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Enhanced Production of Cellulase by *Trichoderma reesei* Using Wheat Straw as a Carbon Source

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Abstract: The objective of this study was the screening of a carbon source and microorganism for the enhanced production of cellulose enzyme system. Comparatively, untreated wheat straw (UWS) proved to be the best carbon source and the presence of lignin offered no hindrance to cellulase production by the fungus. Among single cultures, comparatively, *T. reesei* showed higher specific activity of CMCase and FPase in UWS than *T. viride* and other strains. The co-culture of *T. reesei* and *T. viride* showed better specific CMCase and FPase activity with treated wheat straw (TWS) (3.18 and 2.10) than UWS (1.05 and 1.24). Galactose proved to be a better inducer than glucose or Tween-20 and the specific activity of CMCase (19.21±0.82) and FPase (06.27±0.94) was increased while the production time was decreased from 96 to 72 h. The highest specific cellulase activity was achieved by *T. reesei* at 30±1°C, pH 6 and 2% four-day-old inoculum with 0.2% (w/v) galactose as the inducer. We achieved the objectives of enhanced production of cellulase using inexpensive lignocellulosic biomass from wheat straw to cut down production costs.

Key words: *Trichoderma reesei* · *T. viride* · Wheat straw · Bioethanol · Cellulase · Co-culture

INTRODUCTION

Cellulase is a complex enzyme system that consists of three major types of cellulolytic enzymes, namely endoglucanases (EG, EC 3.2.1.4), exocellobiohydrolases (CBH, EC 3.2.1.91) and β-glucosidases (BGL, EC 3.2.1.21) [1, 2]. Like alkaline protease [3, 4] cellulases have many applications in industrial products such as beverages, food, bakeries, paper, textiles, laundry processing, as well as in the bioconversion of cellulosic plant biomass into ethanol [5]. Lignocellulosic material is converted into glucose by the synergic action of cellulase, which can then be fermented to bioethanol by yeast [6]. Spano (1978) reported that cellulase production was the most expensive step in the production of bioethanol from lignocellulosic plant biomass. A significant reduction in the cost of enzyme production is urgently needed to make second generation bioethanol production economically feasible [7].

Bacteria and fungi can produce cellulases. Most of the cellulase-producing bacteria are anaerobic and do not secrete enzymes into fermentation broth; therefore, researchers are least interested in bacteria for cellulase production. Among the cellulase-producing fungi, *Trichoderma reesei* is considered as the best cellulase-producing fungus and has very strong cellulose-hydrolyzing action. The cellulase enzyme from this fungus is thought to be the best option for industrial processes [8]. Filamentous fungi, particularly the *Trichoderma* species, produce both cellulase and

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xylanase. These fungi are non-pathogenic, aerobic, capable of producing high levels of extracellular enzymes and can be cultivated very easily [9].

Recent research in the field of cellulase enzymology, strain improvement, protoplast fusion, cellulose hydrolysis mechanisms and engineering processes has resulted in bringing the *T. reesei* cellulase enzyme closer to being a commercially useful route toward cellulose hydrolysis. Many industrially useful strains have been developed and characterized [10]. On industrial scale, *T. reesei* can be used to secrete cellulase enzymes to hydrolyze the cellulose from pretreated wheat straw to produce sugars. These sugars can then be fermented by yeast to produce bioethanol. The dilute sulfuric acid method of pretreating wheat straw was effective and promising at commercial level because ethanol production was increased [11]. Furfural and HMF are formed only at very high temperatures, *i.e.*, > 180ºC [9, 11]. Various fungi produce cellulase in different amounts and have different activities [4]. Enhanced and cheaper production of cellulases is a pressing need.

However, no data on cellulase production using UWS and TWS or a comparison of CMCase and FPase in terms of specific activities has been previously reported. Therefore, it is necessary to investigate single and co-culture techniques using wheat straw as a carbon source. In this research work, cellulase production by UWS and TWS were evaluated in batch experiments using *T. reesei, T. viride, T. harzianum* and *A. niger* and the cellulytic efficiency of CMCase and FPase were compared in terms of specific activities. The optimization of process variables (fermentation time, inoculum size, pH, temperature) for the maximum production of cellulases was also investigated in batch experiments. The development of economical indigenous processes for the production of cellulase enzymes will strengthen the technological base of the biofuel industry.

