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CELLULASE PRODUCTION BY TWO FUNGAL STRAINS ISOLATED FROM TAIF IN SAUDI ARABIA

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ABSTRACT

Among 17 fungal isolates isolated from soil of El-hawia, El-hada, El-kaym and Karwa in Taif governorate in Saudi Arabia, two isolates showed high efficacy in producing cellulases enzymes. They were identified to be *Alternaria alternata* and *Aspergillus wentii*. Some factors such as carbon and wheat bran as a raw material, nitrogen, pH and incubation temperature were investigated. Results indicated that glucose and cellulose were the most effective as a carbon source while, urea was the best nitrogen source for cellulases production. Initial pH 5.0 and incubation temperatures at 25 or 35ºC achieved high cellulases production.

INTRODUCTION

Cellulose is the most abundant biomass on the earth. It is the main product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produce biosphere (100 billion dry tons/year).

Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi **(Bahkali, 1996; Magnelli & Forchiassin, 1999; Shin *et al* 2000 and Immanuel *et al* 2006)**. Complete enzymatic hydrolysis of enzyme requires synergistic action of 3 types of enzymes, namely cellobiohydrolase, endoglucanase or carboxymethycellulase (CMCase) and β-glucosidases **(Bhat, 2000)**. Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry detergents for color care, cleaning and anti-deposition; in the food industry for mashing; in the pulp and paper industries for deinking, drainage improvement and fiber modification and they are even used for pharmaceutical applications **(Kirk *et al* 2002 and Cherry & Fidantsef, 2003)**.

MATERIALS AND METHODS

Soil samples

Calcareous soil samples were obtained from El-hawia, El-hada, El-Kaym and Karwa in Taif governorate in Saudi Arabia for isolation of fungi.

(Taif area found in Eastern province of Saudi Arabia, the landscape between Makkah and Taif, is littered with high mountains)

Fungal isolates

Seventeen fungal cultures were isolated from Taif governorate in Saudi Arabia.

Media used

Medium (1): Potato-Dextrose agar (Difco Manual, 1984). It was used for maintenance of fungi .It consists of Potatoe 200.0, Dextrose 20.0, agar 15 g /L, Distilled water 1000.0 ml and pH 5.0.

Medium (2): Czapeck,s Dox broth (Difco Manual, 1984). It has the following composition: Sucrose 30.0, K_2HPO_4 1.0, KCl 0.1, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, $NaNO_3$3.0 g/L, Distilled water 1000.0 ml and pH 5.0.

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This medium was modified by replacing glucose in the basal medium with addition of CMC in concentration of 10 g /L as a substrate to produce cellulase enzyme.

Fungal isolates identification

Identification of the tested fungal isolates was accomplished depending on the morphological characters of fungi using KS 300 measuring program (Carl Zeiss Germany) for performing all measurements and comparing them with those that are present in the identification references **(Gilman,1969 and Domsch *et al* 1980)**.

Fungal inoculum preparation

The fungi were maintained on potato dextrose agar slants at 4°C. To prepare the inoculum, the spores on the slant were suspended in 2 ml medium (10^6–10^7 spores/ml). This was used as the inoculum for the cellulases production medium.

Cultivation

The growth of the culture was carried out in 250 ml Erlenmeyer flask containing 100 ml medium no.2 for fungal strains, supplemented with CMC as a substrate to produce cellulase enzyme. The flasks were sterilized at 121°C for 15 min. The flasks were inoculated with 5% inoculum (v/v) transferred to the production medium, and then incubated on a rotary shaker (150 rpm). The broth after cultivation was used for enzyme studies. **(Dien *et al* 2006)**.

Enzyme assays procedures

Plate enzyme assay screening

At the end of the incubation, the agar medium along with fungal colonies was flooded with an aqueous solution of Congo red (1% w/v) for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter in cm was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity **(Howard *et al* 2003 & Ariffin *et al* 2006)**. The data presented are average of three replicates.

Carboxymethyl cellulose (CMC ase) activity

CMCase activity was assayed using a method described by **Mandels and Weber (1969)**. The activity was estimated using 1% solution of carboxymethlycellulose (CMC) in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 0.5 ml of suitably diluted enzyme solution. The reaction was carried out at 50°C for 30 min. One unit of CMCase activity was expressed as 1 µ mol of glucose liberated per ml enzyme per minute.

