Effect of Incubation Temperature on the Production of Fungal Cellulases

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Abstract:

This study was aimed to investigate the optimum incubation temperature for cellulases production by *Aspergillus niger* AUMC 8485, *Aspergillus flavus* AUMC 8534 and *Emericella nidulans* AUMC 8581.

The obtained results showed that, the optimum incubation temperature for cellulases production as measured by cup-plate clearing zone technique was 35° C for all the studied species. The diameter of clearing zone was 22.0, 24.5, 26.0 mm for *Aspergillus niger*, *Aspergillus flavus* and *Emericella nidulans*, respectively. In case of exo –D-1,4 gluconase (FP-ase) production, the highest enzymatic activity was obtained at 35° C by all the studied fungal species. Meanwhile, the highest specific activity was recorded at 40° C for *A. niger* and at 35° C for *A. flavus* and *E. nidulans*. At the same time, the optimum incubation temperature for endo- ß- D-1,4 gluconase (CMC-ase) production was recorded at 40° C for *A. niger* and *E. nidulans*, and at 35 ° C for *A. flavus*. The highest CMC-ase specific activity was observed at 40° C for all the studied strains. The maximum amount of reducing sugars found in the culture filtrate at 35° C for *A. niger*, and at 40° C for *A. flavus* and *E. nidulans*. Also, the highest amount of soluble protein was recorded at 35° C for *A. niger* and at 30° C for *A. flavus* and *E. nidulans*.

Keywords: Cellulases, Temperature, *Aspergillus niger*, *Aspergillus flavus*, *Emericella nidulans*,.

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Introduction:

Cellulose, the most abundant polysaccharide on earth (Coughlan, 1985) is a linear, unbranched homo-polysaccharide consisting of glucose subunit joined together via β 1-4 glycosidic linkages, being the primary structural material of plant cell wall. Individual cellulose molecules (polymer) vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2002 and Saha *et al*, 2006).

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components including glucose subunits (Jahangeer *et al*, 2005). Cellulase enzymes were divided into three groups according to their mechanism: (1) Exo-ß-1,4 gluconase (C1) was believed to act at first on the crystalline cellulose, modifying it in such a way that subsequent enzyme can hydrolyze the product. (2) Endo-ß-1,4 gluconases (CX). The random acting enzymes will hydrolyze non-crystalline cellulose, soluble cellulose derivatives, and the ß-1,4 oligomers of glucose (Rees and Maguire, 1971 and Okazaki and Moo-Young, 1978). Glucose produced from cellulosic substrate could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol, butanol, methane, amino acid, single-cell protein, etc. (Walsh, 2002). Cellulases have been used for several years in food processing, feed preparation, waste-water treatment, detergent formulation, textile production and in other areas. Additional potential applications include the production of wine, beer and fruit juice. Nevertheless, all these uses are of rather small magnitude compared with cellulase requirements for bioconversion of lignocellulosic biomass to fuel ethanol (Philippidis, 1994).

A battery of enzymes (hydrolytic and oxidative) produced by a variety of fungi and bacteria, work in synergy to perform lignocellulose degradation (Pérez *et al*, 2002). Fungi can degrade cellulose, hemicellulose and lignin in decaying plants by a complex set of excreted enzymes (Gosh and Gosh, 1992). The best-known producers of lignocellulose-degrading enzymes are strains of *Trichoderma* and *Aspergillus* species. Other relevant microorganisms include strains of *Humicola*, *Talaromyces*, *Acrophialophora*, *Thermoascus*, *Bacillus* and *Penicillium* species (Bon and Ferrara, 2007).

Incubation temperature of the fermentation medium is a critical factor has insightful influence on metabolic activities of microorganisms (Gomathi *et al,* 2012). Therefore, the present research was aimed to investigate the optimum incubation temperature required for cellulases production by *Aspergillus niger* AUMC 8485, *Aspergillus flavus* AUMC 8534 and *Emericella nidulans* AUMC 8581.

Materials and Methods:

1. *Fungal species:*

Three fungal species namely: *Aspergillus niger* AUMC 8485, *Aspergillus flavus* AUMC 8534 and *Emericella nidulans* AUMC 8581 were obtained from Assiut University Mycological Center (AUMC). The cultures were maintained at 4°C on potato dextrose agar (PDA) slants containing (g/l): extract from 200 g of potatoes, glucose, 20.0; yeast extract, 1.0; and agar, 20.0 (Ragab,1989).

