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Enzymatic Degradation of Wood Cellulose by Chaetomium Cellulases and its Prevention

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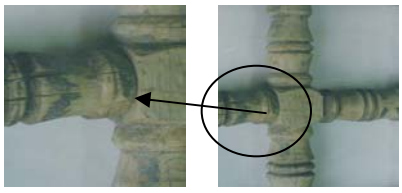
Summary. *Chaetomium globosum* used in this study was isolated from deteriorated monumental wooden objects and tested for its potentiality to produce extra and intracellular cellulases enzymes. The results indicated that *Chaetomium globosum* is a good cellulases producer and produced maximum cellulases components including β-endoglucanases and β-glucosidases enzymes after 20 days period in rotatory shaker (180 rpm) at pH6 and temperature 30°C.Cellulases synthesis was inhibited in the presence of various concentrations of garlic, rosemary and cloves oils. The application of these substances on infected beech wood was studied and examined by using scanning electron microscope (SEM).

Introduction

Chaetomium globosum, as one of the major etiologic agents of wood decay, exhibits very high cellulolytic activity (Unger, et al., 2001) and causes typical soft rot of type1. The fungal growth takes place mainly in the S_2 layer of wood cells, which is only visible microscopically causing a significant loss of wood substance and reduction of wood strength (Daniel and Nilsson, 1988) and (Unger, et al., 2001). Discovery of antimicrobial activities of garlic, rosemary and cloves has a long history and it is reported on different microorganisms (Elnima, et al., 1983), (Fan and Chen, 1999) and (Ghahfarokhi, et al., 2003). The aim of this work is to study the role of *Chaetomium globosum* cellulases in deteriorating wooden objects and to evaluate the inhibitory effects of garlic, rosemary and cloves oils against the cellulolytic activity of this fungus.

Experimental

1- Microorganism: *Chaetomium globosum* used in this study was isolated previously from deteriorated wooden Mashrabeya of wekalt Bazaraa at Al Azhar, Cairo (from Ottoman period) (Fig.1).



(Fig.1): deteriorated wooden object

2- Optimization of cellulases production: Experimental culture flasks each contained 50 ml of Mandels and Weber's medium (1969) plus 0.5 gm of crystalline cellulose (avicel) at initial pH 5 were inoculated by 5% (v/v) of the fungus.

Influence of different incubation periods: The inoculated culture flasks were incubated on a rotatory shaker (180 rpm), for shaking cultivation, or in an incubator, for static cultivation at 30^{o}C for different periods of incubation (7, 10, 15, 20, 25 and 30) days.

Influence of incubation Temperature and initial pH of the medium: The medium was inoculated with tested fungus and incubated at 20, 25, 30 and 40^{o}c. Initial pH was adjusted with 0.1N-NaOH or 0.1N-HCl to different values ranging from 3 to8.

3- Enzyme extraction: At the end of each culture period, the mycelia was harvested by filtration using filter paper what man No. 1 and washed three times with 0.05M cirate-phosphate buffer pH 4.8. The mycelium was resuspended in minimal amount of buffer and was ground with sand. The homogenized mycelium was centrifuged at 5000 rpm for 20min and the supernatant was used for assays of intracellular enzymes.

4- Enzyme assays: Culture filtrates were subjected to extracellular enzyme assays

a) β–1,4 glucanase [Carboxymethyl cellulase (CMC–ase)]: This achieved according to the method of Mandels and weber (1969) where the resulting sugars were determined by Somogyi Reagent (Somogyi, 1952) using glucose as standard. The reaction was carried out by incubated 0.5 ml of enzyme solution with 0.5 ml of 1.0% sodium carboxy methylcellulose, as substrate in buffer at 50^{o} C for 30 min. The reaction was stopped by adding 2 ml of copper reagent, then the mixture was incubated in a boiling water bath for 10 min. 2ml of arsenate reagent was thoroughly mixed with the cooled mixture, then the volume was completed to 25ml with distilled water. The resulting blue colour was measured spectrophotometerically at 520 nm.

b) Cellobiase (β–glucosidase): This was performed according to the method of Berghem and Petterson (1974), where 0.5 ml of enzyme solution was incubated with 0.5 ml of 0.4% cellobiose in buffer at 50^{o} C for 30 min. The reaction was stopped by heating the reaction mixture in a boiling water bath for 3 min. Then 0.2 ml of the reaction mixture was incubated with 2.5 ml of diagnostic kits (glucose/peroxidase kit)

according to Teller (1956) for 15 min at 37^{o}C. The resulting pink colour was measured at 510 nm.

