RESEARCH ARTICLE

MOLECULAR AND MORPHOLOGICAL IDENTIFICATION OF *TRICHODERMA* ISOLATES FROM EGYPTIAN AGRICULTURE WASTES-RICH SOIL

M.H. ISMAIEL, A.M. El ZANATY, and K.S. ABDEL-LATEIF[*]

Department of Genetics, Faculty of Agriculture, Menoufia University, Egypt
*Corresponding author's email: k_dein2001@yahoo.com
Email addresses of co-authors: mohamed.hamed20591@gmail.com, zanaty1966@gmail.com

SUMMARY

Twenty isolates of *Trichoderma* were recovered from lignocellulosic agriculture wastes-rich soil collected from different Egyptian cities (Sadat, Tala, Abo Hamad, Belbeis, Zagazig, Mansoura, Belqas, Kafer-Elshikh, Bella, Tanta, Borg El Arab, Banha, Kafr Shoker, Qalyoub, Shebien Elqanater, Damanhur, Abu al-Matamir, Damietta, Kafr al-Battikh, and Kafr Saad). The *Trichoderma* isolates were first identified morphologically using conidiophore branching type and conidium morphology. Furthermore, molecular identification based on the ITS (internal transcribed spacers) barcode differentiated between *Trichoderma* isolates having 98.6% to 100% identity with two *Trichoderma* species: *T. asperellum* and *T. longibrachiatum*. Qualitative and quantitative tests were used for screening the cellulolytic activity of these isolates. The isolates were screened for cellulase production based on the clearing zone diameters and calorimetrically tested on minimal media supplemented with sugar cane bagasse and rice straw as sole carbon sources. The isolates TM41 (*T. longibrachiatum*) and TM35 (*T. asperellum*) exhibited the highest diameters of clear zones and showed higher Fpase and CMCase activities. Moreover, the isolate TM18 of *T. asperellum* displayed the highest diameters of clear zones and showed higher Fpase and Xylanase activities.

Keywords: *Trichoderma asperellum, Trichoderma longibrachiatum*, ITS primers, cellulase activities, biodegradation, lignocellulosic wastes

Key findings: The molecular identification was more accurate than the morphological identification. TM41 (*T. longibrachiatum*), TM35, and TM18 (*T. asperellum*) exhibited the highest diameters of clear zones and showed higher activities of Fpase, CMCase, and Xylanase.

INTRODUCTION

Cellulosic agriculture wastes represent about 40% of the cell biomass and become a great source of environmental contamination, where many farmers get rid of it by burning (Guruk and Karaaslan, 2020). Cellulose and hemicellulose are the main components of agricultural wastes that can be useful in different industries, such as, paper and the production of biofuels (Camargo *et al.,* 2012; Cova *et al.,* 2018; Torres *et al.,* 2019; Wang *et al*., 2020). Rice, wheat straw, and sugar cane wastes are produced in large quantities annually worldwide, posing a severe environmental challenge (Sari *et al*., 2021).

Each year, China, for example, is anticipated to create approximately two million tons of sugar cane waste (Xu *et al*., 2019). As a result, increased emphasis has been placed globally on eco-friendly and efficient techniques for the enzymatic scarification of cellulose and other plant components (Sari *et al*., 2021).

Trichoderma is one of the most economically viable fungi found in all soil types and decomposing plant tissues (Fahmi *et al.,* 2016). *Trichoderma* species produce a complete set of cellulases vital in the textile, paper, and pulp industries, and the biodegradation of plant lignocellulosic wastes (Ahmed *et al*., 2009). *Trichoderma* cellulolytic enzyme system includes three groups of enzymes: exoglucanases, endoglucanases, and β-glucosidase that act together to convert cellulose into glucose (Bhat and Bhat, 1997; Sukumaran *et al*., 2005; Zhang *et al*., 2014; Guruk and Karaaslan, 2020). For example, *Trichoderma reesei* was used immensely for different industrial purposes, thanks to its exceptional production of large amounts of cellulases (Bischof *et al*., 2016).

Trichoderma was shown to have at least two cellobiohydrolases and five endoglucanases (Srisodsuk, 1994; Saloheimo *et al*., 1997). Cellobiohydrolase 1 (CBH1) is a protein that cleaves cellulose from the ends and accounts for approximately 60% of total protein (Seiboth *et al*., 1992). Meanwhile, endoglucanases (EGs) internally cleave the cellulose strands, producing circa 20% of the extracellular protein (Wood and McCrae, 1982; Miettinen-Oinonen and Suominen, 2002). The endoglucanase II of *Trichoderma* was the superior activity enzyme among all endoglucanases and the most utilized one in the fabrics industry (Samanta *et al*., 2012).

Each CBH and EG convert cellulose to cellobiose, while BGL cleaves cellobiose to glucose (Singhania *et al*., 2010). De Franca *et al*. (2018) revealed that *Trichoderma* has superior activities of EG and CBH but low levels of BGL. The discrimination of *Trichoderma* spp. for use in biological control and biodegradation of lignocellulosic wastes is a critical concern. Using phenotypic criteria to identify *Trichoderma* can yield misleading findings (Fahmi *et al*., 2016). Reports said that ITS sequencing is a highly effective tool for distinguishing between *Trichoderma* species (Savitha and Sriram, 2015; Oskiera *et al*., 2015; Jiang *et al*., 2016; Abdelateif and Bakr, 2018; Marecik *et al*., 2018; Coronado-Ruiz *et al*., 2018; Guruk and Karaaslan, 2020; Li *et al*., 2020; Castrillo *et al*., 2021; An *et al*., 2022). Therefore, the study aimed to identify some isolates of *Trichoderma* spp., recovered from lignocellulosic agriculture wastes-rich soil collected from different areas in Egypt on a molecular level, and to screen their degradation activities.

