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ORIGINAL ARTICLE

Wheat straw hydrolysis by using co-cultures of *Trichoderma reesei and Monascus purpureus* toward enhanced biodegradation of the lignocellulosic biomass in bioethanol biorefinery

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Abstract

Wheat straw (*Triticum aestivum*) is one of the lignocellulosic materials largely available worldwide and could be potentially used for biofuel production. Aiming the cost-effective utilization of wheat straw in the sugar-based biorefineries, co-cultures of *Trichoderma reesei* and *Monascus purpureus* were used for the enzymatic hydrolysis of the wheat straw biomass. The enzymatic breakdown of the dual-fungi-treated wheat straw was chemically analyzed through different enzyme/compositional assays, and the structural modifications were studied through scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR). For hydrolytic enzyme assays, the co-culture treatments resulted in significantly higher values (carboxymethyl cellulase (212.3 U/ml; $p = 0.0173$*), total cellulase (202 U/ml; $p < 0.0001$****), and xylanase (96.7 U/ml; $p < 0.0001$****) when compared with the readings of pure cultures. This hydrolytic activity resulted in the enhanced breakdown of wheat straw exhibiting a significant loss of 45.2% in lignin, 19.18% in cellulase, and 21.84% in hemicellulose contents. Furthermore, SEM and FTIR analysis of the co-culture treatments verified the improved biodegradation of wheat straw. Accumulatively, these results suggest a better approach for the effective use of dual-fungi for the lignocellulosic biomass breakdown and may have applications in bioethanol biorefineries using wheat straw as a sugar feedstock.

Keywords Biodegradation · Lignocellulose · Co-culture · Hydrolysis · Wheat straw

Highlights
- Biodegradation of wheat straw was evaluated using mono- and co-culture of *T. reesei* and *M. purpureus* under solid-state fermentation.
- Hydrolytic enzyme activity of mono- and co-culture treatments was analyzed and compared.
- Compositional analysis and structural modifications of biodegraded wheat straw were observed to compare the structural loss/gain of lignocellulosic constituents.
- A significant enhancement of biodegradation of wheat straw with fungal co-cultures suggested the synergism among the fungi for the cooperative work.

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1 Introduction

In recent years, research interests are more focused on finding renewable energy to substitute the dependence on depletable fossil fuels [1]. Global warming, economic shortfalls, rising world population, and the other drawbacks associated with the usage of fossil fuels [2] have stimulated a road map toward the synthesis of biofuels from plant biomass in an eco-friendly way [3]. Wheat straw (WS), a field residue of cultivated wheat (*Triticum aestivum* L.), offers promising biofuel feedstock largely available worldwide [4]. Utilization of WS for biofuel production not only provides its efficient disposal but also ensures a cheap sustainable substrate supply not competing for the human food chain.

Lignocellulosic biomass (LB) constitutes three major components: cellulose, hemicellulose, and lignin where the lignin provides the structural integrity and cross-links the cellulosic fibrils. WS is mainly composed of polysaccharides (30–45% cellulose, 20–25% hemicellulose), 15–20% lignin, phytic acid, and some minor organic/inorganic compounds. The

depolymerization of these polysaccharides to the energy-rich fermentable sugars is the basis of liquid biofuel synthesis [5], where lignin acts as a barrier to the enzymatic breakdown of the encapsulated cellulose and hence to be removed. This delignification either through physical, chemical, or biological pretreatment is essential for the structural modifications of LB to make it accessible for the subsequent enzymatic hydrolysis and fermentation [5–7]. Biological pretreatment using microbes has gained increasing attention in recent years and is considered an efficient, eco-friendly, cost-effective, and reproducible approach [4, 8].

To date, biological pretreatment of WS has been dominantly reported by using pure cultures of fungi (*Pleurotus* sp. and *Pycnoporus* sp. [9], *Basidiomycetous* and *Irpex lacteus* [10], *Aspergillus niger* [11], and *Trichoderma reesei* [12]). However, reports using mix cultures of fungi are also there (*T. reesei* + *Aspergillus* sp. [13]; *Fusarium oxysporum* + *Saccharomyces cerevisiae* [14]). Co-cultures of the lignocellulolytic fungi could improve the degradation of LB by secreting a blend of hydrolyzing enzymes [15, 16]. Biofuel research should focus on new strategies to optimize the fungal pretreatment and to minimize the drawbacks associated with it such as toxic by-products, unnecessary breakdown, and long cultivation times [17]. In recent years, the prokaryote-derived clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system has emerged as a powerful tool for molecular editing in numerous energy crops. For example, to circumvent the barriers of cell wall complexities of switchgrass (*Panicum virgatum*) for enzymatic hydrolysis and subsequent fermentable sugars release, CRISPR/Cas9 technology has been used to target three 4-coumarate:coenzyme A ligase (*4CL*) genes (*Pv4CL1*, *Pv4CL2*, and *Pv4CL3*) involved in lignin biosynthesis pathway [18]. This tetra-allelic knockout of *4CL* genes resulted in an 8–30% reduction in lignin content in the switchgrass cell wall and indirectly increased (7–11% increased glucose, 23–32% increased xylose) the release of fermentable sugars during biofuel production [18].

Production of cellulases, a very important industrial enzyme used in the bioconversion of LB into bioethanol, textile, paper, and numerous other applications, is attracting attention over the years. Cellulase is a multienzyme complex primarily constituting endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidases (BGL) [19]. WS has been widely used for the cellulase enzyme production in solid-state fermentation [20]. Cellulase production using submerged fermentation is not cost-effective and produces low yields, thus limiting their industrial applications [21]. In contrary, solid-state fermentation using cheap LB substrates represents an effective, low-cost, eco-friendly, and sustainable approach for cellulase production [22]. Cellulase synthesis using individual fungi may not be very efficient for the hydrolysis of WS. However, a blend of cellulases through using cultures of fungal consortia could improve the production technology.

