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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 cellulases 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 luckttowense* 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, EGIV and EGV) and two glucosidases (Saloheimo *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*.,

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2012). It was shown that the promoter of CBH1gene 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; Ávila *et al*., 2019; Perez *et al*., 2019; Alias *et al*., 2021).

This work aimed at screening of some important cellulase genes (CBHI, 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´-TCAACCGCGGACTGGCA TA-3´) and R- (5´-CGACGTCTCGAACTGG GTG-3´) (Shoemaker *et al.*, 1983). Meanwhile, the EGI gene was amplified using the following primers; F- (5´-CCCTCAGTTACACTGCCG TT-3´) and R- (5´-GGGTAGTAGTGGCAT CAGGC-3´). Finally, for the EGII gene was amplified using the primers; F- (5´-CGTCGGCCCGTAGATATCAG-3´) and R-(5´-CTGTATACCCGCCCAACCAG-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 with0.5% 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 (Ismaeil *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

suspension was adjusted to 1×10^6 spores mL^{-1} by using a haemocytometer (Rahnama *et al*., 2013).

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 (10^6 spores/ml), mixed well and incubated at 30℃ 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); Mandels 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):

FPase (U/g) =

$$\frac{Filter\ paper\ activity\left(\frac{U}{ml}\right)*Volume\ of\ extract\ (ml)}{Dry\ weight\ of\ wastes\ (g)}$$

CMCase (U/g) =

$$\frac{CMCase\ activity\ \left(\frac{U}{ml}\right)*volume\ of\ extract\ (ml)}{Wastes\ dry\ weight\ (g)}$$

Xylanase (U/g) =

$$\frac{Xylanase\left(\frac{U}{ml}\right)*volume\ of\ extract\ (ml)}{Wastes\ dry\ weight\ (g)}$$

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;

$$\text{Weight loss (\%)} = \frac{Initial\ weight - final\ weight}{initial\ weight} X100$$

- 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 ℃. 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.

$$\text{Nitrogen (\%)} = \frac{(Vsample - Vblank)*N*14{,}007*100}{m(sample)}$$

Vsample = Volume titrant used for titrating the sample (ml)

Vblank = Volume titrant used for titrating the blank (ml)

N = Normality of titrant

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

level were carried out according to Duncan's multiple range test analysis, the software Costat version 6.3.

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 Xylanse 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.

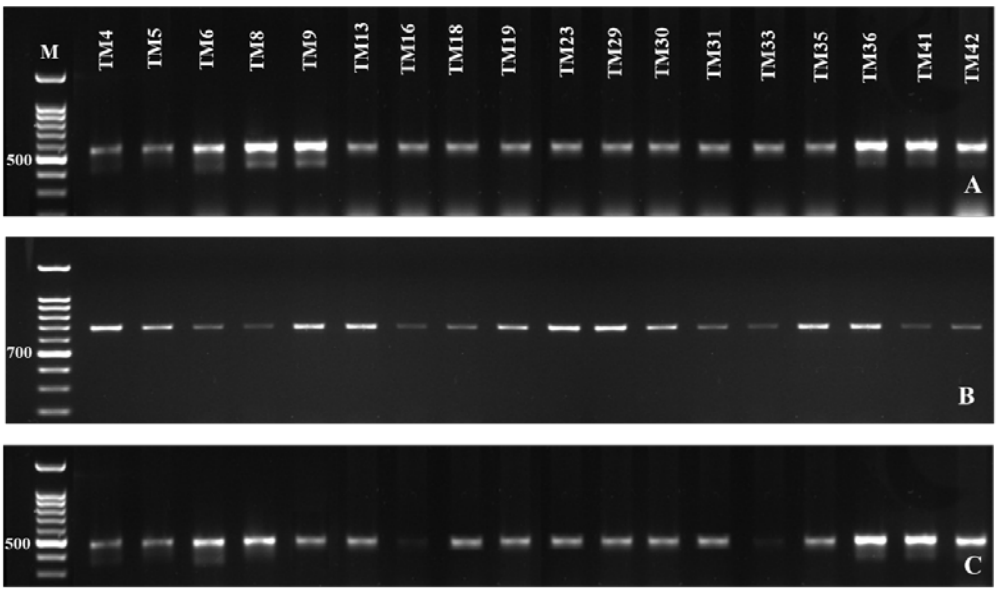

Figure (1). Amplification of cellulase genes. A; Amplification of CBH1 gene, B; amplification of EGI; and C; amplification of EGII gene. Whereas TM13, TM31, TM33, TM36, TM41, TM44 and TM45 are *Trichoderma longibrachiatum* isolates, while TM4, TM5, TM6, TM8, TM9, TM16, TM18, TM19, TM29, TM35 and TM42 are *Trichoderma asperellum* isolates.

