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Production and characterization of cellulolytic enzymes from three fungi

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ABSTRACT

Celluloses are carbohydrate polymers, much abundant as agricultural residues or wastes. They are resistant to enzymatic cleavage in most animals. Researches around the world were therefore directed towards finding methods for hydrolyzing these compounds to obtain glucose. One such approach is enzymatic hydrolysis. Three fungi (*Trichoderma viride, Asperigillus niger* and *Penicillum digitatum*) were used for production of cellulase enzymes, in the present study, under laboratory conditions. Two methods (reducing sugar and viscometry) were used for measuring the enzymetic activity. Carboxymethylcellulose (CMC) was used as a substrates for the enzyme activity. Different substrates were used for enzyme production . The results showed that *A. niger* and *T. viride* gave maximum production of cellulase enzyme after one week. While *P. digitatum* gave it after two weeks. Purification by ammonium sulphate precipitation, showed that the maximum cellulase enzyme activity was at 60% concentration for the three fungi. By using gel electrophoresis, three bands were showing cellulolytic activity. This study showed the importance of fungi as sources of enzymes and recommends that more studies must be done in the field of biotechnology to produce more glucose from natural products by using fungi.

Key word : Celluloses, fungi, enzymes.

INTRODUCTION

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Enzymes are a mubiquitous class of protein molecules that act as biological catalysts, catalyzing many chemical reactions (Timoth, 2001). Out of the 2000 various enzymes that have been described; only a few like cellulolytic, pectolytic and amylolytic enzymes have a wide industrial and biotechnological application (Bhat and Bhat, 1997). An important application of enzymes in food processing is to increase the yield of products from the raw material, and to make it more amenable to treatment particularly in fruit and vegetable processing. Here the use of pectinases, cellulases and hemicellulases assists cell separation and weakening of the cell walls so that the material can be more readily pulped and the yield of juice on pressing will be considerably enhanced (Birch *et al.*, 1981).

The ability to decompose and obtain carbon energy from lignin, cellulose and hemicelluloses is widespread among fungi and bacteria (Klass, 1983). However, fungi have been reported as the main micro-organisms to be enzyme producers, including species of the genera *Trichoderma, Penicillium* and *Aspergillus* (Coral *et al*., 2001).

Cellulases are referred to as a group of enzymes which act together to hydrolyze cellulose (Shin *et al.*, 2000, Ikram *et al*., 2005). Those enzymes degrade the β-1, 4 glycoside bond in cellulose compounds and the cellulosic enzyme system consists of three major components, which are designated as C_1 , C_x and cellobiase (Krishna *et al*., 1998; Rajoka *et al.* 2004).

Factors affecting enzymes production:

Natural inducers of cellulase system have been proposed as early as 1962, and the disaccharide sophorose is since then considered to be the most probable inducer of at least the *Trichodema* cellulase system (Lynd *et al*., 2002). Cellulolytic cultures have been investigated with the use of Avicel and other crystalline celluloses as carbon sources to induce cellulases production (Nogawa *et al*., 2001; Nochure *et al*., 2004). It was found that the optimization of the rate of fermentation of the carbon source substrate, and the

pH of the basal fermentation medium, play a fundamental role in the production of cellulases by *Asperigillus niger* and *Trichoderma viride* (Kohad and Singh, 1993).

MATERIALS AND METHODS

The organisms used as enzymes producers.

Trichoderma viride, was isolated from wood, ***Asperigillus niger,*** was isolated from onion and ***Penicillum digitatum***. was obtained from Sudanese orange fruit. The three isolates were cultured and sub cultured on Potato Dextrose Agar (PDA), as was described by Toole (1995), then purified and identified in Center of Biosciences and Biotechnology, Faculty of Engineering and Technology, University of Gezira.

Medium for enzyme production:

The isolates were grown in 250 ml conical flasks using 75 ml of liquid medium containing the following ingredients (g/L) 1.4 g $(NH4)_2SO_4$; 0.3 g urea ; 2.0 g KH_2PO_4; 0.3 g $MgSO_4.7H_2O$ and 0.3 g $CaCl_2$, trace elements were added as, $FeSO_4$, 1.0 ppm; $ZnCl_2$, 0.8 ppm; $MnSO_4$, 0.5 ppm; $CoCl_2.5H_2O$ 0.5 ppm and $CuSO_4.5H2O$, 0.5 ppm and yeast extracts was also added at 0.1g/liter and 10 g of the carbon substrate source were added per liter. The final pH of the liquid medium was adjusted to 5.3 with KOH. Each flask of the liquid salt medium was inoculated, using sterile cork borer, with 5 disk (of the same diameter, 5mm) of agar on which the fungal mycelia were previously grown. This was necessary to insure that each flask contained the same amount of inoculum.

