Parameters Optimization of Cellulase Zymosynthesis by *Aspergillus flavus* NSPR017 Grown on Pretreated Orange Peels

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Abstract: The potential of *Aspergillus flavus* NSPR016, *Aspergillus flavus* NSPR017 and *Aspergillus flavus* NSPR019 for overproduction of industrial cellulase; adopting cheap and readily available agrowastes as sole carbon substrates under submerged fermentation was investigated. Cellulase production was considerably heightened via physicochemical and nutritional optimization. The effects of several parameters such as carbon and nitrogen substrates, incubation period and temperature, pH and substrate concentration were evaluated. The isolates were screened for cellulase production in mineral medium with carboxymethylcellulose (CMC) supplemented as the sole carbon source. All the tested isolates proved to be cellulase producers with varied rates of enzyme production. However, the highest cellulase production was found with *Aspergillus flavus* NSPR017 and was therefore selected for further optimization studies. Utilization of agrowastes as carbon subtrates instead of CMC for cellulase production was also evaluated. Among tested carbon sources (yam peels, orange peels and wheat bran), orange peels at 5% was found to be the most suitable carbon source. By optimizing the fermentation conditions, maximum cellulase activity was attained at 96 hours, pH 6.5 and temperature 28^{o}C of incubation, 5% orange peels and 0.2% soybeans. The results obtained suggest that lowcost system for hyperproduction of cellulase is achievable for industrial application.

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1. Introduction

Fungal cellulases remained indispensable bio-tools in conversion of agrowastes into value-added products. The cellulase complex secreted by filamentous fungi consists of three major enzymes components, an endo-1,4-β-D-glucanase (EC 3.2.1.4) which attacks β-linkages at random on the amorphous parts of cellulose, a 1,4-β-D-cellobiohydrolase (EC 3.2.1.4) which releases a cellobiose from nonreducing or reducing end, generally from the crystalline parts of cellulose and a 1,4-β-D-glucosidase (EC 3.2.21) which release glucose from cellobiose and short chain cellooligosaccharides, which act synergistically during the conversion of cellulose to glucose (Bhat, 2000; Sun and Cheng, 2002; Xu *et al*., 2011). Cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Bhat, 2000; Sun and Cheng, 2002; Lynd *et al*., 2005; Gilna and Khaleel, 2011).

Agrowastes on the other hand, are food processing wastes, crop wastes and agro-allied industry wastes and other lignocellulosic wastes. Large amounts of lignocellulosic "waste" are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro industries and they pose an environmental pollution problem. Sadly, much of the lignocellulosic waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon (Mabrouk and Ahwany, 2008; Arijit *et al*., 2010). However, efficient utilization of agro-industrial residues such as cassava, sugar beet pulp, coffee pulp/husk, and apple pomase has being increasingly advocated. Several processes have been developed that utilize these as raw materials for the production of bulk chemicals and value-added fine products such as ethanol, single cell protein (SPC), mushroom, enzymes, organic acids, amino acids and biologically active secondary metabolites (Howard *et al.,* 2003; Lynd *et al*., 2005; Ibrahim, 2008). Applications of agro-industrial residues in bioprocesses on the one hand provide alternative substrates, and on the other hand help in solving pollution problems, which their disposal may otherwise cause. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened for their utilization (Howard *et al*., 2003; Mabrouk and Ahwany, 2008).

With such advancement in biotechnoresearches, agricultural wastes are no longer

environmental issue but resources for energy production. 'Waste-to-Wealth' perception of agricultural wastes is a tremendous potential in improving the general state of sanitation, positive environmental actions to reduce green house gas emissions and significantly improve soil fertility, crop yield and reduce global dependence on chemical fertilizers and fossil fuels (Agamuthu, 2009).

Nevertheless, up to date, the production of cellulase, which is one of the key enzymes for agrowaste biodegradation, has been found to be the most expensive step (Kotchoni *et al*., 2003). Thus, there are needs for researches channeled at making biosynthesis of cellulases economically feasible, cost effective, and sourcing of cheap and efficient raw materials and utilization of agrowastes. Various parameters that affect cellulase production require optimization to encourage local production and local industrial development in area of enzyme production.

