ANTIMICROBIAL ACTIVITY OF CHITOSAN ON SOME CELLULOLYTIC FUNGI ISOLATED FROM OLD MANUSCRIPTS AND PAPER QUALITY

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Introduction:

Old manuscripts, papyrus and paper are very important in the history of people and nations. Both papyrus and paper sheets consist essentially of cellulose, which is susceptible to degradation by cellulolytic microorganisms especially fungi and some bacteria (Wang, *et al.*, 1991 and Cvetnic and Pepeljnjak, 2001).

These microorganisms degrade the cellulose to soluble sugars by cellulase enzymes at a moderate temperature and pH. Endoglucanases enzymes cut the cellulose chain in a random fashion whereas, exoglucanases enzymes successively remove single cellobiose or glucose units from the non reducing end of the cellulose chain (Zabel and Morrell, 1992 and Reinikainen, *et al.*, 1995) . β – glucosidase (cellobiase) play an important role in the degradation of the cellulose and in some cases, could constitute the rate limiting step. (Sharma, *et al.*, 1990; Sengupta, *et al.*, 1991; Zable and Morrell, 1992 and Reinikainen, *et al.*, 1995) .

Application of fungicides is far the most effective method to control biodeterioration of paper. However chemicals control programs face imminent problems: first, there are reports of an increasing number of fungicide-resistant strain of paper pathogens. (Jones, 1985 and Horst, *et al.*, 1992) and second, a number of commonly used fungicide such as benomyl are under review in many countries due to health risk concern (FAO/WHO, 1988) .

Thus, there is a growing need to develop alternative approaches for control fungal diseases: one approach that is being actively pursued involves the use of bioactive substances (Benhamou, *et al.*, 1994). Among the most promising bioactive oligosaccharides is chitosan, a mostly deacetylated derivative of chitin occurring in the cell wall of several fungi, which is readily extracted from the chitin of crustacean shell wastes (Hadwiger *et al.*, 1988; Skiak – Braek, *et al.*, 1989 and Goosen, 1997).

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Chitosan is a poly cationic polymer with specific structure and properties. It contains more than 5000 glucosamine unites and is obtained commercially from shrimp and crab shell (Rabea, Entsar, 2003). Previous reports have indicated that when chitosan is dissolved in saline, distilled water or laboratory media it exhibits antimicrobial activity against some strains of filamentous fungi (Allan and Hadwiger, 1979; Kendra and Hadwiger, 1984 and Roller and Covill, 1999), yeasts (Sudarshan, et al., 1992 and Roller and Covill, 1999) and bacteria (Wang, 1992). The antimicrobial activity of chitosan is greater at lower pH values (Roller and Covill, 1999) .

Gupta (2001) reported that when chitosan applied to cellulose by cross linking it gives both antimicrobial and moisture control properties so it is used in textiles industry to prevent bacteria and fungi as well as additives in paper industry to impart wet strength to paper (Wanich pong pan, 2002) .

The objective of this research was first: to determine the causative agent of paper biodegradation, second: to study the inhibitory effect of chitosan against cellulolytic fungi.

Materials and Methods: -

1- Isolation and identification:

Two deteriorated old valuable manuscripts (3rd book of El Mathnawy and Holy Koran, 1820) obtained from the stores of General Egyptian Book Organization (G. E. B. D) Cairo, Egypt Were obtained. The surface of the chosen manuscripts were wiped under asceptic conditions by a sterile wet cotton swab. The surface of potato dextrose agar (PDA) and carboxy methyl cellulose (CMC) Czapek's media poured into plates were wiped under asceptic conditions by the cotton swabs. The petri dishes were incubated at 27 ± 2° C for 7 days.

During the incubation period any emerged fungus was isolated onto PDA slants. Fungi were purified by using the single spore technique of Manandhar, *et al.* (1995) .

The obtained isolates were identified using the microscopic and cultural characteristics according to Gilman (1957); Booth (1971); Nelson, *et al.* (1982) and Barnett and Hunter (1986) .