This is the first report of using a blend of *Trichoderma reesei* and *Monascus purpureus* for the delignification of WS. Among the best-known fungal species for LB biodegradation, *T. reesei* ranks in the top list and has been reported extensively [12, 21–23]. However, *Trichoderma* sp. has a lower potential for the β-glucosidase (BGL) production [24]. In contrary, *Monascus* sp. is a great producer of extracellular BGL [25], yet very limited information is available about *M. purpureus* in the context of its delignification potential. Here, we propose an improvement in the WS biodegradation and cellulase production using a co-culture of *T. reesei* and *M. purpureus* expected to work efficiently. Furthermore, we tried to identify any synergistic interactions between the two fungi strains by comparing the results of co-culture with the actions of individual fungi. A solid-state fermentation system for the individual and co-culture effects of the fungal pretreatment on WS was evaluated based on the compositional and enzyme analysis. The physical and chemical characteristics of the biotreated WS were further investigated for the biodegradation. The experimental flowchart of the current study is graphically depicted in Fig. 1.

2 Material and methods

2.1 Preparation of lignocellulosic substrate

WS from the residue of cultivated wheat (*Triticum aestivum*) cv. "NN Gandhum 1" was collected from the field area (approximate coordinates 31.39502°N, 73.026796°E) of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. The collected WS was dried in a hot-air oven (~ 60 °C) [TR-062CN; NABERTHERM®, Germany] and was shredded to approximately 2-mm particle size with a hammer beater mill [BS hammer mill; Brightsail®, China]. The grounded WS powder (sample) was preserved in sealed plastic bags at room temperature (25 °C) until further use.

2.2 Wheat straw pretreatment by fungal cultures

2.2.1 Microorganism and culture maintenance

Fungal strains of *Trichoderma reesei* (M182) and *Monascus purpureus* (SUB 4880794) were collected from the culture collection lab of the Fermentation Technology Group of the Technical Service Division, NIBGE, Faisalabad, Pakistan. Pure cultures were preserved on potato dextrose agar (PDA) slants and were kept at 4 °C until further use. The spores of *T. reesei* and *M. purpureus* were subcultured on sterile Petri plates (100 mm × 20 mm) containing fresh PDA medium (pH

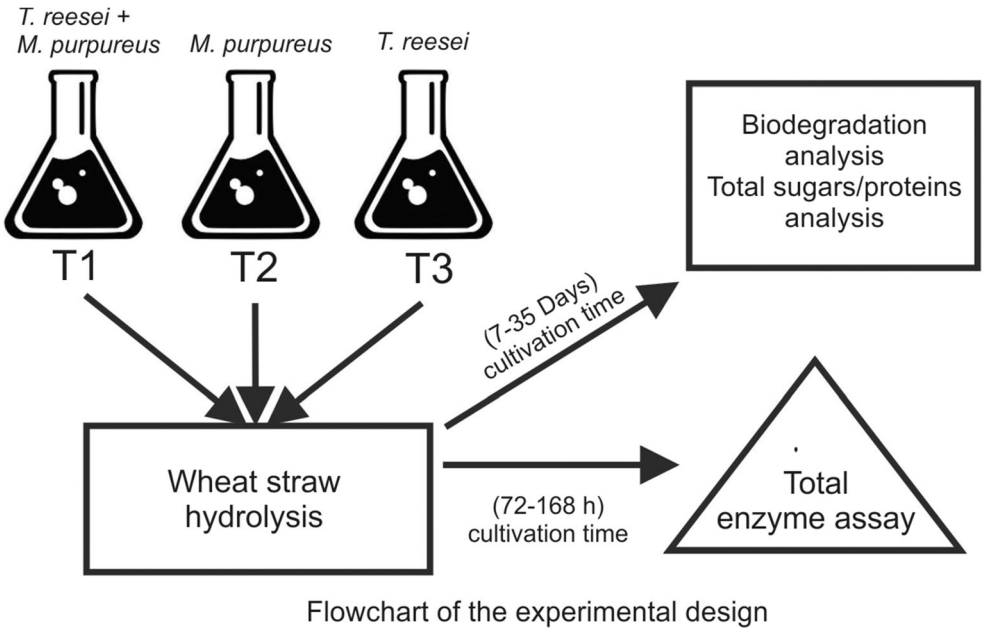

Fig. 1 Schematic flowchart of the experimental design. The treatments (T1, T2, T3) represents the co-culture and pure culture applications of the wheat straw hydrolysis. After fungal pretreatment, the total enzyme assays and biodegradation activities were analyzed accordingly

5.0), incubated upside down at 25 °C under dark for 5 days. All chemicals and reagents used in the current study were of analytical grade and were purchased from Sigma-Aldrich® [Chemie, Germany].

2.2.2 Preparation of fungal spore inoculum

To prepare the spore inoculum, 10 ml of sterile saline water was added to the 5-days old fungi culture plates (PDA media) and were scraped with a sterile spatula. The mixture was sterile filtered using "Miracloth" [Calbiochem®, UK], and the spore count was calculated using a hemocytometer [Hausser Scientific®, USA] assisted with a light microscope. The number of spores per ml of inoculum was calculated using the following equation:

$$\text{No.of}\ \frac{\text{spores}}{\text{ml}} = \frac{\text{Average no.of spores}}{0.021} \times 1000\ \mu\text{l} \times 10\ (\text{fold dilution})$$

After calculating the number of spores, the spore suspensions were transferred to sterile 50-ml tubes and diluted with sterile saline water until a spore count of ~ 10^7 spores/ml was achieved.

