UJMR, Volume 1 Number 1 December, 2016 *ISSN: 2616 - 0668*

https://doi.org/10.47430/ujmr.1611.025

Received: 6th Sept., 2016 **Accepted:** 5th Nov., 2016

Optimization of Cellulase Activity by *Aspergillus niger* and *Penicillium chrysogenum* using Some Agricultural Wastes

***[1]Bukar, A., Abbas, M. I[2]., Milala, M. A[3]., Isa, M. A[2]., Allamin, I. A[2]. And Ismail, H. Y[2].**

[1]Department of Microbiology, Faculty of Science, Bayero University Kano, Nigeria

[2]Department of Microbiology, Faculty of Science, University of Maiduguri, Borno State, Nigeria

[3]Department of Biochemistry, Faculty of Science, University of Maiduguri, Borno State, Nigeria

*Corresponding author: aminubukar80@gmail.com; +2348023567064

Abstract

Cellulases are enzymes used in various manufacturing processes and their production is faced with a number of challenges including high cost of raw materials, low product recovery due to the nature of microbial cells and unfavorable growth conditions leading to high cost of the finished products. This study was therefore designed to investigate the optimum physicochemical conditions that could support the production of large amount of cellulase by *Aspergillus niger* and *Penicillium chrysogenum* using agricultural wastes as substrates. Each of the fungal species was grown in a mineral salt media containing either of paper, sugar cane bagasse or wheat bran as sole carbon source at various concentrations. Cellulase activity was observed to be optimum at 5 % w/v using the substrates after 96 hours incubation. The effect of temperature and pH were then assessed and wheat bran was shown to support optimum cellulase activity (5.36 IU and 3.62 IU) at pH 5 and temperature of 30°Cusing both organisms. Using paper however, the optimum enzyme activity (1.28 IU and 1.26 IU) was observed at pH 6 and temperature ranging from 35°C (*A. niger*) to 45°C (*P. chrysogenum*). pH 5 was observed to be optimum for cellulase activity using sugar cane bagasse but *P. chrysogenum* performed better (1.01 IU) at 40°C than *A. niger* (0.91 IU) at 50°C. Cellulase activity using the two latter substrates was significantly ($p < 0.05$) lower than that of wheat bran under the said conditions. This study therefore, revealed that the two fungal species are excellent cellulase producers under optimum conditions of 30°C and pH 5 using wheat bran at 5% concentration; hence their potentials for large scale cellulase production.

Keywords: Optimum, Cellulase, *A. niger, P. chrysogenum,* Optimization

Introduction

Cellulases are enzymes that break the glycosidic bonds of cellulose microfibrils, releasing oligosaccharides, cellobiose and glucose (Dillon, 2004). These hydrolytic enzymes are not only used in food, drug, cosmetics, detergent and textile industries, but also in wood pulp and paper industry, in waste management and in the medical-pharmaceutical industry (Bhat and Bhat, 1997).

Cellulase is produced by a vast and diverse fungal population, such as the genera *Trichoderma*, *Chaetomium*, *Penicillium*, *Aspergillus*, *Fusarium* and *Phoma*; aerobic bacteria, such as *Acidothermus*, *Bacillus*, *Celvibrio*, *Pseudomonas*, *Staphylococcus*, *Streptomyces* and *Xanthomonas*; and anaerobic bacteria, such as *Acetovibrio*, *Bacteroides*, *Butyrivibrio*, *Caldocellum*, *Clostridium*, *Erwinia*, *Eubacterium*, *Pseudonocardia*, *Ruminococcus* and *Thermoanaerobacter* (Zhang *et al*., 2006).

The production of cellulases by microorganisms occurs, mainly, by bacteria and filamentous fungi, with few reports of production by yeasts. Ascomycetes and imperfect fungi have great importance for degrading cellulose and decomposing soil vegetable residues, being known as brown rot fungi (Sandgren and Hiberg, 2005).

A cellulolytic system based on 'free' enzymes, that act synergistically to complete cellulose degradation, is typically produced by aerobic fungi and bacteria. This enzyme system includes three types of cellulases: i. *Endoglucanases* (EG, endo-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4): hydrolyses at random, β -1,4 glucosidic bonds at internal amorphous sites in the cellulose chains, providing more ends for the cellobiohydrolases to act upon; ii. *Exoglucanases*or *cellobiohydrolases* (CBH, 1,4- β -D-glucancellobiodehydrolase, EC3.2.1.91): act on the reducing (CBH I) or non-reducing (CBH II) ends of cellulose chains, liberating cellobiose; iii. β -*glucosidases*(β -glucosideglycosyl hydrolase or cellobiase, EC 3.2.1.21): hydrolyzecellobiose or cello-oligosaccharides to glucose and are also involved in transglycosylation reactions of β -glucosidic linkages of glucose conjugates (Coughlan and Ljungdahl, 1988).

