum growth temperatures for the majority of fungi has been to fall between temperature(s) 25°C to 30°C. Above 40°C, the growth is being retarded poor and, in some cases, mortality may occur (Sharma and Rajak, 2003). In earlier reports, *F. oxysporum* was found to reach its maximum growth rate at between 25-

30°C (Mogensen *et al*., 2009). *Trichoderma viride* has been reported to reach its maximum growth at 30°C (Ramanathan and Vinodhkumar, 2013).

Plant pathogenic fungi actively kill and degrade plant tissue and utilize liberated carbohydrates and proteins compounds for growth and reproduction (King *et al.*, 2011). The mechanism involved in pathogenicity is by mainly secreting enzymes (King *et al*., 2011) and hence the pathogen was screened for different enzymes.

A number of fungal molecules, like cell wall degrading enzymes (CWDEs), pathogen related proteins and enzymes involved in toxin synthesis, are known to contribute to fungal pathogenicity and virulence (Gonzalez-Fernandez and Jorrin-Novo, 2012).

The present study was following extracellular fungal enzymes on semi-solid agar medium like polygalacturonase (PG), chitinase (CH), cellulase (Cx) at different temperatures. Screening, isolation characterization and production of more efficient extracellular enzyme producing fungi, were considered.

Materials and Methods

Fungal isolates and identification

The tested fungi were isolated from different plant species, locations and the pathogenic fungi were recovered, tested and maintained on slants of potato dextrose agar (PDA) at 4°C. Identification was carried out according to their cultural and morphological features according to the descriptions of Neergaard, (1945), Hansford, (1946), Rifai (1969), Barnett and Hunter (1972), Nelson *et al*., (1983), Carling and Summer (1992). Species identification was run by examining both macroscopic and microscopic features of a seven-day old pure cultures.

Experimental design:

The experimental design was considering media, isolates, temperature, and enzymes, with three replications, each replicate consisted of a single Petri dish (90 mm diameter). Control treatments were all the media not colonized by the fungi isolates.

Modified agar medium for detection of fungal enzymes:

Detection medium (Agrawal and Kotasthans, 2012) was used in principle after modification. Data (Table 1) show the basal medium comprising (g/liter) 0.3 $MgSO_4.7H_2O$, 3.0 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 1.0 citric acid monohydrate, 5 agar, 0.5 Na_2SO_3, 5.0 Peptone, 4.5 colloidal chitin or cellulase or Polygalacturonase or non and 0.15 bromo cresol purple; pH was adjusted to 7.0 and then autoclaved at 121°C for 15 min. (suggested modified solid medium).

It is worth noting that solidification of the agar was made by only 5 g agar and 0.5 g Na_2SO_3. The peptone ingredient was added to give optimal growth.

Fungal isolates growth was evaluated based on the development of the mycelium on the partly solidified suggested medium. The plates were inoculated with a 5 mm-diameter agar disc taken from the actively growing mycelium on PDA medium incubated at 21 and 28°C for 4 days and finally the assay was carried out in three replicates and data were presented as mean.

It is worth noting that the suggested medium was different from that of Agrawal and Kotasthans (2012) in the degree of agar setting of the medium that being added as only 5g agar in addition to 0.5 g Na_2SO_3, sodium sulphite and 5.0g peptone to detect enzyme(s) in partly semi solid matrix, tentatively resembling plant tissues, instead of the usual liquid assays. The components of the assay medium are shown in Table (1).

Table (1): Composition of the medium.

No.	Components of medium/l	Control	Pectin	Cellulose	Chitin
*1	Agar	5.0g	5.0g	5.0g	5.0g
2	Substrate	-	4.5 g	4.5 g	N-Acetyle glucosamine 4.5 g
3	$MgSO_4.7H_2O$	0.3 g	0.3 g	0.3 g	0.3g
4	$(NH_4)_2SO_4$	3.0 g	3.0 g	3.0 g	3.0 g
5	KH_2PO_4	2.0g	2.0g	2.0g	2.0g
6	Citric acid monohydrate	1.0 g	1.0g	1.0g	1.0g
*7	Na_2SO_3	0.5g	0.5g	0.5g	0.5g
*8	Peptone	5.0g	5.0g	5.0g	5.0g
9	Bromo cresol purple	-	0.15 g	0.15 g	0.15 g
10	PH	7	7	7	7

***** The stepwise sequence of the study.

1- Fungal inocula on PDA at pH 7.
2- Fresh culture of fungi in concern propagated and inoculated on different substrates at two different temperature levels, 21 and 28°C for 4 days.
3- Observation of color change from yellow (acid) to purple (alkaline). The color of the medium before autoclaving is purple, shifted to yellow after pH drop caused by autoclaving and again to purple by the action of enzymes.
4- Three replicates for each isolate.
5- Measurement of extracellular enzyme activity on the plate was made in three replicates**.** For each replicate, the diameter of the colony growth and the surrounding halo was carried out. The index of relative enzyme activity (RA) was calculated according to Krishnan *et al*. (2011).

$$\text{Relative enzyme activity (RA)} = \frac{\text{Colored halo diameter - Colony diameter}}{\text{Colony diameter}}$$

6- For the screening it was determined that a RA value of 1 or greater was classified as having significant enzyme activity (Duncan *et al*., 2008).

Enzymes activity:

Cellulase activity (Cx):

Cellulase secretion was detected by growing fungi in the modified agar medium. The plates were incubated at 21 and 28°C for three to seven days. Finally, plates were observed for the formation of yellow-colored haloes around the inoculated discs, following the method of Teather and Wood (1982).

Chitinase activity (CH):

Enzymatic hydrolysis of colloidal chitin was assayed following the release of free N-Acetyleglucosamine (NAG) from colloidal chitin by clearing halo assay method (Frandberg and Schnurer,1998).