2.3 Solid-state fermentation of wheat straw

2.3.1 Preparation of culture media

For solid-state fermentation, 15 sterile Petri plates (100 mm × 20 mm) were prepared containing 5 g of WS powder and 25 ml of Mandel and Sternburg's medium [26] [pH 5.0, KH_2PO_4 (0.2%); urea (0.03%); $MgSO_47H_2O$ (0.03%); $CaCl_2$ (0.03%); peptone (0.075%); yeast extract (0.025%); and trace element solution ($FeSO_47H_2O$ (5 mg/ml), $MnSO_4.4H_2O$ (1.6 mg/ml), $ZnSO_47H_2O$ (1.4 mg/ml), and $CoCl_26H_2O$ (20 mg/ml) as a moistening agent]. The culture-containing petri plates were sterilized in autoclave at 121 °C (15 psi) for 15–20 min.

2.3.2 Treatments and cultivation

A total of three treatments having a spore inoculum of *T. reesei* + *M. purpureus* (T1), spore inoculum of *M. purpureus* (T2), and spore inoculum of *T. reesei* (T3) were used in this experiment. Co-culture spore inoculum of *T. reesei* + *M. purpureus* culture was prepared by thoroughly mixing the equal volume of spore inoculum of each fungus. Each treatment was performed in quintuplicate, and all the readings were measured in triplicate for statistical significance. Each Petri plate containing 5 g of WS powder moistened with the Mandel and Sternburg's medium [26] was inoculated with 5 ml of spore inoculum (~ 10^7/ml of the pure fungal culture of *T. reesei*, *M. purpureus*, or co-culture). Petri plates with respective treatments were incubated at 30 °C for 35 days.

2.3.3 Sampling and crude enzyme extraction

For enzyme assays, 5 ml of fermented WS sample of each treatment was aseptically removed at intervals of 72, 96, 120, 144, and 168 h of cultivation time. For other assays, sampling was performed at intervals of 7, 14, 21, 28, and 35 days of cultivation. After complete sampling, each Petri plate was discarded. For enzyme extraction, the extracted sample was thoroughly mixed with 30 ml of 50 mM citrate buffer (pH 4.8) inside a 100-ml beaker. The mixture was stirred at 4 °C, 300 rpm for 20 min, and then centrifuged at 10,000 rpm for 10 min. Finally, the clear supernatant was filtered and was used as the crude enzyme.

2.4 Enzyme assays

All enzyme assays were spectrophotometrically measured [PerkinElmer®, Waltham, USA], and respective controls were also run along with assays. For total cellulase, filter paper activity (FPase) was used following the "Laboratory Analytical Methods" NREL, USA [27]. For this purpose, 0.5 ml of the diluted crude enzyme [diluted with 1 ml of 50 mM citrate buffer (pH 4.8)] was saturated with a filter paper strip (Whatman No. 1; 1.0 cm × 6.0 cm; ~ 50 mg) in a test tube. Carboxymethyl cellulase (CMCase) activity was used for measuring the endo-β-1,4-glucanase following the method used by [28]. The assay mixture contained 0.5 ml of 1% (w/v) carboxymethyl cellulase solution [diluted with 50 mM citrate buffer (pH 4.8)] and 0.5 ml of crude enzyme. Xylanase activity was measured following the method adopted by [29]. For this purpose, 1% (w/v) xylan was dissolved in 50 mM sodium citrate buffer (pH 4.8). The reaction mixture contained 0.5 ml of 1% xylan and 0.5 ml of crude enzyme solution. The reaction for each assay was incubated at 50 °C for 60 min. Finally, the reactions were ceased by adding 3 ml of DNS (3, 5-dinitrosalicylic acid) reagent and heated for 5 min in boiling water bath for color development. The absorbance for each assay was measured spectrophotometrically at 540 nm. For reference concentration, a standard curve was plotted against the known concentrations of glucose. The enzyme activity was expressed as "U/ml" where "U" represents the amount of enzyme required to liberate 1 μmol of glucose per minute. The enzyme activities were calculated according to the following equation:

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A}{\text{Vol of enzyme}} \times \frac{\text{Std Factor}}{\text{Incubation time}} \times \frac{\text{Dil.factor}}{\text{Vol.of QRM}} \times \text{Reaction.vol.}$$

where
$$\therefore \Delta A = \text{absorbance at spectrophotometer}$$
$$\therefore Std\ factor = 1/\text{slope}$$
$$\therefore Volume\ of\ QRM = \text{volume of DNS}$$

2.5 Total proteins and reducing sugars assay

The total protein content (μg/ml) was measured according to the method described by [30]. For this purpose, sampling was performed at intervals of 7, 14, 21, 28, and 35 days of fungal cultivation. The reaction mixture contains 100 μl of enzyme supernatant (sample) mixed with 1 ml of Bradford reagent in a test tube and was incubated at room temperature for 5 min. Finally, the absorbance was taken at 595 nm using a spectrophotometer. Bovine serum albumin (BSA) protein was used as standard reference, and a standard curve was plotted against the absorbance of the standard solution. The total reducing sugars were measured by following the DNS method [31] to estimate the extent and efficiency of the hydrolysis. The reaction mixture contains 1 ml of enzyme supernatant (sample) mixed with 3 ml of DNS reagent in a test tube and was boiled for 5 min for color development. Then, absorbance was measured spectrophotometrically at 540 nm. A standard curve was plotted against the known concentration of glucose and was used as the standard reference.

2.6 Compositional analysis

The compositional analysis of WS was conducted before and after the experiment following the "Laboratory Analytical Methods" from National Renewable Energy Laboratory (NREL), USA [27]. The effect of biodegradation on the lignin, cellulose, and hemicellulose and moisture contents of untreated and biotreated WS (after 35 days of fungal cultivation) were analyzed.

