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OPTIMIZATION AND CHARACTERIZATION OF CELLULOLYTIC ENZYMES PRODUCED FROM *Gliocladium roseum*

Salem, A.A. and H.M. Abdel-Rahman
Agric. Botany Dept., Fac. Agric. Moshtohor, Benha Univ., Egypt
E-mail: ahmed.nayl@fagr.bu.edu.eg
Corresponding Author: A.A.Salem, Dept., of Agric., Botany, Fac. of Agric., Benha Univ., Egypt.

ABSTRACT

This work deals with optimization the production conditions and characterization of cellulases [Carboxymethyl-cellulase (CMCase), Filter-paperase (FPase) and Cellobiase] produced by *Gliocladium roseum*. Data revealed that Reese and Mandel's basal medium was the best for maximum protein and cellulases secretion by *G. roseum*. Maximum protein and cellulases production were found at 15 g/L of rice straw and 2 g/L yeast extract. Maximum yield of protein, CMCase and cellobiase were obtained after 6 days of incubation, while maximum yield of FPase was obtained after 4 days of incubation. After enzymes concentration with ultrafiltration, specific activity of CMCase, FPase and cellobiase were 5.14, 2.5 and 3.9 U/mg protein, respectively. Optimum temperature for CMCase activity was 55 °C, while 50 °C was optimum for the activity of FPase and cellobiase. CMCase retained about 55.63 % of its activity when incubated at 55 °C for 6 h. While, FPase and cellobiase retained about 15.4 and 14.3 % of the original activity when incubated at 50 °C for 6 h, respectively. The optimum pH for CMCase activity was pH 4. While pH 4.5 was optimum for the activity of FPase and cellobiase. An isoelectric point around 5.0 was detected for CMCase. The molecular weight was calculated to be about 50 KDa.
Keywords: Cellulase, *G. roseum*, production conditions and characterization.

INTRODUCTION

Cellulase serves vast applications in the industries of biofuel, pulp and paper, detergent and textile. With the presence of its three components i.e. Endo-1,4-β D-glucanase [EC.3.2.1.4], Exo-1,4- β-D-glucanase [EC.3.2.1.91] and β-glucosidase [EC. 3.2.1.21], the enzyme can effectively depolymerize the cellulose chains in lignocellulosic substrate to produce smaller sugar units that consist of cellobiose and glucose (Li *et al.,* 2014). Although all cellulolytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds, they show difference in their specificities towards macroscopic properties of substrate (Hong *et al*. 2001). Generally, a typical cellulolytic complex includes a variety of hydrolytic and oxidative enzymes.

Endoglucanases (CMCase) play an important role in the cellulose hydrolysis by cleaving cellulose chains randomly and thus encouraging strong degradation (Cao and Huimin, 2002). The indiscriminate action of endoglucanases progressively increases the accessibility of cellulose chain ends, in this manner increasing the specific surface area of the substrate for exocellulase activity. Endoglucanase attacks the β-1,4 glycosidic bonds

within the amorphous regions of cellulose chains. The products of this attack are oligosaccharides of various lengths and subsequently new chain reducing ends (Lynd *et al*., 2002).

Exoglucanases (FPase) degrade crystalline cellulose most efficiently and act in a processive mode on the reducing or non–reducing ends of cellulose polysaccharide chains, releasing either glucose (glucohydrolases) or cellobiose (cellobiohydrolases) as major products (Lynd *et al.,* 2002).

Cellobiase (β-glucosidases) complete the hydrolysis of cellulose. They hydrolyse cellobiose, a potential inhibitor of cellobiohydrolases (Lemos *et al.,* 2003). The catalytic activity of β-glucosidase is inversely proportional to the degree of substrate polymerization. These enzymes can be grouped as aryl β-D-glucosidases (hydrolysing exclusively aryl-β-glycosides), cellobiases (hydrolysing diglycosides and cellooligosaccharides) or β-glucosidases with wide range of substrate specificities (Bhat and Hazlewood, 2001).

Cellulases and hemicellulases (glycosylhydrolases) are produced by a range of microorganisms, including bacteria, actinobacteria, fungi, and yeast, but fungi appear to be the most efficient producers of extracellular enzymes (Jorgensen *et al*., 2003). The present work focused and aimed to study the characterization of cellulolytic enzymes (CMCase, FPase and cellobiase) produced by *G. roseum* using some lignocellulosic wastes.

MATERIALS AND METHODS

Microorganism

The fungus *G. roseum* was obtained from Agric. Microbiol. Dept., Soil, Water and Environ. Res. Institute, Agric. Res. Centre, Giza, Egypt. The original culture was maintained on potato dextrose agar (PDA) slant at 5˚C.

Lignocellulosic materials

Lignocellulosic materials such as rice straw, wheat straw, bagas, saw dust and corn stalks were obtained from the farm of the Fac. of Agric. at Moshtohor, Benha Unive., Egypt.