Polygalacturonase activity (PG):

Polygalacturonase secretion was detected by growing fungi in agar medium. Clear halo formed around the fungal colony indicates pectinolytic activity (Bijesh *et al*., 2015). The diameter of colonies and clear zones were measured for calculation of relative enzyme (RA) activity.

Effect of temperature:

Temperature is also an important factor that influences the fungal extracellular enzyme activity also depends on the strain variation of the microorganism. The fungal cultures were plated on the suggested modified agar medium, using 0.5 mm cork borer disc, and incubated at both 21°C and 28°C for 4 days.

Statistical analysis:

Data were compared by the analysis of variance according to the procedures of Snedecor and Cochran (1980). Means of all treatments were compared by the least significant difference LSD at 5% level.

Results

Fungal isolation and identification:

The tested fungi were isolated from nine different diseased plants species, i.e., Bean, Cantaloupe, Eggplant, Lettuce, Pea, Pepper, Peanut, Spinach, Tomato collected from seven locations. The isolated fungi were identified according to their cultural and morphological characters as *A. alternata, A. solani, C. lunata, F. solani, F. oxysporum, M. phaseolina, P. lycopersici, R. solani, S. botryosum, T. viride.* and *Th. basicola* (Table 2).

Table (2): Fungi isolated with their respective plant's species and locations.

No.	Fungi	Host	Plant Organ	location
1	*Alternaria alternata* (Fr.) Keissl	Spinach (*Spinacia oleracea)*	leaves	Giza
2	*Alternaria solani* Sorauer	Tomato (*Solanum lycopersicum)*	leaves	Fayoum
3	*Curvularia lunata* (Wakker) Boedijn.	Lettuce (*Lactuca sativa)*	leaves	Sharqiya
4	*Fusarium solani* (Mart.) Sacc.	Pepper (*Capsicum annuum)*	crown	Damiaetta
5	*Fusarium oxysporum* Snyder & Hansen	Cantaloupe *(Cucumis melo*)	stem	Nobariya
6	*Macrophomina phaseolina* (Tassi) Goid.	Bean (*Phaseolus vulgaris*)	root	Qaluobiya
7	*Pyrenochaeta lycopersici* Schneid. & Gerlach.	Tomato (*Solanum lycopersicum)*	root	Qaluobiya
8	*Rhizoctonia solani* Kühn	Eggplant (*Solanum melongena)*	crown	Qaluobiya
9	*Stemphylium botryosum* Wallr	Lettuce (*Lactuca sativa)*	leaves	Nobariya
10	*Trichoderma viride* Pers.	Peanut *(Arachis hypogaea)*	root	Ismailiya
11	*Thielaviopsis basicola* (Berk. & Broome) Ferraris	Pea *(Pisum sativum)*	root	Sharqiya

Colony radial growth:

Data presented in Table (3) and Fig (1) show that the isolated fungi when incubated at 21°C for 4 days grew significantly faster on medium (control) than those amended with different substrates namely pectin, cellulose and chitin. *T. viride, Th. basicola, P. lycopersici, M. phaseolina, R. solani, F. oxysporum, S. botryosum, F. solani, C. lunata, A. solani, A. alternata,* respectively, were recovered and identified. The amended substrates retarded growth significantly compared to control. *T. viride* grew significantly better on pectin (6.0 cm.), followed by *P. lycopersici, M. phaseolina,* and *A. solani* (4.0cm.) respectively. Meanwhile, *F. oxysporum, F. solani, Th. basicola, S. botryosum, R. solani, A. alternata* and *C. lunata* showed limited growth on media amended with pectin, being 2.5 - 3.5 cm. With chitin substrate, however *F. oxysporum, A. solani, F. solani* grew better, being 5.0, 5.0, 4.0 cm., followed by *T. viride, S. botryosum, P. lycopersici* (3.0cm). Furthermore *T. viride, F. oxysporum* and *Th. basicola* grew significantly better on cellulose amended media (4.0cm.) and relatively followed by *A. solani, P. lycopersici* (3.0 and 2.5 cm.), respectively.

Under relatively higher incubation temperature at 28°C for 4 days, seven isolates were grown on media (control) without any substrate in concern *T. viride, Th. basicola, P. lycopersici, M. phaseolina, R. solani, F. oxysporum, F. solani* (9.0cm.). Meanwhile, *S. botryosum, A. solani* recorded (8.5cm.), followed by *A. alternata, C. lunata,* being 8.0 and 7.5 cm. respectively. Approximately similar radial growth was observed for pectin amended media. *T. viride, F. oxysporum, F. solani*, grew significantly better on pectin (9.0 cm.), *P. lycopersici, A. solani* (8.0 cm.) followed at a descending sequence by *S. botryosum, R. solani*, (7.0 cm.) and *A. alternata, M. phaseolina, Th. basicola, C. lunata*, being 6.0, 6.0, 5.0, and 4.0cm., respectively.

On chitin supplemented medium, different growth rates were recorded for the tested fungi. *T. viride, F. solani, R. solani* grew significantly better (8.0cm.) followed by *P. lycopersici, M. phaseolina F. oxysporum*, *A. solani, S. botryosum, Th. basicola, A. alternata,* being 7.0, 7.0, 6.0, 6.0, 6.0, 5.0 and 5.0cm. and last, *C. lunata* (3.0cm.). Furthermore, supplemented cellulose promoted growth, *T. viride, F. oxysporum* recorded 8.0 cm. while *F. solani, S. botryosum* recorded 7.0cm. followed by *A. solani, Th. basicola, R. solani, P. lycopersic*, *A. alternata* (6.5, 6.0, 6.0, 5.0, and 4.0cm.) and to less extend *M. phaseolina, C. lunata* (2.5, and 1.5 cm.) as a weak cellulose decomposer.

