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RESEARCH ARTICLE

INVESTIGATION OF THE CELLULASES PRODUCTION BY *ASPERGILLUS NIGER* NSPR002 IN DIFFERENT CULTIVATION CONDITIONS

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Abstract

This study investigated influence of different process parameters on cellulases production by *Aspergillus niger* NSPR002 under submerged fermentation. The selected fungal isolates coded *Aspergillusniger*NSPR001, *A. niger* NSPR002 and *Fusarium vertilloides* NSPR003were screened for the production of cellulases by submerged cultivation in liquid mineral salt medium in which carboxymethylcellulose (CMC) had been added as the sole carbon source. Cultivation conditions investigated include variation of the carbon and nitrogen sources, pH, incubation temperature and time of incubation.All the tested fungal isolates proved to be producers of cellulases with differences in the rate of biosynthesis. Of all the fungal isolates screened, *Aspergillus niger* NSPR002 displayed the highest cellulases activity of 0.122µmol/min/mL. Several types of agricultural wastes (pawpaw peels, potato peels and cassava peels) were tested as substitutes of CMC in medium for cellulases production by *Aspergillus niger* NSPR002. Among tested agricultural by-products, pawpaw peels at a concentration of 5% was found to be the most effective carbon source. Out of the organic nitrogen sources tested, locust beans meal was observed to yield maximum cellulases activity (0.361µmol/min/mL).The optimum pH, temperature and incubation time were 4.5, 32^{o}C and 72 h, respectively.

Keywords: *Trichoderma viride* NSPR002, cellulases, lignocellulosic substrate, submerged fermentation, process parameters

Introduction

Agrowastes are the most abundant and renewable material produced on earth. Large quantities of agro-wastes are obtained from forests, agricultural practices, and industrial processes, particularly from agro-allied based industries such as breweries, paper and pulp, textile and timber industries (Ilyas*et al.,* 2012). These wastes generally accumulate in the environment as pollutants (Abu *et al.,* 2000). About 2.9 × 103 million tons of lignocellulosic residues are produced from cereal crops and 3 × 103 million tons from pulse and oil seed crops. In addition, 5.4 × 102 million tons is produced annually from crops worldwide (FAO, 2006) and these materials accumulate in enormous amounts (GOP, 2009). Enzymes production from lignocellulosic biomass through the biological route seems to be very attractive and sustainable due to several reasons, the major being the renewable and ubiquitous nature of biomass and its non-competitiveness with food crops (Singhania*et al.,* 2010). The

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polysaccharide component of agrowastes includes cellulose and hemicellulose. Cellulose is produced in large amounts in biosphere (100 billion dry tons/year). It is a linearly condensed polymer consisting of D-anhydroglucopyranose joined by β-1, 4- glycosidic bonds (Zhang and Lynd, 2004; Ilyas*et al.*, 2012). Cellulose, a β (1→4)-linked glucose polymer, is considered to be the primary product of photosynthesis and the most abundant renewable carbon resource in nature (Jarvis, 2003; Zhang and Lynd, 2004). Economic analyses have indicated that the production cost of cellulases is still the major cost factor in the hydrolysis of cellulosic materials to fermentable sugars. It is therefore imperative to improve the production of cellulases in order to make the process more economically viable (Xu*et al.*, 2011).

Cellulases are among the industrially important hydrolytic enzymes and they have a great significance in 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). Cellulose 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). Cellulases are synthesized by fungi belong to the *Chaetomium*, *Aspergillus*, *Penicillium*, *Funsarium*, *Myrothecium* and *Trichoderma* species (Zhang and Lynd, 2004; Akinyele*et al.*, 2013a) and bacteria belong to *Ruminococcus*, *Bacillus*, *Pseudomonas* species (Ohara*et al.*, 2000; Kotchoni*et al.*, 2003; Bakare*et al.*, 2005).