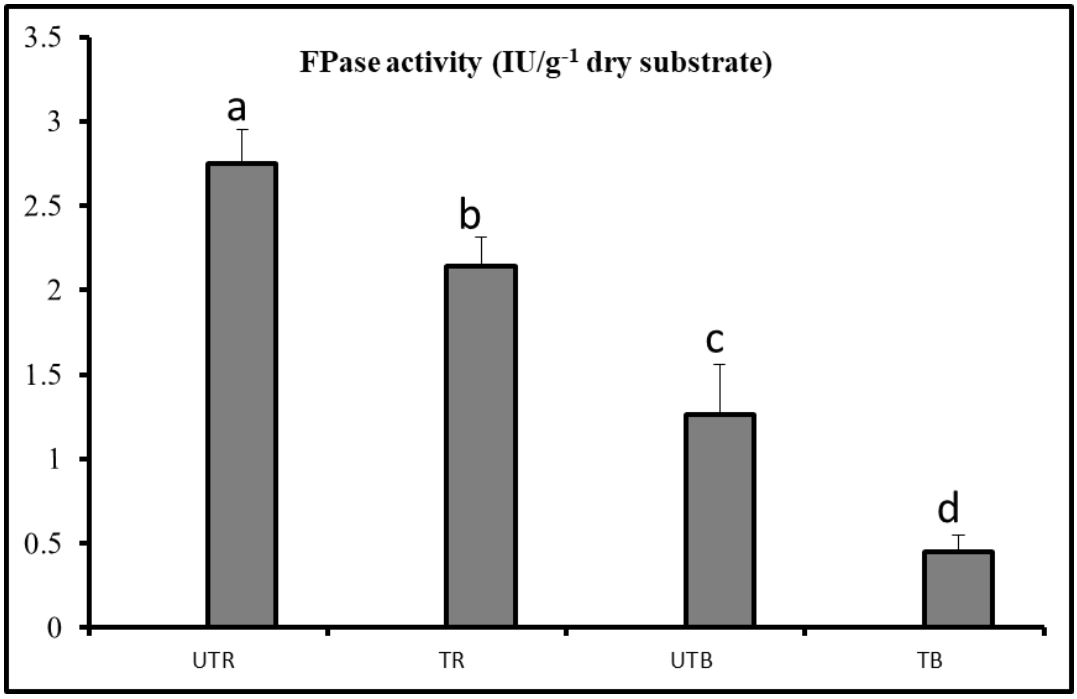

Figure (2). FPase activity (IU/g^{-1}dry substrate) of *Trichoderma asperellum* TM4, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.

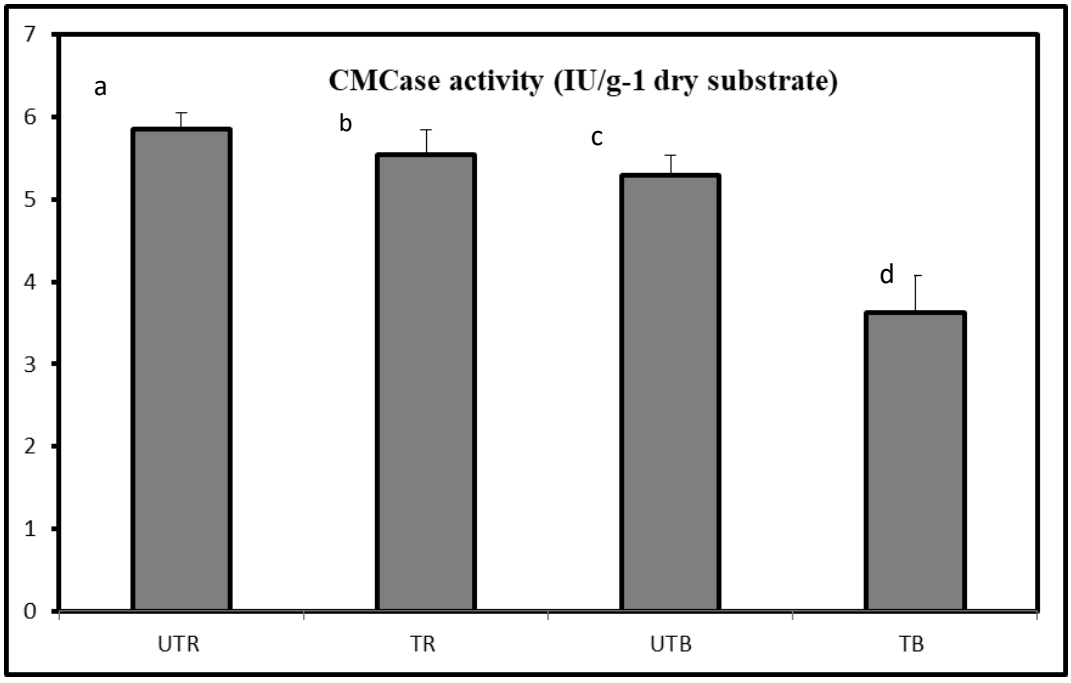

Figure (3). CMCase activity (IU/g^{-1} dry substrate) of *Trichoderma asperellum* TM4, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.

