CHITINASE AND CELLULASE GENES SEQUENCING FOR SOME EGYPTIAN *Trichoderma species* ISOLATED FROM RHIZOSPHERE AND ASSAY OF THEIR ACTIVITY

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Efficient biocontrol strains of the genus *Trichoderma* are being developed as promising biological control agents against plant fungal pathogens based on different mechanisms, such as the production of cell-wall degrading enzymes like chitinases, glucanases and proteases, and utilize the contents of the host hyphae as nutrient source. These biocontrol genes can be easily isolated from chitinolytic strains of *Trichoderma* and characterized (Andre and Monika, 2010).

Chitinases are chitin-degrading enzymes that hydrolyze the β-1, 4-glycosidic bonds between the N-acetyl glucosamine residues of chitin and are widely distributed in nature (Kitamura and Kamei, 2003). *Trichoderma* species are specifically evolved to attack other fungi attributed chiefly due to their ability to produce highly effective chitinases. Different endochitinases, CHIT31, CHIT33 and CHIT36, are produced by various strains of *T. harzianum* which differ in their molecular weight and structure (Viterbo *et al*., 2002; Markovich and Kononova, 2003).

CHIT36 is an endochitinase identified in strain *T. harzianum* TM, which is a 36-kDa protein belongs to the glycoside hydrolase family 18. Homology studies showed that it has 79% nucleotide and 89% amino acid similarity to CHIT37 from *T. harzianum* strain CECT 2413 but without significant similarity to other known *Trichoderma* endochitinases. Expression analysis revealed that *chit36* gene is induced by conditions of stress, colloidal chitin and *N*-acetyl-glucosamine (Sarma *et al*., 2012).

Depending on the strain, the chitinolytic system of *T. harzianum* may contain five to seven individual enzymes (Haran *et al.*, 1995). In the well characterized strain *T. harzianum* TM, this system comprises two β- (1, 4) -N - acetylglucosaminidases (102 and 73 kDa), four endochitinases (52, 42, 33 and 31 kDa), and one exochitinase (40 kDa) (Lorito *et al*., 1993; Haran *et al.*, 1996). The amino acid analysis of endochitinase shows that it belongs to glycosyl hydrolases family 18 which contains two conserved motifs, chitinase family active site ([LIVMFY]- [DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-X-E) and chitin binding

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domain (XXXSXGG) (Terwisscha *et al*., 1996; Renkema *et al*., 1998). In *Trichoderma* whole-gene sequencing reveal between 20 and 36 different genes encoding chitinases, hydrolytic enzymes that are involved in the mycoparasitic attack. *Trichoderma* chitinases chi18-13 and chi18-15 evolve in a manner consistent with rapid co-evolutionary interactions and identifies putative target regions involved in determining substrate-specificity (Ihrmark *et al*., 2010). The chitinase gene (Chi36) of *Trichoderma* strain T53 was amplified by RT-PCR techniques and the sequence analysis of Chi36 gene showed a cDNA of 1,035 nucleotides encoding 344 amino acids (Shih, 2010). Some *Trichoderma* species are very good cellulase producers and therefore they are important for the biotechnological industry. *Trichoderma* offer the complete set of cellulases that are able to hydrolyze lignocellulosic agriculture wastes to glucose, as it could be fermented to many economically important chemicals (Hassan and El-Awady, 2011).

PCR-based technology utilizing pairs of primers specific for CBH I and II genes showed that transcripts of CBH1 were detected when *Trichoderma pseudokoningii* was grown on sophorose and cellulose, but not when grown on glucose, however, CBH II transcripts were detected in cells under all conditions. These results suggest that CBH I is inducible cellulase and the CBH II is expressed at low constitutive level (Wang and Gao, 1999).

Kraková *et al.* (2012) were used a novel PCR-based approach for the detection and classification of potential cellulolytic fungal strains and they found that, the CBH-PCR method demonstrated its discrimination power, and it can be considered as a new molecular system suitable for the classification of fungal strains isolated from different environments. The aim of this study was to determine the chitinase and cellulase activity product by the isolated *Trichoderma spp* as well as detecting their sequences genes using RT-PCR.