Cellulase with its immense importance is being imported for use in Nigeria and other developing countries at a very high cost despite the abundance of raw materials. The local production of such enzyme using locally available agricultural wastes which can serve as substrate, may therefore reduce the cost of importation and encourage self-reliance. In addition, environmental issues and concerns aimed at reducing the ambient pollution may be tackled effectively especially as the world now is focused on search for "clean technologies" to be used in the production of commodities of importance to chemical, energy and food industries.

In a report by Bukar *et al*. (2016), the cellulase producing potentials of *Aspergillus niger, Trichoderma harzianum* and *Penicillium chrysogenum* using wastepaper, sugarcane bagasse and wheat branwas reported. This research was therefore aimed at determining the optimal conditions (pH, temperature and amount of substrate) for the production of cellulase by *Aspergillus niger* and *Penicillium chrysogenum* using wastepaper, sugarcane bagasse and wheat bran.

Materials and methods

Fungal species

This study employed two different fungal species that were earlier screened and showed excellent performances in cellulase production (Bukar *et al*., 2016). The species were *Aspergillus niger* and *Penicillium chrysogenum.*

Pre-treatment of the agricultural wastes

Reduction of particle size was done to make material handling easier and to increase surface/volume ratio. This was carried out by chopping, milling, grinding and shredding of the substrates. The ground substrates were pre-treated using the method of Ali *et al*. (1991) with modification. The substrates 5g/100ml in separate conical flasks (250ml) were soaked in 5% (w/v) NaOH solution in a ratio of 1:20 to delignify them. They were autoclaved at 121^0C for 1 hour. The pre-treated carbon sources were filtered with muslin cloth and residues were neutralised with 1N HCl and washed with distilled water. The pre-treated samples were dried in oven at 120^oC for 6 hours and further broken to powder form in an electric blender.

Media Preparation for Enzyme Production

The fermentation media or mineral salt medium (MSM) was prepared as described by Ali *et al*., (1991) by dissolving the following salt in distilled water (g/litre): KH_2Po_4, 10.5g; $(NH_4)_2So_4$;10.5g;$MgSo_4.7H_2O$, 0.5g; $Cacl_2$, 0.5g; $FeSO_4.7H_2O$, 0.13g; $MnSO_4.7H_2O$, 0.04g;

$ZnSO_4.7H_2O$, 0.04g; $CoCl_2.6H_2O$, 0.0067g; K_2HPO_4, 0.5g; $MnCl_2.4H_2O$, 0.05g; Na_2MO_4, 0.5g; into separate 250 ml conical flasks. Five (5) grams of the treated substrates were added separately to 100ml of MSM the pH of the media was adjusted to 5. The pH was adjusted by addition of NaOH or HCl using pH meter. The media was sterilized in an autoclave at 121^0 C for 15 minutes (Milala *et al.,* 2005).

Culturing of the test organisms

Isolation of Fungal Isolates

Sample collection

Sample of moldy grains (maize, millet, cowpea, and groundnut), soil samples and faeces from the large intestine of cattles were collected from the slaughter house (Abattoir) for the isolation of Fungi as described by Malik (1996).

Primary isolation

The fruiting bodies of the molds from the samples were swabbed and rubbed with a swab stick on the Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) plates to spread spores as described by Cappuccino and Sherman(1999). The plates were examinedfor growth from the third day (72 hours) of inoculation. Colonies from the parent culture were subculturedafter seven days and subcultured until pure cultures were obtained and maintained on slant (PDA).

Secondary isolation

The fungal isolates from Sabouraud Dextrose Agar and Potato Dextrose Agar was shifted separately for further identification by slide culture method as recommended by Awan and Rahman (2002). Each slide was examined under the low (x10) and high power (x40) objectives of microscope for arrangement of hyphae, conidiophore, sterigmata and conidia

Identification of Fungal Isolates

Fungi identified for this study was based on their colony characteristics as well as their vegetative and reproductive structures as observed under bright field microscope

Time Course for Production of Cellulase (length of fermentation)

The media was inoculated with 1ml spore suspension of the fungi with the aid of micropipette and incubated at 30^0C in an orbital shaker at 100 rpm. Samples were withdrawn with the aid of sterile pipette after every 24 hours for 96 hours. These werecentrifuged at 4,000 rpm for 10mins and the supernatant analyzed for the crude enzyme