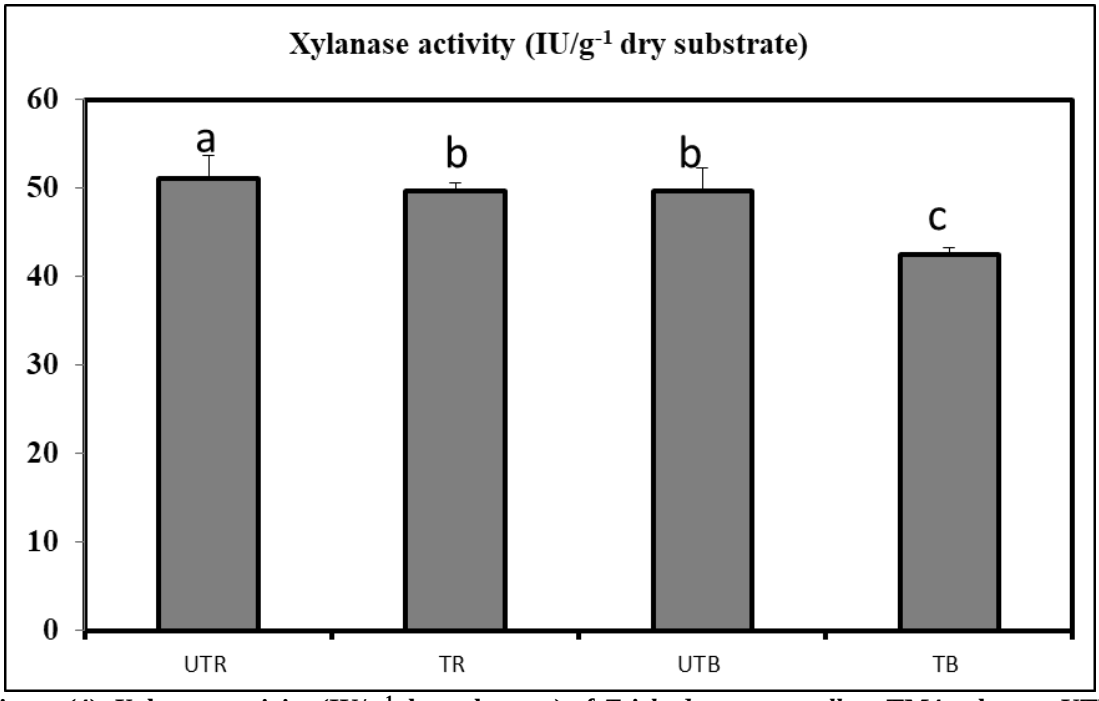

Figure (4). Xylanase activity (IU/g^{-1} dry substrate) of *Trichoderma asperellum* TM4, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.

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.

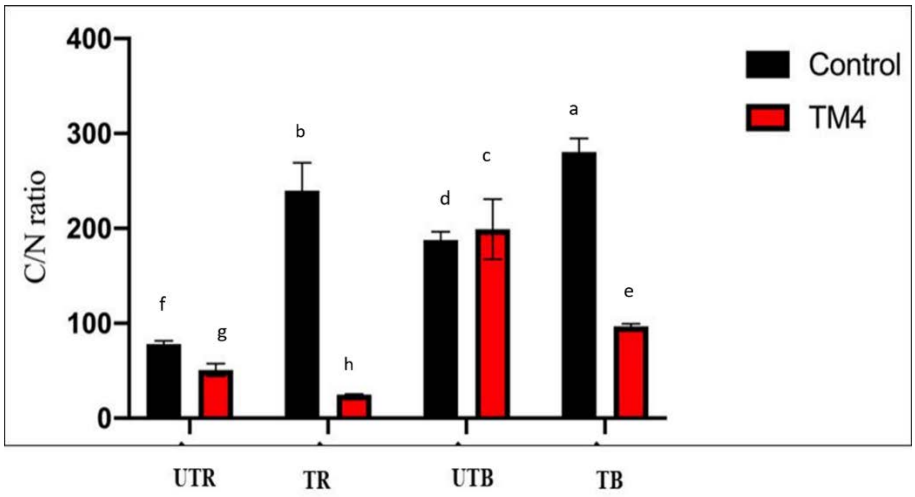

Figure (5). C/N ratio of rice straw and sugarcane bagasse (treated and untreated) inoculated by *Trichoderma asperellum*TM4as compared to control, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali treated sugarcane bagasse). Means with the different letter are significantly different.

