Optimization of cellulase production by *Aspergillus flavus* NSPR017 cultured on pretreated agrowastes

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Abstract

The study was carried out to investigate the potential of the filamentous fungi, *Aspergillus flavus* NSPR016, *Aspergillus flavus* NSPR017 and *Aspergillus flavus* NSPR019 for hyper-production of one of the most highly demanded industrial enzyme cellulase using cheap and readily available agro-wastes as the sole carbon sources under submerged fermentation. Production of cellulase was substantially enhanced through optimization of physicochemical and nutritional parameters. The effect of several kinetic parameters like carbon and nitrogen sources, incubation period, temperature, pH and substrate concentration were evaluated. All the tested fungal isolates produced cellulase in varying degrees. However, highest cellulase production was recorded in *Aspergillus flavus* NSPR017 and therefore selected for further optimization studies. Orange peels was the most effective at 5% concentration of all the agrowastes used. By optimizing the fermentation conditions, maximum cellulase activity was recorded at 96 hours of incubation, pH 6.5, temperature 28°C, 5% orange peels and 0.2% soybeans.

Key words: *Aspergillus flavus* NSPR017, Cellulase, Agricultural wastes, Orange peels

Introduction

Cellulase enzymes provide a key opportunity for achieving tremendous benefits of biomass utilization through the bioconversion of the abundant cellulosic wastes into the simplest carbohydrate monomer and glucose (Rashid *et al*., 2009). Cellulase enzymes, exocellulbiohydrolase and endoglucanase, have wide applications in the textile, paper and pulp as well as the feed industries (Rashid *et al*., 2009). The most well-known application is the use of cellulases in biostoning. They are also used in the treatments of cellulose containing textile materials during their manufacturing and finishing (Hafiz *et al*., 2010). Endoglucanases from cellulose are also important for the degradation of b-glucan in feed which lowers the viscosity of the intestinal contents thereby improving the quality of the feed (Shazia *et al*., 2010). The production of cellulases using various substrates and nutrients by microorganisms has been reported (Shazia *et al*., 2010; Gilna and Khaleel, 2011). Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity. Most of the fungi produce several enzymes with similar cellulolytic activity and members of the filamentous fungi, particularly *Aspergillus* and *Trichoderma* spp. are well known efficient producers of cellulases (Gautam *et al*., 2010; Gilna and Khaleel, 2011). Crude enzymes produced by these microorganisms are commercially available for agricultural and industrial uses (Shazia *et al*., 2010). In general, bacterial cellulases are constitutively produced, whereas fungal cellulases are produced only in the presence of cellulose (Rashid *et al*., 2009).
The present study was carried out to evaluate the effect of different cultural conditions on cellulase production by *Aspergillus flavus* NSPR017 cultured on pre-treated orange peels.

**MATERIALS AND METHODS**

**Fungi Isolates**

*Aspergillus flavus* NSPR016, *A. flavus* NSPR017 and *A. flavus* NSPR019 were obtained from the culture collection centre of the Nigerian Stored Products Research Institute Ilorin, Kwara State, Nigeria. The fungal isolates were maintained on Potato Dextrose Agar (PDA) slants at 4°C in the refrigerator.

**Chemicals and Lignocellulosic Substrates**

All the chemicals used for this study were of analytical grade unless otherwise stated. Orange peels, yam peels and wheat bran were sourced from farms in Akure, Ondo State, Nigeria. The substrates were washed, sun and oven dried at 70°C (Model DHG Heating Drying Oven) for a period of 2 weeks, blended and sieved using 40mm mesh size and stored in air tight transparent plastic containers to keep it moisture free (Hafiz *et al*., 2010).

**Pretreatment of Lignocellulosic Substrates**

Lignocellulosic Substrates (10g) were treated separately with 1000ml of 4% solution of sodium hydroxide for 24hrs 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 sodium hydroxide was further neutralized with diluted hydrochloric acid and they were autoclave at 121°C for 15mins (Muthuvelayudham and Viruthagiri, 2006).