MATERIALS AND METHODS

Trichoderma isolation

Several samples of agricultural wastes-rich soil were collected from different locations in Egypt (Table 1). A serial dilution method was tracked as described by Fahmi *et al*. (2016) to isolate *Trichoderma* spp.

Morphological identification of *Trichoderma* isolates

The *Trichoderma* isolates were characterized morphologically using conidiophore branching type and conidium morphology, as previously reported by Rifai (1969), Barnett and Hunter (1998), and Bissett (1991a, b, c).

Molecular identification of *Trichoderma*

The genomic DNA was isolated following the protocol of Al-Samarrai and Schmid (2000). ITS primers—ITS1 (5'-TCCGTAGGTGAACCTG CGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATGC- 3')—were used to amplify the ITS region, as described by Guruk and Karaaslan (2020). PCR products were first purified using a Montage PCR clean-up kit (Millipore). Sequencing was performed on an Applied Biosystems 3500 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Daejeon, Korea).

Screening of cellulolytic *Trichoderma* isolates

A plate assay method with 1% Carboxy Methyl Cellulose as the sole carbon source was applied for screening and pre-selection of cellulase-positive isolates, as described by Syed *et al*. (2013). The plates were incubated at 28ºC for 96 h and then stained with 1% Congo red solution for 30 min, and finallywashed with 1 M NaCl. The study evaluated the activity by measuring the diameter of the bright orange zones.

Table 1. Details of samples' collection sites, sources, and gene bank accession numbers.

Isolate Code	Location	Crop residues	Soil type	Gene bank accession No.	BLASTn identity (%)	ITS Molecular identification	Morphological identification
TM4	Sadat	Wheat	Clay loam	MW756917.1	100.00	*T. asperellum*	*T. viride*
TM5	Tala	Grape	Limestone saline	MW756918.1	100.00	*T. asperellum*	*T. viride*
TM6	Abo Hamad	Corn	Loamy	MW756919.1	98.63	*T. asperellum*	*T. koningii*
TM8	Belbeis	Rice straw	Clay loam	MW756920.1	100.00	*T. asperellum*	*T. koningii*
TM9	Zagazig	Cotton	Clay	MW756921.1	100.00	*T. asperellum*	*T. viride*
TM13	Mansoura	Rice straw	Loamy	MW756922.1	100.00	*T. longibrachiatum*	*T. viride*
TM16	Belqas	Cotton	Calcareous	MW756923.1	100.00	*T. asperellum*	*T. viride*
TM18	Kafer-Elshikh	Berssem	Clay	MW756924.1	100.00	*T. asperellum*	*T.koningii*
TM19	Bella	Rice straw	Clay loam	MW756925.1	100.00	*T. asperellum*	*T. koningii*
TM23	Tanta	Wheat	Clay loam	0	0	*0*	*T. harizianum*
TM29	Borg El arab	Sugar cane bagasse	Composting	MW756926.1	100.00	*T .asperellum*	*T. viride*
TM30	Banha	Corn	Sandy	0	0	*0*	*T .harizianum*
TM31	Kafr Shoker	Cotton	Loamy	MW756927.1	99.51	*T. longibrachiatum*	*T. harizianum*
TM33	Qalyoub	Wheat	Loamy	MW756928.1	100.00	*T. lnogibrachiatum*	*T. harizianum*
TM35	S. Elqanater	Rice straw	Clay	MW756929.1	100.00	*T. asperellum*	*T. harizianum*
TM36	Damanhur	Wheat	Sandy	MW756930.1	100.00	*T. longibrachiatum*	*T. harizianum*
TM41	Abu al-Matamir	Berssem	Sandy	MW756931.1	100.00	*T. longibrachiatum*	*T. harizianum*
TM42	Damietta	Wheat	Clay	MW756932.1	100.00	*T. asperellum*	*T. viride*
TM44	Kafr al-Battikh	Berssem	Clay	MW756933.1	99.67	*T. longibrachiatum*	*T. harizianum*
TM45	Kafr Saad	Coton	Loamy	MW756934.1	99.51	*T. longibrachiatum*	*T. harizianum*

Production of cellulolytic enzymes

Pre-treatment of sugar cane as substrate

Locally collected sugar cane bagasse was chopped after being washed and milled to 1-2 mm particles. The grounded bagasse was mixed with 0.12 g NaOH/g, dried, and autoclaved at 121°C for 20 min. Finally, the autoclaved bagasse was washed with tap and distilled water until clear, and then dried at 80°C (Guti´errez-Correa and Tengerdy, 1997).

Pre-treatment of rice straw as a substrate

The collected rice straw was suspended in 40 ml of 2% NaOH and was autoclaved at 121°C for 20 min. The autoclaved straw was neutralized by washing with tap water followed by distilled water, and finally, dried at 80°C (Zhang and Cai, 2008).

Cellulase-Enzyme production by *Trichoderma*

A spore suspension of tested isolates (10^6 spores per ml) was prepared after being grown at PDA and used as inoculum. Flasks with Mandel's medium, supplemented with pre-treated cane bagasse and rice straw as sole carbon sources, were inoculated with the inoculum and incubated for seven days at 28°C at 175 rpm/min (Mandels *et al*., 1976). After incubation, the flasks were filtered through a Whatman filter paper No.1, then centrifuged at maximum speed for 15 min at 4°C, then used for enzyme assays.

