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Identification of Endoglucanase Gene Responsible for Cellulose Degradation Using *Aspergillus flavus*

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ABSTRACT

Twenty two of cellulose-degrading fungal isolates were isolated from five different samples; rotted sugar cane bagasse, rotted plant, termites, soil and animal manure. Out of 22 isolates, 21 strains showed hydrolyzing zone on agar plates containing carboxy methyl cellulose after iodine staining. The fungal isolate No. S4, exhibited the highest endoglucanase (CMCase) activity with (0.165 IU/ml) in cellulase production culture. The best exoglucanase (FPase) and endoglucanase (CMCase) of fungal isolate S4 was obtained after incubation at 30°C for 7 days. Sugar cane bagasse (SCB) induced the production of FPase and CMCase with maximum activity of 3.6 fold and 2.2 fold, respectively more than that of the maximum yield in case of carboxy methyl cellulose (CMC). The fungal isolate S4 was identified as *Aspergillus flavus* on the basis of 18S rRNA and ITS region sequence analysis.

INTRODUCTION

Cellulose is the most plentiful renewable bioresource in the world (Macrae *et al.*, 1993).The plenty of lignocellulosic waste makes it an attractive raw material for many industrially commodity products such as paper manufacture, composting and animal feed. Cellulose biodegradation by cellulases, produced by numerous microorganisms, can be used to produce sustainable biobased products (Angenent *et al.*, 2004).New markets for conversion of lignocellulose to alternative bioenergy (e.g. bioethanol and biobutanl) to replace the depleting fossil fuels (Kaylen *et al.*, 2000; Lee 1997; Mitchell 1998; Wheals *et al.*, 1999).

Cellulose, hemicellulose and lignin are the main constituents of lignocellulosic agricultural wastes (Deobald and Crawford 1997).Lignocellulose is a complicated substrate and its biodegradation is not only dependent on environmental conditions, but also the degradative capacity of the microbial degradation (Waldrop et al., 2000).

Different strategies were tested for identifying microorganisms able to biodegrade lignocellulose. Microbial system found in the gut of organisms flourishing on cellulosic biomasses as their major feed. Insects like termites (Isopteran), bookworm (Lepidoptera) are found to have syntrophic symbiotic microflorain their guts responsible for cellulose biodegradation (Dillon and Dillon 2004; Sexana et al., 1993).

Various bacterial and fungal strains, both aerobic and anaerobic, have been reported with cellulosic activities (Milala *et al.*, 2005; Schwarz 2001).

Fungi and bacteria were heavily exploited for testing their abilities to produce a wide variety of cellulases. Traditionally, more focus was given to fungi because of their ability to produce high amounts of extracellular enzymes that can be easily extracted and purified for commercial use. Most fungi are considered as powerful cellulose degraders. The bioconversion of large amounts of lignocellulosic biomass into fermentable sugars has potential application in the area of bio-energy generation (Srivastava *et al.*, 2017).

The cellulosic enzyme system consists of three major components: *endo-ß-glucanase* which randomly break internal glycosidic linkages of the amorphous region of cellulose, liberating oligosaccharides of various lengths, exo-ß-glucanase catalyze the production of either cellobiose or glucose units from the reducing and non-reducing ends of cellulosic fibrils and ß-glucosidase hydrolyze cellobiose and soluble oligosaccharides into glucose (Lynd *et al.*, 2002). There are a lot of genes are responsible for cellulose degradation, for example, *eng1* which encode a high stable endo-ß-glucanase (Hong *et al.*, 2001), *exoP* which encode exo-glucanase (Nakatani *et al.*, 2010), *bgl* which encodes a thermostable β-glucosidase (Xu *et al.*, 2011).

The present work concerned with the isolation of cellulose-degrading fungi from five different samples; rotted sugar cane bagasse, rotted plant, termites, soil and animal manure. The most potent fungal isolate was identified on a molecular level according to 18S rRNA and ITS region sequence. Also, the partial sequence of the endoglucanase gene (*egl B*) was identified and sequenced.

