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Highly Production of Cellulases Enzymes under Submerged State Fermentation for Agricultural Wastes Composting

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ABSTRACT

The current study was designed to isolate and identify cellulose-degrading fungi from Egyptian soils rich in cellulosic compounds, then optimize their cellulose-degrading enzymes production. Among 146 microbial isolates grown on CMC agar medium, 9 isolates were found to be filamentous fungi. Only one isolate was selected as the highest cellulases producer according to the Congo red and cellulase activity assays. This isolate was identified by morphological and genetic characteristics as *Aspergillus niger* MK543209. After that, to optimize the activities of three cellulose-degrading enzymes (Exo-β-glucanase, Endo-β-1,4-glucanase, β-glucosidase) by this strain, the effect of nutritional and environmental factors was studied. Basal mineral salt medium amended with 1% rice straw was the best medium for cellulose-degrading enzymes production under submerged fermentation (SmF). Additionally, bagasse at 30% and urea at 2% were the best carbon and nitrogen sources, respectively. In respect of the environmental conditions, the optimum temperature, pH and incubation period were 45°C, 5.5 and 7 days, respectively.

Keywords: Cellulases, *Aspergillus niger,* SmF, optimization.

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INTRODUCTION

Cellulose is the most abundant agricultural waste and the huge renewable bioresource on earth. It is a long chain of glucose units linked by β-1,4 glucosidic linkages **(Hussain *et al*., 2017).** There are two methods for cellulose converting into glucose: chemical and enzymatic hydrolysis, cellulases were applied as an enzymatic method for this purpose and known as an eco-friendly process because it was accomplished without secondary polluted metabolites **(Sukumaran *et al*. 2005; Juturu and Wu, 2014; Hasanin et al., 2019)**, thus allowing the application of cellulases in various industries, such as pulp and paper industry, textile and bioethanol industry, wine and brewery industry, food industry, animal feed industry, agricultural and detergent industries **(Kuhad *et al*., 2011; Adrio and Demain, 2014; Hasanin et al., 2018)**, pharmaceutical industries and waste management **(Tarek *et al*., 2007).** Cellulolytic microorganisms that can degrade cellulose by producing cellulases enzymes, this cellulosic enzyme system consists of three major enzymes namely; exo-β-glucanases, endo-β-glucanases and β-glucosidase. Exo-glucanases are active on crystallin cellulose and cleave disaccharide units either from non-reducing or reducing end, while endoglucanases are active on the amorphous regions of cellulose and can also hydrolyze substituted celluloses, such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC) internally. Moreover, β-glucosidases can cleave cellobiose and other soluble oligosaccharides to glucose **(Mrudula and Murugammal, 2011)**. Although, cellulases are produced from a wide variety of microorganisms including bacteria, actinobacteria and yeasts, filamentous fungi are preferred for their commercial production, because these fungi produced cellulases with higher level than those obtained from yeast and bacteria **(Anita *et al*., 2009)**. Additionally, most species of genus *Aspergillus* can produce cellulases under different conditions, therefore this genus has the potential to dominate the enzyme industry and it is well known as an efficacious producer for cellulases **(Mrudula and Murugammal, 2011).** Microbiological production of cellulases may be performed by submerged fermentation (SmF) or solid-state fermentation (SSF) cultures **(Farinas, 2018)** using low-cost cellulosic materials as carbon source such as rice straw, saw dust or bagasse **(Buntić *et al*., 2019)**. In this trend, this study was designed to isolate and identify cellulose-degrading fungi from soils rich with cellulosic compounds, then optimize their cellulose-degrading enzymes production.

MATERIALS AND METHODS

Soil samples, enrichment and isolation of cellulose degrading fungi

Five soil samples were collected from agricultural wastes enriching cites. The samples were mixed with different cellulose materials viz. filter paper, sawdust, wheat straw, bagasse and corn stalks as separated treatment and then incubated at room temperature for 4 weeks under moisturizing conditions **(Cappuccino and Sherman, 1999)**.

Ten g of each soil sample was inoculated in 90 ml of carboxy methyl cellulose (CMC) liquid medium (HIMEDIA Co., Germany) containing (g/l): 10.0 CMC, 1.0 KH_2PO_4, 0.5 NaCl, 0.5 $MgSO_4.7H_2O$, 0.01 $MnSO_4.H_2O$, 0.01 $FeSO_4.7H_2O$, 0.3NH_4NO_3, pH 5.5, all flasks were incubated for 2 days at 30±2°C. Then, the serial dilution plating method was used for isolation of cellulolytic fungi. The plates were incubated at 30±2°C for 4 days. At the end of incubation period, all fungal colonies able to utilize cellulose as sole source of carbon were picked up and purified on the same agar medium, pH 5.5±0.2 and maintained at 4°C for further experiments **(Darwesh et al., 2014)**.

