Fuel 262 (2020) 116518

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Full Length Article

Improvement of paper wastes conversion to bioethanol using novel cellulose degrading fungal isolate

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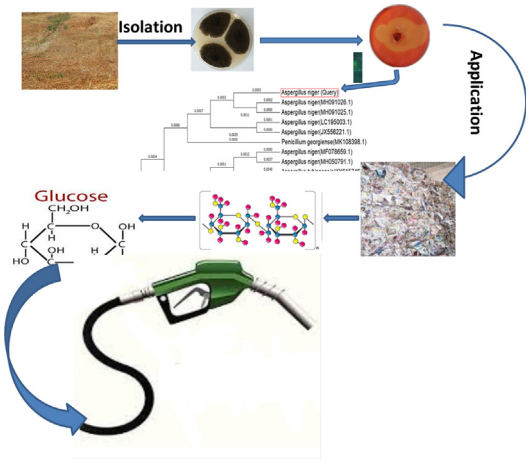
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Cellulases enzymes
Aspergillus niger MK543209
SmF
Bioethanol
Paper wastes

ABSTRACT

The current study was designed to isolate and identify superior cellulose-degrading fungi from Egyptian soils reached with cellulosic compounds, then production of bioethanol from paper wastes using this isolate. Among 9 fungal isolates, one isolate was selected as the highest cellulases producer. This isolate was identified by morphological and genetic characteristics as *Aspergillus niger* MK543209. 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 to the environmental conditions, the optimum temperature, pH and incubation period were 45 °C, 5.5 and 4 days, respectively. The optimized conditions were applied to produce reducing sugars from paper wastes, followed by production of bioethanol as a renewable energy. A 0.9 g/l/h of ethanol were produced as the best value. This study may be considered the initial point for production of renewable energy and protection of environment from wastes.

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https://doi.org/10.1016/j.fuel.2019.116518
Received 17 September 2019; Received in revised form 23 October 2019; Accepted 25 October 2019

1. 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 [1,2]. There are two methods for cellulose conversion 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 is accomplished without secondary polluted metabolites [3–5]. Thus, cellulases enzymes are allowed for application in various industries; pulp and paper industry, textile and bioethanol industries, wine and brewery industry, food industries, animal feed industry, agricultural and detergent industries [6–9], pharmaceutical industries and waste management [10,11]. Cellulolytic microorganisms that can degrade cellulose by producing cellulases enzymes, this process consists of three major enzymes system 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 [12]. Cellulases are produced by a wide variety of microorganisms including bacteria, actinobacteria and yeasts. Also, filamentous fungi are preferred for their commercial production, because their production with higher level than yeasts and bacteria [13]. 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 [12]. Microbiological production of cellulases may be performed by submerged fermentation (SmF) or solid-state fermentation (SSF) cultures [14] using low-cost cellulosic materials as carbon source such as rice straw, saw dust or bagasse [15]. 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 to apply it in biofuel production from paper wastes.

2. Materials and methods

2.1. 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 humidity conditions [16]. Ten grams of each soil sample were inoculated in 90 ml of modified carboxy methyl cellulose (CMC) liquid medium containing (g/l): 20.0 crushed rice straw, 1.0 KH_2PO_4, 0.5 NaCl, 0.5 $MgSO_4{\cdot}7H_2O$, 0.01 $MnSO_4{\cdot}H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, $0.3NH_4NO_3$, pH 5.5, all flasks were incubated for 7 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 [17].

2.2. Screening for cellulases production

2.2.1. Congo red assay

Each fungal isolate (1 cm agar disc) was cultivated on carboxymethyl cellulose agar medium, 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. Subsequently, the dishes were further treated by flooding with 1 M NaCl for 15 min. The formation of a clear zone was the indicator for cellulose degradation [18].

2.2.2. CMCase activity assay

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 (10 g/l) 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 5 days. The crude enzyme was filtered and centrifuged at 11,000 × *g* for 10 min. The enzyme activity was estimated as the method described by Miller [19], in brief, the reaction mixtures were contained 0.2 ml of crude enzyme solution plus 1.8 ml of 1% carboxymethyl cellulose (CMC) in 100 mM sodium phosphate buffer (pH 5.5). Then the reaction was incubated at 50 °C in water bath 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 using standard curve.