2. Materials and Methods

Fungi isolates

Aspergillus flavus NSPR016, *A. flavus* NSPR017 and *A. flavus* NSPR019 were obtained from the culture collection of the Nigerian Stored Products Research Institute Ilorin, Kwara State, Nigeria and maintained on Potato Dextrose Agar (PDA) plates. These were subcultured once in a month by incubation at 30 ±2^{o}C until the entire plates were covered by active mycelium and then stored at 4^{o}C in refrigerator on agar slants.

Chemicals and lignocellulosic substrates

All the chemicals used for this study were of analytical grade unless otherwise stated and produced by Fluka (France), Merk (Germany) and Sigma Chemical Co., (USA). Orange peels, yam peels and wheat bran were procured from farm fields, domestic source and market in Akure, Ondo State, Nigeria and were prepared according to Hafiz *et al.* (2010).

Pretreatment of lignocellulosic substrates

Lignocellulosic substrates (10g) were treated separately with 1000 mL of 4% solution of sodium hydroxide 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 (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^{o}C for 15 min (Muthuvelayudham and Viruthagiri, 2006).

Media preparation and enzyme production

Medium composition described by Mandles and Weber as reported by Acharya *et al.* (2008) was used for submerged fermentation. The media contained (per liter of distilled water): Urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgSO_4.H_2O$ 0.3 g, peptone 1.0 g, $FeSO_4.H_2O$ 5.0 mg, $MnSO_4.H_2O$ 1.6 mg, $ZnSO_4.H_2O$ 1.4 mg, $CoCl_2$ 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 dispensed 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^{o}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^{o}C using refrigerated ultracentrifuge (Centurion Scientific Limited). The supernatant was used as the crude extracellular enzyme source (Gautam *et al*., 2010).

Optimization of culture conditions

Culture conditions are an integral aspect of enzyme production. The present study investigated the effect of nutritional conditions on the production of cellulase by *Aspergillus flavus* NSPR017, adopting different carbon sources (Orange peels, yam peels and wheat bran), organic nitrogen sources (cotton seeds and locust beans) at 0.2%, substrate concentrations (1%, 2%, 3% and 5%), pH ranges (4.5 to 7.5), and temperature (28, 32 and 37^{o}C) according to Gautam *et al*. (2010). The flasks were kept on a rotary shaker (Gallenkamp) at 120rpm at 30 ± 2^{o}C for 4 days of cultivation (Hafiz *et al*., 2010).

Cellulase Assay

Cellulase activity of supernatant collected at the end of each optimization step was determined using a reaction mixture containing 0.5 mL of 0.5% of carboxymethylcellulose as substrate prepared in 0.5 M sodium acetate buffer pH 5.5 according to Acharya *et al.* (2008). The control tube contained the same amount of substrate and 0.5 mL of the enzyme solution heated at 100^{o}C for 15 min. Both the experimental and control tubes were incubated at 50^{o}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 3 mL of 3, 5-dinitrosalicylic acid reagent per tube (Shazia *et al*., 2010). The tubes were incubated for 5 min 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.

Protein and reducing sugar estimation

Protein in the medium was determined by the method of Lowry *et al.* (1951) with Bovine Serum Albumin (BSA) as standard while the amount of reducing sugar in culture filtrate was determined according to Miller (1959).

Statistical Analysis

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

3. Results

Screening of fungal isolates for cellulase production

The selected fungal strains (*Aspergillus flavus* NSPR016, *A. flavus* NSPR017 and *A. flavus* NSPR019) showed the ability to produced cellulase enzyme, protein and liberate reducing sugar on carboxymethylcellulose incorporated into minimal salt medium (Figure 1-3) but with varied rate of enzyme production. The highest cellulase activity (0.110 µmol/min/mL), protein content (0.701 mg/mL) and reducing sugar (0.029 mg/mL) was observed with *A. flavus* NSPR017 after 5 days of incubation on rotary shaker. While, the lowest cellulase activity (0.081 µmol/min/mL) and protein content (0.585 mg/mL) was observed with *Aspergillus flavus* NSPR016. In term of reducing sugar liberation from incorporated substrate, *A. flavus* NSPR019 gave the lowest amount of 0.09 mg/mL.

Effect of different agrowastes on cellulase production

Different agricultural by-products (orange peels, wheat bran and yam peels) supplemented with mineral salt medium for cellulase production and protein content estimation (Figure 4 and 5) showed that, orange peels were the most substrate for the production of cellulase, which gave maximum yield of cellulase activity and protein content of 0.322 µmol/min/mL and 15.76 mg/mL respectively. The cellulase activity of orange peel was observed to be almost 3.0 fold increase compared to carboxymethylcellulose (control). All the tested substrates proved to be novel carbon sources for cellulase production.

