Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 3 (4): 213-224, 2008
ISSN 1816-4935

Production of Cellulase and Pectinase from Some Aquatic Hyphomycetes

M.E. Osman, H.K. Om Kalthoum and A.A. El-Shaphy
Department of Botany, Faculty of Science, Helwan University, Cairo, Egypt

Abstract: The highest total activity of cellulases by *Dactylella aquatica* and *Cylindrocarpon heteronemum* was obtained after 9 days incubation at pH 11-14 and 20-25°C, respectively. The enzyme was produced in the presence of cellulose as a sole carbon source. $CaCl_2$ at level 1000 ppm was the most convent for cellulyltic activity of *D. aquatica* where as growth at this concentration was nearly 1/3 that of control. Although $CaCl_2$ was effective in decreasing cellulyltic activity of *C. heteronemum*. Growth of *D. aquatica* and *C. heteronemum* inhibited by the presence of $CaSO_4$. Polygalacturonase (PG), Pectin Lyase (PL) and pectinmethylestrase (PME) were investigated qualitatively in most active of aquatic fungi.

Key words: Cellulase, aquatic hyphomycetes pectinase

INTRODUCTION

There is a group of deuteromycotina which are known by the conidial state in aquatic habitats, some of these species are also can found in terrestrial habitats. These fungi are referred to as Hyphomycetes. Aquatic Hyphomycetes colonize wood as well as leaves in streams (Kane, 1978; Sanders and Andersen, 1979; Willoughby and Archer, 1973; Khattab, 1991; Shearer and Webster, 1991; Yuen *et al*., 1998). Maximum leaching of sugars from twigs is within 3 weeks of submersion; but some leaching continues for up to 12 weeks (Willoughby and Archer, 1973). To exploit plant cell wall polymers, aquatic hyphomycetes possess enzymes such as pectinases; hemicellulases and cellulases which show ability to degrade lignin (Dwt *et al*., 1992; Schearer, 1992; Rodrigues and Graca, 1997). Aquatic hyphomycetes were investigated for ligninolytic activity (Barlocher and Kendrick, 1974; Sanders and Andersen, 1979; Jones, 1981, 1985; Fisher *et al*., 1983; Dwt *et al*., 1992; Abdel-Raheem, 1992).

The cellulolyltic activity of leaves during different stages of decomposition and at two season of contrasting temperature was examined in an attempt to correlate the patterns of enzymatic activity with the associated microbial community (Sinsabaugh *et al*., 1981; Hodgkiss and Leung, 1986). The mechanism of enzymatic hydrolysis of cellulose by filter paper cellulose (C1) and carboxymethyl cellulose (Cx) involved a two step process with an initial activation by non-hydrolytic enzyme followed by the hydrolytic carboxymethyl cellulase. Existence of the non-hydrolytic enzyme has not been confirmed. The isolation of filter paper cellulase and carboxymethyl cellulase enzymes was attempted by workers and later these were tentatively identified as exo-cellobiohydrolase and endo-1, 4-β-D-glucanase. Both of these enzymes exhibit a distinctive mode of action toward cellulosic substrate. The present concept of crystalline or native cellulose hydrolysis involves three major enzymes as cellobiohydrolase, endoglucanase and β-glucosidase (β-D-glycoside glucohydrolase). The biological decomposition of pectin is an important degradation process. In nature, pectin is degraded by enzymatic systems produced by a wide variety of saprophytic and phytopathogenic microorganisms, including bacteria and fungi (Rombouts and Pilnik, 1980). Pectinases are group of

Corresponding Author: M.E. Osman, Department of Botany, Faculty of Science, Helwan University, Cairo, Egypt

enzymes that hydrolyze pectin by different mechanisms (Fogarty and Kelly, 1982) and are divided into: pectin methylestrase (PME), polygalacturonase (PG) and pectin and pectatelyase (PNI and PL). For each class of enzymes there may be hydrolyze and lyases that clear randomly the appropriate polymers. Polygalacturonases have been isolated from the culture media of various mesophilic fungi such as *Aspergillus japonicus* (Tshii and Yokotsuka, 1972) *Sclerotinia sclrotiorum* (Kenon and Waksman, 1990) and *Colletotrichum lindemuthianum* (English *et al*., 1972; Kenon and Waksman, 1990). The enzymes are secreted by the microorganisms and some of them macerate some plants effectively (Scott-Craig *et al*., 1990; Miyairi *et al*., 1985) obtained a polygalacturonases from the culture medium of *Stereum purpureum* and showed that the enzyme caused silver leaf symptoms on apple trees. Recent studies of polygalacturonases have indicated that this enzyme is involved in the signaling systems of plant that regulated defensive and development at processes (Ryan and Farmer, 1991; Fischer and Bennett, 1991; Ferrari *et al*., 2003; Sicilia *et al*., 2005; Nighojkar *et al*., 2006).

The present study was carried out to investigate aquatic hyphomycetes fungi occurring on colonizing wood as well as leaves in the river Nile posses enzymes such as cellulases and pectinases and show an ability to degrade the cell wall polymers subastances The effect of some factors such as temperature, pH, incubation period, carbon source and heavy metals were carried out on the cellulolytic activity.

MATERIALS AND METHODS

Fungal Isolate

D. aquatica and *C. heteronemum* were isolated from the River Nile (Elquanater Elkhayria) by El- Shafy (2000).

Bioassay Medium for Detection of Pectinase Activity

The procedure used here was that used by Ammar *et al*. (1994)

Pectic Enzyme Assays

The methods used for enzyme assay were these described by Duncan (1969).

Screening for Cellulolytic Activity

The basal media were supplemented with different cellulosic sources, carboxymethyl cellulose or filter paper Whatman No. 1 while were proved to be best sources for the production of cellulase.