Enzymatic assays

One unit of the enzyme was considered as the amount of enzyme required to release 1 μM of glucose or xylose (Miller, 1959).

Exoglucanase activity (**FPase)**

The FPase assay was used to calculate the exoglucanase activity according to Ghose (1987) and Mandels and Sternberg (1976). The assay mixture consists of 1 ml filtrate (enzyme source), 1 ml 50 mM citrate buffer (pH 4.8), and 50 mg Whatman filter paper strips. The mixture was incubated for 60 min at 50°C. The enzymatic reaction was terminated by adding 3 ml DNS (3,5-Dinitrosalicylic acid) reagent to the mixture, followed by boiling for 15 min in a water bath. Finally, the boiled tubes were left to cool in an ice box and the amount of reducing sugar was estimated using a spectrophotometer at 540nm using glucose as a standard. All tests were done in three replicates.

$$\text{FPase activity (IU/ml)} = \frac{mg\ glucose\ released * 1000}{Enzyme\ volume * MWT\ of\ glucose * Time}$$

Where,

MWT: molecular weight of glucose = 180.16
Time: is the incubation time of reaction (60 min)
Specific activity (IU/mg) = 1U(µmol/min/mL)/ Total protein (mg/mL)

Endoglucanase activity (CMCase)

The amount of released glucose was calculated using DNS protocol according to Mandels and Weber (1969), where 1 ml of filtrate was incubated for 30 min at 50°C with 1 ml of 1% carboxymethylcellulose (CMC) prepared in 50 mM citrate buffer (pH 4.8). The reaction was stopped and CMCase activity was estimated as mentioned above with FPase.

$$\text{CMCase activity (IU/ml)} = \frac{mg\ glucose\ released * 1000}{Enzyme\ volume * MWT\ of\ glucose * Time}$$

Where,
MWT: molecular weight of glucose =180.16
Time: is the incubation time of reaction (30 min
Specific activity (IU/mg) = 1U(µmol/min/mL)/ Total protein (mg/mL)

Xylanase assay

The assay is based on the estimation of the amount of reducing sugar released from xylan by the xylanase enzyme (Kinoshita *et al*., 1981). The assay mixture was prepared as follows: 1 ml of the filtrate was mixed with 1 ml birchwood xylan (1% w/v in 50 mM acetate buffer, pH 5.3). The prepared mixture was incubated for 5 min at 50°C. The reaction was stopped and xylanase activity was estimated as mentioned above with FPase using xylose as standard.

$$\text{Xylanase activity (IU/ml)} = \frac{mg\ xylose\ released * 100\text{в}}{Enzyme\ volume * Xylose\ MWT * Time}$$

Where,

MWT: molecular weight of xylose =150.16
Time: is the incubation time of reaction (5 min)
Specific activity (IU/mg) = 1U(µmol/min/mL)/ Total protein (mg/mL)

Data analysis

NCBI database was utilized to match the isolates sequencing data with the published sequences through carrying BLASTn (http://www.ncbi.nlm.nih.gov/). Sequences were submitted to GenBank through Bankit (available on the NCBI home page). The phylogenetic tree was constructed using the UPGMA method using the MEGA 6 program (Tamura *et al.*, 2013). Meanwhile, the enzyme activity data were statistically analyzed using analysis of variance (ANOVA) and comparisons of means at a 5% significance level was carried out according to Duncan's multiple range test analysis, the software Costat version 6.3.

RESULTS

Characterization of *Trichoderma* isolates

Twenty isolates of *Trichoderma* were recovered from agricultural wastes-rich soil, such as, cotton and rice straw, representing various geographic zones in Egypt (Table 1). First, these isolates were morphologically identified based on conidia spores and hyphae branching. Seven isolates were found to belong to *Trichoderma Viride*: TM4, TM5, TM9, TM13, TM16, TM29, and TM42; four isolates classified as *Trichoderma Koningii*: TM6, TM8, TM18; and, TM19 and nine isolates were identified as *Trichoderma Harizianum*: TM23, TM30, TM31, TM33, TM35, TM36, TM41, TM44, and TM45.

Amplification of ITS sequence was achieved using specific primers. The sequencing data were compared with published ITS sequences on the NCBI database. The tested isolates exhibited from 98.63% to 100% identity with two species of *Trichoderma*— seven isolates were identified as *Trichoderma longibrachiatum*: TM13, TM31, TM33, TM36, TM41, TM44 and TM45, and 11 isolates classified as *Trichoderma asperellum*: TM4, TM5, TM6, TM8, TM9, TM16, TM18, TM19, TM29, TM35, and TM42 (Table 1). The phylogeny analysis upheld the ITS identification and classified the isolates of *Trichoderma* into two groups (Figure 1). Frequencies of the four nucleotides: A, T, G, and C in ITS region sequences showed narrow levels of variation (Table 2).
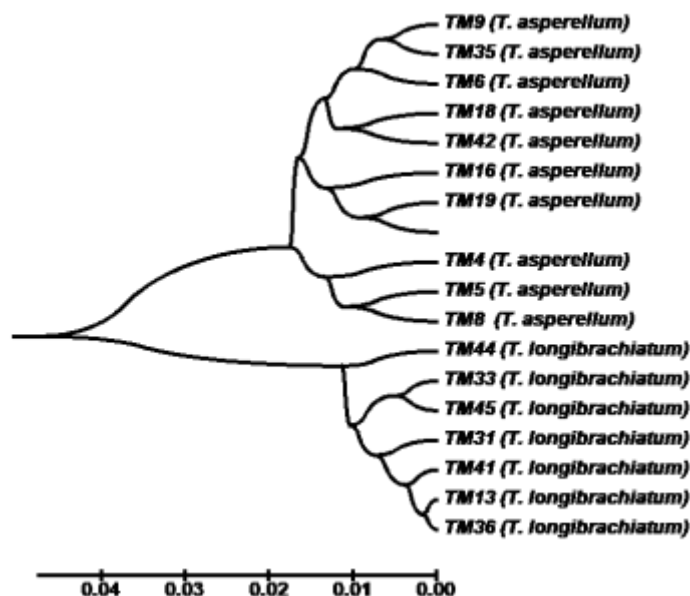
Figure 1. The Phylogenetic tree of the *Trichoderma* isolates based on ITS sequences constructed using MEGA version 6.