MATERIAL AND METHODS

Microorganism: Locally isolated fungal strains of *A. niger, T. viride, T. reesei* and *T. horzianum* were obtained from the Fermentation Biotechnology Laboratory, PCSIR Labs Complex, Ferozpur Road, Lahore, Pakistan. The strains were maintained on a potato-dextrose-agar (PDA) medium. Duplicate slants were prepared at 30°C for four days and were stored at 4°C for later use.

Substrate: Untreated wheat straw (UWS) and acid-treated wheat straw (TWS) (1.5% H_2SO_4) were used as a source of carbon for the production of cellulase enzymes [12, 13]. UWS (lignocellulosic biomass) was obtained from wheat (*Triticum aestivum* L.) crop-2011 grown at the Khokhar Agriculture Farm, at the village SOHIAN, District Gujranwala, Pakistan. The UWS was washed, dried and ground to a powder form (80 to 20 mesh) with a hammer beater mill.

Culture Medium: Modified Vogel's medium (1x) was used as a culture medium [14]. First, 50x Vogel's medium containing (g/dm^3) sodium citrate 150, KH_2PO_4 250, NH_4NO_3 100, $MgSO_4.7H_2O$ 10, $CaCl_2.2H_2O$ 10, biotin solution 5 mL, trace element solution 5 mL and chloroform 2 mL as a preservative was prepared as a stock solution. To 1 part of a 50x stock solution, 49 parts of distilled water was added and the pH was adjusted to 5.9. The resulting solution was referred to as 1x Vogel's medium.

Enzyme Production: 100 ml (1x) of Vogel's medium was added to 250-ml Erlenmeyer flasks numbered F_1, F_2, F_3, F_4, F_5, F_6, F_7, F_8, F_9 and F_{10}. One gram of UWS was added to flasks F_1, F_3, F_5, F_7 and F_9 and 1.0 g of TWS was added to F_2, F_4, F_6, F_8 and F_{10}. After sterilization at 121°C for 15 min, the flasks were allowed to cool and were then inoculated with 2.0 mL of spore suspension (10^6 per mL) of different fungi, *i.e.*, F_1 and F_2 with *T. reesei,* F_3 and F_4 with *A. niger*, F_5 and F_6 with *T. harzianum* and F_7 and F_8 with *T. viride*. Flasks F_9 and F_{10} were co-cultured with *T. viride* and *T. reesei*. The inoculated flasks were incubated at 30±1°C with agitation at 120±5 rpm. Five milliliters of sample aliquot was removed aseptically from each flask after 48, 72, 96, 120 and 144 hours. These samples were centrifuged at 8000 rpm for 8 min at 4 °C to remove unwanted particles and spores. The debris-free supernatant was used as the crude extracellular enzyme source for the estimation of CMCase activity, FPase activity and total protein production.

Effect of Different Process Parameters on the Production and Cellulolytic Activity of Cellulase Produced by *T. reesei* (PCSIR-2011): The optimization of the cultivation conditions of *T. reesei* (PCSIR-2011) for optimal cellulase production using wheat straw as a carbon source was also investigated. In separate batches, the effects of pH, temperature, inoculation size, mixing intensity, inducers and surfactants on the production (total protein) and enzymatic activities of CMCase and FPase were studied.

Carboxymethyl Cellulase "CMCase" Endoglucanase Assay: CMCase activity was measured using the method of Ghose (1987) after slight modifications [15]. An amount of 0.5 mL of 1% (w/v) CMC in 0.05 M phosphate buffer (pH 5.0) as a substrate was incubated with 0.5 mL of enzyme supernatant in test tubes at 37°C for 30 min. Then, 1.0 mL of DNS was added and the solution was kept in boiling water for 10 min. The absorbance was measured at 545 nm. The CMCase activity was measured in terms of International Units (IU), defined as the amount of enzyme that can produce one µmole of glucose per mL per min.

Effect of Inducers and Inhibitors on Growth of *T. reesei*: 100 ml (1x) Vogel medium and 1.0 g of TWS was added to four flasks, which were marked as F_a, F_b, F_c and F_d Then 1.0% (w/v) glucose in F_a, 0.2% (w/v) galactose in F_b and 0.2% (v/v) Tween 20 in F_c were added. In flask F_d 1.0% glucose, 0.2% galactose and 0.2% Tween 20 were added collectively. A ll of the flasks were inoculated with 2.0% *T. reesei* culture and incubated at 30 °C at 140 rpm.

