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Effect of variations in growth parameters on cellulase activity of *Trichoderma viride* NSPR006 cultured on different wood-dusts

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ABSTRACT

Aims: The biotechnology research into agro wastes has been driven by the need to screen organisms for hyper-production of novel extracellular enzymes in which cellulase plays a significant role. Therefore, the aim of the study was to pre-screen selected fungal strains and optimize cultural conditions for cellulase production by *Trichoderma viride* NSPR006 cultured on pretreated sawdust as lignocellulosic substrate.
Methodology and results: The selected fungal isolates namely *Trichoderma viride* NSPR006, *Botrydiplodia* NSPR007 and *Acremonium butyri* NSPR06B obtained from the culture collection of the Nigerian Stored Products Research Institute Ilorin, Kwara State, Nigeria were screened for the production of cellulase in mineral salt medium in which carboxymethylcellulose (CMC) had been incorporated as the sole carbon source. All the tested fungal isolates produced cellulase with differences in the amount of enzyme production. Of all the selected fungal isolates screened, *Trichoderma viride* NSPR006 was found to yield highest cellulase activity compared to the other isolates. Among tested carbon sources, *Pachyslasma tessmani* wood dust at 3% level proved to the best for cellulase production. Of the entire tested organic nitrogen sources, locust beans were observed to yield maximum cellulase activity (0.194 µmol/min/mL). The optimum temperature, incubation time and pH for maximum cellulase production were 28 °C, 72 h and 6.5, respectively.
Conclusion, significance and impact of study: Outcome of this study shows the effectiveness of pre-treatment of wood dust as low cost system for hyper-production of cellulase for industrial application. Also, the work revealed the use of pretreated wood dust as substitute to commercial substrate known to be expensive in cellulase production.

Keywords: Trichoderma viride NSPR006, cellulase, lignocellulosic substrate, sawdust

INTRODUCTION

Lignocellulose biomass is the most abundant organic raw material in the world (Iqbal *et al*., 2010). It constitutes a major portion of agricultural and forest wastes which accumulate in the environment causing pollution problem (Gilna and Khaleel, 2011). The bioconversion of cellulosic materials is now a subject of intensive research as a contribution to the development of large scale conversion process beneficial to mankind (Kumakura, 1997).

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology (Gilna and Khaleel, 2011). Cellulases are widely used in the food, feed, textile and pulp industries (Ojumu *et al*., 2003; Iqbal *et al*., 2010). Cellulase hydrolysis is accomplished with the aid of cellulase enzyme complex which is made up of three classes of enzymes namely exoglucanases, endoglucanases and β-glucosidase (Gautam *et al*., 2010; Iqbal *et al*., 2010). This study deals with the prescreening of *Trichoderma viride* NSPR006, *Botrydiplodia* NSPR007 and *Acremonium butyri* NSPR06B and optimization of cultural conditions for cellulase production by *T.viride* NSPR006 cultured on pretreated sawdust as lignocellulosic substrate.

MATERIALS AND METHODS

T. viride NSPR006, *Botrydiplodia* NSPR007 and *A. butyri* NSPR06B were obtained from the culture collection of the Nigerian Stored Products Research Institute Ilorin, Kwara State, Nigeria. The fungal isolates were maintained on Potato Dextrose Agar (PDA) plates and sub-cultured at regular intervals. They were incubated at 30 ± 2 °C until the entire plates were covered by active mycelium and stored at 4 °C in refrigerator on agar slants.

Alstoma boonei (Ahun), *Ceiba pentandra* (Araba) and *Pachyslasma tessmani* (Eru) wood dusts were procured from three different sawmill in Akure, Ondo State, Nigeria which serve as substrates. The substrates were washed, sun and oven-dried at 70 °C with Model DHG Heating Drying Oven for a period of 2 h, sieved to 40 mm mesh

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size and stored in air tight transparent plastic containers to keep it moisture free (Iqbal *et al.*, 2010).