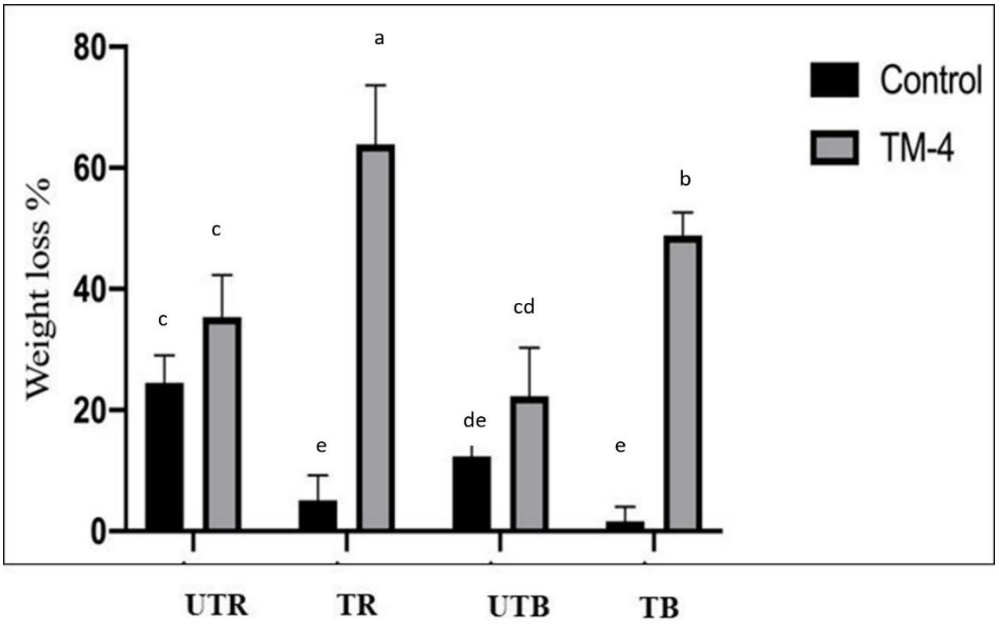

Figure (6). Weight loss of rice straw and sugarcane bagasse (treated and untreated) inoculated by *Trichoderma asperellum*TM4 as compared to control, whereas UTR (untreated rice straw), TR (alkali-treated rice straw), UTB (untreated sugarcane bagasse) and TB (alkali-treated sugarcane bagasse) Means with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

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

Treatments	pH of control	pH after inoculation with *Trichoderma*
Untreated rice	$5.43^{c}\pm 0.015$	$6.25^{a}\pm 0.25$
Alkali-treated rice	$7.58^{a}\pm 0.015$	$4.31^{d}\pm 0.005$
Untreated bagasse	$4.74^{d}\pm 0.110$	$4.73^{b}\pm 0.025$
Alkali-treated bagasse	$6.74^{b}\pm 0.065$	$4.49^{c}\pm 0.010$

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 to 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 السيليوليز والانشطة التحللية للترايكوديرما اسبيريلم من خلال تخمر الحالة الصلبة لقش الارز ومخلف قصب السكر

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الملخص العربى

تم توظيف تفاعل البلمرة المتسلسل باستخدام بادئات متخصصة من اجل الكشف عن جينات السيليوليز المهمة CBH1 و EGI و EGII فى جينومات ١٨ عزلة من عزلات الترايكودرما والمعزولة من تربة مصرية غنية بالمخلفات الزراعية. عزلة ترايكودرما اسبيريلم TM4 والتى اعطت اعلى مناطق تحلل سليولوزى تم انتخابها من اجل تقدير انشطتها السيليولوزية من خلال تخمر البيئة الصلبة على بيئة تشمل قش الارز وقصب السكر كمصادر وحيدة للكربون. النتائج اوضحت ان اعلى انشطة انزيمية ل FPase و CMCase و Xylanase تم التحصل عليها عندما استخدم قش الارز كمصدر وحيد للكربون بالمقارنه مع مخلفات قصب السكر. بالاضافة الى ذلك الانشطة الانزيمية كانت افضل عندما استخدمت مخلفات قش الارز وقصب السكر غير المعاملة بالمقارنة بنفس المخلفات المعاملة قلويا. نسبه الكربون الى النيتروجين انخفضت بينما زادت نسبة الفاقد فى الوزن بشكل ملحوظ فى المعاملات الملقحة بالترايكودرما بالمقارنة بالكنترول غير الملقح. قيم درجات pHفى كلا من مخلفات قش الارز وقصب السكر المعاملين قلويا اتجهت ناحية الحموضة بينما اتجهت درجات pH فى كلا من مخلفات قش الارز وقصب السكرغير المعاملين قلويا ناحية القلوية.

الكلمات الدالة: جينات السيليوليز ، FPase, CMCase, Xylanase