2.3. 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 3 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 [20]. DNA of the fungal isolate was amplified by polymerase chain reaction (PCR) using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCC TCCGCTTATTGATATGC-3′) designed for sequencing [21].

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 [22,23].

2.4. Cellulose degrading enzymes assay

2.4.1. Exo-β-glucanase activity

It is also called Filter paper activity (FPase) and used for total cellulase activity in the culture filtrate according to the standard method described by Hankin and Anagnostakis [24]. Aliquots of appropriately diluted cultured filtrate as enzyme source was added to Whatman no. 1 filter paper strip (1 × 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 [19]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μ mole of glucose by one ml of crude enzyme per ml per min.

2.4.2. Endo-β-1,4-glucanase activity

It also called carboxymethyl cellulase (CMCase), it was measured according to the method detailed by Miller [19] as previously mentioned.

2.4.3. β-glucosidase activity (Cellobiase)

Cellobiase was assayed by the method of Petterson [25]; 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.

Table 1
Chemical analysis of agricultural wastes as carbon source.

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

2.5. Optimization of SmF conditions for maximum cellulases production by *A. niger*

2.5.1. Optimization of the best medium

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; Cellulase production medium (M1) [26]; Basal mineral salt medium amended with 1% rice straw (M2) [27]; Cellulose broth (M3) [28]; Czapek-Dox liquid medium amended with 1% rice straw (M4) [29]; Reese and Mandels basal medium (M5) [30]; Carboxymethyl cellulose (CMC) medium (M6) [18]. After cultivation of *A. niger* into all tested media, the determination of reducing sugars in cell free supernatant was carried out using 3,5 dinitrosalicylic acid method.

2.5.2. Effect of carbon source and concentration

The carbon source of the suitable medium was replaced with various wastes as carbon source; sawdust, bagasse, wheat straw and rice straw to estimate the best carbon source at 10% and the medium pH was adjusted to 6. The chemical composition of the tested wastes is illustrated in Table 1. All flasks were inoculated with standard inoculum of *A. niger* and incubated at 30 ± 2 °C for 5 days. In case of carbon concentration of the best one, Bagasse was applied at different concentrations (10, 15, 20, 25, 30, 35, 40, 45, 50%) to estimate the optimum concentration for maximum enzymes production. The culture supernatant was used for determination of enzyme activity as previously mentioned.

2.5.3. Effect of nitrogen source and concentration

The suitable medium containing bagasse at 30% with pH 6.0 was prepared by replacing native nitrogen with different organic and mineral nitrogen sources *viz.* yeast extract, beef extract, urea, ammonium chloride and sodium nitrate. The above mentioned nitrogen sources were calculated to give equal final nitrogen concentration, irrespective of the chemical constitution [31]. The sterilized media were inoculated with standard inoculum of *A. niger* and incubated at 30 ± 2 °C for 5 days. Cellulases activity was assessed. After that, the most suitable nitrogen source (urea) was applied at different concentrations; 1.0, 1.5, 2.0, 2.5 and 3.0%. Also, the enzymes activity was determined and the best concentration was calculated.

2.5.4. Effect of incubation temperature

The cultures of *A. niger* were incubated at different temperatures; 20, 25, 30, 35, 40, 45 and 50 °C for 5 days. After the end of incubation period, the cell free culture filtrate was used as enzymes source.

2.5.5. Effect of initial pH value

The initial pH value is crucial factor for cellulases production. To manage this factor, the medium pH was adjusted initially at different pH values from 5.0 to 7.0 with interval 0.5°. The media were sterilized and inoculated with standard inoculum of *A. niger* and incubated at 45 °C for 5 days. Cellulases activity was determined to investigate the most suitable initial pH for maximum cellulases production [32].

2.5.6. Effect of incubation period

The optimized medium was sterilized and inoculated with standard inoculum of *A. niger*. Fermentation process was performed for different periods (4, 5, 6, 7, and 8 day) at 45 °C and pH 5.5. The culture supernatant was used for determination of enzymes activity [33].