2. *Mycological procedures:*

2.1. *Preparation of spores suspension:*

Slant surfaces of PDA medium were inoculated with a young culture of fungi, then incubated at 30 °C for 5 days. Spores suspension was prepared by adding ten ml of sterilized distilled water to each slant. The spores were scraped with inoculating loop , then a heavy spores suspension was collected in sterilized Erlenmeyer flask and adjusted to about 1×10^{-6} spores/ml (Ragab,1989).

2.2.*Cultivation of tested fungi:*

Cultivation of tested fungi technique of Al-Mowallad (2008) was applied with some modification as follows : The liquid medium used for growth and production of cellulases was consisted of (g/l): $(NH_4)_2SO_4$, 0.5; L-asparagine, 0.5; KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.2; yeast extract, 0.5; and carboxymethyl cellulose (CMC), 10 (Eggins and Pugh, 1962). The medium was adjusted to pH 5.4. Fifty ml of the medium placed in 250 ml cotton plugged Erlenmeyer flasks and autoclaved at 121°C for 20 min. After cooling at room temperature, each flask was inoculated with one ml of spores suspension and then incubated at different temperatures; 25, 30, 35, 40 and 45°C for 5 days. All the experiments were performed in duplicate.

After incubation period, the contents of each flask were filtered through double layer of cheese cloth to obtain clear extract containing the crude enzyme of cellulases. The crude enzyme was used for determination of exo–D-1,4 gluconase activity, endo-ß-D 1,4 gluconase activity, diameter of clearing zone, reducing sugars and soluble protein.

3. *Enzyme assay:*

3.1. *Clearing zone technique:*

For the purpose of cellulase (s) assay, the cellulase cup plate clearing zone technique of Hankin and Anagnostakis (1977) was applied with some modification as follows: 20 ml aliquots of sterilized cellulose-agar medium containing 0.5% carboxymethyl cellulose (CMC) were poured aseptically in each sterilized Petri-dish. The medium was allowed to cool and solidify. One cup was made per each plate by a sterilized corkborer (10 mm diameter), 0.1 ml of culture filtrate was introduced into the cup, and then, the plates were incubated for 24 hrs. at 30° C. At the end of incubation period, plates were flooded with 0.1% congo red solution for 15 min. and then destained with 1M NaCl. The hydrolysis zones were measured.

3.2. *Exo –D-1,4 gluconase (FP-ase) activity:*

Filter paper cellulase (FP-ase) activity was carried out according to Takao *et al.* (1985). The assay mixture in total volume of 2 ml containing 50 mg (6 × 1 cm) Whatman No.1 filter paper as a substrate (in a 0.1 M acetate buffer solution pH 5) and 1 ml of enzyme filtrate. The mixture incubated at 50-55°C for 60 min.

The released reducing sugars was determined by the dinitrosalicylic acid (DNS) method described by Miller (1959). One unit of FP-ase activity is defined as the amount of enzyme that released 1 μ mole of reducing sugars from the substrate per minutes under the test conditions.

3.3. *Endo-β-D 1,4 gluconase (CMC-ase) activity:*

Carboxymethyl cellulase (CMC-ase) activity was determined according to Takao et al., (1985). The assay mixture in total volume of 2 ml was containing 1 ml of 1% CMC (in a 0.1 M acetate buffer solution pH 5) as a substrate and 1 ml of enzyme filtrate. The mixture was then incubated at 50-55°C for 10 min. and the released reducing sugars was determined by the (DNS) method according to Miller (1959). One unit of CMC-ase activity is defined as the amount of enzyme that released 1 μ mole of reducing sugars from the substrate per minutes under the test conditions.

4. *Reducing sugars determination:*

Reducing sugars contents were determined by the method described by Miller (1959) as follows: Three ml of DNS reagent (10 g dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulfite, 10 g sodium chloride and add water to 1 L) were added to two ml of aliquot filtrate in a tightly capped test tube. The mixture was heated at 90° C in a water bath for 15 min. to develop the red-brown color. One ml of 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance recorded by spectrophotometer at 575 nm.