5- Effect of garlic, rosemary and cloves oils on cellulases production and activity: Various concentrations of the above substances (0, 1, 2, 5 and 10%) were used. The medium was inoculated and incubated as discussed before. To study the effect of these oils on cellulases activity, the culture filtrate of the control was subjected to incubation separately with 10% garlic, 10% rosemary and 5% cloves at different time intervals.

6- Effect of fungal infection on wood samples: Specimens of beech wood were sprayed with *Chaetomium globosum* spores and incubated in a desicator with 100% relative humidity for two months at 30^{o}C. Then, they were examined by SEM. Moreover, α-cellulose contents were gravimetrically estimated according to (Markblatt IV/29 Zell-cheming).

7- Effect of treatment with garlic, rosemary and cloves oils: Specimens of beech wood were treated separately by 10% garlic & rosemary oils and 5% cloves oil then sprayed with fungal spores, incubated and tested as discussed before.

Results and Discussion

1- Optimization of cellulases production: Cultural conditions have a great effect on the production of extra and intracellular cellulases components (Sandhu and Kalra, 1985), (Nigam and Prabhu, 1991), (Darwish, 2001) and (Darwish, et al., 2005).

Influence of incubation periods: The results in table (1) showed that *Chaetomium globosum* had the ability to produce cellulases components under both static and shaking conditions (Unger, et al., 2001). The maximum productions of the two cellulases components were obtained after 25 days for stationary cultures and 20 days for shaking cultures. In shake flasks, the activity was high compared with stationary cultures and this high activity may be due to the better aeration (Nigam and Prabhu, 1991), (Ismail and Sahab, 2004) and (Darwish, et al., 2005).

Influence of pH and temperature: The results in tables (2 and 3) showed that *Chaetomium globosum* could produce both extra and intracellular cellulases at a wide pH range and the maximum yield was obtained at pH6. The best enzyme activities

were supported at 30°C. These data are in agreement with those reported by other researchers (Gersonde and Kerner-Gang, 1976), (Unger, 2001) and (Darwish, et al., 2005).

Table 1: Effect of incubation periods on cellulases production.

Condition	Periods (days)	Final pH	Dy* wt (gm)	CMC-ase		Cellobiase	
				C.F. (U/ml)	My. ext (U/gm)	C.F. (U/ml)	My. ext (U/gm)
Stationery	7	6.8	0.481	0.011	0.08	0.012	0.05
	10	6.6	0.470	0.015	0.192	0.015	0.07
	15	6.3	0.463	0.07	0.233	0.02	0.14
	20	6.5	0.457	0.08	0.262	0.023	0.152
	25	6.9	0.441	0.145	0.291	0.03	0.182
	30	7.0	0.438	0.139	0.162	0.025	0.11
Shaking	7	6.5	0.490	0.08	0.437	0.03	0.252
	10	6.5	0.485	0.105	0.520	0.04	0.472
	15	6.4	0.481	0.170	0.882	0.045	0.693
	20	6.5	0.489	0.175	1.329	0.14	0.833
	25	6.6	0.356	0.07	0.620	0.105	0.583
	30	6.7	0.366	0.069	0.391	0.093	0.371

C.F. = Culture filtrate, My. ext. = Mycelial extract
* Dry weight of mycelium and residual cellulose (gm / 50 ml culture)