FPase Activity Assay: FPase activity was measured as described by Ghose (1987) [15]. Fifty milligrams of Whatman No. 1 filter paper pieces of 1×3 cm size were added to a test tube. Then, 1 mL of crude enzyme extract and 1 mL of acetate buffer (100 mM, pH 4.8) was added and the test tube was heated at 45°C for 50 min. Next, 3 mL of DNS was added and the test tube was kept in boiling water for 10 min, following Miller (1959) [16]. The optical density (OD) was measured at 545 nm using a spectrophotometer (Spectro UV-VIS, UVS-2800 Lambomed, USA). The glucose curve was used as a standard. One international unit (IU) of FPase activity was defined as 1 µMol of glucose liberated per mL per min.

Protein Assay: The total protein content was measured using the method of Lowery *et al*., (1951) using bovine serum albumin (BSA) protein as the standard [17]. First, 0.5 mL ofenzyme supernatant was mixed with 2.5 mL of an alkaline copper reagent (ACR) at room temperature for 10 min; then, 0.5 mL of freshly prepared folin reagent was added and the solution was allowed to stand for 30 min. The OD was measured at 600 nm.

Statistical Analysis: Important data were subjected to analysis of mean, variance and standerd deviation (SD) using Standard Deviation Calculator "live". (http://www.mathsisfun.com/data/standard-deviation-calculator.html) Each value was average of three replicates of same sample. ± indicates standard deviation (SD) among the replicates.

RESULTS AND DISCUSSION

To explore the cellulase production capability of fungi after different time intervals, (48, 72, 96, 120 and 144h) five batches of enzyme production were designed. Cellulase enzyme activities (CMCase and FPase activities and total protein production) were estimated from fermentation broths and the results are shown in Table 1.

Estimation of CMCase and FPase Activities and the Total Protein Produced by 48 Hour Fermentation Culture: In this batch, maximum CMCase activity of *T. reesei* was 86.5 IU in UWS and 49.1 IU in TWS. The CMCase activity shown by *T. viride* in the UWS was 82.1 IU and *A. niger* in the TWS was 28.4 IU. The overall CMCase activity was higher in the UWS compared to the TWS. Among the four strains of fungi (*T. reesei, T. viride, T. harzianum* and *A. niger)*, *T. reesei* was the best cellulase producer fungus after 48 h of fermentation. A co-culture of *T. reesei* and *T. viride* showed good CMCase activity, with activities of 177.6 IU and 105.3 IU for TWS and UWS, respectively (Table 1).

The FPase activity was the highest with *T. reesei,* while it was the second highest with *T. viride*, *i.e*., 161.3 and 88.7 IU with UWS and 56.1 and 65.2 IU with TWS, respectively. *T. harzianum* showed the minimum FPase activity after 48 hrs of fermentation. In the case of the co-culture, the maximum FPase activity was observed with TWS in comparison to UWS: 206.2 and 52.4 IU, respectively. Therefore, the co-culture of the two fungi, *T. reesei* and *T. viride*, proved to be successful.

Table 1 also shows that the maximum amount of protein (201.5 µg/mL) was produced by *A. niger* in flask F_4, containing TWS and the minimum amount of protein (101.1 µg/mL) was produced by *T. viride* in flask F_7, containing UWS. In the co-cultured flasks, F_9 and F_{10}, production of protein was 186.2 and 142.1 µg/mL, respectively.

Estimation of CMCase and FPase Activities and the Total Protein Produced by 72 Hour Fermentation Culture: CMCase and FPase activities and total protein production were estimated from a 72h old culture broth. The maximum CMCase activity of *T. reesei* was observed to be 257.1 IU in UWS and 105.2 IU in TWS, while higher CMCase activity was shown by *T. viride* in the UWS (154 IU), compared to that in TWS and the minimum activity (112.2 IU) was shown by *T. harzianum* in the UWS. The overall CMCase activity was higher in the UWS) in comparison to TWS. Of all four strains of fungi, *T. reesei* and *T. viride*

Table 1: Timeline Study of Cellulase Production by Various Fungal Strains using Untreated and 1.5% H_2SO_4-Treated Wheat Straw at 30°C, 5.0 pH and 120 rpm in Flasks

