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Full Length Research Paper

Effect of acid hydrolysis of *Garcinia kola* (bitter kola) pulp waste on the production of CM-cellulase and β-glucosidase using *Aspergillus niger*

Caritas U. Okafoagu[1] and Humphrey C. Nzelibe[2]*

[1]National Agency for Food and Drugs Administration and Control, Kaduna Area Laboratory, Kaduna, Nigeria.
[2]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria.

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Effect of acid hydrolysis of bitter kola (*Garcinia kola*) pulp wastes on the production of CM-cellulase and β-glucosidase using *Aspergillus niger* was investigated. Hydrolysis for 3 h with 2.5 M sulphuric acid yielded highest fermentable sugar. Acid hydrolysis enhanced CM-cellulase and β-glucosidase levels by 500% at 96 h and 200% at 120 h, respectively. Acid hydrolysed *G. kola* pulp wastes is a better substrate for the production of CM-cellulase and β-glucosidase compared with maize and rice husks.

Key words: Bitter kola pulp wastes, CM-cellulase, β-glucosidase, *Aspergillus niger,* acid hydrolysis.

INTRODUCTION

Cellulose is the most abundant organic molecule in the biosphere. It occurs in almost pure form and in combination with other materials such as lignin and hemicellulose in plant pulps, leaves, and stalks. Cellulose is a biopolymer consisting of thousands of glucose units linked with β-glucosidic bonds, and the resulting biopolymers are associated by means of hydrogen bonding. For this reason, cellulose exhibits structural features such as crystalline sections and amorphous parts (Saka and Ueno, 1999). Cellulose is the most abundants organic source of food, fuel and chemicals (Spano et al., 1975; Henrissat, 1994; Saka and Ueno, 1999). However, its usefulness is dependent upon its depolymerization to fermentable sugar using microbial system (Akin-Osanaiye and Nzelibe, 2005). Effect of acid hydrolysis has been reported in wheat straw and wheat bran (Dahot and Noomrio, 1996; Beatriz et al., 2004). Many fungi and bacteria secrete a multicomponent enzyme system called cellulase that exhibits the ability to saccharify cellulose (Wood et al.*,* 1986). The hypothesis acceptable now indicates that three, rather than two enzymes are essential for the decomposition of cellulosic biomass. Endo-β-glucanase (1,4-β-D-glucan glucanohydrolase or CMCase) acts randomly on cellulose chains yielding glucose and cello-oligosaccharides. Exo-β-glucanase (1,4-B-D-glucan cellobiohydrolase or Avicelase) attacks the non-reducing end of cellulose yielding cellobiose while β-glucosidase (cellobiase) finally hydrolyses cellobiose to glucose.

The crystallinity and lignification limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents (Fan et al*.,* 1987). However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported (Tang et al., 1996; Kumakura, 1997; Wu, 1997; Depaula et al*.,* 1999; Kansoh et al., 1999). Pre-treatment of cellulose opens up the structure and removes secondary interaction between glucose chains

*Corresponding authors E-mail: zeechuks@yahoo.com. Tel: +2348036005184.

(Fan et al., 1987; Tang et al., 1996). High-yield cellulase production by *Trichoderma reesei* Zu-02 on corn cob residue has been reported (Xia and Shen, 2003). Cellulase production level of 5.6×10^{-2} U/ml using *Aspergillus flavus* on bagasse pre-treated with caustic soda was reported (Solomon et al., 1999). Ojumu et al. (2003) reported high cellulase activity using *Aspergillus niger* with the value 7.4×10^{-2} U/ml for sawdust, as well as 5.7×10^{-2} and 5.0×10^{-2} U/ml for bagasse and corn cob, respectively. Wheat straw and rice were delignified and employed as substrates for cellulase production by *A. niger* CS14. Maximum production of β-glucosidase and CM-cellulase by *Aspergilluis fumigatus* grown on sulphuric acid and hydrochloric acid pretreated wheat straw substrate in comparison to nitric acid and perchloric pretreated wheat straw was also reported (Dahot and Noomrio, 1996). The pattern of cellulase enzyme production by this organism in the wheat straw and rice husks media showed an optimum time of 72 h (128 U/ml) and 96 h (120 U/ml), respectively (Raji et al., 1998). Cellulase production depends on the type of substrate, pretreatment and strain of microorganism used (Ali et al., 1991). The present study was therefore carried out to investigate the effect of acid hydrolysis of bitter kola pulp wastes on CM-cellulase and β-glucosidase production using *A. niger*.

MATERIALS AND METHODS

Plant material and microorganism

Garcinia kola was seperated from the pulp. One kilogram of the pod was dried in the solar dryer and ground into powder using a blender. The sample was packaged and then stored in the deep freezer at 4°C. The *A. niger* was isolated from the soil and identified in Microbiology Department laboratory, Ahmadu Bello University, Zaria. The *A. niger* was added into a freshly prepared potato dextrose agar (PDA) and left for four days. Tween 80 (2%) solution was prepared and sterilized for one hour. *Aspergillus niger* spores were transferred into the solution to get uniform suspension. Number of spores was counted with binocular using haemocytometer, and the spore load was 9.55×10^{7}/ml.

