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ISOLATION AND IDENTIFICATION OF A CELLULOLYTIC FUNGUS FROM ANIMAL MANURE.

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Fungi are considered the best cellulose decomposers in nature. More than 80% of cellulose degradation in nature is accomplished by fungus. The most know fungal species that degrade cellulose are members of the Basidiomycota (Baldrian and Valášková, 2008), Ascomycota (Hernández *et al.*, 2018), and Chytridiomycota encountered in the rumen of some animals (Gleason *et al.*, 2011). Fungi are recognized for their ability to secrete large quantities of extracellu-

lar cellulase making them preferable for industry. Cellulases have been successfully produced in diverse industrial sectors from different fungal species such as *Aspergillus* (Bansal *et al.*, 2012), *Trichoderma* (Ellilä *et al.*, 2017), and *Penicillium* (Prasanna *et al.*, 2016). A various number of cellulase enzymes have been produced from fungal species such as *Aspergillus niger*. (Mrudula and Murugammal, 2011), *Aspergillus ornatus* (Toor and Ilyas, 2014), *Aspergillus terreus* (Gao *et al.*, 2008).

The cellulase production from *Trichoderma* species are widely known, but it lacks significant levels of β-glucosidase. As a result, the enzyme cocktails from *Aspergillus* species are used instead though with lower titers but contains higher activities of β-glucosidase (Gusakov, 2011). Cellulose degrading enzymes isolated from *Aspergillus* genus members have been extensively studied and the genomic organization of many of the enzymes has been reported (de Vries and Visser, 2001).

Agricultural waste can be an economically sustainable and renewable source of energy. The achievability of agricultural waste as a resource of energy mainly depends upon its hydrolysis of the lignocellulosic biomass for the production of desired metabolites and biofuels. The effective converting of agricultural wastes to fermentable sugars offers outstanding benefits such as improving strategic security, decreasing the trade deficits, better environmental quality, and securing a sustainable energy resource supply (Kamm and Kamm, 2004). Cellulose is one of the most abundant polysaccharides and the key component of plant biomass. Cellulose biodegradation by cellulases and cellulosomes is preferred over chemical degradation due to the absence of sugar degradation in the enzymatic process (Sari *et al.*, 2017).

Three major components constitute the cellulosic enzyme system. First, the endo-ß-glucanase (EC 3.2.1.4) breaks internal glycosidic linkages of the amorphous region of cellulose, yielding glucose and cello-oligo saccharides. Second, the exo-ß-glucanase (EC 3.2.1.91) catalyzes the production of either cellobiose or glucose units from the reducing and non-reducing ends of the cellulosic fibril. Finally, the ß-glucosidase (EC 3.2.1.21) hydrolyze cellobiose into glucose. These enzymes work synergistically to degrade cellulose to glucose units that can be used in many applications (Lynd *et al.*, 2002).

Solid-state fermentation (SSF) is a favored method for the growth of microorganisms on moist solid supports, including inert carriers and insoluble substrates. This technology has been long-established for the efficient production of cellulase enzymes using cultivating conditions of fungi that simulate their natural environment (Sukumaran *et al.*, 2009). SSF is preferred over the submerged fermentation (SmF), because of the higher concentration of products, decreased production cost, simpler fermentation equipment, minor effluent production, and less waste

output. Many reports studied the use of cheap and easily available substrates like bagasse and lignocellulose waste materials, for reducing the cost of cellulases production (Yoon *et al.*, 2014). Other studies tested the solid-state fermentation of *Aspergillus terreus* using agricultural waste (Gao *et al.*, 2008; Ismail and Hassan, 2020).

The current report describes the isolation of cellulose-degrading fungal strain, AGE-1, from cow manure. The strain was molecularly identified using 18S rRNA gene and ITS as *Aspergillus terreus*. AGE-1 fungal strain was tested for the cellulases induction under various parameters such as pH, incubation temperature, incubation time, carbon sources. Moreover, its ability to cellulases production using solid-state fermentation was optimized.

MATERIALS AND METHODS

Isolation and qualitative screening of cellulolytic activity of isolated fungal strain

In this study, cellulose-degrading fungal isolates were collected from cow rumen. Serial dilutions of the sample were cultured onto potato dextrose agar plates and incubated for 7 days at 30ºC. Single colonies were picked and sub-cultured to obtain a pure isolate. Stock cultures were maintained on potato dextrose agar slants at 4ºC (Devi and Kumar, 2012). Cellulase producing fungi were screened on selective carboxymetyl cellulose agar plates containing (g/L): $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$ $7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose sodium salt 10.0, peptone 0.2, agar 17.0. Plates were spot inoculated with a spore suspension of pure culture and incubated at 30ºC. After three days, plates were flooded with 1% Congo red solution for 15 minutes then destained with 1M NaCl solution for 15 minutes. The diameter of the zone of decolorization around each colony was measured (Teather and Wood, 1982).

