



Production of Cellulase by *Trichoderma viride* via Submerged Fermentation using Corncob as Substrate

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ABSTRACT

Cellulolytic enzymes are commonly used in lignocellulosic biomass conversion, textile, and paper and pulp industries. However, the high cost of cellulase and the high demand has led to increase researches to explore agro-residues for cost-effective production. This study was aimed at isolating *Trichoderma viride* from decaying wood and soil, and testing the isolates for cellulase production via submerged fermentation using corncob as substrate. Twenty-one (21) soil samples were collected from three different locations (botanical garden, refuse dump site and decaying wood) and used in the study. Colonies suspected to be *Trichoderma viride* were identified by microscopy of lactophenol cotton blue-stained preparation and slide culture technique, respectively. Isolates confirmed to be *Trichoderma viride* were then screened for cellulase production on a mineral salt medium containing carboxymethyl cellulose as an inducer. The isolates were further tested for cellulase production using corncob as the substrate via submerged fermentation. Out of the 21 samples analysed, 4 (19%) were positive for *Trichoderma viride* based on cultural and microscopic characteristics. The highest occurrence of *Trichoderma viride* (57%) was recorded from decaying wood samples, while no occurrence (0%) was observed from the botanical garden and refuse dumpsite soils. All four isolates obtained produced cellulase, with *T. viride* DW6 having the highest zone of cellulose hydrolysis (61.5 ± 2.12 mm) and *T. viride* DW1 having the least (58.5 ± 2.12 mm). The highest enzyme activity was recorded from *T. viride* DW4 (3.41 ± 0.46 U/ml) and the lowest from *T. viride* DW1 (1.77 ± 0.52 U/ml) using corncob as substrate. *Trichoderma viride* is abundantly present in decaying wood, and such isolates have high cellulase-producing ability, as wood material is rich in cellulose. This preliminary study provides information on the isolation of *T. viride* from decaying wood and their potential for cellulase production using corncob as substrate.

Keywords: *Trichoderma viride*; cellulase; decaying wood; isolation; production; corncob

Introduction

Cellulase refers to a class of enzymes produced primarily by fungi, bacteria and protozoan that catalyses the hydrolysis of cellulose (Abubakar and Oloyede, 2013). Cellulase is a complex of three

types of enzymatic complexes: endoglucanase (carboxymethyl cellulase), exoglucanase, and β -glucosidase. Cellulases are the enzymes that hydrolyse β -1,4 linkages in cellulose chains. Cellulose is a linear polymer of D-glucose units linked by a 1,4- β -D-glucosidic bond and is

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crystalline (El Baz *et al.*, 2018). Cellulose is the main constituent of plants and thus, the most abundant biopolymer on earth, comprising approximately 35–50% of plant dry weight (Ja'afaru, 2013; Islam and Roy, 2018).

Filamentous fungi are mainly the source of cellulases and hemicellulases. *Trichoderma* and *Aspergillus* species have most commonly been used to produce these enzymes. They secrete these enzymes into the medium and have higher activities than those produced by bacteria. This makes fungal enzymes more attractive for various industrial processes (Shakshi, 2016). Crude enzymes produced by these microorganisms are commercially available for agricultural and industrial uses (Pandey *et al.*, 2015). *Trichoderma* species are free-living, filamentous fungi that multiply rapidly on many substrates such as soil, decaying wood materials, and root colonisers (Grondona *et al.*, 1997; Hewavitharana *et al.*, 2018). *Trichoderma viride* is a filamentous, asexual spore-producing ascomycetic fungus that belongs to the class Deuteromycetes (Iqbal *et al.*, 2017). As a saprophytic mould, it is found naturally in soil and can grow directly on wood, compost or dead mushroom (Awojobi *et al.*, 2013).