2.6. Converting of paper wastes to bioethanol

Paper wastes were collected and used as a source of bioethanol. The sterilized wastes were inoculated by 4 days old *A. niger* (growing into optimized cultural conditions). The fermentation moisture was adjusted and saved to 30% using synthetic medium (K_2HPO_4, 1 g/l; KH_2PO_4, 0.5 g/l; NaCl, 0.1 g/l; yeast extract, 0.2 g/l) and incubated at 45 °C. The mixture was remixed every 6 h under septic conditions. Samples for FPase assay, CMCase assay, cellulose amount and reducing sugars production were collected every 6 h. The cellulose amounts were determined by converting it to reducing sugars [34]. Reducing sugars products were measured according to the method detailed by Miller [19]. The final products are used to produce bioethanol as biofuel.

3. Results and discussion

3.1. Isolation and screening of microorganisms

Enrichment technology was applied in this step using deferent cellulosic wastes to increase the number of cellulolytic microbes. 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 [1]. Also, it was clear that the production of cellulases has been reported in a wide variety of bacteria [35] and fungi [13,36]. However, filamentous fungi are celebrated for commercial production of cellulases due to their capability to produce high amounts of these enzymes compared to another microbial community [12].

The isolated fungi were screened for their cellulose decomposition activity using two assays; Congo red and cellulase activity assays. Results illustrated in Fig. 1A indicated that all nine isolates were able to produce cellulases on Congo red plates which estimated by clear zone diameter (cm) that ranged from 0.5 to 3.0 cm. Moreover, data in Fig. 1B graphically illustrated that all nine isolates were able to produce cellulase which ranged from 1.13 to 3.72 (μ/ml). Generally, the largest clear zone diameter and the highest cellulase activity were recorded by the isolate No. CF2, so, this isolate was selected as the most potent producer for identification. Similar results were demonstrated by Mrudula and Murugammal [12] 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 [15]. Fungal cellulases (carboxy methyl cellulase, cellobiose hydrolases and β-glucosidas) have different specificity and mode of actions compared to bacterial cellulases [37,38].

3.2. Identification of the most potent fungal isolate (CF2)

The isolate number CF2 showed the highest cellulases activity, afterward; it was selected as the most potent isolate and subjected for identification. The morphological identification of this isolate showed that colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidial heads are large, globose, dark brown, becoming radiate and tending to split into several loose columns with age. Conidiophore stipes is smooth-walled, hyaline or turning dark towards the vesicle. When viewed under the microscope, it consists of a smooth and colorless conidiophores and spores. Also, the molecular biology techniques were applied to confirm morphological and cultural characterization. The genomic DNA was isolated and purified using isopropyl method [39]. The ITS genes were amplified by PCR technology and sequenced. The obtained sequences were compared with sequences available in GenBank using BLAST

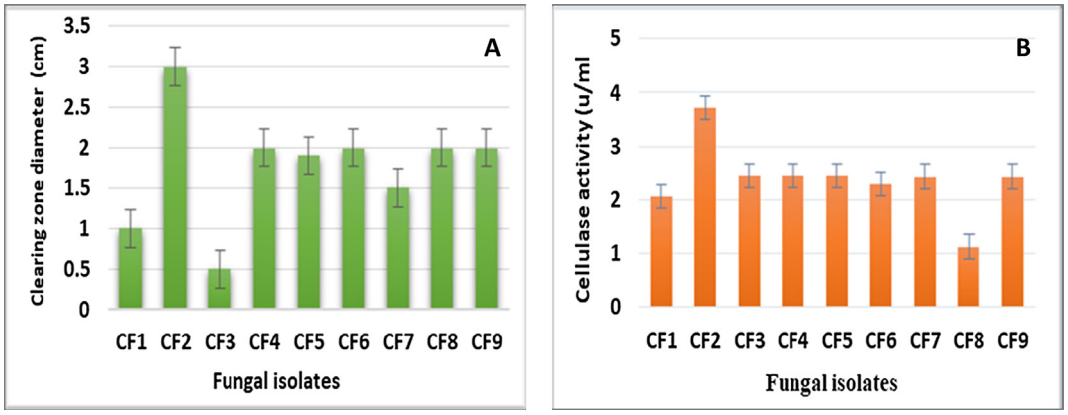

Fig. 1. Screening of fungal isolates for production of cellulases enzymes using Congo red assay (A) and Cellulase activity assay (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