Chemical and reagents

Carboxymethylcellulose (CMC), salicin, sodium potassium tartarate, dinitrosalicylic acid, sulphuric acid, hydrochloric acid and standard glucose were of analytical grade and products of British Drug House (BDH) Poole, England and Sigma Chemicals (USA).

Acid hydrolysis

Ten grams of the solar dried sample were weighed into 250 cm^3 conical flask and 100 ml of distilled water added. Two milliliters each of sample were taken and added into six test tubes. Two milliliters of distilled water and 4.0 ml of 2.0 M sulphuric acid were added to each flask except one which was taken as control. The flasks were covered with aluminum foil paper and placed in a boiling water bath. A test tube was removed hourly, neutralized with 4.0 ml of 0.4 M sodium hydroxide and the concentration of reducing sugar released was determined by dinitrosalicylic acid (DNS) method described by Miller (1959).The procedure was repeated using 2.5 M and 5.0 M sulphuric acid and 2.0, 2.5 and 5.0 M hydrochloric acid. Hydrolysis was carried out for 5 h in duplicates.

Cm-cellulase and β-glucosidase production and assay

The enzyme production was as described by Dahot and Noomrio (1996). 100 g of dried sample each were weighed into two 2.0 L conical flasks and 1.0 L sulphuric acid (0.5%) was added to one conical flask (acid treated) while 1000 ml distilled water was added to the other (control). The flasks were covered with aluminium foil paper and heated for 2 h on flame. The flasks were autoclaved for 30 min, allowed to cool and the sample filtered through Whatman no.1 paper. 50 ml of solubilized acid hydrolysed sample was added into nine 250 cm^3 conical flask. The flasks were covered with aluminium foil and autoclaved for 20 min and were allowed to cool at room temperature. 1.0 ml of 9.33×10^{7} spores' suspension of *A. niger* were added aseptically to each flask and incubated at 30ºC. A flasks was removed every 24 h and 1.0 ml of digest added into a test tube. 1.0 ml of 1% carboxymethylcellulose (CMC) substrate was added and the tube was covered with aluminium foil and incubated at 30ºC for one hour. The concentration of reducing sugar released was determined by DNS method and cellulase activity was determined as reported by Mandels et al. (1976) as amount of enzyme that released 1 μ moles of glucose per ml from CMC under the assay conditions. The experiment was carried out for nine days in duplicates. The same was repeated for control sample. The procedure was repeated using maize and rice husks. The β-glucosidase activity was determined as reported by Sternberg et al. (1977) as the amount of enzyme that released one micromole of glucose per ml from cellobiose under the standard assay condition.

RESULTS AND DISCUSSION

The concentration of reducing sugar released by 2.0, 2.5 and 5.0 M sulphuric and hydrochloric acids was maximum at 3 and 5 h hydrolysis, respectively (Table 1). This shows that sulphuric acid is preferable for the hydrolysis of bitter kola pulp waste (Fan et al., 1987). 2.5 M sulphuric acid released the highest fermentable sugar. Several pretreatment methods are reported in the literature such as physical, chemical and enzymatic but sulphuric acid of about 0.5% is usually used at 150 – 185°C (Dahot and Noomrio, 1996).

Effect of acid hydrolysis of bitter kola pulp wastes on CM-cellulase and β-glucosidase production levels is shown on Table 2. Acid hydrolysis significantly ($P<0.05$) increased CM-cellulase and β-glucosidase production levels by 500% at 96 h and 200% at 120 h, respectively. This is higher than what was reported previously (Dahot and Noomrio, 1996). This could be due to the nature of agrowaste, pretreatment or the species of *Aspergillus* used (Ali et al., 1991). Acid hydrolysed bitter kola pulp waste is a better substrate for cellulase and β-glucosidase production. The effect of acid hydrolysis of bitter kola pulp on CM-cellulase level compared with the values obtained from maize and rice husks is shown in

Table 1. Effect acid hydrolysis (2.0M, 2.5M, 5.0M Sulphuric acid and hydrochloric acid respectively) of bitter kola pulp on concentration of reducing sugar.

Time (hr)	Concentration of reducing sugar (g/100g)					
	Sulphuric acid			Hydrochloric acid		
	2.0M	2.5M	5.0M	2.0M	2.5M	5.0M
0	15.9±0.1	15.9±0.1	15.9±0.1	15.9±0.1	15.9±0.1	15.9±0.1
1	21.7±0.1	43.6±0.4	30.4±0.9	33.0±0.5	42.1±1.1	42.4±0.2
2	30.4±0.9	61.2±0.4	41.4±1.2	38.3±1.6	43.4±1.7	47.4±0.1
3	84.2±0.3	96.4±0.2	55.1±1.4	55.0±0.4	51.5±0.1	51.7±0.4
4	68.1±0.1	84.8±0.4	46.5±0.5	31.8±0.9	58.5±0.1	54.8±0.9
5	57.1±0.4	69.3±0.01	43.1±0.0	42.7±0.7	68.4±1.0	69.4±0.2

Results are expressed as means ± SD of duplicate analysis

Table 2. Effect of acid hydrolysis of bitter kola pulp on cellulase and β-glucosidase production level.