MATERIALS AND METHODS

Trichoderma species

The chitinolytic enzymes producing *Trichoderma* was previously isolated by Hassan *et al*. (2014) from rhizosphere of bean, cowpea, cucumber, wheat and faba bean plants and it was identified as *T. koningii* and *T. harzianum*. The GenBank accession number for the ITS region of rDNA sequence of *Trichoderma* strains is KC200070, KC200071, KC200073, KC200074 and KC200075 for *Trichoderma koningii* FUE3, *T. koningii FUE5*, *T. koningii FUE6*, *T. koningii FUE9* and *T. harzianum FUE15*, respectively.

Production and assay of chitinase activity

For chitinase production, *Trichoderma* strains were grown on PDA plates. Three agar discs inoculum of 5 mm

for each strain were grown in 200 ml of Czapeck-Dox medium supplemented with 10% glucose in 500 ml flask and incubated for 96 h at 25°C. The mycelium was harvested by filtration through filter paper and washed several times with 2% of $MgCl_2$ and distilled water transferred to Czapeck-Dox medium supplemented with 1.5% colloidal chitin and incubated at 100 rpm on a rotary shaker for 96 h at 25°C. The spectrophotometric assay of chitinase was carried out according to the procedure developed by Ulhoa and Peberdy (1991), using N-acetyl-D-glucosamine (GlcNAc) as standard with minor modifications. One ml of fungal filtrate was incubated with 1 ml of 0.5% colloidal chitin for 24 h at 40°C with shaking, the mixture will centrifuged at 4000 rpm for 5 min. one ml of fungal filtrate was boiled for 3 min at 100°C and mixed with 1 ml of 0.5% colloidal chitin was prepared according to Mathivanan (1995) for used as negative control. The mixture will centrifuged at 4000 rpm for 5 min. and 1.5 ml of the supernatant was added to 1.5 ml of the modified reagent (dinitrosalicylic acid 1%, phenol 0.2%, sodium sulfite 0.05% and sodium hydroxide 1%.). The mixture was heated for 5 min in a boiling water bath and 0.5 ml of 40% Rochelle salt (potassium sodium tartrate solution) was added to stabilize the color. After cooling to room temperature (using running tap water), the color intensity was measured using a spectrophotometer at 575 nm. One unit of enzyme activity was defined as the amount of enzyme required for the formation of 1 µ mole of the N-acetyl-glucosamine in one ml of the reaction, under the standard assay conditions (Mathivanan *et al*., 1998).

Production and assay of cellulase activity

The strains of *Trichoderma*, which versatility of chitinase and cellulase production was used. The mycelia from these strains previously grown for 96 h in 200 mL of Czapeck-Dox medium supplemented with 10% glucose in 500 mL flask. Harvested mycelia were washed several times with 2% of $MgCl_2$ and distilled water transferred to Czapeck-Dox medium supplemented with 1% carboxymethyl cellulose. The culture medium was incubated at 100 rpm on a rotary shaker at 25°C. After the incubation period (six days), the mycelium was harvested using centrifugation. Activity of cellulases was assayed by incubating 0.5 ml of culture filtrate with 1 mL 0.5 mM sodium citrate buffer pH 4.8 and 1 mL of 1% (w/v) carboxymethyl cellulose at 50°C for 10 min (Melo *et al*., 1997). The reaction was stopped by addition of alkaline dinitrosalicylic acid (Miller *et al*., 1960), and absorbance was read at 540 nm.

RNA isolation from Trichoderma species

For RNA isolation, *Trichoderma* strain was grown in 250 ml shacking flasks containing 150 ml Czapek-Dox medium supplemented with 10% glucose at 200 rpm on a rotary shaker for 96 h at 28°C. Mycelia were collected after 96 hours by Whatman (No. 1) filter paper and washed several times by (2%) $MgCl_2$ and then inoculated into Czapek-Dox medium supplemented with 1.5% colloidal chitin

(De la Cruz and Liobell, 1999). Cells were harvested after 42 hours of growth and frozen in liquid nitrogen. Frozen mycelium was ground to a fine powder and suspended in 5 volumes of guanidine isothiocyanate, 0.5% Na-lauryl sarcosinate, 25 mM sodium citrate pH 7.0 and 0.1 M ß-mercaptoethanol (Sambrook and Russell, 2001). The isolated RNA was treated with DNaseI and purified by the RNeasy Mini Elute Cleanup kit 1741985 (Roche).