MATERIAL AND METHODS

Isolation and Screening of Cellulose Degrading Fungi:

In this study, cellulose degrading fungi were isolated from five samples collected from different areas in Egypt (rotted sugar cane bagasse, rotted plant, termites, soil and Animal Manure). All samples were rich in cellulose. Serially diluted samples were spread on the surface of potato dextrose agar medium and incubated for 7 days at 30ºC. The Colonies were picked and sub-cultured to obtain pure culture. Stock of fungal cultures were maintained on potato dextrose agar at 4ºC (M. Charitha Devi, 2013).

Qualitative Screening of Cellulytic Activity of Isolated Fungi:

Cellulase producing fungal isolates were screened on selective carboxy metyl cellulose (CMC) (1%) agar medium containing (g/L); $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$.$7H_2O$ 0.5, KCl 0.5, carboxy methyl cellulose sodium salt 10.0, peptone 0.2 and agar 17.0. The Plates were inoculated with spore suspension of pure fungal cultures and incubated at 30 ºC. After 3 days, the plates were flooded with gram's iodine stain (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 3 to 5 minutes(Kasana *et al.*, 2008). The diameter of decolorization zone around each well was measured.

Quantitative Screening of Cellulytic Activity of Isolated Fungi:

Sugar cane Bagasse was collected from local market, washed, dried then milled to small particle sizes about 2 mm. the milled bagasse was mixed with 0.12% NaOH, then autoclaved at 121°c for 20 min. The autoclaved bagasse was washed by tap water to remove the excess of NaOH till it become neutral, then dried at 80°C (Gutierrez-Correa and Tengerdy, 1997).

The pure cultures of twenty one fungal isolates were harvested with sterile distilled water for inoculums preparation, which were used for cellulase production. The 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 ; $MgSO_4 \cdot 7H2O$, 0.3g ; peptone , 1.0g ; Tween 80, 0.2% ; $CoCl_2 \cdot 6H_2O$, 2.0mg ; $FeSO_4 \cdot 7H2O$, 5.0mg ; $MnSO_4 \cdot 2H_2O$, 1.6mg ; $ZnSO_4 \cdot 7H_2O$,

1.4mg ; Distilled water ,up to 1000 ml. the basal medium was supplemented with 1 gm of treated sugar cane bagasse. 100 ml of the production medium (pH 5.5) in 250ml Erlenmeyer flask was inoculated with 2 ml of fungal spore suspension containing 10^6 spores/ml. The flask was incubated at 30^oC with an agitation speed of 170 rpm in rotary shaking incubator. After 7 days, the culture filtrate was collected, centrifuged at 6000 rpm for 15 min and the supernatant was used as a source of crude cellulase enzyme (Vega *et al.,* 2012).

Enzymes Assay*:*

The crude enzyme extracts from all fungal isolates were assayed for CMCase and FPase activities using the method of Ghose (1987). The reaction mixture for determining CMCase activity consisted of 1 ml of appropriately diluted crude enzyme extract and 1 ml of the substrate (1% CMC prepared in 100mM sodium acetate buffer, pH 4.8). The reaction mixture for FPase assay consisted of 1 ml of enzyme and 1 ml of 100mM sodium acetate buffer (pH 4.8) containing 50mg (1x6cm) of Whatman filter paper no.1. The reaction was carried out by incubating the contents at 50°C for 30 min in case of CMCase and for 60 min in case of FPase assay. The reactions were terminated by the addition of 3 ml of dinitro Salicylic acid followed by boiling for 10 min for the development of color. Finally, the amount of released reducing sugars was determined spectrophotometrically at 540nm, by comparing the results with a glucose standard curve. The activity of enzyme was expressed in U/ml. One unit (IU) of enzyme activity is defined as the amount of enzyme required to release 1µmol of glucose in the reaction mixture per minute under specified conditions.

Optimization of Cellulase Production Using the Most Potent Fungal Isolate*:*

To select the suitable temperature and incubation period for fermentative production of the enzyme the selected fungal strains were incubated at varying temperatures ranged from 25°C - 37°C, and the incubation period ranged from 3 - 7 days, while all other parameters were kept constant.