Effect of incubation period on cellulase production

The flasks were incubated at different time duration; 24, 48, 72, 96 and 120 h and cellulase activity expressed in terms of percentage relative activity were 25.93 %, 69.1%, 80.28%, 100%, 25.1% respectively (figure 6). Thus, at 96 h of incubation, maximum degradation was obtained. The production of enzyme increased with increase in fermentation period and beyond the optimum incubation period (96 h), a decline in enzyme production was observed. Time course profile of protein production by *A. flavus* NSPR017 is shown in Figure 7. Protein production progressively increased with increase in incubation period until an optimum production (2.394 mg/mL) was attained at 72 h. Subsequent increase in incubation time beyond 72 h resulted into a decline in production of protein.

Effect of incubation temperature on cellulase production

The effect of incubation temperatures (28^{o}C, 32^{o}C and 37^{o}C) on cellulase biosynthesis and protein production by *A. flavus* NSPR017 on pretreated orange peels under submerged state fermentation is shown in Table 1. There was gradual decrease in cellulase activity and protein content with increase in incubation temperature. However, optimum cellulase activity and protein content was obtained at 28^{o}C.

Table 1: Optimization of temperature

Temperature (0^{o}C)	Cellulase activity (µmol/min/mL)	Protein content (mg/mL)	Specific activity (µmol/min/mg)	Percentage relative activity (%)
28^{0}C	0.322±0.01	2.043±0.02	0.158	100
32^{0}C	0.281±0.03	1.985±0.01	0.142	0.116
37^{0}C	0.149±0.01	1.284±0.01	0.116	46.27

(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 8). 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 on cellulase production

Cellulase activity and protein content estimation was studied by varying the concentration of orange peels (Fig.9 and10). 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) and protein content (2.86 mg/mL). Thus, the optimum substrate concentration for maximum production of cellulase was obtained at 5%. It was observed that protein content increased progressively with increase in substrate concentration until optimum protein content (3.269 mg/mL) was recorded at 3% and above this, a decline was recorded. However, all substrate concentrations utilized in this study gave appreciable yield of cellulase activities and protein contents.

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 (0.337 µmol/min/mL) (Figure 11). However, the lowest cellulase production was obtained in ammonium sulphate $(NH_4)_2S0_4$. 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. The biosynthesis of protein by *A. flavus* NSPR017 was evaluated (Figure 12). The highest protein content was observed in cotton seeds (1.634 mg/mL) followed by locust beans (1.284 mg/mL) while the lowest was recorded in ammonium sulphate.

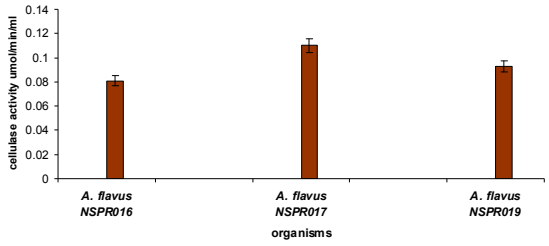

Figure 1. Screening of fungal isolates for cellulase production on carboxymethylcellullose (CMC). They were grown on mineral salt medium with tested substrate (10 g/L) and incubated at 30±2°C for 5 days.

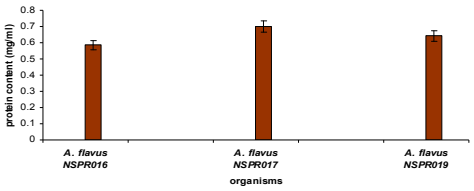

Figure 2: Protein content in culture filtrate inoculated with different fungal isolates at 30±2°C for 5 days.

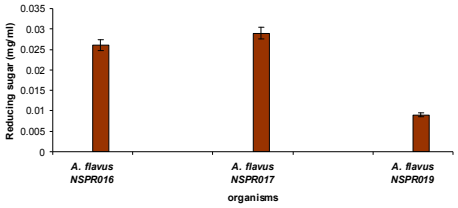

Figure 3. Amount of reducing sugar liberated in culture filtrate of different fungal isolates at 30±2°C for 5 days.

