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Degradation of Agro-Waste by Cellulase from *Aspergillus candidus*

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Abstract: Rice husk, millet straw, guinea corn stalk and sawdust were used as fermentation feed substrate for the evaluation of cellulase activity secreted by *Aspergillus candidus*. The substrates were pretreated with 5% NaOH (alkaline treatment) and autoclaved . From the fermentation studies, rice husk, millet straw and guinea corn stalk feed substrates showed the highest cellulase activity of 7.50, 6.88 and 5.84 IU, respectively. The effect of pH showed that optimal pH for maximum cellulase activity varied in each of the substrates used. Rice husk and millet straw had maximum enzyme activity at pH 5, while guinea corn stalk and sawdust had maximum activity at pH 3 and 4, respectively. From this study, *Aspergillus candidus* holds the potential of converting lignocellulose materials into products of commercial and industrial values such as glucose and other biofuels.

Key words: Cellulase, *Aspergillus candidus*, lignocellulose materials

INTRODUCTION

The recognition that environmental pollution is a worldwide threat to public health has given rise to a new massive industry for environmental restoration. Biological degradation, for both economic and ecological reasons, has become an increasingly popular alternative for the treatment of agricultural, industrial, organic as well as toxic waste.

These wastes have been insufficiently disposed leading to environmental pollution (Fabiyi and Ogunfowora, 1991). Plant lignocellulosics as organic substances are subject to attacks by biological agents such as fungi, bacteria and insects (Highley *et al*., 1987). Acids can breakdown the long chains in cellulose to release the sugars through hydrolysis reaction, but because of their high specificity, cellulase can achieve higher yield of glucose from cellulose (Wyman, 2004). A portion of pretreated biomass can be used to feed a fungus or other organism that produces cellulase that can then be added to pretreated solids to release glucose from cellulose (Wyman, 2008). Filamentous fungi which use cellulose as carbon source possess the unique ability to degrade cellulose molecules in plant lignocellulose. Although, a large number of microorganisms are capable of degrading cellulose, only a few of these produce significant quantities of cell-free enzymes capable of completely hydrolyzing crystalline cellulose *in vitro* (Immanuel *et al*., 2006).

Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. Fungal genera like *Trichoderma* and *Aspergillus* are known to be cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use. The genus *Aspergillus* species attack cellulose producing significant amount of cell free cellulase capable of hydrolyzing cellulose into fermentable soluble sugars such as glucose; an important raw material in chemical industries (Wainwright, 1992). *Aspergillus* and *Trichoderma* specie are well known efficient producers of cellulases (Peij *et al*., 1998). Several studies have been carried out to produce cellulolytic enzymes from biowaste degradation

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process by many microorganisms including fungi such as *Trichoderma*, *Penicillium* and *Aspergillus* species etc., by Mandels and Reese (1985) and Hoffman and Wood (1985).

Cellulase is used to modify the surface properties of cellulosic fibers and fabric in order to achieve a desired surface effect (Kotchoni *et al*., 2003). Cellulase has been used to degrade environmental wastes such as plant wastes (lignocellulosics). Cellulase as an industrial enzyme is imported for use in Nigeria. Therefore, its production using readily available sources (example plant residues) will help reduce importation costs. It is against this background, that this study was carried out to evaluate the cellulase activity of *Aspergillus candidus* on various agro-forestry residues as feed substrates and to determine the effects of pH on cellulase activity.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used in the study were of analytical grade.

Source of Fungus

Aspergillus candidus was isolated from decayed wood in the Plant Pathology Laboratory of the Crop Protection Department, Faculty of Agriculture, University of Maiduguri, Nigeria. It was maintained on Potato Dextrose Agar (PDA) at 4°C.

Source of Lignocellulose

The agro-forestry residues, rice husk, millet straw, guinea corn stalk and sawdust used in the study were obtained on farmlands, sawmills around Maiduguri and from the cattle market known as kasuwan shanu in Maiduguri, Borno state, Nigeria. They were oven-dried at 65°C for two days to reduce the moisture content. They were pulverized to mesh size powder.

Substrate Pretreatment

The ground powdered substrates were pretreated by a modification of Ali *et al*. (1991) method. The substrates 5 g/100 mL in separate conical flasks (250 mL) were soaked in 5% (w/v) NaOH solution in a ratio of 1:20 to delignify them. They were then autoclaved at 121°C for 1 h. The pretreated substrates were filtered with muslin cloth and residue was neutralized with 1N HCl and washed with distilled water. This was then oven-dried at 65°C.