2.7 Biodegradation assays

2.7.1 Scanning electron microscope (SEM) analysis

For morphological characterization of the microstructural changes due to the fungal biodegradation of WS, scanning electron microscope (SEM) was used [JSM- 6360LVPRIME, JEOL®, USA, fitted with Carl Zeiss Ultra Plus lens]. The instrument was operated between a working distance of 5–10 mm, 10 kV acceleration voltage, and with different magnification range (× 200–2.50 KX). SEM images of untreated and biotreated samples were obtained on black carbon tape and precoated with gold using a sputter coater following [32].

2.7.2 Fourier transform infrared (FTIR) analysis

To investigate the modifications in the functional groups of the biotreated WS, Fourier transform infrared (FTIR) spectrometer analysis was used [IRAffinity-1, Shimadzu®, Japan, equipped with a 2-m gas cell and a DTGS KBr detector]. Two milligrams of each untreated and biotreated WS sample was grounded in an agate mortar and was mixed with 200 mg of spectroscopic grade KBr, pressed to produce 13-mm diameter pellets. The spectra of untreated and biotreated WS were determined in the wave number range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} [33]. For each sample, all the readings were performed in triplicate, and a background spectrum was obtained to measure the response of the FTIR without samples.

2.8 Statistical analysis

Results are presented as a bar, continuous line plots, in which the mean ± standard error of the mean of three

individual replicates is presented. Statistical analyses were performed using GraphPad Prism 7 software. A two-way ANOVA with Tukey's or Sidak's multiple comparison posttest was used for comparing the means of more than 2 groups. Results were considered significant ($p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, $p < 0.0001 = ****$, and ns = nonsignificant).

3 Results and discussion

3.1 Enzyme extraction from the biodegraded wheat straw

Lignocellulosic substrates have been pretreated with various physical/chemical methods [34–36] to facilitate the

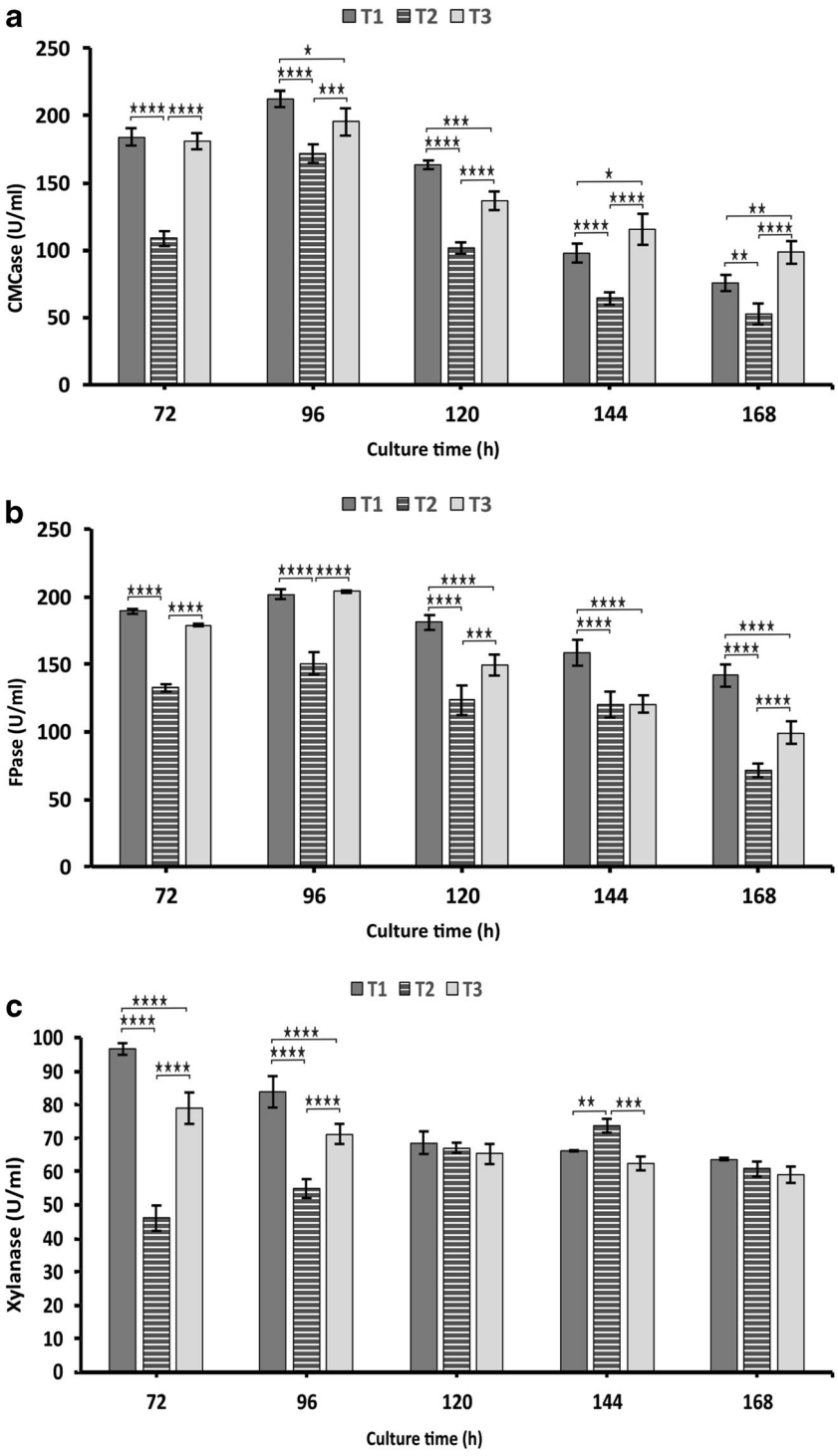

Fig. 2 Enzyme activity of enzyme from (10^7/ml) spore suspension cultures of fungi (T1, co-culture of *T. reesei* + *M. purpureus*; T2, culture of *M. purpureus*; T3, culture of *T. reesei*) cultivated with untreated WS as a carbon source. The enzyme activity is expressed as "U/ml" where "U" represents the amount of enzyme required to liberate 1 μmol of glucose per minute under the assay conditions (incubation time 30 min; pH 4.5; temperature 50 °C). The bar values represent the average mean (error bar = standard deviation) of 3 biological replicates. A two-way ANOVA with Tukey's multiple comparison posttest was performed on log-transformed data using GraphPad Prism 7, where * represents the significant values, i.e., $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$; and $p < 0.0001 = ****$. The bar values sharing no line represent nonsignificant values

subsequent microbial biodegradation through opening the plant cell wall complexities. However, to avoid the cost associated with the pretreatment process, the current study was aimed to evaluate the co-cultivation of cellulolytic fungi for digesting the untreated WS, ensuring an economical cost-effective approach to use the cellulosic feedstock in bioethanol biorefineries [37].