Screening for cellulase production

Congo red assay

Each fungal isolate (1 cm agar disc) was cultured on carboxymethyl cellulose agar medium **(Ariffin et al., 2006)**, and incubated for 4 days at 30±2°C. At the end of incubation period, all dishes were flooded with an aqueous solution of Congo red (1% w/v) for 15 min., then Congo red solution was poured off, and the dishes were further treated by flooding with 1M NaCl for 15 min. The formation of a clear zone indicated for cellulose degradation.

Cellulase activity assay (CMCase)

To confirm cellulose-degrading potential of the selected fungal isolates, each fungal isolate was grown in 150 ml Erlenmeyer flask contained 50 ml of basal salt medium amended with CMC as sole carbon source.

The pH of the medium was adjusted to 6.5. Each flask was inoculated with agar discs of 7 days old culture from PDA plates and incubated at 30±2°C for 4 days. The crude enzyme was filtered and centrifuged at 11000 xg for 10 min. The enzyme activity was estimated as the method described by **Miller (1959):**
The reaction mixture containing 0.2 ml of crude enzyme solution plus 1.8 ml of 1% carboxymethyl cellulose (CMC) in 100 mM sodium phosphate buffer (pH 3.5). The reaction mixture was incubated at 50°C for 25 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and placing the reagent tubes in water bath at 100°C for 15 min for color development. The OD was measured at 575 nm and the concentration of reducing sugars was determined and calculated as glucose.

Identification of the potent fungal isolate

The selected cellulolytic fungus was identified according to its morphological characteristics and molecular biology techniques. The morphological characteristics were examined using light microscope (Olympus cx41) after 4 days of growth on PDA agar plates. For molecular identification, fungal mycelium from a 3 days old culture in potatoes dextrose broth (PDB) was harvested using Whatman No. 1 filter paper. The total genomic DNA was extracted using CTAB protocol **(Eida *et al*., 2018)**. DNA of the fungal isolate was amplified by polymerase chain reaction (PCR) using ITS1 (5´- TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC - 3´) designed for sequencing (**Elshahawy *et al*., 2018**).

The identification was achieved by comparing the contiguous DNA sequence with data from the reference and type strains available in public databases GenBank using the BLAST program (National Centre for Biotechnology Information) (http://www.ncbi.nlm.nih.Gov /BLAST). The obtained sequences were aligned using Jukes Cantor Model and the isolate was registered in Gen Bank **(Barakat et al., 2017)**.

Cellulose degrading enzymes assay
Exo-β-glucanase activity (EC 3.2.1.91)

It also called Filter paper activity (FPase) and used for total cellulase activity in the culture filtrate according to the standard method **(Hankin and Anagnostakis, 1975)**. Aliquots of appropriately diluted cultured filtrate as enzyme source was added to Whatman no. 1 filter paper strip (1 x 6 cm, 50 mg) immersed in one milliliter of 0.05 M sodium citrate buffer of pH 4.8. After incubation at 50±2°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method **(Miller, 1959)**. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µ mole of glucose from one ml of crude enzyme per ml per min.

Endo-β-1,4-glucanase activity (EC 3.2.1.4)

It also called carboxymethyl cellulase (CMCase), it was measured according the method by **Miller (1959)** as previously mentioned.

β-glucosidase activity (Cellobiase) (EC 3.2.1.21)

Cellobiase was assayed by the method of **Petterson (1975)**: 0.5 ml of crude enzyme was added to 1.0 ml of 0.4% cellobiose dissolved in 0.05 *M* citrate-phosphate buffer (pH 4.8). The reaction mixture was incubated at 50°C for 30 min. Then, the released glucose was estimated by dinitrosalicylic acid (DNS) method **(Miller, 1959)** as abovementioned.

Optimization of SmF conditions for maximum cellulase production by *Aspergillus niger*
Effect of different media

Six media were used for screening the highest production of cellulose degrading enzymes by *A. niger*, the following used media were adjusted to pH 6:

M1: Cellulase production medium (Camassola and Dillon, 2007).
M2: Basal mineral salt medium amended with 1% rice straw (Chen and Wayman, 1991).
M3: Cellulose broth (Bagga *et al*., 1990).
M4: Czapek-Dox liquid medium amended with 1% rice straw (Coral *et al*., 2002).
M5: Reese and Mandels basal medium (Reese and Mandels, 1963).
M6: Carboxymethyl cellulose (CMC) medium (Ariffin *et al*., 2006).