Fermentation and optimization studies

Five media were used to study the production of cellulolytic enzymes by *G. roseum*, the used media were: Cellulose broth, (Bagga *et al.*, 1990); basal mineral salt medium containing 1% cellulose, (Chen and Wayman, 1991); Reese and Mandel's basal medium, (Reese and Mandel, 1963); Cellulase production medium (Camassola and Dillon, 2007) and Czapek-Dox liquid medium containing 1% cellulose, (Coral *et al*., 2002), each medium was adjusted to pH 6.

About 95 mL of the production medium were dispended into 250 mL Erlenmeyer flasks, sterilized and inoculated with 5 mL of a 5-days-old fungal inoculum. The inoculated flasks were incubated with shaking at 150 rpm and 25˚C. The cultures were centrifuged at 4000 rpm for 30 min at 4˚C. The supernatant was used for measurement of cellulase activity. Optimization of cellulases production by *G. roseum* was carried out in the most suitable medium which modified by replacing carbon sources with different lignocellulosic wastes i.e. rice straw, wheat straw, bagas, saw dust and corn stalks). In addition, different nitrogen sources (peptone, yeast extract,

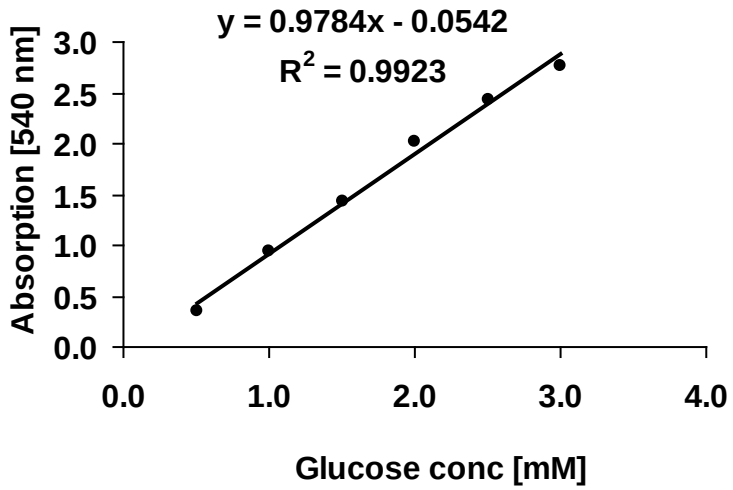
ammonium sulphate, ammonium nitrate and sodium nitrate) and their concentrations and time course (2, 4, 6, 8, and 10 days) were tested.

Enzymes and protein determination

Carboxymethyl-cellulase (CMCase) activity was assayed using a modified method described by Wood and Bhat (1998) with some modifications. 0.2 mL of culture filtrate was added to 1.8 mL of 1% CMC prepared in 0.05M sodium citrate buffer (pH 4.8) in a test tube and incubated at 40°C for 30 minutes. The reaction was terminated by adding 3.0 mL of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. One milliliter of Rochelle salt solution (40 g Rochelle salt in distilled water to make the volume 100 mL) was then added to stabilize the colour. The absorbance was recorded at 575 nm against the blank (of 0.05M sodium citrate buffer). One unit of CMCase activity was expressed as 1 µmole of glucose liberated per mL enzyme per minute.

Filter-paperase (FPase) activity was assayed according to the method explained by Wood and Bhat (1998) with some modifications. Briefly, the methods are similar to the CMCase assay method, but the substrate used was Whatman no. 1 filter paper strip (1 x 3 cm) soaked in 1.8 mL 0.05M sodium citrate buffer (pH 4.8). The samples were incubated at 40°C for 60 minutes.

Cellobiase activity was deterimined by a modification of the method described by Berghem and Petterson (1974). 0.5ml o f culture filtrate was added to 1.0 mL of 0.4% cellobiose dissolved in 0.05 M citrate-phosphate buffer (pH 4.8). The reaction inixture was incubated at 50°C for 30 min. The released glucose was determined dinitrosalicylic acid method as abovementioned.



Standard curve of glucose

Reducing sugars analysis was conducted based on the method as described by Miller, (1959). In this method, 2 mL of diluted sample was added to 3 mL of DNS and boiled for 15 minutes. After boiling, 1 mL of Rochelle salt was added. The absorbance was recorded at 575 nm using spectrophotometer against the blank of distilled water. Protein concentration was determined according to Lowry *et al.*, (1951). Enzyme specific activity (U/mg) = Enzyme activity (U/mL) / Protein concentration (mg/mL).

Enzymes concentration

Culture of *G. roseum* was centrifuged at 4000 rpm for 30 min at 4°C and the clear supernatant was used as source of crude enzyme. Cellulases were concentrated by ultrafiltration technique (Jumbosep™ Centrifugal Devices, exclusion limit 10 kDa) at 3000 rpm and 4 °C. Following the ultrafiltration, protein concentration and cellulases activity in the retentate and filtrate were measured.