Table (3): Screening for enzyme detection of isolates grown on modified agar plates.

Fungi	Radial growth (cm) after 4 days							
	21°C				28°C			
	Control	Poly galacturonase PG	Cellulase Cx	Chitinase CH	Control	Poly galacturonase PG	Cellulase Cx	Chitinase CH
A. alternata	4.5	2.5	1.5	1.5	8.0	6.0	4.0	5.0
A. solani	5.0	4.0	3.0	5.0	8.5	7.0	6.5	6.0
C. lunata	5.5	2.5	1.0	2.5	7.5	4.0	1.5	3.0
F. solani	6.0	3.0	2.0	4.0	9.0	8.0	7.0	8.0
F. oxysporum	7.0	3.5	4.0	5.0	9.0	9.0	8.0	6.0
M. phaseolina	8.0	4.0	1.0	1.0	9.0	6.0	2.5	7.0
P. lycopersici	9.0	4.0	2.5	3.0	9.0	8.0	5.0	8.0
R. solani	7.5	2.5	1.0	2.5	9.0	8.0	6.0	7.0
S. botryosum	6.5	3.0	1.0	3.0	8.5	7.0	7.0	6.0
T. viride	9.0	6.0	4.0	3.0	9.0	9.0	8.0	8.0
Th. basicola	9.0	3.0	4.0	2.5	9.0	5.0	6.0	5.0
LSD at 5%	1.30	0.85	0.68	1.22	0.96	1.33	1.00	0.99

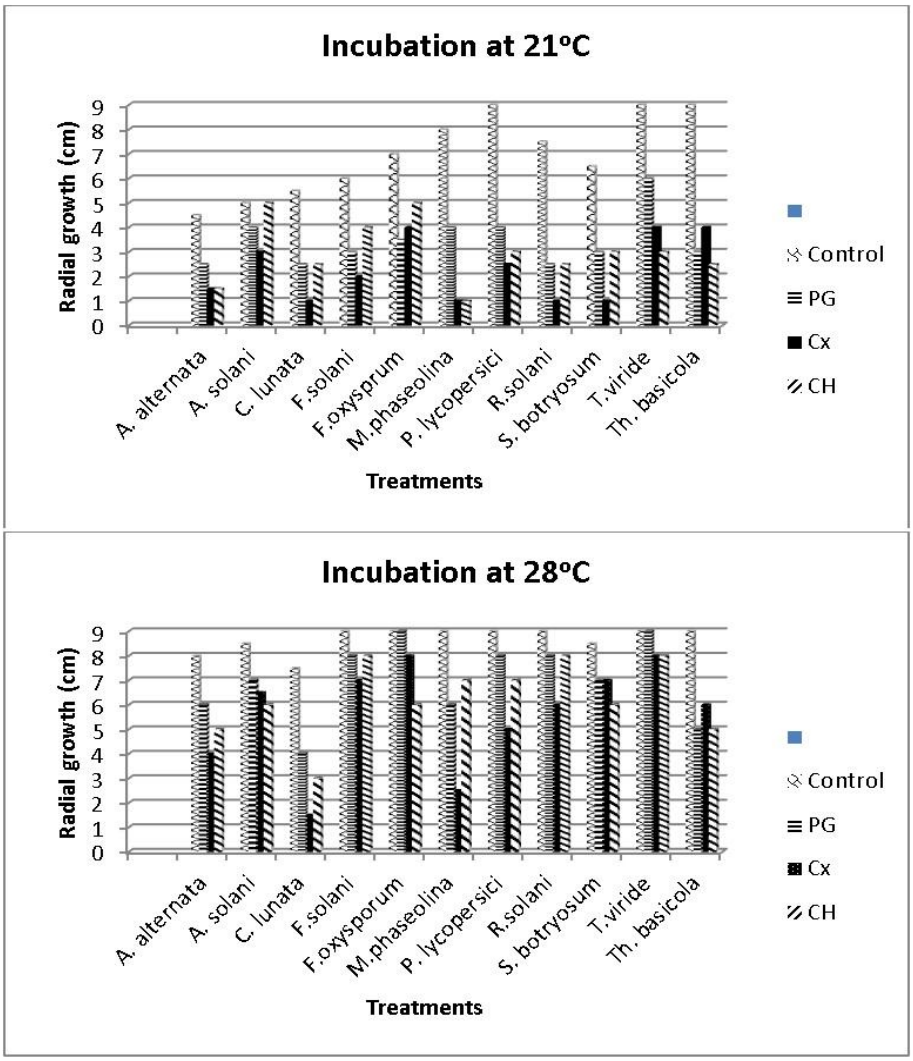

Fig (1): Radial growth of isolates grown on the different supplements at 21°C and 28°C for 4days.

Enzymes secretion:

Determination of enzyme secretion related to time of inoculation, temperature and different substrates was carried out over a period of four days incubation. A distinct plate-colored halo of corresponding to enzyme(s) secretion is observed as compared to control. The results in (Fig.2) show the highest specific activity.

Enzymes detection at 21°C:

Results presented in Table (4), Fig (2 a and b) and Fig. (3 a, b, and c) show that polygalacturonase (PG) detection secreted by different fungi *i.e., T. viride, P. lycopersici, F. oxysporum* and *Th. basicola* after 4 days incubation at 21°C, showed the large colored halos of hydrolysis by (PG) secretion, being 9.0 cm. as indicated by change of color to purple followed by descending sequencer, *M. phaseolina* (7.0 cm.), *S. botryosum* (6.0 cm.), *A. solani* (5.0 cm.), *C. lunata* (4.5 cm.), *F. solani, A. alternata* (4.0 cm.) and the least secretion was detected for *R. solani*, being 0.5cm.