Cellulase production by *T. viride* could be improved by substituting carbon sources with sustainable and cost-effective lignocelluloses or agricultural wastes. The main components of agricultural wastes consist of 40-50% cellulose, 20-30% hemicellulose and 10-15% lignin, which can contribute to the large-scale production of cellulose (Iqbal *et al.*, 2011; Anwar *et al.*, 2014). These wastes are widespread and found abundantly in nature, making them the largest renewable and fermentable carbohydrates. Thus, using them in various industries is of great interest (Ho, 2015).

Most large-scale fermentation processes are performed via submerged cultures, and it is the best process for cellulase production by *Trichoderma* species (Florencio *et al.*, 2015). Industrially, important enzymes have traditionally been obtained

from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH, agitation/aeration, inoculum sizes, incubation period and nutrients (Mrudula and Murugammal, 2011).

Although several fungi capable of producing cellulase had been discovered for decades, new isolates that are hyper-producers with high stability are yet to be exploited (Akinyele *et al.*, 2013). Search for new isolates of *T. viride* locally and utilising them for cellulase production, which is in high demand, will go a long way in boosting local industries and the economy by reducing importation and increasing the availability of the enzyme.

There is presently little or no commercial production of cellulases for large-scale industrial use in Nigeria. Annually, a tremendous amount of money is spent on importing this enzyme (Egbere *et al.*, 2014). Production of cellulase enzyme with high activity level using agro residues would help in the cost-effective production of this important enzyme (Gupta *et al.*, 2015). Thus, this study aimed to isolate *Trichoderma viride* from soil and decaying wood and utilise the isolates for cellulase production using corncob as substrate via submerged fermentation.

Materials and Methods

Collection of Samples

Twenty-one (21) samples were collected from different locations, namely, refuse dump sites, botanical garden soils and decaying wood. Seven samples (10 grams each) were separately collected from each location from a depth of 3-5cm using a spatula into a clean polythene bag (Kulkarni and Gupta, 2013). The samples were labelled appropriately and brought to the Main Teaching Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria, for further analysis.

Preparation of Media

Potato dextrose agar

Potato dextrose agar (PDA) used in this study was of analytical grade (Oxoid™) and was prepared according to the manufacturer's instruction.

Mineral salt medium

Mineral salt medium supplemented with carboxymethyl cellulose (CMC) was used for the primary screening and contained the following; 0.2g KCl, 0.2g MgSO₄.7H₂O, 0.8g (NH₄)₂HPO₄, 0.2g NaH₂PO₄, 0.003g CaCl₂.2H₂O, 0.001g FeSO₄.7H₂O, 0.002g ZnSO₄.7H₂O, 3g CMC and 6g of agar in 300ml of distilled water. The mixture was heated to dissolve before sterilising in an autoclave at 121.5 °C for 15mins. For cellulase production under submerged fermentation, 10g of corncob was used as the source of carbon in place of CMC (Muhammed, 2013).

Isolation and Identification of *Trichoderma viride*

Isolation of *Trichoderma viride*

Ten grams (10g) of the soil and decaying wood samples were suspended in 90ml of distilled water. A tenfold serial dilution was carried out to 10⁻⁸. Aliquots of 0.1 ml from the 10⁻¹ and 10⁻³ dilutions were separately inoculated on freshly prepared PDA by spread plate technique in triplicate. Streptomycin (0.03 g/L) was added to the molten medium after autoclaving to inhibit bacterial growth while permitting the selective isolation of fungi. The inoculated plates were incubated aerobically at room temperature for 3-5 days and examined for growth (Bairagi, 2016). The isolates were sub-cultured to obtain pure isolates and preserved on a PDA slant at 4 °C for subsequent use.

Identification of *Trichoderma viride*

Cultural characterisation

The isolated fungi were identified after growth on the PDA medium by observing their macroscopic

characteristics (colour, texture, appearance, and diameter of colonies).

Microscopic characterisation

Wet mount

A pinch of the isolated fungi was teased on a clean grease-free slide containing one drop of Lactophenol cotton blue and covered with a coverslip. The slides were examined using 10× and 40× objectives of a compound microscope for microscopic appearance (Ja'afaru, 2013).