After that, determination of reducing sugars (glucose) was carried out using 3,5 dinitrosalicylic acid method (Miller *et al*., 1959) as mentioned above.

Effect of carbon source and concentration

The carbon source of the suitable medium was replaced with various carbon wastes source such as sawdust, bagasse, wheat straw and rise straw to estimate the best carbon source at 10% and the medium pH was adjusted to 6. All flasks were inoculated with standard inoculum of *A. niger* and incubated at 30±2°C for 4days. Then, the suitable carbon source (Bagasse) was applied at different concentrations ranged from 15, 20, 25, 30, 35, 40, 45, to 50% to estimate the optimum concentration for maximum enzyme productions. The culture supernatant was used for determination of enzyme activity as previously mentioned.

Table 1. Chemical analysis of carbon source materials used in the experiment.

Parameters	Organic carbon (%)	Total N (%)	C/N ratio
Sawdust	36 c	0.39	92.3
Bagasse	53 c	0.65	81.5
Rice straw	43 c	0.56	76.8
Wheat straw	40 c	0.45	88.9

Effect of nitrogen source and concentration

The suitable medium containing bagasse at 30% and pH was adjusted to 7.0 was prepared by replacing native nitrogen with different organic and mineral nitrogen sources *viz.* yeast extract, beef extract, urea, ammonium chloride and ammonium molybdate. The abovementioned nitrogen sources were calculated to give equal final nitrogen concentration, irrespective of the chemical constitution. The flasks were sterilized and inoculated with standard inoculum of *A. niger* and incubated at 30±2°C for 4days. Cellulase activity was determined. After that, the most suitable nitrogen source (urea) was applied at different concentrations namely 1.0, 1.5, 2.0, 2.5, to 3.0% were added to the optimized medium. After that the culture supernatant was used for determination of enzyme activity as previously mentioned.

Effect of incubation temperature

The suitable medium was sterilized and inoculated with standard inoculum of *A. niger*, and incubated at different temperatures from 20, 25, 30, 35, 40, 45, and 50°C for 4 days. Then, at the end of incubation period, the cell free culture filtrate was used as enzyme source as previously mentioned.

Effect of pH

The most suitable medium for cellulase production was adjusted to different pH values from 5.0 to 8.0 with interval 1.0. The flasks were sterilized and inoculated with standard inoculum of *A. niger* and incubated at 45°C for4 days. Cellulase activity was determined to investigate the most suitable initial pH for maximum cellulase production. The culture supernatant was used for determination of enzyme activity as previously mentioned.

Effect of incubation period

The optimized medium was sterilized and inoculated with standard inoculum of *A. niger*. The optimized factors already concluded were considered. Fermentation process was performed for different periods (4, 5, 6, 7, and 8 day) at 45°C. The culture supernatant was used for determination of enzyme activity as previously mentioned.

RESULTS AND DISSCUSSION

Isolation and screening of microorganisms

Among 146 microbial isolates from different soil samples, nine of them were morphologically classified as fungal isolates, this due to that soil contains an assortment of cellulolytic microorganisms including bacteria, fungi and actinobacteria **(Hussain *et al.*, 2017)**. Also, it was clear that the production of cellulase has been reported in a wide variety of bacteria **(Immanual *et al.*, 2006)** and fungi **(Anita *et al.*, 2009 and Sarao *et al.*, 2010)**. However, filamentous fungi are celebrated for commercial production of cellulases due to their capability to produce highest amounts of these enzymes compared to those obtained from yeast and bacteria **(Mrudula and Murugammal, 2011)**.

The isolated fungi were screened for their cellulose decomposition activity using two assays, Congo red and cellulase activity assays. Results illustrated by Fig. 1(a) indicated that all nine isolates were able to produce cellulase on Congo red plates which estimated by clear zone diameter (cm) that ranged from 2.3 to 7.0 cm. Moreover, data in Fig. 1(b) graphically illustrated that all nine isolates were able to produce cellulase

which ranged from 1.13 to 3.72 (u/ml). Generally, the largest clear zone diameter and the highest cellulase activity were recorded by the isolate No. CF2 (Fig. 2), so, this isolate was selected for identification. Similar results were demonstrated by **Mrudula and Murugammal (2011)** who selected the cellulase producing fungal colony that showed largest halo forming zone when all plates were flooding with Congo red (0.1% w/v), followed by de-staining with NaCl (0.1 M).