		48 h Fermentation			72 h Fermentation			96 h Fermentation			120h Fermentation			144 h Fermentation		
Sr. No.	Flask No.	CMCase Activity	FPase Activity	Total Protien	CMCase Activity	FPase Activity	Total Protien	CMCase Activity	FPase Activity	Total Protien	CMCase Actvity	FPase Activity	Total Protien	CMCase Actvity	FPase Activity	Total Protien
1	F_1	86.5	161.3	162.3	257.1	127.9	192.6	637.9	315.1	239.9	439.4	313.5	430.7	217.3	337.9	451.8
2	F_2	49.1	56.1	157.6	105.2	124.5	191.3	244.9	303.5	251.9	254.7	160.7	272.5	257.5	101.7	348.3
3	F_3	79.5	66.8	116.7	133.2	68.4	239.9	342.4	243.6	210.2	201.0	220.8	293.9	199.5	123.9	336.5
4	F_4	28.4	51.4	201.5	131.9	59.1	255.5	210.3	299.6	278.5	182.8	215.9	364.2	143.5	119.8	276.4
5	F_5	80.8	44.1	150.4	112.2	98.2	187.8	114.5	106.4	221.5	202.3	79.6	280.5	157.5	75.4	351.1
6	F_6	39.7	24.0	188.3	151.9	105.8	197.8	161.3	224.1	212.2	206.9	127.3	276.4	219.6	74.8	304.9
7	F_7	82.1	88.7	101.3	154.3	109.0	167.7	427.7	426.2	240.4	207.3	272.3	243.1	198.1	125.9	262.2
8	F_8	53.7	65.2	115.2	122.3	89.4	179.2	231.2	401.5	247.4	180.2	235.9	249.5	176.3	121.3	285.0
9	F_9	105.3	52.4	186.2	212.7	94.1	208.7	236.3	277.1	224.1	197.3	125.0	244.8	98.2	95.3	255.2
10	F_{10}	177.6	206.2	142.1	560.9	242.5	174.3	680.4	448.1	213.9	458.8	386.6	325.1	231.4	244.4	365.9

Note: (I) Flasks were inoculated with a 2.0% spore (10^6 per mL) suspension of fungi, *i.e.*, F_1 and F_2 with *T. reesei*, F_3 and F_4 with *A. niger*, F_5 and F_6 with *T. harzianum* and F_7 and F_8 with *T. viride*. Flasks F_9 and F_{10} were co-cultured with *T. viride* and *T. reesei*

(II) IU/mL/min is simply represented as IU in the paper text.

(III) All values are averages of triplicates

were the best cellulase-producing fungi after 72 h of fermentation. A co-culture of *T. reesei* and *T. viride* gave better results with TWS than with UWS; the CMCase activitities were 560.9 and 212.7 IU, respectively. Table 1 also shows that after 72 h of fermentation, *T. reesei* produced more FPase activity than *T. viride* with both UWS and TWS. *A. niger* produced the minimum FPase activity after 72 h of fermentation. In the co-culture, FPase activity was increased in TWS compared to UWS, with 242.5 and 94.1 IU, respectively. Therefore, the co-culture of two fungal strains, *T. reesei* and *T. viride*, was proven to be a successful attempt to improve the CMCase and FPase activities. The highest protein production (255.5 µg/mL) was detected in the *A. niger* culture containing TWS and the minimum protein production (167.7 µg/mL) was exhibited by the *T. viride* culture containing UWS. In the co-cultured flasks, F_9 and $F_{10,}$ the production of total protein was 208.7 and 174.3 µg/mL, respectively.

Estimation of CMCase and FPase Activities and the Total Protein Produced by 96 Hour Fermentation Culture: In this batch after 96 h of fermentation, the maximum CMCase activity estimated in the single cultures was of *T. reesei*, at 637.9 IU in the UWS and 244.9 IU in the TWS. The second highest CMCase activity was shown by *T. viride* in the UWS (427.7 IU) and the lowest activity was shown by *T. harzianum* in the UWS, at 114.5 IU. Note that after 96 h of fermentation, the CMCase activity of *A. niger* was also very high; it was 342.4 and 210.3 IU with UWS and TWS, respectively. Once again, the overall CMCase activity was higher in UWS than it was in TWS. Among the four strains of fungi, *T. reesei* and *T. viride* are the best cellulase-producing fungi after 96 h of fermentation with UWS. A co-culture of *T. reesei* and *T. viride* gave better results with TWS as compared to UWS and the CMCase activity was 680.4 and 236.3 IU, respectively.

Table 1 also shows that after 96 h of fermentation, the FPase activity was the highest in the *T. reesei* culture and the second highest in the *T. viride* culture. Their FPase activity was observed to be 315.1 and 303.5 IU with UWS and 426.2 and 401.5 IU with TWS, respectively. The minimum FPase activity was shown by *T. harzianum* after 96 h of fermentation in flasks F_5 and F_6. In the co-culture, higher FPase activity was seen with TWS as compared to UWS, at 448.1 and 277.1 IU, respectively. The co-culture of two fungi, *T. reesei and T. viride*, proved to be successful in improving the CMCase and FPase activity.