Effect of temperature on cellulases activity and thermal stability

The concentrated enzymes preparation was assayed for CMCase, FPase and cellobiase activity at temperature range of 30-60 °C for 25 min. Thermal stability was detected by incubating the enzyme at its optimum temperature for different periods intervals, starting from 2 to 12 h. Samples were removed periodically every 2 h. and assayed for residual activity under standard assay conditions.

Effect of pH on cellulases activity

Enzymes activity were measured after incubating the reaction mixtures for 25 min at different pH values, ranging from pH 3 to 5 using 0.05 M sodium acetate buffer.

Isoelectric focusing (IEF)

Isoelectric point was detected according to the methods of Kluepfel, (1988). Polyacrylamide gel (IEF-gel, Serva) with immobilized pH gradient ranging from 3 - 10 was used. 5 µL of marker (Servalyt®, precote® 3-10) and 6 µL as sample were individually applied to a flatbed electrophoresis apparatus. In parallel, 8 µL samples were used for the activity staining. Protein bands were visualized by staining for 1 h. with 0.2 % Coomassie Brilliant Blue CBB-R250 dye in methanol-acetic acid-water solution (4:1:5, by volume). The same solution without dye was used for the destaining process. For the activity staining, the gel was placed on 1% agarose plate containing 0.1 % hydroxyethylcellulose and incubated at 40 °C for 4-5 h. The agarose plate was then stained with 0.1% congo red solution for 30 min and destained in 1 M NaCl for 15 min. Clear zone (unstained) on the agarose plate indicates the presence of endoglucanase activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Active IEF band was cut, ground in 60 µL ultrapure water and mixed with 10µL loading buffer. 50 µL as sample was applied to polyacrylamide gel electrophoresis. 5µL protein marker for SDS-PAGE (Roti®-Mark STANDARD Roth) was used. Protein bands were visualized by staining for 1 h. with 0.2 % Coomassie Brilliant Blue CBB-R250 dye in methanol-acetic acid-water solution (4:1:5, by volume). The same solution without dye was used for the destaining process. A plot of log molecular weight versus relative mobility (Rf)

of standard proteins was used to estimate molecular weight of endoglucanase enzyme, Laemmli, (1970).

RESULTS AND DISCUSSION

Effect of different media on protein and cellulases production

Five different media were tested for their ability to support growth, protein and cellulases secretion by *G. roseum* (Fig. 1). Data indicated that Reese and Mandel's basal medium was the best medium for protein and cellulases (CMCase, FPase and cellobiase) production. This may be attributed to the presence of Tween 80 within the constituent of the medium, which supports the release of the enzyme into the culture medium.

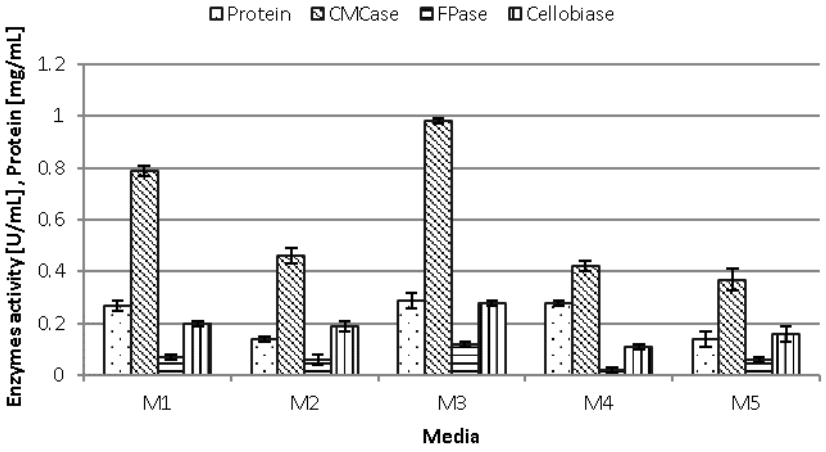


Fig.1. Effect of different media on protein content and cellulases production by *G. roseum.*

M1: Cellulose broth.

M2: Cellulase enzyme production medium.

M3: Reese and Mandel's basal medium

M4: Basal mineral salt medium containing 1% cellulose.

M5: Czapek-Dox liquid medium containing 1% cellulose

The obtained results are in agreement with those reported by Vyas (2004) who demonstrated that the addition of Tween 80 as surfactant led to higher cellulase activities. Moreover, Tween 80 facilitates the release of cellulases into the medium by causing an increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes. Nicholas *et al.,* (2016) studied the nutrient control for stationary phase cellulase production in *Trichoderma reesei* Rut C-30 and stated that the addition of Triton X-100 as a surfactant led to high fungal pellet formation, in addition a stationary phase cellulase production period in excess of 300 h was achieved, with a constant enzyme production rate of 7 ± 1 FPU/g^{-h}.