Quantitative screening of cellulolytic activity of isolated fungi

Purified fungal isolates were tested for cellulases production on Sugar cane Bagasse as a sole carbon source. Sugar cane Bagasse was washed, dried then milled to small particle sizes; about 2 mm long. The milled bagasse was mixed with 0.12% NaOH and autoclaved at 121ºC for 20 min. The autoclaved bagasse was washed again with tap water to remove the excess of NaOH till it becomes neutral and dried at 80ºC (Gutierrez-Correa and Tengerdy, 1997).

One gram of treated Sugar cane Bagasse (in 250 mL Erlenmeyer flask) was supplemented with 100 ml basal mineral medium contained (g/L): (KH_2PO_4, 2g; $(NH_4)_2SO_4$, 1.4 g; Urea, 0.3g; $CaCl_2 \cdot 2H_2O$, 0.3g; $MgSO_4 \cdot 7H_2O$, 0.3g; peptone, 1.0g; Tween 80, 0.2%; $CoCl_2 \cdot 6H_2O$, 2.0 mg; $FeSO_4 \cdot 7H_2O$, 5.0mg; $MnSO_4 \cdot 2H_2O$, 1.6 mg; $ZnSO_4 \cdot 7H_2O$ 1.4 mg., Two ml of fungal spore suspension (10^6 spores/ml) were inoculated into a 100 ml of the cellulases

production medium (pH 5.5) and incubated at 30ºC and agitated at 170 rpm in a rotary shaking incubator. After seven days, the culture filtrate was collected by centrifugation (6000 rpm for 15 min) and the supernatant was used as a source of crude cellulase enzyme (Vega *et al.*, 2012).

Enzymes assay

The fungal isolates were assayed for CMCase and FPase activities according to Ghose, (1987). One milliliter of diluted crude enzyme extract was mixed with one milliliter of the substrate (1% CMC prepared in 100 mM sodium acetate buffer, pH 4.8) to determine CMCase activity. One milliliter of diluted crude enzyme extract was mixed with one milliliter of 100 mM sodium acetate buffer (pH 4.8) containing 50mg (1x6cm strip) of Whatman filter paper no.1 for the determination of the FPase activity. The reactions were incubated at 50ºC for 30 min and 60 min in the case of CMCase and FPase assay, respectively. The released reducing sugar was estimated spectrophotometrically at 540nm by the addition of 3,5-dinitrosalicylic acid (DNS) with glucose as standard. The activity of the enzyme was expressed in IU/ml; one unit (IU) of enzyme activity is defined as the amount of enzyme required to release 1 µmol of glucose in the reaction mixture per minute under the specified conditions.

Effect of initial medium pH, temperature and incubation temperature on cellulase production.

Different pHs ranged from 4-10, varying temperatures ranged from 30ºC - 37ºC, and different incubation periods ranged from 4-10 days, were tested for determining the best conditions for cellulases induction. Each parameter was tested individually while all other parameters were kept constant (Vega *et al.*, 2012). Sugar cane bagasse (SCB) and carbon methylcellulose (CMC), were tested as a sole carbon source in a basal mineral media at 30ºC and 170 rpm. The production media was used and inoculated by 3 ml (1 x 10^7 spores/ml) and all the flasks were incubated in a shaking incubator at 170 rpm (New Brunswick, USA). Enzyme assays of both FPase and CMCase were determined as described above.

Molecular identification of fungal strain

For molecular identification, genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (ThermoFisher®, California, USA). Two specific primers ITS5 (5-TCCTCCGCTTATTGATATGC-3), ITS4 (5-GGAAGTAAAAGTCGTAACAAGG-3) (Zheng *et al.*, 2009) in the Internal Transcribed Spacer (ITS) region of the rRNA were used for PCR amplification of a 600 bp. The *18S rRNA* gene was amplified using this pair of forward and reverse primer NS1 (18S) (5-GTAGTCATATGCTTGTCTC-3) and NS4 (18S) (5-CTTCCGTCAATTCCTTTAAG-3)

(Panzer *et al.*, 2015). PCR was performed using the standard reaction mixture (50 µl) containing; 1× PCR buffer, 1.5 mM MgCl2, 200 mM of each dNTPs, 15 pmol of each primer, 1 U of Taq polymerase enzyme (Promega® Corporation, Madison, USA), and 50 ng of DNA template. The PCR was carried out as follows: primary denaturation for 5min at 94ºC; 30 cycles of denaturation at 94ºC for 30 s; annealing at 58°C for 30 s, and extension at 72ºC for 60s; and an additional extension for 5min at 72°C. DNA sequencing of the purified PCR products was performed using an ABI BigDye Terminator V3.1 cycle sequencing kit and 3130xl genetic analyzer (Applied Biosystem, USA).