Table 2. Nucleotide frequencies of ITS sequences.

Isolates code	Full length	A%	T%	G%	C%	(G+C)%
TM4	577	20.62	24.26	29.64	25.48	55.1
TM5	580	20.52	24.48	29.48	25.52	55
TM6	580	20.69	24.14	29.48	25.69	55.2
TM8	581	20.31	24.44	29.60	25.65	55.2
TM9	578	20.24	24.39	29.58	25.78	55.4
TM13	609	22.00	20.85	31.36	25.78	57.1
TM16	606	20.63	24.09	29.54	25.74	55.3
TM18	574	20.38	24.56	29.79	25.26	55.1
TM19	583	20.07	24.70	29.50	25.73	55.2
TM29	576	20.31	25.00	29.69	25.00	54.7
TM31	615	21.79	21.79	31.06	25.37	56.4
TM33	609	22.00	21.18	31.36	25.45	56.8
TM35	583	20.58	24.53	29.33	25.56	54.9
TM36	605	22.15	20.33	31.57	25.95	57.5
TM41	612	21.73	21.73	31.05	25.49	56.5
TN42	567	20.46	24.69	30.34	27.87	55.6
TM44	608	22.04	21.22	31.41	25.33	56.7
TM45	619	21.65	21.97	30.86	25.53	56.4

Measurement of cellulase activities

Qualitative cellulase assays of Trichoderma isolates

Based on the diameters of the clearing zone recorded, seven isolates: TM41, TM4, TM35, TM18, TM36, TM42, and TM19, exhibited the highest hydrolysis zones. Moreover, the isolates TM8 and TM13 gave the lowest hydrolysis zones, whereas the others showed moderate hydrolysis zones (Figure 2).

Quantitative cellulase assays

The tested isolates were submitted to colorimetric tests to determine their extracellular hydrolytic cellulases enzyme activities on filter paper, β-1,4-endoglucanase (CMCase), and Xylanase on minimal media, supplemented with sugar cane bagasse and rice straw as sole carbon sources. Measuring absorbance at 540 nm assayed the liberated reducing sugar. For Fpase activity, the isolates TM9, TM41, TM35, TM13, and TM33 exhibited
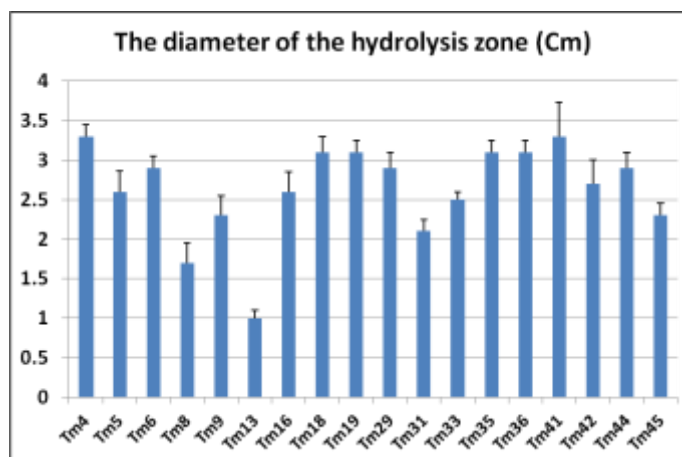
Figure 2. The hydrolysis zones (Cm) produced by *Trichoderma* isolates on CMC medium.

Table 3. Fpase activities of *Trichoderma* isolates on medium supplemented with bagasse and rice straw as sole carbon sources.