On the other hand, Czapek-Dox liquid medium containing 1% cellulose gave the lowest CMCase activity and protein content. Moreover, basal mineral salt medium containing 1% cellulose showed low activity for FPase and cellobiase when used for growing of *G. roseum*. Consequently, Reese and Mandel's basal medium was used for the succeeding experiments.

Carbon source

The effect of different carbon sources on cellulase secretion and protein content of *G. roseum* was studied. Five different lignocellulosic materials (wheat straw, bagas, rice straw, corn stalks and saw dust) were tested (Fig.2). The highest cellulases activity (CMCase, FPase and cellobiase) and protein content were observed with using rice straw as carbon source.

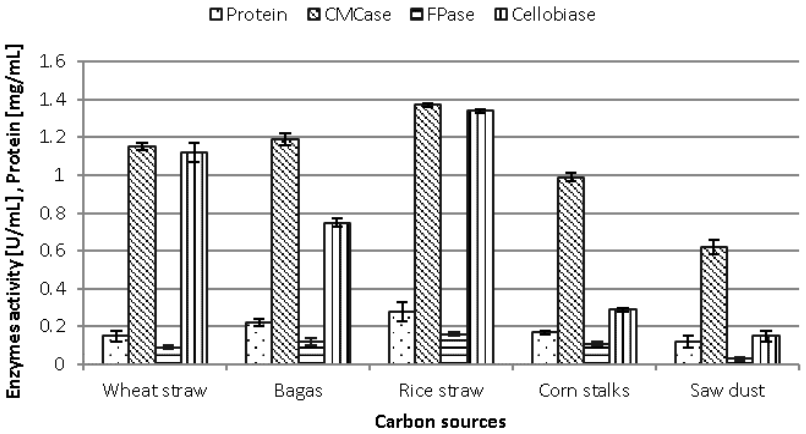


Fig.2. Effect of different carbon sources on protein content an cellulases production by *G. roseum.*

On the other hand, saw dust showed the lowest cellulases activity and protein content when used as carbon source. The obtained results are in harmony with those of Hanpeng *et al*. (2015) who recorded that the majority of extracellular proteins were cellulose-degrading enzymes induced by agricultural wastes. Moreover, large amount of FPase (1.4 U/mL), CMCase (2.0 U/mL) and β-glucosidase (2.7 U/mL) activities were produced when *Penicillium oxalicum* GZ-2 was grown on rice straw.

Rice straw concentrations

Different concentrations of rice straw (5 – 25 g/L) were added to Reese and Mandel's basal medium to study their effect on cellulases (CMCase, FPase and cellobiase) production and protein content (Fig. 3). Data show gradual increase of cellulase activity and protein content reaching a maximum activity at 15 g/L rice straw.

The production of hydrolytic enzymes is directly related to the available substrate (Nybroe *et al.* 1992). Endoglucanase is an inducible enzyme system. Therefore, an increase in the concentration of a particular substrate may stimulate the microorganisms' specific enzyme production. In addition,

Narasimha *et al*. (2006) stated that the production of cellulase from *A. niger* in response to cellulose concentration.

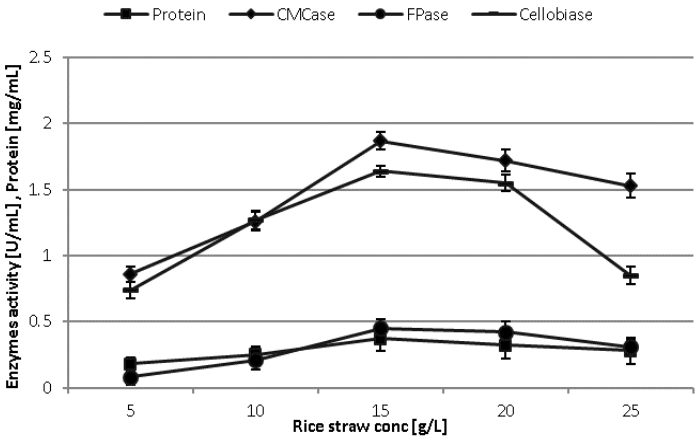


Fig.3. Effect of rice straw concentration on protein content and cellulases production by *G. roseum*.

Effect of the nitrogen source

The effect of various nitrogen sources on cellulases activity and protein content by *G. roseum* using Reese and Mandel's basal medium was illustrated in Fig. 4. Among the various organic and inorganic nitrogen sources, yeast extract was found to be optimal for cellulases and protein production. On the other hand, inorganic nitrogen sources (ammonium nitrate and sodium nitrate) yielded the lowest protein content and cellulolytic activity.