Pretreatment of lignocellulosic substrates

Lignocellulosic substrates (10 g) were treated separately with 1000 mL of 4% solution of sodium hydroxide (NaOH) for 24 h in Petri dishes at room temperature prior to autoclaving. The substrates were washed with distilled water until it is neutral to litmus paper and dried at 70 °C (Model DHG Heating Drying Oven) to constant weight. The effect of NaOH was further neutralized with diluted hydrochloric acid (HCl) and they were autoclave at 121 °C for 15 min (Muthuvelayudham and Viruthagiri, 2006).

Media preparation and enzyme production

Medium composition described by Mandles and Weber as reported by (Iqbal *et al*., 2010) was used for submerged fermentation. The media contained (per liter of distilled water): Urea 0.3 g, $(NH4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgSO_4 \cdot H_2O$ 0.3 g, peptone 1.0 g, $FeSO_4 \cdot H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg and carboxymethylcellulose (CMC) 10.0 g. pH of the media were adjusted to 6.5 with pH meter (Denver Instrument, Model 20 pH/ Conductivity meter) prior sterilization. Then, 100 mL of the liquid medium was placed in 250 mL Erlenmeyer flask and sterilized by autoclaving 121 °C for 15 min. This was cooled and inoculated with 10 discs of 8 mm diameter of the organism from PDA culture plates using sterile cup borer. The flasks were incubated at 30 ± 2 °C for 5 days on a rotary shaker (Gallenkamp) at 120 rpm. Sterile basal medium supplemented with carboxymethylcellulose without inoculating with fungal strain served as the control. Crude enzyme preparation was obtained by centrifugation at 5000 rpm for 10 min at 4 °C using refrigerated ultracentrifuge (Centurion Scientific Limited). The supernatant was used as the crude extracellular enzyme source.

Cellulase assay

Enzyme activity of supernatant collected at the end of each optimization step was determined using Spectrophotometer (Lab-Tech Digital Colorimeter) by the method of Acharya *et al.* (2008). The reaction mixture contained 0.5 mL of 0.5% of CMC as substrate prepared in 0.5 M sodium acetate buffer with pH 5.5. The control tube contained the same amount of substrate and 0.5 mL of the enzyme solution heated at 100 °C for 15 min. Both the experimental and control tubes were incubated at 50 °C for 30 min. At the end of the incubation period, tubes were removed from the water bath (Lamfield Medical England Model DK-600), and the reaction was terminated by addition of 2 mL of 3, 5- dinitrosalicylic acid (DNSA) reagent per tube (Shazia *et al*., 2010). The tubes were incubated for 5 min in a boiling water bath for colour development and were cooled rapidly. The activity of reaction mixture was measured against a reagent blank at 540 nm. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentration of glucose. Unit enzyme activity was defined as the amount of enzyme required for liberating 1 µM of glucose per milliliter per minute and was expressed as µM/mL/min.

Optimization of culture conditions for cellulase production

Effect of different wood dusts (carbon sources) for cellulase production.

Effects of various carbon compounds namely: *A. boonei*, *C. pentandra* and *P. tessmani* wood dusts were used in this study with CMC serving as control. The broth was distributed into 250 mL flasks containing 50 mL optimized medium and 0.5% of each carbon sources was added before inoculation of the strain and after culture inoculation, the flasks were incubated at 30 ± 2 °C for 3 days at 120 rpm in shaken condition (Gautam *et al*., 2010).

Effect of pH and temperature on cellulase production

The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium at different levels in the range of pH 4.5 to 7.5 using different buffers. In oder to determine the effective temperature for cellulase production by the *T. viride* NSPR006, fermentation was carried out at 28 °C, 32 °C and 37 °C.

Effect of incubation period on cellulose production

Fermentation period was an important parameter for enzyme production by *T. viride* NSPR006. In this study, fermentation experiment was carried out up to 120 h and production rate was measured at 24 h intervals. Cellulase assay was carried out according to standard assay procedures (Gautam *et al*., 2010).

Effect of organic nitrogen sources and different concentration of Pachyslasma tessmani *wood dust on cellulase production*

Evaluation of selected organic nitrogen sources was carried out to determine the appropriate organic nitrogen source for cellulase production by the *T. viride* NSPR006. The fermentation medium was supplemented with organic compounds (soybeans, cotton seeds and locust beans) at 0.2% level, replacing the prescribed inorganic nitrogen source (ammonium sulphate) of the fermentation medium (Gautam *et al*., 2010). Different concentrations of the substrate (1%, 2%, 3% and 5%) were added to the basal salt medium in separate conical flasks for cellulase production replacing the prescribed carbon source of the fermentation medium. The flasks were kept on a rotary shaker (Gallenkamp) at 120 rpm at 30 ± 2 °C for 3 days of cultivation (Iqbal *et al*., 2010).