Solid-state fermentation was performed using the pure and co-cultures of *T. reesei* and *M. purpureus* under similar cultivation conditions (WS substrate 5 g; pH 4.5; temperature 30 °C), and the enzyme activities were measured at 72, 96, 120, 144, and 168 h of cultivation time. The co-cultivation of fungal strains (*T. reesei* + *M. purpureus*, T1 treatment) showed a significantly higher level of cellulolytic enzyme activity when compared with the single cultivation of each fungus (Fig. 2). For carboxymethyl cellulase (CMCase) activity, it was interesting to note that the co-cultivation of *T. reesei* + *M. purpureus* (T1) exhibited significantly higher ($p < 0.0001$****) enzymatic activity (220 U/ml) than individual cultures of *M. purpureus* (T2 treatment) and *T. reesei* and at all levels of cultivation time (Fig. 2a). The lower CMCase activity of *M. purpureus* might be attributed to the complex lignocellulosic nature of untreated WS as *Monascus* sp. tends to exhibit slow growth in lignocellulosic substrates having higher lignin contents [38]. However, in comparison with individual *T. reesei* (T3 treatment) cultures, T1 results were significant at 96 h (212.3 U/ml; $p = 0.0173$*) and 120 h (163.3 U/ml; $p = 0.0002$***) of cultivation time (Fig. 2a). Interestingly, the CMCase activity of *T. reesei* (T3) exhibited a significantly higher value at 144 h (115.5 U/ml; $p = 0.0117$*) and 168 h (98.3 U/ml; $p = 0.0013$**) of cultivation time as compared to T1 (97.6 U/ml at 144 h; 75.6 U/ml at 168 h) (Fig. 2a). The reasons for the higher CMCase activity by individual *T. reesei* culture might be due to the optimum assay conditions such as pH 4.0–5.0 [39] or the preferred carbon source [40, 41]. *Trichoderma* spp. have been dominantly used for cellulase production at industrial levels [42].

The total cellulase was estimated using FPase activity, and the results were very interesting. Overall, from 72 to 168 h of cultivation time, the FPase activity of T1 (*T. reesei* + *M. purpureus*) was significantly higher ($p < 0.0001$****) 200 U/ml than values of T2 and at 120–168 h values of T3 treatment (Fig. 2b). A similar increase in total cellulolytic enzyme was reported by Adsul [40], where they utilized the co-cultures of fungal strains (*P. janthinellum*, *T. reesei*, and *A. tubingensis*) for WS hydrolysis and showed increased enzyme activities during co-cultures as compared to the individual fungal strains. Another study also reported the remarkably higher values of cellulases (FPase, CMCase) using co-cultures of *T. reesei* and *P. oxalicum*as, potential cooperative decomposers of rice straw [43].

The xylanase activity of *T. reesei* + *M. purpureus* (T1) was significantly higher ($p < 0.0001$****) (96.7 U/ml) at 72 h and (83.8 U/ml) at 96 h as compared to the single cultures of T2 (*M. purpureus*) or T3 (*T. reesei*) (Fig. 2c). T3 treatment also produced significant results at 72 and 96 h of cultivation time when compared with the T2 (Fig. 2c) and in accordance with some previous studies reporting higher xylanase activity by *T. reesei* [39, 44]. Interestingly, the xylanase activity of T2 (*M. purpureus*) at 120 h showed a significant higher value (73.8 U/ml; with T1 $p = 0.0081$**; with T3 $p = 0.0001$***) when compared with T1 (66.3 U/ml) or T3 (59.1 U/ml) (Fig. 2c). The gradual increase in xylanase activity of *M. purpureus* with cultivation time reflects the adaptation of fungi with the lignocellulosic substrate to break down the hemicellulose inside the plant cell wall.

The enzymatic activities (CMCase, FPase, and Xylanase) of co-cultures of *T. reesei* + *M. purpureus* obtained in the