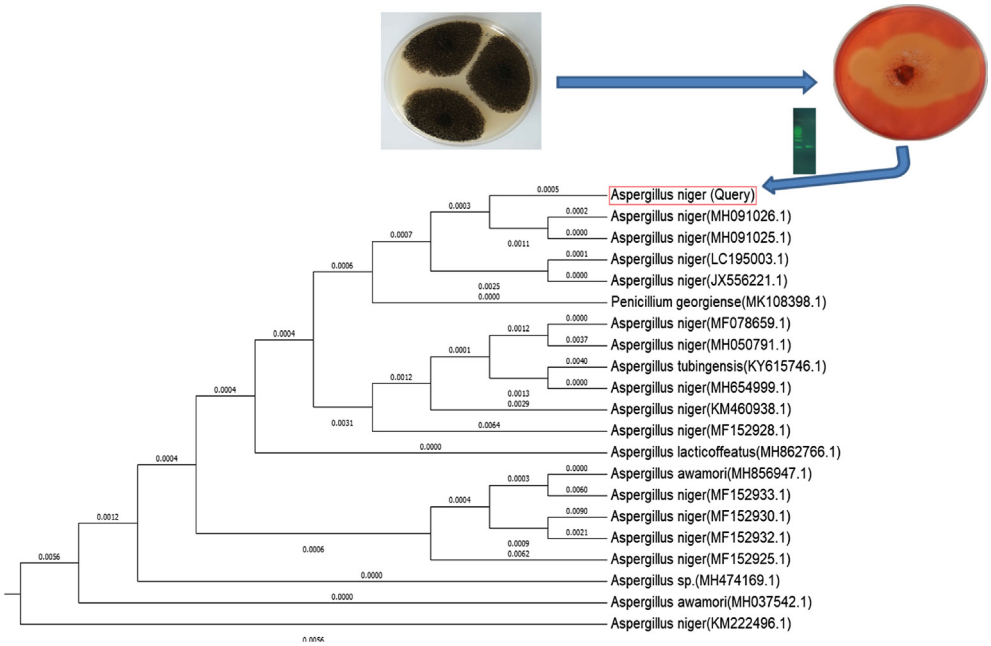

Fig. 2. Phylogenetic relationship between the obtained ITS sequence of *Aspergillus niger* and their related strains in GenBank.

Table 2
Effect of different fermentation media on cellulases production by *A. niger* MK543209.

Media	CMCase activity (μ/ml)	FPase activity (μ/ml)	Cellobiase activity (μ/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

program (http://www.ncbi.nlm.nih.Gov/BLAST) 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 closed to the type strains of *Aspergillus* genus deposited in culture collection centre of National Centre for Biotechnology Information (Fig. 2). So, this strain was named as *Aspergillus niger* CF2. In this way, Devanathan et al. [40] had isolated cellulose-degrading fungi and based on the colony morphology and microscopic observation, the strain was identified as *Aspergillus niger*. Moreover, Mrudula and Murugammal [12] demonstrated that almost all fungi of genus *Aspergillus* synthesize cellulase, therefore this genus has the potency to predominate the enzyme industry.

3.3. Optimization of submerged fermentation conditions for maximum cellulases production by A. niger CF2

3.3.1. Selection of the best fermentation medium

The first component of fermentation technology should be optimized is cultivation medium, because, this component is considered the base of other fermentation conditions [41]. Also, this process is still one of the most critically explored phenomenon that is approved before any large scale metabolite production, and hold many challenges too [42]. From data presented in Table 2 it was clearly that *A. niger* MK543209 gave cellulose degradation activity and produced the 3 enzymes assays; CMCase, FPase and cellobiase in different used media. CMCase, FPase and cellobiase enzymes were noted higher activity with basal mineral salt medium containing 1% cellulose (M2) and recorded 4.36, 2.69 and 2.17 μ/ml. This means that the medium contains the best enzymes inducers for production of high amount of the 3 enzymes. So, the M2 medium was chosen for the following experiments. In contrast, Salem

Table 3
Effect of different carbon sources on cellulases production by *A. niger* MK543209.

Carbon sources at 10%	CMCase activity (μ/ml)	FPase activity (μ/ml)	Cellobiase activity (μ/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

[43] used 5 different media to study the effect of different fermentation media on cellulases activity by *Trichoderma viride*, results showed Reese and Mandels basal medium gave higher cellulases activity. On the other hand, Sohail et al. [44] reported *A. niger* MS82 was grown in basal medium supplemented with 1% (w/v) of CMC, the production of endoglucanase and β-glucosidase reach their maximum.