Designing of primers for Trichoderma chitinase and cellulase sequences

Primers were designed for coding the genes of *Trichoderma* chitinase and cellulase, these primers designed and produced by AUGCT Company, China according to the sequence of chitinase (chit36) and Cellobiohydrolase (cbh1) sequence of *Trichoderma* spp, which available from GenBank database of NCBI site. Various *Trichoderma* spp. chitinase and cellulase sequences were downloaded and multiple sequence alignment was done by using CLUSTAL X1.81 software. From the multiple sequence alignment, degenerate primers were designed based on the consensus sequences obtained. The degenerative primers designed used for chitinase and cellulase genes amplification were listed in Table (1).

RT-PCR detection of chitinase and cellulase genes using specific primer

Reverse transcription (RT)-PCR was performed using one step RT-PCR kit, produced by Biotech corporation, China in an Eppendorf thermal cycler using a 2X power Taq Master mix for 25 cycles per reaction with the gene-specific primers listed in Table (1). PCR product (8 µl) was mixed with loading buffer (2 µ1) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis (Viterbo *et al*., 2002).

Sequencing of the chitinase and cellulase gene

Each chit36 and cellobiohydrolase (CBH) amplicons obtained from specific PCR was separated in 2.5% agarose gel and added ethidium bromide for running buffer. The expected chitinase bands (1039 bp, one band per strain) and the expected cellobiohydrolase bands 204 bp, one band per strain were excised from the gel and DNA was eluted using QIAquick Gel Extraction Kit (Qiagen). Sequencing products were resolved on HiSeq 2000/2500 (Macrogen, a sequencing company). The obtained Chit36 and Cbh1 sequences were compared directly with those in GenBank by BLAST search (http://blast.ncbi.nlm.nih.gov/ Blast. cgi). The contigs were constructed with http://pbil.univ-lyon1.fr/cap3.php**.** The nucleotide sequences were translated with Xpasy program.

Sequence alignment and phylogentic analysis

Chitinase and cellulase sequences from different *Trichoderma* strains were retired from uniprot database

(http://www.pir.uniprot.org). Multiple sequence alignment was generated using Clustal W (http://www.ebi-ac.uk/ Clustal W). Computer analysis of the sequences was carried out and the deduced amino acid sequence from each Chit36 and Cbh1 genes was obtained by BLASTP Network Service (NCBI) and alignment of this amino acid sequence was done by clustal method (Larkin *et al*., 2007).

RESULTS AND DISCUSSION

Chitinase production by Trichoderma

The results presented in Table (2) showed that *Trichoderma* strains I18, FUE9, FUE6, FUE3, FUE5 and FUE15 exhibited significant activities of chitinase compared to control. Chitinase produced by some *Trichoderma* species are the key enzyme in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi. These enzymes may be important in the destruction of plant pathogens and could be used as the basis of screening for potential biocontrol agents (De La Cruz *et al*., 1992). Among fungal chitinases, three types of enzymes are recognized based on their action on chitin substrates (Sahai and Manocha, 1993). These chitinolytic enzymes are exochitinase, endochitinase and N-acetylglucosaminidase. The combined action of all these chitinolytic enzymes can degrade chitin to its monomers, N-acetylglucosamine.

Cellulase production by Trichoderma

Results presented in Table (3) indicated that the maximum amount of cellulase 0.075 mg/ml excreted by FUE5 strain. On the other hand, the minimum amount of cellulase 0.024 mg/ml excreted by FUE15 strain. Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity (Tomme *et al*., 1988; Lynd *et al*., 2005). Since the cellulase enzyme was a complex enzyme, so, there were many genes to code cellulase enzyme. The enzymatic degradation of waste cellulose by cellulolytic microorganisms has been suggested as a feasible alternative for the conversion of cellulosic wastes in to fuel ethanol, (Lowe *et al*., 1987). Microorganisms of the genera *Trichoderma* and *Aspergillus* are thought to be cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use. However, attempts to use these enzymes in the degradation of cellulytic wastes have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition of the enzymes.