Phylogenetic analysis

The raw sequences (forward and reverse) *18S rRNA* gene and ITS region of the rRNA gene were assembled with Sequencher version 4.0.5 (Gene (Gene Code Corporation, Ann Arbor, MI, USA). The 18S rRNA and ITS assembled contigs were blasted for similarities to DNA sequences deposited in the GenBank using BLASTN program and submitted to the GenBank nucleotide sequence database (NCBI). The sequences were aligned using ClustalW version 1.8 (Altschul *et al.*, 1997). Further phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) version7 (Kumar *et al.*, 2016), using the maximum likelihood methods (ML) and 1000 bootstrap replication to assess branching confidence. The Jones-Taylor-Thornton (JTT) model was selected as the substitution model.

Solid-state fermentation

A 7 days fungus maintained on potato dextrose agar (PDA) slants and incubated at 30ºC was used to prepare spore suspension by scratching the growing fungus in each slant with 10 ml distilled water containing 0.1% tween 80. Spores of fugal strain (5.33×10^7, total amount) were used to inoculate 100 mL of modified Mandels and Weber medium (Szijártó *et al.*, 2004) in 500 mL conical flasks, incubated at 30ºC and shaken 200 rpm for fungus propagation. After 3 days, 10 mL of culture containing the growing fungus mycelia (3.8 g/L) was transferred into 1 L Erlenmeyer flasks containing 200 mL of media supplemented with Avicel CE-15 (10 g/L, microcrystalline cellulose, (FMCBiopolymer, Philadelphia, USA).

For testing the carbon sources, five grams of the dry carbon source was mixed with the mineral solution (gram per liter distiller water: $(NH_4)_2SO_4$ 3.5 g, KH_2PO_4 3 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCl_2$ 0.5 g) in 250 ml Erlenmeyer flasks, which were all sterilized for 30 min at 121ºC in succession (Juhász *et al.*, 2005).

RESULTS AND DISCUSSION

Isolation, and screening of cellulase producing fungal isolates

Twelve fungal isolates were obtained based on their ability to grow on basal mineral medium containing sugar-

cane bagasse pulp as the sole carbon source. These fungal isolates were endoglucanase producers judged by showing hydrolyzing clear zones on agar plates containing CMC-Na and iodine staining (Kasana *et al.*, 2008). The hydrolyzing zone diameter and colony diameter were recorded for all tested isolates (Table 1) which confirmed that all isolated fungal isolates were able to grow and secrete endoglucanase.

The quantitative screening using (3,5-dinitrosalicylic acid) DNS method (Adney and Nrel, 2008) showed that all twelve fungal isolates produced exoglucanase (FPase) and endoglucanase (CMCase) (Table 1). The Fungal isolates showed FPase activity ranged from 0.006 to 0.41 U/ml, while the CMCase activity ranged from 0.007 U/ml to 0.142 U/ml. Fungal isolate AGE-1 showed the highest CMCase activity with 0.143 IU/ml in addition to 0. 41 U/ml for FPase, and was further characterized.

Some studies propose a direct relation between log enzyme concentration and the diameter of the cleared zone in the Congo-red staining method (Teather and Wood, 1982; Florencio *et al.,* 2012). Other studies showed that the isolated microorganisms did not follow this pattern (Liang *et al.*, 2014). In the present study, the isolates showed a direct relationship between the enzyme concentration and the diameter of the cleared zone in agreement with Teather and Wood, (1982) and Florencio *et al.,* (2012).

Optimization of Culture Conditions for Enzyme Production

Cellulases production or induction of FPase and CMCase from AGE-1 isolate were assessed at different pH values (Fig. 1A). The results clearly showed that the optimum pH for FPase and CMCase activity were pH 6.0. The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual fall in the activity. The maximum values at pH 6.0 were 1.44 U/ml, and 0.88 U/ml for CMCase and FPase, respectively. Our results are in the same range as those reported for FPase and CMCase produced by *Aspergillus terreus* (Shahriarinour *et al.*, 2011). Whereas, the FPase and CMCase production by *Aspergillus niger* MS82 were maximal when the initial culture pH was between 6.0 or 7.0 (Sohail *et al.*, 2009).