Induction Strength of Cellulase Production by Different Carbon Sources:

Each of SCB and CMC was tested separately; a sole carbon source in a basal mineral media at 30 °C and 170 rpm for 7 days. Enzyme assay of both FPase and CMCase was determined for each carbon source. A comparison between SCB and CMC as inducer for cellulase production was carried out.

Genomic DNA Isolation, PCR Amplifications and Gene Sequencing:

Genomic DNA of S4 was isolated using GeneJET Genomic DNA Purification Kit (ThermoFisher®, California,USA). PCR-amplified with the universal primers for ITS region using ITS5 (TCCTCCGCTTATTGATATGC), ITS4 (GGAAGTAAAAGTCGTAACAAGG) and 18S rRNA using NS1 (18S) (GTAGTCATATGCTTGTCTC) and NS4 (18S) (CTTCCGTCAATTCCTTTAAG). For partial sequence of the endoglucanase gene (*egl B*), primers eglB-F (5' ATCTCAACCAAGCAGCCATT-3') and eglB-R (5' CCAGGATATCCAGCATACCC-3') were used (Mahmoud *et al.,* 2016). PCR was performed using the standard reaction mixture (50 µl) containing; 1× PCR buffer, 1.5 mM $MgCl_2$, 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 94°C; 30cycles of denaturation at 94°C for 30s; annealing at 58 °C for 30 s, and extension at 72°C for 60s; and an additional reaction for 5min at 72°C. The PCR products were detected on 0.8% agarose gel to confirm its purity, quantity, and size. The DNA sequencing reaction of PCR amplification was carried out using an ABI BigDye Terminator V3.1 cycle sequencing kit and ABI PRISM 310 genetic analyzer (Applied Biosystem, USA).

Phylogenetic Analysis:

Sequence reads were edited and assembled using the DNASTAR software (Lasergene, Madison, USA). The research of

Sequence similarity was performed at the National Center for Biotechnology Information (NCBI) server using BLASTN to identify ITS region and BLASTX to annotate the proteins encoded by endoglucanase gene. The sequences were aligned using ClustalW version 1.8 (Altschul *et al.,* 1997), and was subjected to phylogenetic analysis. Phylogenetic trees were constructed using the maximum likelihood methods (ML) in MEGA version7 (Kumar, Stecher, and Tamura 2016), and 1000 bootstrap replication to assess branching confidence. For ITS region, phylogeny was constructed based on nucleotide sequences, and many phylogenies were constructed based on amino acids sequences. Using Mega 7 software, maximum likelihood fits with 48 different amino acid substitution models were tested. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the best substitution pattern. The JTT model was selected as it gives the lowest BIC scores.

RESULTS AND DISCUSSION

Isolation and Primary Screening for Selection of Cellulose Degrading Fungi:

Out of twenty two cellulose-degrading fungal isolates, which were cultured in basal mineral medium containing sugarcane bagasse pulp as the sole carbon source 21 fungal isolates showed hydrolyzing zones on agar plates containing CMC-Na and Iodine staining. The diameters of hydrolyzing zone were listed in Table 1.

Table 1: Assessment of hydrolyzing zone of cellulose-degrading fungal isolates.

Code	clear zone	Source	Location
BEZF2	++	Sugar cane bagasse	Cairo
S2	+	Soil	Ismailia Governorate
S4	+++	Soil	Ismailia Governorate
S12	+++	Soil	Ismailia Governorate
BEZ11	++	Sugar cane bagasse	Cairo
S13	+++	Soil	Ismailia Governorate
BEZF4	+++	Sugar cane bagasse	Cairo
BEZF8	+++	Sugar cane bagasse	Cairo
BEZF3	+++	Sugar cane bagasse	Cairo
S1	++	Soil	Ismailia Governorate
TF4	+++	Termites	Ismailia Governorate
TF9	+++	Termites	Ismailia Governorate
TF1	+++	Termites	Ismailia Governorate
TF3	+++	Termites	Ismailia Governorate
TF2	+++	Termites	Ismailia Governorate
TF8	+++	Termites	Ismailia Governorate
MF1	++	Animal manure	Ismailia Governorate
TF6	+	Termites	Ismailia Governorate
TF11	-	Termites	Ismailia Governorate
TF7	+	Termites	Ismailia Governorate
TF5	++	Termites	Ismailia Governorate
TF10	+	Termites	Ismailia Governorate