Lynch *et al.* (1981) found that *Trichoderma* produced cellulase, β-(1-3)-glucanase and chitinase enzymes and degraded the glucans in the walls of the plant pathogens.

RT-PCR detection of chitinase gene using specific primer

The first step in the isolation of chitinase gene from *Trichoderma* strains was the amplification of the cDNA with the primers which designed according to the sequence of chit36 (accession number AY028421). One fragment with an ap-

proximately length of 1039 bp was amplified from *Trichoderma* DNA of strains FUE3, FUE5, FUE6, FUE9, FUE15 and I18 (Fig. 1) and it was observed that specific band appeared in all tested *Trichoderma* strains. Upon DNA sequencing analysis, the full length chitinase gene isolated from *Trichoderma* strains had 1039 bp, encoding 344 amino acids.

Sequence alignment and phylogentic analysis of the chitinase gene

A homology search utilizing the computer program Blast p, revealed high homology between *Trichoderma* strains chit36 and several chi36 gene of other fungi, including ABG56440-*T. asperellum* (Sulistyowati *et al.*, 2005), ABC48784-*T. asperellum* (Severgnini, 2006), AAL01372- *T. harzianum* (Viterbo *et al*., 2002) and ABO14715-*T. atroviride* (Yazdanpanah *et al*., 2007) higher than 96%. The 1032 bp of chi36 gene encodes for 344 amino acids. The GenBank accession number for the chit36 sequence of *Trichoderma* is KC333417, KC333418 and KC333419 for *Trichoderma koningii* FUE3, *T. koningii* FUE5 and *T. koningii* FUE9, respectively.

Multiple alignment of the deduced amino acid sequence with related fungal proteins was performed with the CLUSTAL W 2.1 program. *Trichoderma* strains chit36 was compared with the previously reported chitinase sequences of ABG56440-*T. asperellum*, ABC48784-*T. asperellum*, AAL01372-*T. harzianum* and ABO14715-*T. atroviride* (Fig. 2). The sequences of these chitinases were obtained from the GenBank with the following accession numbers {ABO14715-*T. atroviride*, ABG56440- *T. asperellum*, ABC48784-*T. asperellum*, AAL01372-*T. harzianum*, *T. koningii*-I18, *T. koningii*-FUE3, *T. koningii* -FUE5 and *T. koningii* -FUE9}.

The dendrogram of *Trichoderma* species was divided into two clusters (Fig. 3). The first cluster included I18 strain, while the second cluster was divided into two sub-clusters. The first sub-cluster was divided into two sub-subclusters. The first sub-subcluster included ABG56440-*T. asperellum* and ABC48784-*T. asperellum* species. The second sub subcluster included ABO14715-*T. atroviride*, *T. koningii*-FUE3, *T. koningii*-FUE5 and *T. koningii*-FUE9 strains. The second subcluster included AAL01372-*T. harzianum* strain.

Through sequence analysis, chi36 can be grouped in the glycoside hydrolase family 18. The GH18 (glycosyl hydrolase, family 18) type II chitinases hydrolyze chitin, an abundant polymer of beta-1, 4-linked N-acetylglucosamine (GlcNAc) which is a major component of the cell wall of fungi and the exoskeleton of arthropods. The structure of the GH18 domain is an eight-stranded beta/alpha barrel with a pronounced active-site cleft at the C-terminal end of the beta-barrel (Karlsson and Stenlid, 2008).

RT-PCR detection of cellulase gene (CBH) using specific primers

The first step in the isolation of cellulase gene from *Trichoderma* strains was the amplification of the cDNA with

the primers were designed according to the sequence of cbh1 (accession number AFD01232). One fragment with an approximately length of 204 bp was amplified from *Trichoderma* DNA of FUE15 strain (Fig. 4) and it was observed that specific band appeared in tested *Trichoderma* species for cellulase gene. Upon DNA sequencing analysis, the length partial cellulase gene isolated from *Trichoderma harzianum* FUE15 strain had 204 bp, encoding 68 amino acids.