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Statistical Analysis

Data presented on the average of three replicates (± SE) are obtained from their independent experiments.

RESULTS AND DISCUSSIONS

The selected fungal isolates namely: *T. viride* NSPR006, *Botrydiplodia* NSPR007 and *A. butyri* NSPR06B were cultured in minimal salt medium supplemented with carboxymethylcellullose (CMC) (10 g/L) and incubated at 30 ± 2 °C for 5 days (Figure 1). The three strains of fungi used in this study showed the ability to produce cellulase on CMC of commercial potential with significant different in the rate of enzyme production. In all the tested strains, *T. viride* NSPR006 has the highest cellulase activity of 0.243 µmol/min/mL followed by *Botrydiplodia* NSPR007 (0.072 µmol/min/mL) while the lowest was recorded for *A. butyri* NSPR06B (0.046 µmol/min/mL). The differences in the amount of enzyme produced by each of the isolate suggest that production rate depends on the genetic composition of the microorganisms (Gautam *et al*., 2010). *T. viride* NSPR006 was therefore selected for further studies because of its high cellulase activity.

Effect of different wood dusts (carbon sources) on cellulase production

Different wood dusts (*A. boonei*, *C. pentandra* and *P. tessmani* wood dust) were supplemented with Mandel and Weber medium for cellulase production (Figure 2). Of all the wood dusts tested, *P. tessmani* wood dust was found to be the best substrate for cellulase production, which gave maximum yield of 0.531 µmol/min/mL cellulase activity. *P. tessmani* wood dust was observed to be almost 2.119 fold increase compared to carboxymethylcellulose (control). However, all the tested substrates proved to be suitable carbon sources for cellulase production. There was variation in the amount of cellulase produced when wood dusts were substituted in culture medium. The large variation in cellulase yield may be due to the nature of cellulose or hemicellulose, presence of some components (activators or inhibitors) in these materials and variations in the substrate accessibility (Mabrouk and Ahwany, 2008). Among these, *P. tessmani* wood dust was found to be the most suitable wood dust for cellulase production and it was therefore selected for optimization studies as a carbon source replacing CMC.

Effect of incubation period on cellulase production

Time course profile (Figure 3) for the cellulase production was studied from 24 to 120 h. The production of enzyme (% relative activity) increased with increase in fermentation period and reached maximum (0.233 µmol/min/mL) at 72 h of incubation time. Subsequent increase beyond the optimum time (72 h) resulted into a decline in percentage relative activity. A decrease in activity is always found to be associated with depletion of nutrients or accumulation of some autotoxic products of organism in the media (Ikram *et al*., 2005; Iqbal *et al*., 2010). In addition, the medium components were initially more susceptible to fungal digestion and make a rapid rise in enzyme biosynthesis. But with prolongation of cultural time, the susceptible portions were completely hydrolyzed by microorganism which inhibited the enzyme secretion pathways (Gautam *et al*., 2010). Other related factors, such as the nature of the microorganism and the physiological conditions of the media, are also considered to be important during production (Jian and Jichu, 2006; Malik *et al*., 2010; Shazia *et al*., 2010).

Effect of incubation temperature on cellulase production

To reveal the effect of different incubation temperatures on cellulase production by *T. viride* NSPR006 on pretreated *P. tessmani* wood dust under submerged state fermentation, experiments were conducted at 28 °C, 32 °C and 37 °C and the cellulase activities were found to be 0.069, 0.048, 0.066 µmol/min/mL, respectively. Thus, maximum cellulase production was observed at 28 °C (Table 1). The influence of temperature on enzyme production is related to the growth of the organisms (Ahmed *et al*., 2009). Hence, the temperature depends on whether the culture is mesophilic or thermophilic. Among the fungi, most cellulase production studies have been done with mesophilic fungi within the temperature range of 25-37 °C (Lu *et al*., 2003; Gautam *et al*., 2010).