5. *Soluble protein determination:*

Soluble protein contents were determined by the method described by Lowry *et al.* (1951) as follows: one ml of aliquot filtrate was pipette into a test tube, then 5 ml of fresh reagent (50 ml of 2% Na_2CO_3 in a 0.1 N NaOH solution + 0.5 ml of 0.5% $CuSO_4$-$5H_2O$ + 0.5 ml of 1% sodium potassium tartarate) were added and left for 10 min. at room temperature. After that 0.5 ml of Folin reagent (1M) was added rapidly and mixed for 30 min. at room temperature. The intensity of color was determined as O.D at 750 nm.. The protein content was calculated from a standard curve of bovine serum albumin solution.

Results and Discussion:

Generally, temperature affects the rate of all biological activities of microorganisms, nutrient uptake and enzyme synthesis. Therefore, an experiment was conducted to find out the effect of different incubation temperatures (25, 30, 35, 40 and 45° C) on production of cellulases by *Aspergillus niger* AUMC 8485, *Aspergillus flavus* AUMC 8534 and *Emericella nidulans* AUMC 8581

1. Cellulase *clearing zone:*

Data presented in Table (1) and illustrated in Fig. (1) showed that all the studied fungal species exhibited their highest celluolytic activity when incubated at 35° C. Maximum diameter of cellulase cup-plate clearing zone observed by *A. niger*, *A. Flavus* and *E. nidulans* were 22.0, 24.5 and 26.0 mm respectively. Lower values of clearing zone diameter were obtained when fungi were incubated at lower or higher than the optimum temperature (35□ C).

Table (1): Effect of incubation temperature on the cellulytic activity of the studied fungal species.

Fungal strain	Clearing Zone Diameter (mm)				
	25 ºC	30 ºC	35 ºC	40 ºC	45 ºC
Aspergillus niger	19.0	18.0	22.0	21.0	ND
Aspergillus flavus	18.0	19.0	24.5	24.0	ND
Emericella nidulans	20.0	25.0	26.0	23.5	22.5

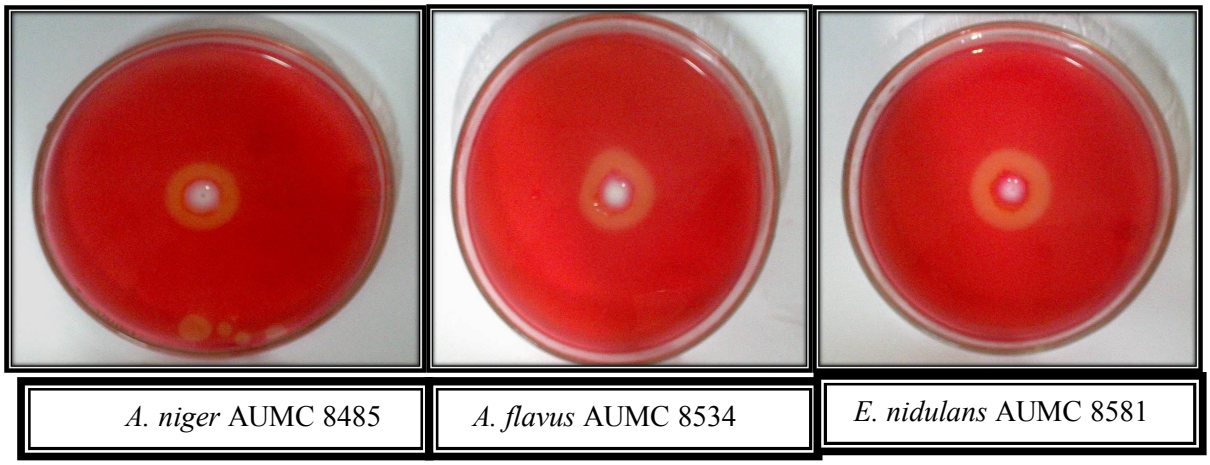

Fig. (1): Diameter of clearing zones produced by the studied fungal species at 35 º C.

