SCREENING OF CELLULASE GENES AND DEGRADING ACTIVITIES OF TRICHODERMA ASPERELLUM VIA SOLID-STATE FERMENTATION OF RICE STRAW AND SUGARCANE BAGASSE

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ABSTRACT: PCR based on specific primers was employed for detection of important cellulase genes (CBH1, EGI and EGII genes) in the genomes of eighteen Trichoderma isolates recovered from Egyptian soil rich with the agricultural wastes. The Trichoderma asperellum (Tm4) isolate that exhibited the highest hydrolysis zone was selected for evaluation their cellulase activities via solid state fermentation on media supplemented with rice straw and sugarcane bagasse as sole carbon sources. The results showed that the highest FPase, CMCase, Xylanase activities were obtained when rice straw was used as sole carbon source as compared to sugarcane bagasse. In addition, the cellulase activities were better in each the untreated rice straw and sugarcane bagasse than the alkali pretreated rice straw and sugarcane bagasse. The C/N ratio was decreased, while the weight loss was increased in the treatments inoculated with Trichoderma as compared to control. Moreover, the pH values of each alkali pretreated rice straw and sugar cane bagasse were shifted toward the acidity. Meanwhile, the pH values of untreated rice straw were shifted toward alkali state.

Key words: Cellulase genes, FPase, CMCase and Xylanase

INTRODUCTION

Accumulation of the agricultural wastes such as rice straw and sugarcane bagasse residues each year in huge amounts around the world represent a critical challenge facing all the humanity (Sari et al., 2021). Xu et al. (2019) reported that China produces about two million tons of sugar cane wastes each year. In addition, rice straw is one of the most copious agricultural remnants in the world (Binod et al., 2010; Rahnama et al., 2016). It must be mentioned that India cultivated in 2016 approximately 43.19 million hectares of rice producing 280 million tons of wastes (Sarangi et al., 2021). Moreover, the European countries produce 180 million tons of wastes yearly via biogas production (Alias et al., 2022). Since the agricultural wastes as rice straw contain cellulose, hemicellulose and lignin, therefore this makes it difficult to degrade (Kaur et al., 2019). Hence, farmers discard their agricultural remnants by combustion and this mean pollution of the environment.

Biological fermentation of agricultural cellulosic wastes to provide important substances like glucose and ethanol is critical issue not only for industrial purposes but also for conserving the environment (Häkkinen et al., 2012; Cova et al., 2018; Torres et al., 2019; Wang et al., 2020).

Trichoderma sp. has complete arsenal of cellulosases that can convert cellulose to glucose in ecofriendly way and with low cost (Chokhawala et al., 2015). Trichoderma cellulolytic system includes three groups of enzymes, exoglucanases, endoglucanases and β-glucosidase (Bhat and Bhat, 1997; Sukumaran et al., 2005; Zhang et al., 2014; Guruk and Karaaslan, 2020). It is well known that the content of cellulases of fungi and their efficiency are very dissimilar. For example, Chrysosporium lucktowense has one gene of each exoglucanases and endoglucanases. Although, the cellulolytic system of T. reesei contain two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGV and EGV) and two glucosidases (Salohimeo et al., 1997; Srisodsuk, 1994; Hong et al., 2001; Foreman et al 2003; Azimova et al., 2016).

CBH1 is substantial enzyme that degrade the cellulose from the ends, and represent about 60% of total protein (Seiboth et al., 1992; Horn et al., 2009; Cova et al., 2018).
It was shown that the promoter of CBH1 gene is classified as one of the strongest promoters (Beier et al., 2022). Meanwhile, EGI and EGII attack the cellulose polymer internally and represent more than 20% of the extracellular protein (Wood and McCrae, 1982; Miettinen-Oinonen and Suominen, 2002; Karkehabadi et al., 2008; Horn et al., 2012; Chokhawala et al., 2015). The EGII of Trichoderma was proved to be excellent enzyme and is frequently used in fabrics industry (Samanta et al., 2012).

Production of cellulases from agricultural residues requires physical and chemical processings (Sánchez, 2009). Solid-state fermentation (SSF) is the fermentation of the solid waste in presence of little water (Prabhu et al., 2022). SSF is characterized with high cellulase levels with temperature constancy and low cost (Holker and Lenz, 2005; Singhania et al., 2009; Saqib et al., 2010; Rahnama et al., 2013; Avila et al., 2019; Perez et al., 2019; Alias et al., 2021).