Narasimha (2006) used saw dust as carbon source for the production of cellulase by *A. niger* with optimum cellulase activity obtained at 28 °C. Similar result was reported in the research findings of Acharya *et al.* (2008) when sawdust was optimized for cellulase production by *A. niger*. Different optimal temperatures were reported by many workers, Nguyen and Quyen (2010) reported 30 °C, Jamal and Alam (2010) reported 30 °C and Gautam *et al*, (2010) reported 45 °C as optimal temperatures. Different temperature optimal reported by these workers suggested that the cellulase production depends on the strain variation of the microorganisms (Gautam *et al*., 2010).

Effect of pH on cellulase production

The effect of pH (4.5-7.5) of the culture on the biosynthesis of cellulase by *T. viride* NSPR006 was studied (Figure 4). There was slight increase in specific activity with increase in pH until maximum (0.239 µmol/min/mg) specific activity was reached at pH 6.5. Further increase in pH resulted in a sharp reduction of cellulase biosynthesis by the organism. Hence, pH of 6.5 was optimized for the maximum production of cellulase. Enzyme production varies with changes in physical parameters such as temperature and pH of the production medium. Any change in these parameters induces

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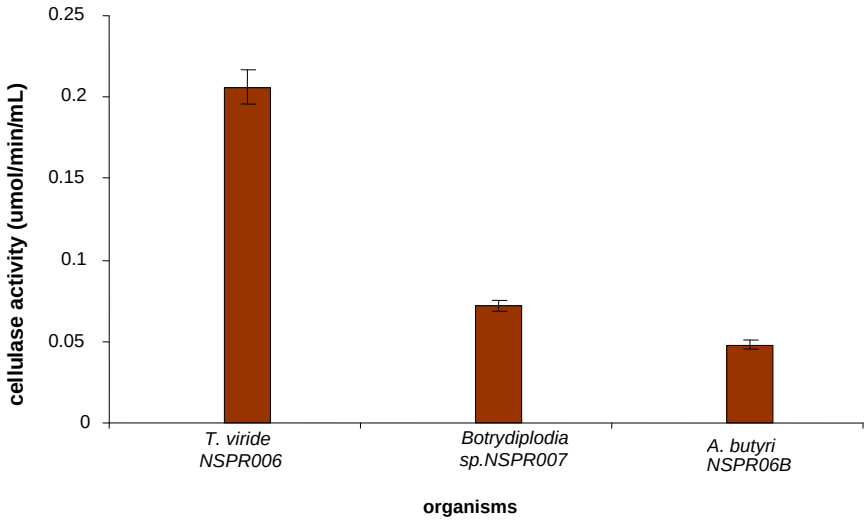

Figure 1: Microbial screening of selected fungal isolates for cellulase production on carboxymethylcellullose (CMC). They were grown on mineral salt medium with tested substrate (10 g/L) and incubated at 30 ± 2 °C for 5 days.