2. Exo –*D-1,4 gluconase (FP-ase) activity:*

Fig. (2) showed that 35° C was the most optimal temperature giving maximal exo –D-1,4 gluconase activity for all tested fungal strains. It was 0.455, 0.577 and 0.718 U\ml for *A. flavus, A. niger* and *E. nidulans*, respectively. Production of exo –D-1,4 gluconase was increased gradually with increasing the incubation temperature from 25 to 35º C and then turned to the gradual decrease with increasing the incubation temperature from 35 to 45° C. These results were agree with that obtained by Darwish (1995) and Sohail *et al.* (2009) who found that the optimum temperature for cellulase production by *A. niger* was 35° C.

The specific activity of exo –D-1,4 gluconase (FP-ase) reached to the maximum value at 35° C for each *A. flavus* and *E. nidulans* giving about 1.217 and 1.666 U\mg protein, respectivally. Moreover, the optimum temperature for *A. niger* was 40° C, which gave 1.203 U\mg protein (Fig. 3).

3. *Endo-ß-D 1,4 glucanase (CMC-ase) activity:*

Results obtained in Fig. (4) showed that the activity of endo-ß-D 1,4 glucanase (CMC-ase) increased as the incubation temperature increased, reaching the maximum value at 40° C for each *A. niger* and *A. flavus* which produced 0.756 and 0.370 U\ml, respectively. While it was 45°C for *E. nidulans* (0.809 U\ml). The same trend was observed in case of endo-ß-D 1,4 glucanase (CMC-ase) specific activity which recorded the maximum value at 40°C for each *A. flavus* and *A. niger* and at 45° C for *E. nidulans* (Fig. 5).

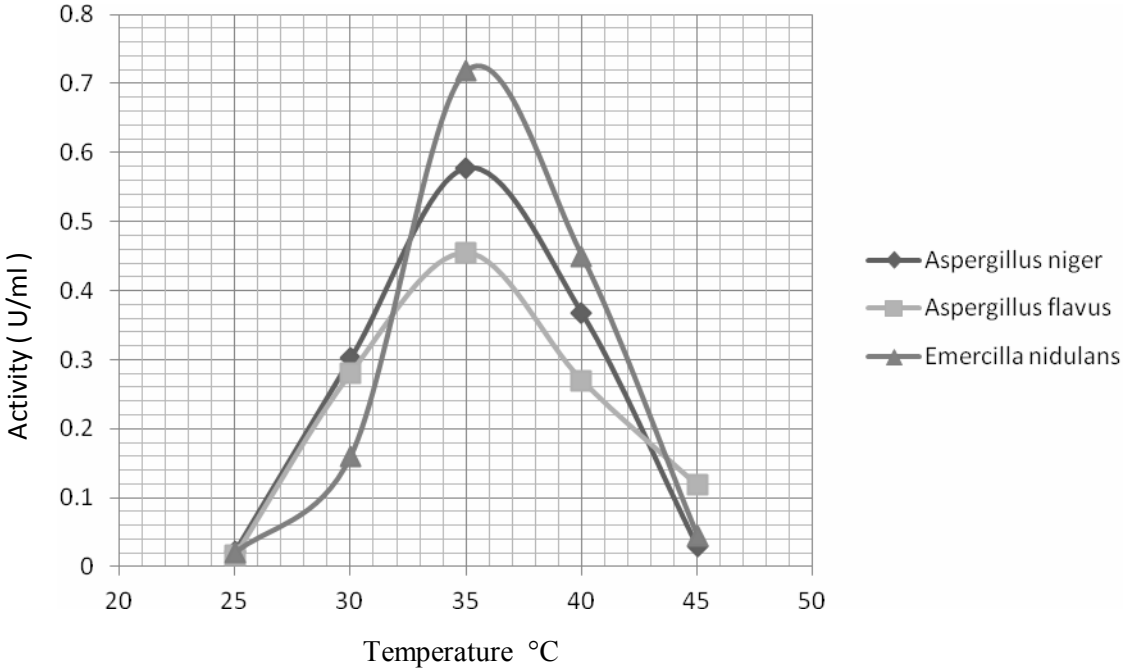

Fig. (2): Effect of incubation temperature on exo-gluconase activity by tested fungi (U\ml).