Detection of cellulase (Cx) secreted by different fungi after 4 days incubation at 21°C showed that *Th. basicola* produced the largest halo of hydrolysis with Cx, being 9.0 cm. diameter followed by *T. viride, F. oxysporum* (6.0 cm.), and to less extent for *A. solani* (4.0 cm.), *P. lycopersici* (3.5cm.) and *F. solani* (3.0 cm.). The failure of production, however, was reported for *A. alternata, C. lunata, S. botryosum, M. phaseolina* and *R. solani*, (0.5cm.).

Chitinase (CH) detection under the same conditions for *F. oxysporum* showed distinct colored halo of hydrolysis by CH, being 7.0 cm. followed by *F. solani* (6.0 cm.), *A. solani, P. lycopersici* (5.0 cm.) and to less extent by *T. viride* (4.5cm.), *Th. basicola, S. botryosum* (4.0 cm.), *C. lunata* (3.0 cm.). The failure of detection, however, was reported for *A. alternata, R. solani, M. phaseolina* that did not show any change in color.

Enzymes detection at 28°C:

Polygalacturonase (PG) detection secreted by different fungi after 4 days at high incubation temperature at 28°C, favored the growth of *T. viride, P. lycopersici, F. solani, F. oxysporum, M. phaseolina, R. solani, A. solani, S. botryosum, Th. basicola, A. alternata* produced large halo of hydrolysis with PG, being 9.0 cm. and to less extent by *C. lunata,* (6.0 cm.).

Cellulase (Cx) secration by different fungi after 4 days incubation at 28°C, is shown in Table (4). *T. viride, P. lycopersici, F. oxysporum, F. solani, A. solani, S. botryosum, Th. basicola* showed wide halo of hydrolysis, being 9.0 cm. followed by *A. alternata* (6.0 cm.) and to less extent *M. phaseolina* (3.5cm.), and impotent Cx secretion by *R. solani, C. lunata* (0.5cm.) indicating failure of production under the conditions of the experiment.

Chitinase (CH) production by different fungi after 4 days incubation at 28°C was reported by *F. oxysporum, F. solani, A. solani, T. viride, P. lycopersici, R. solani, S. botryosum, M. phaseolina, Th. basicola* as large zone of hydrolysis with chitinase, being 9.0 cm. followed by *A. alternata, C. lunata* (7.0 and 5.0 cm.).

All isolates tested were able to grow in the media with CMC as carbon sources and produced cellulolytic enzymes; however, their production potential was variable and was less active compared to polygalacturonase and chitinase.

Table (4): Comparative determination of enzyme activity of the tested fungi incubated at different temperature levels.

Fungi	Enzymes activity at 21°C						Enzymes activity at 28°C					
	Polygalacturonase (PG)		Cellulase (Cx)		Chitinase (CH)		Polygalacturonase (PG)		Cellulase (Cx)		Chitinase (CH)	
	Colony	Clear halo	Colony	Clear halo	Colony	Clear halo	Colony	Clear halo	Colony	Clear halo	Colony	Clear halo
A. alternata	2.5	4.0	1.5	0.5	1.5	0.5	6.0	9.0	4.0	5.0	5.0	7.0
A. solani	4.0	5.0	3.0	4.0	5.0	5.0	7.0	9.0	6.5	9.0	6.0	9.0
C. lunata	2.5	4.5	1.0	0.5	2.5	3.0	4.0	6.0	1.5	0.5	3.0	5.0
F. solani	3.0	4.0	2.0	3.0	4.0	6.0	8.0	9.0	7.0	9.0	8.0	9.0
F. oxysporum	3.5	9.0	4.0	6.0	5.0	7.0	9.0	9.0	8.0	9.0	6.0	9.0
M. phaseolina	4.0	7.0	1.0	0.5	1.0	0.5	6.0	9.0	2.5	3.5	7.0	9.0
P. lycopersici	4.0	9.0	2.5	3.5	3.0	5.0	8.0	9.0	5.0	9.0	8.0	9.0
R. solani	2.5	0.5	1.0	0.5	2.5	0.5	8.0	9.0	6.0	0.5	7.0	9.0
S. botryosum	3.0	6.0	1.0	0.5	3.0	4.0	7.0	9.0	7.0	9.0	6.0	9.0
T. viride	6.0	9.0	4.0	6.0	3.0	4.5	9.0	9.0	8.0	9.0	8.0	9.0
Th. basicola	3.0	9.0	4.0	9.0	2.5	4.0	5.0	9.0	6.0	9.0	5.0	9.0
LSD at 5%	0.85	1.22	0.68	0.96	1.22	1.30	1.33	1.53	1.00	1.82	0.99	1.08

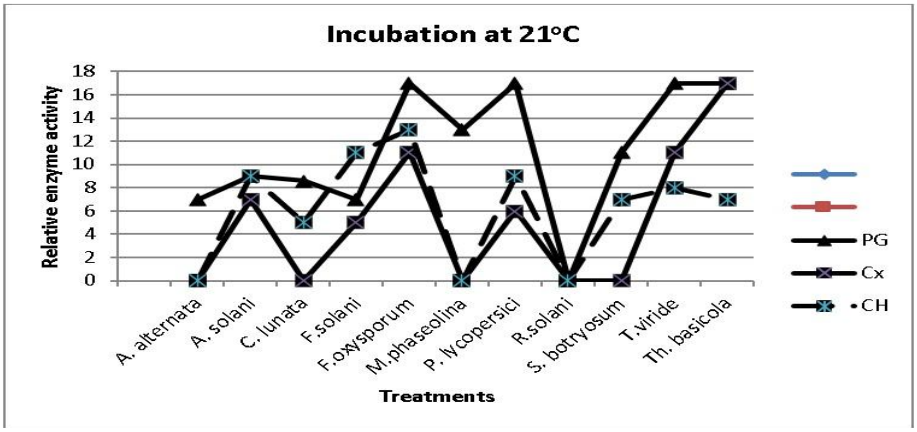

Fig. 2(a): Enzyme secretion pattern by isolated fungi at 21°C PG, Cx, and CH.