**Media Preparation and Enzyme Production**

Medium composition described by Shazia *et al*. (2010) was used for submerged fermentation. The media contained (per liter of distilled water): Urea 0.3 g, (NH₄)₂SO₄ 1.4 g, KH₂PO₄ 2.0 g, CaCl₂ 0.3 g, MgSO₄·H₂O 0.3 g, peptone 1.0 g, FeSO₄·H₂O 5.0 mg, MnSO₄·H₂O 1.6 mg, ZnSO₄·H₂O 1.4 mg, CoCl₂ 2.0 mg and carboxymethylcellulose (CMC) 10g. 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 120rpm. Sterile basal medium supplemented with carboxymethylcellulose without organism served as the control. Crude enzyme preparation was obtained by centrifugation at 5000rpm for 10mins at 4°C using refrigerated ultracentrifuge (Centurion Scientific Limited). The supernatant was used as the crude extracellular enzyme source (Gautam *et al*., 2010).

**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 0.5ml of 0.5% of CMC as substrate prepared in 0.5M sodium acetate buffer pH 5.5. The control tube contained the same amount of substrate and 0.5ml of the enzyme solution heated at 100°C for 15mins. Both the experimental and control tubes were incubated at 50°C for 30mins. 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 3ml of 3, 5-dinitrosalicylic acid reagent per tube (Shazia et al., 2010). The tubes were incubated for 5mins in a boiling water bath for color development and were cooled rapidly. The activity of reaction mixture was measured against a reagent blank at 540nm. 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 1μM of glucose per milliliter per minute and was expressed as μM/ml/min.

**Optimization of Culture Conditions for Cellulase Production**

The optimization for cellulase production was performed based on the modification of environmental and nutritional parameters. The effect of environmental factors was determined by modification of pH of the fermenting medium in the range of (4.5, 5.5, 6.5 and 7.5), cultivation temperature in the range of (28, 32 and 37°C) and incubation period in the range of (24, 48, 72, 96 and 120 hrs).

**Statistical Analysis**

Data collected were subjected to Analysis of Variance(ANOVA) and means separated by Duncan’s New Multiple Range Test.

**RESULTS**

**Microbial screening of selected fungal isolates for cellulase production on carboxymethylcellulose (CMC).**

The selected fungal isolates (*Aspergillus flavus* NSPR016, *A. flavus* NSPR017 and *A. flavus* NSPR019) were screened for their ability to secrete cellulose on carboxymethylcellulose incorporated into minimal salt medium (Figure 1). After 5 days of incubation on rotary shaker, the highest cellulase activity (0.110μmol/min/ml) was observed with *A. flavus* NSPR017. The three strains of fungi used in this study showed the ability to produce cellulase on CMC with significant differences in the rate of enzyme production. All the strains were observed to yield cellulase activities with all liberating more than 0.080μmol/min/ml. However, the lowest cellulase activity (0.081μmol/min/ml) was observed with *Aspergillus flavus* NSPR016.

**Effect of different agrowastes (carbon sources) on cellulase production**

Different agricultural by-products (orange peels, wheat bran and yam peels) were supplemented with mineral salt medium for cellulase production (Figure 2). Of all the agrowastes tested, orange peels were found to be the best substrate for the production of cellulase, which gave maximum yield of cellulase activity of 0.322 μmol/min/ml. The cellulase activity of orange peel was observed to be almost 3.0 fold increase compared to carboxymethylcellulose (control).

**Effect of incubation period on cellulase production**

In Figure 3, the flasks were incubated at different time duration; 24, 48, 72, 96 and 120 hours and cellulase activity expressed in terms of percentage relative activity were 25.93 %, 69.1%, 80.28%, 100%, 25.1% respectively. Thus, at 96 hours of incubation, maximum yield was obtained. The production of enzyme increased with increase in fermentation period and beyond the optimum incubation period (96hrs) a decline in enzyme production was observed.

**Effect of incubation temperature on cellulase production**

The effect of incubation temperatures (28°C, 32°C and 37°C) on cellulase biosynthesis by *A. flavus* NSPR017 on pretreated orange peels under submerged state fermentation is shown in Table 1. There was gradual decrease in cellulase activity with increase in incubation temperature. However, optimum cellulase activity was obtained at 28°C.
Figure 1. Microbial screening of selected fungal isolates for cellulase production on carboxymethylcellulose (CMC).