The results illustrated by Fig.(1) clearly show that CMCase production, expressed as enzyme activity, gradually increased as the incubation period increased reaching the maximum at the seventh day, 1.44 U/ml ±0.02 when incubated at 30ºC. The maximum activity for CMCase was 1.30 U/ml ± 0.03 on day 7 when the fungal isolate incubated at 34ºC, and 0.966 U/ml ± 0.05 when incubated at 37ºC. A slow decrease of the CMCase was observed on the following days (Fig. 1B-D). The FPase gradually increased as the incubation period increased reaching the maximum on the seventh day, 0.88 U/ml ±0.03 when incubated at ºC. The maximum activity for FPase was on day 7 and

it reaches 0.783 U/ml ± 0.03 when the isolate incubated at 34 °C and reached 0.482 U/ml ± 0.09 when incubated at 37ºC (Fig. 1 B-D). Our results are in accordance with Sohail *et al.,* (2009); and Shahriarinour *et al.,* (2011), which confirmed the decrease of FPase and CMCase production with the increase of temperature.

Sugarcane bagasse and carboxymethyl cellulose were tested for their effects on cellulases production. Data presented in Fig. (2) showed that Bagasse was the better sole source of carbon for cellulases production as the activity was 0.74 U/ml for FPase and 1.29 U/ml for CMCase (Fig. 2).

Identification of AGE-1 using ITS and *18S rRNA*

The sequence of Internal Transcribed Spacer (ITS) region of *rRNA* gene on the AGE-1 genome was deposited in NCBI under the Accession No LC593154.1. The molecular analysis of the ITS region of the AGE-1 fungal isolate exhibited maximum homology with *A. terreus* strain SF 981 (accession number MT530257.1), *A. terreus* strain SF 938 (accession number MT530214.1), *A. nomius* strain F_1 (accession number MN700028.1) and *A. niger* strain AY01 (accession number MT771988.1). Maximum likelihood (ML) phylogenetic tree based on ITS region was generated and determined the evolutionary relatedness of the fungal isolates AGE-1 (Fig. 3). Phylogenetic analysis showed that the AGE-1 isolate was grouped with type strains belonging to *A. terreus*, *A. nomius* and *A. niger* (Fig. 3).

For more accurate molecular identification, the *18S rRNA* gene of the AGE-1 isolate was amplified and sequenced. The sequence was deposited in NCBI with Accession No LC593155.1. Nucleotide BLAST of the *18S rRNA* of the AGE-1 fungal isolate showed the highest similarity with *A. terreus* strain 23A (accession number MN995500.1), *A. terreus* strain AZM03 (accession number MH550051.1), *A. terreus* strain 8F (accession number MN995485.1), and *A. terreus* strain 6Fb (accession number MN995482.1). Maximum likelihood (ML) phylogenetic tree based on 18S rRNA was generated and confirmed the identification of the AGE-1 isolate as *A. terreus* strain (Fig. 4). Phylogenetic analysis showed that the AGE-1 isolate grouped with strains belonging to *A. terreus*. The AGE-1 was grouped in a separate clade with *A. terreus* strains, which is separated from the *A. nomius* strains. The phylogentic tree showed that *A. niger* strains, *A. fumigatus* and *A. flavus* form a separate clades that are separated from the AGE-1 strain (Fig. 4).

The use of *18S rRNA* genes, *26S rRNA* genes, *5.8S rRNA* genes, *28S rRNA* genes and ITS sequences of different fungi for the study of their genetic relationship is a well-established tool (Sugita *et al.*, 2002; Woo *et al.*, 2010). In our report, the use of ITS phylogeny could not differentiate between the closely related *Aspergillus* strains, while the use of *18S rRNA*

phylogeny resolved this tack. This is contrary to another study that suspected the ability of *18S rRNA* gene to identify *Aspergillus* down to the species and suggested the use of ITS region for this purpose (Henry *et al.*, 2000).

Optimization of FPase and CMCase production using solid-state fermentation.

SSF process is a very important fermentation strategy in countries that have huge plant biomass wastes. *Aspergillus* spp. and *Rhizopus* spp were frequently used for the production of α-amylase, lipase, caseinase, and cellulases. The two species were preferred due to their ubiquity and modest nutritional requirements. Lignocellulosic waste such as banana peels, date wastes, rice straw, sugarcane bagasse and many others were used for the biosynthesis of the aforementioned enzymes (Acourene and Ammouche, 2012 and Aliyah *et al.*, 2017). AGE-1 fungal strain was tested for the FPase and CMCase production using SSF (Table 2). The results are illustrated in Table. (2) which showed that CMCase production reached the maximum expression, 235 U per one gram of dry carbon source when incubated at^{o}C and pH7. The same condition showed the maximum expression of FPase at 92 U per one gram of dry carbon source (Table 2).

Testing carbon sources for Fpase and CMCase production by AGE-1 Fungal strain using solid-state fermentation.