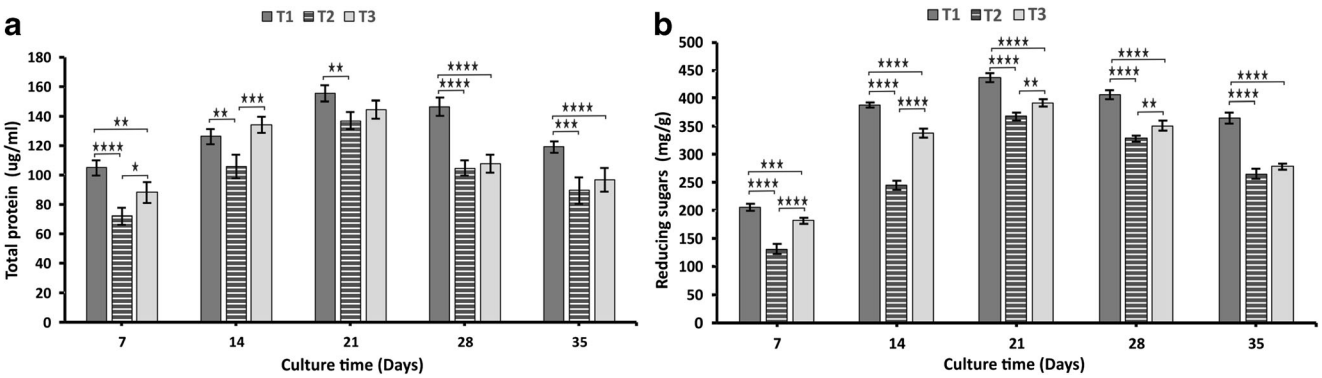

Fig. 3 Total protein and reducing sugars from (10^7/ml) spore suspension cultures of fungi (T1, co-culture of *T. reesei* + *M. purpureus*; T2, culture of *M. purpureus*; T3, culture of *T. reesei*) cultivated with untreated WS as a carbon source. The total protein is expressed as "μg/ml" and reducing sugars are expressed as "mg/g" released under the given assay conditions. The bar values represent the average mean (error bar = standard deviation) of 3 biological replicates. A two-way ANOVA with Tukey's multiple comparison posttest was performed on log-transformed data using GraphPad Prism 7, where * represents the significant values, i.e., $p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; and $p < 0.0001$ = ****. The bar values sharing no line represent nonsignificant values

Table 1 Composition analysis of untreated/biotreated WS sample

Treatments	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Moisture (%)
Untreated WS (control)	39.1 ± 1.3a	24.5 ± 0.4a	17.7 ± 0.5a	9.6 ± 0.3b
T1-treated WS	31.6 ± 0.8c	19.1 ± 0.6c	9.7 ± 0.04c	10.6 ± 0.5a
T2-treated WS	34.4 ± 0.8b	21.4 ± 0.3b	10.5 ± 0.5b	10.1 ± 0.2ab
T3-treated WS	33.5 ± 1.1b	20.9 ± 0.6b	11.05 ± 0.8b	9.8 ± 0.4ab

*The data values are average means of three replicates (± = standard deviation)

*Samples having different letters are significantly different, $p < 0.05$ by ANOVA

current study exhibit a significant data for WS decomposition and in line with several studies reporting co-cultures of fungal strains showing higher degradation efficiency [40, 43, 45].

3.2 Amount of released sugars and total protein contents

The effect of co-cultivation of fungal strains on the amount of total reducing sugars and total proteins liberated during hydrolysis was estimated at various intervals (7, 14, 21, 28, and 35 days) of cultivation time (Fig. 3). With regard to the single fungal treatments (T2 and T3), the co-culture of *T. reesei* + *M. purpureus* (T1) exhibited the total protein in a range from 105 to 155.5 μg/ml and the reducing sugars in a range from 206.1–437.1 mg/g (Fig. 3). During the 5 weeks of cultivation time, a gradual increase in the concentration of total protein and reducing sugars was observed with maximum values at the 21st days; afterward, a slow decline was observed. A similar trend of increasing reducing sugars during the biotreatment of WS was reported previously, where a maximum value of 439 mg/g was reported at 14th days of cultivation time [46]. Importantly, the co-culture cultivation of T1 treatment showed a significantly higher ($p < 0.0001$****) values of total protein (Fig. 3a) and reducing sugars (Fig. 3b) when compared with single fungal cultivation either T2 or T3.

The effectiveness of these fungal co-cultures could be extended to another lignocellulosic substrate decomposition. A fungal consortium constituting 3 or more fungal strains or a blend of fungal and bacterial cultures can also be further investigated. However, a microbial consortium comprising multiple organisms may face difficulties in establishing a useful culture due to differences in growth requirements, competition, stability, and phenolics toxicity in the medium [47].

3.3 Compositional analysis of biotreated wheat straw

The biodegradation of biotreated WS was determined through compositional analysis before and after the fungal treatment. Table 1 shows the chemical composition of biotreated WS (after 35 days of fungal cultivation) in comparison with the untreated WS (control) and represents the biodegradation potential of different treatment applications. Depolymerization of WS lignocellulose was observed through loss in cellulose, hemicellulose, and lignin contents of the biotreated WS. A significant decrease ($P < 0.05$) of 19.18% and 21.84% of cellulose and hemicellulose was observed in case of T1 (co-culture of *T. reesei* + *M. purpureus*) when compared with control, indicating the release of trapped cellulosic fibrils within WS and their utilization for the release of sugars. Importantly, lignin removal 45.2% was significantly higher in the case of co-culture T1, as compared to monocultures, 40.7% in case *M. purpureus* T2 and 37.6% in the case of *T. reesei* T3. Overall, among all treatments, T1 performed better and in line with other results presented in the

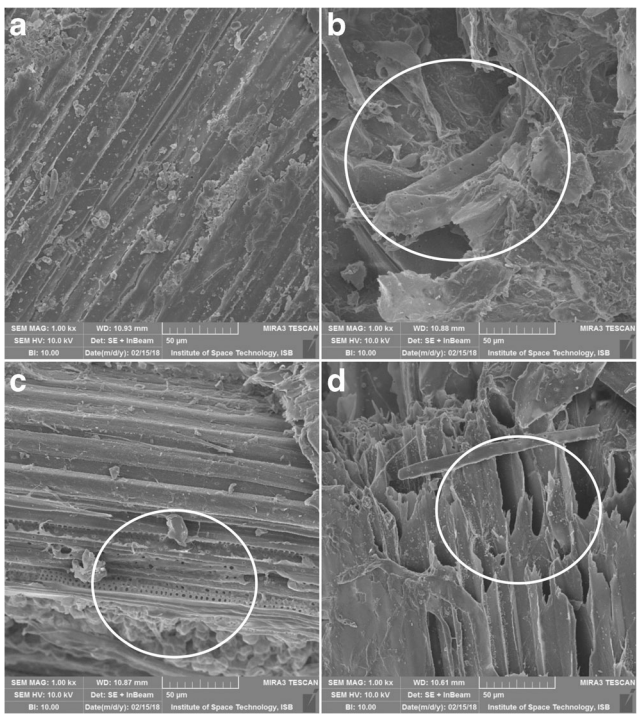

Fig. 4 Scanning electron microscope (SEM) image (1.00 kx magnification) indicating the inaccessible and packed structure of untreated and biotreated wheat straw. **a** Untreated wheat straw and biotreated WS samples of **b** T1 (co-culture of *T. reesei* + *M. purpureus*), **c** T2 (culture of *M. purpureus*), and **d** T3 (culture of *T. reesei*) at the 35th day of cultivation time. The white circle represents the biodegraded structure after fungal pretreatment