Cellulase is a simple, linear polymer built up from glycosyl units connected by β (1-4) linkages. These linear chains can vary in length and often consist of many thousands of units. Within the biosphere, there is an enormous of cellulose through large-scale production and degradation. Though chemically simple, cellulose is physically complex with both crystalline and amorphous regions and a number of different enzymes are required for its efficient hydrolysis. Cellulose is degraded in nature by the concerted action of several synergistically functioning enzymes. Depending on their mode of action, cellulolytic enzymes fall into one of two main groups, endogluconase or cellobiohydrolase. The complete degradation of cellulose to glucose requires the action of at least three types of enzymes: endo-ß-1, 4-glucanase, exo-ß-1, 4-glucanase (cellobiohydrolase) and ß-glucosidase (Sarah *et al*., 2007).

The PCR assay described by Aro *et al.* (2005) was able to detect in a rapid way the potential cellulolytic ability of fungal strains isolated from cultural heritage items. This assay is based on the amplification of the cellobiohydrolase (cbh-I) gene. The cbh genes are widely spread in fungal kingdom both in ascomycota and basidiomycota.

Sequence alignment and phylogentic analysis of the cellulase gene

Multiple alignment of the deduced amino acid sequence with related fungal proteins was performed with the CLUSTAL W 2.1 program. *Trichoderma harzianum* FUE15 isolates cbh1 was compared with the previously reported cellulase sequences of AFD01232-*T. harzianum* and ADH04808-*T. harzianum* (Fig. 5).The results showed that, using pairwise alignment, the homology found between the *T. harzianum* FUE15 cbh-1 and celullase protein from AFD01232-*T. harzianum* was 97.1%. The homology between the FUE15-*T. harzianum* and the ADH04808-*T. harzianum* (94.2%). The less homology between the AFD01232-*T. harzianum* and ADH04808-*T. harzianum* was 94.06%.

The dendrogram of *Trichoderma strains* was divided into two clusters (Fig. 6). The first cluster included *T. harzianum* FUE15 and AFD01232-*T. harzianum*, while the second cluster included ADH04808-*T. harzianum.*

As previously mentioned, there were three types of cellulases that are endoglucanases, cellobiohydrolases and β-glucosidases. Thus the cellulase enzyme was a complex enzyme, not a single one,

there were many genes to code cellulase proteins (Li *et al*., 2010).

SUMMARY

Hydrolytic enzyme producing *Trichoderma* species have long been recognized as an agent for controlling plant diseases caused by various phytopathogenic fungi. A study was intended to identify highly chitinase and cellulose isolated *Trichoderma* producer from the rhizosphere. Chitinase gene isolated from *Trichoderma* strains FUE3, FUE5, FUE6, FUE9, FUE15 and I18 had one fragment with length of 1039 bp, encoding 344 amino acids. Cellulase gene isolated from *Trichoderma* strais FUE15 had one fragment with length of 204 bp, encoding 68 amino acids. The results presented showed that *Trichoderma* strains I18, FUE9, FUE6, FUE3, FUE5 and FUE15 exhibited significant activities of chitinase compare to control. The high potent cellulase producer was detected by FUE5 being 0.075 mg/ml whereas, the lowest figure produced by FUE15 being 0.024 mg/ml.

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Table (1): Primers used in RT-PCR amplification of chitinase and cellulase genes and their sequences.

Primer name	Sequence	Product size	Accession no.
chit36 F chit36 R	5'-CATGACACGCCTTCTTGACG-3' 5'-ATTTCTAACCAATGCGAGTAAGC-3'	1039 bp	AY025421
Cbh1 F Cbh1 R	5'-CAGTCACCCAACTCCAAGGT-3' 3'- GCTAGCGCATCTGGTAGGTC -5'	204 bp	AFD01232

Table (2): Specific activity of chitinase produced by *Trichoderma* culture filtrate.

No.	Protein concentration (g/dl)	Chitinase activity (mg /ml) (Mean ± SE)	Specific activity (u/g protein)
C*	1.055	0.0053± 0.0007 ^{F}	0.0050
I18**	1.000	0.0180±0.0000 ^{A}	0.0180
FUE9	0.950	0.0170±0.0012 ^{AB}	0.0179
FUE5	0.925	0.0147±0.0015 ^{ABCDE}	0.0159
FUE6	0.955	0.0150± 0.0000 ^{ABC}	0.0157
FUE3	1.015	0.0147± 0.0015^{ABCDE}	0.0145
FUE15	0.980	0.0137±0.0009 ^{BCDEF}	0.0140

* Boiled fungal filtrate ** Reference strain *T. koningii*

Table (3): Specific activity of celullase produced by *Trichoderma* culture filtrate.