Table 2: Influence of different pH on the production of cellulases components*

Initial pH	Final pH	Dry wt (gm)	CMC-ase		Cellobiase	
			C.F. (U/ml)	My. ext (U/gm)	C.F. (U/ml)	My. ext (U/gm)
3	4.5	0.337	0.085	2.350	0.090	1.767
4	5.2	0.339	0.163	1.540	0.125	4.280
5	5.9	0.277	0.230	2.210	0.355	10.804
6	6.2	0.239	0.245	1.840	0.375	11.840
7	6.5	0.334	0.095	0.683	0.365	9.913
8	6.9	0.417	0.055	2.040	0.213	7.906

* This experiment was done under shaking conditions at incubation period 20 days using beech wood as a substrate.

Table3: Influence of different temperatures on the production of cellulases components*

Initial pH	Final pH	Dry wt (gm)	CMC-ase		Cellobiase	
			C.F. (U/ml)	My. ext (U/gm)	C.F. (U/ml)	My. ext (U/gm)
20°C	6.1	0.325	0.121	0.913	0.172	2.710
25°C	6.4	0.283	0.192	1.421	0.233	7.210
30°C	6.3	0.215	0.253	2.010	0.363	11.310
40°C	5.9	0.361	0.081	0.832	0.153	2.410

* This experiment was done under shaking conditions at incubation period 20 days & pH6 using beech wood as a substrate.

2- Effect of garlic, rosemary and cloves oils on cellulases production and activity:

Effect on cellulases production: The results in table (4) showed that inhibition of extracellular glucosidases enzymes reached to maximum of 100% for both garlic and rosemary at 10% v/v concentration and 5% for cloves. While complete inhibition of intracellular glucosidases enzymes was achieved at 10% v/v for all the tested oils. On the other hand, Extracellular endoglucanases enzymes were inhibited completely at 10% of all tested oils. 10% of both garlic and rosemary oils decreased the production of intracellular endoglucanases to 13.1 and 23.62% respectively. While 10% cloves inhibited the production of this enzyme completely. The inhibitory effect of these substances on fungi were studied by other workers (Appleton and Tansey, 1975), (Elnims, et al., 1983), (Bilgrami, et al., 1992), (Fan and Chen, 1999) and (Ghahfarokhi, et al., 2003).

Table 4: Effect of different concentrations of garlic, rosemary and cloves on cellulases production*

Substance	Concentration %	% of the relative activity			
		CMC-ase		Cellobiase	
		C.F.	My. ext.	C.F.	My. ext.
Control	0	100	100	100	100
Garlic	1	40.20	47.08	75.10	42.30
	2	32.80	44.49	52.32	35.19
	5	18.01	30.20	24.13	21.26
	10	0.00	13.10	0.00	0.00
Rosemary	1	52.21	57.70	82.10	25.97
	2	33.35	37.64	75.32	24.43
	5	12.23	36.61	43.01	23.59
	10	0.00	23.62	0.00	0.00
Cloves	1	55.10	92.73	52.31	59.27
	2	42.31	77.32	31.92	41.74
	5	15.03	41.91	0.00	14.56
	10	0.00	0.00	0.00	0.00

* This experiment was done using beech wood as a substrate initial pH=6, incubation temperature= 30°C, incubation period= 20 days under shaking conditions.

Effect on cellulases activity: The results in table (5) clearly indicated that garlic, rosemary and cloves had a negatine effect on cellulases activities and these activities were inhibited more by cloves as compared with both garlic and rosemary oils.

Table 5: Effect of garlic, rosemary and cloves and the time of incubation on the activity of cellulases components

Substance conc.(%)	Time (min)	% of Residual CMC-ase activity	% of Residual cellobiase activity
-	0.0	100	100
Garlic (10%)	15	78.57	72.85
	30	51.78	68.31
	45	40.42	31.11
	60	16.2	00.0
Rosemary (10%)	15	83.2	58.57
	30	57.14	51.42
	45	42.85	41.32
	60	38.21	28.21
Cloves (5%)	15	71.42	57.14
	30	40.85	35.71
	45	18.57	00.00
	60	0.00	00.00

3- Estimation of α-cellulose content: Results in table (6) showed that *Chaetomium globosum* displayed considerable variations in its effects on cell-wall constituents during decay development. The observed decrease in α-cellulose is related to breakdown some of long cellulosic chains to smaller units by the action of exoenzymes produced from the fungus. These smaller chains have the ability to dissolve in 17.5% NaOH (wt/wt). The results also showed that garlic, rosemary and cloves had a positive effect on α-cellulose value i.e., the wood decay is retarded or prevented.