3.3.2. Selection of the best agriculture waste as the carbon source

The main factor for choosing the best carbon source is the rate of carbon source for metabolization and how it influences the synthesis of biomass and metabolites production. To test that, 4 agriculture wastes were examined as carbohydrate source. Results illustrated in Table 3 proved that *A. niger* CF2 is 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 FPase and cellobiase activities were noted when rice straw was applied as a carbon source. Moreover, data showed that the enzymes activities were ranged from 1.67–2.57, 1.44–2.48 and 0.99–1.75 μ/ml for CMCase, FPase and cellobiase, respectively when bagasse applied as a sole carbon source. In this trend, Mrudula and Murugammal [12] reported that among the 5 substrates screened, coir waste gave the maximum cellulases 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. [45] reported that saw dust, corn cobs and bagasse were found to be the best substrates for cellulases production by *A. flavus*. On the other hand, Oberoi et al. [46] reported kinnow pulp as the best substrate for cellulase production by *A. flavus* in Solid State Fermentation. 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* [47].

3.3.3. Effect of different bagasse concentrations

As graphically illustrated by Fig. 3, *A. niger* 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

Table 4
Effect of nitrogen source on cellulases enzymes production by *A. niger* MK543209.

Nitrogen sources	CMCase activity (μ/ml)	FPase activity (μ/ml)	Cellobiase activity (μ/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
Sodium nitrate	5.74	3.89	1.05

that the activities of the three estimated enzymes was gradually increased from 10% to reach maximum activity at 30% then decreased gradually. The lowest activities were recorded when bagasse was applied at 50%. Additionally, CMCase activity was ranged from 1.78 to 8.92 μ/ml, while, FPase and cellobiase activities were ranged from 1.07 to 4.81 and 0.81–3.64 μ/ml, respectively.

3.3.4. Choosing the best nitrogen source

Nitrogen is an important element for enzymes and other microbial proteins syntheses [33]. For that, the best N source is the material initiates enzymes production. In the current study, 5 nitrogen sources were screened to select the best one for cellulases production. 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, the lowest values of the three estimated enzymes were recorded when *A. niger* cultured on medium containing sodium nitrate. Whereas, the highest values were recorded when *A. niger* cultivated on medium containing urea followed by ammonium chloride. So, urea was applied the N source. In contrast, Gilna and Khaleel [48] reported that among nitrogen sources (peptone, urea, yeast extract and sodium nitrate), yeast extract was the best one to enhance the activity of FPase and CMCase by *Aspergillus fumigatus*. Different results were noted by other researchers [12,40,49] who reported that among different nitrogen sources, peptone supported maximum cellulases production followed by beef extract, yeast extract and casein.

3.3.5. Effect of different urea concentrations

Urea as organic N was applied as nitrogen source for production of cellulases enzymes by *A. niger* MK543209 and used by 5 concentrations to screen the best one. The results were illustrated in Fig. 4, it was clear that *A. niger* MK543209 is 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. So, 2.0% urea was chosen as the optimum concentration of nitrogen source.

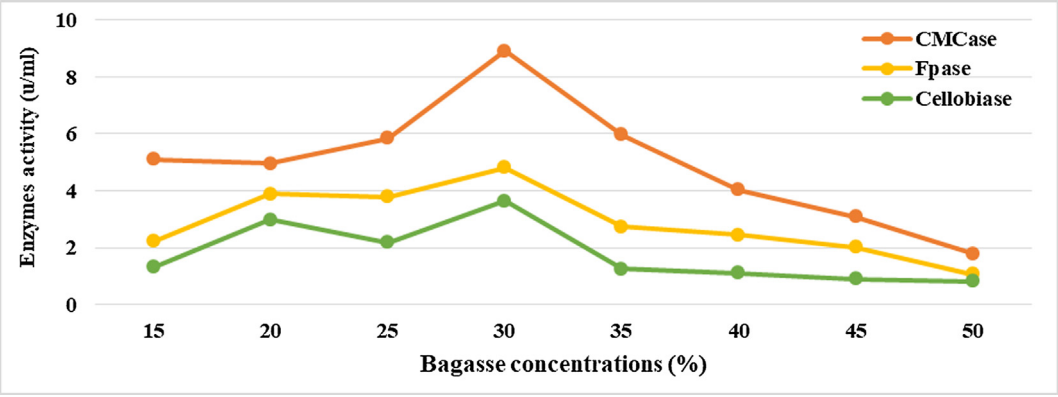

Fig. 3. Effect of bagasse concentrations on cellulases enzymes production by *A. niger* CF2.