According to (Bland and Douglas, 1977) the basic medium used for cellulase production composed of the following ingredients (g L^{-1}). Urea, 0.3; KH_2PO_4; $2(NH_4)_2SO_4$ 1.4; $MgSO_4 7H_2O$, 0.3; cellulose, 5; peptone, 1; $FeSO_4.7H_2O$, 1; $MnSO_4.7H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.7; $COCl_2$, 2 and distilled water 1 L.

Detection of Cellulase Activity in Liquid Media

This method was measured as described by Mandels *et al*. (1976a).

Factors Affecting Cellulase Activity

In this experiment two isolates of *Dactylella aquatica* and *Cylindrocarpon heteronemum* which were the highest cellulase producers among the other isolated from aquatic fungi tested were chosen (Table 1). The cellulolytic activities of chosen fungi were investigated under different environmental factors. Factors such as pH, temperature incubation period, carbon source and heavy metals were considered.

Table 1: Cellulolytic activities of isolated fungi on solid medium

Fungal species	Diameter of clear zone (mm)
Anguillospora longissima	2.7
Achlya sp.	0.7
Alternaria sp.	2.5
Aspergillus candidum	2.2
Aspergillus clavatus	2.4
Aspergillus flavus	2.7
Aspergillus fumigatus	2.9
Aspergillus niger	3.6
Candida aquatica	1.9
Cylindrocarpon candidum	3.3
Cylindrocarpon didymum	3.6
Cylindrocarpon heteronemum	4.1
Cylindrocarpon ianthothele	2.8
Dactylella aquatica	3.1
Flagellospora curvula	2.4
Fusarium chlamidosporium	3.1
Fusarium merismoides	3.1
Fusarium oxysporium	3.6
Fusarium poae	3.3
Fusarium tumidum	2.9
Heliscus lugdunensis	2.3
Infundibura adhaerens	2.0
Penicillium sp.	2.9
Rhizopus sp.	6.0
White sporodochial fungus	1.8

Effect Hydrogen Ion Concentration

The effect of pH on cellulase enzymes production by *Dactylella aquatica* and *Cylindrocarpon heteronemum* were determined at pH range from 3, 5, 7, 9, 11 and 14 using 0.1 N NaOH or 0.1 HCl.

Effect of Temperature

The effect of temperature on enzyme production was tested at 20, 25, 30, 35, 40, 45 and 50°C.

Effect of Incubation Period

Cellulase activity was determined for different incubation period, at zero time 3, 5, 7, 9, 12 and 15 days.

Effect of Carbon Source on Cellulolytic Activity

The basal medium was contained 50 mL and weighting different carbon source as glucose, sucrose, starch, autoclaved, inoculated with 1 mL of spore suspension and incubate at optimum temperature.

Effect of Heavy metals

Prepare the basal medium and weight different molarity of heavy metals as calcium, copper, manganese, magnesium and cobalt (mg L^{-1}) and adjust optimum pH thin distributed the medium in a conical flasks, each containing 50 mL and autoclave as usual. Inoculated each flask with 1 mL spore suspension of *Dactylella aquatica* and *Cylindrocarpon heteronemum* then incubate at optimum temperature for optimum incubation period and filter the mycelia keep the filtrate cool until use for the assay.

Cellulolytic Activity Assay

Cellulase was measured in terms of filter paper cellulase activity (Cl), carboxymethyl cellulose (Cx) by Mandel *et al*. (1976a).

Determination of Filter Paper Cellulase (Cl)

To 50 mg filter paper (Watman No. 1), 1 mL sodium acetate buffer pH 4 and 0.5 mL enzyme filtrate were added. The mixture was incubated at 50°C for 30 min (Sternberg, 1976; Nisizawa *et al*., 1978). The liberated reducing sugars were measured as glucose using 1 mL of 3,5 dinitrosalcylic acid reagent.

Determination of Carboxymethyl Cellulose (Cx)

The method was based on that described by Su (1976).

Determination of β-Glucosidase (β-G)

To 0.5 mL of filtrate enzyme culture 0.5 mL of 1% salicine was added and incubated for 30 min at 50°C the liberated reducing sugars were measured as glucose using 3,5 dinitrosalicylic acid reagent.

Determination of Reducing Sugar in the Crud Enzyme Filtration

To 0.5 mL enzyme filtrate, 1 mL of 1% 3, 5 dinitrosalicylic acid reagent was added in a test tube, then heated for 3 min in boiling water bath, cooled in ice bath. Absorbency reading was taken at 540 nm using speckol spectrophotometer pure glucose was prepared as reference for constructing the calibration curve (Willis and Scott, 1988).

Enzyme Precipitation Using Ammonium Sulphate

Grow the organisms on the optimum growth; then filter the culture and transfer the filtrate into fresh tube or tubes. According to the table of ammonium sulfate apply the different concentration as, Zero, 10, 20, 30, 40, 50% as follow for each concentration. Calculate the needed grams of ammonium sulfate according to the used volume of filtrate. Add the salt gradually and shake gently, after complete dissolving of the salt, leave the tube to stand for 30 min at 15000 rpm at 4°C. Transfer the supernatant into fresh tube and keep the ppt (as No. 1). The supernatant is Sl. To the supernatant add more grams of ammonium sulfate according to the next wanted concentration dissolve gently and leave to stand at room temperature for 30 min. Then centrifuge for 20 min at 15000 rpm. at 4°C after centrifugation, transfer the supernatant into fresh and keep as ppt No. 2. The supernatant is S2. Carry on all the wanted concentration following the same steps as before. All the obtained ppts. were dissolved in equal volumes of the used buffer. See the activity of the enzyme in the ppts. Solution and in the different supematants (S1, S2, S3,.....).

Statistical Analysis

Analysis of variances were performed with Program Minitab Throughout the result were calculated. All replicates were repeated at lest 6 times.

