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Effects of different parameters on cellulase production by *Trichoderma harzianum* TF2 using solid-state fermentation (SSF)

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ABSTRACT Solid-state fermentation is one of the easiest and cheapest methods for producing microbial bioactive compounds. *Trichoderma harzianum* has long been recognised as one of the potential fungi for this purpose. *Trichoderma* sp. were isolated from banana rhizosphere using the soil dilution method and later screened for their ability to produce cellulases using filter paper activity (FPase) and the carboxylmethyl cellulase (CMCase) test. *Trichoderma* sp. were also subjected to one factor change at a time to determine the effects of different parameters on cellulase production. It was observed that *T. harzianum* TF2 showed the ability to produce higher cellulase activity when wheat bran was used as the substrate. The results showed that 38.5 U/g of cellulase was produced with the use of wheat bran coupled with an incubation temperature of 28 °C and moisture content of 60%. *T. harzianum* TF2 showed good potential for use as a culture for cellulase production in this study due to its higher cellulase production under solid-state fermentation, with the possibility of its application to industry.

KEYWORDS cellulase; compost; microbial fermentation; solid-state fermentation; *Trichoderma harzianum*

1. Introduction

Cellulase has been well known for its economically important ability. Due to this, cellulase had been attracting a lot of attention from researchers all over the world (Singhania et al. 2010; Darabzadeh et al. 2019; Han et al. 2020). Currently, cellulase derived from microorganisms are gaining their market shares rapidly, however fungal cellulase are more intensively studied because of their high enzyme productivity and also applicability in the industries (Darabzadeh et al. 2019). Production of cellulase from fungal can be done either using submerged fermentation or solid state fermentation (SSF). However, due to the simplicity and economical way for enzymes production, SSF was well accepted by the industry (Hölker and Lenz 2005).

The used of solid state fermentation was well employed and accepted in develop countries because it uses their excess agricultural wastes, which could be a source of pollution and turn it into a good source of substrate for enzyme production (Irfan et al. 2014). The use of these wastes will reduce the cost of production by 40%-60% (Wen et al. 2005). The most used substrates were wheat bran, rice husk, rice bran, sawdust and others (Irfan et al. 2014; Tambichik et al. 2018).

A few well know fungal that were widely used in cellulase production are *Trichoderma* sp. and *Aspergillus* sp. (Kittanan et al. 2018; Triwahyuni et al. 2018; Wang et al. 2020). Sari et al. (2013) demonstrated the used of *Trichoderma reesei* and *Aspergillus niger* in producing cellulases using rice straw from Indonesia. However, we believe that apart from *T. reesei*, *T. harzanium* can also be used to produce cellulases with higher or similar cellulase activity. *Trichoderma harzianum* can be found in abundance in all soil and has been studied by many researchers for its broad potential as plant biological control, industrial enzymes producers and composting (Pandey et al. 2014; Kumar et al. 2015; Triwahyuni et al. 2018). Haq et al. (2006) showed that *T. harzianum* can utilize several agricultural by-products to produce cellulase by estimating the CMCase and FPase activities. Pandey and Srivastava (2015) and Zhang and Yang (2015), also showed that *T. harzianum* can be used to produce enzyme cellulase via SSF utilizing agriculture waste such as wheat bran by determining both CMCase and FPase activities.

The aim of this study was to study the optimum substrates, temperature and moisture content for used in SSF for the production of industrial important enzyme cellulase from *Trichoderma* sp. isolated from organic soil.

2. Materials and Methods

2.1. Isolation of Trichoderma sp. from soil

Trichoderma sp. was isolated from the banana rhizosphere of an organic banana plot situated at MARDI Organic Farm at MARDI headquarters Serdang, Selangor, Malaysia. The soil samples were collected and put into a zip lock bag for transportation back to the laboratory. Upon reaching, 10 g of the soil sample was weighed and put into a 250 mL Erlenmeyer flask containing 100 mL of sterile distilled water and agitated for 1 h at 250 rpm. After 1 h, 150 µL of the soil suspension was pipetted to a fresh new Potato Dextrose Agar (PDA) plate. The plate was incubated at room temperature of 28±2 ºC for 7 days. Emerging *Trichoderma* sp. was picked up using an inoculation loop and transferred to a new PDA plate.