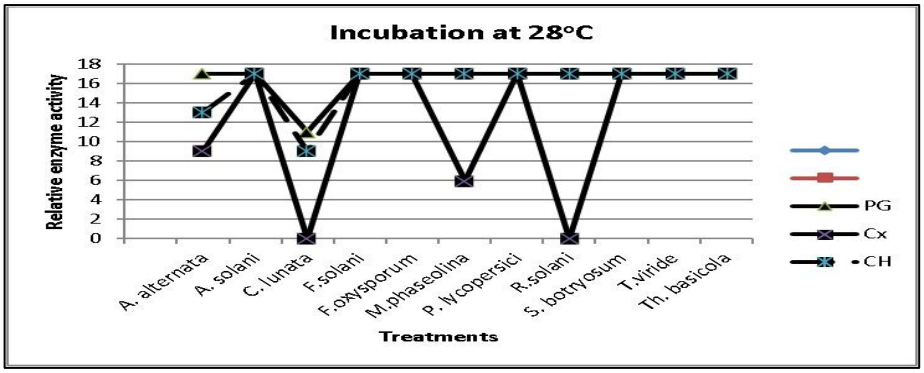

Fig. 2(b): Enzyme secretion pattern by isolated fungi at 28°C. PG, Cx, and CH.

Relative enzyme activity (RA) of the isolated fungi:

Data in Table (5) show the maximal relative enzyme activity **(RA),** polygalacturonase (PG), cellulase (Cx), chitinase (CH) of each of *T. viride*, *Th. basicola*, *F. oxysporum*, *F. solani*, *A. solani*, *P. lycopersici*, *S. botryosum* after incubation at 28°C for four days. The results show clearly that no secretion could be recognized for *A. alternata*, *C. lunata*, *M. phaseolina*, *R. solani* and *S. botryosum* at low temperature (21°C) for the fungi Cellulase (Cx), polygalacturonase (PG) and chitinase (CH).

Table (5): Relative enzyme activity (RA) of the isolated fungi after incubation at two different temperature levels.

Fungi	Relative enzyme activity (RA)					
	21°C			28°C		
	Poly galacturonase (PG)	Cellulases (Cx)	Chitinase (CH)	Poly galacturonase (PG)	Cellulases (Cx)	Chitinase (CH)
A. alternata	7.0	0.0	0.0	17.0	9.0	13.0
A. solani	9.0	7.0	9.0	17.0	17.0	17.0
C. lunata	8.6	0.0	5.0	11.0	0.0	9.0
F. solani	7.0	5.0	11.0	17.0	17.0	17.0
F. oxysporum	17.0	11.0	13.0	17.0	17.0	17.0
M. phaseolina	13.0	0.0	0.0	17.0	6.0	17.0
P. lycopersici	17.0	6.0	9.0	17.0	17.0	17.0
R. solani	0.0	0.0	0.0	17.0	0.0	17.0
S. botryosum	11.0	0.0	7.0	17.0	17.0	17.0
T. viride	17.0	11.0	8.0	17.0	17.0	17.0
Th. basicola	17.0	17.0	7.0	17.0	17.0	17.0
% high activity	54.5	27.3	18.2	100.0	81.8	100.0