Cellulases are produced by a wide number of microorganisms including fungi and bacteria during their growth on cellulosic compounds **(Buntić *et al*., 2019)**. Fungal cellulases (carboxy methyl cellulase, cellobiose hydrolases and β-glucosidas) have different specificity and mode of actions compared to bacterial cellulases **(Quintanilla *et al*. 2015; Liu and Kokare 2017)**.

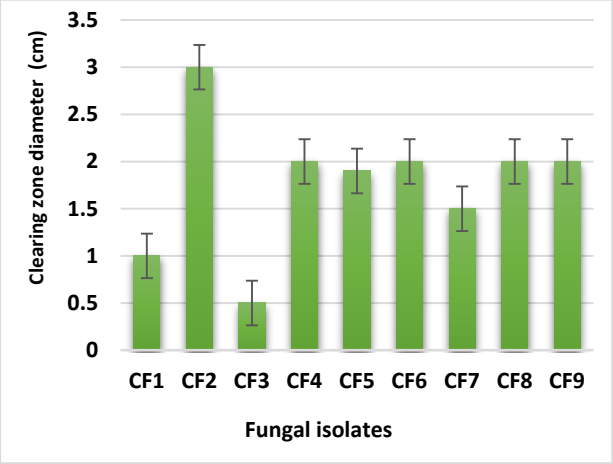

Fig. 1a. Congo red assay

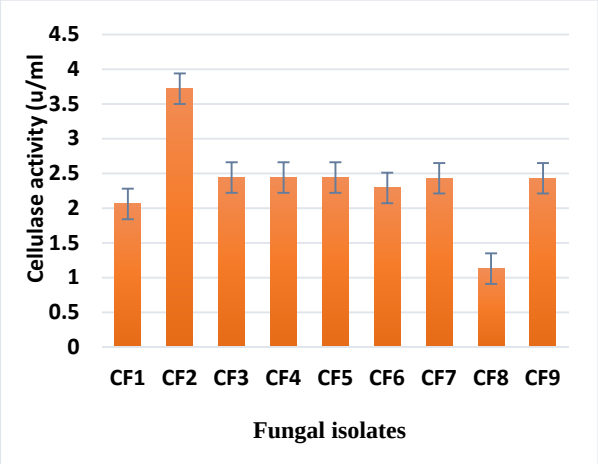

Fig. 1b. Cellulase activity assay

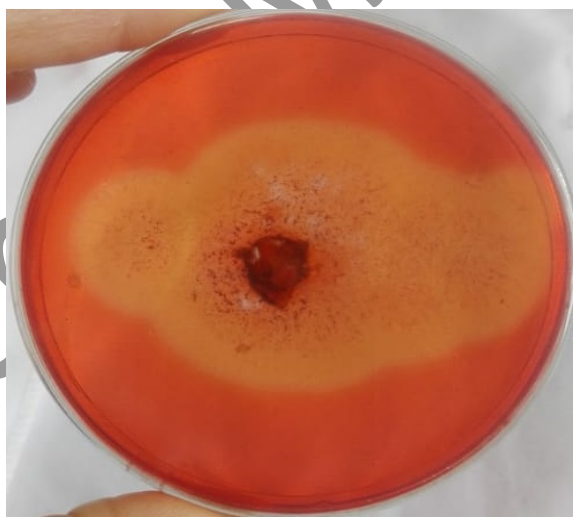

Fig. 2. Clear zone on CMC by isolate No. CF2

Identification of the more potent fungal isolate (CF2)

The isolate number (CF2) that show the highest cellulase activity was selected as the more potent isolate and analysed for its 18S rRNA gene. The genomic DNA of tested strains was isolated and purified using isopropyl method. The 18S rRNA gene was amplified by PCR technology using forward and reverse primers and the PCR product was purified. The size of PCR product was approximately 1000 bp. After sequencing for 18S rRNA gene, the obtained sequences were compared with sequences available in GenBank using BLAST program (NCBI web page) and the similarity percentage of this isolate accounted for 99.85% % with strain *Aspergillus niger*. The sequences of this strain were submitted to GenBank and recorded under accession

number MK543209. The phylogenetic relationship showed that this strain very close to the type strains of *Aspergillus* genera (Fig. 3) deposited in culture collection centre of National Centre for Biotechnology Information. So, this strain was *Aspergillus niger* CF2.

Similar results were conducted by **Devanathan *et al*. (2007)** who isolated cellulose-degrading fungi and based on the colony morphology and microscopic observation the strain was identified as *Aspergillus niger*. Moreover, **Mrudula and Murugammal (2011)** demonstrated that almost all fungi of genus *Aspergillus* synthesize cellulase, therefore this genus has the potency to predominate the enzyme industry. Also, they reported that *Aspergillus* spp. and *Trichoderma* spp. are well known as efficient producers for cellulases.