Table 2 FTIR spectrum analysis of biotreated WS at various culture time

Wavelength (cm^{-1})	Functional group linkage	Absorbance at 7th day			Absorbance at 14th day			Absorbance at 21st day			Absorbance at 28th day			Absorbance at 35th day		
		T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
3301	–OH stretching-phenolic/hydroxyl (lignin)	0.887	0.957	0.892	0.870	0.945	0.889	0.903	0.947	0.901	0.912	0.955	0.931	0.901	0.966	0.959
2919	C–H des/symmetric stretching (lignin)	0.923	0.989	0.925	0.901	0.977	0.915	0.929	0.963	0.931	0.945	0.976	0.948	0.931	0.980	0.973
1730	C=O stretching-acetyl/carbonyl (lignin)	0.970	0.943	0.971	0.949	0.935	0.954	0.958	0.958	0.956	0.961	0.974	0.968	0.956	0.985	0.984
1620	C=C stretching-aromatic ring (lignin)	0.951	0.916	0.939	0.924	0.911	0.931	0.932	0.936	0.930	0.947	0.964	0.953	0.934	0.977	0.977
1511	C=C stretching-aromatic ring (lignin)	0.929	0.939	0.945	0.927	0.946	0.940	0.943	0.945	0.941	0.989	0.984	0.963	0.945	0.980	0.979
1422	C–H2 symmetric bending (cellulose/hemicellulose)	0.903	0.943	0.932	0.901	0.933	0.914	0.915	0.923	0.918	0.983	0.944	0.946	0.922	0.972	0.972
1370	C–H asymmetric bending (cellulose)	0.895	0.910	0.931	0.874	0.904	0.907	0.906	0.918	0.915	0.983	0.895	0.940	0.919	0.970	0.969
1317	C–H2 wagging (cellulose)	0.942	0.964	0.928	0.909	0.941	0.9	0.901	0.911	0.908	0.923	0.922	0.935	0.911	0.969	0.967
1201	C–O–C asymmetric stretching (cellulose)	0.889	0.909	0.926	0.886	0.902	0.902	0.905	0.909	0.890	0.929	0.895	0.913	0.904	0.970	0.960
1030	C–O stretching (hemicellulose)	0.778	0.733	0.792	0.707	0.717	0.673	0.678	0.715	0.667	0.733	0.837	0.731	0.711	0.918	0.883
897	C–O–C stretching-β-glycosidic linkage (cellulose)	0.882	0.961	0.896	0.837	0.914	0.857	0.858	0.870	0.852	0.871	0.898	0.876	0.866	0.959	0.940

*T1 represents treatment 1, i.e., WS cultivated with co-culture of *Trichoderma reesei* and *Monascus purpureus*

*T2 represents treatment 2, i.e., WS cultivated with single culture of *Monascus purpureus*

*T3 represents treatment 3, i.e., WS cultivated with single culture of *Trichoderma reesei*

current study suggesting the cooperative actions of fungi to degrade WS efficiently. These results are inconsistent with these findings [35, 46, 48, 49].

3.4 Scanning electron microscope (SEM) analysis

The biodegradability of WS was morphologically analyzed by SEM before and after fungal cultivation. Figure 4 shows the SEM micro-surface and structural degradation of WS in a comparison of untreated and fungal treated WS (T1, T2, and T3) after 35 days of cultivation time. The untreated WS exhibited a rough, continuous, and compact surface (Fig. 4a), inaccessible for enzymatic saccharification, and that is why it needed pretreatment. Compared to the untreated, a significant change in surface morphology in the form of channeling, fracture, and the irregular texture was observed in fungal hydrolyzed WS (Fig. 4b, c, d) and in accordance with SEM analysis of biotreated WS reported previously [37, 46, 50]. Importantly, a more obvious change in surface morphology and structural breakdown was observed in T1-biotreated WS caused by the co-culture of *T. reesei* and *M. purpureus* (Fig. 4b), indicating the texture modifications by the synergistic actions of fungal strains and in line with previous studies [51]. From SEM analysis, it could be proposed that T1-treated WS showed enhanced potential for WS surface depolymerization (Fig. 4b) and correlates with the higher enzymatic hydrolysis of T1 as compared to T2 and T3 mentioned above (Fig. 2). This also confirms the surface accessibility of fungal strains to penetrate deeply into the lignocellulosic substrate and facilitates the subsequent hydrolysis process. A number of recent studies have shown the biodegradability of WS using fungal cultures through SEM analysis [37, 50, 52], however limited to the usage of single fungal cultures.

3.5 Fourier transform infrared (FTIR) analysis

The biodegradation of fermented WS was further evaluated through FTIR analysis, and the results were compared according to differences in absorbance at representative peaks (4000–500 cm^{-1}), linked with the modifications of different functional groups (cellulose, hemicellulose, and lignin) [37, 53] (Table 2; Fig. 5). The FTIR spectra revealed a significant reduction in the absorbance/intensity of biotreated WS which represents the change in lignocellulosic structure (Fig. 5). Table 2 shows the variations in biodegradability among T1 (WS cultivated with co-culture of *T. reesei* + *M. purpureus*), T2 (WS cultivated with single cultures of *M. purpureus*), and T3 (WS cultivated with single cultures of *T. reesei*) from the differences in absorbance at different cultivation times.