" – " : **No** clear zone; " + ": clear zone from **1cm to 2 cm**; " ++ ": clear zone from **2 cm to 3 cm**; " +++ ": clear zone is**more than 3 cm.**

Enzymes Assay:

All of twenty two isolates were screened quantitatively for cellulase activity, (FPase and CMCase) using DNS method (Miller 1959). The Fungal isolates showed FPase activity in a range from (0.130 IU/ml) to (0.003 IU/ml), while the CMCase activity was ranged from (0.165 IU/ml) to (0.001 IU/ml). Fungal isolate S4 showed the highest CMCase activity with (0.165 IU/ml) in addition to (0.048 IU/ml) for FPase. As matched to previous study; *Aspergillus sp.* is

reported as a powerful fungi having cellulose degrading activity by 0.01 IU/ml for FPase and 0.6 IU/ml for CMCase. Florencio *et al.*,(2016) used 30 g/l of glucose and treated sugar cane bagasse as microbial enhancer for cellulase induction; however in this study treated sugar cane bagasse was only used as sole carbon source.

Table 2: Assessment of activity of both Exoglucanase (FPase) and Endoglucanase (CMCase) of the cellulose degrading isolates.

Strain	FPase activity (IU/ml)	CMCase activity (IU/ml)
S4	0.048	0.165
TF2	0.085	0.096
S12	0.099	0.092
S1	0.050	0.083
BEZF4	0.130	0.080
TF9	0.020	0.076
TF1	0.038	0.076
MF1	0.025	0.073
TF6	0.045	0.071
BEZ11	0.088	0.070
TF4	0.038	0.069
TF7	0.034	0.064
TF5	0.028	0.061
S13	0.068	0.056
S2	0.085	0.043
BEZF3	0.010	0.030
TF10	0.003	0.025
BEZF2	0.053	0.004
TF8	0.006	0.004
TF3	0.006	0.002
BEZF8	0.062	0.001

Effect of Temperature and Incubation Time on the Activity of Fpase and Cmcase Using the Isolate S4 :

As shown in the results of Figures 1 and 2, the best activity of both FPase and CMCase was obtained at 30 °C after 7 days. The activity of FPase and CMCase was declined when the incubation temperature and incubation time were not optimal. Santos *et al.*, (2017) reported that the optimum temperature for *Aspergillus sydowii* CBMAI 934 was 30 °C and the optimum incubation time was 7 days, which matches with the results in this study.

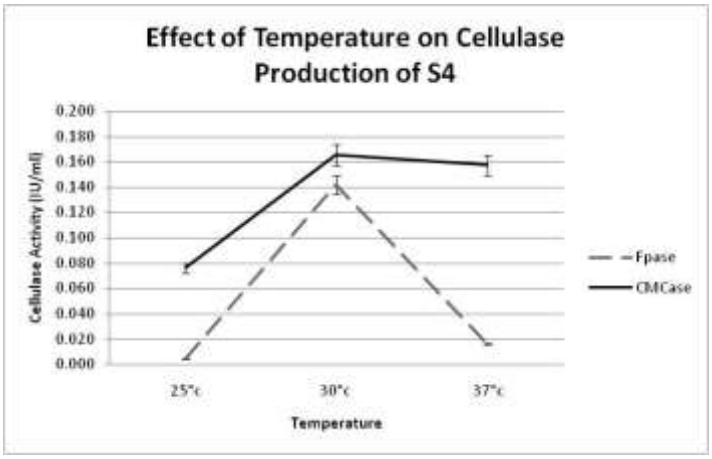

Fig. 1: Effect of Temperature on Cellulase Production using fungal isolate S4.