Filter-paperase (FPase) activity

The activity of FPase was assayed according to the method explained by **Mandels and Weber (1969)**. This method is similar to the CMCase assay method, but the substrate was Whatman No. 1 filter paper strip (1 x 6 cm) soaked in 1 ml 0.05 M sodium citrate buffer (pH 4.8). The samples were incubated with 0.5 ml enzyme solution at 50°C for 1 h. One unit of FPase activity was determined as 1 µ mol of glucose liberated per ml enzyme per minute.

β-Glucosidase activity

One-tenth ml of the culture supernatant was incubated with 0.5 ml of 0.05 M acetate buffer (pH 5) containing 2.5 mg cellobiose. After incubation at 50 ºC for 10 min the glucose released was measured by the glucose oxidase peroxidase method **(Zaldívar *et al* 2001)**.

Determination of sugars

The total amount of non-reducing sugars (as cellulose) was determined by the glucose oxidase peroxidase kit from (BIO-ADWIC) EL NASR PHARMACEUTICAL CHEMICALS Co. (Egypt).

Effect of different carbon sources

The appropriate carbon source was selected by replacing the original carbon substrate of the basal medium with equivalent carbon amount of each of the tested carbon sources (Glucose, Carboxymethycellulose, Cellobiose and Cellulose).Also, local raw material such as wheat bran (Cellulose 28%, total nitrogen 4.8 %) was used as a source of carbon for cellulase production by the most efficient cellulase producing fungi.

Pretreatment of wheat bran (raw material)

Wheat bran was pretreated with 4% solution of sodium hydroxide (2000 ml/ 100 g substrate), autoclaved at 121°C for 30 min. The material recovered by filtration, was washed with distilled water until neutralilty (pH 7.0) and dried at 65°C to constant weight.

Effect of different nitrogen sources

To detect the best nitrogen source for cellulase production by selected strains, the prescribed nitrogen source of the fermentation medium was replaced by equivalent nitrogen amount of each of the tested organic [Yeast extract, , Peptone, Urea] and inorganic [$NaNO_3$, NH_4Cl & $(NH_4)_2SO_4$] nitrogen sources.

Effect of initial pH

Five values of pH ranged between 3.0 and 7.0 were chosen for studying their effects on cellulase enzyme to select the most suitable pH for cellulases production.

Effect of incubation temperature

To determine the optimum temperature for cellulases production, fermentation was carried out at various temperatures in the range of 20, 25, 28, 35 or 40ºC.

Dry weight

The mycelial dry weight or biomass from the liquid culture was determined by filtering off and washing the mycelium and drying it at 80ºC for 24h.

RESULTS AND DISCUSSION

Isolation of cellulase producing fungi

A number of 17 isolates of fungi were isolated from different sources of soil from Taif governorate in Saudi Arabia. Screening of fungal isolates for cellulases activities was conducted by using Congo red test as a preliminary study for selecting the cellulases producers. After 7 days of incubation, all 17 fungal isolates showed signs of growth on CMC agar and demonstrated positive results in the Congo red test. Since the sole carbon source in CMC agar was carboxymethylcellulose (CMC), therefore the result of the test was a strong evident that cellulases were produced in order to degrade cellulose. The isolates were identified by morphological and taxonomic characteristics as genera of: *Alternaria, Aspergillus, Fusarium, Rhizopus* and *Penicillium*.

Data presented in **Table (1)** clearly show that *Aspergillus* sp. (10) and *Alternaria* sp. (17) were the most efficient genera selected according to the high ratio of clear zone diameter to colony diameter being 1.50 and 1.56 for *Aspergillus* sp. (10) and *Alternaria* sp. (17), respectively, and was selected for further studies. These results are in agreement with **Peij *et al* (1998)** who stated that filamentous fungi particularly *Aspergillus* and *Trichoderma* spp. are well known as efficient producers of cellulases. **Mes-Hartree *et al* (1988); Hanif *et al* (2004) and Milala *et al* (2005)** also reported that cellulase production was *higher* upon growth of *Trichoderma harzianum* and *Aspergillus niger* on cellulosic substrates.

Identification of the most efficient isolates in cellulase production

Identification of the fungal isolates was accomplished depending on colonial characters of the pure culture, microscopic characters and dimensions of informative character of each fungal isolate using a specific program for measurements Axio Vision 4.7 (with help of computerized Carl Zeiss microscope Axioplane 2) and comparing them with those that are present in the identification references **(Gilman, 1969 and Domsch *et al* 1980)**.