This work aimed at screening of some important cellulase genes (CBH1, EGI and EGII) using PCR specific primers in eighteen Trichoderma isolates collected from different lignocellulosic agricultural wastes represent different zones of Egypt. Meanwhile, evaluation of cellulase and xylanase activities of T. asperellum (Tm4) isolate under SSF conditions in medium supplemented with rice straw and sugarcane bagasse as sole carbon source.

**MATERIALS AND METHODS**

**Trichoderma isolates**

Eighteen isolates of Trichoderma were recovered from agricultural wastes-rich soil represent several governorates in Egypt and were identified before as indicated by Ismaeil et al. (2022). Seven isolates were belong to Trichoderma longibrachiatum (TM13, TM31, TM33, TM36, TM41, TM44 and TM45), while eleven isolates were identified as Trichoderma asperellum (TM4, TM5, TM6, TM8, TM9, TM16, TM18, TM19, TM29, TM35 and TM42).

**DNA extraction**

The genomic DNA was isolated from all tested isolates following the protocol of Al-Samarrai and Schmid (2000).

**Amplification of cellulase genes**

The primers for the amplification of CBH1 gene were; F- (5´-TCAACCCGGGACTGGCA TA-3´) and R- (5´-CGACGTCTCGAACTGG GTG-3´) (Shoemaker et al., 1983). Meanwhile, the EGI gene was amplified using the following primers; F- (5´-CCCTCAAGTTACACTGC CG TT-3´) and R- (5´-GGGTAGTAGTGCGCAT CAGGC-3´). Finally, for the EGII gene was amplified using the primers; F- (5´-CGTCGGCCGCGTAGATATCAG-3´) and R- (5´-CTGTATACCCGCGCAACCAG-3´). Each the EGI and EGII primers were designed from DNA sequences on NCBI site (http://www.ncbi.nlm.nih.gov/). PCRs were carried out in 25μl reaction mixtures using the following conditions: Initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 2 min, 59°C for 1 min, and 3 min at 72°C. A final extension was done at 72°C for 7 min. Amplified DNA products were detected by electrophoresis in 1.5% agarose gel at 100V for 2 h.

**Solid state fermentation**

**Substrates preparation**

Rice straw and sugarcane bagasse wastes collected from delta region of Egypt were drilled to 5 mm particles. The two substrates were treated with 0.5% NaOH at 1: 10 ratio (1 g substrate in 10 ml of NaOH) and autoclaved at 121°C for 20 min. Finally, the autoclaved materials were washed with tap water and distilled water till neutrality and dried as shown previously by Rahnama et al. (2013). The untreated rice straw and sugarcane bagasse were used as controls.

**Trichoderma inoculum preparation**

The isolate of T. asperellum Tm4 (isolated from soil rich with wheat crop residues in Sadat city, Egypt) That gave the highest diameters of inhibition zones on medium containing carboxy methyl cellulose was selected and used as inoculum (Ismail et al., 2022). The isolate was grown on Potato Dextrose Agar (PDA) for seven days. The agar surface was washed with sterilized distilled water and the spores
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Fermentation process

Eight grams of each sugarcane bagasse and rice straw (alkali-treated and untreated) were added into 500 ml flasks containing Mandel’s medium (Mandels *et al.*, 1974). Each flask was inoculated with 15 ml spore suspension (10⁶ spores/ml), mixed well and incubated at 30°C for 15 days. The fermented materials were filtered using a cloth cheese and the filtrates were centrifuged at 5000 rpm/min for 20 min and re-filtered using 0.45μm sterile membrane filter. Uninoculated rice straw and sugarcane samples were used as controls. All tests were carried in triplicates.

Cellulase and xylanase activities

The filtrates were used for estimating the cellulase and xylanase activities as described previously by Ghose, (1987); Mandel and Sternberg (1976); Mandels and Weber (1969); Kinoshit *et al.* (1981). Whereas one unit of the enzyme was considered as the amount of enzyme required to release 1 μ mol of glucose or xylose/min and expressed as a unit of enzyme activity per gram fermented substrate (U/g):

\[
\text{FPase (U/g)} = \frac{\text{Filter paper activity (U/ml) \times Volume of extract (ml)}}{\text{Dry weight of wastes (g)}}
\]

\[
\text{CMCase (U/g)} = \frac{\text{CMCase activity (U/ml) \times volume of extract (ml)}}{\text{Wastes dry weight (g)}}
\]

\[
\text{Xylanase (U/g)} = \frac{\text{Xylanase (U/ml) \times volume of extract (ml)}}{\text{Wastes dry weight (g)}}
\]

Chemical characterization of substrates

The following parameters; weight loss, pH, organic carbon, organic nitrogen and C/N ratio were determined:

- **Weight loss**

  The solid residues were dried at 70°C and the weight loss % was calculated as follows;

  \[
  \text{Weight loss (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100
  \]

- **pH**

  pH was determined using a digital pH meter.