Cellulase production is usually dependent on the nature of the carbon source in the culture medium. The utilization of agricultural wastes as a carbon source for the production of cellulase instead of using the refined cellulosic carbon source can reduce the cost of the production and make the enzyme production economically feasible. The ability of *A. terreus* AGE-1 for the cellulases production in the SSF using four different relatively cheap components as carbon source was investigated. The effect of sugarcane bagasse (SCB), wheat straw, ground sesame and rice hey as carbon source on the cellulase biosynthesis on A. *terreus* AGE-1 was recorded (Fig. 5). Results showed that sugarcane bagasse (SCB) is the best sole source of carbon for cellulase production as cellulase activity was 62 U for FPase and 288 U per one gram of dry carbon source for CMCase (Fig. 5). Wheat straw showed similar values for the cellulases production, as it produced 58 U for FPase and 274 U per one gram of dry carbon source for CMCase (Fig. 5).

The productivity of the CMCase and FPase using the isolated strain is comparatively high, indicating the efficiency of the isolated strain to utilize bagasse as a carbon source for the production of cellulase. Our results are in accordance with the results of Gao *et al.*, (2008), which shows similar values for CMCase and FPase using bagasse in the solid-state fermentation of *Aspergillus terreus* M11. Various reports favored the utilization of rice straw as the fermentation carbon source in fungal production (Aggarwal *et al.*, 2017). Sugar cane bagasse is stored in a huge amount in one place in Egypt, the sugar company. The utilization of sugar cane bagasse as the fermentation carbon source in the fungal production of cellulases will be a reliable

source and can reduce its disposal of environmental pollution hazards.

SUMMARY

Successful conversion of lignocellulose waste to fermentable sugars should open the door for the production of many desired metabolites. Fungi are the best cellulose decomposers in nature. The present study describes the isolation of cellulose-degrading fungal strain, AGE-1, from cow manure. AGE-1 fungal strain was tested for the cellulases production under various pHs, incubation temperatures, incubation time, and carbon sources. The AGE-1 isolate showed the maximum activity for CMCase of 1.44 U/ml ± 0.02 on day 7 when the fungal isolate was incubated at 30 °C. The maximum activity for FPase was on day 7 and it reaches 0.88 U/ml ± 0.03 when the isolate incubated at 30ºC. The strain was molecularly identified using the 18S rRNA gene and ITS as *Aspergillus terreus*. The strain was tested for cellulases production using solid-state fermentation and showed maximum expression of FPase and CMCase at 30ºC and pH7. Various agricultural wastes were tested as substrates for cellulase production by solid-state fermentation, and sugarcane bagasse (SCB) was the best sole source of carbon for cellulase production followed by wheat straw. The isolated strain can be used as a starting step for degrading agricultural waste that can be used for the production of biofuel and the production of desired metabolites from cellulose.

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Table (1): Evaluation of Exoglucanase (FPase) activity and Endoglucanase (CMCase) activity of the cellulose-degrading fungal isolates.

Isolate code	D/d	FPase (U/ml)	CMCase (U/ml)
AGE-1	37/3.1	0.41 ± 0.002	0.143 ± 0.002
AGE-2	28/2.4	0.038 ± 0.001	0.0.75 ± 0.003
AGE-3	21/1.9	0.024 ± 0.002	0.061 ± 0.004
AGE-4	24/2.2	0.024 ± 0.01	0.062 ± 0.001
AGE-5	11/2.1	0.016 ± 0.003	0.034 ± 0.003
AGE-6	17/3.0	0.018 ± 0.002	0.031 ± 0.002
AGE-7	14/2.4	0.010 ± 0.003	0.026 ± 0.004
AGE-8	25/2.5	0.045 ± 0.001	0.085 ± 0.004
AGE-9	21/2.1	0.028 ± 0.001	0.071 ± 0.007
AGE-10	10/2.5	0.012 ± 0.002	0.029 ± 0.002
AGE-11	9/2.0	0.01 ± 0.001	0.012 ± 0.002
AGE-12	7/2.1	0.006 ± 0.002	0.007 ± 0.005

"*D*/*d*": hydrolyzed zone diameter/colony diameter on agar media containing CMC as sole carbon source;

Table (2): Evaluation of Exoglucanase (FPase) activity and Endoglucanase (CMCase) activity of the cellulose degrading fungal isolates using SSF.