Assay of cellulase Activity

Cellulase activity was determined using spectrophotometer by measuring the increase in glucose concentration from the hydrolysis of β 1-6 glycosidic linkages (bonds) of cellulose (Ali *et al*., 1991). The cultured sample was centrifuged at 4,000 rpm for 10 minutes, to remove the mycelia and supernatant was used for assaying enzymatic activity. The cellulase activity was assayed by estimating the amount of glucose released upon hydrolysis of cellulose from agricultural waste substrate. Glucose released was determined at 24 hour intervals. One unit of cellulase activity was defined as the amount of enzyme which released 1 unit of reducing sugar measured as glucose per minute under the assay condition. Therefore:

Glucose concentrations =

$$\frac{\text{Absorbance of test} \times \text{Concentration of standard}}{\text{Absorbance of standard}}$$

Estimation of glucose released was by the use of Glucose oxidase kits (Randox) obtained from Sigma laboratory limited, Germany.

Effect of varying substrate concentrations

Different concentrations of the substrates including 2.0w/v, 4.0, 6.0and 8.0 % w/v was added to the mineral salt media in separate conical flasks thereby serving as the fermentation media. The optimal concentration of the substrates for enzyme production was determined by measuring the activity of cellulase produced after 96 hours at various substrate concentrations.

Effect of varying pH on cellulase activity

Both of the organisms were inoculated in to a series of the fermentation media separately with the pH of 3.0,4.0, 5.0, 6.0, 7.0 and 8.0.The media was incubated for 96 hour at 30°C and optimal pH for enzyme production were determined.

Effect of varying temperature on cellulase activity

The optimal temperature for enzyme production was determined by growing the fungi in fermentation media incubated at different temperature of 25,30, 35, 40, and 50°C. Cellulase activity of the crude enzyme was determined after 96 hours of incubation.

Statistical analysis

Data obtained were analyzed using one way ANOVA and the significant difference was determine using Duncan Multiple Range Test (DMRT) at P<0.05 level of confidence.

Results

The effect of different substrate concentrations on cellulase production by *Aspergillus niger* after 96 hours is shown in Figure 1. Wheat bran had the highest activity of cellulase (5.36 IU) at 5% substrate concentration. This was followed by paper and sugarcane bagasse which produced highest (1.28 IU and 0.27 IU) cellulase activity at the same substrate concentrations respectively. Generally, low activity was observed with higher percentages of substrate concentration. There was statistically significant differences between the enzyme activity using different concentration of the substrates (P<0.05).

Figure 2 shows the effect of different substrate concentrations on cellulase activity by *Penicillium chrysogenum* after 96 hours. The same pattern of activity as that of *A. niger* was observed in this case also. Highest and lowest cellulase activities recorded in paper were 1.26 IU and 0.19 IU at 6% and at 4% substrate concentration respectively. With Sugarcane bagasse, the highest activity of cellulase enzyme was 1.013IU at 6% and the lowest activity was 0.246IU at 4% substrate concentrations. With Wheatbran however, highest and lowest cellulase activities (3.62 IU and 0.35 IU) were observed at 5% and 8% substrate concentrations respectively. Cellulase activity using wheat bran was also shown to be statistically significant (p<0.05).

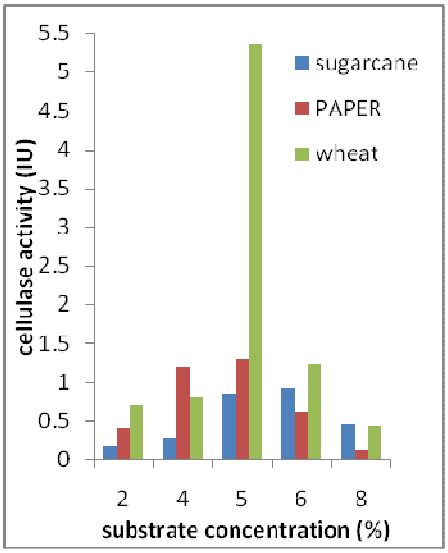

Figure 1: Effect of Substrate Concentration on Cellulase Activities using *A. niger*