2- Selection of cellulose degrading microorganisms:

The fungal isolates were screened for their cellulolytic activity by streaking on agar slants of the basal salt medium containing 1 % (w/v) CM Cellulose. The most active isolates that give cellulolytic activity were propagated on agar slants with the same constituents.

3- Effect of Chitosan:

Purified chitosan (obtained from National Reserch Centre, Cairo, Egypt) was dissolved in 0.25 N HCl by heating with constantly agitation. The solution was adjusted to pH 5.5 by adding sodium hydroxide 1 N, then 1 ml of tween 80 was added (El – Ghaouth, *et al.*, 1991).

a) **Radial growth**: the effect of chitosan on the radial growth of *F.oxysporum* and *T.harzianum* was studied using agar plates. An disc (5 mm-dm.) from pure cultures of tested fungi was placed in the center of a PDA plate containing chitosan at 0.0, 0.38, 0.75, 1.5, 3.0 and 4.5 mg / ml. Plates were incubated at 27 ± 2° C for seven days and the two diameters of every dish were measured. Four replicates were used for each particular treatment.

b) **Spore production**: Spores of tested fungi were harvested by scraping them of the agar with the aid of glass rod and distilled water. The mycelium and the spores mixture was double filtered through cheesecloth and resulting filtrate was adjusted to 20 ml. The number of spores/ml of filtrate was determined using Neubauer haemacytometer.

c) **Spore germination:** Cultures of 10 days old of tested fungi were flooded with sterile distilled water containing 0.1 % tween 80 and were gently agitated to remove the spores. PDA plates amended with chitosan were prepared as mentioned before.

Germination of 100 spores per plate was determined micro scopically as method described by El – Ghaouth, *et al.* (1992). A spore was considered germinated when the length of the germ tube equaled or exceeded the length of the spore.

d) **Paper properties:** Different concentrations of chitosan (0, 3.0 and 4.5 mg / ml) were applied on specimens of the tested paper (filter paper whatman No. 1). Chitosan was applied with a wide brush in one layer on the paper surfaces and kept to dry, then sprayed with fungal spores suspended in sterile distilled water. Paper specimens were incubated in a desiccator with 100 % relative humidity for one month at 28° C then, were scored visually for fungal invasion and decaying. Moreover, some physical and mechanical properties were recorded according to Annon (1951).

4- <u>Enzyme assays</u>:

Experimental cultures were made in 250 ml Erlenmeyer flasks each containing 50 ml of sterilized Mandels and weber's medium (1969) plus 0.5 gm filter paper at initial pH 5 and inoculated by 5 % (v / v) of inoculum of the tested fungi. The inoculated flasks were incubated on a rotatory shaker (180 rpm) at 30° C for 20 days. Culture filtrates were subjected to enzyme assays; β – 1,4 glucanase and β – glucosidase. Final pH and the biomass production of the cultures were also determined.

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 % soluim carboxy methyl cellulose, as substrate in 0.05 M citrate-phosphate buffer (pH 4.8) at 50° 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. 2 ml of arsenate reagent was thoroughly mixed with the cooled mixture, then the volume was completed to 25 ml with distilled water. On similar lines, the blank was prepared by adding citrate-phosphate buffer instead of the substrate. The resulting blue colour was measured at 520 nm in a spectrophotometer (BAUCH LOMB spectronic 2000 spectrophotometer). One unit of enzyme was defined as the amount of enzyme necessary to librate one μ mole of glucose under the assay conditions.

b) Cellobiase (β – glucosidase):

This was performed by a modification of 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 0.05 M citrate – phosphate buffer (pH 4.8), as substrate at 50° C for 30 min. The reaction was stopped by heating the reaction mixture in a boiling water bath for 3 min. The blank was prepared by using citrate-phosphate buffer instead the substrate.

The activity of the enzyme was determined by measuring the concentration of the released glucose by incubating 0.2 ml of the reaction mixture with 2.5 ml of diagnostic kits (glucose / peroxidase kit; according to Teller (1956) for 15 min at 37° C. The resulting pink colour was measured at 510 mm.

Statistical analysis :

Data obtained were subjected to analysis of variance according to procedures obtained by Snedcor and Cochran (1980).