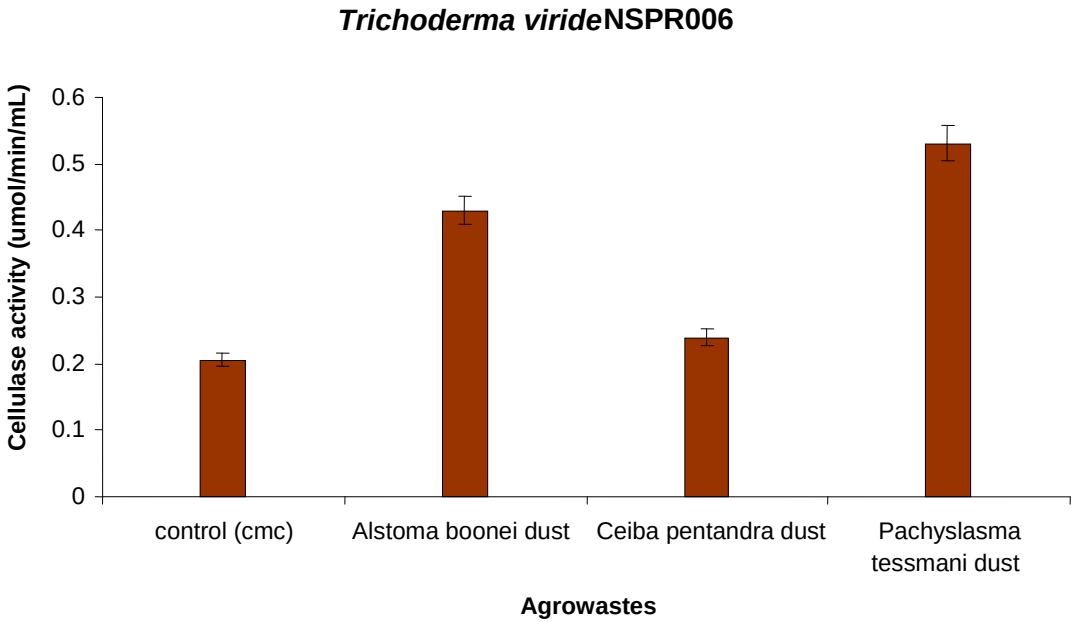

Figure 2: Effect of different wood dust as carbon source on the production of cellulase by *Trichoderma viride* NSPR006.

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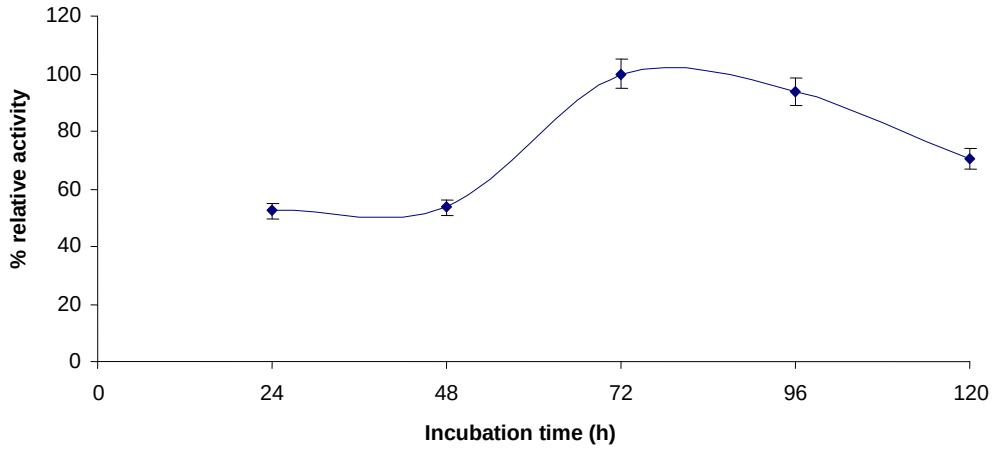

Figure 3: Time course of the cellulase production by *Trichoderma viride* NSPR006 using wood dust (*Pachyslasma tessmani*) as carbon source.

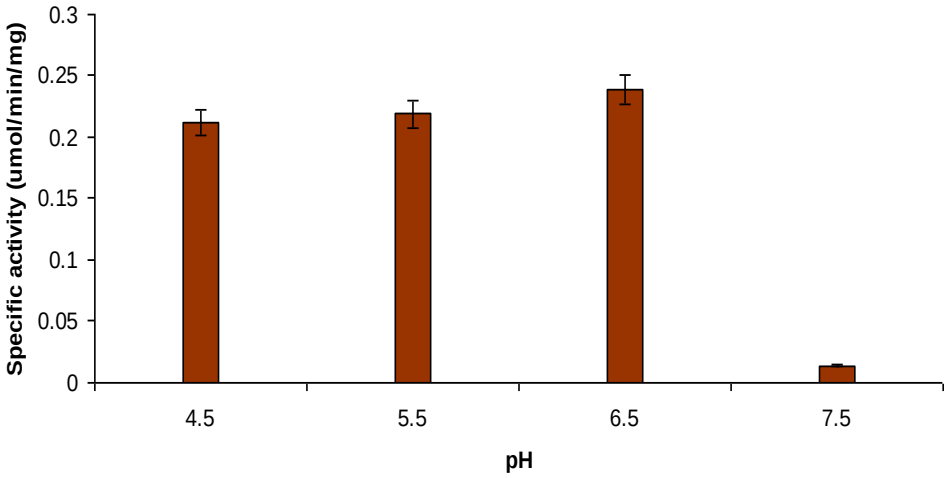

Figure 4: Effect of initial pH on cellulase production.