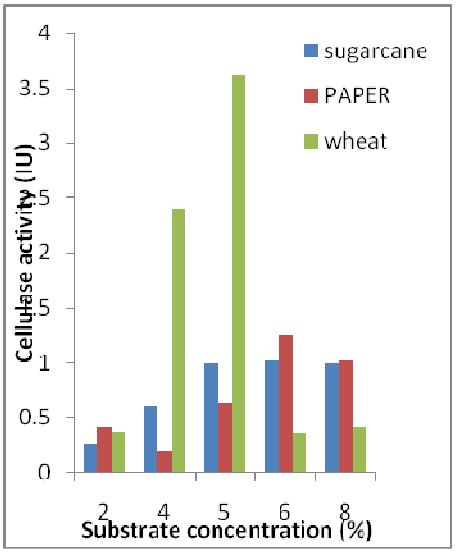

Figure 2: Effect of Substrate Concentration on Cellulase Activities using *P. chrysogenum*

Results in Figure 3 shows the effect of pH on the activity of cellulase produced by *A.niger* using different substrates. Wheatbran gave the highest (3.21 IU) activity at pH 5 whereas paper produced the lowest (0.25 IU) cellulase concentration at pH 8. For *P. chrysogenum* also, cellulase activity was optimum (3.11 IU) at pH 5 using wheat bran, followed by sugar cane bagasse (0.62 IU) at pH 4 while the lowest (0.03 IU) was recorded using paper at pH 4. Wheat bran supported significant ($p<0.05$) cellulase activity compared to the other substrates (Figure 4). The effect of temperature however showed that, 30°C was optimum for both the organisms using wheat bran. Using sugar cane bagasse, 40°C (*P.chrysogenum*) and 50°C (*A.niger*) were shown to be optimum. Cellulase activity was more pronounced between 35 and 40°C using paper as substrate (Figures 5 and 6). Table 1 summarizes the best growth conditions for optimum cellulase production using the substrates and corresponding fungal species.