Table (6): Effect of treatment by garlic, rosemary and cloves on α-cellulose content.

Wood sample	α-cellulose Value (%)	α-cellulose change (%)	
Control (1)	47.70	0.00	-Uninfected, untreated wood.
Wood (2)	33.93	-17.75	-Infected wood.
Garlic (3)	41.10	-8.53	-Treated wood by (10%) garlic.
Rosemary (4)	34.20	-17.40	-Treated wood by (10%) rosemary.
Cloves (5)	43.80	-5.06	-Treated wood by (5%) cloves.

4- Examination by SEM: Fig (2b) indicated clearly the ability of the hyphae of *Chaetomium globosum* to penetrate the wood and form fruit bodies (cleistothecuim) inside the cavities of wood cells. Also, there are many other stages of growth (mycelium, open fruit bodies releasing spores). Formation of these fruit bodies ensures survival and spread of the fungus (Ungers, et al., 2001). Ability of this fungus to colonize the wooden materials could be attributed to its activity in secreting cellulases

enzymes that degrade cellulose exists in wood cell walls (Zabel and Morrell, 1992). Fruit bodies were absent in Figs. (2c, 2d&2e) where treatment with garlic, rosemary and cloves were applied. In case of Fig. (2e) where cloves were used there are many clear areas free from mycelium and spores indicating that cloves have a great antimicrobial effect against *Chaetomium globosum.* In case of treatment with rosemary Fig. (2d) there are some mycelium and few spores. When garlic was used as in Fig. (2c) no spores and only few mycelium can be seen.

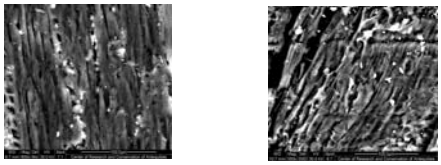


Fig. (2a): control (uninfected, untreated wood)

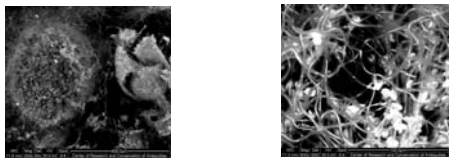


Fig. (2b): infected wood

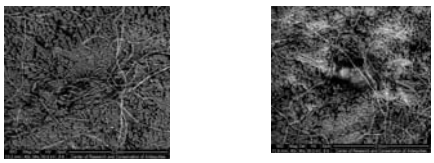


Fig. (2c): treated wood by garlic

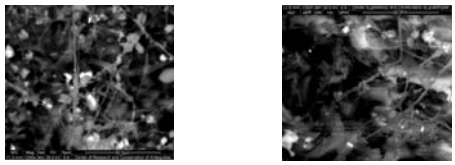


Fig. (2d): treated wood by rosemary

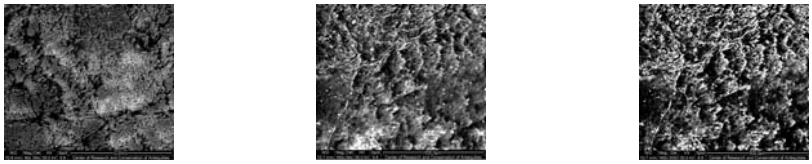


Fig. (2e): treated wood by cloves

Conclusion

It is clear from this study that cellulases activities of *Chaetomium globosum* are important factors in deterioration of wooden objects; so, their inhibition by garlic, rosemary and cloves indicates that these substances may have potential values for treatment of deteriorated wood.

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