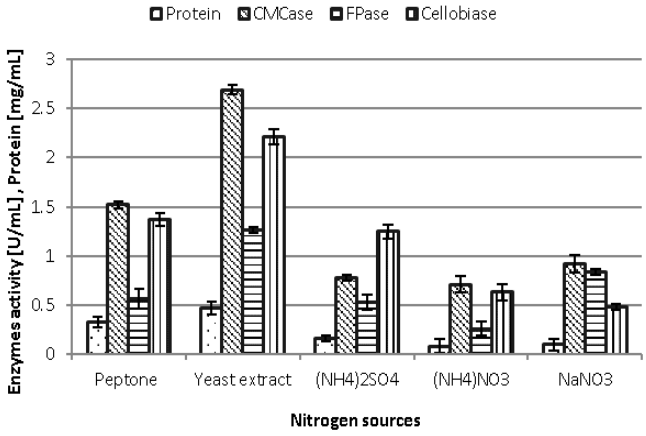


Fig.4. Effect of different nitrogen sources on protein content and cellulases production by *G. roseum.*

Previous experiments on the effect of various nitrogen sources on cellulase production demonstrated a substantial increase in the enzyme activity when the media were supplemented with yeast extract (Vyas, 2004).

Effect of different concentrations of yeast extract

Reese and Mandel's basal medium was supplemented with 1 – 3 g/L yeast extract. Cellulases activity and protein content were determined (Fig. 5). Data show that yeast extract at concentration of 2 g/L resulted in maximum enzymes (CMCase, FPase and cellobiase) activity and protein content by *G. roseum*.

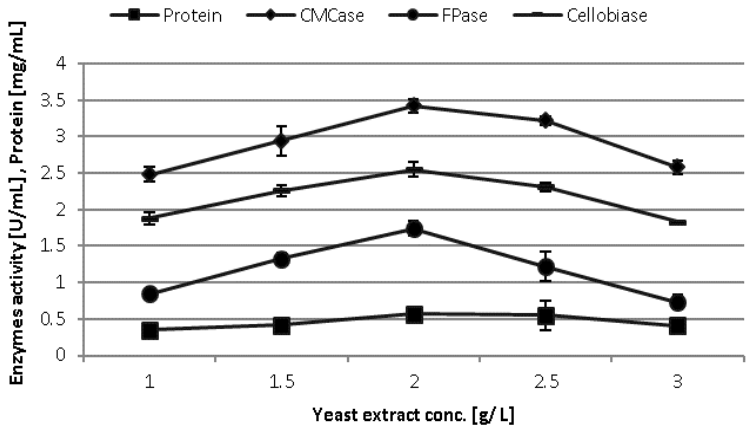


Fig.5. Effect of yeast extract concentration on protein content and cellulases production by *G. roseum.*

Effect of the cultivation period

The time course of cellulases production (CMCase, FPase and cellobiase) and protein content were studied to determine the point of time with maximum activity (Fig. 6). Enzymes activity gradually increased with increasing the culture period. Maximum protein content, CMCase and cellobiase activities were obtained after 6 days of cultivation. While, FPase activity showed its maximum activity after 4 days of incubation.

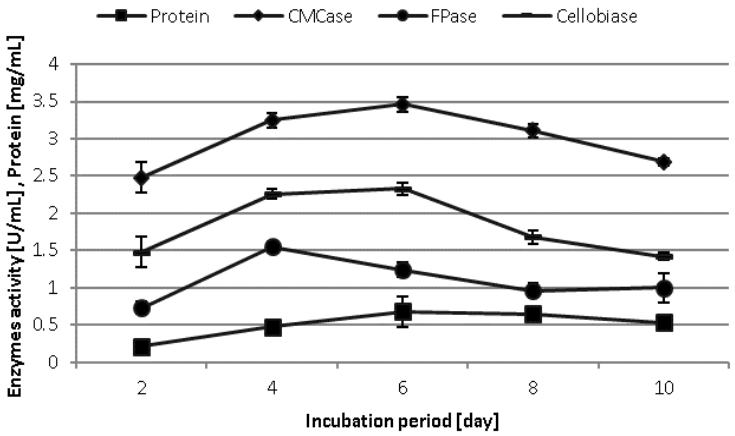


Fig.6. Effect of incubation period on protein content and cellulases production by *G. roseum*.

These results are in good agreement with those reported by Camassola and Dillon, (2007) who obtained the highest activity of an endoglucanase from *Penicillium echinulatum* after 4 days of cultivation. Garcia-Kirchner *et al*., (2002) grew *Penicillium sp.* and *Aspergillus terreus* for 6 days for the maximum yield of cellulolytic and xylanolytic activities. Moreover, Nitin *et al.,* (2015) studied the production of cellulase from the marine fungus *Cladosporium sphaerospermum* through solid state fermentation (SSF) using the common green seaweed *Ulva fasciata*. The maximum cellulase production was obtained after 4 days of incubation.

Concentration of cellulases (CMCase, FPase and cellobiase) produced by *G.roseum*

The culture supernatant of *G. roseum* was concentrated by ultrafiltration (Table1). Protein yields after concentration was 4.5 mg/mL which was sufficient for analysis by means of isoelectric focused electrophoresis.