Substrate/	Bagasse		Rice straw	
Isolate	Activity (IU/mL)	Specific activity (IU/mg)	Activity (IU/mL)	Specific activity (IU/mg)
TM4	$0.060^{fgh} \pm 0.001$	0.084 ± 0.007	$0.022^{h} \pm 0.001$	0.036 ± 0.001
TM5	$0.081^{abcd} \pm 0.002$	0.041 ± 0.000	$0.036^{g} \pm 0.005$	0.034 ± 0.001
TM6	$0.059^{gh} \pm 0.002$	0.100 ± 0.000	$0.016^{h} \pm 0.002$	0.012 ± 0.002
TM8	$0.055^{h} \pm 0.004$	0.083 ± 0.005	$0.036^{g} \pm 0.005$	0.032 ± 0.001
TM9	$0.066^{efg} \pm 0.000$	0.083 ± 0.000	$0.113^{a} \pm 0.001$	0.061 ± 0.002
TM13	$0.077^{cd} \pm 0.006$	0.066 ± 0.010	$0.076^{c} \pm 0.002$	0.058 ± 0.001
TM16	$0.061^{efgh} \pm 0.001$	0.056 ± 0.001	$0.070^{cd} \pm 0.001$	0.056 ± 0.001
TM18	$0.091^{a} \pm 0.001$	0.062 ± 0.002	$0.057^{e} \pm 0.001$	0.054 ± 0.001
TM19	$0.084^{abc} \pm 0.004$	0.046 ± 0.000	$0.058^{e} \pm 0.001$	0.052 ± 0.001
TM29	$0.079^{bcd} \pm 0.000$	0.041 ± 0.001	$0.059^{e} \pm 0.001$	0.050 ± 0.001
TM31	$0.061^{efgh} \pm 0.010$	0.086 ± 0.000	$0.014^{h} \pm 0.001$	0.045 ± 0.001
TM33	$0.072^{de} \pm 0.002$	0.061 ± 0.001	$0.074^{c} \pm 0.002$	0.059 ± 0.001
TM35	$0.086^{abc} \pm 0.004$	0.073 ± 0.004	$0.087^{b} \pm 0.008$	0.090 ± 0.004
TM36	$0.071^{de} \pm 0.001$	0.087 ± 0.001	$0.062^{de} \pm 0.001$	0.038 ± 0.001
TM41	$0.089^{ab} \pm 0.000$	0.063 ± 0.003	$0.094^{b} \pm 0.005$	0.062 ± 0.003
TM42	$0.071^{de} \pm 0.003$	0.061 ± 0.003	$0.043^{fg} \pm 0.002$	0.012 ± 0.001
TM44	$0.085^{abc} \pm 0.001$	0.044 ± 0.002	$0.035^{g} \pm 0.003$	0.061 ± 0.001
TM45	$0.070^{def} \pm 0.004$	0.077 ± 0.002	$0.047^{f} \pm 0.005$	0.043 ± 0.001

the highest enzyme activities on media supplemented with rice straw (Table 3). Meanwhile, the isolates TM6, TM31, TM8, and TM44 showed the lowest enzyme activities. The isolates TM18, TM41, TM35, and TM19 showed the highest enzyme levels on media supplemented with sugar cane bagasse, whereas the isolates TM8 and TM6 gave low levels of cellulase enzymes.

Regarding CMCase activity, the isolates TM44, TM31, and TM41 exhibited the highest enzyme activities on media supplemented with rice straw, whereas the isolates TM29, TM33, and TM45 showed the lowest enzyme activities (Table 4). The isolates TM6, TM9, TM45, TM5, TM8, and TM35 showed the highest enzyme activities on media supplemented with sugar cane bagasse, but the isolates TM19, TM18, and TM41 gave low levels of cellulase enzymes.

Concerning Xylanase activity, the isolates TM4, TM5, TM18, and TM6 exhibited the highest enzyme activities on media supplemented with rice straw. Meanwhile, the isolates TM36 and TM45 showed the lowest enzyme activities (Table 5). In addition, the isolates showed a closed amount of enzyme activities on media supplemented with sugar cane bagasse, but the activity, in general, was less than the activities recorded on media supplemented with rice straw.

Table 4. CMCase activities of *Trichoderma* isolates on medium supplemented with bagasse and rice straw as sole carbon sources.

Substrate/ Isolate	Bagasse		Rice straw	
	Activity (IU/mL)	Specific activity (IU/mg)	Activity (IU/mL)	Specific activity (IU/mg)
TM4	0.351[g] ± 0.002	0.412 ± 0.006	0.464[bc] ± 0.003	0.355 ± 0.008
TM5	0.396[bc] ± 0.002	0.394 ± 0.017	0.453[bcd] ± 0.003	0.365 ± 0.014
TM6	0.426[a] ± 0.002	0.316 ± 0.003	0.398[de] ± 0.003	0.380 ± 0.017
TM8	0.389[c] ± 0.004	0.286 ± 0.004	0.331[fg] ± 0.003	0.299 ± 0.014
TM9	0.418[ab] ± 0.007	0.344 ± 0.006	0.296[ghI] ± 0.003	0.252 ± 0.011
TM13	0.378[cde] ± 0.005	0.209 ± 0.015	0.320[fg] ± 0.003	1.038 ± 0.133
TM16	0.381[cd] ± 0.015	0.399 ± 0.007	0.430[bc] ± 0.016	0.342 ± 0.015
TM18	0.292[i] ± 0.001	0.357 ± 0.003	0.416[cde] ± 0.019	0.434 ± 0.038
TM19	0.268[i] ± 0.002	0.370 ± 0.005	0.247[gh] ± 0.019	0.155 ± 0.019
TM29	0.309[hI] ± 0.009	0.377 ± 0.006	0.127[j] ± 0.003	0.213 ± 0.022
TM31	0.315[hI] ± 0.021	0.254 ± 0.003	0.511[ab] ± 0.010	0.338 ± 0.015
TM33	0.363[efg] ± 0.008	0.262 ± 0.013	0.162[j] ± 0.003	0.292 ± 0.041
TM35	0.360[efg] ± 0.005	0.255 ± 0.004	0.361[ef] ± 0.020	0.346 ± 0.060
TM36	0.353[ef] ± 0.003	0.274 ± 0.001	0.306[fgh] ± 0.019	0.315 ± 0.073
TM41	0.325[h] ± 0.005	0.412 ± 0.001	0.465[bc] ± 0.003	0.349 ± 0.007
TN42	0.377[cde] ± 0.005	0.420 ± 0.001	0.296[ghI] ± 0.003	0.273 ± 0.045
TM44	0.359[efg] ± 0.010	0.300 ± 0.006	0.534[ab] ± 0.048	0.287 ± 0.020
TM45	0.396[bc] ± 0.008	0.294 ± 0.003	0.238[i] ± 0.046	0.072 ± 0.021

Table 5. Xylanase activities of *Trichoderma* isolates on medium supplemented with bagasse and rice straw as sole carbon sources.