Fermentation Media

The fermentation medium or Mineral Salt Medium (MSM) was prepared as described by Ali *et al*. (1991) by dissolving the following salts in distilled water (g mL^{-1}):$ZnSO_4.7H_2O$ 0.04, KH_2PO_4 10.5, $MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.5, $FeSO_4.7H_2O$ 0.13, $MnCl_2.4H_2O$ 0.005, K_2HPO_4 0.5, $NaMbO_4$ 0.5 and $(NH_4)_2SO_4$ 0.5. This was then autoclaved at 121°C for 1 h.

Five gram of the pretreated substrates were added separately to 100 mL of mineral salt medium and again sterilized at 121°C for 15 min. This medium provides nitrogen and mineral element requirements of the organism. The pretreated cellulosic substrates served as carbon and energy sources as well as inducers of the enzyme.

Inoculation

The spores of the 4 day old *Aspergillus candidus* maintained on Potato-Dextrose Agar (PDA) at 4°C were washed with 2 mL of 0.2% Tween 80 solution into 250 mL conical flask. The flasks were incubated at 30°C in orbital shaker at 200 rpm for a period of 10 days.

Enzyme Assay

The cellulase activity was assayed by estimating the amount of glucose released upon hydrolysis of cellulose from the culture medium by the organism. Glucose released was determined at 24 hourly intervals.

One unit of cellulase activity was defined as the amount of enzyme which released one micromole per minute of glucose in 30 min under the specified assay conditions. Therefore,

$$\text{Glucose concentration (mg dL}^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

Values obtained were converted to international unit.

Estimation of glucose released was by the use of glucose oxidase kits obtained from Randox Laboratories Ltd., UK.

Effect of pH

The working pH of the culture medium was varied from pH 3-5 using phosphate buffer. The optimum pHs for each of the cellulosic substrates were evaluated.

Statistical Analysis

All determinations were carried out in triplicates and the data obtained were expressed as Mean+SD and were subjected to analysis of variance and Tukey-Kramer multiple comparison test to observe any significant difference.

RESULTS

As the period of fermentation increased, enzyme activities also increased. Peak levels were obtained on the 7th day (168 h) for rice, millet and sawdust feed substrates with activities of 7.50, 6.88 and 4.79 IU, respectively. Peak level of cellulase activity was obtained on the 8th day (192 h) for guinea corn feed substrate with an activity of 6.88 IU (Table 1). *Aspergillus candidus* fermentation culture in the rice husk medium had the highest level cellulase activity, millet and guinea corn media had the same levels with peak levels obtained on different days (7th and 8th day), respectively. Cellulase activity in the sawdust medium was significantly different ($p<0.001$) at the peak periods when compared to the activities obtained in the other media.

pH had varying effects on cellulase activities in each of the cultures. Sawdust medium had the highest level of cellulase activity at pH 4 with an activity of 9.14 IU; guinea corn medium had the highest cellulase activity at pH 3 with an activity of 5.66 IU (Table 2).

Table 1: Cellulase activity of rice, millet, guinea, lorn and sawdust at different periods of fermentation

Period of fermentation (h)	Cellulase activity of feed substrates (IU)			
	Rice	Millet	Guinea corn	Sawdust
24	-	-	-	-
48	1.62±0.72	1.88±0.63	1.88±063	2.08±0.72
72	1.25±0.00	1.67±0.72	1.67±0.73	2.08±0.72
96	3.54±0.36	2.71±0.36	3.33±0.22	2.71±0.36
120	4.38±0.63	4.59±0.36	4.58±0.72	3.13±1.08
144	6.25±0.00	4.76±4.76	6.46±0.36	2.92±0.72
168	7.50±0.00[a]	6.88±0.63[a]	5.84±0.36[a]	4.79±0.36[b]
192	5.83±0.72	4.79±0.19	6.88±0.36	3.13±0.00
216	5.84±0.37	4.92±0.14	5.84±0.36	3.14±0.36
240	5.43±0.37	3.96±0.36	5.00±0.00	1.25±0.00

The results are presented as Mean±SD. Values with different superscript are significantly different. While values with the same superscript are not significantly different ($p>0.05$)