**Aspergillus flavus NSPR017**

![Graph showing cellulase activity](image)

**Figure 2**: Effect of different agrowaste on cellulose production

![Graph showing relative activity over incubation time](image)
Figure 3: Time course of the cellulase production by *Aspergillus flavus* NSPR017 using orange peels as carbon source.
Table 1: Optimization of temperature

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Cellulase activity (µmol/min/ml)</th>
<th>Protein content (mg/ml)</th>
<th>Specific activity (µmol/min/mg)</th>
<th>Percentage relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C</td>
<td>0.322±0.01</td>
<td>2.043±0.02</td>
<td>0.158</td>
<td>100</td>
</tr>
<tr>
<td>32°C</td>
<td>0.281±0.03</td>
<td>1.985±0.01</td>
<td>0.142</td>
<td>0.116</td>
</tr>
<tr>
<td>37°C</td>
<td>0.149±0.01</td>
<td>1.284±0.01</td>
<td>0.116</td>
<td>46.27</td>
</tr>
</tbody>
</table>

(Values are means of three replicates, ± = standard deviation)

**Effect of pH on cellulase production**

The specific activity of cellulase by *A. flavus* NSPR017 was studied by varying the pH of the fermentation media from 4.5 to 7.5 (Figure 4). Maximum specific activity of cellulase (0.393 µmol/min/mg) was achieved when the pH of basal medium was kept at 6.5. At pH 7.5 the specific activity dropped to about 22.65% of that obtained at pH 6.5.

**Effect of different concentrations of orange peels (carbon source) on cellulase production**

Cellulase activity and protein content estimation was studied by varying the concentration of orange peels (Figure 5). Different concentrations of orange peels were used for the enzyme production ranging from 1 to 5% with the exclusion of 4%. Of these, 5% orange peels were optimized for maximum production of cellulase (0.967 µmol/min/ml). Thus, the optimum substrate concentration for maximum production of cellulase was obtained at 5%.

**Effect of organic nitrogen sources on cellulase production**

In this present work, different organic nitrogen sources such as cotton seeds, locust beans and soybeans were added separately to the fermentation medium at 0.2% concentration replacing ammonium sulphate (inorganic) from mineral salt medium. Among all the organic nitrogen sources tested; soybeans gave maximum production of cellulase (0.3370.194 µmol/min/ml) (Figure 6). However, the lowest cellulase production was obtained in ammonium sulphate (NH₄)₂S0₄. The cellulase activity obtained from soybeans was almost 2.011 higher than the ammonium sulphate (control). The use of ammonium sulphate as inorganic nitrogen source caused a reduction in enzymatic activity to about 45.10% of that obtained with soybeans.
**Figure 4:** Effect of pH variation on cellulase production

**Figure 5:** Effect of varying substrate concentrations on cellulase production

**Figure 6:** Effect of different nitrogen sources on the cellulase production

Akinyele et al., 2011  
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DISCUSSION

Three fungal isolates were screened in minimal salt medium supplemented with CMC with differences in the amount of cellulase produced by each of the isolate. The differences suggest that the rate of cellulase produced depends on the genetic composition of the microorganisms (Gautam et al., 2010). Aspergillus flavus NSPR017 was therefore selected for further studies because of its high cellulase activity. Capacity of an organism to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria and fungi and these characters are restricted to a few species among several major taxa (Hafiz et al., 2010) however, Trichoderma sp. (Jamal and Alam, 2010; Gautam et al., 2010), Penicillium sp. (Han et al., 2009); Sporotrichium sp (Sukumaran et al., 2005) and Aspergillus sp (Hafiz et al., 2010) have been reported to have cellulolytic activity.

Agricultural by-products such as corn cob, wheat straw, rice straw, bagasse were utilized in previous studies for cellulase production (Ojumu et al., 2003; Ikram et al., 2006; Omojasola et al., 2008). In the present study, the natural waste materials were utilized effectively as major carbon source for the production of cellulase by selected fungal isolate and the substitution of CMC with different agrowastes resulted in a maximum cellulase activity. There was variation in the amount of cellulase produced when agrowastes were substituted in culture medium. The large variation in cellulase yield may be due to the fact that its provided adequate amount of nutrients like proteins, carbohydrates, fat, fibres, ash, trace elements, various amino acids and porosity for oxygen supply (Bakri et al., 2003; Ikram et al., 2006).