Slide culture technique

Aseptically, with a pair of forceps, a sterile filter paper was placed in a petri dish. A U-shaped glass rod was then placed on the filter paper. Four (4ml) of sterile water was poured to moisten it completely. Using forceps, a sterile slide was placed on the U-shaped rod. A scalpel was gently flamed for sterilization and used to cut a 1-by-1cm square block of agar from the PDA plate. The agar block was picked by inserting the scalpel and carefully transferring it aseptically onto the slide. Using a sterile wire loop, four sides of the agar were then inoculated with spores of mycelial fragments of the fungi examined. A sterile coverslip was aseptically placed on the upper surface of the agar cube. The plates were then incubated at room temperature for 72 hours. The slide culture was then examined under a microscope using 10× and 40× objectives for observation of the microscopic characteristics of the mould (Rosana *et al.*, 2014).

Screening of *Trichoderma viride* Isolates for Cellulase Production

Primary screening

Primary screening was performed by plate assay method in mineral salt medium supplemented with 1% Carboxymethyl cellulose (CMC) (MSM-carboxymethyl cellulose). A pinch of the mycelia plucked from a 5-day-old culture plate of pure *T. viride* isolate was placed at the centre of the agar plates. The inoculated plates were incubated at

room temperature for 5 days. The cultured plates were flooded with 0.01% of 15 ml Congo red solution and allowed to stand for 15 minutes. It was de-stained with 1% NaCl for 15min, after which it was decanted, and the diameter of the zone of cellulose hydrolysis was measured in millimetres (mm) (Ramanjaneyulu, 2015). The screening was carried out in three biological replicates and results were expressed as means \pm standard deviation.

Cellulase production using submerged fermentation (SmF)

Inoculum preparation

Spore suspensions were prepared by adding 10 ml of 2.5% Tween-80 to a 5-day-old slant culture of the *T. viride* isolates. A sterilised wire loop was used to dislodge the fungal spores aseptically and then shaken vigorously to obtain a homogenised spore suspension. The spores in the suspension were counted using a haemocytometer to obtain a concentration of 1.0×10^6 spores/ml (Irfan *et al.*, 2014).

Submerged fermentation (SmF)

The *T. viride* isolates were cultured in a 250 ml Erlenmeyer flask using a mineral salts medium with corncobs as the sole carbon source. The pH of the medium was adjusted to 5.0 using HCl and NaOH with the aid of a pH meter. A hundred millilitres (100ml) of the medium was transferred into 250ml Erlenmeyer flasks and autoclaved at 121.5 °C for 15 minutes. Ten millilitres (representing 10% of the production medium) of the prepared spore suspensions containing 1.0×10^6 spores/ml were introduced into the mineral salt medium and incubated at room temperature on a rotary shaker for 5 days. After incubation, the medium was filtered through Whatman No.1 filter paper to extract the crude cellulase enzyme. The filtrates were centrifuged at 10,000g for 20mins and the supernatant was used as the source of crude cellulase enzyme produced by *Trichoderma viride* (Nair *et al.*, 2008).

Cellulase enzyme assay

Carboxymethyl cellulase (CMCase) activity was determined by measuring the releasing sugar in a reaction mixture containing 0.1 ml of culture filtrate and 0.9 ml of 1% carboxymethyl cellulose (CMC) in 0.1 M citrate buffer (pH 5.0). The mixture was incubated at 50 °C for 30 min in a water bath for enzymatic reaction and then terminated by adding 1 ml of 3,5-dinitrosalicylic acid (DNSA) reagent. The tubes were further incubated in a boiling water bath at 100 °C for 5 minutes. The mixture was cooled rapidly to room temperature, and the absorbance was read at 540 nm using a spectrophotometer (Muhammed, 2013). One unit (U) of CMCase activity is expressed as 1 μ mol of reducing sugar (glucose equivalent) released per minute per millilitre of enzyme solution.