Trial	pH (Fermenter)	Temp	Humidity / moisture	Cmcase	FPase
1	6.5	30	75%	215	78
2	6.5	35	75%	200	75
3	7.0	30	75%	235	92
4	7.0	35	75%	220	76
5	7.5	30	75%	222	68
6	7.5	35	75%	210	67
7	8.0	30	75%	195	61
8	8.0	35	75%	189	60

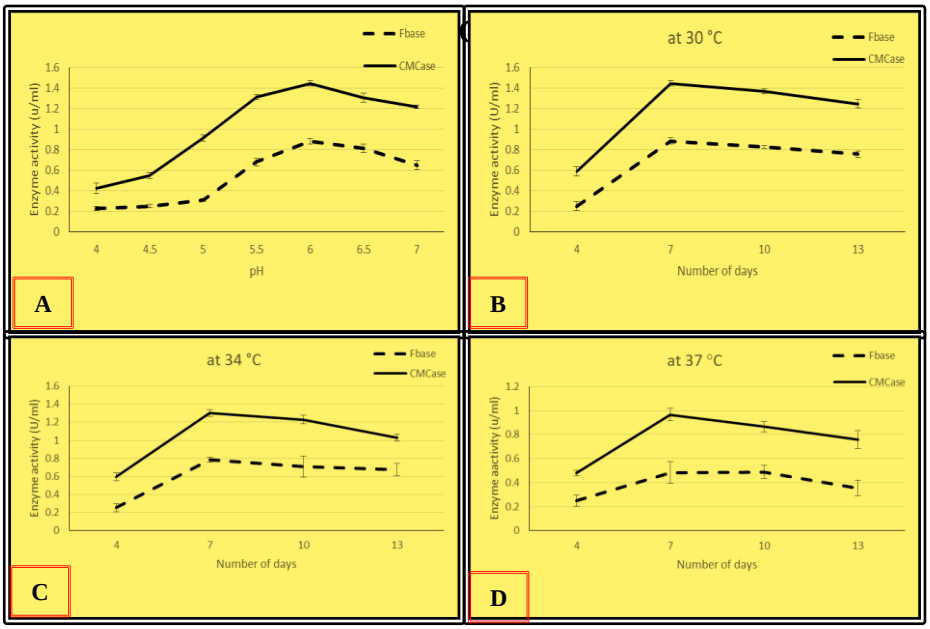

Fig. (1): Effect of pH and incubation time at different incubation temperatures on the activity of FPase and CMCase for *A. terreus* AGE-1. (**A):** Effect of different pH, at 30ºC, on the production of cellulases by *A. terreus* AGE-1. Effect of incubation period on the production of cellulases at incubation temperature30ºC (**B**), 34ºC (**C**) andº37ºC (**D**).

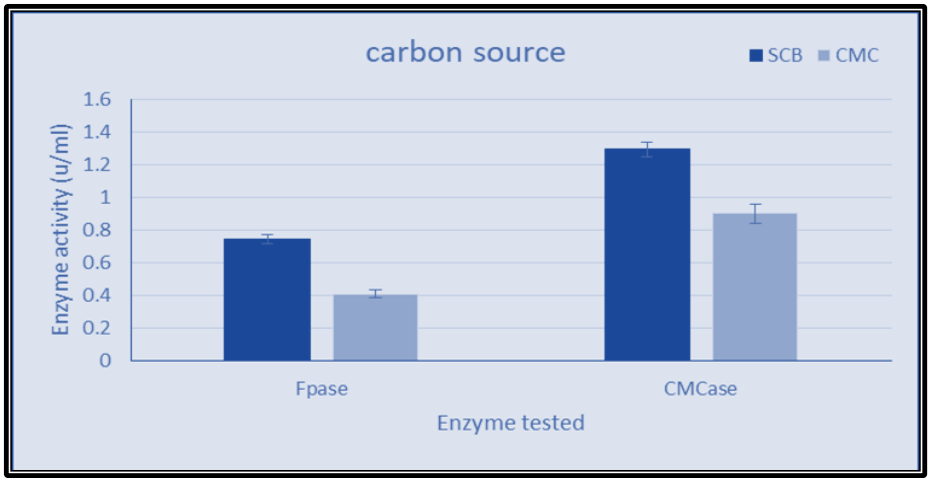

Fig. (2): Effect of SCB and CMC as sole carbon sources for the induction of FPase and CMCase.

