

RESEARCH ARTICLE

Optimization of Cellulase Production and Biodegradation of *Artemisia annua* L. wastes by *Aspergillus niger* and *Trichoderma viride*

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Abstract

Aspergillus niger and *Trichoderma viride* isolated from soils of *Artemisia annua* L. plantations were used for the production of cellulases using submerged state fermentation (SmF). *Artemisia annua* process waste was used as the substrate for the fermentation. The fermentation broth was further assayed for various parameters including effects of incubation period, different concentrations of Carboxymethyl cellulose (CMC), incubation temperature and pH on enzyme production. The results indicated that the test fungi had their peak enzyme activity of 126 and 913 IU/mL respectively on the 3rd d of incubation. At 3% conc. of CMC, *A. niger* and *T. viride* had their highest enzyme production of 181.5 and 222.6 IU/mL respectively. The lowest enzyme production of 127.3 and 138.9 IU/mL was recorded for the two fungi at 0.5% conc. of CMC. *Aspergillus niger* had its peak of 166.2 IU/mL at incubation temperature of 30°C and 189 IU/mL for *T. viride* at 50°C. The optimum pH for enzyme production for *A. niger* and *T. viride* was pH 3 producing 227 and 239 IU/mL enzymes respectively.

Keywords: *Aspergillus niger*, *Trichoderma viride*, *Artemisia annua*, cellulose, enzyme activity.

Introduction

Agricultural wastes contain a high proportion of cellulosic matter comprising of hemicelluloses and lignocelluloses which are easily decomposed by a combination of physical, chemical and biological processes (Kadarmoidheen *et al.*, 2012). Utilization of renewable, economical, abundantly available agro-waste for the production of useful products is an increasing trend especially in a time of rising energy prices. Cellulose is the most abundantly available biomass on earth. It is the main product of photosynthesis in terrestrial environments and most abundant renewable bioresource produced in biosphere (Bhat, 2000). Siddiqui *et al.* (2000) reported that cellulose is commonly degraded by enzyme cellulases which comprise of endoglucanases. Although a few bacteria and Actinomycetes have also been reported to have cellulase activity, fungi are the main cellulase producing microorganisms. Several studies have been carried out to produce cellulolytic enzymes in organic waste degradation process by several microorganisms including fungi such as *Trichoderma* sp., *Penicillium* sp. and *Aspergillus* sp. respectively. The crude enzymes produced by these microorganisms are commercially available for agricultural use (Peij *et al.*, 1985; Lee and koo, 2001; Wainwright, 2010; Coughlan, 2011). The use of fungi in biodegradation processes has been emphasized because of their capability to produce high amounts of cellulases and hemicellulases which are secreted into the medium for easy extraction and purification (Tuomela *et al.*, 2000).

Aspergillus attack cellulose producing significant amount of cell free cellulase capable of hydrolyzing cellulose into fermentable soluble sugars such as glucose; an important raw material in chemical industries (Wainwright, 2010). During composting, the capacity of thermophilic microorganisms to assimilate organic matter depends on their ability to produce the enzymes needed for degradation of the substrate (Tuomela *et al.*, 2000). Cellulases have found application in the food, textile, laundry, baking, brewing, pulp and paper industries from biomass and genetic engineering (Bhat, 2000; Kirk *et al.*, 2002). The lignin component of cellulosic substrates as well as the crystalline nature of cellulose makes it inaccessible to cellulose and there is a need for a synergistic action of cellulases for the complete hydrolysis of cellulose. The present study was therefore designed to evaluate the ability of cellulolytic strains of *Aspergillus niger* and *Trichoderma viride* to produce cellulases for upgrading of *Artemisia annua* wastes using submerged fermentation (SmF).

Materials and methods

Isolation and identification of fungi from soil samples: *Aspergillus niger* and *Trichoderma viride* were isolated by primary selection from the top 10 cm soil samples collected from different locations of *Artemisia annua* plantation. The isolation was done using serial dilution and pour plate method of Rahna *et al.* (2012). Serial dilutions of the soil samples were made. One mL of 10⁻⁴ dilution was plated on sterile Petri plates containing Potato Dextrose Agar (PDA) media.

Identification of each of the fungal isolates was done using identification manual according to Samson *et al.* (1984), Nagamani *et al.* (2006) and Domsch *et al.* (2007).