RESULTS

The ability of fungal isolates to hydrolyze cellulose and pectin are shown in (Table 1). A strong cellulalyltic ability were shown by *Cylindrocarpon heteronemum*, *Dactylella aquatica.* However, the ability of these isolates to produce pectinolytic enzymes were variable. The most active isolates were *Rhizopus* sp., *C. heteronemum*, *F. chlamidosporium*, *F. merisopedium* and *C. candidum.* The remaining species had limited cellulalyltic and pectinolytic ability.

Effect of Temperature on Cellulase Activity

The maximum dry biomass production in *Dactylella aquatica* was obtained at 25°C. Biomass markedly decreased by the rise in temperature (Table 2).

Table 2: Effect of different temperatures on biomass (dry weight g L^{-1}) and cellulolytic activity (μ mL^{-1}) of *Dactylella aquatica* and *Cylindrocarpon heteronemum*

	Dactylella aquatica							*Cylindrocarpon heteronemum*						
		Enzyme activites (μ mL^{-1})							Enzyme activites (μ mL^{-1})					
Temperature (°C)	Biomass (g)	C1	Cx	βG	TA	PC	SA	Biomass (g)	C1	Cx	βG	TA	PC	SA
15	0.7	136.60	129.7	146.9	413.2	29.8	13.9	1.5	124.50	150.5	133.8	408.8	64.3	6.3
20	1.1	163.60	144.4	156.4	464.4	38.5	12.1	2.9	157.00	142.7	138.7	438.4	78.7	5.5
25	2.3	165.60	277.8	131.6	575.0	54.9	10.5	1.3	158.00	128.8	137.5	424.3	98.2	2.2
30	1.0	162.00	200.8	124.1	486.9	37.2	13.1	1.2	157.00	125.0	133.7	415.7	110.0	3.7
35	0.9	161.30	135.6	121.6	418.5	30.3	13.8	0.8	133.60	123.2	132.0	388.8	80.2	4.8
40	0.6	151.80	133.7	120.2	405.7	29.0	14.0	0.8	133.50	120.3	129.0	382.8	73.4	5.2
45	0.3	148.90	131.6	118.9	399.4	25.8	15.5	0.4	126.30	119.1	123.7	389.1	61.5	6.3
50	0.2	120.50	118.3	117.9	356.7	18.8	19.0	0.2	120.50	118.6	122.8	361.9	60.0	6.0
LSD 0.01		73.06							38.65					
LSD 0.05		46.58							24.64					

C1: Filter paper cellulase, Cx: Carboxymethyl cellulase, βG: β-glucosidase: salicinase, TA: Total Activity, PC: Protein Concentration, SA: Specific Activity (μ mg^{-1} protein), LSD: Least Significant Difference

On the other hand, filter paper cellulase (Ci) and β-glucosidase showed insignificant differences among the studied range of temperature (15-50°C). However, carboxymethyl cellulase (Cx) activity increased significantly and reached maximum activity (277.8 μ L^{-1}) at 25°C, the rise in the incubation temperature to 50°C resulted in a significant decrease in the activity and reached about 43% that recorded at 25°C. In general, 25°C was the most convenient temperature for the production of total cellulases (575 μ L^{-1}) in *Dactylella aquatica*. Higher temperature decreased total activity significantly. The highest specific activity (18.9 μ mg^{-1}) has been observed at temperature 50°C which produced the least total activity (356.7 μ L^{-1}).

In case of *Cylindrocarpon heteronemum*, the response of cellulases to the various studied temperatures was not very clear, in case of filter paper cellulase (Ci), activity was increased significantly in the temperature range (20-30°C), then started to decline and reached its minimal value at 50°C. On the other hand, carboxymethyl cellulase (Cx) showed its highest activity at temperature range (15-20°C), then the activity decreased to reach its lowest value at 50°C. B-glucosidase activity showed insignificant differences in the range of tested temperature. Maximum growth represented by dry biomass (2.9 g L^{-1}) obtained when the cultures incubated at 20°C. The highest total activity was also obtained at 20°C (438.4 μ L^{-1}).

Effect of pH on Cellulase Activity

Cellulolytic activity of *Cylindrocarpon heteronemum* and *Dactylella aquatica* was investigated under a wide range of pH (3-14). Although growth was markedly inhibited under conditions of high acidity or alkalinity, cellulases were not affected in the same manner observed for biomass production. pH 7 was the best for maximum production of biomass in case of *Dactylella aquatica* (2.1 g L^{-1}) while pH 5 produced maximum biomass in case of *Cylindrocarpon heteronemum* (2.5 g L^{-1}).

The results of (Table 3) showed clearly that β-glucosidase activity was insignificantly changed under all tested pH values (3-14). In case of *Dactylella aquatica* slight increase in C1 activity was observed at pH 5, while Cx activity significantly increased at pH 7. In general total cellulases activity significantly increased at pH 7 with activity (417.8 μ L^{-1}) compared to (370 μ L^{-1}) at pH 3 and (374.4 μ L^{-1}) at pH 14.

In case of *Cylindrocarpon heteronemum*, the most obvious observation was the significant increase in C1, which reached (161.1 μ L^{-1}) at pH 5. Total activity was also maximum (418.9 μ L^{-1}) at pH 5. Variable values of specific activity were obtained under different pH conditions, pH 11 and 14 showed the highest values in both fungi.