2.2. Screening for cellulase activity produce by Trichoderma sp.

Trichoderma sp. was grown in Potato Dextrose Broth (PDB) for 12 days. Every day a flask would be used for the cellulase activity test until day 12 of the incubation. The culture filtrate was used for filter paper activity (FPase) and carboxylmethyl cellulase (CMCase) test according to the method recommended by Ghose (1987). For FPase activity, Whatman number 1 filter paper was cut into a 1×5 cm strip after that 1.5 mL of the culture filtrate and 0.5 mL buffer (0.05 M citrate buffer, pH 4.5) were added to it and incubated for 1 h at 50 ºC. The hydrolysis process was terminated by the addition of 3 mL of dinitro-salicylic acid (DNS) solution, followed by 5 min of boiling. After 5 min, the solution was left to cool (Miller 1959). When it was cooled, 20 mL of distilled water was added and the absorbance was read at 540 nm using a nanodrop reader. While for CMCase activity, the method was the same, except the filter paper used in the FPase was replaced with 5 mL of CMCase solution (1%). Tests were repeated for every day until day 12. Glucose was used as standard and enzyme activity was expressed as the amount of enzyme required to liberate 1 µmol of product at 50 °C. One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate 1 µmol of product at 50 °C.

2.3. Molecular identification of Trichoderma sp.

Isolation of genomic DNA from *Trichoderma* sp. was done using the QIAamp® DNA Mini Kit following protocol suggested by Qiagen (Qiagen 2020). After that polymerase chain reaction (PCR) was conducted using ITS primer. The reaction was performed in a 25 µL final volume containing 0.1 µg of genomic DNA, 10 pM of each primer (ITS4 and ITS5), 1 × Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U of Taq DNA polymerase. PCR thermal cycle parameters used were 94 °C for 3 min followed by 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 35 s at 72 °C and a final extension at 72 °C for 7 min (Lu et al. 2012). The PCR products were later subjected to purification using QIAquick PCR Purification Kit (Qiagen 2020). The purified PCR products were later sent for sequencing at Apical Scientific Sdn. Bhd., Selangor. The results obtained were then compared with the databases from National Center for Biotechnology Information (NCBI).

2.4. Experimental design

The best cellulase producer of *Trichoderma* sp. was chosen for SSF process. The effect of *Trichoderma* sp. on each of the following parameters was conducted by changing one factor at one time. All experiments were conducted in a triplicate manner and the means of the results were compared with one-way ANOVA and considered significant when $p < 0.05$.

2.5. Preparation of Trichoderma sp. spores

The *Trichoderma* sp. was grown on the petri dish for 7 days. After 7 days the plate was flooded with sterile distilled water (dH_2O) and spores were harvested using a sterile glass scraper. The harvested spores were then measured for their concentration using a hemocytometer and adjusted using dH_2O to obtain a concentration of 10^7.

2.6. Solid extraction of cellulase

The weight of the fermented solid substrate was determined and dH_2O was added into the flasks, approximately five times of the solid substrate weight. The mixture was then stirred with a magnetic stirrer for 30 min at 700 rpm. After that, the mixture was centrifuged at 8000×g for 15 min (Sigma 4K10, B. Braun, Germany) and the supernatant separated was taken as the enzyme extract and used in FPase and CMCase tests.

2.7. Effect of different agriculture waste on cellulase production

Three agricultural wastes were used in this study (wheat bran, rice bran and rice husk). Five millilitter of a mixture containing 10^7 of *Trichoderma* sp. spores was inoculated into a conical flask containing 20 g of each agriculture waste while moisture content and temperature were maintained at 50% and 28 °C respectively. Results were observed every day for 12 days and the cellulases (FPase and CMCase) activity was measured using the DNS method.