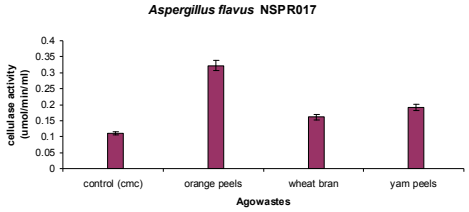

Figure 4 : Effect of different agriculture by-products as carbon source on the production of cellulase by *Aspergillus flavus* NSPR017. Y-error bars indicate the standard deviation among the three replicates.

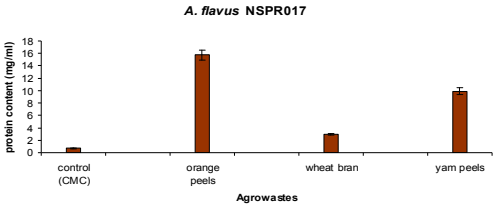

Figure 5: Protein content in culture filtrate inoculated with *Aspergillus flavus* NSPR017 at 30±2°C for 5 days.Y-error bars indicate the standard deviation among the three replicates.

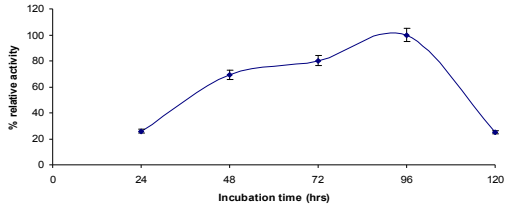

Figure 6: Time course of the cellulase production by *Aspergillus flavus* NSPR017 using orange peels as carbon source. Y error bars indicate the standard deviation among the three parallel replicates.

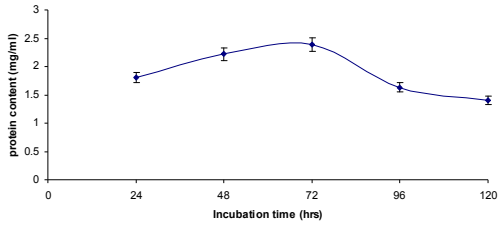

Figure 7: Time course profile of the protein production by *Aspergillus flavus* NSPR017 using orange peels as carbon source. Y error bars indicate the standard deviation among the three parallel replicates

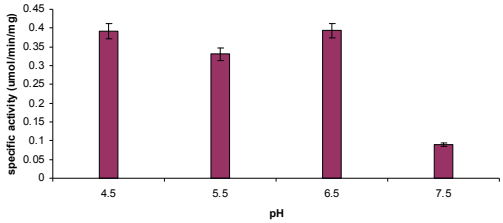

Figure 8: Effect of initial pH on cellulase production

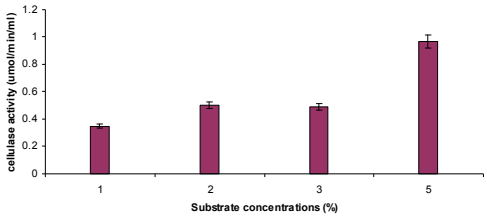

Figure 9: Effect of varying substrate concentrations on cellulase production by *A. flavus* NSPR017

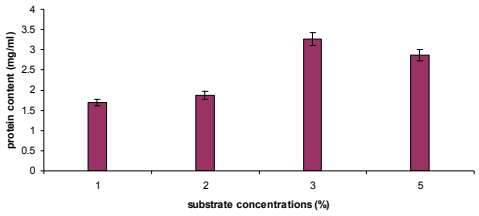

Figure 10: Protein content from different substrate concentrations

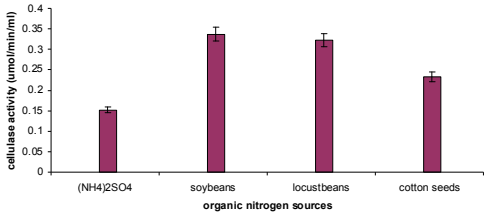

Figure 11: Effect of different nitrogen sources on the cellulase production by *A. flavus* NSPR017 in shake flasks. Y error bars indicate the standard deviation among the three parallel replicates