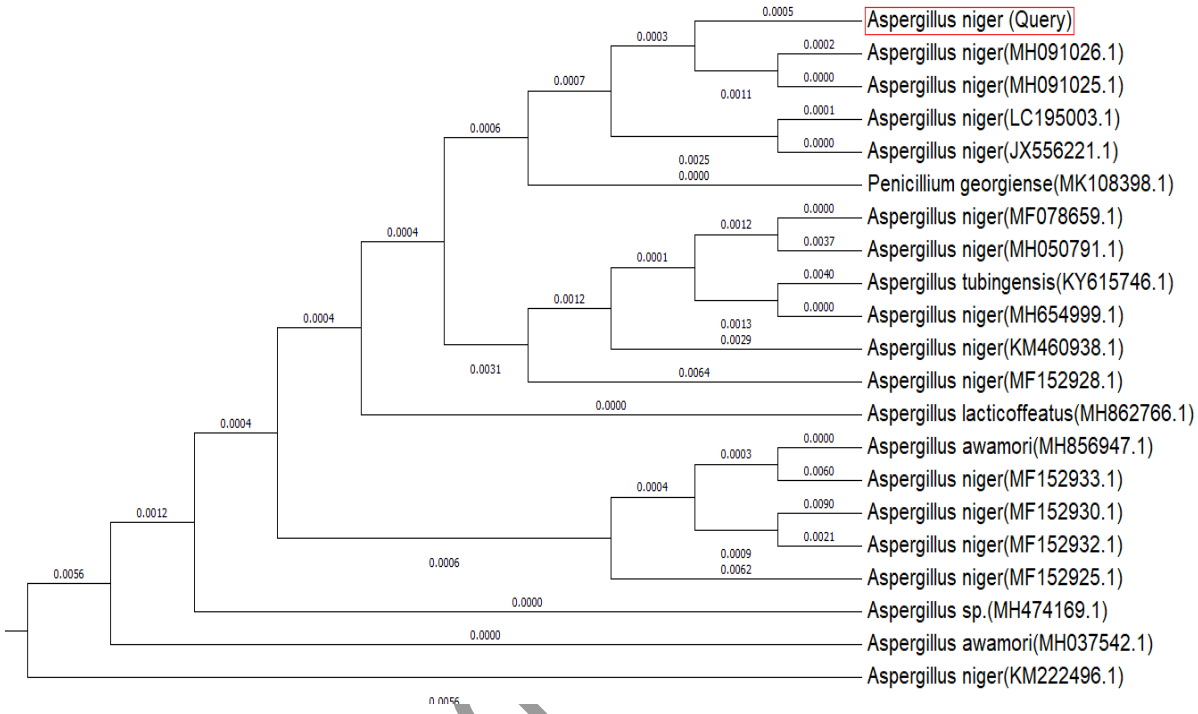

Fig. 3. The phylogenetic tree of *Asperigillus niger*

Optimization of submerged fermentation conditions for maximum cellulase production by *Aspergillus niger* MK543209

Effect of different fermentation media

From data presented in **Table (2)** it was clearly that *A. niger* MK543209 gave cellulose degradation activity using three enzymes assays namely, CMCase, FPase and cellobiase in different used media. CMCase activity was ranged from 1.66 to 4.36 (u/ml), the highest and the lowest activity was recorded using basal mineral salt medium containing 1% cellulose (M2) and Czapek-Dox liquid medium (M4), respectively. Same trend of results was recorded in case of FPase and cellobiase activities which showed that *A. niger* MK543209 gave highest activities when cultured in M2 and lowest activity when cultured in M4. On the other hand, Reese and Mandels basal medium (M5) gave higher CMCase and cellobiase activities than cellulase production medium (M1) while, reverse results were recorded with FPase activity in the same media. So, the basal mineral salt medium containing 1% cellulose (M2) was chosen for the following experiments. In contrast, **Salem (2010)** used five different media to study the Effect of different fermentation media on cellulases activities by *trichoderma viride*, results showed Reese and Mandels basal medium gave higher cellulases activities. On the other hand **Sohail *et al* (2009)** reported A. nigerMS82 was grown in basal medium supplemented with 1% (w/v) of CMC, the production of endoglucanase and β-glucosidase reaches its maximum

Table 2. Effect of different fermentation media on cellulose degrading enzymes activity by *A. niger* MK543209.