2.8. Effect of different moisture content on cellulase production

Trichoderma sp. spores concentration of 10^7 was inoculated into a conical flask containing 20 g of each agriculture waste according to the moisture content desired (20%–80%) and the temperature was maintained at 28±2 °C. Results were observed for 12 days and the cellulases (FPase and CMCase) activity was measured using the DNS method.

2.9. Effect of different temperatures on cellulase production

Five mililitter of *Trichoderma* sp. spore of 10^7 was inoculated into a conical flask containing 20 g of each agricul-

ture waste. Moisture content was set at 50% while temperature was set at 28 °C, 35 °C, 45 °C and 55 °C. Results were observed for 12 days and the cellulases (FPase and CMCase) activity was measured using the DNS method.

2.10. Determination of cellulase production using optimized condition

Trichoderma sp. spores of 10^7 was inoculated into a conical flask containing 20 g optimized substrate and incubated under optimized moisture content and temperature for 7 days. Cellulases (FPase and CMCase) activity was measured using the DNS method.

3. Results and Discussion

3.1. Isolation, cellulase screening and identification of Trichoderma sp.

A total of 30 *Trichoderma* sp. were isolated from the soil samples. All the *Trichoderma* sp. was screened for their ability to secrete FPase and CMCase activity which will gave a representative on the total cellulase activity secreted by *Trichoderma* sp. Out of the 30 isolates of *Trichoderma* sp. isolated, only five isolates of the *Trichoderma* sp. showed high FPase and CMCase activity of 7.4 U/mL–9.5 U/mL and 7.5 U/mL–10.9 U/mL respectively at day 6 of incubation (Table 1). Based on the results of the five potential isolates of *Trichoderma* sp. that we obtained (Table 2), *Trichoderma harzanium* TF2 was chosen for further investigation due to its highest cellulases pro-

TABLE 1 Potential Trichoderma sp. with cellulase producing activity (mean±SD)

Isolate no.	Species ID	FPase activity (U/mL)	CMCase (U/mL)	Total cellulase (FPase + CMCase activity) (U/mL)
TF2	*Trichoderma harzianum*	7.267±0.094	8.867±0.287	16.134±0.800
TF7	*Trichoderma* sp.	6.400±0.216	7.800±0.408	14.200±0.700
TF10	*Trichoderma harzianum*	6.833±0.170	8.000±0.478	14.833±0.584
TF16	*Trichoderma viride*	7.533±0.170	5.467±0.403	13.000±1.033
TF23	*Trichoderma* sp.	5.433±0.287	6.067±0.170	11.500±0.317

TABLE 2 Five potential isolates of *Trichoderma* sp.