Table 3: Effect of different pH values on biomass (dry weight g L^{-1}) and cellulolytic activity (μ mL^{-1}) of *Dactylella aquatica* and *Cylindrocarpon heteronemum*

	Dactylella aquatica							*Cylindrocarpon heteronemum*						
		Enzyme activites (μ mL^{-1})							Enzyme activites (μ mL^{-1})					
pH	Biomass (g)	C1	Cx	βG	TA	PC	SA	Biomass (g)	C1	Cx	βG	TA	PC	SA
	0.1	122.7	120.0	127.2	369.9	23.1	16.0	0.4	120.8	120.7	125.8	367.3	44.1	8.30
5	1.7	138.8	132.0	130.8	401.7	40.4	9.9	2.4	161.1	119.1	138.7	418.9	48.8	8.50
7	2.1	128.1	153.4	126.3	407.8	45.1	9.1	1.4	129.2	136.5	137.1	402.8	45.3	8.90
9	0.2	124.4	132.8	137.3	394.5	22.1	17.8	1.3	126.6	138.7	131.8	397.1	25.2	15.76
11	0.1	122.0	122.5	133.8	378.2	15.2	24.9	0.8	121.6	138.2	130.7	390.5	20.4	19.14
14	0.1	121.9	118.7	133.7	374.4	10.1	37.1	0.4	121.3	119.2	127.9	367.4	17.8	20.64
LSD 0.01		16.0							20.711					
LSD 0.05		10.2							13.206					

C1: Filter paper cellulase, Cx: Carboxymethyl cellulase, βG: β-glucosidase: Salicinase, TA: Total Activity, PC: Protein Concentration, SA: Specific Activity (μ mg^{-1} protein), LSD: Least Significant Difference

Table 4: Effect of incubation periods on biomass (dry weight g L^{-1}) and cellulolytic activity (μ mL^{-})[1] of *Dactylella aquatica* and *Cylindrocarpon heteronemum*

	Dactylella aquatica							*Cylindrocarpon heteronemum*						
Incubation period		Enzyme activites (μ mL^{-1})							Enzyme activites (μ mL^{-1})					
(days)	Biomass (g)	C1	Cx	βG	TA	PC	SA	Biomass (g)	C1	Cx	βG	TA	PC	SA
3	1.4	118.20	118.3	119.2	355.5	62.3	5.7	2.1	121.50	121.7	118.2	361.4	95.61	3.78
5	2.5	120.50	121.0	130.7	372.2	100.7	3.7	3.0	131.40	130.8	128.2	390.4	98.83	3.95
7	2.9	127.20	136.8	131.7	395.7	132.2	3.0	4.2	134.50	135.0	131.0	400.5	132.40	3.03
9	3.2	131.60	133.0	131.6	396.2	123.7	3.2	4.8	174.80	138.7	135.1	448.6	284.50	1.58
12	4.5	200.00	146.9	168.4	515.1	211.8	2.4	3.7	150.70	146.9	130.8	428.4	220.90	1.94
15	4.0	137.30	131.9	152.2	421.4	123.2	3.4	3.2	120.40	120.3	129.0	369.7	92.78	3.98
LSD 0.01		31.13							25.24					
LSD 0.05		19.85							16.09					

C1: Filter paper cellulase, Cx: Carboxymethyl cellulase, βG: β-glucosidase: Salicinase, TA: Total Activity, PC: Protein Concentration, SA: Specific Activity (μ mg^{-1} protein), LSD: Least Significant Difference

Effect of Incubation Period

Table 4 shows the effect of incubation period on cellulases as well as biomass production and specific activity. It is obvious that growth of *Dactylella aquatica* reached its maximal growth after 12 days of incubation (4.5 g L^{-1}), while maximum growth (4.8 g L^{-1}) was observed after 9 days of incubation in case of *C. heteronemum*. The results showed clearly that cellulases, especially C1 and β-glucosidase significantly increased by the increase in incubation period and reached their maximum activity (200 and 168.4 μ L^{-1}, respectively) in 12 days of incubation in case of *Dactylella aquatica*. Total activity showed the same trend and produced maximum value (515 μ L^{-1}) after the same period of incubation.

In case of *Cylindrocarpon heteronemum*, the most observed increase was in case of C1, which reached its maximum activity (174.8 μ L^{-1}) in 9 days of incubation. Total activity was at its highest level (448.6 μ L^{-1}) after the same period of incubation. It is clear that cellulolytic activity in both fungi was related to fungal growth.

Effect of Different Carbon Sources

Four carbon sources, representing mono, di- and polysaccharides were examined for their ability to induce cellulolytic activity in *D. aquatica* and *C. heteronemum* (Table 5). The most effective carbon source was cellulose, which resulted in total activity as much as 5 times that of starch and 10 times as that in the presence of sucrose. β-glucosidase activity represented about 40% that of the total activity. Although growth was highest in the presence of glucose yet cellulases were not detectable in the filtrate.

Table 5: Effect of carbon sources on biomass (dry weight g L^{-1}) and cellulolytic activity (µ mL^{-1}) of *Dactylella aquatica* and *Cylindrocarpon heteronemum*

	Dactylella aquatica							*Cylindrocarpon heteronemum*						
		Enzyme activites (µ mL^{-1})							Enzyme activites (µ mL^{-1})					
Carbon source	Biomass (g)	C1	Cx	βG	TA	PC	SA	Biomass (g)	C1	Cx	βG	TA	PC	SA
Glucose	3.8	ND	ND	ND	0	0	0	5	ND	ND	ND	0	0	0
Sucrose	1.6	57.2	89.5	74.0	220.7	257.6	0.8	2.7	66.20	43.9	38.7	148.8	399.2	0.3
Starch	1.1	22.8	66.4	33.6	122.8	232.3	0.5	1.9	41.00	26.3	82.9	150.2	187.8	0.4
Cellulose	2.6	322.6	369.8	442.2	1134.6	772.8	1.5	3.9	139.33	119.1	122.0	380.4	98.8	2.6
LSD 0.01		33.6							11.90					
LSD 0.05		25.5							9.00					

ND: Not Determined, C1: Filter paper cellulase, Cx: Carboxymethyl cellulase, βG: β-glucosidase: salicinase, TA: Total Activity, PC: Protein Concentration, SA: Specific Activity (µ mg^{-1} protein), LSD: Least Significant Difference

Table 6: Effect of different $CaCl_2$ concentrations on biomass (dry weight g L^{-1}) and cellulolytic activity (µ mL^{-1}) of *Dactylella aquatica* and *Cylindrocarpon heteronemum*