Among all the single-strain cultures, the highest total protein production (278.5 µg/mL) was estimated in flask F_4 containing TWS and the lowest protein production (210.2 µg/mL) was shown by *A. niger* in flask F_3, containing UWS. In the co-cultured flasks, F_9 and F_{10}, the total protein production was 224.1 and 213.9 µg/mL, respectively.

Estimation of CMCase and FPase Activities and the Total Protein Produced by 120 Hour Fermentation Culture: The highest CMCase activity of *T. reesei* was 439.4 IU in UWS and 254.7 IU in the TWS, while the second highest CMCase activity was shown by *T. viride* in the UWS (207.3 IU) and the lowest activity was shown by *A. niger* in the TWS (182.8 IU). The overall CMCase activity was higher in UWS than it was in TWS. Among the four single strains of fungi, *T. reesei* was the best strain to produce a cellulose enzyme after 120 h of fermentation.

A co-culture of *T. reesei* and *T. viride* showed better results in TWS in comparison with UWS and the CMCase activity was 458.8 and 197.3 IU, respectively. Thus, co-culturing in UWS is not a suitable technique for cellulase enzyme production.

T. reesei had the maximum Fpase activity, while *T. viride* was second in its yield. Their FPase activities were 313.5 and 160.7 IU with UWS and 272.3 and 235.9 IU with TWS, respectively. *T. harzianum* showed the minimum yield of FPase activity after 120 h of fermentation. In the co-culture, the yield of FPase activity was seen to be higher when using TWS than when using UWS, with values of 386.6 and 125.0 IU, respectively. Therefore, the co-culture of two fungi strains, *T. reesei* and *T. viride*, was proven to be a successful technique to improve the enzyme activities.

After 120 hours of fermentation among the single-strain cultures, the highest total protein production (430.7 µg/mL) was estimated in flask F_1, containing UWS and the lowest protein production (243.1 µg/mL) was shown by *T. viride* in flask F_7, containing TWS. In the co-cultured flasks, F_9 and F_{10}, the total protein production was 244.8 and 325.1 µg/mL, respectively.

Estimation of CMCase and FPase Activities and the Total Protein Produced by 144 Hour Fermentation Culture: After the 6th day (144 h), the CMCase and the FPase activity was decreased in almost all of the flasks and increased total protein production was observed. Table 1 indicates that the production of protein gradually increased day by day. However, the CMCase and FPase activities increased gradually up to 96 h and then decline up to 144 h of fermentation. The effect of the incubation period has been studied and was noted to be optimum at 96 h for maximum cellulase activity [18, 19]. Beyond the 4th day of incubation, the enzyme activity was decreased, perhaps due to the depletion of essential nutrients or the accumulation of toxic metabolites [20]. Similar results, *i.e.*, the maximum enzyme production was achieved on the 4th day of incubation and then the activity was decreased on the 5th and 6th day, were reported by Das *et al*., (2010) [21]. In our study, it was noted that fermentation after the 4th day reduced the growth as well as the enzyme yield. Lee *et al*., (2011) reported that the growth rate of *T. reesei* was slower than that of *A. niger* [22], therefore, the FPase production reached the maximum level after the 5th day of fermentation with *A. niger* and after the 6th day of fermentation with *T. reesei* [22]. The cellulase production of 250 to 430 IU/g of cellulose was recorded in the solid-state fermentation of wheat straw with *T. reesei* QMY-1 by Chahal *et al*. (1984) [23].

Over all comparison of five batchs of enzyme production (Table 1) shows that among all of the single-strain cultures, *T. reesei* gave the best yield of CMCase activity (637.9 IU) after 96 h at 30 °C in modified (1x) Vogel's medium at a pH of 5.9 using UWS as the carbon source. Similarly, among all of the single-strain cultures, *T. viride* was the second best in producing CMCase activity (427.7 IU) after 96 h at 30 °C in modified (1x) Vogel's medium at a pH of 5.9 using UWS as the carbon source. Under similar conditions of temperature, fermentation time and pH, the co-culture of *T. reesei* and *T. viride* gave the highest CMCase activity, *i.e.*, 680.4 IU. Thus, *T. reesei* was considered the best strain to produce cellulase enzyme and was further used for the optimization of the process parameters and mutagenesis. King *et al*., (2011) reported that *T. reesei* is highly cellulolytic and is a major industrial microorganism for commercial cellulases, xylanases and cell wall-degrading enzymes [24]. Kumar *et al*., (2008) reported that the co-cultivation of fungi in fermentation can increase the amount of the required component of the cellulase enzyme complex [19]. The results of this study corroborate both of the above mentioned studies.