For commercial production of cellulases, filamentous fungi are mostly preferred because the enzymes produced by these fungi are more efficient as compared to those obtained from bacteria (Irfan*et al.*, 2011). Cellulases are the third largest industrial enzyme in the world, which is also gaining rejuvenated interests due to its applications (Singhania*et al.*, 2010). However, the high cost of production of these enzymes has hindered the industrial application of cellulose bioconversion. One of the different approaches to overcome this hindrance is to make continuous search for organisms with secretion of cellulose enzymes in large amounts and to optimize enzymes production with them

The aim of this study was to screening selected fungal isolates strains provides from culture collection of the Nigerian Stored Products Research Institute Ilorin, Kwara State, Nigeria as active producers of cellulases and evaluating the influence of some fermentative conditions upon yield of enzymes production.

Materials and methods

Microorganisms

Aspergillus niger NSPR001, *A. niger* NSPR002 and *Fusarium vertilloides* NSPR003 strains were obtained from culture collection of the Nigerian Stored Products Research Institute Ilorin, Kwara State, Nigeria. The pure cultures were maintained on Potato Dextrose Agar (PDA) medium and subcultured once in a month. They were incubated at 30 ± 2^{o} C until the entire plates were covered by active mycelium and then stored at 4^{o}C.

Agro-wastes treatment

Pawpaw peels, potato peels and cassava peels were procured from farm fields, domestic source and market in Akure, Ondo State, Nigeria which serve as substrates. The substrates were washed and oven-dried at 70°C with DHG Heating Drying Oven (Jianqyin Linqlinq Machinery Co., Limited, China)for a period of 2 h, and then milled and sieved to 40 mm mesh size and stored in air tight transparent plastic containers to keep it moisture free (Iqbal*et al.*, 2010).

Agricultural wastes used as substitutes of carboxyl ethyl Cellulose (CMC) (10g) were treated separately with 1000 mL of 4% NaOH solution 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^{o}C in DHG Heating Drying Oven (JianqyinLinqlinq Machinery Co., Limited, China)to constant weight. The alkaline effect was further neutralized with diluted HCl and then the mixture was autoclaved at 121^{o}C for 15 min (Muthuvelayudham and Viruthagiri, 2006).

Fermentative media preparation and submerged cultivation for cellulases production

Medium composition described by Mandles and Weber (1969) was used for submerged fermentation. The media contained (g/L): peptone 1.0, urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $CaCl_2$ 0.3, $MgSO_4.H_2O$ 0.3 , $FeSO_4.H_2O$ 5.0 mg, $MnSO_4.H_2O$ 1.6 mg, $ZnSO_4.H_2O$ 0.0014 g, $CoCl_2$ 0.002 g and CMC 10g. pH of the media were adjusted to 6.5 with a pH/conductivity meter Model 20 Denver Instrument (Systronics Limited, India) 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 biomass obtained by cultivation on PDA using sterile cup borer. The flasks were incubated at 30 ±2°C for 5 days on a rotary shaker (Gallenkamp, Model RS-12, Singhla Scientific Industries, India) at 120 rpm. Sterile basal medium supplemented with CMC without organism served as the control. Crude enzyme preparation was obtained by centrifugation at 5000 rpm for 10 min at 4°C using refrigerated ultracentrifuge (Model KBM-70, Centurion Scientific Limited, Germany). The supernatant was used as the crude extracellular enzymes source (Gautam *et al.*, 2010).

Screening of agro-wastes (carbon sources) for cellulases production

Effects of various carbon compounds namely: pawpaw peels, potato peels and cassava peels were screened 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 were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 3 days and after culture inoculation, the flasks were incubated at 30 ± 2 °C, for 3 days, at 120 rpm, insubmerged conditions of cultivation on rotary shaker (Gallenkamp, Model RS-12, Singhla Scientific Industries, India)(Gautam*et al.*, 2010).