Substrate/ Isolate	Bagasse		Rice straw	
	Xylanase activity (IU/mL)	Xylanase-specific activity (IU/mg)	Xylanase activity (IU/mL)	Xylanase-specific activity (IU/mg)
TM4	3.007[a] ± 0.027	3.149 ± 0.040	5.304[a] ± 0.042	4.060 ± 0.084
TM5	2.732[a] ± 0.060	2.524 ± 0.021	5.123[b] ± 0.031	4.129 ± 0.158
TM6	2.915[a] ± 0.029	3.006 ± 0.633	4.635[b] ± 0.030	4.426 ± 0.186
TM8	2.786[a] ± 0.038	2.979 ± 0.027	4.312[def] ± 0.030	3.897 ± 0.165
TM9	2.627[a] ± 0.008	3.222 ± 0.234	3.429[j] ± 0.030	2.911 ± 0.113
TM13	2.894[a] ± 0.539	1.679 ± 0.020	3.317[j] ± 0.027	10.768 ± 1.363
TM16	2.438[a] ± 0.016	3.170 ± 0.021	4.603[c] ± 0.008	3.659 ± 0.067
TM18	2.785[a] ± 0.601	2.907 ± 0.031	4.836[b] ± 0.152	5.038 ± 0.381
TM19	2.789[a] ± 0.049	3.041 ± 0.087	4.404[cde] ± 0.145	2.752 ± 0.220
TM29	2.895[a] ± 0.219	3.232 ± 0.043	4.086[fg] ± 0.145	6.839 ± 0.767
TM31	2.531[a] ±0.002	2.272 ± 0.010	4.268[ef] ± 0.090	2.827 ± 0.135
TM33	2.885[a] ± 0.005	2.022 ± 0.163	3.609[ij] ± 0.091	6.522 ± 0.969
TM35	2.926[a] ± 0.023	0.545 ± 0.082	3.473[ij] ± 0.093	3.305 ± 0.489
TM36	2.894[a] ± 0.084	1.888 ± 0.042	3.022[k] ± 0.090	3.080 ± 0.614
TM41	2.910[a] ± 0.004	2.823 ± 0.023	3.933[gh] ± 0.038	2.951 ± 0.070
TN42	2.802[a] ± 0.131	3.012 ± 0.044	3.753[hI] ± 0.041	3.459 ± 0.566
TM44	2.791[a] ± 0.290	1.887 ± 0.003	4.040[fgh] ± 0.264	2.173 ± 0.100
TM45	0.845[b] ± 0.130	2.186 ± 0.101	2.937[k] ± 0.082	0.850 ± 0.123

DISCUSSION

The *Trichoderma* isolates were identified both morphologically and on the molecular level. The results of molecular identification were completely different compared with morphological identification. Earlier studies indicated that molecular identification is more accurate than morphological identification, which can lead to deceptive results (Fahmi *et al*., 2016; Li *et al*., 2020). In the study, ITS primers successfully distinguished the different species of *Trichoderma***.** These results agree with previous studies that stated the strength of ITS as a smart tool to identify *Trichoderma* spp. (Oskiera *et al*., 2015; Savitha and Sriram, 2015; Wu *et al*., 2017; Abdel-lateif and Bakr, 2018; Guruk and Karaaslan, 2020; Castrillo *et al*., 2021).

Fast evaluation of the cellulolytic activities for the tested isolates was done based on the clearing zone assay. All isolates exhibited clearing zones with different diameters reflecting the importance of this

assay in screening and pre-selection of cellulase-positive isolates. Various studies used this method in screening the different *Trichoderma* cellulolytic isolates (Sazci *et al*., 1986; Khokhar *et al*., 2012; Syed *et al*., 2013; Castrillo *et al.,* 2021; Suirta *et al*., 2021).

Enzymatic hydrolysis requires the cooperation of exoglucanases, endoglucanases, and beta-glucosidase for the successful degradation of cellulose (Zhang *et al*., 2014) thus, FPase, CMCase, and Xylanase activities were evaluated colorimetrically on minimal media supplemented with sugar cane bagasse and rice straw as sole carbon sources. Interestingly, some isolates, such as, TM41 (*T. longibrachiatum*), TM4, and TM35 (*T. asperellum*) that exhibited high diameters of clear zones also showed higher activities of Fpase and CMCase. Moreover, isolates like TM18 of *T. asperellum* that exhibited high diameters of clear zones, showed higher activities of Fpase and Xylanase.

The activities obtained for both qualitative and quantitative assays were very close and consistent with those obtained by Sazci *et al*. (1986). Castrillo *et al*. (2021) stated that there is congruence between the qualitative and the quantitative methods and advised to rely on the clearing zone assay as a fast, inexpensive, and sensitive test for screening a high number of isolates. Marecik *et al*. (2018) tested 123 strains of *Trichoderma* for the degradation of cellulose and xylan. About 30 strains exhibited higher levels of cellulase and xylanase activities compared with the reference strain. The difference among *Trichoderma* isolates in their cellulolytic activities may be due to the variation in their genetic content, the origin of the isolates, and the number of cellulase enzymes produced by the fungus.