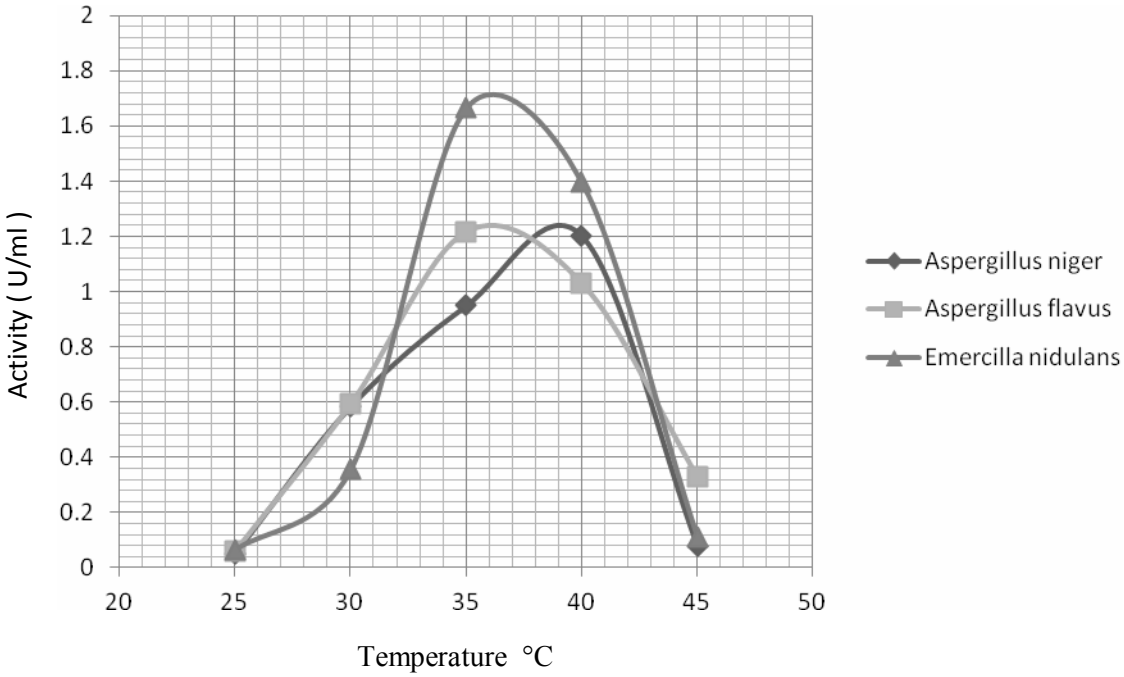

Fig.(3): Effect of incubation temperature on exo-gluconase specific activity by tested fungi (U\mg protein).