No.	Protein concentration (g/dl)	Celullase activity(mg /ml) (Mean ± S. E)	Specific activity (u/g protein)
C*	1.738	0.005± 0.0006 ^{E}	0.003
I18**	1.999	0.029 ± 0.0050 ^{BC}	0.015
FUE5	1.824	0.075± 0.0049 ^{A}	0.041
FUE9	1.914	0.035± 0.0009 ^{B}	0.018
FUE3	1.949	0.029± 0.0029 ^{BC}	0.015
FUE6	1.939	0.028± 0.0006 ^{BCD}	0.014
FUE15	1.939	0.024± 0.0009 ^{CDE}	0.012

* Boiled fungal filtrate ** Reference strain *T. koningii*

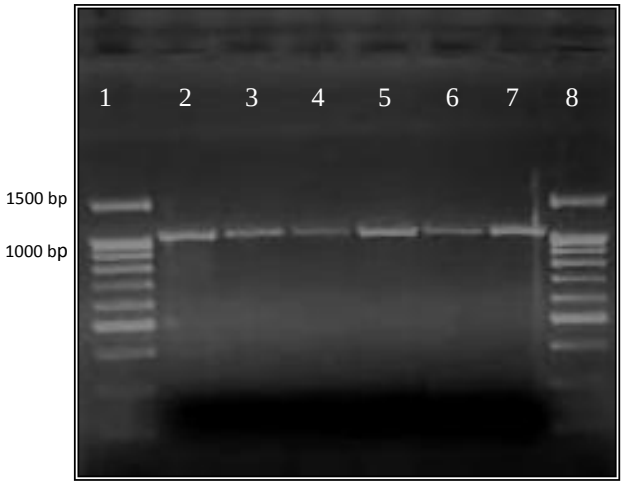

Fig. (1): Agarose gel analysis of specific-PCR products from amplification of chitinase gene of *Trichoderma* isolates. Lane 1- 100 bp DNA Ladder, Lane 2-FUE3, Lane 3- FUE5, Lane 4-FUE6, Lane 5- FUE9, Lane 6- FUE15, Lane 7-I18 and Lane 8- 100 bp DNA Ladder.

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|ABG56440.1|T.asperellum MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
|ABC48784.1|T.asperellum MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
|AAL01372.1|T.harzianum  MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
|ABO14715.1|T.atroviride MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
FUE3-T.koningii         MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
FUE9-T.koningii         MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
FUE5-T.koningii         MTRLLDASFLMLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
I18-T.koningii          MTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDG
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|ABG56440.1|T.asperellum AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
|ABC48784.1|T.asperellum AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
|AAL01372.1|T.harzianum  AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
|ABO14715.1|T.atroviride AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
FUE3-T.koningii         AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
FUE9-T.koningii         AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
FUE5-T.koningii         AANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDT
I18-T.koningii          SANGVHPGFGWTPIENPIIKQNGYNVINAAFPVILSDGTVLWENDMAPGT
                        :************************************.********.*

|ABG56440.1|T.asperellum QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
|ABC48784.1|T.asperellum QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
|AAL01372.1|T.harzianum  QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
|ABO14715.1|T.atroviride QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
FUE3-T.koningii         QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
FUE9-T.koningii         QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
FUE5-T.koningii         QVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQ
I18-T.koningii          QVATPAEMCAAKAAGATILLSIGGATAGIDLSSSTVADKFIATIVPILKQ
                        ********* ************************:***************

|ABG56440.1|T.asperellum YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
|ABC48784.1|T.asperellum YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
|AAL01372.1|T.harzianum  YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
|ABO14715.1|T.atroviride YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
FUE3-T.koningii         YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
FUE9-T.koningii         YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
FUE5-T.koningii         YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNFGLTMA
I18-T.koningii          YNFDGIDIDIETGLVNSGNIKTLSTSQANLIRIIDGVLAQMPSNFGLTMA
                        **************.*****:******:**********************