Effect of type and concentrations of carbon and nitrogen sources on cellulases production

Soybeans, cotton seeds and locust beansat 0.2% concentration were replacing the prescribed inorganic nitrogen source (ammonium sulphate) of the fermentation medium. Different concentrations of the pawpaw peels ranged from 1.0 % (w/v) to 5.0 % (w/v) were added to the basal salt medium for cellulases production replacing the prescribed carbon source of the fermentation mediums (Hafiz *et al.*, 2010).

Effect of pH and temperature on cellulases production

The pH of the fermentative medium wasvaried from 4.5 to 7.5. The cultivation took place in submerged conditions at 30 ± 2°C, for 3 days, at 120 rpm. In order to determine the optimum incubation temperature for cellulases production, fermentation was carried out at 28°C, 32°C and 37°C respectively (Gautam *et al.*, 2010).

Effect of cultivation time on cellulases production

In optimal conditions before established the production of enzyme was studied during 120h of cultivation. The cellulases activity was measured at regular intervals of 24h, and the period of maximum enzyme production was determined (Milalaet *al.*, 2005).

Cellulase assay

Enzyme activity was determined using the method recommended by Acharyaet *al.* (2008). The reaction mixture contained 0.5 mL of 0.5% of CMC as substrate prepared in 0.5 M sodium acetate buffer pH 5.5 and 0.5 mL of enzyme extract. The control sample 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 samples 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, Labnics Equipment, United Kingdom), and the reaction was terminated by addition of 3 mL of 3, 5- dinitrosalicylic acid (DNSA) reagent per tube (Shaziaet *al.*, 2010). The tubes were incubated for

5 min in a boiling water bath for colour development and then were cooled rapidly. The activity of reaction mixture was measured against a blank sample at wavelength of540 nm. The concentration of glucose released by enzymes 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 1µM of glucose per millilitre per minute, in analysed conditions of reaction and was expressed as µM/mL/min.

Protein content determination Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard (Ghose, 1987).

Statistical analysis

Microsoft excel (Microsoft corporation, USA) was used to analyze data on the average of three replicates (±SE) obtained from three independent experiments.

Results and discussion

Screening of fungal strains based on the ability for cellulases production on CMC

All the tested fungal strains were able to produce extracellular cellulase in submerged fermentation although with differences in the rate of enzymes production. Among the screened isolates, the strain *A. niger* NSPR002 showed the highest level of cellulase activity of 0.12µmol/min/mL, after 5 days of cultivation in submerged conditions, while strains *Aspergillus niger* NSPR001 and *Fusarium vertilloides* NSPR003 displayed cellulase activities of 0.09 and 0.08µmol/min/mL respectively (Figure 1). Cellulases production had been reported using a variety of moulds by submerged fermentations (Hanif*et al*., 2004; Narasimha *et al.*, 2006; Gilna and Khaleel, 2011; Xu *et al.*, 2011; IIyas *et al*., 2012). The differences in the amount of enzyme produced by each of the isolate suggest that production rate depends on the genetic characteristics of the microorganisms and also on the conditions of fermentation (Mabrouk and El Ahwany, 2008; Gautam *et al*., 2010; Akinyele *et al*., 2013a). *Aspergillus niger* NSPR002 strain was therefore selected for further studies because of its competitive cellulase activity.