	Dactylella aquatica							*Cylindrocarpon heteronemum*						
		Enzyme activites (µ mL^{-1})							Enzyme activites (µ mL^{-1})					
Concentration (mg L^{-1})	Biomass (g)	C1	Cx	BG	TA	PC	SA	Biomass (g)	C1	Cx	BG	TA	PC	SA
Control	4.5	269.30	148.4	166.4	584.1	411.7	1.4	4.8	174.8	188.7	135.1	498.6	308.2	1.6
250 ppm	0.2	163.90	151.4	158.8	474.2	342.6	1.3	1.4	162.7	123.0	167.4	453.1	145.2	3.1
500 ppm	0.5	183.90	160.1	151.7	494.8	348.5	1.4	1.4	148.5	122.2	164.8	435.5	108.7	4.0
1000 ppm	3.5	267.50	160.8	144.7	572.9	213.1	2.6	1.0	139.0	121.4	153.0	413.4	69.7	5.8
1500 ppm	0.2	183.80	144.1	135.4	463.3	164.9	2.8	0.6	130.4	120.5	133.8	384.8	125.3	4.2
2000 ppm	0.1	120.50	132.1	125.5	378.1	148.9	2.5	0.2	122.1	119.7	124.7	366.6	54.5	6.7
LSD 0.01		62.46							29.0					
LSD 0.05		47.5							22.0					

ND: Not Determined, C1: Filter paper cellulase, Cx: Carboxymethyl cellulase, βG: β-glucosidase: salicinase, TA: Total Activity, PC: Protein Concentration, SA: Specific Activity (TA/PC) (u mg^{-1} protein), LSD: Least Significant Difference

The same trend has been observed in case of *C. heteronemum*. The presence of cellulose markedly induced cellulylytic activity, which produced (380.4 µ L^{-1}) while in other carbon sources, totals activities were 150.2 and 148.2 µ L^{-1} for starch and sucrose, respectively. In both fungi Specific Activity (SA) was higher in case of cellulose than in the other used carbon sources.

Effect of Calcium Chloride

In Table 6 variable responses of tested fungi in the presence of different concentrations of calcium chloride as well as under control conditions. In case of *D. aquatica*, filter paper cellulase C1 activity significantly decreased by the addition of $CaCl_2$ to the growth medium. All tested $CaCl_2$ concentrations showed the same response with the exception of the concentration (1000 ppm). Total activity, at this concentration was nearly the same as that under control conditions without the addition of $CaCl_2$. All detected fractions of cellulases were more or less as the same levels of controls.

Higher concentrations resulted in a marked decrease in activity, which was about 40%, that of the control when added at the level 1500 ppm. It seems that $CaCl_2$ at level 1000 ppm was the most convenient for cellulolytic activity of *D. aquatica*, the growth at this concentration was nearly 1/3 that of control.

On the other hand, in the case of *C. heteronemum* growth was markedly inhibited by the addition of $CaCl_2$ salt to the growth medium, the lowest used concentration (250 ppm) produced nearly 1/3 that of the dry biomass produced under control conditions (in absence of $CaCl_2$). The developed growth under the stress of high concentration of Cad; (2000 ppm) was only 5% that of the control. Activities of filter paper cellulase C1 and carboxymethyl cellulase Cx were reversibly related to $CaCl_2$

Table 7: Effect of different $CaSO_4$ concentrations on biomass (dry weight g L^{-1}) and cellulolytic activity (u mL^{-1}) of *Dactylella aquatica* and *Cylindrocarpon heteronemum*

	Dactylella aquatica							*Cylindrocarpon heteronemum*						
		Enzyme activites (μ mL^{-1})							Enzyme activites (μ mL^{-1})					
Concentration ($mg\ L^{-1}$)	Biomass (g)	C1	Cx	βG	TA	PC	SA	Biomass (g)	C1	Cx	βG	TA	PC	SA
Control	4.5	269.3	148.4	166.4	584.1	411.7	1.4	4.8	174.80	188.7	135.1	498.6	308.2	1.6
250 ppm	1.5	183.1	150.0	159.2	492.3	521.3	1.1	2.3	134.20	130.4	145.2	409.8	510.1	0.8
500 ppm	1.2	154.7	139.0	145.4	439.2	447.5	0.8	1.9	126.50	159.9	132.6	419.1	755.3	1.6
1000 ppm	0.5	138.8	129.6	123.7	392.1	377.8	1.1	1.2	124.40	144.8	128.7	398.0	251.7	9.0
1500 ppm	0.3	121.6	126.6	121.8	378.0	291.8	1.3	0.9	120.10	132.7	121.6	374.4	146.0	5.1
2000 ppm	0.0	ND	ND	ND	ND	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.0	0.0
LSD 0.01		29.0							26.36					
LSD 0.05		22.0							20.06					

ND: Not Determined, C1: Filter paper cellulase, Cx: Carboxymethyl cellulase, βG: β-glucosidase: salicinase, TA: Total Activity, PC: Protein Concentration, SA: Specific Activity (TA/PC), LSD: Least Significant Difference

Table 8: Pectenasic activites of isolated fungi on solid medium

Fungal species	Diameter of clear zone (mm)
Anguillospora longissima	1.5
Achlya sp.	1.2
Alternaria sp.	2.3
Aspergillus candidum	1.9
Aspergillus clavatus	1.7
Aspergillus flavus	2.0
Aspergillus fumigatus	2.2
Aspergillus niger	2.4
Candida aquatica	1.9
Cylindrocarpon candidum	3.9
Cylindrocarpon didymum	2.9
Cylindrocarpon heteronemum	4.0
Cylindrocarpon ianthothele	2.8
Dactylella aquatica	1.7
Flagellospora curvula	1.3
Fusarium chlamidosporium	4.3
Fusarium merismoides	3.9
Fusarium oxysporium	2.7
Fusarium poae	2.5
Fusarium tumidum	2.1
Heliscus lugdunensis	1.6
Infundibura adhaerens	1.1
Penicillum sp.	2.6
Rhizopus sp.	6.1
White sporodochial fungus	2.3