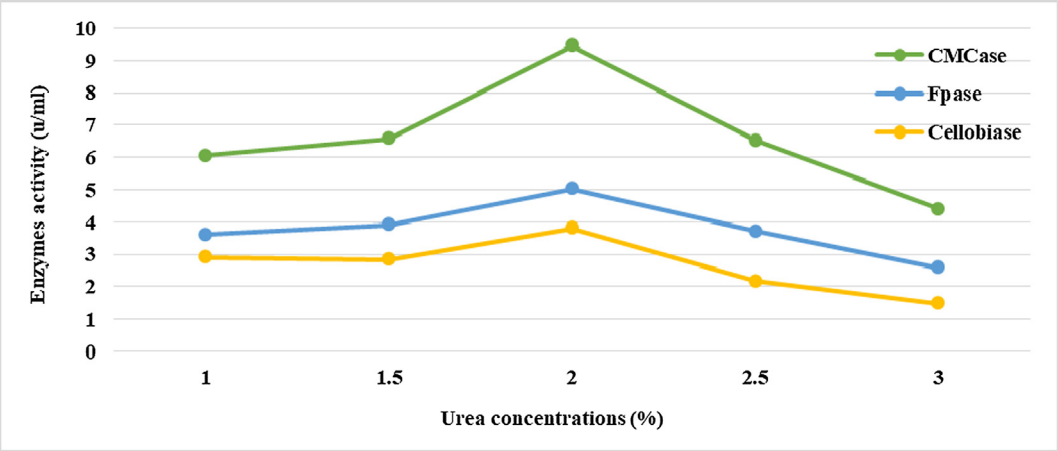

Fig. 4. Effect of urea concentrations on cellulose degrading enzymes production by *A. niger* CF2.

3.3.6. Optimization of initial pH value

The pH value of microbial growing media is considered one of the most important factors for microbial growing as well as enzymes production [50]. This is not means the optimum growth pH value is considered the optimum degree for enzymes production [5]. The pH of the growth medium plays an important role in enzyme secretion by microorganisms. In this study, five different pH values ranged between 5 and 7 were applied. High enzyme production was obtained at pH 5.5 (Table 5) and reached to 11.1, 5.70, and 4.52 μ/ml for endoglucanase, exoglucanase and β-glucosidase enzymes, respectively. So, pH 5.5 was applied for the following experiments. Similar results were presented by Gupta et al. [51], who estimated the cellulase production by *Aspergillus glaucus* XC9 at different pH values and found the highest activity at pH 5.5. In contrast, Gilna and Khaleel [48], who estimated the cellulase production by *A. fumigatus* at different pH values and reported that the highest FPase and CMCase activities were at pH 6.5. Different result was recorded by Sohail et al. [44] who reported that among different pH values, pH 4.0 supported maximum β-glucosidase and CMCase activities under submerged conditions.

3.3.7. Effect of incubation temperature on cellulases production

Incubation temperature plays an important role in the metabolic activities of all microorganisms. In this experiment, 7 temperature degrees were examined and results represented in Table 6, which showed that *A. niger* CF2 had the ability to degrade cellulose compound under wide range of incubation temperatures (20–50 °C). The highest activity of the three estimated enzymes were recorded when the fungal strain incubated at 45 °C with values of 12.6, 6.30 and 4.35 μ/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 activity at 45 °C then decreased sharply. While, Gilna and Khaleel [48] tested the effect of various incubation temperatures (25, 30, 32, 35 and 37 °C) on CMCase, FPase production by *A. fumigatus* and reported the optimum temperature was found to be at 32 °C. This may be replied to the behavior of the used strain, because, the applied strain in this study considered as thermophile.