Table1.Concentration of cellulases (CMCase, FPase and cellobiase) produced by *G. roseum.*

	Initial supernatant	Retentate
Volume (mL)	1000	55
Activity (U/mL)		
CMCase	2.17	23.16
FPase	0.98	11.35
Cellobiase	1.83	17.53
Protein (mg/mL)	0.53	4.5
Specific activity (U/mg)		
CMCase	4.1	5.14
FPase	1.8	2.5
Cellobiase	3.5	3.9

The obtained specific activities of retentate were 5.14, 2.5 and 3.9 U/mg for CMCase, FPase and cellobiase, respectively.

Effect of temperature on cellulases activity and thermal stability

Temperature optima and the thermal stability of cellulases (CMCase, FPase and cellobiase) were studied over a certain period. The loss of enzyme activity depends both on time and temperature, it is therefore important to investigate the stability of the enzyme with respect to these factors. To estimate the temperature stability, the residual activity after incubation for 12 h. at the enzymes temperature optima was determined under standard conditions.

The obtained data was illustrated in Figs.7, 8. The enzymes preparations were active in a broad temperature range of 30 °C to 60°C.

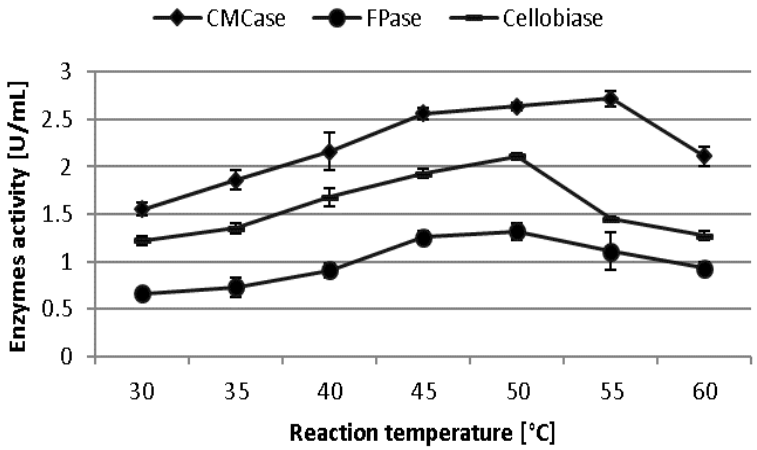


Fig.7. Effect of the temperature on cellulases activity

The optimum temperature for CMCase activity was 55 °C. Moreover, 50 °C was optimum for the activity of FPase and cellobiase. CMCase retained about 55.63 % of its activity when incubated at 55 °C for 6 h. While, FPase and cellobiase retained about 15.4 and 14.3 % of the original activity when incubated at 50 °C for 6 h, respectively. These results are in good agreement with those reported by Rao *et al.* (2003). Moreover, Hanpeng *et al*. (2015) studied the production and characterization of cellulase from *Penicillium oxalicum* GZ-2 and found that the optimum temperature for the maximum activity of CMCase and cellobiase were 50 °C and 60 °C, respectively.

The use of thermostable enzymes to carry out hydrolysis at high temperature is advantageous because they speed up the reaction rate and prevent microbial contamination (Raza and Ur-Rehman, 2009). Moreover, the loss of enzyme activity can be attributed to protein denaturation which are in accordance with those obtained by Wang *et al.* (2008) who stated that the loss of enzyme activity at elevated temperatures is a consequence of thermal denaturation of protein.

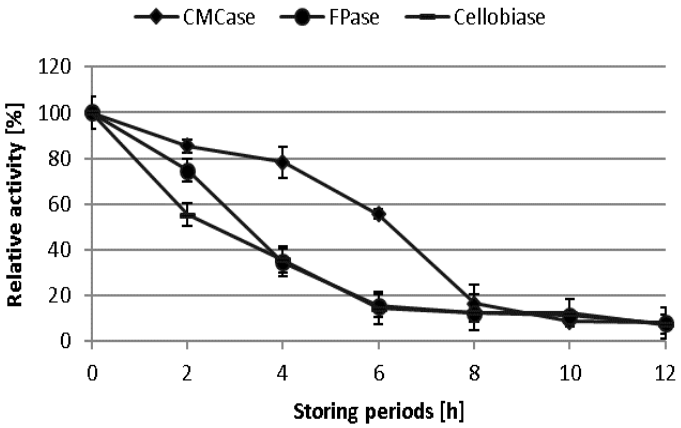


Fig.8. Thermal stability of cellulases

Effect of pH on cellulase activity

The ionization state of amino acid residues of an enzyme depends on the pH value. Since catalytic activity is dependent on the state of ionization of these residues, enzyme activity is consequently pH dependent. Enzymes are often active over a narrow pH range with a specific pH optimum at which their catalytic activity is maximal (Wilson, 2000). Therefore, it was of interest to determine the pH optima of the cellulases (CMCase, FPase and cellobiase) under investigation (Fig.9).