Results

Isolation and Characterisation of *Trichoderma viride*

Figure 1 shows the macroscopic appearance of *Trichoderma viride* on PDA after 5 days of incubation. Four (4) isolates were confirmed to be *Trichoderma viride* isolated from decaying wood based on cultural and microscopic characteristics as presented in Table 1. The percentage occurrence of the *Trichoderma viride* isolated from the three different sampling locations is shown in Table 2 with an occurrence of 57% observed for samples from decaying wood, while no occurrence was observed from botanical garden and refuse dumpsite soil samples. Furthermore, out of the 21 total samples analysed, only 4 (19%) were positive for *Trichoderma viride*.

Primary Screening of *Trichoderma viride* for Cellulase Production

Isolates capable of producing cellulase were identified by the clear zone indicating cellulose hydrolysis as shown in Figure 2. Of the four isolates screened, *T. viride* DW6 and DW4 had similar diameters in the zone of cellulose hydrolysis (61.5

mm and 61 mm respectively), while 58.5 and 59 mm of the zone of cellulose hydrolysis were observed for *T. viride* DW1 and DW2 as presented in Table 3.

Cellulase Production by *T. viride* via Submerged Fermentation using Corncob as Substrate

The cellulase produced was quantified and reported as the cellulase activity of the crude enzyme as shown in Figure 3. Table 4 shows the activity of the cellulase enzyme produced by the *Trichoderma viride* isolates, with *T. viride* DW4 and DW6 isolates having the highest cellulase activity of 3.45 ± 0.46 U/ml and 3.09 ± 0.00 U/ml respectively, while *T. viride* DW1 had the least cellulase activity of 1.77 ± 0.52 U/ml.



Figure 1: Macroscopic appearance of *Trichoderma viride* on PDA after 5 days' incubation

Table 1: Cultural and Microscopic Characteristics of the *Trichoderma* Isolates

Isolate's code	Cultural Characteristics			Microscopic Characteristics		Identity
	Colour change of colony	Colony texture	Colour of reverse	Conidiophore	Phialides	
DW1	White-Dark Green	Granular	White	Highly Branched, Short and Erect	Flask-Shaped	<i>Trichoderma viride</i>
DW2	White-Dark Green	Granular	Yellow Pigment	Highly Branched, Short and Erect	Flask-Shaped	<i>Trichoderma viride</i>
DW4	White-Light Green	Granular	Yellow Pigment	Highly Branched, Short and Erect	Flask-Shaped	<i>Trichoderma viride</i>
DW6	White-Light Green	Granular	White	Highly Branched, Short and Erect	Flask-Shaped	<i>Trichoderma viride</i>

Key: DW; Decaying wood

Table 2: Occurrence of *Trichoderma viride* in Various Soil Samples Collected n= 21

Sample Location	Number of Samples Collected	Number Positive (%)
Botanical garden	7	0 (0)
Refuse dump site	7	0 (0)
Decaying wood	7	4 (57)
Total	21	4 (19)

KEY: n = Total number of samples collected



Figure 2: Zone of cellulose hydrolysis by *Trichoderma viride* on MSM-carboxymethyl cellulose agar

Table 3: Cellulase Production by *Trichoderma viride* Isolates on solid medium

Isolate's Code	Diameter of Zone of cellulose hydrolysis (mm)
DW1	58.5 ± 2.12
DW2	59 ± 1.41
DW4	61 ± 1.41
DW6	61.5 ± 2.12

KEY: DW; Decaying wood



Figure 3: Carboxymethyl cellulase activity of the crude enzyme produced by *Trichoderma viride* under submerged fermentation

Table 4: Cellulase Production by *Trichoderma viride* Isolates via Submerged Fermentation using Corncob as substrate