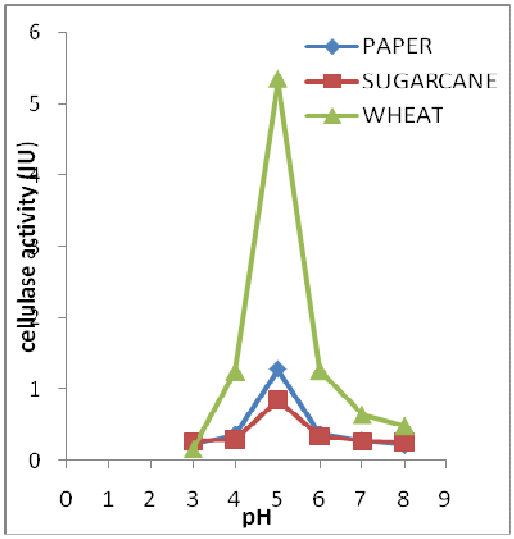

Figure 3: Effect of pH on cellulase activity using *A. niger*

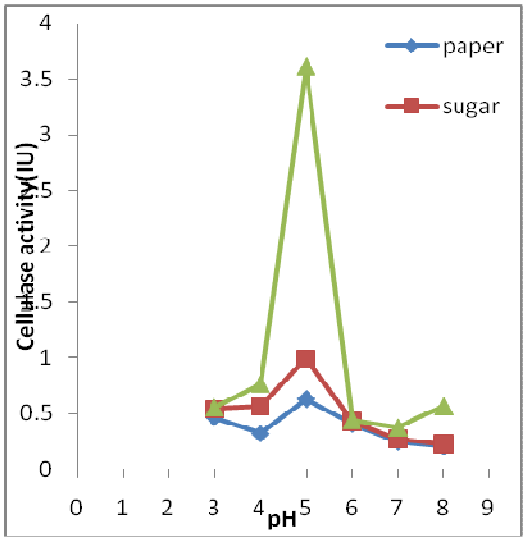

Figure 4: Effect of pH on cellulase activity using *P. chrysogenum*

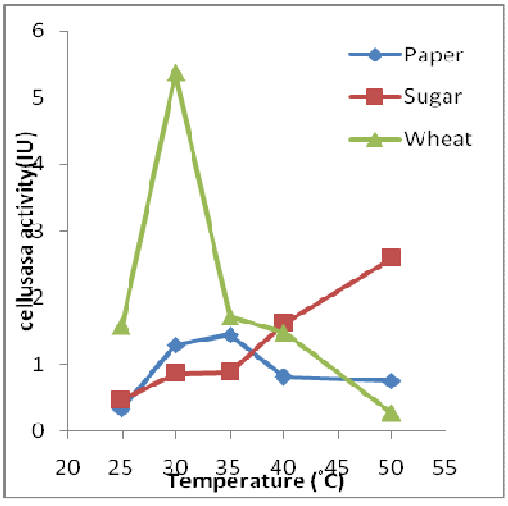

Figure 5: Effect of Temperature on Cellulase activityusing *A. niger*

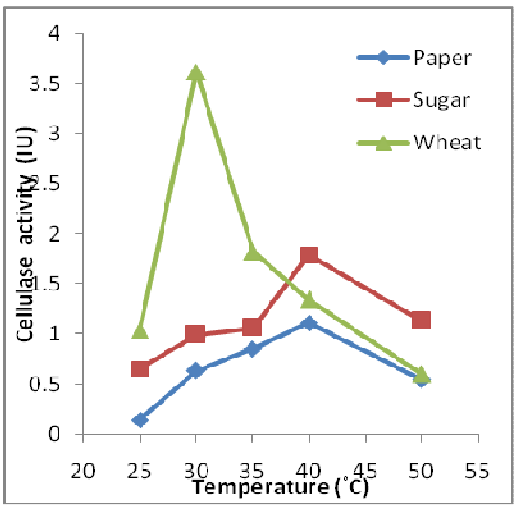

Figure 6: Effect of Temperature on Cellulase activityusing using *P. chrysogenum*

Table 1: Summary of growth conditions for optimum cellulase production by *A.niger* and *P. chrysogenum* using wastepaper, sugarcane bagasse and wheat bran

Fungi	Substrates	pH	Substrate concentration (%)	Temperature (^oC)	Time (Hours)	Cellulase activity (IU)
A. niger	Paper	6	5	35	96	1.28
	Sugarcane bagasse	4	5	50	96	0.91
	Wheat bran	5	5	30	96	5.36
P.chrysogenum	Paper	6	5	40	96	1.26
	Sugarcane bagasse	6	5	40	72	1.01
	Wheat bran	5	5	30	72	3.62

Discussion

Substrate concentration of 5% was shown to support optimum enzyme activity. Substrate concentrations have caused significant variation in enzyme activity. This can be explained to be as a result of availability of more cellulose at 5% concentration. A decrease in enzyme activity beyond optimum concentration (5%) of substrate may be due to unavailability of active binding sites or inhibitors. This is supported by the findings of Gbekeloluwa and Moo-young (1991) who reported the inhibitory effect of accumulated cellobiose and cellodextrin of low degree polymerization. The decrease may also be due to depletion of other nutrients (mineral salts) in the medium, other than the energy source or due to the specific binding of the enzymes with the substrates as observed by Grey and Neelakantan (1983) and Gilkes *et al.* (1984).

The optimum pH for cellulase activity ranged between pH4-6 (Table 1). This pH range has been shown to support maximum cellulase activity using agricultural wastes. Substrates type might have also affected the activity of the enzyme within the pH range as most agricultural wastes were reported to support optimum cellulase activity within this range. This is in agreement with the findings of Omojasola and Jilani (2008) which stated that optimum glucose liberated from the agricultural waste using *A. niger* was produced at pH 4.5 and 6. Also this is in line with the findings of Beldman *et al.* (1985) who reported that *Aspergillus* species grow and metabolize well in acidic pH medium between pH3-5. The instability of these enzymes at lower or very high pH was due to the fact that they are proteins which are denatured at extreme pH levels. This is in line with the works of Ali *et al.* (1991) and Steiner *et al.* (1994).

Cellulase production was optimum at 30^oC using wheat bran by *A. niger* and *P. chrysogenum*. Cellulase activity due to *P. chrysogenum* was optimally observed at 40^oC for paper and sugar cane bagasse. With *A. niger* however, 30^oC and 50^oC was observed to be optimum for paper and sugar cane bagasse respectively. The temperature range reflects the mesophilic nature of the microorganisms producing the enzyme. This is in line with the work of Baig *et al.* (2004), who reported that cellulase activity is mostly optimum at moderate temperatures. It is also in line with the findings of Ogundero (1982) who reported that culture of filamentous fungi were found to be more active at temperature range of 35^oC -50^oC. Cellulase production using these substrates reflects the fact that filamentous fungi are naturally excellent protein (enzymes) secretors and can produce industrial enzymes in feasible amounts (Bergquist *et al.*, 2002).

Conclusion

This study highlighted the potentials of wheat bran, sugarcane bagasse and waste paper as possible raw materials for cellulase production using *Aspergillus niger* and *Penicillium chrysogenum. P. chrysogenum* was shown to perform better using paper and sugarcane bagasse when compared with *Aspergillus niger* using the same substrates. The optimum conditions for enzyme production were 5% substrate concentration, 30^{o}C temperature and pH 5. Wheat bran was shown to be the best substrate for high cellulase production under the said conditions.

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