Table 9: Pectinolytic activity

Fungal sp.	Polygalactuonase (PG)	Pectin layase (PL)	Pectin estrase (PE)
Cylindrocarpon heteronemum	±	±	-
Cylindrocarpon candidum	±	±	-
Fusarium chlamidosporium	-	±	-

concentration, their activities decreased significantly with increase in $CaCl_2$ concentration and reached minimal value when growth medium amended with (15 00 ppm) $CaCl_2$. However, β-glucosidase activity was significantly higher at low concentrations (250 and 500 ppm) and started to decline by increasing concentration to reach the lowest value at (1500 ppm) $CaCl_2$. In general, calcium chloride was effective in decreasing cellulolytic activity of *C. heteronemum*.

Effect of Calcium Sulphate

The results of (Table 7) showed the response of tested fungi towards the addition of different concentrations of $CaSO_4$ to growth medium. The trend in this experiment is very clear.

Growth and biomass production markedly inhibited by the presence of $CaSO_4$ in growth medium and such inhibition in activity increased by the increase in $CaSO_4$ concentration and at the highest applied one (2000 ppm), growth completely ceased in *D. aquatica* and *C.. heteronemum*. Also total cellulolytic activity as well as all detected fractions of cellulases (C1, Cx and β-G) followed the same trend as in growth. Addition of $CaSO_4$ to growth medium markedly decreased cellulases activity. The activity decreased with the activity of both fungi. However, Cx fraction in the case of *D. aquatica* showed significant increase in activity (178.9 μL^{-1}) compared to (148.4 μL^{-1}) of the control, then decreased at higher concentration (1000 ppm). In case of *C. heteronemum* the activity of Cx fraction showed slight decrease than control.

No activity has been detected when $MgSO_4$ applied in higher concentration than (1000 ppm) in case of *D. aquatica* and (500 ppm) in case of *C. heteronemum*.

Pectinase Activity

Table 8 showed the ability of the isolates to produce pectinolytic enzymes, which were variable as detected by the formation of a clear zone around the fungal growth on solid medium. *Infindepera adhaerens* had the least activity, while *Rhizopus* sp., *C. heteronemum*, *Fusarium chlamidosporium*, *F. merisopedium* and *C. candidum* were the highest pectinase producers.

Polygalactoronase (PG), Pectin Lyase (PL) and Pectin Estrase (PE) were investigated qualitatively in the most active isolates (Table 9). PE was not detected in the three tested isolates, while PG and PL were detected in *C. heteronemum* and *C. candidum*. PL was only detected in *F. chlamidosporium*.

DISCUSSION

The results of the present study showed that cellulolytic activity of *Cylindrocarpon heteronemum* and *Dactylla aquatica* respond variably to temperature and hydrogen ion concentration. Maximum activity was maintained at 20°C and pH 5 in case of *C. heteronemum* while 25°C pH 7 were optimum for the activity in case of *D. aquatica*. The results are in agreement with that of Mohamed (1998) which showed that pH 5 was the most suitable for maximum production of cellulases in case of *Aspergillus fumigatus* and *Penicillin notatum*. Also, Sayed (1989) reported that pH value between 4-5 was convenient for enzyme formation by *A. flavus* and the shift in pH level over this range resulted in a decrease in the activity. Hazaa (1985) showed that pH between 5-7 was optimum for cellulases activity in *Micropolyspora thermogluca*. In *Rhizopus* sp. optimum pH for cellulases was 5.5 as reported by Kassim (1983) and Taj Alden and Alkenany (1993). Considering incubation temperature variable results obtained by Mohamed (1998), optimum temperature for cellulolytic activity was 27.5 and 35°C for *P. notatum* and *A. fumigatus*, respectively.

Au *et al*. (1992) reported that tested aquatic Hyphomycetes showed higher cellulolytic activity in the winter than summer leaf litter. On the other hand, Parado and Forchiassin (1999) found that temperature between 50-55°C was the optimal temperature for cellulase system in *Nectria catalinensis* while Mandeep *et al*. (1994) recorded the optimum temperature (28°C) for the secretion of the 3 components of cellulase complex *in vitro* and pH value 6. The type of carbon source in the growth medium is very important factor in the synthesis of cellulases. Crystalline cellulase was the most effective carbon in inducing cellulases in the present study, while in case of glucose no cellulases were detected.

The present study results are in agreement with that of Coughlan and Ljungdahl (1988) and Gritzali and Brown (1979). They found that cellulase formation by *T. reesei* was inducable by cellulase repressed by monosaccharides such as glucose. The same findings were reported by Merivuori *et al*. (1984) in the same fungus. Also, Hazaa (1985) and Tsao and Chaiang (1983) reported that cellulase

was a good inducer for cellulases by *Micropolyspera thermogluca*. On the other hand, Robert and Christian (1991) suggested that cellulase formation by *T. reesei* is not at all repressed by carbon sources and that formation of cellulase on lactose is most probably due to induction.

It has been shown that calcium is required for growth and development of certain Oomycetes (Giffm, 1966; Cameron and Lejohn, 1972). Also, it is widely implicated in several biochemical processes (O'Day, 1990; Jacson and Heath, 1989). So it can be accepted as an essential micronutrient of fungi. Also, magnesium requirements may be considered essential for enzyme activation and ATP metabolism, in general both required in very low concentrations. The present study showed clearly the inhibitory effect of the tested salts of calcium and magnesium on fungal growth and cellulalytic activity specially when applied in high concentrations. The inhibitory effect of high concentrations of calcium and magnesium salts may be attributed to the interference with the utilization of other essential microelements by fungal cells. Also, the shift on pH toward more acidity by the presence of sulphate and chloride ions may be considered as on important factor in decreasing the tested fungi (the recorded optimum pH was between 5-7).