The peaks around 3301 cm^{-1} are attributed to the intramolecular hydrogen bonding (–OH stretching) [54]. The decrease in the absorbance at 3301 cm^{-1} of all samples from day 7 to day 14 clearly exhibits the depolymerization of lignin chains in the fermented WS (Table 2). The lowest absorbance was observed at day 14 in T1 samples as compared with other days (21, 28, and 35) (Table 2) and in accordance with early lignin degradation [55]. The maximum activity at day 14 might be due to the higher rate of hydroxylation during that period. The peaks near 2919 cm^{-1} are assigned to the methylene group of C–H des/symmetric stretching [56], and the lowest absorbance in T1 implies the breakdown of lignin side chains [57]. Overall, the gradual decrease in absorbance of every sample implies the cleavage of the methylene groups in the

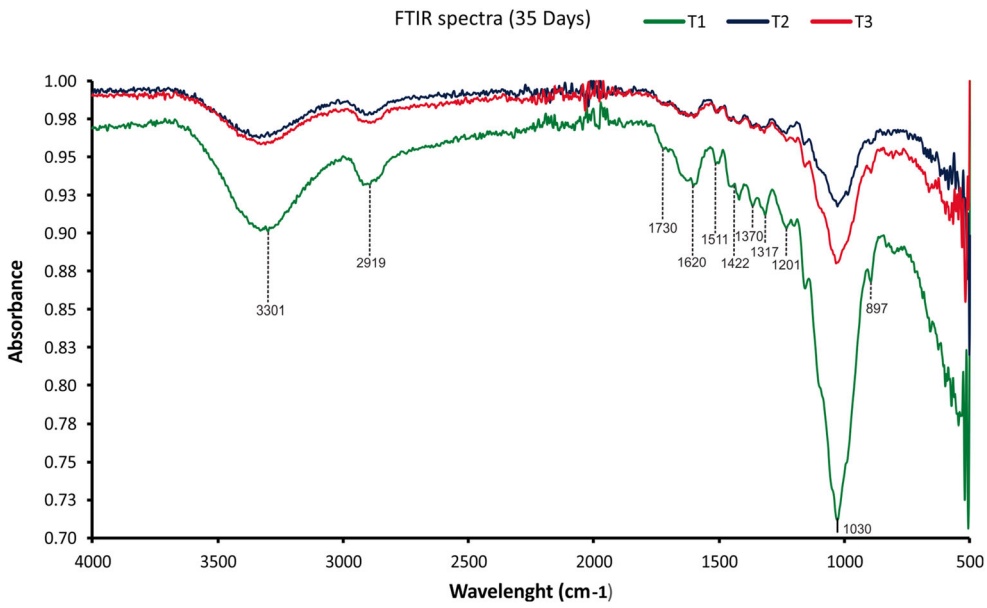

Fig. 5 FTIR spectra of biotreated WS samples of T1 (co-culture of *T. reesei* + *M. purpureus*), T2 (culture of *M. purpureus*), and T3 (culture of *T. reesei*) at the 35th day of cultivation time

lignocellulose substrate (Table 2) and in accordance with the results reported earlier [33]. The peaks from 1511 to 1730 cm^{-1} are further associated with different functional groups of lignin characteristics [58, 59]. From day 7 to day 14, the trend in a decrease in absorbance was observed as T1 > T3 > T2 (Table 2) and then an indeterminate trend till the 35 day. This might be due to that the coadaptation of *T. reesei* + *M. purpureus* (T1) at day 14 was optimum and resulted in enhanced oxidation of the methylene group, thus degradation of the lignin [60]. These results are in line with the findings of [33], where a similar trend of lignin degradation was observed at day 20 when a co-culture of *Trichoderma viride* and *Aspergillus niger* was used for rice straw biodegradation. The peaks from 1317 to 1422 cm^{-1} are associated with C–H stretching of cellulose/hemicellulose [37], and a significant decrease in absorbance in T1 as compared to T2 and T3 exhibits the breakdown of cellulose/hemicellulose after biotreatment (Table 2; Fig. 5). The peaks from 897 to 1201 cm^{-1} are associated with C–O–C, C–O, and C–O–C stretching of the phytate contents and β-glycosidic linkage of the cellulose/hemicellulose [33] and were significantly decreased in T1, implying the removal of Phytic acid and partial breakdown of polysaccharides (Fig. 5; Table 2).

From FTIR data, the changes in absorbance over different fungal cultivation times indicate the biodegradation of lignin, cellulose, and hemicelluloses in WS. The co-culture of *T. reesei* + *M. purpureus* exhibited a higher biodegradation potential as compared to the single cultures of fungal strains and in accordance with other results of SEM, enzyme activity, and reducing sugars released overall.

4 Conclusion

In summary, we investigated the role of *T. reesei* and *M. purpureus* for WS pretreatment and found interesting results with regard to fungal hydrolytic enzyme activities, the release of proteins/sugars, and the biodegradation potential. Several attempts with the use of single fungi for WS biodegradation have been reported with different success rates. Here, we employed a dual-fungi approach and utilized a co-culture of *T. reesei* + *M. purpureus* for WS biodegradation using solid-state fermentation. The results were very interesting and showed significantly enhanced biodegradation of WS in comparison to the individual actions of each fungus, thus suggesting a synergism among the different fungi for a cooperative decomposition. Further optimization of the cultivation assay could lead to further improvement of the process. This is a first report of using a co-culture of *T. reesei* and *M. purpureus* for WS biodegradation and proves the efficacy of co-culture technique. This study may have a crucial impact on its usage in bioethanol biorefinery on a sustainable basis using lignocellulosic feedstocks.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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