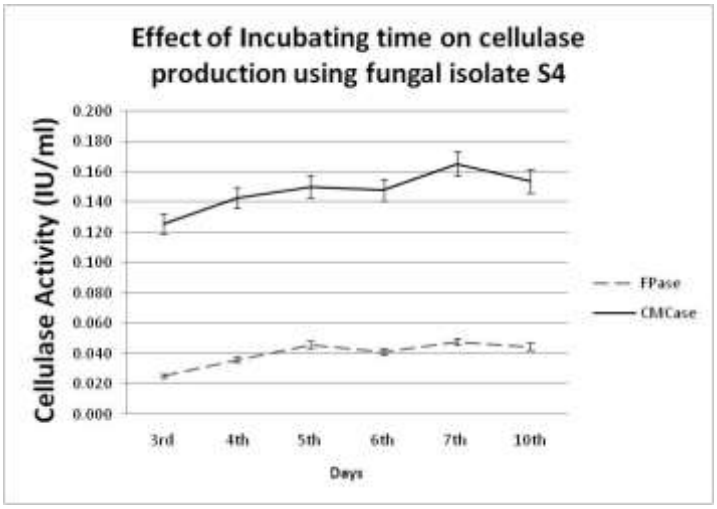

Fig. 2: Effect of incubating time on cellulase production using fungal isolate S4.

In the same study *Aspergillus sydowii* CBMAI 935 showed an optimum temperature at (40 °C) after 3 days of incubation time. These indicate that the optimum factors for optimum cellulase yield are variable on the level of strain.

Induction Strength of Cellulase Production by Different Carbon Sources:

As shown in the results of Figure, (3), there is an increasing of FPase production in case of SCB more than CMC with 3.6 folds using fungal isolate S4. The results showed that cellulase enzymes are produced by fungal isolate S4, whether it has grown on natural source like SCB or artificial source like CMC. Also, CMCase production showed more activity in SCB than CMC with 2.2 folds using fungal isolate S4 (Figure. 3). In the previous studies, SCB also showed a powerful induction for cellulase activity more than CMC reported by (Padilha *et al.*, 2015 and Liang *et al.*, 2014). On the other hand, another study reported that CMC induce cellulase enzyme more than SCB (Belal, 2013). SCB showed more induction for cellulase enzymes than CMC. This indicated that natural cellulose induces cellulase production more than synthetic cellulose.

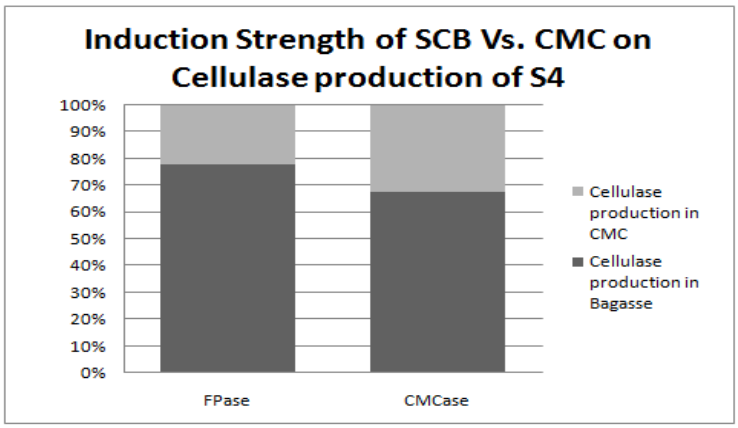

Fig. 3: Induction Strength of SCB Vs. CMC for Cellulase production of using fungal isolate S4.

Identification of Selected Isolate S4 :

Nearly full-length 18S rDNA gene (1,027 bp) and ITS region (596 bp) were successfully amplified and sequenced (Figure, 4). The 18SrRNA and the ITS sequences were submitted to the GenBank under accession number LC385526.1 and LC385527.1, respectively. The maximum likelihood (ML) phylogenetic trees based on 18S and ITS were generated to determine the evolutionary relatedness of the fungal isolate S4.