Isolate no.	Species ID	Sequence
TF2	Trichoderma harzianum	tttacaactc ccaaacccaa tgtgaacgtt accaaactgt tgcctcggcg ggatctctgc cccgggtgcg tcgcagcccc ggaccaaggc gcccgccggr rgaccaacct aaaactctta ttgtataccc cctcgcgggt ttttttttat aatctgagcc ttctcggcgc ctctcgtagg cgtttcgaaa atgaatcaaa actttcaaca acggatctct tggttctggc atcgatgaar aacgcagcga aatgcgataa gtaatgtgaa ttgcaraatt cagtgaatca tcgaatcttt aacgcacat tgcgcccgcc agtattctgg cgggcatgcc tgtccgagcg tcatttcaac cctcgaaccc ctccgggggg tcggcgttgg ggatcggccc tcccttagcg ggtggccgtc tccgaaatac agkggcggtc tcgccgcagc cc
TF7	Trichoderma sp.	tgaggaagta aaagtcgtaa caaggtctcc gttggtgaac cagcggaggg atcattaccg agtttacaac tccaaaccc aatgtgaacg ttaccaaact gttgcctcgg cgggaactca tgccccgggt gcgtcgcagc cccggaccaa ggcgcccgcc ccaggaccaa ccaaaactct ttttgtatac cccctcgcgg gttttttata atctgagcct tctcggcgcc tctcgtaggc gtttcgaaaa tgaatcaaaa ctttcaacaa cggatctctt ggttctggca tcgatgaaga acgcagcgaa atgcgataag taatgtgaat agcagaattc agtgaatcat cgaatctttg aacgcacatt gcgcccgcca gtattctggc gggcatgcct gtccgagcgt catttcaacc ctcgaacccc tccggggggt cggcgttggg gatcggcctt ccttcacggg ggccgtctcg
TF10	Trichoderma harzianum	tcttggtcat ttagaggaag taaaagtcgt aacaaggtct ccgttggtga accagcggag ggatcattac cgagtttaca actcccaaac ccaatgtgaa cgttaccaaa ctgttgcctc cgcgggatct ctgccccggg tgcgtcgcag ccccggacca aggcgcccgc cggaggacca accaaaactc ttttgtata ccccctcgcg ggtttttat aatctgagcc ttctcggcgc ctctcgtagg cgtttcgaaa atgaatcaaa actttcaaca acggatctct tggttctggc atcgatgaag aacgcagcga aatgcgataa gtaatgtgaa ttgcagaatt
TF16	Trichoderma viride	ctgttgcctc ggcggggtca cgccccgggt gcgtcgcagc cccggaacca ggcgcccgcc agagggacca accaaactct ttctgtagtc ccctcgcgga cgttatttct tacagctctg agcaaaaatt caaaatgaat caaaactttc aacaacggat ctcttggttc tggcatcgat aagaacgcat gcgaaatgcg ataagtaatg tgaattgcag aattcagtga atcatcgaat ctttgaacgc acattgcgcc cgccagtatt ctggcgggca tgcctgtccg agcgtcattt caaccctcga acccctccgg gggtccggcg ttggggatcg ggaacccta agacgggatc ccggccccga aatacagtgg cggtctcgcc gcagcctctc atgcgcagta gtttgcacaa ctcgcaccgg gagcgcggcg cgtccacgtc cgtaaaacac ccaacttctg aaatgttgac ctcggatcag gtaggaatac
TF23	Trichoderma sp.	aaactgttgc ctcggcgggg tcacgccccg ggtgcgtcgc agccccggaaccaggcgccc gccggaggga ccaaccaaac tctttctgta gtcccctcgc ggacgttatt tcttacagct ctgagcaaaa attcaaaatg aatcaaaact ttcaacaacg gatctcttgg ttctggcatc gatgaagaac gcagcgaaat gcgataagta atgtgaattc cagaattcag tgaatcatcg aatctttgaa cgcacattgc gcccgccagt attctggcgg gcatgcctgt ccgagcgtca tttcaaccct cgaacccctc cgggggtcg gcgttgggga tcgggaaccc ctaagacgcg atcccggccc cgaaatacag tggcggtctc gccgcagcct ctcctgcgca gtagtttgca caactcgcac cgggagcgcg gcgcgtccac gtccgtaaaa cacccaactt ctgaaatgtt gacctcggat caggtaggaa tacccgctga acttc

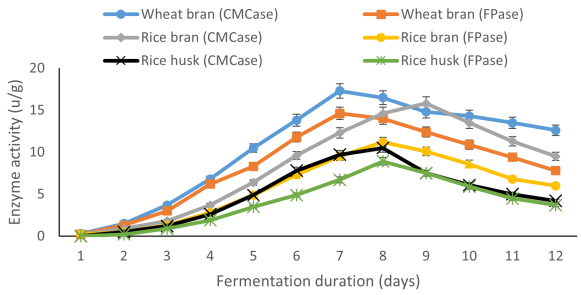

FIGURE 1 Cellulase production by *Trichoderma harzianum* TF2 using wheat bran; rice bran and rice husk under 12 days of Solid State Fermentation. The mean values were significantly different in ANOVA with $p < 0.05$.

duction activity.