Table 5
Effect of the initial pH value on cellulases production by *A. niger* MK543209.

pH values	CMCase activity (μ/ml)	FPase activity (μ/ml)	Cellobiase activity (μ/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

Table 6
Effect of incubation temperature on cellulases production by *A. niger* CF2.

Incubation temperature (°C)	CMCase activity (μ/ml)	FPase activity (μ/ml)	Cellobiase activity (μ/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

3.3.8. Effect of different incubation period

Clearly, the incubation period is directly affects enzymes production. In this trend, the maximum yield of cellulases was tested at different incubation period and the data was illustrated in Table 7. *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, the data indicated that CMCase activity was increased gradually till reached the maximum after 7 days then decreased sharply. On the other hand, FPase and cellobiase activities were hesitating because they decrease after 4 days then increased after 7 days then decreased again. Generally, 4 days was the best incubation period based on data recorded. 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 [52]. In the same trend, Gautam et al. [53] estimated the best incubation period for highest cellulases activity by *A. niger* and found that 4th day was the optimum incubation period to produce FPase and cellobiase.

3.3.9. Conversion of paper wastes to biofuel

Waste paper is produced daily by large amounts globally, some of it

Table 7
Effect of incubation period on cellulases production by *A. niger* MK543209.

Incubation period (days)	CMCase activity (μ/ml)	FPase activity (μ/ml)	Cellobiase activity (μ/ml)
4	8.34	7.22	5.44
5	6.70	1.61	1.16
6	7.84	1.18	0.97
7	10.8	2.72	2.04
8	2.12	1.68	1.55

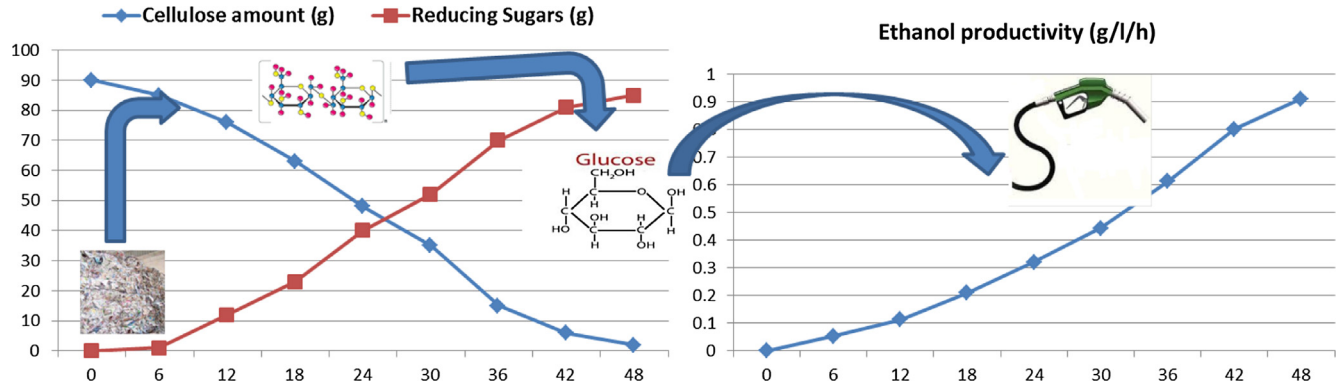

Fig. 5. Conversion of paper wastes to bioethanol.

is recycled. Also, it can be applied as a feedstock for ethanol production because it's reached with cellulose and not needing any pretreatment processes [54]. It consisted about 95% cellulosic materials and negligible lignin, because that, it is applied in this study to produce ethanol. Hundred grams of paper wastes were fermented to reducing sugars using *A. niger* and the resulted sugars were converted to ethanol using *Saccharomyces cerevisiae* (Fig. 5). The amount of reducing sugars was increased gradually due to decomposition of cellulose which decreased in amount. The maximum value of ethanol productivity was recorded (0.9 g/l/h) after 48 h of fermentation. Finally, in this research, the wastes were converted to bioethanol to solve energy problem as economic value.

4. Conclusion

In the current research, we try to solve problems of conventional energy shortage and produce renewable one through applying eco-friendly technology. The paper wastes were converted to reducing sugars using superior new fungal isolate (*Aspergillus niger* MK543209) and then bioethanol was produced from resulted reducing sugars as biofuel resource.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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