The efficieny of cellulase can also be expressed more accurately in term of specific activities of CMCase and FPase (Table 2). A careful and thorough analysis of Table 2 indicates that among all of the single-strain-inoculated flasks (F_1-$F_{8)}$ in all five batches (48 to 144 h), *T. reesei* demonstrated the highest CMCase (2.66) and FPase (1.81) specific activity in the UWS after 96 h of culture at 30 °C (Table 2).

T. viride showed the second highest CMCase (1.78) and FPase (1.77) specific activity in flask F_7 with UWS after 96 h of culture at 30 °C. Gulsher et al. (2010) indicated that the production of CMCase and FPase by *T. reesei* was much greater than that of *A. niger* using 2.0% NaOH-treated sugar cane baggase [25]. Our results corroborate the hypothesis of Reczey *et al*., (1996) [26], who claimed that lignin in the wheat straw does not inhibit but rather increases cellulase enzyme production, indicating that delignified plant biomass was not as suitable for a substrate as non-delignified plant biomass. Microorganisms may produce mixture of endocellulase, exocellulase [25] and β-glucosidase in good proportion and also other enzymes like xylanases or laccases may be

produced in small quantity depending upon the composition of substrate [27]. Shankar and Isaiarasu (2011) reported that maximum cellulase activity was observed at pH 6, temperature 37 °C, 0.5% Tween-20, 72 h and 2% inoculum [28].

Factors that Affect Enzyme Production

Effect of pH on Cellulase Production by *T. reesei*: Proper pH control is also necessary for the optimum yield of enzymes from dilute H_2SO_4-pretreated wheat straw. Fig. 1 represents the effect of a change in the pH of the fermentation medium. The maximum specific CMCase & FPase activity of *T. reesei* was noted at pH 6. It was reported by Immanuel *et al*., (2011) that the pH of cellulase varies from 3 to 9 [29]. They also reported that cellulase produced by *A. niger* showed the maximum activity at pH 5 with coir waste substrate, but the enzyme yield was the highest at pH 6 using saw dust as the substrate. The FPase activity was observed to be the highest at pH 7 and 6 while using coir waste and saw dust, respectively.

Effect of Temperature on Cellulase Production by *T. reesei*: The effect of temperature on the production of CMCase and FPase by *T. reesei* was also studied (Fig. 2). Among the various temperatures tested, *i.e.*, 25, 28, 30, 35 and 37°C, the maximum specific activity of CMCase (2.74) and FPase (1.89) was obtained at 30 °C. The minimum specific activity of CMCase (1.65) and FPase (1.31) was obtained at 37°C. Gautam *et al*. (2011) reported that the optimum temperature and pH of the medium for cellulase production by *A. niger* was 40 °C and 6.0, whereas those for the production of cellulase by *Trichoderma sp*. were 45 °C and 6.5, respectively [30].

Effect of Shaking Flasks (rpm) on Cellulase Production by *T. reesei*: The effect of shaking the flasks on the specific activity of CMCase and FPase from *T. reesei* was also studied. The results are shown in Fig. 3. Among different speeds of shaking the flasks, such as 100, 120, 140, 160 and 180 rpm, during 96 h of fermentation, the maximum specific activity of CMCase (2.75) and FPase (1.83) was obtained at 140 rpm [29]. According to Shankar and Isaiarasu (2011), the rate of shaking of the fermentation broth is an important factor for the production of enzymes [28].

Effect of Inoculum on Cellulase Production by *T. reesei*: The initial inoculum percentage in the fermentation media is a critical factor in fermentation processes. In our study, the percentage of inoculum played a very important role in the production of CMCase and FPase by *T. reesei* using wheat straw as the substrate. In this study, 1.0, 1.5, 2.0, 2.5 and 3.0% inoculums were tested and the maximum specific activity of CMCase (2.79) and FPase (1.93) was noted when 2.0% of the four-day-old inoculum was used, as shown in Fig. 4.