Media	CMCase activity (u/ml)	FPase activity (u/ml)	Cellobiase activity (u/ml)
M1	3.01	2.69	1.07
M2	4.36	2.69	2.17
M3	2.42	1.96	0.99
M4	1.66	1.02	0.80
M5	3.68	2.53	1.59
M6	2.07	1.66	0.86

Effect of different carbon sources

Results illustrated in Table (3) proved that *A. niger* MK543209 able to use different raw materials as a sole carbon source. Also, data indicated that the CMCase, FPase and cellobiase activities recorded highest values when bagasse was applied in medium as a sole carbon source followed by sawdust. On the other hand, the lowest value of CMCase activity was recorded when wheat straw was applied while the lowest FaseP and cellobiase activities were recorded when rice straw was applied as sole carbon sources. Moreover, data showed that the enzymes activities were ranged from 1.67-2.57, 1.44-2.48 and 0.99-1.75 u/ml for CMCase, FPase and cellobiase, respectively. So, bagasse was applied as a sole carbon source in the following experiments. In this trend, Mrudula and Murugammal (2011) reported that among the 5 substrates screened, coir waste gave the maximum cellulase production when fermented with *Aspergillus niger* under SmF, also, considerable amounts of enzyme were recorded on wheat bran and rice bran.

Similarly, Ojumu *et al*. (2003) reported that saw dust, corncobs and bagasse were found to be the best substrates for cellulase production by *Aspergillus flavus*. On the other hand, Oberoi *et al*. (2008) reported kinnow pulp as the best substrate for cellulase production the same fungus in SSF. Additionally, sugarcane bagasse, wheat bran and rice bran have been employed for production of cellulase using a variety of microorganisms such as *Trichoderma*, *Aspergillus*, *Penicillium* and *Botrytis* (Pandey *et al*., 1999).

Table 3. Effect of the different carbon sources on cellulose degrading enzymes activity by *A. niger* MK543209.

Carbon sources at 10%	CMCase activity (u/ml)	FPase activity (u/ml)	Cellobiase activity (u/ml)
Sawdust	2.06	1.98	1.46
Bagasse	2.57	2.48	1.75
Rice straw	1.82	1.44	0.99
Wheat straw	1.67	1.54	1.14

Effect of different bagasse concentrations

As graphically illustrated by Fig. (4), *A. niger* MK543209 was able to use bagasse at different concentrations (10, 15, 20, 25, 30, 35, 40, 45 and 50%) and gave variable activities of CMCase, FPase and cellobiase. It was clear that the activities of the three estimated enzymes was gradually increased from 10% to reach their maximum at 30% then decreased gradually. The lowest activities were recorded when bagasse was applied at 50%. Additionally, CMCase activity was ranged from 1.78-8.92 while, FPase and cellobiase activities were ranged from 1.07-4.81 and 0.81-3.64 u/ml, respectively.

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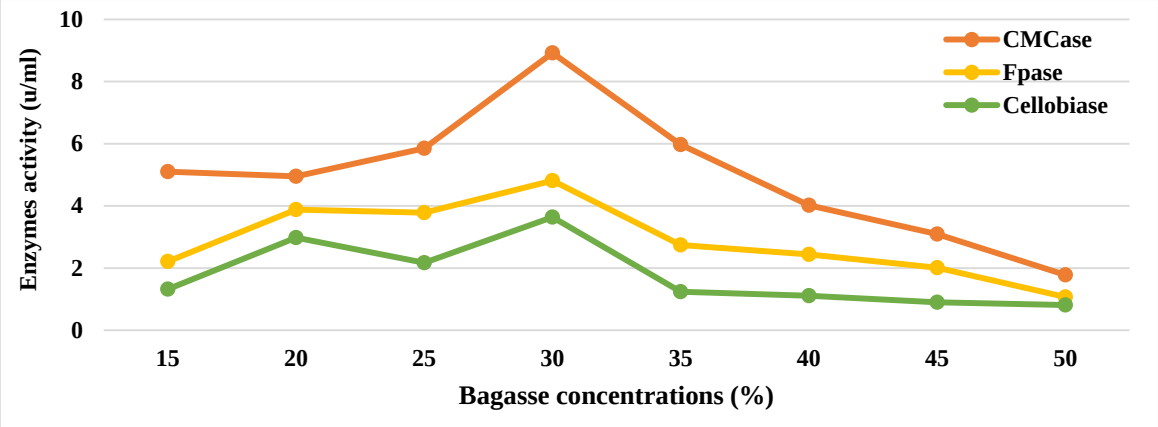

Fig. 4. Effect of bagasse concentrations on cellulose degrading enzymes activity by *A. niger* MK543209.