Effect of carbon source

An experiment was carried out to investigate the effect of different carbon sources such as glucose, carboxymethycellulose (CMC), cellobiose, cellulose and wheat bran (as raw material) on the production of cellulase enzyme. Five carbon sources were used as shown in **Table (2)** .Data presented in **Table (2)** show that medium containing glucose gave the highest yield of cellulase activity being 2.244 & 2.229 U/ml of CMCase, 2.228 & 2.233 U/ml of FPase while, the medium containing cellulose gave the highest β- glucosidases being 8.738 and 6.562 U/ml of by *Alternaria alternata* (17) and *Aspergillus wentii* (10), respectively. Regarding to the use of raw material as a sole source of carbon, in case of *Alternaria alternata* (17) ,the yield of the FPase enzyme was highest on wheat bran being 2.315 U/ml .While, in *Aspergillus wentii* (10) the highest β-glucosidases being 3.158 U/ml on wheat bran.

Table 1. Hydrolysis ratio of carboxymethycellulose (CMC) by fungal general incubated at 28°C for 7 days

Fungal genera	Growth diameter (cm)	*Cellulolysis diameter (cm)	**Cellulolysis ratio
1- Alternaria			
Alternaria sp. (1)	1.0	1.2	1.2
Alternaria sp.(3)	1.1	0.9	0.82
Alternarial sp. (4)	1.2	0.8	0.67
Alternaria sp. (9)	0.6	0.7	1.17
Alternaria sp. (11)	0.8	1.0	1.25
Alternaria sp. (17)	0.9	1.4	1.56
2- Aspergillus			
Aspergillus sp. (5)	1.2	0.9	0.75
Aspergillus sp. (10)	1.2	1.8	1.50
Aspergillus sp. (12)	1.7	1.0	0.59
3- Fusarium			
Fusarium sp. (7)	1.1	0.9	0.82
Fusarium sp. (8)	1.5	1.1	0.73
Fusarium sp. (13)	1.2	0.8	0.67
Fusarium sp. (14)	0.8	0.5	0.63
Fusarium sp. (15)	0.1	0.7	0.07
4- Rhizopus			
Rhizopus sp. (2)	1.3	0.5	0.38
5- Penicellium			
Penicellium sp. (6)	0.8	0.7	0.88

*** Cellulolysis diameter = Clear zone diameter –Grwoth diameter**
****Cellulolysis ratio = Cellulolysis diameter ÷ Grwoth diameter**

Table 2. Effect of carbon sources on production of cellulase enzyme by *Alternaria alternata* (17) and *Aspergillus wentii* (10) using shake flasks as a batch culture

* Different carbon * sources	*Alternaria alternata (17)*				*Aspergillus wentii (10)*			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPase	β-glucosidases		CMCase	FPase	β-glucosidases
Glucose	0.506	2.244	2.228	2.746	0.603	2.229	2.233	5.373
Carboxymethycellulose (CMC) (Control)	0.110	2.169	2.193	1.887	0.295	2.223	2.174	3.276
Cellobiose	0.463	2.110	2.031	2.814	0.212	1.813	1.623	3.216
Cellulose	0.412	2.205	2.155	8.738	0.397	1.914	1.470	6.562
Wheat bran	0.315	1.963	2.315	2.121	0.204	1.352	1.259	3.158

Table 3. Effect of nitrogen sources on the production of cellulase enzyme by *Alternaria alternata* (17) and *Aspergillus wentii* (10) using shake flasks as a batch culture

Different Nitrogen Sources	*Alternaria alternata* (17)				*Aspergillus wentii* (10)			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPase	β-glucosidases		CMCase	FPase	β-glucosidases
Yeast extract	0.408	0.972	0.014	1.285	0.359	2.247	2.012	1.157[e]
Peptone	0.283	1.491	1.101	2.138	0.317	2.238	2.185	2.695
Urea	0.310	2.241	2.252	6.857	0.286	2.266	2.247	7.870
$NaNO_3$	0.292	2.235	2.244	4.981	0.267	1.627	2.028	5.424
NH_4Cl	0.344	2.232	2.187	4.395	0.285	2.160	2.204	5.459
$(NH_4)_2SO_4$	0.610	2.154	2.190	6.653	0.189	2.217	2.169	7.415