3.2. Effect of different agriculture waste on cellulase production

From the obtained data, it was observed that wheat bran showed a better source for the secretion of enzyme cellulase compared to rice bran and rice husk (Figure 1). The use of wheat bran showed an increase of 23.6% and 64.4%, respectively ,in the cellulases activity production (FPase and CMCase activity) by *T. harzianum* TF2 when compared to rice bran and rice husk. These results were in accordance to a study conducted by Haq et al. (2006), whereby they observed that *T. harzanium* KM07 produced 16 U/g of total cellulases in wheat bran, but the amount decreases when they are using rice bran (13 U/g) and rice husk (12 U/g). According to Nochur et al. (1993), the amount of nutrients in wheat bran, such as protein 1.32%, carbohydrate 69.0%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca 0.05%, P 0.35%, Mg 0.17%, S 0.12% and K 0.45% might be one of the factors that influence the fungal growth and subsequently better cellulases production when wheat bran was used as substrate in the fermentation. This statement was further supported by Brijwani et al. (2010) and Kittanan et al. (2018) when these researchers stated that an increased in the secretion of cellulase by *Trichoderma* sp. was due to the presence of wheat bran which contained high protein and starch. This was noted by Kittanan et al. (2018), when wheat bran was added to copra waste, the cellulase activity produced by *Trichoderma reesei* increased from 0.31 FPU/g–5.23 FPU/ g dry substrate at day 2 and 0.42U/g–5.18 U/ g dry substrate at day 6 of solid state fermentation respectively. Triwahyuni et al. (2018) also showed that *Trichoderma* sp. T004 isolated from Indonesian soil produces highest cellulase activity (0.52 FPU/mL) when wheat bran was used. This further established the reason why wheat bran was the best substrate for cellulase production by *Trichoderma* sp.

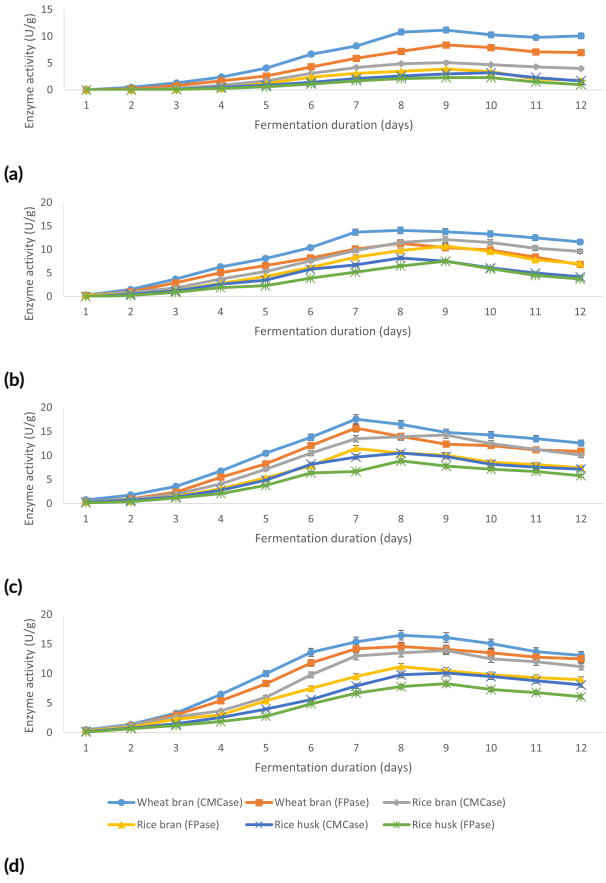

FIGURE 2 Cellulase production by *Trichoderma harzianum* TF2 with (a) 20%; (b) 40%; (c) 60%; (d) 80% moisture content under 12 days of Solid State Fermentation. The mean values were significantly different in ANOVA with p< 0.05.