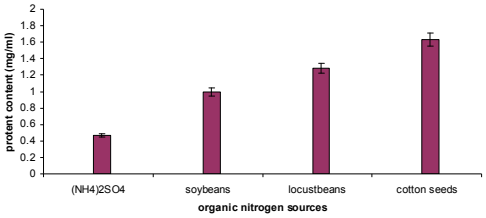

Figure 12: Protein content from different nitrogen sources. Y error bars indicate the standard deviation among the three parallel replicates

4. Discussions

Screening of fungal isolates for cellulase production

The differences obtained in the enzyme synthesis 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. A capacity to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria and fungi. The characters are restricted to a few species among several major taxa (Hafiz *et al*., 2010). The important cellulolytic fungus like *Trichoderma* sp. (Jamal and Alam, 2010; Gautam *et al*., 2010), *Penicillium* sp. (Han *et al*., 2009); *Sporotrichium* sp (Sukumaran *et al*., 2005); *Aspergillus* sp (Hafiz *et al*., 2010) have been reported to have cellulolytic activity.

Effect of different agrowastes on cellulase production

For optimum production of cellulase, the selected agrowastes were given alkaline pretreatment with 2N NaOH at 4%, autoclaved at 121^{o}C for 15 min, in an Erlenmeyer flask to ensure proficient deprivation of lignocellulosic content of wastes to get optimum cellulase production (Hafiz *et al*., 2010). The pretreatment of lignocellulosic wastes has been reported by many researchers (Hafiz *et al*., 2010; Gilna and Khaleel, 2011). The enzymatic conversion of cellulosic materials requires some form of pretreatment to improve cellulose accessibility and digestibility. It was apparent that the most effective pretreatment was one which allowed the substrate to be hydrolysed partially and completely, using a minimum amount of enzyme (Godliving, 2009). It was observed that pretreatment causes the removal of lignocellulosic contents including lignin and hemicelluloses successfully, which at the same time induces the loosening in structure of lignin and destroy the crystallinity of cellulose that improves the porosity characteristics of substrates

(Muthurelayedham and Viruthagiri, 2006; Gilna and Khaleel, 2011).

Agricultural by-products such as corn cob, wheat straw, rice straw, bagasse and so on were used in previous studies for cellulase production (Ojumu *et al.,* 2003; Ikram *et al*., 2006; Omojasola *et al*., 2008). Although, the raw materials are cheaper, pretreatment is generally required to improve the utilizability of lignocellulosic materials and the cost is considerable (Mahro and Timm, 2007; Foyle *et al.,* 2007; Godliving, 2009). In view of the above facts, in the present study, the natural waste materials were utilized effectively as major carbon substrate for the production of cellulase by selected fungal isolate. The substitution of CMC in the culture medium 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 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). Orange peels, yam peels and wheat bran were the agrowastes utilized in this study. Of all the substrates tested, orange peels were found to be the most suitable substrate for the production of cellulase which gave maximum yield of cellulase activity. The selection of orange peels might 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).

Effect of incubation period on cellulase production

The effect of incubation period on cellulase production was estimated for 120 h. The enzyme was found to increase steadily with increase in incubation time. Maximum production was observed after 96 h 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).

Effect of incubation temperature on cellulase production

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 ^{o}C (Lu *et al*., 2003; Gautam *et al*., 2010). In the present investigation, 28^{o}C was an optimum temperature. The optimum temperature obtained from this study correlated with the finding of Narasimha *et al*. (2006), who reported maximum cellulase activity at 28^{o}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 a strain *Aspergillus niger*. As the temperature 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 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).

Effect of pH on cellulase production

The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very critical 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.

Effect of different concentrations of orange peels on cellulase production

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). A dynamic influencing feature that affects the yield and initial hydrolysis rate of cellulase is substrate concentration (Hafiz *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 enzymes formation (Liu and Yang, 2007; Singhania *et al*., 2007). In this present

study, orange peels at 5% 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 that of *Aspergillus* spp as reported by Abo-State *et al*. (2010). 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).

Effect of organic nitrogen sources on cellulase production

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 not 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). Hence, it should be used in proper concentration for an optimum production of cellulase.

Based on the results obtained, we were able to established that the optimized culture conditions (96 h, pH 6.5 and temperature 28^{o}C, 5% orange peels and 0.2% soybeans) for *Aspergillus flavus* NSPR017 could be exploited in lowcost system for hyperproduction of industrial cellulase at bioreactor level for commercialization. Thus, recommended that the isolate should be subjected to strain improvement studies to fully harness its potential to scale up production of cellulase.

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