Table 1: Optimization of temperature.

Temperature (0 °C)	Cellulase activity (µmol/min/mL)	Protein content (mg/mL)	Specific activity (µmol/min/mg)	Percentage relative activity (%)
28 °C	0.439±0.005	6.238±0.05	0.07	75.43
32 °C	0.582±0.010	7.473±0.01	0.078	100
37 °C	0.474±0.03	5.955±0.02	0.080	81.44

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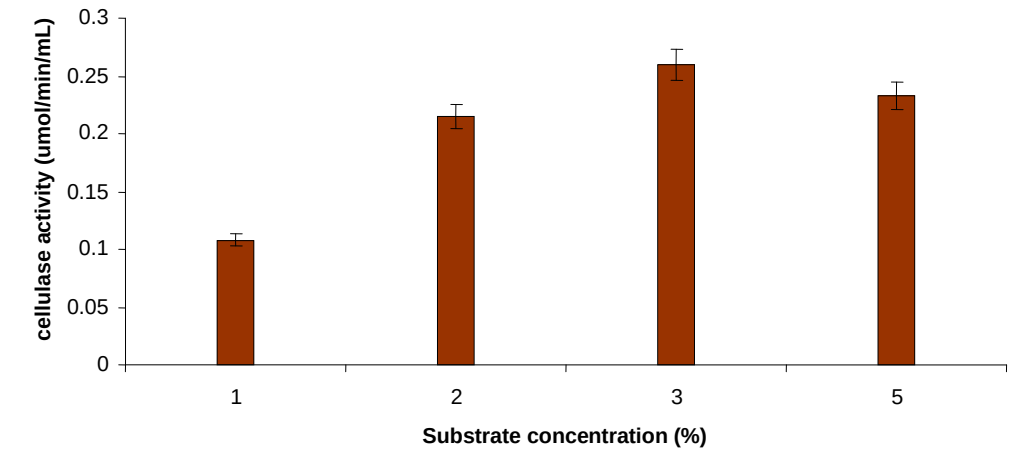

Figure 5: Effect of varying substrate concentrations on cellulase production by *Trichoderma viride* NSPR006.

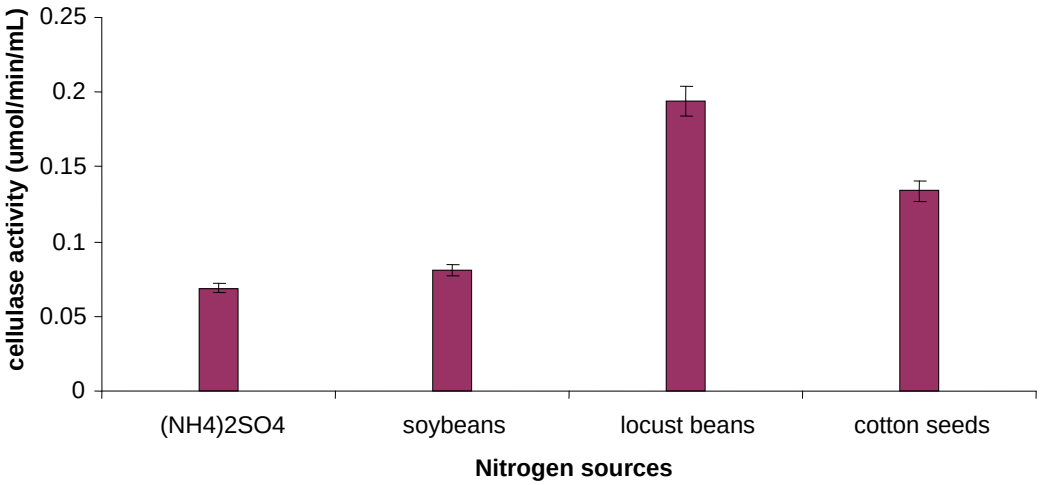

Figure 6: Effect of different nitrogen sources on the cellulase production by *Trichoderma viride* NSPR006 in shake flasks.

morphological changes in microbes and in enzyme secretion (Bodade *et al*., 2010). The effect of pH is related to the growth and metabolic activities of the organism. A change in pH affects the ionization of essential active site amino acid residues that are involved in substrate binding and catalysis. The ionization of these residues may cause distortion of the active site cleft and hence may indirectly affect enzyme activity (Bobade *et al*., 2010).