|ABG56440.1|T.asperellum PETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
|ABC48784.1|T.asperellum PETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
|AAL01372.1|T.harzianum  LETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
|ABO14715.1|T.atroviride PETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
FUE3-T.koningii         PETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
FUE9-T.koningii         PETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
FUE5-T.koningii         PETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
I18-T.koningii          PETAYVTGGSITYGSIWGSYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSG
                         *****************:*******************************

|ABG56440.1|T.asperellum DSYAAGTVQGFIAQTDCLNAGLTIQGTTIKVPYDMQVPGLPAQSGAGGGY
|ABC48784.1|T.asperellum DSYAAGTVQGFIAQTDCLNAGLTIQGTTIKVPYDMQVPGLPAQ-------
|AAL01372.1|T.harzianum  DSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGY
|ABO14715.1|T.atroviride DSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGY
FUE3-T.koningii         DSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGY
FUE9-T.koningii         DSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGY
FUE5-T.koningii         DSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGY
I18-T.koningii          DSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGY
                                  **********************:*******************

|ABG56440.1|T.asperellum MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTFGDNLLTRIG
|ABC48784.1|T.asperellum ----------------------------------------------
|AAL01372.1|T.harzianum  MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTLGDNLLTRIG
|ABO14715.1|T.atroviride MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTFGDNLLTRIG
FUE3-T.koningii         MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTFGDNLLTRIG
FUE9-T.koningii         MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTFGDNLLTRIG
FUE5-T.koningii         MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTFGDNLLTRIG
I18-T.koningii          MNPSLVGQAWDHYNGALKGLMTWSINWDGAGNWTFGDNLLTRIG
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Fig. (2): Protein sequence alignment of *Trichoderma* chitinase gene using Clustal W 2.1 multiple sequence alignment.

An * (asterisk) indicates positions which have a single, fully conserved residue.

A: (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

A: (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

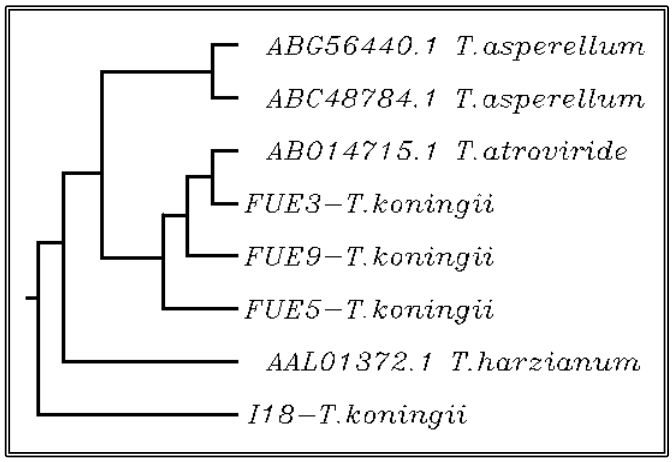

Fig. (3): Phylogenetic tree showing the relationship between *chi36*gene from *Trichoderma* strains. The tree was constructed using the Clustal w 2.1 multiple sequence alignment programs.Rooted phylogentic tree (UPGMA).

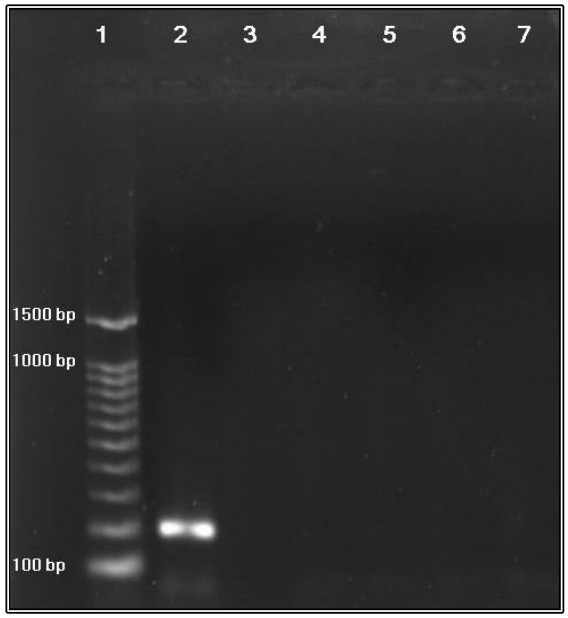

Fig. (4): Agarose gel analysis of specific-PCR products from amplification of cellulase gene of *Trichoderma* strains. Lane 1- 100 bp DNA Ladder, Lane 2 - FUE15, Lane 3 - FUE3, Lane 4 - FUE5, Lane 5 - FUE6, Lane 6 - FUE9 and Lane 7 - I18.