Inoculum preparation: The spore suspension of the fungi used as inoculum in the present study was prepared from a 7 d old slant by adding 10 mL of sterile water to it. The spores were scratched with the help of a sterilized wire loop to make a homogeneous suspension. Spore count was measured with the help of Haemocytometer. The inoculum size of 1×10^{-4} cfu was used for the experiments.

Substrate preparation: *Artemisia annua* process waste used as the substrate for cellulase production was collected from *A. annua* plantation in Gangnum, Langtang South Local Government Area of Plateau State. The waste was oven dried at 60°C until the moisture content was removed. The dried waste was pulverized and stored in sample bottles for future use.

Biodegradation of *A. annua* wastes and production of crude enzyme source using SmF: Submerged Fermentation (SmF) method of Lennox *et al.* (2010) was used for the experiment. Two 250 mL conical flasks were used for each of the test fungus. About 100 mL of already prepared basal medium containing (g/L) KH_4PO_4 , 1.4; NH_4NO_3 , 1.0; KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; H_2O , 1000 mL was introduced into the flasks. A weight of 5 g of *A. annua* process waste was added. The flasks were plugged with cotton wool and autoclaved. After cooling, 5 mL of spore suspension of each of the test fungus was added into one of the conical flasks and incubated at 25°C for 5 d. One of the flasks was not inoculated with the spore suspension and thus served as control.

Effect of incubation period on cellulase production: The method of Jeffries (1996) was used for the experiment. A volume of 2 mL of culture filtrate from the incubated flasks was harvested at 24 h interval for 5 d by centrifugation at 6000 rpm for 15 min. The cell free culture supernatant was used as crude enzyme source. A volume of 0.5 mL of enzyme source was taken in a test tube, 0.5 mL of 0.05 M citrate buffer (pH 4.8) was added and 0.5 mL of 1% Carboxymethyl cellulose (CMC) was added and incubated at 30°C for 30 min. The reaction was stopped by the addition of 1 mL of 3,5-Dinitrosalicylic acid (DNSA) reagent. The mixture was boiled for another 10 min and cooled in water for color stabilization. Boiled enzyme extract from the control flask served as blank and D-glucose served as standard. Readings were taken using spectrophotometer at 540 nm, for the 5 d of incubation. Enzyme activity expressed in international units (IU) is defined as micromoles (μmol) of glucose released per min per mL of culture filtrate.

After 5 d incubation period, the fermented broth was centrifuged at 10000 rpm for 10 min and the supernatant was assayed for enzyme activity.

Effect of different concentrations of CMC on enzyme production: The modified method of Jaafaru and Fagade (2010) was used for the assay. The reaction mixture contained 0.5 mL of enzyme source was taken in a test tube, 0.5 mL of 0.05 M sodium citrate buffer (pH 4.8) was added and 0.5 mL of different concentrations (0.5, 1, 2 and 3%) of Carboxymethyl cellulose (CMC) was added and incubated at 30°C for 30 min. The reaction was stopped by the addition of 1 mL of DNSA solution. The mixture was boiled for 10 min and cooled in water for color stabilization and was read at 540 nm. Enzyme activity expressed in international units (IU) is defined as micromoles (μmol) of glucose released per min per mL of culture filtrate.

Effect of different incubation temperatures on cellulase production: For optimum temperature for cellulase production, the reaction mixture was incubated at different temperatures of 25-50°C for 30 min using the method of Sami *et al.* (2008). One mL of crude enzyme for each isolates was collected separately and 1 mL of 0.05 M sodium citrate buffer (pH 4.8) was added to the crude enzyme. One mL of 1% CMC was also added and incubated. DNSA reagent was added to stop the reaction. The reaction mixture was heated for 10 min. After cooling, the mixture was read at 540 nm using spectrophotometer as described by Jeffries (1996).

Effect of pH on the production of cellulase: The optimum pH for enzyme production was determined using the method of Sami *et al.* (2008) by incubating 1 mL crude enzyme, 1 mL 1% CMC in 1 mL 0.05 M sodium citrate buffer at different pHs (3-9) for 30 min at 50°C. Reducing sugars thus released were estimated by the Dinitrosalicylic acid reagent method at 540 nm using spectrophotometer as described by Jeffries (1996).