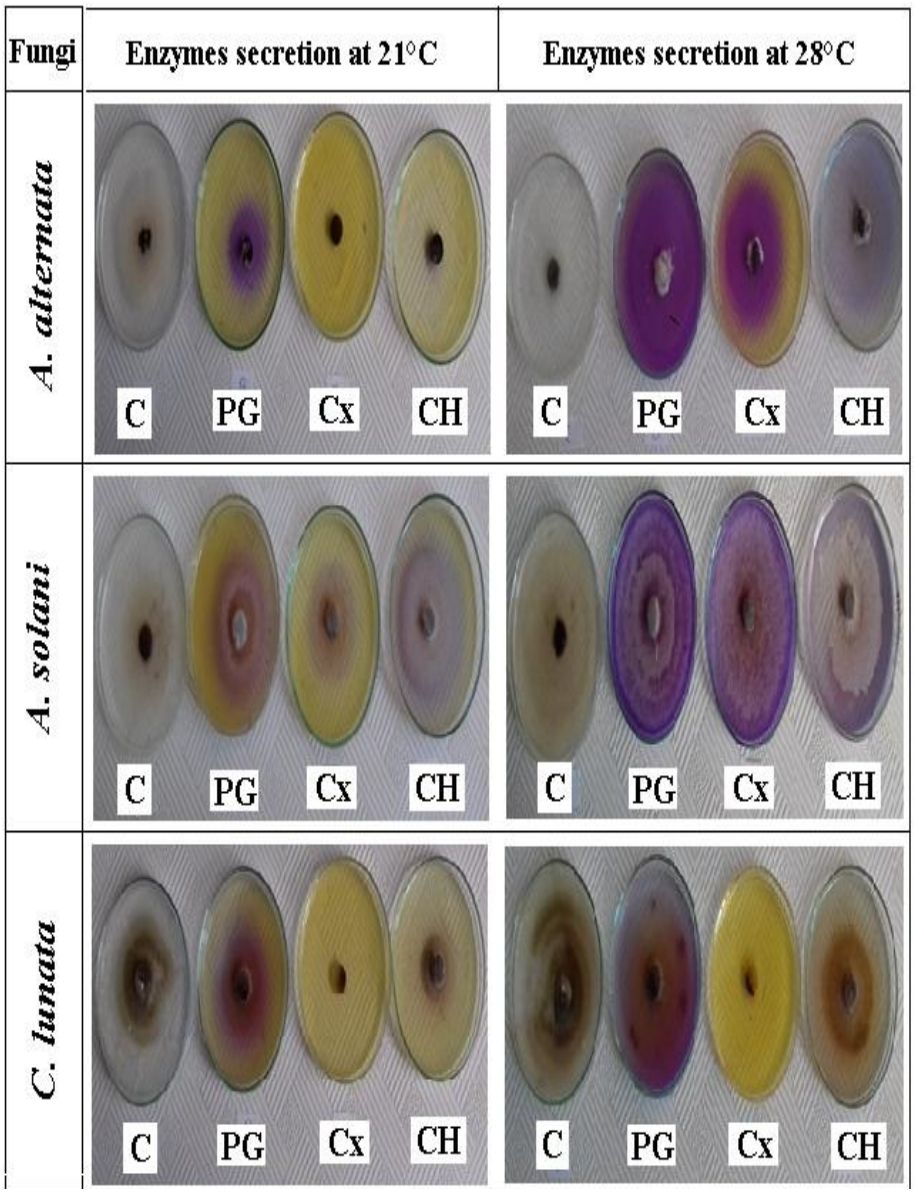

Fig. (3a): Modified agar assay for the detection of (PG), (Cx) and (CH) secreted by *A. alternata*, *A. solani* and *C. lunata* grown at 21 and 28°C as indicated by color change after substrate hydrolyses.

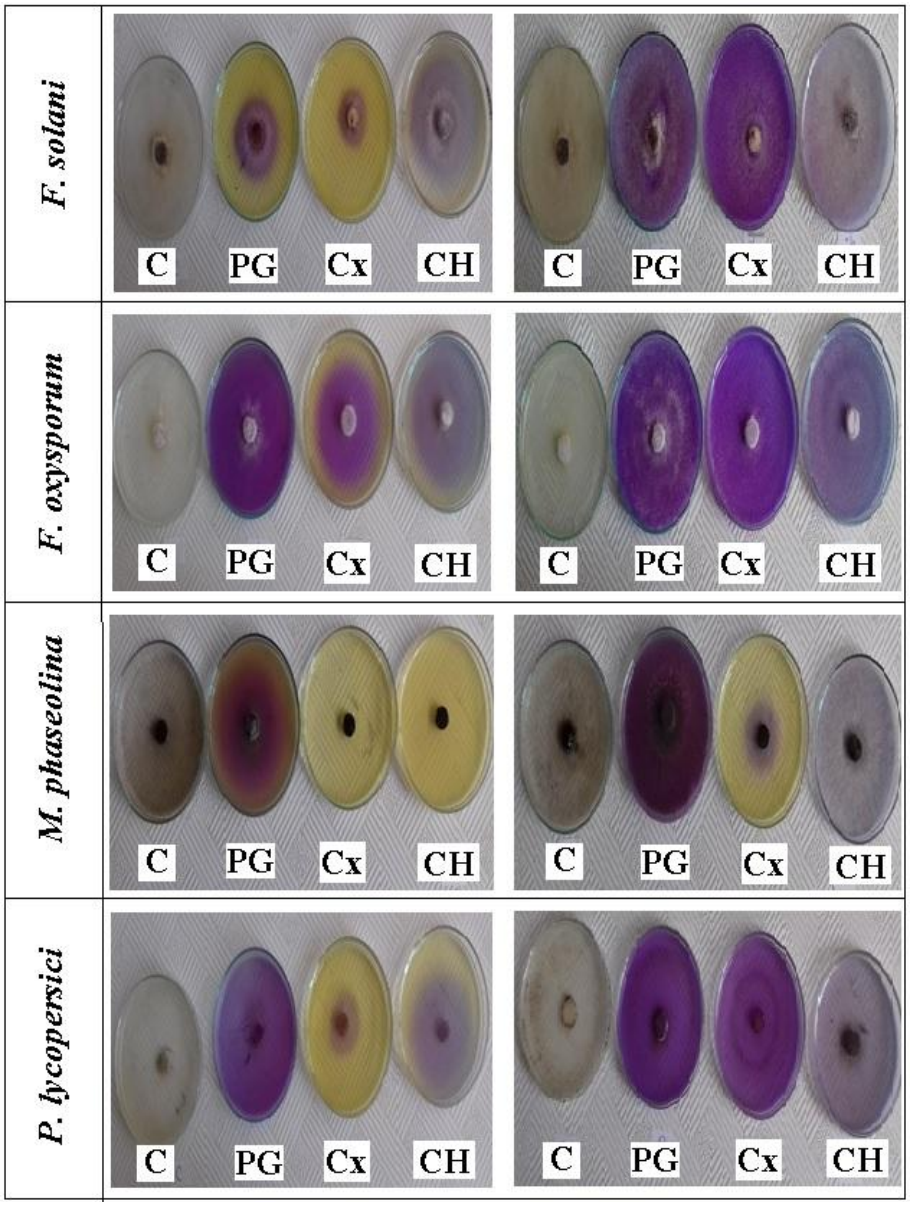

Fig. (3b): Modified agar assay for the detection of (PG), (Cx) and (CH) secreted by *F. solani, F. oxysporum, M. phaseolina* and *P. lycopersici* grown at 21 and 28°C as indicated by color change after substrate hydrolyses.