Effect of Inducers on Cellulase Production by *T. reesei*: Certain compounds when added in proper amounts in the fermentation medium can enhance the specific activity of enzymes. The effect of the addition of glucose, galactose and Tween 20, separately and in combination, was checked. After 48 h of fermentation, a dense growth was observed in flask F_d. A very noticeable growth was seen in flask F_b, containing 0.2 % (w/v) of galactose and greenish spores were found on the walls of the flasks and also were floating on the surface of the medium in the flask. In flask F_a, the growth of *T. reesei* was good but less than that in flasks F_b and F_d. The growth rate of *T. reesei* in flask F_c was not good after 48 h and the contents showed the least growth. The order of growth was observed as $F_d > F_b > F_a > F_c$.

The results were different when the specific activities of the CMCase and FPase were calculated after 72 and 96 h, as shown in Table 3. Specific activities of CMCase and FPase were at a maximum after 72 h, rather than after 96 h. The highest specific activity of the CMCase and FPase was noted in F_b, containing 0.2% (w/v) galactose, yet dense growth was seen in F_d. Thus, a combination of glucose, galactose and Tween 20 had a positive effect on the growth of *T. reesei*, but a negative effect on the specific activity of cellulase, acting as an inhibitor. The order of the specific activity in the different flasks was observed as $F_b > F_a > F_c > F_d$.

Sadaf *et al*., (2005) reported that a high yield of cellulase was obtained at 37 °C and pH 4.8 after 7 days of fermentation by *A. niger* [31]. They also noted that cellulose acts as an inducer, while glucose acts as an inhibitor. According to Sun and Cheng (2005), Tween 20 showed an inhibitory effect, even at very a low concentration of 0.1% [32]. Among the most potential cellulolytic organisms, Trichoderma sp. is very important, whose hypercellulolytic mutant strains secrete large amount of cellulases. This fungus produces a complete set of cellulases including ß-glucosidase. Different species of Trichoderma appear to be most promising microorganisms for industrial production of cellulases [33].

Table 2: Timeline Study of the Specific Activity of CMCase and FPase Produced by Various Fungal Strains using Untreated and 1.5% H_2SO_4-TWS

		Specific Activity After 48 h		Specific Activity After 72 h		Specific Activity After 96 h		Specific Activity After 120 h		Specific activity After 144 h	
Sr. No.	Flask No.	CMCase	FPase	CMCase	FPase	CMCase	FPase	CMCase	FPase	CMCase	FPase
1	F1	0.53	0.99	1.34	0.66	2.66	1.81	1.02	0.89	0.48	0.75
2	F2	0.31	0.36	0.55	0.65	0.97	1.69	0.93	0.59	0.74	0.29
3	F3	0.68	0.57	0.56	0.56	1.63	1.15	0.68	0.75	0.59	0.37
4	F4	0.16	0.26	0.52	0.23	0.76	1.08	0.51	0.59	0.52	0.43
5	F5	0.54	0.30	0.58	0.52	0.52	0.48	1.54	0.29	0.45	0.22
6	F6	0.21	0.13	0.77	0.89	0.76	1.05	1.08	0.46	0.72	0.25
7	F7	0.81	0.88	0.92	0.65	1.78	1.77	0.85	1.12	0.76	0.48
8	F8	0.47	0.56	0.68	0.50	0.93	1.62	0.73	0.94	0.61	0.43
9	F9	0.57	0.28	1.02	0.45	1.05	1.24	0.80	0.51	0.39	0.38
10	F10	1.25	1.45	3.21	1.39	3.18	2.10	1.41	1.18	0.63	0.67

Note: All values are averages of triplicates. These were calculated from triplicate measurements on the same sample using http://www.mathsisfun.com/data/standard-deviation-calculator.html.

Table 3: Effect of inducers and inhibitors on cellulase activity

	Specific Activity After 72 h		Specific Activity After 96 h	
Flask No.	CMCase	FPase	CMCase	Fpase
F_a	13.21±0.62	05.04±0.56	05.45±0.14	02.54±0.02
F_b	19.21±0.82	06.27±0.94	16.24±0.62	05.17±0.68
F_c	02.23±0.47	04.01±0.17	05.52±0.26	03.98±0.12
F_d	2.14±0.05	04.37±0.12	01.11±0.03	01.80±0.06

Note;- Where standard deviations are presented, these were calculated from triplicate measurements on the same sample using http://www.mathsisfun.com/data/standard-deviation-calculator.html.