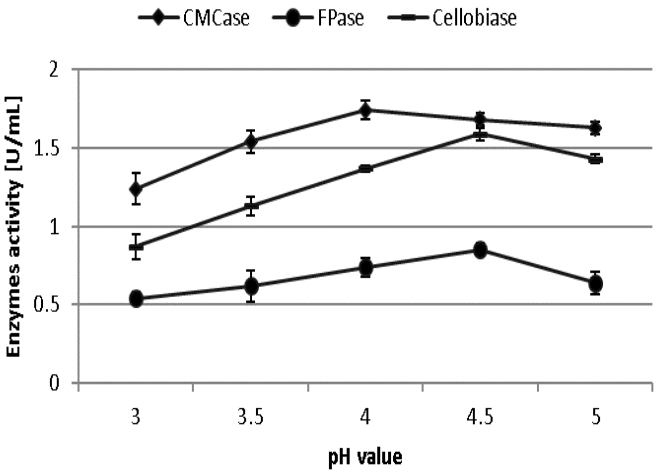


Fig.9. Effect of pH on cellulases activity

Cellulases were active in a broad pH range of 3.0 to 5.0. pH 4.0 was favorable for the activity of CMCase while FPase and cellobiase showed their maximum activity at pH4.5.

These results are in good agreement with those of Coral *et al*. (2002). They studied the pH dependence of an endocellulase from a wild type strain of *A. niger* and reported a broad activity range of 3.0 - 9.0 with a maximum activity at pH 4.5. In addition, endoglucanase showed high stability for pH 5 when incubated for 12 h. While, Dongyang *et al.*, (2011) studied the production of thermostable cellulase from *Aspergillus fumigatus* Z5 under solid-state fermentation and its application in degradation of agricultural wastes. They recorded pH5 to be optimum for maximum CMCase activity.

Isoelectric focusing (IEF)

Endoglucanase produced by *G. roseum* was analyzed by isoelectric focusing electrophoresis (IEF) with Coomassie and activity staining (Fig. 10). A clear (unstained) zone on the agarose plate indicated the presence of endoglucanase activity. An isoelectric point (pI) around 5.0 was determined for the endoglucanase produced by *G. roseum*.

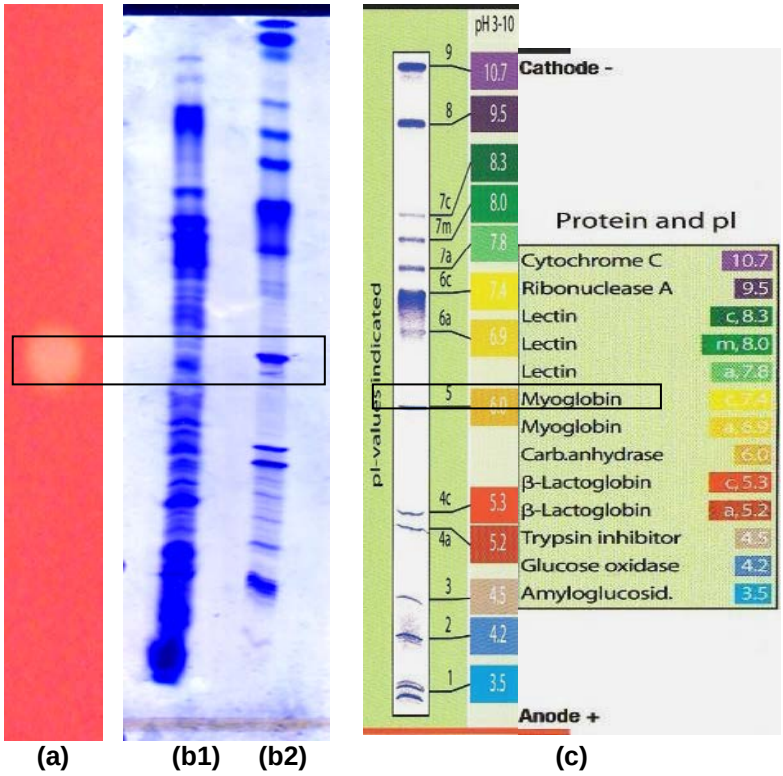


Fig. 10. Analysis of endoglucanase (*G. roseum*) by IEF electrophoresis, (a): activity staining with congo red, (b1, b2): Coomassie staining of sample (6µL) and protein marker (5µL), respectively. (c): protein marker.

SDS –PAGE analysis

A plot of log molecular weight versus relative mobility (Rf) of standard proteins was used to estimate molecular weight of endoglucanase produced by *G. roseum*. Single band was detected on the SDS-PAGE gel (Fig. 11). The molecular weight was calculated to be about 50 KDa.