Enzyme activity measurement:

After incubation for the required time, culture filtrates were centrifuged at 16,000 r.p.m for 15 minutes. The filtrates were then aseptically collected in sterile McCartney bottles and stored in 10 ml portions at -20°C before being assayed. Cellulase activity was

measured using two methods; the decrease in viscosity and Spectrophotometric method, using Carboxymethylcellulose (CMC) as a substrate. The cellobiase activity was measured by the Spectrophotometric method only. Both autoclaved enzymes and 0 times reaction mixtures containing active enzyme were employed as controls.

The viscosity reducing method

For the viscosity reducing method the reaction mixture contained; 8.0 ml w/v substrate (1%) in 0.1M buffer, 1.0 ml of 0.01 $CaCl_2$ (0.001M) and 1.0 ml enzyme. On the addition of the enzyme, the reaction mixture was mixed by being blown through the viscometer and then the viscosity was measured at different intervals of time. The enzyme activity was expressed as 100/t, where t is the time in minutes for 50% reduction in the viscosity of the reaction mixture. All viscometers were calibrated against water before being used. The blow time of 10 ml water was considered as 100% reduction in viscosity (Abdel-Rahim, 1980).

Spectrophotometric method:

0.45 ml of 1% CMC in sodium citrate buffer (0.1M) was added to 0.05 ml of enzyme preparation (filtrate). The mixture was then incubated for 15 minutes at 55^0 C in a controlled water bath. The reaction mixture was removed from the water bath and immediately 0.5 ml of 3,5- dinitrosalicylic acid reagent were added. The mixture was then heated in a boiling water bath for 5 minutes and cooled to room temperature. Distilled water was added to make a 5 ml final volume. Absorbance of the sample was determined with the spectrophotometer (Double beam spectrophotometer, model UV-150-02) at 540 nm as was described by (Abdel-Rahim, 1980).

A calibration curve (Fig. 1) was obtained with a series of glucose solutions containing between 5 - 50µg / ml and the enzyme activity expressed as µg reducing groups (glucose equivalent) released.

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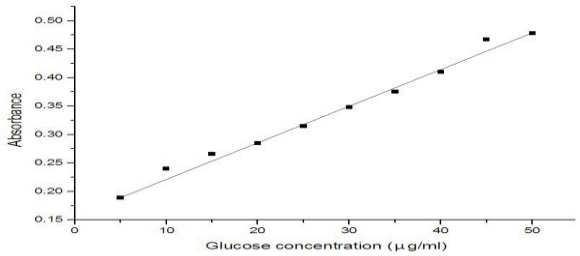

Fig (1). Standard curve of glucose concentrations.
X= v – 0.163 / 0.006 Where X: Absorbance Y: concentration

Effect of different substrates on enzyme production

Cellulose, cellobiose, CMC, filter paper and cotton fiber were used individually, as substrates for enzyme production. The substrates were added to the liquid medium at 0.1%. The filtrate of each culture was assayed for enzyme activity.

Effect of incubation time on enzyme production

Flasks of the liquid salt medium inoculated with the three fungi were incubated at different incubation times ranging from one week to four weeks and samples were drown weekly. The filtrates of each culture were tested for cellulase enzyme activity.

3.6. Partial purification by ammonium sulphate precipitation

The crude enzyme filterates of the three tested fungi were filtered through a sintered glass funnel and each filtrate was treated with 20%, 40%, 60%, and 80% saturation of ammonium sulphate. The ammonium sulphate concentrations were slowly added, with stirring, to 100 ml enzyme solution until all the ammonium sulphate dissolved. The solution was centrifuged at 6000 r.p.m for 20 minutes; the supernatant carefully discarded leaving the pellet, which was dissolved in 10 ml of 0.1M acetate buffer (acetic acid – NaOH) and was tested for the cellulase enzymes activities. Both The viscosity reduction and the spectrophotometric methods were used.