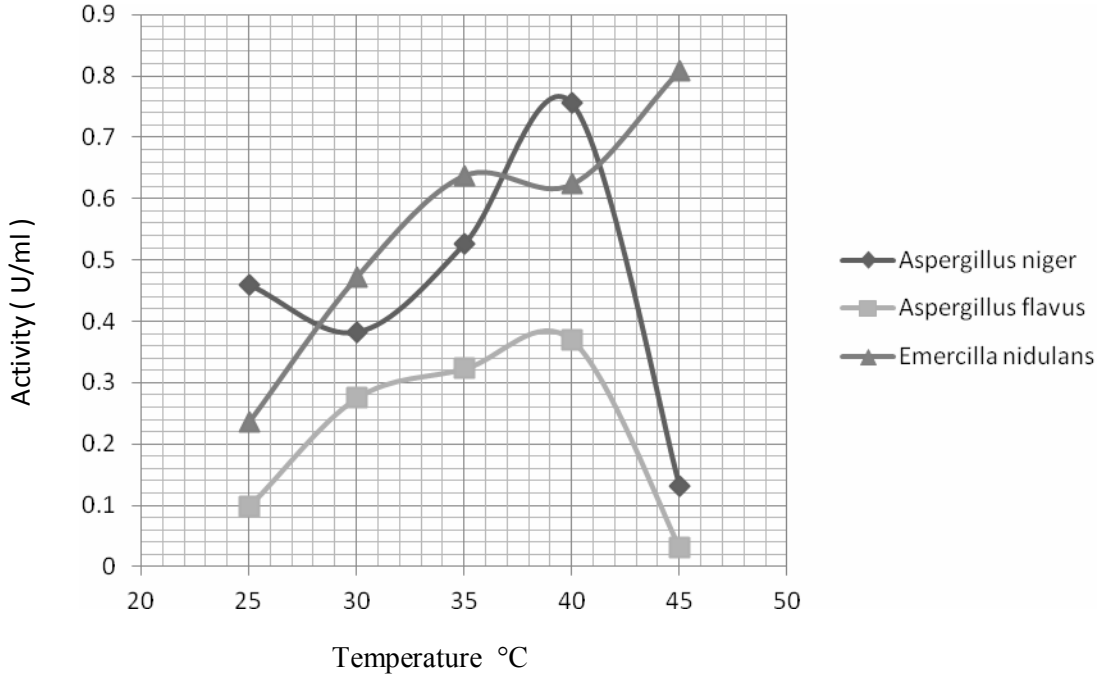

Fig. (4): Effect of incubation temperature on endo-gluconase activity by tested fungi (U\ml).

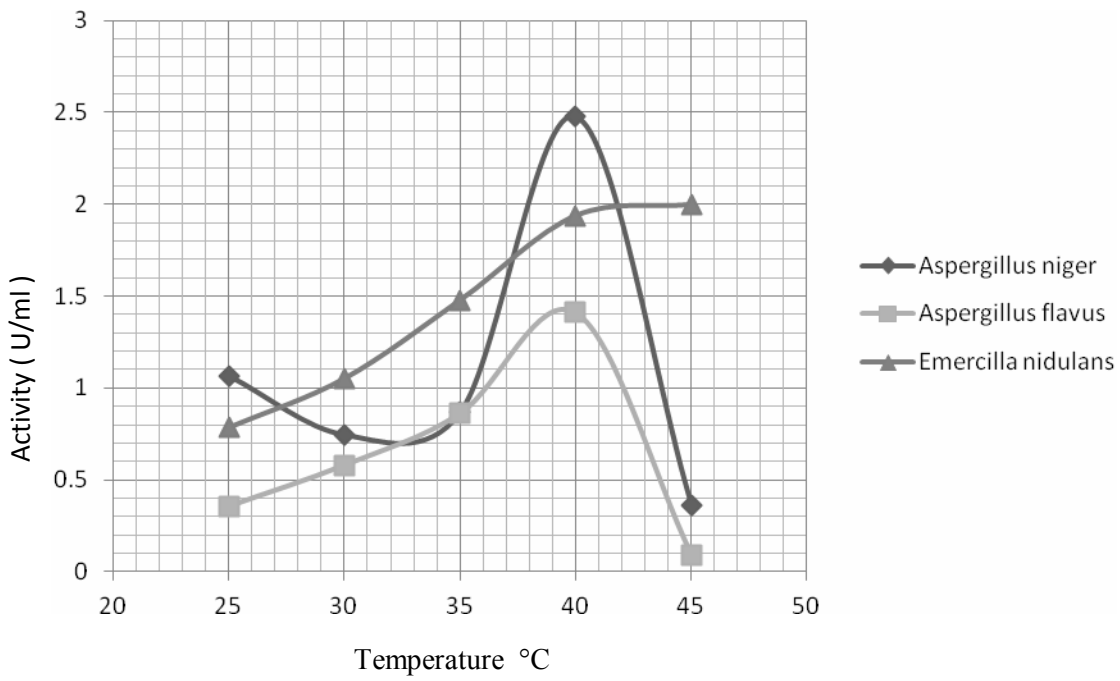

Fig. (5): Effect of incubation temperature on endo-gluconase specific activity by tested fungi (U\mg protein**).**

In conclusion, the optimum incubation temperature for the three fungal species recorded at 35° C. The activity of cellulases enzymes produced by the three fungal species was varied.

4. **Reducing *sugars:***

Data in Table (2) showed the amount of reducing sugars at different temperature degree (25, 30, 35, 40 and 45° C). For *A. niger*: the maximum amount of reducing sugars was 220.20 µg\ml at 35° C, while the minimum value was 90 µg\ml at 25° C. The maximum amount of reducing sugars produced from *E. nidulans* and *A. flavus* were 244.00 and 233.00 µg\ml at 40° C. Meanwhile, the minimum reducing sugars amount by *A. flavus* and *E. nidulans* was 27.47 and 101.00 µg\ml at 25 and 45° C, respectively.