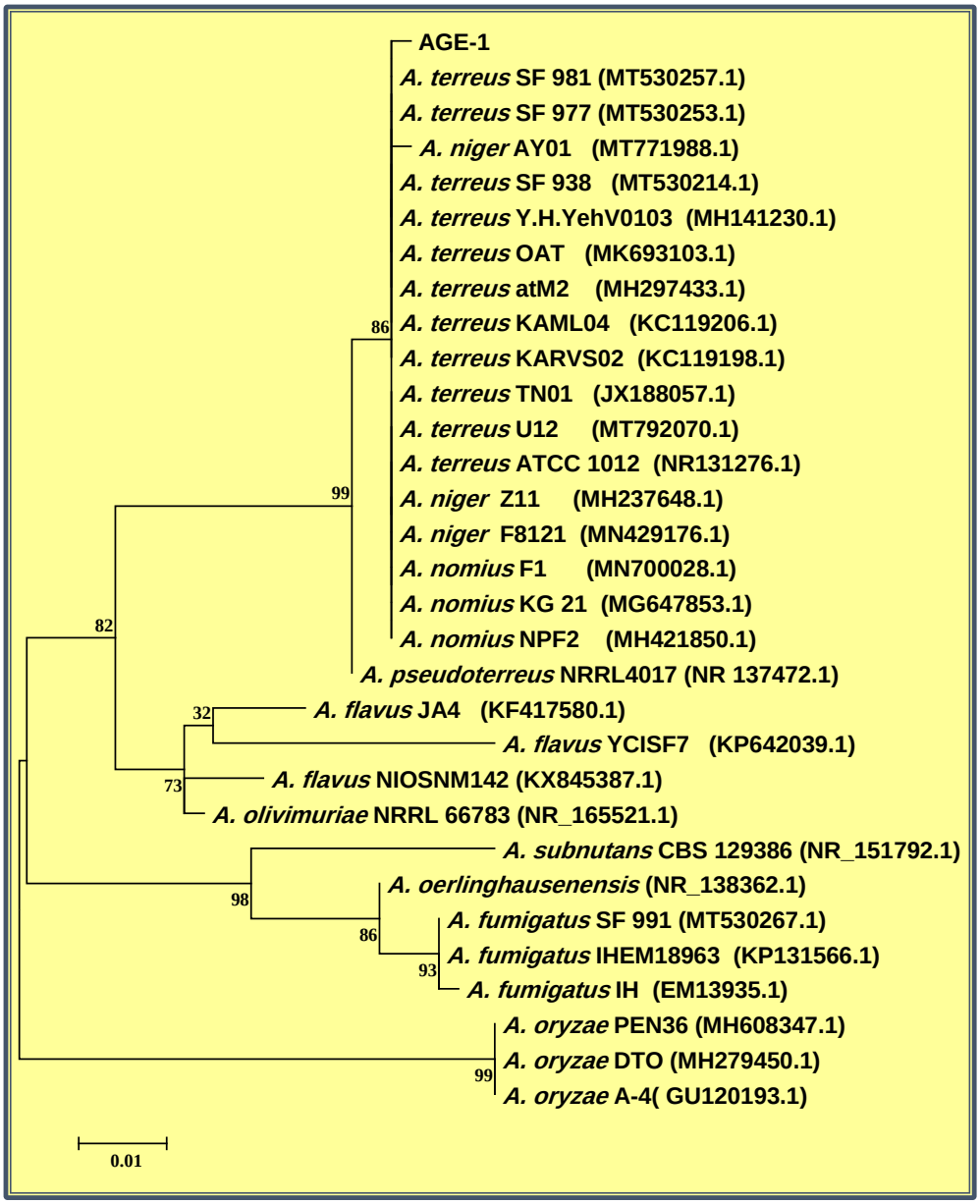

Fig. (3): Phylogenetic tree based upon the Internal Transcribed Spacer (ITS) region of *rRNA* gene of the AGE-1obtained by maximum likelihood (ML) method. GenBank accession numbers are indicated next to the strain name. Bootstrap values were calculated from 1000 trees and shown at the branching points.