3.3. Effect of different moisture content on cellulase production

According to Liu and Yang (2007), the optimal moisture content for solid state fermentation should be 40%–60% (v/w). In this study, it was observed that at 60% of moisture content (Figure 2c), the production of cellulases activity by *Trichoderma harzianum* TF2 was induced from 23.8 U/g to 33.3 U/g when moisture content increased from 40% to 60% (v/w) (Figure 2b and Figure 2c). According to Liu and Yang (2007), an increase moisture content at a certain level will caused the cellulases enzyme production to decrease. This is because the moisture content will reduce the surface area of the substrate and this will affect the accessibility of the air to the substrate, thus affecting the growth and metabolism of the microbes (Liu and Yang 2007). Irfan et al. (2014), concurred with the statement made by Liu and Yang (2007), when they observed that at the ratio of 11:10 (mL:g) *Trichoderma viride*-IR05 produces 64.3 U/g of xylanase activity, but when the ratio of water was increased to 13:10 (mL:g) the activity was reduced to 55 U/g. In this study, it was also observed that the cellulase activity reduced from 33.3 U/g to 29.6 U/g in wheat bran when the moisture content was further increased from 60%–80% (v/w) (Figure 2c and Figure 2d). At 20% (v/w) moisture content it was observed that *T. harzanium* TF2 required 9 days to colonize the substrate and to obtain optimum cellulase activity of 19.6 U/g in wheat bran (Figure 2a). This is much lower when we com-

pared to cellulase activity at 60% (v/w), which is 33.3 U/g in 7 days of fermentation (Figure 2c). Another research done by Sachdev et al. (2018) noted that *Trichoderma lixii* growth at optimum when 68.87% of moisture content was used. This further justified that *Trichoderma* sp. grows well in moisture content of approximately 60%.

3.4. Effect of different temperature on cellulase production

The optimum temperature for the highest cellulase production was when *Trichoderma* sp. was grown at 28 °C (Figure 3). It was observed that, at 28 °C (Figure 3a), *Trichoderma harzianum* TF2 produced 32.6 U/g of cellulases activity (total of CMCase and FPase activity) when wheat bran was used however, when the temperature was increased to 45 °C (Figure 3c) the cellulases activity was drastically decreased to 16.1 U/g for the same substrate. Darabzadeh et al. (2019), reported that at 30 °C production of cellulase was 1.16 U/g but decreased to 0.85 U/g when the temperature was increased to 35 °C. This indicates that the optimum temperature for the enzymatic reaction to take place should be around 25-30 °C. Our findings fit well in this region. According to Ali et al. (2017), fungi grow best at 25–30 °C. However, this differs from the genus, species and strain of the fungus. Singh et al. (2014), in their study, observed that *T. harzianum* produces the highest biomass at 25 °C –30 °C. According to work done by Iqbal et al. (2010), CMCase activity increases until the temperature reached 35 °C and decreased as the temperature increased further. In our study, it was observed that CMCase activity do increase until 35 °C but the incremental was just 0.1U/g. However, beyond 35 °C all cellulases activity (CMCase and FPase) reduced and this concurred with Iqbal et al. (2010).

3.5. Cellulase production using optimized conditions

Trichoderma harzianum TF2 showed an optimum cellulase activity (CMCase and FPase) of 38.5 U/g when SSF was conducted under the combined optimized condition (Figure 4). This showed an incremental of approximately 19% compared to cellulases activity produced when optimization was done for each parameter only. Sari et al. (2013), reported that the combination of *T. reesei* with rice straw as substrate only gives a reading of 1.80 IU/mL of cellulase activity which much lower than the results showed by *T. harzanium* TF2 in this study. Haq et al. (2006), indicated that their formulated condition was only able to give an incremental of 11% in cellulase production for *T. harzanium* KM07 used. In a study conducted by Rahnama et al. (2013), *T. harzanium* SNRS3 produces 117.56 U/g of cellulase activity (6.25 U/g of FPase and 111.31 U/g of CMCase), the results obtained were high compared to the results we obtained in this study. However, it was noticed that the FPase activity obtained was low compared to 17.7 U/g obtained in this study. The ability of different isolates of *T. harzanium* to react to their optimized condition will produce different increment rates in their cellulases activity. The current conditions used