Time (hr)	Enzyme production level (U/ml/hr)			
	CM-Cellulase		β-glucosidase	
	Control	Acid hydrolysed	Control	Acid hydrolysed
24	34.4±2.1	36.1±1.3	27.8±2.3	47.4±2.3
48	152.1±0.4	73.6±1.4	81.5±0.4	84.5±0.4
72	37.6±2.3	100.3±0.4	89.0±0.4	150.6±0.4
96	55.6±2.3	264.7±2.3	164.8±0.5	175.7±1.0
120	63.8±2.3	245.9±2.6	152.1±0.4	309.4±1.0
144	75.5±0.4	166.3±0.5	151.1±0.4	263.2±0.4
168	40.8±2.2	89.0±4.4	89.7±0.5	221.5±2.3
192	68.7±2.3	21.3±2.3	37.6±2.3	162.2±1.0
216	80.1±2.3	9.8±2.3	14.7±2.2	154.7±2.3

Results are expressed as means ± SD of duplicate analysis

Table 3. Effect of acid hydrolysis of bitter kola pulp on CM-cellulase level compared with the values from maize and rice husks. (IU/ml/hr).

Time(hr)	Control			Acid hydrolysed		
	Bitter Kola pulp	Maize husks	Rice husks	Bitter kola pulp	Maize husks	Rice husks
24	27.8±2.3	65.8±2.3	69.0±1.2	47.4±2.3	72.2±2.3	85.0±2.3
48	81.5±0.4	69.0±1.0	85.0±2.3	84.5±0.4	78.6±1.2	88.2±1.0
72	89.0±0.4	85.0±0.8	91.4±0.8	150.6±0.4	88.2±1.0	65.8±0.8
96	164.8±0.5	88.2±2.3	78.6±1.2	175.7±1.0	85.0±2.3	72.2±2.3
120	152.1±0.4	91.4±1.2	65.8±2.3	309.4±1.0	88.2±0.8	78.6±1.0
144	151.1±0.4	94.6±0.4	69.0±0.4	263.2±0.4	91.4±2.3	85.0±2.3
168	89.7±0.5	94.6±2.3	85.0±2.3	221.5±2.3	88.2±0.4	97.8±0.8
192	37.6±2.3	85.0±0.8	91.4±0.8	162.2±1.0	78.6±2.3	104.2±0.4
216	14.7±2.2	81.8±0.4	88.2±2.3	154.7±2.3	81.8±2.3	110.7±2.3

Cellulase activities are expressed as means ± SD of duplicate analysis, (IU/ml/hr)

Table 3. Acid hydrolysis of bitter kola pulp waste significantly ($P<0.05$) increased cellulase production and was higher by a factor of 310 and 370% at 96 h for maize and rice husks, respectively. Also, the effect of acid

Table 4. Effect of acid hydrolysis of bitter kola pulp on β-glucosidase level compared with the values from maize and rice husks.(IU/ml/hr).

Time(hr)	Control			Acid hydrolysed		
	Bitter Kola pulp	Maize husks	Rice husks	Bitter kola pulp	Maize husks	Rice husks
24	34.4±2.1	65.8±2.2	69.0±2.3	36.1±1.3	72.2±2.3	85.0±2.3
48	351±0.4	69.0±1.2	85.0±0.8	73.6±1.4	78.6±1.3	88.2±1.2
72	37.6±2.3	85.0±2.3	91.4±1.2	100.3±0.4	88.2±2.3	65.8±0.8
96	55.6±2.3	88.2±1.0	78.6±0.4	264.7±2.6	85.0±0.8	72.2±0.4
120	63.8±2.3	91.4±2.3	65.8±2.3	245.9±2.6	88.2±0.4	78.6±2.3
144	75.5±0.4	94.6±0.4	69.0±0.8	163.3±0.5	91.4±2.3	85.0±1.7
168	40.8±2.2	94.6±2.3	85.0±1.2	89.0±0.4	88.2±1.2	97.8±2.3
192	68.7±2.3	85.0±1.2	91.4±2.3	21.3±2.3	78.6±0.8	104.2±1.2
216	80.1±2.3	81.8±2.3	88.2±2.3	9.8±2.3	81.8±2.3	110.7±2.3

B-glucosidase activities are expressed as means ± SD of duplicate analysis(IU/ml/hr).

hydrolysis of bitter kola pulp on β-glucosidase level compared with the values obtained from maize and rice husks is shown on Table 4. Acid hydrolysis of bitter kola pulp waste significantly ($P<0.05$) increased β-glucosidase production and was higher by a factor of 350 and 400% at 120 h when compared with maize and rice husks respectively. This showed that bitter kola pulp waste is a better substrate for CM-cellulase and β-glucosidase production compared with maize and rice husks.

In conclusion, this study revealed that pretreatment of bitter kola pulp wastes with 2.5 M sulphuric acid for 3 h enhanced cellulase and β-glucosidase production. Acid hydrolysis achieved over 94% depolymerization of the cellulosic materials to fermentable sugar.

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