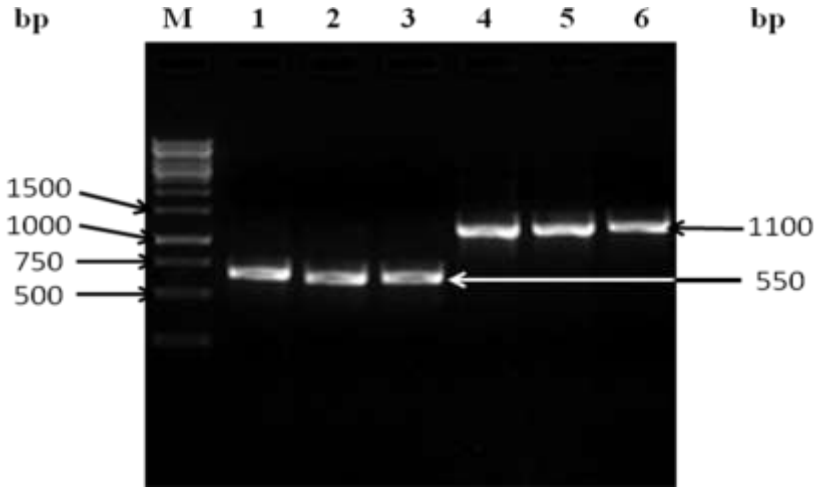

Fig. 4: PCR amplification of NS and ITS genes for sample (S4).
M: ladder 1kbp DNA (thermoscientific), lanes 1 to 3 are triplicate of ITS region and lanes 4 to 6 are triplicate of 18SrRNA.

According to the sequences similarity, the S4 isolate is closely related to the defined species within the genus *Aspergillus*. ML tree based on 18rRNA sequences confirmed the high degree of similarity of S4 isolate with *Aspergillus flavus* strain azm015 (Accession number MH107055.1), *Aspergillus oryzae* strain 112822 (Accession number EU583496.1), *Aspergillus oryzae* strain RIB40 (Accession number XR2735719.1), and *Aspergillus flavus* strain TZ1985 (Accession number GU953210.1) (Figure 5).

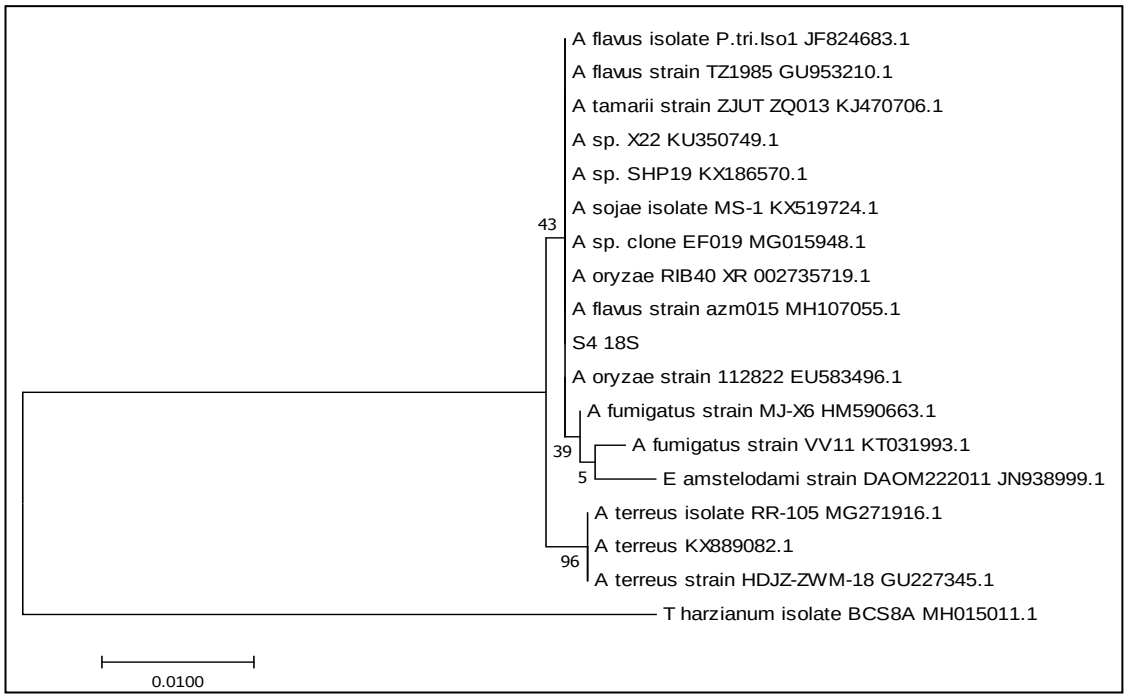

Fig. 5: ML tree based on 18S rRNA sequences, showing the relationships among cellulose-degrading fungi and recognized species of the genus *Aspergillus*. Bootstrapvalues are indicated for each node (1000 replicates). Sequence retrieved in this study is shaded. Abbreviations used, A: *Aspergillus*; S4: the promising strain of this study.