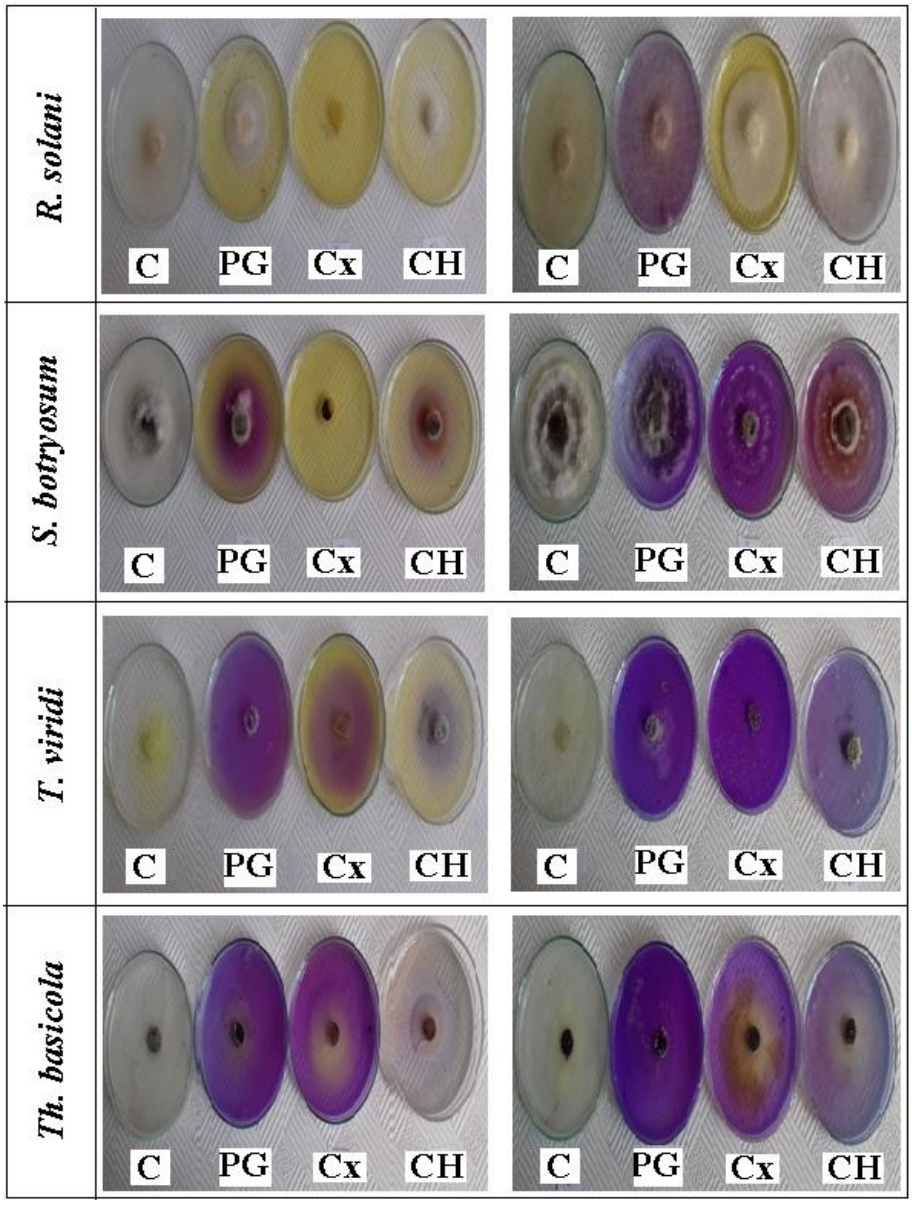

Fig. (3c): Modified agar assay for the detection of (PG), (Cx) and (CH) secreted by *R. solani, S. botryosum, T. viride* and *Th. basicola* grown at 21 and 28°C as indicated by color change after substrate hydrolyses.

Discussion

Propagation media to detect secretion of extracellular enzymes produced by fungi isolated from different plant organs were evaluated in earlier tired basically in broth cultures.

Modified semi solid agar medium showed that polygalacturonase (PG) was the first actively enzyme secreted at high temperature 28°C that is being correlated with degradation of a plant tissues along with other enzymes involved in degradation process as chitinase and cellulase.

The conclusive remarks made on this study revealed that incubation temperature is of paramount importance for growth and secretion of degradation enzymes in general. In this study, it has been shown that the most appropriate temperature for growth and enzyme(s) secretion was 28°C and to less extend at 21°C, though the majority of fungi prefer. The pectin supplemented medium supported maximum linear growth for *T. viride* (6.0 cm.).

The first one with cellulose medium, however *T. viride, F. oxysporum, Th. basicola* supported less growth (4.0 cm.) and with chitin medium the isolates of *F. oxysporum, A. solani* (5.0 cm.) at 21°C compared to maximum linear growth for all isolates, being 9.0cm. at 28°C for growth of *F. oxysporum, F. solani, M. phaseolina, R solani, T. viride,* and *Th. basicola.*

Pectin supplemented medium showed that *T. viride* and *F. oxysporum* gave maximum linear growth, being 9.0 cm. and cellulose medium showed that, the maximum linear growth (8.0 cm.) was for *T. viride* and *F. oxysporum* similar to chitin medium with *T. viride, F. solani* and *P. lycopersici* (8.0 cm.) at 28°C.

In this regard, Stelica *et al*. (2015) recorded similar results for *F. oxysporum*. Moreover, Ali and Vidhale (2013) reported that optimum temperature is the essential factor for microorganism production of essential enzymes necessary for suppression of cell viability. Similar conclusion was reported by Mishra and Khan (2015) who found that the optimum temperature for growth range of *T. viride* was ranging between 20°C - 30ºC and Arfarita *et al.,* (2016) who reported that the optimum temperature for growth of *T. viride* was found to be between 25-27°C.

Eleven different fungal isolates were grown in a synthetic medium with different carbon sources alternatively employed to assess the production of different cell wall-degrading enzymes. Polygalacturonase (PG) activity reached its highest level on total isolates assayed at 28°C followed by chitinase and cellulase.