Effect of different nitrogen sources

Data presented in Table (4) indicated that all nitrogen sources enhanced the production of cellulose-degrading enzymes by *A. niger* MK543209 compared to control. Also, data showed that the lowest values of the three estimated enzymes were recorded when *A. niger* cultured on medium containing ammonium molybdate. Whereas, the highest values were recorded when *A. niger* cultured on medium containing urea followed by ammonium chloride. So, urea was applied for the following experiments. In contrast, Gilna and Khaleel (2011) reported that among nitrogen sources (peptone, urea, yeast extract and sodium nitrate), yeast extract was the best to enhance the enzyme activity FPase and CMCase by *Aspergillus fumigatus*.

Different results were recorded by other researches such as Mrudula and Murugammal (2011) who reported that among different nitrogen sources, peptone supported maximum cellulase production followed by beef extract, yeast extract and casein. These results agreed with reports of Kathiresan and Manivannan (2006) and Devanathan *et al*. (2007) for production of cellulase by *Penicillium fellutanum* and *Aspergillus niger*, respectively in submerged fermentation.

Table 4. Effect of the different nitrogen source on cellulose degrading enzymes activity by *A. niger* MK543209.

Nitrogen sources	CMCase activity (u/ml)	FPase activity (u/ml)	Cellobiase activity (u/ml)
Urea	9.12	4.98	3.39
Yeast extract	6.13	4.26	2.19
Beef extract	6.09	4.07	2.12
Ammonium chloride	6.60	4.42	2.78
Ammonium molybdate	5.74	3.89	1.05

Effect of different urea concentrations

From results illustrated by Fig. (5), it was clear that *A. niger* MK543209 able to degrade cellulose at different concentrations of urea. Moreover, the activities of all estimated enzymes were gradually increased from 1.0% to reach their maximum values at 2.0% and then gradually decreased. Data indicated that *A. niger* CF2 recorded CMCase, FPase and cellobiase activities at 4.40-9.46, 2.58-5.03 and 1.46-3.80 u/ml, respectively. So, urea at 2.0% was chosen as the optimum concentration for the following experiments.

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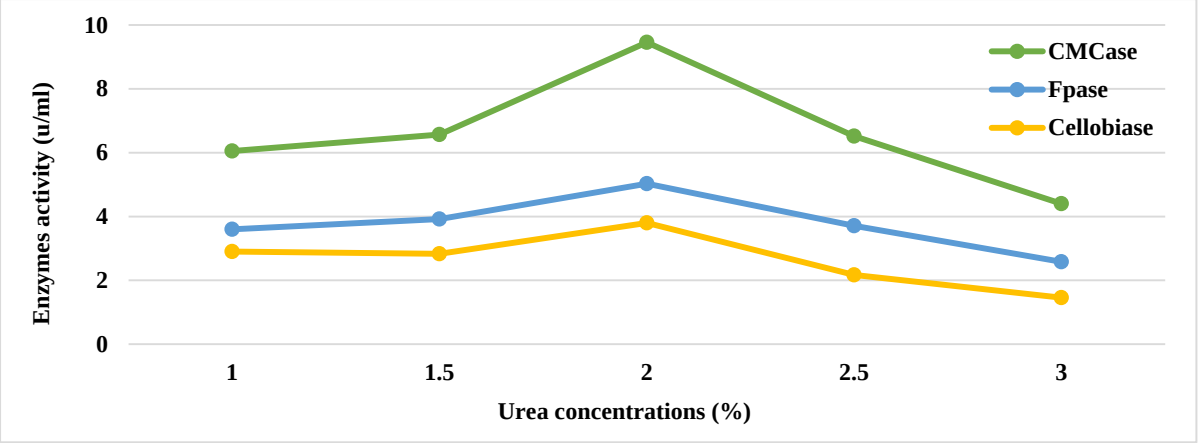

Fig. 5. Effect of urea concentrations on cellulose degrading enzymes activity by *A. niger* MK543209.

Effect of different pH values

The pH value of microbial growing media is considered the one of the most important factor for microbial growing as well as enzymes production (Ray and Behera, 2017). This is not means the optimum growth pH value is considered the optimum degree for enzymes production (Hasanin et al., 2019).