Results and Discussions

Isolation, Identification and frequency of the isolated fungi :

Fifty five purified fungal isolates were obtained from old manuscripts samples (Fig. 1) & (Table 1). The fungal genera could be arranged on the basis of their frequent occurrence as follows:

Penicillium (32.73 %), *Aspergillus* (27.27 %), *Alternaria* (16.36 %), *Trichoderma* (14.54 %) and *Fusarium* (9.09 %) of the total fungal count.

Eight species belonging to five genera of the 55 isolates were identified and classified as: *Alternaria tenuis, Aspergillus candidus, A.flavus, A.niger, Fusarium oxysporum, Penicillium funiculosum, Trichoderma harzianum* and *T.viride*. The same fungi were also recorded by Mahmoud, *et al.* (1980); Sahaba (1988); Zyska, *et al.* (1995) and Wang, *et al.* (1999).

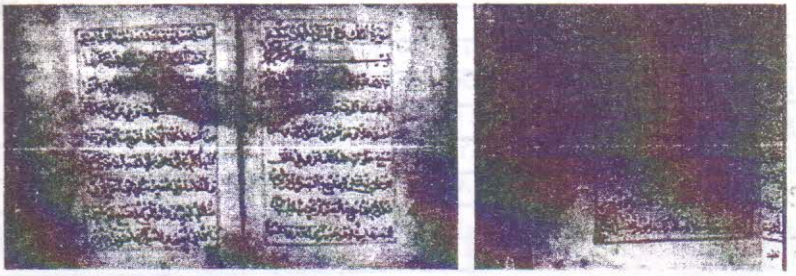

Fig. (1): deteriorated pages samples caused by fungi.

Table (1) occurrence and frequency of fungi isolated from old manuscripts :

Sam / Isolated fungi	3rd Book of El Mathnawy	Holy Koran (1820)	Total isolates	Frequncy %
	No. of isolates			
Penicillium	7	11	18	32.73
Aspergillus	6	9	15	27.27
Alternaria	3	6	9	16.36
Trichoderma	5	3	8	14.54
Fusarium	3	2	5	9.09
Total			55	

Trichoderma harzianum and *Fusarium oxysporum* obtained from old manuscripts were chosen for the following test as they gave good growth on CMC-Czapek's medium (Table 2).

Table (2) Fungal species associated with deteriorated old manuscripts, and their capability in decomposing carboxymethyl cellulose (CMC) :

Fungal species	No. of tested isolates	Growth on CMC-Czapek's medium			
		Not effective	Feeble effective	Moderate effective	Highly effective
Alternaria tenuis	9	4	2	3	-
Aspergillus candidus	3	1	2	-	-
Aspergillus flavus	7	4	3	-	-
Aspergillus niger	5	2	-	1	2
Fusarium oxysporum	5	-	-	-	5
Penicilluim funiculosum	18	8	6	2	2
Trichoderma harzianum	5	-	-	1	4
Trichoderma viride	3	-	-	2	1
Total	55	19	13	9	14

Effect of chitosan on the inhibition of fungal growth:

This experiment was undertaken to determine the effect of chitosen as agar amendment on the inhibition fungal growth of *T.harzianum* and *F.oxysporum*.

Table (3) shows the effect of chitosan on the mycelial linear growth, spore production and spore germination.

Data indicated that the antifungal activity of chitosan against tested fungi growth was significantly increased as the concentration of chitosan was increased (figures 2&3). Similar results were also reported by other investigators [Wang, 1992; Bautista, *et al.*, 2003 and Rabae Entsar, *et al.*, 2003]. Chitosan has been reported to inhibit the growth of several fungi [Stossel and Leuba, 1984; Hirano and Nagao, 1989 and El-Ghaouth, *et al.*, 1991].