- **Organic carbon**

  The organic carbon was estimated by adding known amount of the dried samples in a weighed silica crucible. The samples were incubated in a muffle furnace for 2 h at 60 °C. Followed with desiccation, cooling and finally it was weighed (ash weight). The total percent of organic matter was estimated by calculating the difference of the samples dry weight and ash weight. Then organic carbon was calculated by dividing the organic matter percent by the factors 1.724 (Jackson, 1973).

- **Total nitrogen**

  Nitrogen in the samples was estimated by the micro kjeldahl method (Jackson, 1973). 0.5 g of the dried sample was digested using 10 ml of conc. sulphuric acid in the presence of 0.3g of catalytic mixture (potassium sulphate, copper sulphate and selenium powder in the ratio (50:10:1) in the micro Kjeldahl digestion unit. The digested samples were diluted with 80 ml distilled water and distilled after the addition 50 ml of NaOH (40%).

  The ammonia evolved was trapped in boric acid (2%) mixed indicator solution and titrated against 0.1 N HCl. The nitrogen content was calculated from the volume of acid consumed.

  \[
  \text{Nitrogen (\%)} = \frac{(\text{Vsample} - \text{Vblank}) \times N \times 14.007 \times 100}{\text{m(sample)}}
  \]

  \[
  \text{Vsamp} = \text{Volume titrant used for titrating the sample (ml)}
  \]

  \[
  \text{Vblank} = \text{Volume titrant used for titrating the blank (ml)}
  \]

  \[
  \text{N} = \text{Normality of titrant}
  \]

  \[
  \text{m sample = Weight sample (mg)}
  \]

- **C/N ratio**

  The C/N ratio was calculated by dividing percentage of organic carbon by percentage of organic nitrogen.

Statistical analysis

The data were analysed statistically by ANOVA to determine standard error of means and comparisons of means at a 5% significance.
Results

Amplification of cellulase genes

PCR was employed for detection of important cellulase genes (CBH1, EGI and EGII) in the genomes of eighteen Trichoderma isolates as shown in Figure 1. The primers utilized for amplification of CBH1 gene yielded one band (500 bp) in all tested isolates (Figure 1, A). Meanwhile, for EGI, the PCR produced one band (750 bp) that appeared in all isolates (Figure 1, B). Finally, one band of approximately 500 bp was detected in all isolates with amplification of the EGII gene (Figure 1C).

Cellulase activities via solid-state fermentation

Cellulase and Xylanase activities of Trichoderma asperellum (Tm4) isolate were evaluated after growth on solid fermentation medium contain rice straw and sugarcane bagasse as sole carbon sources. Each waste was divided into two treatments (alkali treated and untreated). FPase, CMCase and Xylanase activities were estimated in culture filtrates after fifteen days of fermentation.

FPase activities (units per gram dry substrate Ug⁻¹) were calculated in culture filtrates produced after fermentation. Higher activity of FPase (2.749 IU/g⁻¹) was recorded when untreated rice straw was used as substrate compared to 2.14 IU/g⁻¹ with the alkali pretreated rice. Meanwhile, FPase activity (1.26 IU/g⁻¹) was obtained for Trichoderma isolate grown on medium supplemented with untreated sugarcane bagasse as compared to 0.452 IU/g⁻¹ with alkali pretreated sugarcane bagasse (Figure 2). For CMCase activity, the untreated rice straw showed the highest activity, followed with the treated rice straw, then the untreated sugar cane bagasse and finally, the treated bagasse group with 5.85, 5.54, 5.29 and 3.62 (IU/g⁻¹) respectively, as indicated in Figure 3. Finally, for Xylanase activity, the untreated rice straw showed the highest activity, followed with the alkali-treated rice straw, then the untreated sugar cane bagasse and finally, the treated bagasse group with 51.15, 49.70, 49.65 and 42.55 (IU/g⁻¹), respectively as indicated in Figure 4.