CONCLUSIONS

Twenty isolates of *Trichoderma* were recovered from agricultural wastes-rich soil, such as, cotton and rice straw representing various geographic zones in Egypt. These isolates were identified on a morphological and molecular level. The molecular identification was more accurate than the morphological identification. Qualitative and quantitative tests screened the cellulolytic activity of these isolates on media supplemented with sugar cane bagasse and rice straw as sole carbon sources. TM41 (*T. longibrachiatum*), TM35, and TM18 (*T. asperellum*) exhibited the highest diameters of clear zones and showed higher activities of

Fpase, CMCase, and Xylanase. Further investigation must be done on the promised isolates to confirm their superior cellulolytic activities and apply them on an industrial level.

REFERENCES

Abdel-lateif KS, Bakr RA (2018). Internal transcribed spacers (ITS) based identification of *Trichoderma* isolates and biocontrol activity against *Macrophomina phaseolina*, *Aspergillus niger* and *Meloidogyne incognita*. *Afr. J. Microbiol. Res.* 12(30): 715-722.

Ahmed S, Huma Saleem AB, Saadia M, Jamil A (2009). Production and purification of cellulose degrading enzymes from a filamentous fungus *Trichoderma harzianum*. *Pak. J. Bot.* 41(3): 1411-1419.

Al-Samarrai T, Schmid J (2000). A simple method for extraction of fungal genomic DNA. *Lett. Appl. Microbiol*. 30: 53-56.

An XY, Cheng GH, Gao HX, Li XF, Yang Y, Li D, Li Y (2022). Phylogenetic analysis of *Trichoderma* species associated with green mold disease on mushrooms and two new pathogens on *Ganoderma sichuanense*. *J. Fungi.* 8: 704.

Barnett HL, Hunter BB (1998). Illustrated genera of imperfect fungi. 4th Edition. St. Paul, MN, APS Press 223.

Bhat M, Bhat S (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv*. 15: 583-620.

Bischof RH, Ramoni J, Seiboth B (2016). Cellulases and beyond: The first 70 years of the enzyme producer *Trichoderma reesei*. *Microbiol. Cell. Fact.* 15(1):106.

Bissett J (1991a). A revision of genus *Trichoderma*, II. Infrageneric classification. *Can. J. Bot.* 69: 2357- 2372.

Bissett J (1991b). A revision of genus *Trichoderma*, III. Section Pachybasium. *Can. J. Bot.* 69: 2372-2417.

Bissett J (1991c). A revision of genus *Trichoderma*, IV. Section *Longibrachiatum*. *Can. J. Bot.* 69: 924- 931.

Camargo FA, Innocentini-Mei LH, Lemes AP, Moraes SG, Duran N (2012). Processing and characterization of composites of poly (3-hydroxybutyrate-co-hydroxyvalerate) and lignin from sugar cane bagasse. *J. Compos. Mater.* 46(4): 417-425.

Castrillo ML, Bich GA, Amerio NS, Rodríguez MD, Zapata PD, Villalba LL (2021). Assessment of cellulase complex secretory capacity of *Trichoderma* strains and morphological and molecular identification of the isolate with the highest enzymatic secretion capacity. *J. Microbiol. Biotechnol. Food. Sci.* 10(5): e1357.

Coronado-Ruiz C, Avendaño R, Escudero-Leyva E, Conejo-Barboza G, Chaverri P, Chavarría M (2018). Two new cellulolytic fungal species

isolated from a 19th-century art collection. *Sci. Rep*. 8*:* 7492*.*

Cova TF, Murtinho D, Pais AACC, Valente AJM (2018). Combining cellulose and cyclodextrins: Fascinating designs for materials and pharmaceutics. *Front. Chem*. 6: 271.

De Franca PD, Pereira N Jr, de-Castro AM (2018). A comparative review of recent advances in cellulases production by *Aspergillus*, *Penicillium* and *Trichoderma* strains and their use for lignocellulose deconstruction. *Curr. Opin. Green. Sustain. Chem.* 14: 60-66.

Fahmi AI, Eissa RA, El-Halfawi KA, Hamza HA, Helwa MS (2018). Identification of *Trichoderma* spp. by DNA barcode and screening for cellulolytic activity. *J. Microbiol. Biochem. Technol*. 8: 202-209.

Ghose TK (1987) Measurement of cellulase activities. *Pure. Appl. Chem.* 59: 257-268.

Guruk M, Karaaslan M (2020). Production and biochemical characterization of cellulase enzyme by *Trichoderma* strains from Harran plain. *Int. J. Life. Sci. Biol.* 3(3): 258-274.

Gutierrez-Correa M, Tengerdy RP (1997). Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation. *Biotechnol. Lett*. 19(7): 665-667.

Jiang H, Zhang L, Zhang JZ, Ojaghian MR, Hyde KD (2016). Antagonistic interaction between *Trichoderma asperellum* and *Phytophthora capsici in vitro*. *J. Zhejiang Univ. Sci. B*. 17: 271-281.

Khokhar I, Haider MS, Mushtaq S, Mukhtar I (2012). Isolation and screening of highly cellulolytic filamentous fungi. *J. Appl. Sci. Environ. Manag*. 16 (3): 223-226.

Kinoshita S, Seki T, Kishimoto M, Taguchi H, Kitpreechavanich V, Nagai S (1981). Annual Reports of International Centre Cooperative Research and Development in Microbial Engineering, Osaka University, Japan. 4: 408-410.