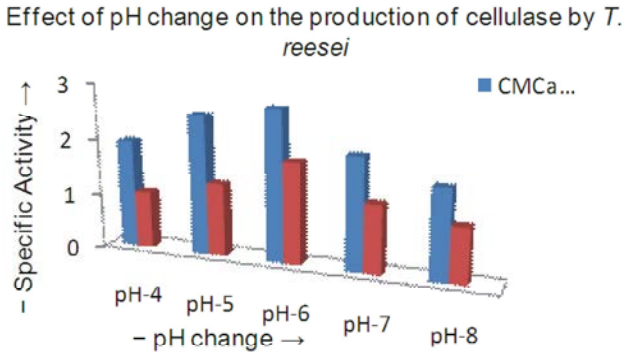

Fig. 1: Study of the effect of a change in pH on cellulase production by *T. reesei*

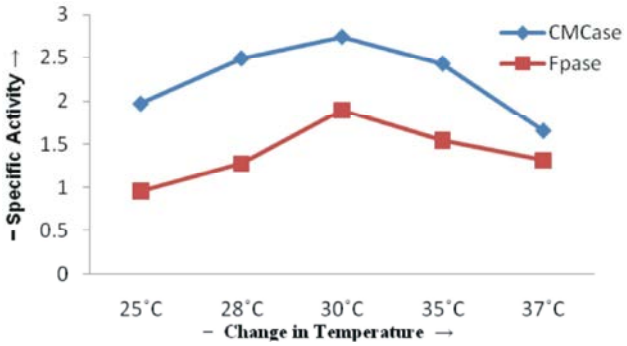

Fig. 2: Effect of change of temperature on cellulase production by *T. reesei*

This study demonstrated that *T. reesei* is a promising platform for the production of cellulases for the destruction of lignocelluloses. The supremacy of the cellulases of *T. reesei* in hydrolyzing the lignocellulose of plant biomass to fermentable sugars was established. An overall economical process must include a high cellulase yield, a high specific activity, a relatively inexpensive substrate and a short production time, most of which were achieved in the present study.

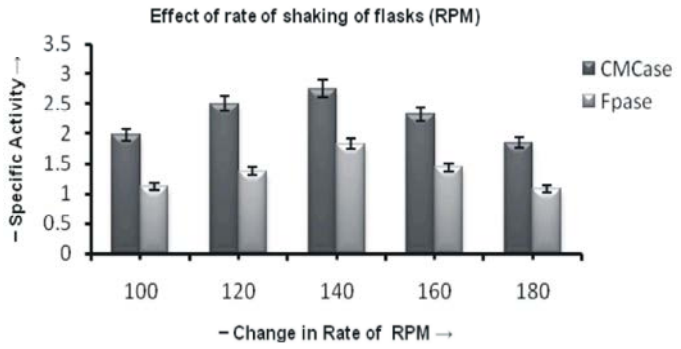

Fig. 3: Effect of speed of shaking flasks (rpm) on cellulase production by *T. reesei*

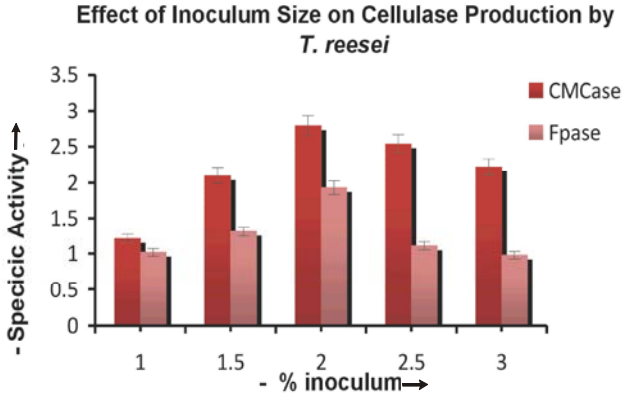

Fig. 4: Study of the effect of inoculum on cellulase production by *T. reesei*

CONCLUSIONS

In this study, it was established that untreated lignocelluloses were the best substrate for the production of cellulases of a high specific activity. *T. reesei* produced a higher yield of cellulase than did other fungal strains. Co-cultivation of fungi in fermentation cultures increased the yield of the cellulase enzyme complex. A combination of glucose, galactose and Tween 20 had a negative effect on production of the cellulase enzyme complex; therefore, they act as inhibitors. galactose induces the production of the cellulase enzyme complex. Fermentation conditions were optimized and a notable reduction in time for cellulase production was noted *i.e.* 72 h, 30°C, pH 6, 140 rpm and 2% inoculum. The objective of enhaced production of cellulase was achieved.

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