Results and discussion

The two fungal strains used in the study were isolated from soils of *Artemisia annua* plantation using standard microbiology techniques. These two fungal strains were selected considering their maximum cellulolytic activity on plate assay using basal salt medium supplemented with 2% (w/v) Carboxymethyl cellulose. Using their morphological characteristics, the fungi were identified as *Aspergillus niger* and *Trichoderma viride* (Fig. 1). These two fungal species have been reported as the most predominant and most frequently isolated from natural habitats especially the one containing cellulosic waste materials (Ogaraku, 2010; Guatam *et al.*, 2012). Many fungi capable of degrading cellulose synthesize large quantities of extracellular cellulases that are more efficient in depolymerising the cellulose substrate.

Fig. 1a. Colony of *A. niger* on PDA; b. Structure showing the conidiophore and the conidial head (X100).

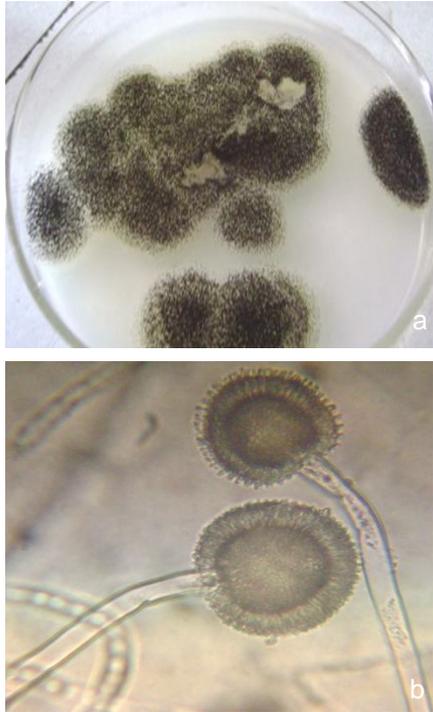
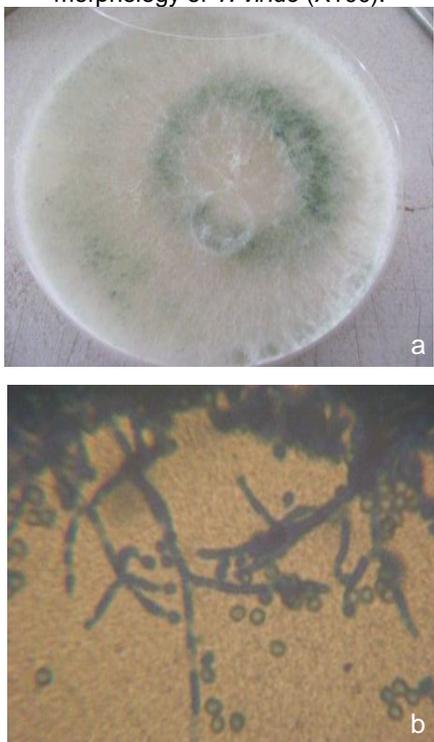


Fig. 2a. Colony of *T. viride* on PDA; b. Spore morphology of *T. viride* (X100).



Most commonly studied cellulolytic fungal species include *Trichoderma*, *Humicola*, *Penicillium* and *Aspergillus* species (Guatam *et al.*, 2009). Other researchers who isolated different array of cellulolytic fungi included Duncan *et al.* (2006) who screened 72 fungal species for cellulase activity using the Carboxymethyl cellulose (CMC) plate assay technique.

Effect of incubation period on enzyme production (IU/mL) during biodegradation: The liquid state fermentation carried out on the test fungi indicated maximum enzyme activity. Their peak enzyme activity was recorded after the 3rd d of incubation after which enzyme production declined. The highest peak of 913 IU/mL was observed in *T. viride* while that of *A. niger* was 126 IU/mL (Fig. 3). The growth assay carried out on the two fungal species showed that they were able to use the *A. annua* waste residues as sole carbon source indicating that they synthesized the cellulolytic enzymes required for the hydrolysis of the cellulosic wastes materials with eventual release of reducing sugars. The results as shown in Fig. 3 indicated that the two species liberated higher quantities of sugars from the 1st d (24 h) of incubation steadily until the 3rd d of the incubation time (72 h) with *T. viride* reaching a maximum of 913 IU/mL and *A. niger*, reaching a maximum of 126 IU/mL. The sugar production then declined steadily until the culture completion time which was the 5th d (120 h). The results of this research work are similar to that of Al-Taweil *et al.* (2009) and Guatam *et al.* (2012) who reported cellulase production in *Aspergillus* and *Trichoderma* strain. The high accumulation of sugar observed in the medium containing *T. viride* could be attributed to the high enzymatic hydrolysis rate as a result of the cellulolytic activity of the fungi. It could also be that the *T. viride* strain has a low consumption rate of the sugar accumulated in the medium as a result of the cellulose hydrolysis, thereby promoting higher levels of sugars without high consumption tendency.