Fungal pectinases are very important in maceration of plant leaves and other plant fragments and the present investigation showed the presence of pectinases in variable amounts in the isolates detected during the study. Pectinases and cellulases are very important in leaf maceration and degradation, so favorable conditions are required to degrade plant fragments and make them available as feed for water inhabitants.

In general, aquatic habitat of moderates conditions, i.e., temperature between 20-30°C, pH between 5.5-7.5, optimum. concentration of salts will maintain good conditions for growth and enzymatic activity and consequently water fungi will be able to degrade plant fragment and render them available for utilization by living organisms.

REFERENCES

Abdel-Raheem, A.M., 1992. Studies on aquatic phycomycetes and aquatic hyphomycetes in upper Egypt. Ph.D Thesis, Faculty of science (Sohag), Assiut University.

Ammar, M.S., S.S. Louboudy, M.S. Azab, A.A. El-Deeb and M.M. Afifi, 1994. Pectinolytic activity and identification of the fungal flora of Tut-Ankhmen Tomb (TAT) allowed grow under Solid State Fermentation (SSF) condition. Al-Azhar Bull. Sci.

Au, D.W.T., I.J. Hodgkiss and L.P. Vrijmoed, 1992. Fungi and cellulolytic activity associated with decomposition of *Bauhinia purpurea* leaf litter in a polluted and unpolluted Hong Kong waterway. Can. J. Bot., 70: 1071-1079.

Barlocher, F. and B. Kendrick, 1974. Dynamic of the fungal populations on leaves in a stream. J. Ecol., 62: 761-791.

Bland, S.M. and E.E. Douglas, 1977. Semi-quantitative plate assay for determination of cellulase production by Trichoderma uiride. Applied Environ. Microbial., pp: 179-183.

Cameron, L.E. and H.B. Lejohn, 1972. On the involvement of calcium in amino acid transport and growth in the fungus Achlya. J. Biol. Chem., 244: 4729-4739.

Coughlan, M.P. and L.G. Ljungdahl, 1988. Comparative Biochemistry of Fungal and Bacterial Cellulolytic Enzyme System, Beguin, P. and J. Millte (Eds.). Biochemistry and Genetics of Cellulose Degradation. Academic Press, Inc., Ltd., London.

Duncan, J.M., 1969. Mutational studies of virulence Erwinia atroseptica. Ph.D Thesis, Glasgow University Glasgow.

Dwt, A.U., I.J. Hodgkiss and L.L.P. Vrigmoed, 1992. Fungi and cellulolyltic activity associated with decomposition of Bauhinia purpurea leaf litter in a polluted and unpolluted Hong Kong water way. Can. J. Bot., 70: 1071-1079.

El-Shafy, A.A., 2000. Eco physiological studies of aquatic fundi isolated from River Nile. M.Sc. Thesis, Faculty of Sceences Heluan University.

English, P.D., A. Maglothin, K. Keegstra and P. Albersheim, 1972. A cell wall degrading endopolygalacturon-ase secreted by Colletotrichum lindemuthia-num. Plant Physiol., 49: 293-297.

Ferrari, S., D. Vairo, M.A. Frederick, F. Cervone and G. Lorenzo, 2003. Tandemly duplicated Arabidopsis genes that encode polygalacturonase inhibiting proteins or regulated coordinately by different signal transduction pathways in response to fungal infection. The Plant Cell, 15: 93-106.

Fischer, R.L. and A.B. Bennett, 1991. Role of cell wall hydrolases in fruit ripening.. Annu. Rev. Physiol. Mol. Biol., 42: 675-703.

Fisher, P.J., R.A. Davey and J. Webster, 1983. Degradation of lignin by aquatic and aero-aquatic Hyphomycetes. Trans. Br. Mycol. Soc., 80: 166-168.

Fogarty, W.M. and C.T. Kelly, 1982. Microbiol enzymes and biotechnology. Applied Sci. London, pp: 131-182.

Giffin, D.H., 1966. Effect of electrohgtes on differentiation in *Achlya* sp. Plant Physiol., 41: 1254-1256.

Gritzali, M. and R.D. Jr. Brown, 1979. *Trichoderma* relationship between purified extracellular enzymes and cellulose grown cells. Adv. Chem. Ser., 181: 237-260.

Hazaa, M.M., 1985. Studies on the microbial degradation of some agriculture residues. M.Sc. Thesis, Bot. Fac. Sci. Zagazig University.

Hodgkiss, I.J. and H.C. Leung, 1986. Cellulase associated with mangrove leaf decomposition. Bot. Mar., 29: 467-469.

Jacson, S.I. and I.B. Heath, 1989. Effect of exogenous calcium ions on tip growth, intracellular Ca^ concentration and actin arrays in Hyphae of fungus Saprolegnia ferax. Exp. Mycol., 13: 1-2.

Jones, E.B.G.., 1981. Observations on the Ecology of Lignicolous Aquatic Hyphomycetes. In: The Fungal Community, Wicklow and G.C. Carroll (Eds.). Marcel Dekker New York, pp: 731-742.

Jones, E.B.G., 1985. *Candida aquatica* sp. nov., isolated from water scums. Antonie van Leeuwenhoek, 32: 228-232.

Kane, D., 1978. The effect of sewage effluent on the growth of microorganisms in the marine environment. Ph.D Thesis, C.N.A.A., Portsmouth Polytechnic.

Kassim, E.A., 1983. Cellulase activity of *Rhizopus* sp. Egypt Microbiol., pp: 171-178.

Kenon, K.P. and G. Waksman, 1990. Common amino acid domain among endopolygalacturonase of ascomycetesfungi. Applied Environ. Microbial., 56: 522-528.