3.7. Gel electrophoresis separation

T. viride and *P. digitatum were* grown in the basal medim using CMC as a substrate. Then After growth, the culture filtrates were separated by the centrifugation process. The filtrate collected from each culture was dissolved individually in 50 ml of polyethyleneglycol 10% and dialyzed overnight using dialysis tubing cellulose (membrane size 43mm × 27mm). For the determination of molecular weight, the enzyme preparation and known molecular weight markers were subjected to electrophoresis The sample solution and the markers were loaded to the gel with 10-30µl by pipette, and the gel electrophoresis was started by turning on power, at first 15 minutes at the voltage of

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60 volt then turn to 120 volt for 1 hour. Then the gel will be removed from between the glass plate. Finally, the gel was stained by staining solution (over night), the dye that is not bound to the sample was removed by destaining solution and the gel obtained was photographed and scanned, and then the molecular weight of individual enzyme fraction was detected by referring to the standard curve of molecular weight of the markers.

RESULTS

Effect of different substrates on cellulase enzymes production:

The three fungi (*A. niger, P. digitatum* and *T. viride*) were grown, individually on the salt medium to which cellulose, cotton fiber, filter paper and carboxymethylcellulose (CMC) were added to induce cellulolytic enzymes. The maximal yields of the cellulase enzymes were found when CMC was used as an inducer substrate (Table, 1). The effect of cotton fiber and filter paper as inducers resulted in the lowest enzyme induction, while the cellulose powder gave an intermediate effect. The culture filtrates of *A. niger* showed higher production than both *T. viride* and *P. digitatum*. However, the *P. digitatum* showed the lowest production of the cellulase enzyme (Table, 1). The same results were obtained when the reducing groups method was used (Table, 2). Also CMC was found to be the best inducer for cellobiase enzyme produced by the three fungi (Table, 3).

Table (1). Effect of different substrates on cellulase enzyme activity Produced by three fungi using the viscometeric method.

Substrate	*A. niger*	*P. digitatum*	*T. viride*
Cellulose powder (Avicel)	6.99	8.5	13.00
Filter paper	7.00	14.9	19.04
Cotton fiber	7.00	20.0	19.01
CMC	3.50	6.99	10

Table (2). Effect of different substrates on cellulase activity(mg/ml) produced by the three fungi using the reducing sugar method.

Substrate	*A. niger*	*P. digitatum*	*T. viride*
Cellulose powder (Avicel)	44.5	19.5	21.1
Filter paper	34.5	12.8	9.5
Cotton fiber	17.8	9.5	11.2
CMC	71.2	62.8	49.5

Table (3). Effect of different substrates on Cellobiase activity (mg/ml) produced by the three fungi.

Organism	Cellobiase activity (mg/ml)			
	CMC	Cotton fiber	Filter paper	cellulose
T. viride	55.6	25.6	34.5	27.8
A. Niger	78.8	27.8	37.0	42.8
P. digitatum	72.5	21.1	35.5	40.1

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Effect of incubation time on the enzyme production:

The three fungi (*A. niger, P. digitatum* and *T. viride*) were cultured for the enzyme production on the medium described above. The prepared cultures were incubated at 30^0C for 4 weeks, and samples were drown weekly. The filtrates of each culture were used tested for cellulase enzyme activity. Results on Fig. (2) and Fig (3) showed that *A. niger* and *T.viride* gave maximum production of the cellulase enzyme after one week, while, the higher activity of the cellulase enzyme produced by *P. digitatum* was detected after two weeks.

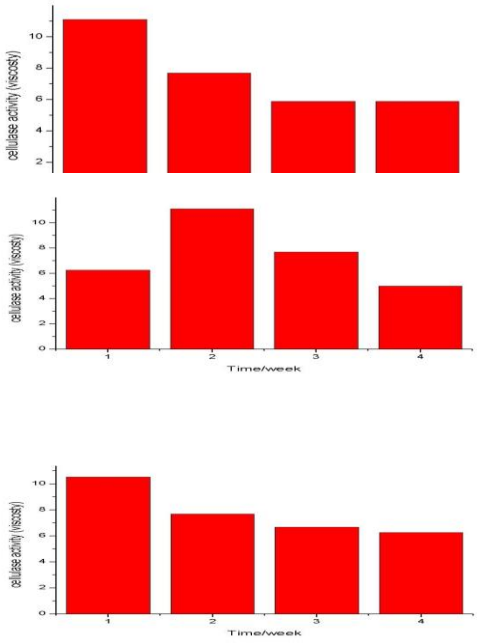

Fig. (2). Effect of the incubation time on cellulase activity produced by *A niger*, (B) *P. digitatum*, (C) *T. viride*. Using the viscometric method.