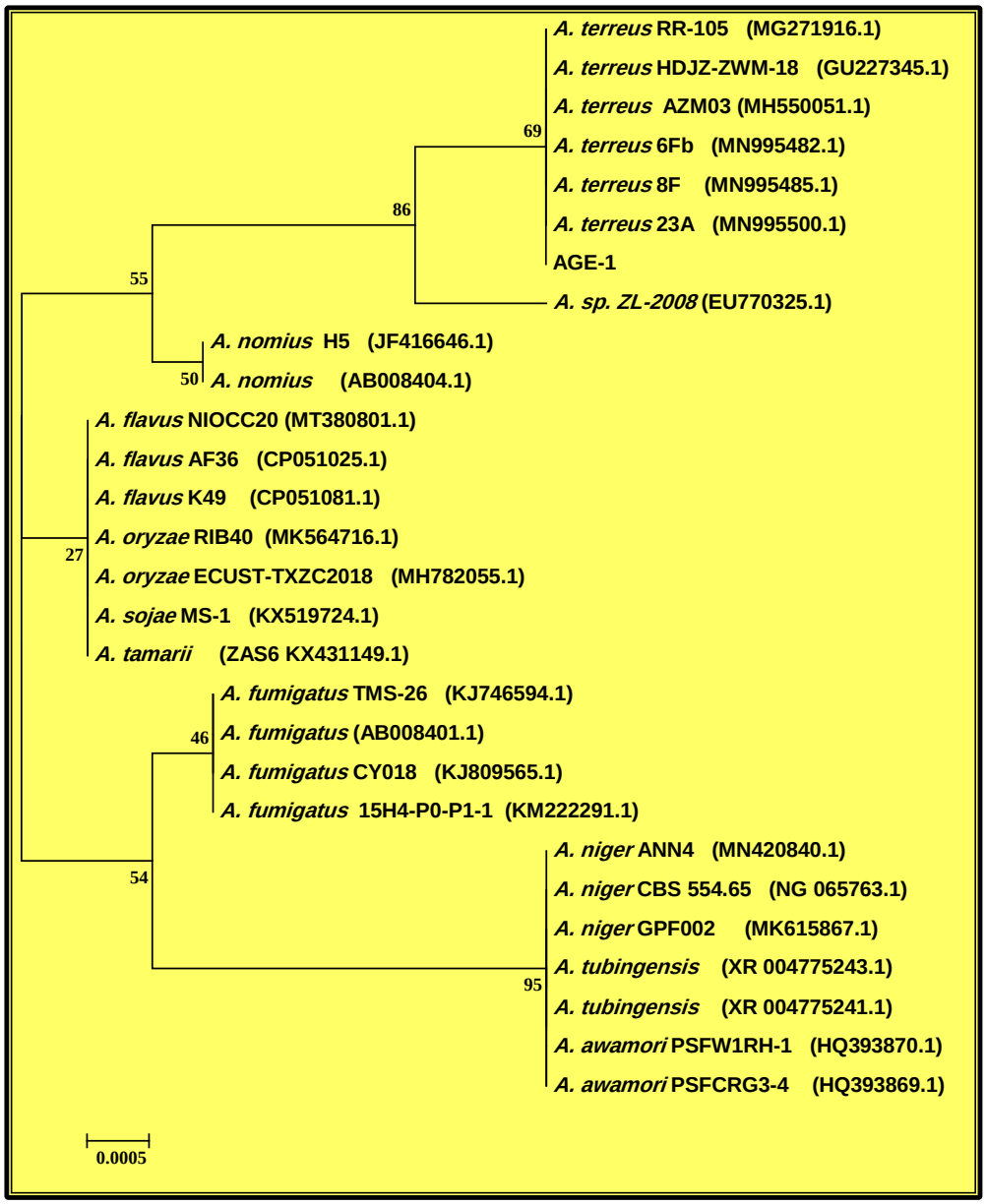

Fig. (4): Phylogenetic tree based upon the *18S rRNA* gene of the AGE-1obtained by maximum likelihood (ML) method. GenBank accession numbers are indicated next to the strain name. Bootstrap values were calculated from 1000 trees and shown at the branching points.

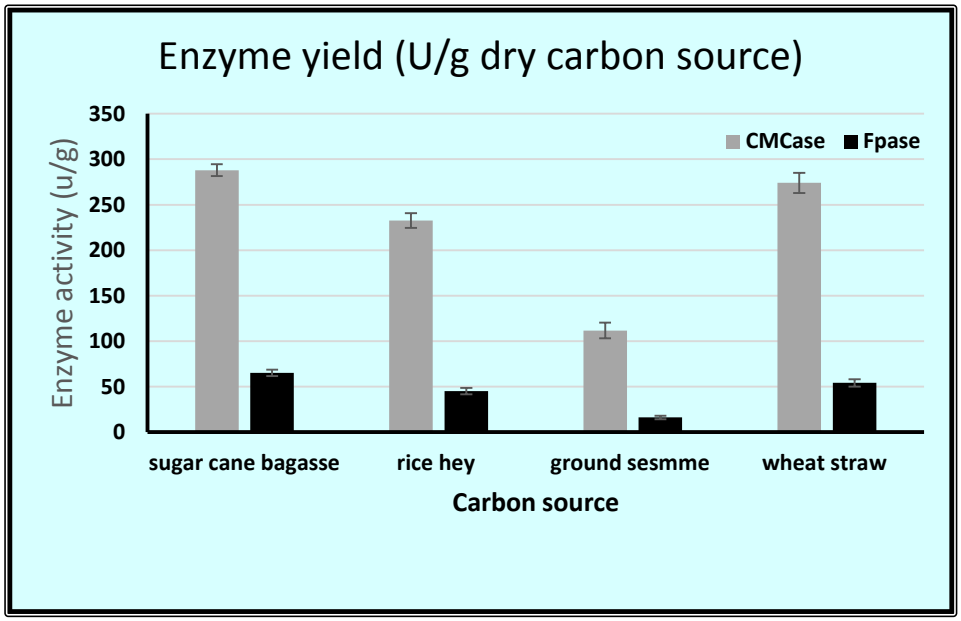

Fig. (5): Effect of sugarcane bagasse, wheat straw, ground sesame and rice hey as sole carbon sources for the induction of FPase and CMCase using SSF.