Isolate's code	Cellulase Activity (U/ml)
DW1	1.77 ± 0.52
DW2	2.68 ± 0.84
DW4	3.45 ± 0.46
DW6	3.09 ± 0.00

KEY: DW; Decaying wood

Discussion

Many microorganisms are capable of degrading cellulose, but only a few of these microbes produce sufficient quantities of enzymes capable of completely hydrolysing cellulose. Fungi are the main lignocellulose degrading microorganisms due to the production of a wide variety of extracellular enzymes such as cellulases. In this study, four (4) *T. viride* were isolated from decaying wood samples and identified based on cultural and morphological characteristics. All the *T. viride* isolates were obtained from decaying wood with no isolate obtained from the soils of the botanical garden and refuse dump site. This observed isolation of *T. viride* from decaying wood and not soils from botanical gardens and refuse dumpsites might be due to the richness of cellulose in wood and their ability to produce high extracellular wood degrading enzymes. The finding in this study is in line with previous studies by Ja'afaru (2013) where *T. viride* was isolated from decaying wood and sawdust. This is also similar to the findings of Blaszczyk *et al.* (2016) where they isolated *Trichoderma* species from decaying woods with *Trichoderma viride* having the highest percentage occurrence of 53% of all isolates.

T. viride is considered to be one of the most important producers of cellulase (Iqbal *et al.*, 2011; Irshad *et al.*, 2013). They have been used industrially for enzyme production using submerged fermentation (SmF). Some of the enzymes produced by *Trichoderma viride* that are

of industrial importance are cellulase (El Baz *et al.*, 2018; Zahra *et al.*, 2020), α -amylase (Mahmood and Rahman, 2010), lipase (Kashmiri *et al.*, 2006) and chitinase (Felse and Panda, 2000). All four (4) isolates screened for cellulase production using the plate assay method demonstrate cellulase-producing ability. There were observed differences in cellulase produced on the solid medium between the *T. viride* isolates, however, the difference was not significant. This observed variation in cellulose hydrolysis between the isolates might be due to the difference in metabolic capabilities of the different strains. This finding is similar to the findings of Khokhar *et al.* (2012) where several fungal species were isolated and differences were observed in terms of their cellulase production potential, with *T. viride*, *T. harzianum* and *T. koningii* isolates having the highest zone of hydrolysis of carboxymethyl cellulose of 90 ± 0.0 mm.

Cellulase production using lignocellulosic biomass such as corncob is considered cost-effective compared to synthetic media. Thus, the *T. viride* isolates were tested to produce cellulase using corncob as a substrate under SmF. The highest enzymatic activity was recorded in *T. viride* DW4 (3.45 ± 0.46 U/ml) and the lowest was recorded in *T. viride* DW1 (1.77 ± 0.52 U/ml). These observed differences between the isolates might be attributed to differences in metabolic capabilities of the different isolates of *T. viride*. Previous studies by Kiro (2010) recorded maximum enzyme activity of 2.222 U/ml using *T. viride* with wheat straw as substrate under solid-state fermentation (SSF). Furthermore, the finding of this study is corroborated by studies in other fungi such as *Aspergillus niger* by Mrudula and Murugammal (2011), who reported cellulase activity of 2.04 U/ml by *Aspergillus niger* using coir waste as substrate; however, the cellulase activity was recorded for a different fungus.

Conclusion

In the present study, *T. viride* were isolated, screened and compared for their ability to hydrolyse cellulose. Four (4) *T. viride* strains were readily isolated from decaying wood with a percentage occurrence of 57%. All four (4) isolates of *T. viride* obtained in this study were found to have the ability for cellulase production under submerged fermentation using corncob as the substrate, with *T. viride* DW4 having the highest cellulase activity of 3.45 ± 0.46 U/ml. The findings reported in this study provide information on the isolation of *T. viride* from decaying wood and the potential for cellulase production using corncob as substrate. Thus, paving the way for further research towards improving the cellulase production potential of *T. viride* DW4.

Conflict of interest

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

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