Also, addition of chitosan to the agar medium led to sever inhibition to spore germination and spore production reaching 82.15% , 42.29% , 11.54% , 0.0% and 5.1 , 3.4 , 2.3 , 1.0 , 0.7 / ml for *F.oxysporum*, while for *T.harzianum* they reach 83.41% , 72.05% , 26.39% , 25.06% , 0% and 2.7 , 2.1 , 1.6 , 1.2 , 0.4 /ml at 0.38 , 0.75 , 1.50 , 3.00 and 4.5 mg / ml of chitosan respectively .

Similar inhibitory effects of chitosan were recorded by Benhamou, *et al.* (1994) and El-Gaouth, *et al.* (1997).

Complete inhibition of germination, however was achieved at a concentrations of 3.00 mg / ml for *F.oxysporum* and4.5 mg / ml for *T.harzianum*, indicating that chitosan is fungicidal rather than fungistatic. In this regard it is worth to note that Rabea, Entsar, *et al.* (2003) reported that chitosan has been shown to be fungicidal against several fungi. The minimum inhibitory concentrations reported for specific target organisms range from 0.0018 % to 1.00 % and are influenced by a multitude of factors such as pH of the growth medium, the degree of polymerization of chitosan and the presence or absence of interfering substances such as lipid and proteins .

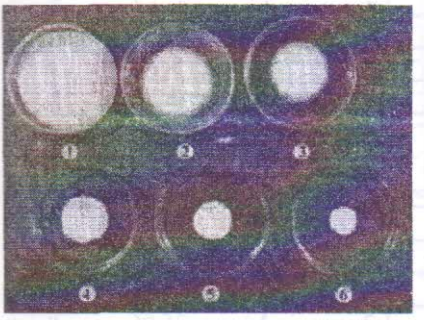

Fig. (2): effect of different concentrations of chitosan on linear growth of *F.oxysporum*

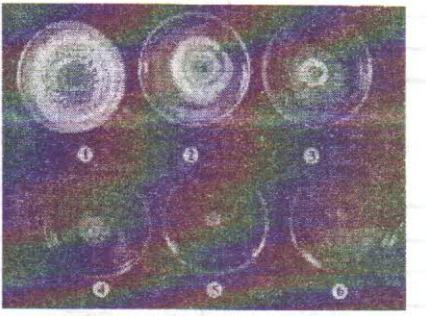

Fig. (3): effect of different concentrations of chitosan on linear growth of *T.harzianum*

Table (3) : Effect of different concentrations of chitosan on growth, spore production and germination of *T.harzianum* and *F.oxysporum*:

Parameters / Chitosan Conc.	Linear Growth (mm)	Spore production (numbers/ml)	Germination %
Fusarum oxysporum			
0.00	90	12.3	100
0.38	79.3	5.1	82.15
0.75	73.3	3.4	42.29
1.50	53.0	2.3	11.54
3.00	40.3	1.0	0.00
4.50	34.3	0.7	0.00
Trichoderma harzianum			
0.00	90	6.6	100
0.38	59.5	2.7	83.41
0.75	53.0	2.1	72.05
1.50	42.8	1.6	26.39
3.00	22.5	1.2	25.06
4.50	16.0	0.4	0.00
L. S. D. at 50 %	Conc. = 3.00 Fungi = 1.73 Interaction = 4.25	9.28 5.36 13.13	4.46 2.57 6.31

Effect of chitosan on the production and activity of cellulases enzymes:

The experiment was made to examine the effect of different concentrations of chitosan on the excretion of carboxymethyl cellulase and cellobiase.

The results in Table (4) indicated that the effect of chitosan on the production of CMC – ase was more than its effect on the production of cellobiase of both *T.harzianum* and *F.oxysporum*. Also, addition of chitosan to cellulases medium led to reduction of *T.harzianum* CMC – ase production to 17.3 % & 9.61 % and whose cellobiase production to 34.44 % & 2.22 % at 3 & 4.5 mg/ml chitosan respectively. On the other hand CMC – ase production of

F.oxysporum was completely inhibited at 4.5 mg/ml and its cellobiase production decreased to 37.5 % & 12.5 % at 3 & 4.5 mg/ml of chitosan respectively.