Screening of agro-wastes (carbon sources) for cellulase production

The effect of different carbon sources on cellulase production was studied by using basal medium supplemented with CMC as the control (Figure 2). In new fermentative media, the CMC was replaced by equal amount (5%) of different agricultural by-products, which included pawpaw peels, potato peels and cassava peels as natural substrates that might be useful for the production of enzyme in a commercial scale. Pawpaw peels was the most preferable carbon source yielding a maximum extracellular cellulase activity of 0.64 µmol/min/mL. This activity was 5.03 fold higher than the value obtained during cultivation on medium containing CMC. On the other hand, the lowest cellulase activity was obtained in culture containing potato peels where it showed an activity lower than that of pawpaw peels by 61%. Differences in biosynthesis potential displayed by this organism on different substrates can be attributed to the difference in substrates chemical composition which is an important parameter of the fermentation. Generally, the production of cellulases and hemi-cellulases have been shown to be inducible and affected by the nature of substrate (Kang *et al.*, 2004). Different kinds of agro industrial wastes have been utilized as carbon sources for cellulase production (Gautam *et al.*, 2010; Sherief *et al.*, 2010; Lee *et al.*, 2010). Pawpaw peels was therefore selected for further studies as novel substitute of CMC. The selection of pawpaw peels might be due to the fact that it provided adequate amount of nutrients like proteins, carbohydrates, fat, fibres, ash, trace elements, and various amino acids, have a good porosity for oxygen supply (Ikram*et al.*, 2006).

Effect of different substrate concentrations on cellulases production

Cellulase activity was studied by varying the concentration of pawpaw peels varying from 1 to 5% (Figure 3). Thus, the optimum substrate concentration for maximum production of cellulase was obtained at 5%. At this concentration of pawpaw peels gave maximum cellulase activity

(1.11µmol/min/mL) and protein content (9.63 mg/mL). It was observed that all the substrate concentrations used for optimization study supported the production of cellulases with all producing more than 0.24µmol/min/mL activity.

The carbon is an important factor affecting cells growth and product formation of microorganisms. Carbon sources may have either repressing or inducing effects on enzyme production (Gupta *et al.*, 2010). A dynamic influencing feature that affects the yield and initial hydrolysis rate of cellulose is substrate concentration (Hafiz *et al.*, 2010).

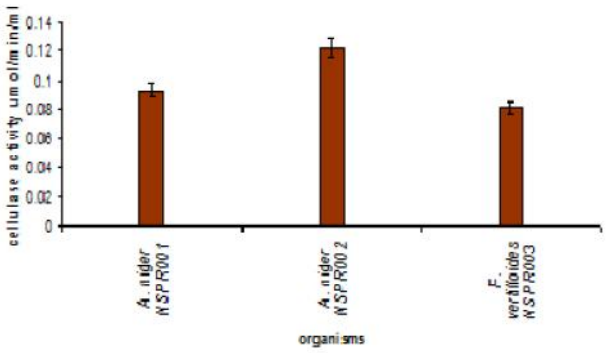

Figure 1.*The cellulose hydrolysis potential of the tested fungal strains by cultivation on submerged conditions on medium with 5% carboxymethylcellullose (CMC)*

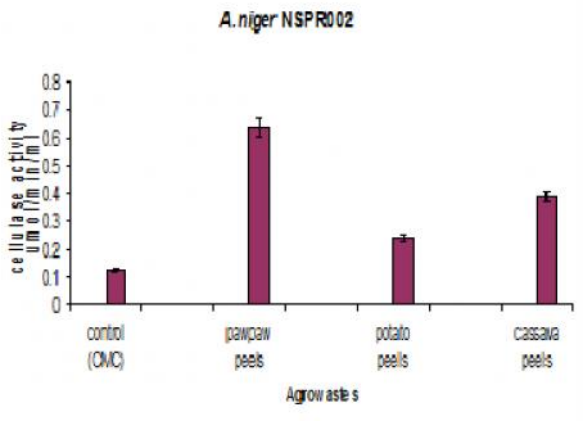

Figure 2. *Effect of different agrowastes used as carbon source on the production of cellulase by AspergillusnigerNSPR002*

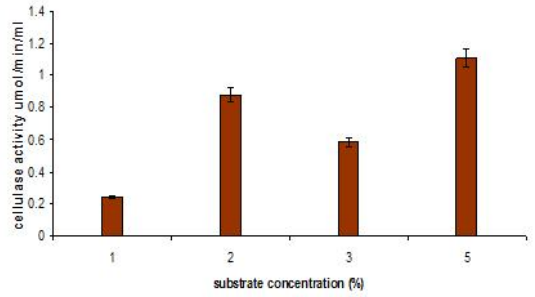

Figure 3.*Effect of varying of pawpaw peels concentrations on cellulases production by AspergillusnigerNSPR002*

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 enzymes formation (Liu and Yang, 2007; Singhania *et al.*, 2007). This result matched with other reports that the optimum substrate concentration for cellulases production by a strain of *Trichoderma* spp. was 5% (Gautam *et al.*, 2010). A 5% optimum substrate concentration was also reported by Abo-State *et al.* (2010) for *Aspergillus* spp.