Figure (1). Amplification of cellulase genes. A; Amplification of CBH1 gene, B; amplification of EGI; and C; amplification of EGII gene. Whereas TM13, TM31, TM33, TM36, TM41, TM44 and TM45 are Trichoderma longibrachiatum isolates, while TM4, TM5, TM6, TM8, TM9, TM16, TM18, TM19, TM29, TM35 and TM42 are Trichoderma asperellum isolates.
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Figure (2). FPase activity (IU/g⁻¹ dry substrate) of *Trichoderma asperellum* TM4, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.

Figure (3). CMCase activity (IU/g-1 dry substrate) of *Trichoderma asperellum* TM4, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.
Chemical analysis of wastes

The C/N ratio was calculated after growing of *Trichoderma* isolate on rice straw and sugarcane bagasse (treated and untreated) as compared to control. The treated rice straw showed the lowest percentage of C/N %, followed with the untreated rice straw treatment, then the treated sugar cane bagasse and finally the untreated bagasse group with 24.92, 50.92, 96.72 and 199.14%, respectively, as compared to control (Figure 5). In addition, the percentage of weight loss was estimated after 15 days of incubation for rice straw and sugar cane bagasse (treated and untreated). The reduction in total dry weights was indicator for the good growth of the inoculated fungus. The results showed a high reduction of total weight in the treatments inoculated with *Trichoderma* as compared to control. The treated rice straw showed the highest reduction in weight loss, followed with the alkali-treated bagasse, then the untreated rice and, finally the untreated bagasse with 63.88, 48.86, 35.36 and 22.30%, respectively as compared to the control (Figure 6).

The change in pH values during the solid-state fermentation process was recorded for each rice straw and sugarcane bagasse (treated and untreated). Data in Table (1) showed that the pH values of each treated rice and sugarcane bagasse were changed from 7.58, 6.35 (control) to 4.31, 4.49 (inoculated with *Trichoderma*), respectively. Meanwhile, the pH values of each untreated rice and sugarcane bagasse were changed from 5.43, 4.74 (control) to 6.25, 4.73 (inoculated with *Trichoderma*), respectively.

Discussion

PCR was succeeded for detection of important cellulase genes (CBH1, EGI and EGII genes) in the genomes of tested *Trichoderma* isolates as shown in Figure 1. The primers used for the amplification CBH1 yielded a band of approximately 500 bp in all of tested isolates (Figure 1A), this band is the same band obtained previously by Shoemaker et al. (1983). For EGI and EGII, the PCR produced bands of approximately 750 bp, 500bp in all of tested isolates (Figure 1B, 1C), respectively. The sizes
of these bands were confirmed through alignments of primer sequences with the published sequences of cellulase genes on NCBI database (http://www.ncbi.nlm.nih.gov/). Previous studies indicated that Trichoderma sp. have two exoglucanase genes (CBHI and CBHII), five genes of endoglucanases (EGI, EGII, EGIII, EGIV and EGV) and two genes of β-glucosidases (Srisodsuk, 1994; Saloheimo et al., 1997; Hong et al., 2001; Foreman et al, 2003; Azimova et al., 2016). CBH1 is critical gene with strong promoter which encode for enzyme that hydrolyze the cellulose from the ends, and represent about 60% of total protein (Seiboth et al., 1992; Horn et al., 2012; Beier et al., 2022). Meanwhile, EGI and EGII encode for endoglucanases which attack the cellulose chains internally and represent more than 20% of the extracellular protein (Wood and McCrae, 1982; Miettinen-Oinonen and Suominen, 2002; Karkehabadi et al., 2008; Horn et al., 2012; Chokhawala et al., 2015).

Rapid test based on the diameters of hydrolysis zones was done for selection the highest isolates in hydrolysis of cellulose (Ismeil et al., 2022). The Trichoderma asperellum (Tm4) isolate exhibited the highest hydrolysis zone. Based on these results, this isolate was selected for evaluation of their cellulase activities via solid state fermentation. Previous studies employed this technique in screening the efficient cellulolytic Trichoderma isolates (Sazci et al., 1986; Khokhar et al., 2012; Syed et al., 2013; Castrillo et al., 2021).

Our results showed that the higher FPase, CMCase, Xylanase activities were obtained when untreated rice straw was used as sole carbon source as compared to alkali pretreated rice straw. Rahnama et al. (2013) reported that the untreated rice straw gave higher activity of FPase, CMCase, β-glucosidase, and xylanase on media contain untreated rice straw as compared to alkali-pretreated rice. X-ray analysis explained that the alkali treatment increase the crystallinity of cellulose in the treated rice straw and therefore this requires more cellulases for its hydrolysis as compared to the untreated rice straw (Rahnama et al., 2013). In general, it must be mentioned that using of rice straw as substrate was better than sugarcane bagasse for FPase, CMCase, Xylanase activities in solid state fermentation medium. Badhan et al. (2007) showed that the higher activity of FPase, CMCase, β-glucosidase were obtained when rice straw was used as substrate, while the lower activities were obtained with wheat straw, bagasse and corn cob substrates.