ML tree based on ITS sequences showed a similar pattern to the 18SrRNA phylogenic tree. The fungal isolate S4 showed a similarity to *Aspergillus flavus* strain F (Accession number MG857638.1), *Aspergillus oryzae* strain Ao-1 (Accession number MH127461.1), Aspergillus oryzae strain RIB40 (Accession number AP007173.1), and *Aspergillus flavus* strain JT1 (Accession number MG734749.1) (Figure 6). The use of sole molecular marker has been criticized because of the possibility of genetic recombination and horizontal gene transfer and low phylogenetic power (Gomes Germano *et al.*, 2006; Santillana *et al.*, 2008). The two phylogenic trees showed a similar pattern. The fungal isolate S4 was identified as *Aspergillus flavus*.

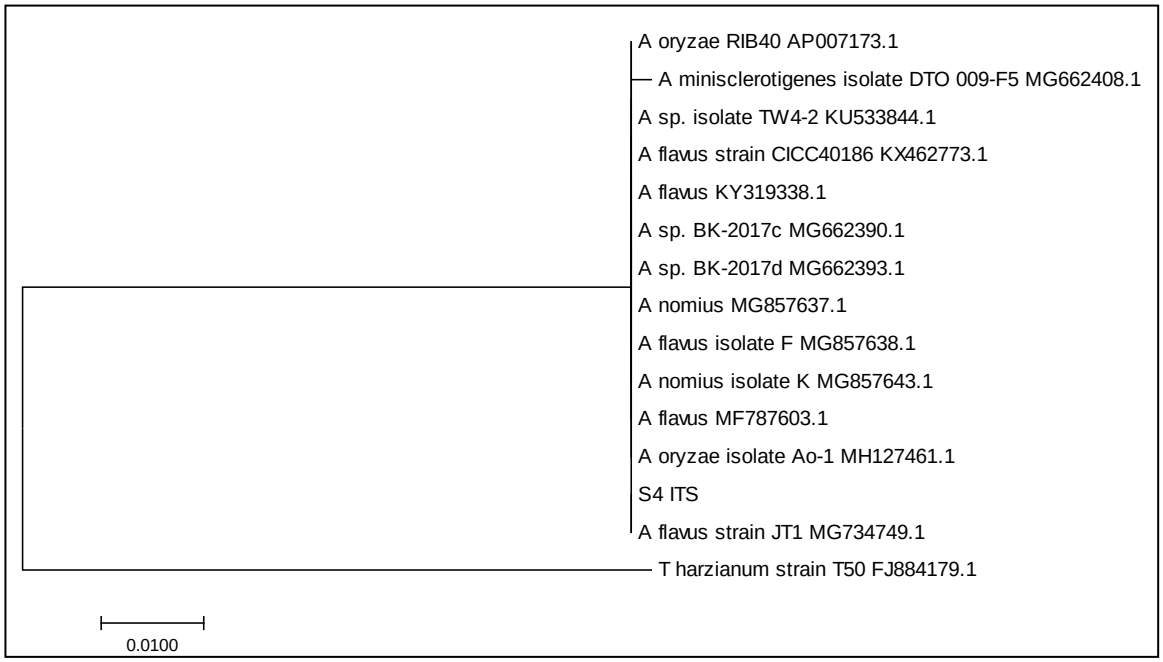

Fig. 6: ML tree based on ITS sequences, showing the relationships among cellulose-degrading fungi and recognized species of the genus *Aspergillus*. Bootstrapvalues are indicated for each node (1000 replicates). Sequence retrieved in this study is shaded. Abbreviations used, A: Aspergillus; S4: the promising strain of this study.