Ten isolates, *i.e., F. oxysporum, F. solani, M. phaseolina, R solani, T. viride, Th. basicola, A. alternata, A. solani, S. botryosum, P. lycopersici* produced the highest level of polygalacturonase (PG), seven isolates *i.e., T. viride, Th. basicola, A. solani, F. oxysporum, F. solani, P. lycopersici* and *S. botryosum* produced the highest level

of cellulase (Cx) level activity and nine isolates produced the highest level of chitinase (CH), *i.e. T. viride, Th. basicola, A. solani, F. oxysporum, F. solani, P. lycopersici , S. botryosum, M. phaseolina* and *R. solani at* 28°C.

Baayen *et al*. (1997) reported that *F. oxysporum* causing vascular wilt disease is characterized by a severe degradation of vascular tissue, the amount of PG activity was correlated highly with the development of the disease. Ahmad *et al*. (2006) mentioned that in several pathogens including *M. phaseolina*, pectinase was the highly activated before cellulase enzyme that initiating the process of cell wall degradation.

Kaur *et al*. (2012) reported that one of the isolates of *M. phaseolina* was a potential source of several hydrolytic enzymes, such as cellulase, hemicellulase and amylase. The CMCase of *F. oxysporum*, displayed significant activity within a temperature range of 25 - 37°C with maximum activity at 28°C as reported by Dar *et al*. (2013). The optimum production of cellulases by *T. viride* has been also reported at 35°C (Kandari *et al*. 2013).

Relative enzyme activity (RA) after 4 days incubation at 28°C for eleven isolates produced maximum polygalacturonase (17), nine isolates produced maximum chitinase (17) followed by *A. alternata* (13) and *C. lunata* (9) while seven isolates produced maximum cellulase (17) followed by *A. alternata* (9) and *M. phaseolina* (6) while non produced were *C. lunata* and *R. solani* (0.0).

Screening of extracellular % high activity enzymes pathogenic fungi, at 28°C, PG, CH (100.0%), and Cx (81.8%). However, the percentage of activity of enzymes of pathogenic fungi, at 21°C was low, PG gave 54.5% compared to, CH 18.2% and Cx 27.3% indicating a sharp dropin growth rate of fungi.

Conclusion

A comparison of the radial growth of fungi on modified semi-solid medium and its influence on specific enzymes secretion of the eleven isolates tested showed that both (PG) and (CH) had high growth rates and produced the highest enzyme secretions at either 21°C or 28°C.

The results presented in this paper gave information’s on the possible detection of different enzymes produced in non-liquid form, tentatively with similar consistency of plant tissues.

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الأجار النصف متصلب للكشف عن إنزيمات الفطريات

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تم عزل وتعريف ١١ عزله من الفطريات الممرضه للنبات وهى كالتالى *A. alternata, A. solani, C. lunata, F. solani, F. oxysporum, M. phaseolina, P. lycopersici, R. solani, S. botryosum, Th. basicola,* وبالاضافه الى الفطر *T. viride* إجريت الدراسه بهدف إختبار عدد من الفطريات لإنتاج إنزيمات البولى جلاكتورنيز والسليوليز والشيتينيز على البيئه نصف صلبه على درجه الحراره ٢١ و ٢٨°م. وكانت النتيجه: أن كل الفطريات المختبره لها القدره على إفراز البولى جلاكتورنيز والشيتينيز بنسبه ١٠٠% والسليوليز بنسبه ٨١,٨ % على درجه حراره ٢٨°م. بينما على درجه حراره ٢١°م فقد إختلفت النتائج و كانت أعلى الفطريات انتاجا للبولى جلاكتورنيز: *T. viride, P.lycopersici, Th. basicola, F. oxysporum, M. phaseolina,* S. *botryosum,* ويمثل عددها ٥٤,٥% واقلهم انتاجا هو الفطر *R. solani* الذي يمثل بنسبه ٩,١% وأربعه فطريات متوسط الانتاج وتمثل ٣٦,٤%.

وكان أعلى انتاج للسليوليز بواسطه الفطريات *T. viride, Th. basicola,* و *F. oxysporum* *وتمثل* ٢٧,٣% وأقلهم انتاجاً بنسبه ٤٥,٥% *A. alternata, C. lunata, M. phaseolina, R. solani* و *S. botryosum* والباقى متوسطه الانتاج وتمثل ٢٧,٣% من اجمالي الفطريات المختبرة.

كما وجد أن اعلى انتاج للشيتينيز كان من الفطريات *F. solani, F. oxysporum* *والتي تمثل* ١٨,٢% واقلهم انتاجا الفطريات *A. alternata, M. phaseolina, R. solani* وتمثل ٢٧,٣% وباقى الفطريات متوسطه الانتاج بنسبه ٥٤,٥%. وعند حساب نسبه النشاط الانزيمى للفطريات ، كانت اعلى نسبه لعدد سبع فطريات تمثل ٦٣,٦% من العدد الكلى للفطريات المفرزه للانزيمات البولى جلاكتورينيز والسليوليز والشيتنيز عند درحه ٢٨°م مئويه وهم الفطريات *A. solani, F. solani, F. oxysporum, P. lycopersici, S. botryosum, T. viride, Th. basicola* بينما وجد أن أربعه فطريات كان لها نشاط إنزيمى فى درجتى الحراره ٢١ و ٢٨°م ولنفس فتره التحضين ٤ ايام وهم *F. oxysporum, P. lycopersici, T. viride, Th. basicola*.

إتضح أن بيئه الأجار النصف متصلب (٥ جم اجار/ لتر) أفضل للكشف الحقيقي عن إنزيمات الفطريات مثل البولى جلاكتورينيز والسليوليز والشيتينيز. وأن درجه الحراره لها تاثير إيجابى لنشاط الفطريات فى افراز الانزيمات المختلفه ،وتحتاج هذه النقطه دراسات اعمق حيث انه ليس من الشائع تقدير الانزيمات فى البيئه المتصلبه.