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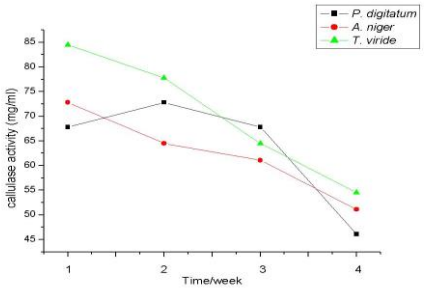

Fig. (3). Effect of the incubation time on cellulase activity produced (mg/ml) by the three fungi using the reducing sugar method

Partial purification of the cellulase enzymes:

The filtrates of the three fungi (*A. niger*, *P. digitatum* and *T. viride*) were fractionally precipitated with ammonium sulphate. Then the reaction mixtures were assayed with the reducing sugar method. The results presented on Fig. (4) showed that, increasing the concentration of ammonium sulphate resulted in the precipitation of more cellulase enzymes of the three fungi up to 60% of concentration).

Estimation of Molecular weight of the cellulase enzymes produced by The fungus *T. viride* by Gel Electrophoresis separation

The molecular weight of the cellulase enzyme produced by the fungus *T. viride* was determined by SDS – PAGE as described above. The results of the analyses of the enzyme showed that twelve bands have been detected on the gel (Plate, 1). Some of them have higher molecular weights than that known for the cellulases (above 160,000). But three of them have molecular weights in the range of the cellulase enzymes molecular weights (2) reported. These bands were estimated to be about 64,000 for band (1); 47,000 for band (2) and 39,000 for band (3).

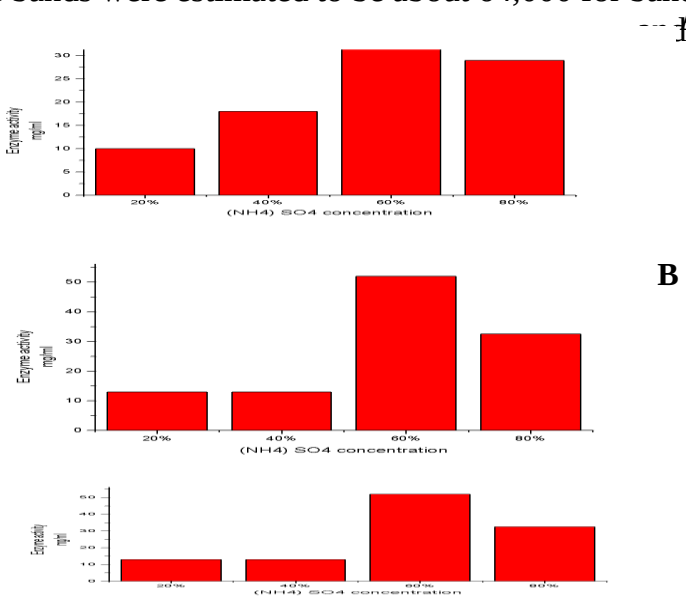

Fig. 4. Partial purification of cellulases produced by the three fungi by Ammonium sulphate (A) *A. niger* (B*) P. digitaum* and (C) *T. viride*

DISCUSSION

The three fungi (*T. viride*, A. *niger* and *P. digitatum*) were grown, individually on a salt medium to which either cellulose, cotton fiber, filter paper or carboxymethylcellulose (CMC) to induce cellulolytic enzymes. The maximum yield of cellulase enzymes were found when CMC was used as an inducer substrate. This result was in agreement with that presented by Ahmed *et al.* (2009) and Sibtain *et al*. (2009), who reported that the CMC seem to be the strong inducer of cellulase of *T. harizianum*. It also agreed with Niranjane *et al*. (2007) who observed that the highest yields of cellulases produced by *Phlebia gigantean* were on CMC. Shanmugapriya *et al.* (2012) and Ponnuswamy and Prakash (2012) and Devendra *et al.* (2012) place that among the synthetic carbon substrate tested, maximum (coir waste, sawdust and CMC) carboxymethylcellulase production was found for exit CMC different microorganisms.