In the present study, maximum cellulase activity was recorded at pH 6.5. Shazia *et al*., (2010) reported maximum cellulase activity at pH 5.5 by *T. viride* among the tested pH range between 4.5 and 6.5, Malik *et al*., 2010 also reported a similar result of maximum CMCase and FPase activity at pH 5.5; Coral *et al*. (2002) reported maximum CMCase activity at pH 7.5 by *Aspergillus niger* (Z10, wild type strain) among the tested pH range between 4.0 and 9.0. Similarly, the optimal pH of 6.0 to 7.0 for maximum protease-resistant cellulase activity in *A. niger* was reported by Akiba *et al.* (1995).

Effect of different concentration of *P. tessmani* wood dust (carbon source) on cellulase production

As shown in Figure 5, different concentrations of *P. tessmani* wood dust was added separately to basal medium and evaluated individually. 1%, 2%, 3% and 5% substrate concentrations were used and maximum cellulase activity of 0.260 µmol/min/mL was obtained at 3% substrate concentration. It was observed that enzyme production increased with increase in substrate

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concentration up to 3% and subsequent increase resulted into gradual fall in production. A dynamic influencing feature that affects the yield and initial hydrolysis rate of cellulose is substrate concentration (Iqbal *et al*., 2010). Low substrate concentration results in an increase in yield and reaction rate of the hydrolysis while, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme formation (Liu and Yang, 2007; Singhania *et al*., 2007). In this present study, *P. tessmani* wood dust at 3% level proved to be the best for cellulase production by *T. viride* NSPR006. This result was in conformity with other reports. The optimum substrate concentration (sawdust) for cellulase production by a strain of *Aspergillus niger* was 3% (Acharya *et al*., 2008) and 3% optimum substrate concentration was also reported by Nguyen and Quyen (2010) for *Aspergillus awamori* (VTCC)-F099. Although, different optimal substrate concentrations had been reported by many researchers, this could be attributed to the chemical nature and nutrient availability of the used substrates (Omojasola *et al*., 2008; Gautam *et al*., 2010).

Effect of organic nitrogen sources on cellulase production

The different organic nitrogen sources (soybeans, locust beans and cotton seeds) were evaluated for cellulase production by *T. viride* NSPR006 in comparison with ammonium sulphate (inorganic) (Figure 6). The fermentation medium was supplemented with each of these nitrogen sources at a level of 0.2%. Among all the organic sources tested, locust bean was observed to give the optimum cellulase production (0.194 µmol/min/mL) while other organic nitrogen sources yielded cellulase with all liberating more than 0.080 µmol/min/mL. However, the lowest cellulase production was obtained in ammonium sulphate $(NH_4)_2SO_4$. The cellulase activity obtained from locust beans was almost 2.056 higher than the ammonium sulphate (control). In this study, locust bean was selected as the best organic nitrogen source. There are no intensive reports on the use locust bean as a novel substitute to ammonium sulphate for cellulase production. It was due to the fact that locust beans provided both the ammonium as well as sulfate ions for conidial cell growth and enzyme production (Mekala *et al*., 2008). Mangat and Mandahr (1998) showed that nitrogen sources greatly influence cellulases biosynthesis hence they should be used with proper concentration.

Cellulose is abundant in nature and awaited to be converted into more valuable products used for mankind. Several microorganisms are capable of converting cellulose into simple carbohydrates had been discovered for decades. However, newly fungal isolates that are hyper-producer with high stability are yet to be exploited.

CONCLUSION

The *T. viride* NSPR006 showed potential to convert cellulose into reducing sugars which could be readily used in many applications such as animal foods and feed stock for production of valuable organic compounds. The cellulase production from *P. tessmani* wood dust was highest at 3% substrate concentration, 72 h of incubation time with an initial pH 6.5 and 28 °C.

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