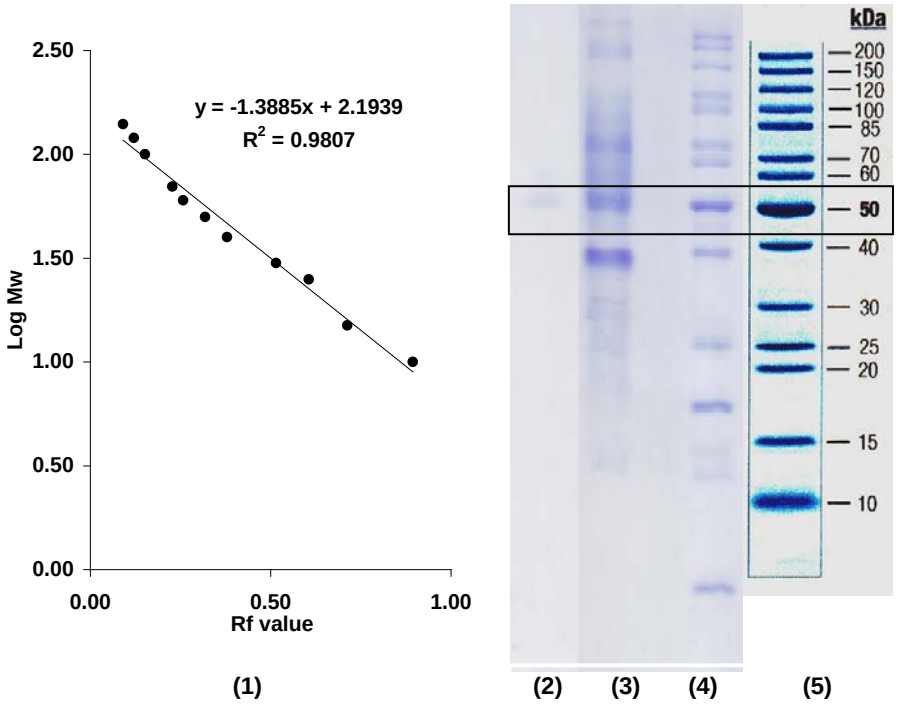


Fig. 11. SDS-PAGE analysis of endoglucanase secreted by *G. roseum*. (1): A plot of log molecular weight versus relative mobility (Rf) of standard proteins; (2): Single active band; (3): The original culture of *G.roseum*; (4): Molecular mass marker; (5): Original marker

CONCLUSION

From the previous work it can be concluded that, fungi are the most suitable cellulase producers attributing to its ability to produce a complete cellulase system. *G. roseum* was used for cellulases production using rice straw as a carbon source which imposes lower cost and enables the production of cellulase with higher titre.

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انتاج و خصائص الإنزيمات المحللة للسليولوز من فطر *Gliocladium roseum*

احمد عبد الخالق سالم و هاني محمد احمد عبدالرحمن

قسم النبات الزراعي – كلية الزراعة بمشتهر – جامعة بنها – مصر

يهتم هذا العمل بإنتاج و دراسة خصائص الإنزيمات المحللة للسليولوز بأنواعها (CMCase, FPase and cellobiase) بواسطة فطر *Gliocladium roseum*. اوضحت الدراسة افضلية بيئة Reese and Mandel's basal medium للحصول علي اعلي انتاجية من المحتوي البروتيني و الإنزيمات المحللة للسليولوز بأنواعها الثلاثة من فطر *G. roseum*. أعلي إنتاجية من المحتوي البروتيني و النشاط الانزيمي تم الحصول عليها عند إستخدام 15جم/لتر من قش الارز و 2جم/لتر مستخلص خميرة. كذلك تم الحصول علي أقصي إنتاجية من المحتوي البروتيني و إنزيمات CMCase , cellobiase بعد 6 أيام من التحضين. بينما أقصي إنتاجية من FPase فكانت بعد 4 أيام من التحضين. بعد تركيز الإنزيم بإستخدام تقنية الـ Ultrafiltration كان النشاط التخصصي Specific activity لإنزيمات CMCase, FPase and cellobiase 5.14 و 2.5 و 3.9 وحدة/مجم بروتين، علي التوالي. درجة الحرارة المثلي لنشاط إنزيم CMCase كانت 55 م° ، علاوه علي ذلك كانت درجة حرارة 50م° المثلي لنشاط إنزيمات FPase , cellobiase. إحتفظ إنزيم CMCase بحوالي 55.63 % من نشاطه الأصلي عند التحضين علي 55 م° لمدة 6 ساعات. بينما FPase , cellobiase احتفظوا بحوالي 15.4 و 14.3 % من النشاط الأصلي عند التحضين علي 50 م° لمدة 6 ساعات ، علي التوالي. فيما يخص درجة الـ pH ، وجد ان pH 4 هو الأمثل لأعلي نشاط لإنزيم CMCase بينما pH 4.5 كان الأمثل لنشاط إنزيمات FPase , cellobiase . تم تحديد درجة التعادل الكهربي لإنزيم CMCase و كانت حوالي 5 اما الوزن الجزيئي فكان 50 كيلودالتون.