Table 2: Cellulase activity of rice, millet, guinea, lorn and sawdust at different pH

	Cellulase activity of feed substrates (IU)			
pH range	Rice	Millet	Guinea corn	Sawdust
3	4.84±0.81	3.49±0.47	5.65±0.00	6.25±0.52
4	3.22±0.81	3.49±0.47	5.11±0.47	9.14±0.47
5	5.95±0.52	4.57±0.47	4.57±0.42	7.02±0.41

All values are in Mean±SD of three determinations

Millet and rice media had highest cellulase activities of 4.52 and 5.95 IU, respectively at pH 3. Sawdust culture medium had the highest cellulase activity when the pH of fermentation media was varied, compared to the activities obtained in the other media but did not show any significant difference ($p>0.005$).

DISCUSSION

Cellulase activity of *Aspergillus candidus*, using rice husk, millet straw, guinea corn stalk and sawdust as cellulosic substrates showed the highest enzyme activity was on the 7th day (168 h) for rice, millet and sawdust media and on 8th day (192 h) for the guinea corn stalk medium after 240 h fermentation period. At 168 h period, rice husk medium had the highest cellulase activity of 7.50 IU; while millet and guinea corn substrates had activities of 6.88 and 5.84 IU. However, sawdust feed substrate had a significantly low cellulase activity of 4.79I U. The differences in the cellulase activity observed in the different culture media could be attributed to the differences in the chemical composition and concentration of other macromolecules such as lignin and hemicellulose that exists in natural association with cellulose. The sawdust used was obtained from hardwood, had higher lignin content when compared to the other feed substrates which belong to the Cereal family. Lignin which forms a physical seal around cellulose inhibits cellulase from hydrolyzing the cellulose and hence this may affect the cellulase secreted by the organism as can be observed with sawdust substrate. This observation is in relation with the findings of Ojumu *et al*. (2003), who reported a low cellulase activity. Again, alkali pretreatment of sawdust cellulosic material may not be an efficient method of accessing cellulose by cellulase enzyme as reported by Gharpuray *et al*. (1983). The differences observed in the cellulase activity could also be attributed to the facts that, although the residues are distinctive in outward appearance, these materials all comprise of about 40-50% cellulose, 20-30% hemicellulose with lesser amounts of lignin in cereals and herbaceous plants but higher in forestry residues (sawdust) (Wyman, 2008). More efficient technologies like steam explosion pretreatment must be applied to completely degrade lignin and gain access to the cellulose molecules. Although, these substrates were pretreated to delignify them, only a significant percentage of the total lignin content was removed. The organism through cascades of enzymatic actions must therefore degrade the remaining to enable its unlimited access to the cellulose molecule. As reported by Kirk *et al*. (1980), combination of biological and chemical agents enables the complete degradation of lignin. Organic acids such as oxalate produced by the organism inhibit lignin peroxidase, a lignin degrading enzyme and this could also be responsible for the differences in the cellulase activity (Kirk *et al*., 1980).

Another factor that may lead to the differences in cellulase activity is the production of non-specific by-products other than glucose by unspecified side reactions. These by-products promote glucose degradation and reduce its yield. Pretreatment of sawdust for example enhances the production of proteins which leads to a corresponding increase in ethanol production from glucose as reported by Ryu and Lee (1983). The submerged fermentation culture method used in this study may also contribute to the differences in cellulase activity. A comparison of solid state fermentation and submerged fermentation has shown that submerged method has shearing forces which rupture mycelial cells and deactivate enzymes, thus enzyme activity is decreased (Wase *et al*., 1985). The decrease in

cellulase activity shown by the fungus after attaining its maximum peak period of enzyme secretion could be attributed to so many other factors. Products of cellulase action on cellulose (cellobiose and glucose) inhibit enzyme secretion. Depletion of carbon and nitrogen sources causes starvation and hence the fungus may not grow and cellulase activity is growth related as reported by Dosoretz *et al*. (1990). The effect of pH on cellulase secretion shows that each substrate supports a particular pH for maximum enzyme secretion. Beldman *et al*. (1985) reported that *Aspergillus* species grow and metabolize well in acidic pH medium between pH 3-5. Linder and Teeri (1996) also proposed that cellulases produced by filamentous fungi rely on several aromatic amino acids for high binding capacity to cellulose surface and this might enhance enzyme activity.

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