Khattab, O.H., 1991. Further studies on aquatic Hyphomycetes communities in the River Teign. Ph.D Thesis, Faculty of science, Menoufeya University.

Mandeep, K., C.L. Mandahar and M. Kaur, 1994. Influence of cultural conditions on production of cellulolytic enzymes by *Helminthosporaium maydis* Research. Bull. Punjab Uni. Sci., 44: 1-4.

Mandels, M., R. Andreoti and E. Roche, 1976a. Measurement of saccharifying cellulase. Biotechnol. Bioeng. Symp., 6: 21-33.

Merivuori, H., K.M. Siegler, J.A. Sands and B.S. Montenecourt, 1984. Regulation of cellulose biosynthesis and secretion in fungi. Biochem. Soc. Trans., 13: 411-414.

Miyairi, K., T. Okuno and K. Sawai, 1985. Purification and properties of endopolygalacturonase 1, from *Stereum purpureum*, a factor. Inducing silver-leaf symptoms on appte trees. Agric. Biol. Chem., 49: 1111-1118.

Mohamed, S.M., 1998. Environmental changes and activities of cellulose and lignocellulose degrading fungi. M.Sc. Thesis, Faculty of Science, Tanta University.

Nighojkar, S., Y. Phanse, D. Sinha, A. Nighojkar and A. Kumar, 2006. Production of polygalacturonase by immobilized cells of Aspergillus niger using orange peel as inducer. Process Biochem., 41: 1136-1140.

Nisizawa, K., T. Kanad and K. Wakabayash, 1978. Mutarotation of hydrolysis products by different types of exocellulase from Trichoderma uiride. J. Biochem., 83: 1625-1630.

O'Day, D., 1990. Calcium as on intracellular messenger in eucaryotic microbes. American Society for Microbiology Washington DC., pp: 418.

Parado, A.G. and F. Forchiassin, 1999. Influence of temperature and pH on cellulase activity and stability in *Nectria catalnensis* Revista-Argentina- de-Microbiologia, 31: 31-35.

Robert, M. and P. Christian, 1991. Carbon Source control of cellobiohydrolase I and II formation by *Trichoderma reesei*. Applied Environ. Microbiol., pp: 630-635.

Rodrigues, A.P.L. and M.A.S. Graca, 1997. Enzymatic analysis of leaf decomposition in fresh water by selected aquatic Hyphomycetes and terrestrial fungi. Sydowia, 49: 60-173.

Rombouts, F.M. and W. Pilnik, 1980. Pectic Enzymes. In Economic Microbiology, Rose, A.H. (Ed)., Academic Press, London, 5: 227-282.

Ryan, C.A. and E.E. Farmer, 1991. Oligo saccharide signals in plant. Acurrent assessment. Annu. Rev. Physiology. Mol. Biol. V., 42: 651-674.

Sanders, P.F. and J.M. Andersen, 1979. Colonization of wood blocks by aquatic Hyphomycetes. Transactions of the Britsh Mycological Society, 72: 103-107.

Sayed, A.S., 1989. Effect of nutritional conditions celluolase activity of A, flavus. Egypt. J. Microbiol., 24: 223-234.

Scott-Craig, J.S., D.G. Panaccione, F. Cervon and J.D. Walton, 1990. Endopolygalacturonase is not required for pathogenicty of *Cochliobolus carbonum* on maiz. Plant Cell, 2: 1191-1200.

Scheaer, Ch., 1992. *Massarina tetrapola* sp. Nov., the teleomorph of Tetrapoa Aristata. Mycol. Res., 95: 128-129.

Shearer, C.A. and J. Webster, 1991. Aquatic Hyphomycetes communities in the River Teign IV. Twig colonization. Mycol. Res., 95: 413-420.

Sicilia, F., J. Fernandez-Rrcio, C. Caprari, G. Delorenzo, D. ernTsoglou, C. Felice and L. Federici, 2005. The polygalacturonase-inhibiting protein PGIP2 of *Phaseolus vulgaris* has evolved a mixed mode of inhibition of endo-polygalacturonase PG1 of *Botrytis cinerea*. Plant Physiol., 139: 1380-1388.

Sinsabaugh, R.L., Ill, E.F. Benfield and A.E. Lmkins, III, Copenhagen, 1981. Cellulase activity associated with the decomposition of leaf litter in a wood land stream. Oikos, 36: 184-190.

Sternberg, D., 1976. Recent advances in cellulase technology. J. Ferment. Technol., 54: 267-286.

Su, T., 1976. Comments on papers presented at cellulase production sessions. Biotechno. Bioeng. Symp., 6: 83-89.

Taj-Alden, S.J. and K.I. Alkenany, 1993. Properties of the cellulolytic system from *Asperagillus* sp. Mycolo. Res., 97: 15-22.

Tsao, G.T. and L.C. Chaiang, 1983. Cellulose and Hemicellulose Technology. In: Phytochemistry. Vol. 4, Fungal Technology, Smith, J.E., D.R. Berry and B. Kristiansen (Eds.), Edward Aranold, London, pp: 269-326.

Tshii, S. and T. Yokotsuka, 1972. Purification and properties of endopolygalacturonase from *Aspergillus japonicus*. Agric. Biol. Chem., 36: 1885-1893.

Willis, A. and T. Scott, 1988. Purification of cellulose degrading enzyme. Methods of enzymology part *A. cellulose* and *hemicellulose*, 160: 200-264.

Willoughby, L.G. and J.F. Archer, 1973. The fungal flora of fresh water stream and its colonization pattern on wood. Further observations on *Tricladium giganteum* Iqbal. Trans. Br. Mycol. Soc., 65: 77-82.

Yuen, T.K., K.O. Hyde and I.J. Hodghiss, 1998. Physiological growth parameters and enzyme production in tropical fresh water fungi. Material and Organisms, I: 1-16.