5. Soluble protein:

The amount of soluble protein was shown in Table (3). The results illustrated that soluble protein content of A. *niger* was increased with the incubation temperature increasing until reached to the maximum value (0.607 mg\ml) at 35° C and turned to decrease another time. While each of *A. flavus* and *E. nidulans* had the maximum amount of soluble protein at 30° C it was 0.473 and 0.449 mg/ml, respectively.

Table (2): Effect of incubation temperature on the production of reducing sugars by tested fungi.

Fungal strain	Reducing sugars (µg\ml)				
	25 ºC	**30 ºC**	**35 ºC**	**40 ºC**	**45 ºC**
Aspergillus niger	90.00	203.07	220.20	198.67	218.47
Aspergillus flavus	27.47	102.82	102.43	233.00	88.67
Emericella nidulans	208.03	123.08	218.87	244.00	101.00

Table (3): Effect of incubation temperature on the production of soluble protein by tested fungus.

Fungal strain	Soluble protein (mg\ml)				
	25 ºC	**30 ºC**	**35 ºC**	**40 ºC**	**45 ºC**
Aspergillus niger	0.432	0.515	0.607	0.305	0.365
Aspergillus flavus	0.275	0.473	0.373	0.262	0.357
Emericella nidulans	0.302	0.449	0.431	0.322	0.405

Previous studies indicated that production of cellulases was dependent on fungal strain as well as cultivation conditions. Maximum enzyme production was recorded by *A. fumigatus* at 40° C on coir waste, and at the same time, 50° C was optimum for maximum cellulase production by *A. niger* on saw dust waste (Immanuel *et al,* 2007). On the other hand, Ali and Saad El-Dein (2008) obtained maximum cellulase production by *A. niger* and *A. nidulans* at 35 and 30° C, respectively. Also, Singh *et al* (2009) defined 30° C as the optimum temperatures for cellulase production by *A. heteromorphus.*

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تأثير درجة حرارة التحضين على انتاج انزيمات السليوليز الفطرية

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الملخص:

تهدف هذه الدراسة الى اختبار وتحديد درجات حرارة التحضين المثالية لإنتاج انزيمات السليوليز بواسطة فطريات *Aspergillus niger* AUMC 8485، *Aspergillus flavus* AUMC 8534 و *Emericella nidulans* AUMC 8581 .

وقد أوضحت النتائج المتحصل عليها أن درجة الحرارة المثلى لإنتاج انزيمات السليوليز كانت ٣٥ °م لكل الأنواع محل الدراسة والتي قدرت بطريقة الأطباق المصبوبة حيث كان قطر الهالة الشفافة هو ٢٢,٠ ، ٢٤,٥ ، ٢٦,٠مم لكل من *A. niger* ، *A. flavus* و *E. nidulans* على التوالى. بالنسبة لإنتاج انزيم exo-D-1,4-gluconase كان أعلى نشاط انزيمي له عند ٣٥ °م بواسطة كل السلالات الفطرية، أما بالنسبة لأعلى نشاط نوعي للإنزيم فقد أنتج عند ٤٠ °م بواسطة فطر *A. niger* وعند ٣٥ °م بواسطة كل من *A. flavus* و *E. nidulans*. في نفس الوقت سجلت درجة الحرارة المثلى لإنتاج انزيم endo-D-1,4-gluconase عند ٤٠ °م لكل من *A. niger* و *E. nidulans* وعند ٣٥ °م لفطر *A. flavus* . أعلى نشاط نوعي لانزيم endo-D-1,4-gluconase قدر على ٤٠ °م لكل السلالات محل الدراسة. أعلى إنتاجية من السكريات المختزلة في مرشح المزرعة كانت عند ٣٥ °م لفطر *A. niger* وعند ٤٠ °م لكل من *A. flavus* و *E. nidulans*. كذلك سجلت أعلى انتاجية من البروتين الذائب عند ٣٥ °م لفطر *A. niger* وكانت عند ٣٠ °م لكل من *A. flavus* و *E. nidulans*.