Fig. 3. Effect of incubation period on cellulase production.

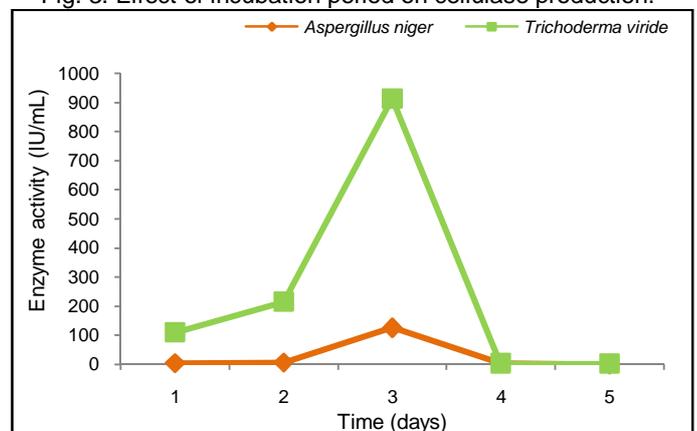


Fig. 4. Effect of different concentrations of CMC on cellulase production.

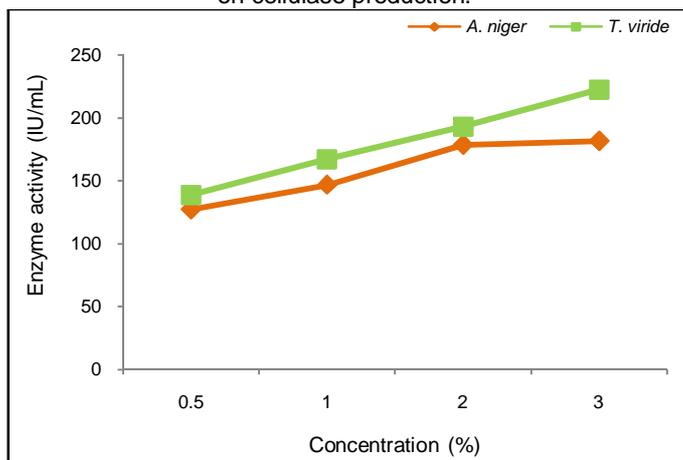
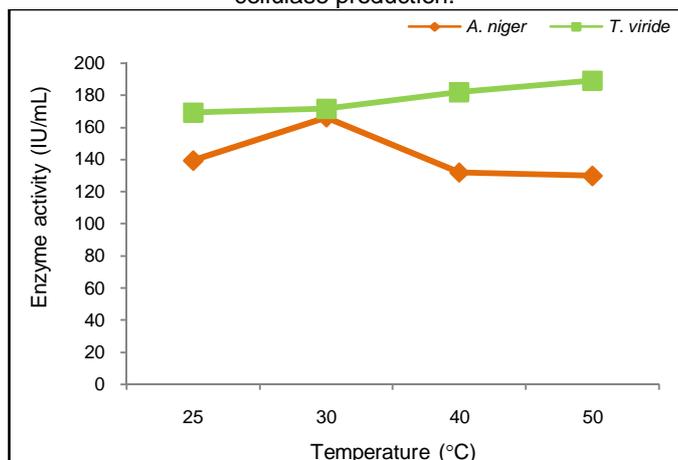


Fig. 5. Effect of incubation temperature on cellulase production.