Effect of organic nitrogen sources on cellulases production

Ammonium sulphate (control) used as an inorganic nitrogen source in the basal medium was replaced by soybeans, locust beans or cotton seeds (Figure 4).

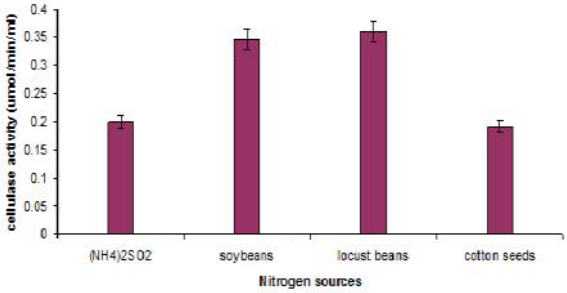

Figure 4: Effect of different nitrogen sources on the cellulases production by Aspergillusniger NSPR002 in submerged fermentation

Of the entire tested organic nitrogen source, locust beans was observed be most effective, the maximum yield of cellulase activity of

0.36µmol/min/mL was obtained, followed by soybeans and the lowest cellulase activity was recorded for cotton seeds. The use of ammonium sulphate as inorganic nitrogen source caused a reduction in enzymatic activity to 44.60% comparing with those obtained with locust beans. However, all the organic nitrogen sources used had better cellulase activity than ammonium sulphate except cotton seeds. The best cellulase activity obtained when locust bean was used could be due to the fact that locust beans provided both the ammonium as well as sulphate ions for conidial cell growth and enzyme production (Akinyele *et al.*, 2013a).

Effect of pH and temperature on cellulases production

The inoculated flasks were incubated for 3 days at different incubation temperatures (28°C, 32°C and 37°C) in submerged cultivation conditions (Table 1). The maximum cellulase activity was achieved at an incubation temperature of 32°C. At higher or lower incubation temperatures, the enzyme activity showed a lower value. Maximum specific activity of 0.32 µmol/min/mg was achieved when the pH of basal medium was kept a 4.5 (Figure 5). The pH values higher than 4.5 had an adverse effect on the specific cellulase activity of crude extract produced by *A. niger*NSPR002. At pH 7.5 the specific activity reduced to 80.40% of that obtained at pH 4.5. The temperature of the fermentation medium is one of the vital factors that have deep influence on the yield and quality of the biosynthesis products (Ahmed *et al.*, 2009; Hafiz *et al.*, 2010). The optimum temperature obtained from this study correlated with the finding of Gilna and Khaleel (2011), who reported maximum cellulase activity at 32°C when *Aspergillus fumigatus* was cultured on selected lignocellulosic wastes under liquid state fermentation. Mekala *et al.* (2008) reported an optimum temperature of 33°C. As the temperature was further increased, there was a gradual reduction in the enzyme production. This may be due to the fact that higher temperature denatures the enzymes. High temperature may also lead to inhibition of microbial growth (Shazia *et al.*, 2010). Many workers have reported different optimal temperatures for cellulase production either in shake or in bioreactor studies using *Aspergillus* spp. suggesting that the optimum temperature for cellulases production also depends on the differences within the same genus of the same fungus (Akinyele *et al.*, 2013a).

