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

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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-Elsikh, Bella, Tanta, Borg El Arab, Banha, Kafir Shoker, Qalyoub, Shebben Elqanater, Damahur, 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.

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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).


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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'-TCCGTAAGTGAAACTGC CGG-3') and ITS4 (5'-TCTCCGCTTATTGAT ATGC- 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 finally washed with 1 M NaCl. The study evaluated the activity by measuring the diameter of the bright orange zones.
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 ground 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 (Gutiérrez-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 (10⁶ spores per ml) was prepared after being grown at PDA and used as inoculum. Flasks with Mandel's medium, supplemented with pretreated 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.

 FPase activity (IU/ml) = \frac{\text{mg glucose released} \cdot 1000}{\text{Enzyme volume} \cdot \text{MW of glucose} \cdot \text{Time}}
Where,

\[ \text{MWT: molecular weight of glucose} = 180.16 \]
\[ \text{Time: is the incubation time of reaction (60 min)} \]
\[ \text{Specific activity (IU/mg)} = \frac{1U(\mu\text{mol/min/mL})}{\text{Total protein (mg/mL)}} \]

**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.

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

Where,

\[ \text{MWT: molecular weight of glucose} = 180.16 \]
\[ \text{Time: is the incubation time of reaction (30 min)} \]
\[ \text{Specific activity (IU/mg)} = \frac{1U(\mu\text{mol/min/mL})}{\text{Total protein (mg/mL)}} \]

**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.

\[ \text{Xylanase activity (IU/ml)} = \frac{\text{mg xylose released} \times 100}{\text{Enzyme volume} \times \text{Xylose MWT} \times \text{Time}} \]

Where,

\[ \text{MWT: molecular weight of xylose} = 150.16 \]
\[ \text{Time: is the incubation time of reaction (5 min)} \]
\[ \text{Specific activity (IU/mg)} = \frac{1U(\mu\text{mol/min/mL})}{\text{Total protein (mg/mL)}} \]

**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).
Table 2. Nucleotide frequencies of ITS sequences.

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<th>Isolates code</th>
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<th>T%</th>
<th>G%</th>
<th>C%</th>
<th>(G+C)%</th>
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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...
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.
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 Karasaan, 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

<table>
<thead>
<tr>
<th>Substrate/Isolate</th>
<th>Bagasse Activity (IU/mL)</th>
<th>Bagasse Specific activity (IU/mg)</th>
<th>Rice straw Activity (IU/mL)</th>
<th>Rice straw Specific activity (IU/mg)</th>
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<td>TM4</td>
<td>3.007 ± 0.027</td>
<td>3.149 ± 0.040</td>
<td>5.304 ± 0.042</td>
<td>4.060 ± 0.084</td>
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<td>TM5</td>
<td>2.732 ± 0.060</td>
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<td>5.123 ± 0.031</td>
<td>4.129 ± 0.158</td>
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<tr>
<td>TM6</td>
<td>2.915 ± 0.029</td>
<td>3.006 ± 0.633</td>
<td>4.635 ± 0.030</td>
<td>4.426 ± 0.186</td>
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<td>2.786 ± 0.038</td>
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<td>4.312 ± 0.030</td>
<td>3.897 ± 0.165</td>
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<tr>
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<td>2.627 ± 0.008</td>
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<td>3.429 ± 0.030</td>
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<td>2.894 ± 0.053</td>
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Table 5. Xylanase activities of *Trichoderma* isolates on medium supplemented with bagasse and rice straw as sole carbon sources.
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; Suíra 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. Mareck 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