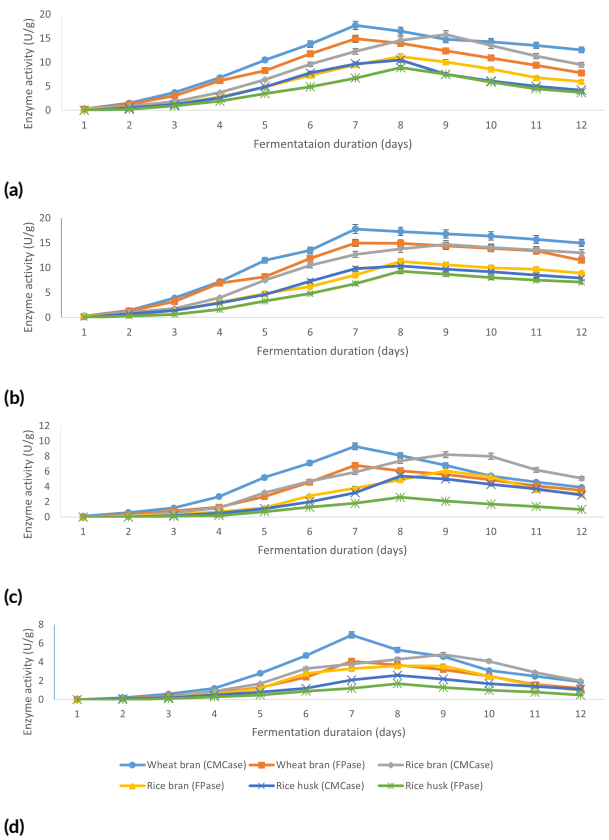

FIGURE 3 Cellulase production by *Trichoderma harzianum* TF2 with (a) 28 °C; (b) 35 °C; (c) 45 °C; (d) 55 °C incubation temperature under 12 days of Solid State Fermentation. The mean values were significantly different in ANOVA with $p < 0.05$

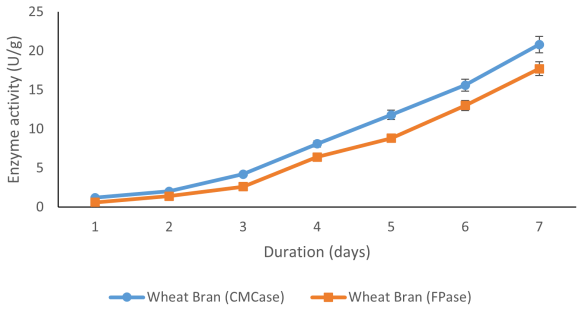

FIGURE 4 Cellulase (FPase and CMCase) production by *Trichoderma harzianum* TF2 using wheat bran with 60% moisture content at 28 °C. The mean values were significantly different in ANOVA with $p < 0.05$.

for *T. harzianum* TF2 indicated that these were the most suitable conditions for *T. harzanium* TF2 to produce cellulases activity at this moment. There might be other parameters that will increase the cellulase activity of *T. harzanium* TF2 however, those parameters were not yet tested in this study. From all the test conducted it was noted that cellulase production increase as the biomass of *T. harzanium* TF2 increased. Darabzadeh et al. (2019), stated that *T. reesei* biomass production correlates with the enzyme production.

4. Conclusions

Trichoderma harzanium TF2, isolated from banana rhizosphere, produced a high cellulases activity of 38.5 U/g when wheat bran was used as the substrate, incubated at 28±2 ºC and moisture was kept at 60% (v/w).

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Authors' contributions

JLSH was involved in setting up experiment, conducting experiments and analyzing results. The author also wrote the manuscript. HH involved in setting up of experiment and conducting experiment on isolation, screening and identification of *Trichoderma* sp.

Competing interests

There is no competing interest in relevant to this submitted article.

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