Li JX, Zhang F, Jiang DD, Li J, Wang FL, Zhang Z, Wang W, Zhao XQ (2020). Diversity of cellulase-producing filamentous fungi from Tibet and transcriptomic analysis of a superior cellulase producer *Trichoderma harzianum* LZ117. *Front. Microbiol*. 11: 1617.

Mandel M, Weber J (1969). Exoglucanase activity by microorganisms. *Adv. Chem*. 95:391-414.

Mandels M, Andreotti R, Roche C (1976). Measurement of saccharifying cellulase. *Biotechnol. Bioeng. Symp.* 21-33.

Mandels M, Sternburg D (1976). Recent advances in cellular technology. *J. Ferment. Technol*. 54: 267-286.

Marecik R, Błaszczyk L, Biegańska-Marecik R, Piotrowska-Cyplik A (2018). Screening and identification of *Trichoderma* strains isolated from natural habitats with potential to cellulose and xylan degrading enzymes production*. Polish. J. Microbiol.* 67(2): 181-190.

Miettinen-Oinonen A, Suominen P (2002). Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. *Appl. Environ. Microbiol.* 68(8): 3956–3964.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31(3): 426-248.

Oskiera M, Szczech M, Bartoszewski G (2015). Molecular identification of *Trichoderma* strains collected to develop plant growth-promoting and biocontrol agents*. J. Hortic. Res*. 23(1): 75-86.

Rifai MA (1969). A revision of the genus *Trichoderma*. *Mycol. Papers*. 116: 1-116.

Saloheimo M, Nakari-Setälä T, Tenkanen M, Penttilä M (1997). cDNA cloning of a *Trichoderma reesei* cellulase and demonstration of endoglucanase activity by expression in yeast. *Eur. J. Biochem*. 249(2): 584-591.

Samanta S, Asitava Basu A, Halder UC, Sen SK (2012). Characterization of *Trichoderma reesei* Endoglucanase II expressed heterologously in *Pichia pastoris* for better biofinishing and biostoning. *J. Microbiol*. 50(3): 518-525.

Sari RM, Torres FG, Troncoso OP, De-la-Torre GE, Gea S (2021). Analysis and availability of lignocellulosic wastes: Assessments for Indonesia and Peru. *Environ. Qual. Manag.* 30: 71-82.

Savitha MJ, Sriram S (2015). Morphological and molecular identification of *Trichoderma* isolates with biocontrol potential against *Phytophthora* blight in red pepper. *Pest. Manage. Hortic. Ecsyst*. 21(2): 194-202.

Sazci A, Radford A, Erenle K (1986). Detection of cellulolytic fungi by using Congo red as an indicator: A comparative study with the dinitrosalicylic acid reagent method. *J. Appl. Bocteriol.* 61: 559-562.

Seiboth B, Messner R, Gruber F, Kubicek CP (1992). Disruption of the *Trichoderma reesei cbh2* gene coding for cellobiohydrolase II leads to a delay in the triggering of cellulase formation by cellulose. *J. Gen. Microbiol.* 138(6): 1259-1264.

Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A (2010). Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme. Microb. Technol.* 46(7): 541-549.

Srisodsuk M (1994). Mode of action of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. Finland: VTT Publications, Espoo. ISBNs: 951-38-4626-1.

Sukumaran RK, Singhania RR, Pandey A (2005). Microbial cellulases-production, applications and challenges. *J. Sci. Ind. Res.* 64: 832-844.

Suirta IW, Suarsana IN, Swantara IMD, Proborini MW (2021). Morphological and molecular characterization of the pathogenic fungi

isolated from purple eggplant originating from Bali, Indonesia. *SABRAO J. Breed. Genet.* 53(3): 459-467.

Syed S, Riyaz-Ul-Hassan S, Johri S (2013). A novel cellulase from an endophyte, *Penicillium* Sp. NFCCI 2862. *Am. J. Microbiol. Res.* 1(4): 84-91.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.

Torres FG, Mayorga JP, Vilca C, Arroyo J, Castro P, Rodriguez L (2019). Preparation and characterization of a novel starch–chestnut husk biocomposite. *SN. Appl. Sci.* 1(10): 1-7.

Wang BT, Hu S, Yu XY, Jin L, Zhu YJ, Jin FJ (2020). Studies of cellulose and starch utilization and the regulatory mechanisms of related enzymes in fungi. *Polymers* 12(3): 530.

Wood TM, McCrae SI (1982). Purification and some properties of a (1→4)-β-d-glucan glucohydrolase associated with the cellulase from the fungus *Penicillium funiculosum*. *Carbohydr. Res*. 110(2): 291-303.

Wu Q, Sun R, Ni M, Yu J, Li Y, Yu C, Dou K, Ren J, Chen J (2017). Identification of a novel fungus, *Trichoderma asperellum* GDFS1009, and comprehensive evaluation of its biocontrol efficacy. *PloS. One.* 12(6): e0179957.

Xu Q, Ji T, Gao SJ , Yang Z, Wu N (2019). Characteristics and applications of sugar cane bagasse ash waste in cementitious materials. *Materials* 12: 39.

Zhang Q, Cai W (2008). Enzymatic hydrolysis of alkali-pretreated rice straw by *Trichoderma reesei* ZM4-F3. *Biomass. Bioenerg*. 32: 1130-1135.

Zhang Z, Liu JL, Lan JY, Duan CJ, Ma QS, Feng JX (2014). Predominance of *Trichoderma* and *Penicillium* in cellulolytic aerobic filamentous fungi from subtropical and tropical forests in China, and their use in finding highly efficient β-glucosidase. *Biotechnol. Biofuels*. 7: 107-121.