Table (4): Effect of chitosan concentrations on the production of CMC – ase and cellobiase by *T.harzianum* and *F.oxysporum* using filter paper as a substrate:

Organism	Chitosan Conc. (mg/ml)	Final pH	Dry weight (9/50 ml culture)	% of the relative activity	
				CMC - ase	cellobiase
T.harzianum.	0.0	7.2	0.580	100	100
	3.0	5.4	0.423	17.30	34.44
	4.5	5.3	0.553	9.61	2.22
F.oxysporum.	0.0	7.3	0.720	100	100
	3.0	4.8	0.713	15.38	37.5
	4.5	4.6	0.786	0.00	12.5

To prove if the effect of chitosan on decreasing the levels of the enzymes resulting from its effect on the production only and / or its activity too, the culture filtrates of the control of both *T.harzianum* and *F.oxysporum* were subjected to incubation with 4.5 mg/ml chitosan at different time intervals.

The results in Table (5) clearly indicated that chitosan had a great inhibition effect on the enzyme activities. CMC – ase and cellobiase of *F.oxysporum* showed complete inhibition after 30min incubation period. On the other hand, CMC – ase of *T.harzianum* was completely inhibited after 45 min while, the activity of cellobiase reduced to 42.22 % after 60 min.

Table (5) : Effect of (4.5 mg/ml) chitosan concentration and the time of incubation on the activity of CMC – ase and cellobase of *T.harzianum* and *F.oxysporum*:

Organism	Time (min)	% of Residual CMC – ase activity	% of Residual Cellobiase activity
T.harzianum.	0.0	100	100
	15	7.69	68.88
	30	5.76	60.00
	45	0.00	46.66
	60	0.00	42.22
F.oxysporum.	0.0	100	100
	15	25	12.5
	30	0.00	0.00
	45	0.00	0.00
	60	0.00	0.00

Effect of chitosan on the paper properties :

The experiment was done to determine the effect of treatments with chitosan on physical and mechanical properties comprising air permeability, tensile strength and folding endurance of filter paper fungly infected by *T.harzianum* and *F.oxysporum.*

The results obtained are recorded in Table (6). Data showed that both *T.harzianum* and *F.oxysporum* had a deteriorating effect on filter paper. The observed decrease in tensile strength and folding and increase in air permeability is related to break down some of long cellulosic chains to smaller units by the action of exoenzymes produced from the micro organisms (Zabel and Morrell, 1992 and Reinikainen, *et al.*, 1995). The results also showed that chitosan has a positive effect on the paper properties. It increases the strength of paper as binds to cellulose by cross linking giving antimicrobial and moisture control properties (Gupta, 2001) and impart strength to it (Wanich pang pan, 2002).

Table(6): Effect of Treatments with chitosan on physical and mechanical properties of filter paper fungly infected by *T.harzianum* and *F.oxysporum*:

Organisms	Samples	Tensile strength kg/15 mm		Folding endurance		Air permeability $cm^3/cm^2/sec.$
		In machine direction	In cross direction	In machine direction	In cross direction	
T.harzianum.	Control	3.4	2.4	1005	95	1.13
	T_1	3.2	2.5	118	45.5	1.27
	T_2	3.7	2.6	437.3	235	1.21
	T_3	4.1	2.5	832	67.8	1.17
F.oxysporum.	Control	3.4	2.4	1005	95	1.13
	F_1	3.2	2.4	63.7	37.5	1.44
	F_2	3.4	2.6	618.3	308	1.41
	F_3	3.9	2.6	1820	100.5	0.956

- Control : uninfected, untreated paper.
- T_1 : paper infected by *T.harzianum.*
- F_1 : paper infected by *F.oxysporum.*
- T_2 & F_2 : paper treated by 3 mg/ml chitosan.
- T_3 & F_3 : paper treated by 4.5 mg/ml chitosan.

In conclusion, this study demonstrates the potential of chitosan, a nontoxic compound, to suppress the deteriorating effect of celluloytic fungi. This potential value appear to be attributable to the combination of the antifungal and strengthening properties of chitosan. These unique properties may very well make chitosan an ideal antifungal against biodeterioration of valuable manuscripts considering its nontoxic effect on the environment.

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