In this study, it was also found that *A. niger* and *T. viride* gave maximum production of the cellulase enzyme after one week. However, Swelim *et al.* (2010) reported that the maximum cellulase enzyme of *T. viride*, *A. terreus* and *A. flavus* had been reached after one week but soluble protein increased as incubation period increased. Garg and Neelakantan (1981) and Gautam *et al*. (2010) reported that the highest cellulase activity had been recorded after 7 days for *Trichoderma* sp, and *A. terreus*. But Ali *et al.* (2011) reported that *T.viride* and *A. niger* produced maximum cellulase enzymes after 10 days of incubation. According to Nwodo *et al.* (2005) the maximum activity at the incubation time, varied with the different organisms. On the other hand, *P. digitatum* showed the maximum cellulase activity after two weeks. The difference may due to the nature of the organism.

The filtrates of the three fungi (*A. niger*, *T. viride* and *P. digitatum*) were fractionally precipitated with ammonium sulphate. The results showed that, increasing the concentration of ammonium sulphate up to (60%) resulted in the precipitation of more

cellulase enzymes of the three fungi. The results agreed with what was reported by Nayera *et al*. (2011) for *Streptomyces halsteadii*. On the other hand, Jabbar *et al*. (2004) reported that complete precipitation of the crude carboxymethylcellulase of *Arachniotus citrinus* occurred at 75% saturation of ammonium sulphate at 0ºC. While Siddig *et al.* (2000) reported that the carboxymethylcellulase from *A. niger* was precipitated between 45-65% ammonium sulphate saturation at 0ºC.

To determine the molecular weight of the cellulase enzyme. *T.viride* was grown in the liquid –salt medium containing %1 CMC as the sole carbon source. The culture was filtrated, and the crude enzyme preparation was subjected to SDS-PAGE. During the electrophoresis of the enzyme, three bands showing cellulolytic activity were detected, the molecular weights of these bands were calculated to be 64.000 for band (1); 47.000 for band (2) and 39.000 for band (3). These values fall within the range of molecular weight reported by Bledman *et al*. (1985) for cellulase of *T. viride*. While, other cellulases from different organisms were tested to determined their molecular weights, Morozova *et al.* (2010) reported that the filtrate of *P. verruculosum*, have nine bands, five of them were endo-glucanases with molecular weights 23,000, 33,000, 39,000, 52,000 and 71,000 and the last four were cellobiohydrolases with molecular weights 50,000, 55,000, 60,000 and 66,000. Christina and Sunil (2012) showed that the analysis of partially purified endo and exoglucanase from *A. niger* have two protein bands with molecular weights 33,000 and 24,000, respectively. Devendra *et al.* (2012) reported that the purified carboxymethylcellulase of P*aeibacillus polymyxa* have two bands with molecular weights of 26.500 and 34.000. It was reported that these proteins may be isoenzymes or the different subunits of the same enzyme protein (Hurst,1977).

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EDITORIAL

إنتاج وتوصيف خواص الانزيمات المحللة للسيليوز من ثلاث فطريات

أمينة أ. عبد الوهاب و عوض م. عبد الرحيم

الملخص

هي بوليميرات كاربهيدراتية شائعة بكثرة كبقايا ومخلفات زراعية . وهي روابط يصعب تحليلها انزيمياً في معظم الحيوانات . البحوث حول العالم اتجهت لإيجاد طرق لتحليل هذه المركبات للحصول على الجلوكوز . تمثلت احدى الطرق في التحليل الانزيمي و استخدمت ثلاثة من الفطريات

Asperigillus niger) و*Penicillum digitatum* و (*Trichoderma viride*

لإنتاج انزيمات السيلولوز استخدمت طريقتان (السكريات المختزلة و اللزوجة) لقياس نشاط الانزيمين . استخدمت المركب كاربوكسي ميثيل سيليولوز كمواد اساس لقياس النشاط الانزيمي . استخدمت مواد مغذية مختلفة لإنتاج الانزيمات . أظهرت النتائج أن الفطريات *A niger* و *T. viride* قد أعطت أعلى انتاج لانزيمات السيولوز بعد أسبوع واحد من بينما أعطى الفطر *P. digitatum* أعلى انتاج للسيليوليز بعد اسبوعين ، أظهرت تقنية فصل الانزيمات جزئياً بواسطة كبريتات الامونيوم، حيث وجد أن اعلى نشاط لانزيم كان عند التركيز 60% . وباستخدام الفصل الكهربي الهلامي وجد ان هناك ثلاثة حزم لها نشاط سيليوليزي. هذه الدراسة تؤكد أهمية الفطريات اعلاه كمصادر للانزيمات وتوصى الدراسة بإجراء المزيد من البحوث في مجال التقنية البيولوجية لإنتاج الجلوكوز من مصادر طبيعية بإستخدام الفطريات .