Apart from this, some environmental factors are also influenced the growth of the organisms as well as maximum production of enzymes will be at certain optimum temperature, pH, time duration and so on (Immanuel et al., 2006). The effect of incubation period on cellulase production was estimated for 120 hours. The enzyme was found to increase steadily with increase in incubation time. Maximum production was observed after 96 hours and beyond this, the enzyme production substantially decreased, probably due to the depletion of essential nutrients in the media and/or accumulation of toxic secondary metabolites produced by the fungus itself (Gautam et al., 2010).

The cultivation temperature has a marked influence on the growth rate as well as on the level of cellulase production (Arijit et al., 2010). Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic (Ahmed et al., 2009; Gautam et al., 2010). Among the fungi, most cellulase production studies have been done with mesophilic fungi within the temperature range 25 to 37°C (Lu et al., 2003; Gautam et al., 2010). In the present investigation, 28°C was an optimum temperature. The optimum temperature obtained from this study correlated with the finding of Narasimha (2006), who reported maximum cellulase activity at 28°C when Aspergillus niger was cultured on pretreated sawdust. Similar result was reported by Acharya et al. (2008) when pretreated sawdust was optimized for cellulase production by Aspergillus niger. Many workers have reported different optimal temperatures for cellulase production either...
in shake or in fermentor studies using *Aspergillus* spp. suggesting that the optimum temperature for cellulase production also depends on the differences within the same genus of the same fungus (Hafiz *et al*., 2010).

The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of enzymes (Gupta *et al*., 2010). A pH regulatory system may be especially important. Apart from the regulatory effect on gene expression, cultivation pH can also affect fungal morphology greatly (Gupta *et al*., 2010). Thus, development of an optimal pH control strategy is helpful in obtaining higher protein productivity. Here in the present study, it was found that pH 6.5 is optimum in case of *Aspergillus flavus* NSPR017 as an organism for cellulase production. Similar result was reported by Gautam *et al*. (2010) they found that the cellulase production was optimum at pH 6.5 for *Trichoderma viride* under submerged fermentation. Contrary to this, Pham *et al*. (2010) showed that the optimum pH for cellulases production from strain of *Aspergillus niger* VTCC-F021 was 5.0. Acharya *et al*. (2008) reported pH optimum that fall between 4.0-4.5 for cellulase enzyme from *A. niger*. Coral *et al*. (2002) reported pH optimal for a cellulase production by an *A. niger* strain was 4.5 and 7.5.

The carbon is an important factor affecting cell growth and product formation of microorganisms. Carbon source may have either repressing or inducing effect on enzyme production (Gupta *et al*., 2010). In this present study, orange peels at 5% level proved to be the best for cellulase production by *Aspergillus flavus* NSPR017. This result matched with other reports that the optimum substrate concentration for cellulase production by a strain of *Trichoderma* spp. was 5% (Gautam *et al*., 2010) and 5% optimum substrate concentration was also reported by Abo-State *et al*. (2010) for *Aspergillus* spp. Although, different optimal substrate concentrations had been reported by many researchers and this could be attributed to the chemical nature and nutrient availability of the used substrates (Gautam *et al*., 2010).

Most industrially used microorganisms can utilize inorganic or organic nitrogen sources. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates and as amino acids, protein or urea. It was found that the growth was faster with the supply of organic nitrogen, and a few microorganisms also were found to have absolute requirement for amino acids (Ray *et al*., 2007). However, amino acids are more commonly added as complex organic nitrogen sources which are non-homogenous, cheaper and readily available. In the present study, soybeans at 0.2% level proved to be the best organic nitrogen source for cellulase production by *Aspergillus flavus* NSPR017. It was due to the fact that soybeans provided both the ammonium as well as sulfate ions for conidial cell growth and enzyme production (Mekala *et al*., 2008).

**CONCLUSION**

Orange peel was the most suitable substrate for cellulase production when compared with others agro-wastes as it gives highest yield of enzyme. Cost of production of enzyme is the major impediment that has reduced it application for various industrial processes. To reduce cost of production, the lignocellulosic substrates are used instead of commercial substrates due to their reasonable cost and high enzyme production capacity. The reduction in cost paves an economically easy way application in food, feed, paper and pulp, detergent and bio-ethanol production. It is an important issue to deal with the residue both the comprehensive utilization of lignocellulosic
resources and for the prevention of environmental pollution.

REFERENCES


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