Characterization of Endoglucanase Gene of S4 Strain:

Endoglucanse gene of the fungal isolate S4 was amplified using specific primers (Mahmoud *et al.*, 2016). For *Aspergillus sp., eglB* gene was amplified with 356 bp (Figure, 7); the sequence of the amplified gene was submitted to the GenBank under accession number LC385522.1.

As shown in the results of Figure, (8) ML tree based on concatenated protein eglb sequences confirmed the high degree of similarity of fungal isolate S4 with *Aspergillus oryzae* strain RIB 40 (Accession number XP 001818463.1), *Aspergillus oryzae* strain (Accession number BAD72778.1). The phylogenic tree showed that, isolated eglb of the promising strain was identified as a gene of endoglucanase gene, which belong to glycosyl hydrolase family.

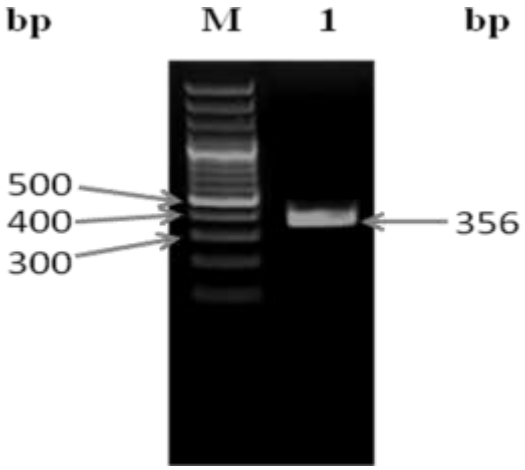

Fig. 7: PCR amplicon of *eglB* gene, lane M (ladder 100 bp plus DNA), and lane 1 (eglB gene of S4).

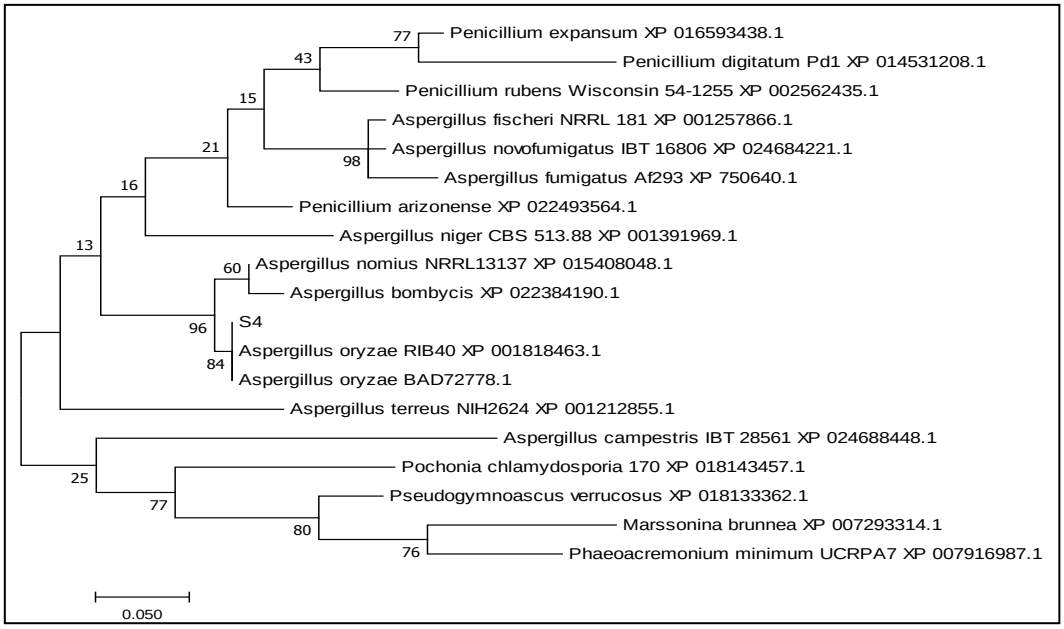

Fig. 8: ML tree based on concatenated protein eglb sequences, showing the relationships among cellulose-degrading fungi and recognized species of the genus *Aspergillus*. Bootstrapvalues are indicated for each node (1000 replicates). Sequence retrieved in this study is shaded. Abbreviations used, S4: the promising strain of this study.

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