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|AFD01232.1|T.harzianum  ----------------MQQVCTQQAETHPPLTWQKCTASGCTAQSGSVVL
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  MYRKLAAISAFLAAARAQQVCTQQAETHPPLTWQKCTASGCTAQSGSVVL

|AFD01232.1|T.harzianum  DANWRWTHDTKSTTNCYDGNTWSSTLCPDDATCAKNCCLDGANYSGTYGV
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  DANWRWTHDTKSTTNCYDGNTWSSTLCPDDATCAKNCCLDGANYSGTYGV

|AFD01232.1|T.harzianum  TTSGDALTIQFVTQSNVGSRLYLMATDTTYQEFTLSGNEFSFDVDVSQLP
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  TTSGDALTLQFVTASNVGSRLYLMANDSTYQEFTLSGNEFSFDVDVSQLP

|AFD01232.1|T.harzianum  CGLNGALYFVSMDADGGKSKYPGNAAGAKYGTGYCDSQCPRDLKFINGQA
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  CGLNGALYFVSMDADGGQSKYPGNAAGAKYGTGYCDSQCPRDLKFINGQA

|AFD01232.1|T.harzianum  NVDGWQPSSNNANTGIGNHGSCCSEMDIWEANSISEALTPHPCEDVGQTM
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  NVEGWEPSSNNANTGVGGHGSCCSEMDIWEANSISEALTPHPCETVGQTM

|AFD01232.1|T.harzianum  CSGDSCGGTYSDDRYGGTCDPDGCDWNPYRLGNTSFYGPGSSFTLDTTKK
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  CSGDACGGTYSNDRYGGTCDPDGCDWNPYRLGNTSFYGPGSSFALDTTKK

|AFD01232.1|T.harzianum  LTVVTQFATNGAISRYYVQNGVKFQQPNAQVGSYSGNTINADYCAAEQTA
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  LTVVTQFATDGSISRYYVQNGVKFQQPSASVGSYTGNTINTAYCAAEQTA

|AFD01232.1|T.harzianum  FGGTSFTDKGGLAQINKAFQGGMVLVMSLWDDYSVNMLWLDSTYPANATG
FUE15-T.harzianum       --------------------------------------------------
|ADH04808.1|T.harzianum  FGGTSFTDKGGLAQINKAFQGGMVLVMSLWDDYAVNMLWLDSTYPTNATA

|AFD01232.1|T.harzianum  -TPGAKRGSCSTSSGVPAQVEAQSPNSKVVFSNIRFGPIGSTGGNTGSNP
FUE15-T.harzianum       ----------------------GQSPNSKVVFSNIRFGPIGSTGGNTGSNP
|ADH04808.1|T.harzianum  STPGAKRGSCSTSSGVPAQVEAQSPNSKVIYSNIRFGPIGSTGGNTGSNP
                                            .*******::********************

|AFD01232.1|T.harzianum  PGTSTTRAPPSSTGSSPTATQTHYGQCGGTGWGGPTICASGYTCQVLNPF
FUE15-T.harzianum       PGTSTTRAPPSSTGSSPTATQTHYGQCGGTGWGGPTRCAS----------
|ADH04808.1|T.harzianum  PGTSTTRAPPSSTGSSPTATQTHYGQCGGTGWTGPTRCASGFTCQVLNPF
                        ******************************** *** ***

|AFD01232.1|T.harzianum  YSQCL
FUE15-T.harzianum       -----
|ADH04808.1|T.harzianum  YSQCL
```

Fig. (5): Protein sequence alignment of *Trichoderma* cellulase gene using Clustal W 2.1 multiple sequence alignment.

Fig. (6): Phylogenetic tree showing the relationship between cellulose genes from *Trichoderma* strains. The tree was constructed using the CLUSTAL W 2.1 multiple sequence alignment programs. Rooted phylogentic tree (UPGMA)