Table 4. Effect of initial pH on the production of cellulase enzyme by *Alternaria alternata* (17) and *Aspergillus wentii* (10) using shake flasks as a batch culture

Initial pH	*Alternaria alternata* (17)				*Aspergillus wentii* (10)			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPae	β - glucosidases		CMCase	FPase	β-glucosidases
3.0	0.209	2.169	2.193	1.887	0.098	2.202	1.88 7	4.495
4.0	0.242	2.194	2.208	2.862	0.202	2.257	1.831	4.561
5.0	0.312	2.261	2.245	4.988	0.368	2.631	2.031	5.488
6.0	0.293	2.115	2.172	3.462	0.274	1.939	1.143	3.480
7.0	0.288	2.008	2.079	3.120	0.262	1.082	1.036[i]	3.096

Table 5. Effect of incubation temperature on the production of cellulase enzyme by *Alternaria alternata* (17) and *Aspergillus wentii* (10) using shake flasks as a batch culture

Incubation temperature°C	*Alternaria alternata* (17)				*Aspergillus wentii* (10)			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPase	β-glucosidases		CMCase	FPase	β-glucosidases
20	0.174	2.217	2.144	1.410	0.136	1.446	2.185	2.151
25	0.223	2.214	2.220	2.640	0.209	2.244	2.049	4.520
28 (Control)	0.297	2.231	2.256	4.977	0.276	2.661	2.102	5.312
35	0.281	2.112	2.125	3.245	0.290	1.998	2.228	3.199
40	0. 201	2.002	2.109	3.200	0.205	1.824	2.061	2.239

Effect of nitrogen source

To evaluate the effect of nitrogen source on cellulase formation, the nitrogen source in the basal medium was replaced by different nitrogen sources. Data revealed that the supplementation of organic and inorganic nitrogen sources stimulated the cellulase yield and activity. Using of organic N sources responded in the positive cellulase activity more than the inorganic ones. Among the tested complex N sources, the effectiveness in supporting cellulase production and cellulolytic activity. Results recoded in **Table (3)** indicate that the sources of nitrogen greatly affected the production of cellulase enzyme. Urea was the best nitrogen source for *Alternaria alte*rnata (17) and *Aspergillus wentii* (10) giving 2.241 & 2.266 U/ml of CMCase, 2.252 & 2.247 U/ml of FPase and 6.857 & 7.870 U/ml of β-glucosidases, respectively.

These results support the findings obtained by **Ganguly and Mukherjee (1995)** who studied the effect of nitrogen sources on the production of cellulases by *Pencillium purpurogenum*, where the organism enabled to use both inorganic and organic nitrogen sources for growth and production of the enzyme. Also, **Spiridonov & Wilson (1998) and Sun *et al* (1999)** who found that NH_4 compounds are the most favorable nitrogen sources for protein and cellulase synthesis.

Effect of pH

In **Table (4)**, five values of pH ranged between 3.0 and 7.0 were chosen for studying their effects on cellulase production by *Alternaria alternata* (17) and *Aspergillus wentii* (10). The highest yield of cellulase activity being 2.261 & 2.631 U/ml of CMCase, 2.245 & 2.031 U/ml of FPase and 4.988 and 5.488 U/ml β- glucosidases by *Alternaria alternata* (17) and *Aspergillus wentii* (10), respectively on pH 5.0 . These results were in agreement with **Coral *et al* (2002)** who found that the CMCase activity of *Aspergillus niger* Z10 has a broad pH range between 3.0 and 9.0. The enzyme showed two major activity peaks at pH 4.5 and 7.5. This result was probably due to the presence of two isoenzymes or subunits in the enzyme preparation.

Effect of incubation temperature

An experiment was conducted to find out the effect of different degrees of incubation temperatures ranged from 20 to 35ºC on cellulase production by *Alternaria alternata* (17) and *Aspergillus wentii* (10). Results in **Table (5)** show that the temperatures from 25 to 35ºC achieved high cellulase production in different degrees of incubation .These results are in agreement with Nipa *et al* (2006) who found that the optimum temperature for CMCase production by the fungus might lie between 27° and 37°C. While **Muthuvelayudham and Viruthagiri (2006)** reported that the maximum cellulase activity was obtained at 28°C by *Trichoderma reesei.*

In the present study, it could be concluded that carbon and nitrogen sources, pH values and incubation temperatures play an important role in the production of cellulase enzyme by *Alternaria alternata* (17) and *Aspergillus wentii* (10).

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