Figure (5). C/N ratio of rice straw and sugarcane bagasse (treated and untreated) inoculated by Trichoderma asperellum Tm4 as compared to control, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.
Figure (6). Weight loss of rice straw and sugarcane bagasse (treated and untreated) inoculated by *Trichoderma asperellum* TM4 as compared to control, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali-treated sugarcane bagasse) Means with the different letter are significantly different.

Table (1). pH values before and after inoculation with *Trichoderma asperellum* TM4.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH of control</th>
<th>pH after inoculation with <em>Trichoderma</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated rice</td>
<td>5.43± 0.015</td>
<td>6.25± 0.25</td>
</tr>
<tr>
<td>Alkali-treated rice</td>
<td>7.58± 0.015</td>
<td>4.31± 0.005</td>
</tr>
<tr>
<td>Untreated bagasse</td>
<td>4.74± 0.110</td>
<td>4.73± 0.025</td>
</tr>
<tr>
<td>Alkali-treated bagasse</td>
<td>6.74± 0.065</td>
<td>4.49± 0.010</td>
</tr>
</tbody>
</table>

Means with the different letter, per each column, are significantly different.

The C/N % was decreased in the treatments inoculated with *Trichoderma* as compared with control. The same observation was mentioned before by Hu *et al.* (2012) who indicated that the total organic carbon and C/N ratio were decreased during the fermentation process due to the degradation of substrate carbon. Moreover, our study indicated that the percentage of weight loss in rice straw and sugarcane bagasse after 15 days of incubation with *Trichoderma* was higher than the non-inoculated control. These results were in harmony with these obtained by Sarangi *et al.* (2021) who indicated that there was 20% reduction of rice straw during composting with *Trichoderma*. It’s unknown why the weight loss in rice straw was more than in sugarcane bagasse may be due to the difference in their cellulose structure.

The pH values were estimated on rice straw and sugarcane bagasse (alkali-treated and untreated) and compared to control. The pH values of each alkali-treated rice and sugarcane bagasse were shifted toward the acidity. Meanwhile, the pH values of untreated rice were shifted to alkali state. Rahnama *et al.* (2016) reported that the cellulase enzymes are more active in the acidic region. Moreover, Hu *et al.* (2012) mentioned that pH was decreased after 29 days from fermentation of *Pangolagrass* with several microorganisms.
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حصر جينات السيلولوز والأنشطة التحليلية للترايكوديرما اسبيريلم من خلال تخم الحالة الصلبة لقش الأرز ومختلف قصب السكر

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الملخص العربي

تم توظيف تفاعل البلمرة المشتقة باستخدام بادرات متخصصة من أجل الكشف عن جينات السيلولوز المهمة CBH1 و CBH2 في جينومات 18 عزلة من عزلات الترايكوديرما والمعزولة من تربة مصرية غنية بالمخلفات الزراعية. عزلة EGI والتي أعطت أعلى متعاطم تحل سيلولوزي تم اتخاذها من أجل تقييم اخشنتها السيلولوزية من ترايكوديرما اسبيريلم TM4 خلال تخم البيئة الصلبة على بيئة تشمل قش الأرز وقصب السكر كمصادر وحيدة للكربون. النتائج أوضحت أن أعلى انشطة تم التحصل عليها عندما استخدم قش الأرز كمصادر وحيدة للكربون بالمقارنة مع مخلفات قصب السكر. بالإضافة إلى ذلك الاختلاف الإحصائي كانت أفضل عندما استخدمت مخلفات قش الأرز وقصب السكر غير المعالمة بالمقارنة بنفس المخلفات المعالمة قلياً. نسبة الكربون إلى النتروجين انخفضت بينما زادت نسبة الفائق في الوزن بشكل ملحوظ في المعالمة الملفحة بالترايكوديرما بالمقارنة بالكنتروغير الملفحة. قم درجات pH في كلا من مخلفات قش الأرز وقصب السكر المعالمين قلياً اتجهت ناحية الحموضة بينما اتجهت درجات pH في كلا من مخلفات قش الأرز وقصب السكر غير المعالمين قليا ناحية القلوية.

الكلمات الدالة: جينات السيلولوز ، FPase ، CMCase ، Xylanase