Table 1. *Variation oncellulases biosynthesis byAspergillusniger NSPR002 in different temperatures during submerged fermentation*

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±0.01	0.07	75.43
32°C	0.582±0.010	7.473±0.01	0.078	100
37°C	0.474±0.010	5.955±0.02	0.080	81.44

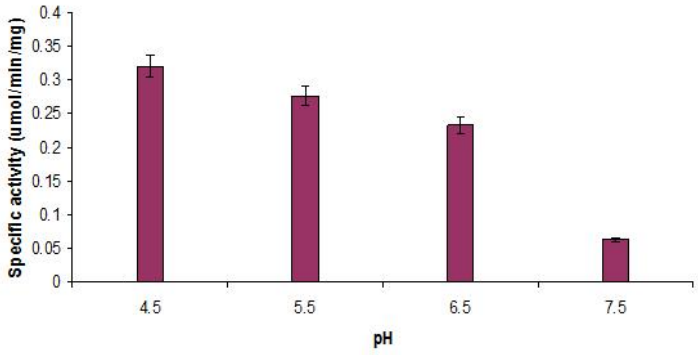

Figure 5.*Theeffect of pH on cellulase production by Aspergillus niger NSPR002 during submerged fermentation*

Different optimal pH values had been reported for different microorganisms by many workers (Acharya *et al.*, 2008; Akinyele *et al*., 2013a). The Pham *et al.* (2010) showed that the optimum pH for cellulases production from *Aspergillus niger* VTCC-F021 strain was 5.0. Acharya *et al.* (2008) reported pH optimum that fall between 4.0-4.5 for cellulase enzymes from *A. niger* strains. Coral *et al.* (2002) reported pH optimal for a cellulases production by an *A. niger* strain was 4.5 and 7.5. Such differences may appear because of differences within the same genus. In addition, no comparative investigations have been published on the cellulases from these organisms but the difference appears to be small as difference in morphology between the species (Gautam *et al.*, 2010).

Effect of incubation period on cellulases production

Time course profile (Fig.6) for the production of cellulases in submerged cultivation system with selected strain *A. niger* NSPR002 was evaluated between 24 to 120 h, and at an interval of 24 h.

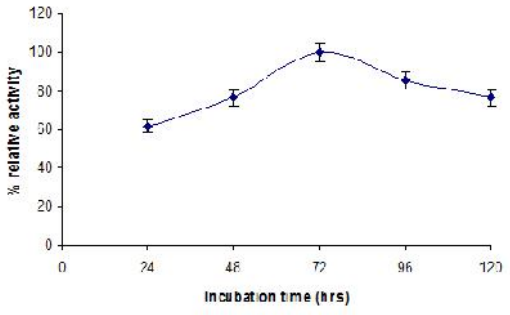

Figure 6. *Time course of the cellulases production by Aspergillusniger NSPR002 using 5% pawpaw peels as single carbon source*

Cellulase activities were expressed in terms of percentage relative activity for the incubation periods as (24, 48, 72, 96 and 120 h) 61.69 %, 76.62%, 100%, 85.70%, 76.62%, respectively. Enzyme activity increased with increase in fermentation period and reached maximum (0.96 µmol/min/mL) at 72 h of fermentation. Further increase in the incubation period beyond the optimum time (72h) resulted in the decreased production of cellulases. The decrease in the percentage relative activity of *A. niger*NSPR002 strain after 72 h of incubation might be due to the depletion of nutrients and accumulation of other by-products like proteases in the fermentation medium initiating autolysis of cells (Gautam *et al*., 2010; Olaniyi *et al.*, 2013).

Conclusions

Three fungal strains and different agro wastes were studied in order to obtain celullases with practical importance. The *A. Niger* NSPR002 strain showed be a potential producer of cellulolytic enzymes which could be readily used in many applications for production of valuable organic compounds by wastes bio-valorisation. The cellulases production with selected stain is effective by submerged fermentation by cultivation on medium contain 5%pawpaw peels and 0.2% locust beans as nitrogen source, after 72 h of cultivation at pH 4.5 and30°C.

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