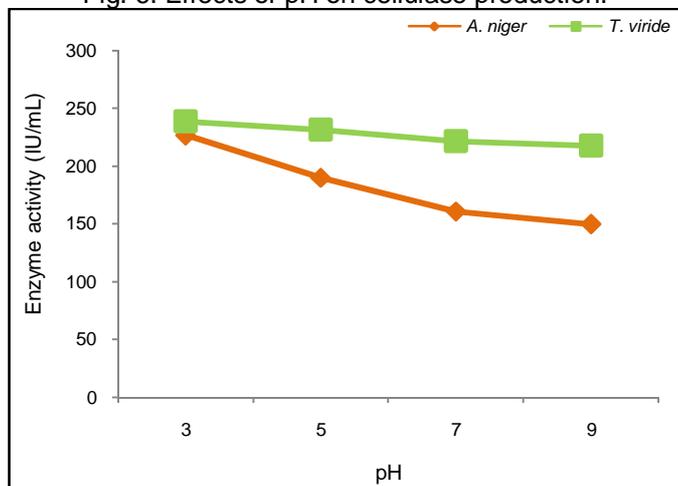


Effect of different concentration of CMC on cellulase production: The effect of different concentration of Carboxymethyl cellulose ranging from 0.5%, 1%, 2% and 3% on enzyme production of *A. niger* and *T. viride* was evaluated. *Aspergillus niger* indicated that there was a progression as concentration increased (Fig. 4). Cellulase synthesis was influenced by the concentration of CMC in the production medium. The highest concentration of 3% yielded more enzymes of 181.5 IU/mL and 222.6 IU/mL for *A. niger* and *T. viride* respectively. At the concentration of 0.5%, enzyme production was lowest with 127.3 IU/mL and 138.9 IU/mL respectively for *A. niger* and *T. viride*. Similar results were obtained by Pushelkar *et al.* (1995) who observed maximum enzyme production by *A. terreus* when grown at cellulose concentration of 1%. In fungi, the production of cellulolytic enzymes is subject to transcriptional regulation by available carbon sources. Carboxymethyl cellulose has proved to be a strong inducer of cellulase and it has been reported that endoglucanase was induced by CMC (Padmavathi, 2012). The findings of this study is not in conformity with that of Jaafaru and Fagade (2010) who reported highest enzyme activity at CMC concentration of 1% in *Aspergillus niger*, below or above which enzyme activity declined. These variations could be attributable to differences in the fungal strains as well as the media composition used in the studies.

Optimum temperature of incubation of 30°C in *A. niger* has been reported by other researchers (Garg and Neelakantan, 1981; Singh *et al.*, 1988; Jaafaru and Fagade, 2010). Sivakumaran (2014) reported optimum temperature of incubation of 50°C for *Trichoderma* species thereby conforming to the findings of this work. It is pertinent to note that the enzymes produced during this assay are thermotolerant in nature and further increase in temperature above 50°C will bring about denaturation of the enzymes.

Effect of different incubation temperature on cellulase production: Temperature of incubation ranging from 25, 30, 40 and 50°C had effects on cellulase production in *A. niger* and *T. viride*. *Aspergillus niger* had its peak of enzyme production of 166 IU/mL at 30°C, enzyme production declined after that. The highest enzyme production of 189 IU/mL was recorded for *T. viride* at 50°C (Fig. 5). Temperatures below 25°C and above 50°C could result to decline in enzyme production in *T. viride*. Also, for *A. niger*, temperatures below 25°C and above 30°C may cause reduction in enzyme production.

Fig. 6. Effects of pH on cellulase production.



Effects of pH on cellulase production: The test fungi were subjected to different pHs ranging from 3, 5, 7, and 9 in order to determine the optimum pH for enzyme production. *Aspergillus niger* and *T. viride* had their peak enzyme production at pH 3 with 227 IU/mL and 239 IU/mL respectively and least enzyme production at pH 9 producing 150 IU/mL and 218 IU/mL respectively (Fig. 6). This is an indication that the maximum enzyme production for both species is at pH 3, although significant levels of cellulase production was also recorded at other pH up to that of pH 9.

This agrees with the observation of Steiner *et al.* (1994) who reported that cellulase synthesis take place below pH 3 and above pH 9 inactivates the enzyme and may affect their production. Padmavathi *et al.* (2012) obtained similar results for *Aspergillus terreus* and *Mucor plumbeus* using *Lantana camara* leaves as sole carbon source.

Conclusion

Production of cellulases from *Artemisia annua* agro-wastes under submerged fermentation method was studied using *Aspergillus niger* and *Trichoderma viride* having observed that the two fungal species have the ability to convert cellulose into reducing sugars. *Artemisia annua* agro-waste is a cheap residue which can be used as a substrate for enzyme production thereby reducing the cost of enzyme production and enzymatic conversion of waste residues into fermentable sugar. Further work is being carried out to find the optimum fermentation conditions of purified forms of the cellulases produced by *Aspergillus niger* and *T. viride* for biotechnological applications in various industries.

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