In this study, five different pH values ranged between 5and 7g/L. High enzyme production was obtained at pH 5.5, Data presented in Table (9) and fig.(8). Showed the enzymes activity was reached to 11.1, 5.70, and 4.52 U/ml for endoglucanase, exoglucanase and β-glucosidase respectively, so, pH value 5.5 was applied for the following experiments. . Similar results were presented by Xu et al. (2006), who estimated the cellulase production by Aspergillus glaucus XC9 at different pH values, results showed that cellulase production were highest at pH 5.5. In contrast, Gilna and Khaleel (2011), who estimated the cellulase production by Aspergillus fumigatus at different pH values ranged between 5.0 and 9.0, results showed that FPase and CMCase activities were highest at pH 6.5. Different result was recorded by Sohail et al. (2009) who reported that among different pH values, pH 4.0 supported maximum β-glucosidase and CMCase activities under submerged conditions. pH of the growth medium plays an important role in enzyme secretion by microorganisms. Changes in pH value which observed during the growth of microorganisms also effects enzymes stability in the medium (Gupta et al., 2015).

Table 6. Effect of the different pH values on cellulose degrading enzymes activity by *A. niger* MK543209.

pH values	CMCase activity (u/ml)	FPase activity (u/ml)	Cellobiase activity (u/ml)
5.0	6.50	4.27	3.74
5.5	11.1	5.70	4.52
6.0	7.82	4.87	2.85
6.5	6.15	3.16	1.89
7.0	2.75	2.29	1.49

Effect of different incubation temperature

Incubation temperature plays an important role in the metabolic activities of all microorganisms. In this experiment, results in Table (5) showed that *A. niger* MK543209 was able to degrade cellulose under wide range of incubation temperatures 20-50°C. The highest activities of the three estimated enzymes were recorded when the fungal strain was incubated at 45°C with values of 12.6, 6.30 and 4.35 u/ml for CMCase, FPase and cellobiase, respectively. Also, data demonstrated that the lowest CMCase activity was recorded on 20°C while the lowest activities of FPase and cellobiase were recorded at 50°C. Generally, the activities of the three estimated enzymes were gradually increased till reached to their maximum at 45°C then decreased sharply. So, the optimum temperature for incubation 45°C was applied in the following experiments. In contrast, Gilna and Khaleel (2011) tested the effect of various incubation temperatures (25, 30, 32, 35 and 37°C) on CMCase, FPase activities by *Aspergillus fumigatus*, results showed that the optimum temperature was found to be 32°C.

Table 5. Effect of the different incubation temperature on cellulose degrading enzymes activity by *A. niger* MK543209.

Incubation temperature (°C)	CMCase activity (u/ml)	FPase activity (u/ml)	Cellobiase activity (u/ml)
20	4.17	3.51	2.39
25	7.22	3.67	2.51
30	7.80	3.72	2.55
35	7.84	5.78	2.72
40	8.06	5.47	2.24
45	12.6	6.30	4.83
50	4.55	2.86	1.24

Effect of different incubation period

Clearly, the incubation period is directly related with the enzymes production by microorganisms. In this trend, data in Table (7) proved that *A. niger* MK543209 gave the highest CMCase activity after 7 days of incubation while the highest activities of FPase and cellobiase were recorded after 4 days of incubation. Also, data indicated that CMCase activity was increased gradually till reached the maximum after 7 then decreased sharply. On the other hand, FP and cellobiase activities were hesitating because they decrease after 4 days then increased after 7 days then decreased again. Generally, 7 days was best incubation period and applied in the following experiments. This may be due to the depletion of nutrients in cultural medium which affect the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Kang *et al*., 2004). On the other hand, Gautam *et al*. (2011) who estimated the best incubation period for highest cellulase activity by *Aspergillus niger*, and found that 4th days was the optimum FPase and cellobiase incubation period.

Table 7. Effect of the different incubation period on cellulose degrading enzymes activity by *A. niger* MK543209.

Incubation period (days)	CMCase activity (u/ml)	FPase activity (u/ml)	Cellobiase activity (u/ml)
4	8.34	7.22	5.44
5	6.70	1.61	1.16
6	7.84	1.18	0.97
7	14.8	2.72	2.04
8	2.12	1.68	1.55

Conclusion

From the previous results it can be concluded that the Egyptian soils are rich in cellulose-degrading microorganisms especially filamentous fungi such as *Aspergillus* species. Nine isolates were morphologically classified as fungi and screened for their cellulose-degrading ability by Cngo-red and cellulase activity assays. Among them only one isolate which highly cellulose degrading fungus was identified according morphological and genetic characteristics as *Aspergillus niger* MK543209. The nutritional and environmental conditions for this strain was optimized for highest CMCase, FPase and cellobiase activities, and found to be Basal mineral salt medium amended with 1% rice straw was the best medium. Additionally, bagasse at 30% and urea at 2% were the best carbon and nitrogen sources, respectively. In respect of the environmental conditions, the optimum temperature, pH and incubation period were 45°C, 5.5 and 7 days, respectively.

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