

ENZYMATIC SACCHARIFICATION OF SOME AGRO-INDUSTRIAL CELLULOSIC WASTES BY CELLULASE PRODUCED FROM A MIXED CULTURE OF *ASPERGILLUS NIGER* AND *SACCHAROMYCES CEREVISIAE* GROWN ON SORGHUM POMACE

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Abstract

Production of cellulase by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisiae* using sorghum pomace as nutrient source was investigated. Sorghum pomace was further supplemented with mineral elements to evaluate its effect on cellulase production. All the sorghum pomace media recorded significantly ($P < 0.05$) higher level of cellulase enzyme (2.06–4.06 units/ml) than that of carboxymethyl-cellulose medium (1.72 units/ml). Mixed culture fermentation significantly ($P < 0.05$) enhanced higher cellulase production than mono culture media. However, mineral supplementation significantly ($P < 0.05$) suppressed cellulase production. Cellulosic substrates were significantly ($P < 0.05$) more susceptible to crude enzyme from sorghum pomace than that of carboxymethyl cellulose

1. Introduction

Cellulases are undefined extracellular enzyme mixture produced by various microbes (fungi and bacteria), insects and lower animals which can hydrolyse cellulose (Shin and Yan, 1996). Cellulase activity in vivo is not mediated by a single enzyme, but rather by a complex system of several enzymes which act synergistically (Eveleigh, 1987; Lamed *et al.*, 1987; Gbelekeloluwa and Moo-Young, 1991). The cellulase complex forms a unique structure which has been called "cellulosome" and appears to be ubiquitous among cellulolytic micro-organisms (Lamed *et al.*, 1987). Cellulosome associated with fungi are Endo I, 4- β -glucanase, Exo-1, 4- β -glucanase and β -glucosidase (Eveleigh, 1987; Coughlan, 1990).

Cellulase is an industrially useful enzyme in brewing, chemical, textile, paper, food and pharmaceutical industries (Brown *et al.*, 1987). Despite the wide application of cellulases it is heavily imported into Nigeria.

Use of local substrates for cellulases production has been actively investigated (Udotong, 1997; Raji *et al.*, 1998; Abu *et al.*, 2001, 2003) and a number of agro- industrial wastes have been shown to be good substrates for cellulase production. However there is a dearth of information on the application of the enzyme produced (Udotong, 1997).

This paper reports on the preliminary investigation of the application of crude cellulase to cellulosic hydrolysis and the effect of mixed culture and mineral supplementation on cellulase production and cellulose hydrolysis.

2. Materials and Methods

Organism

Aspergillus niger sl.1 and *Saccharomyces cerevisiae* used were isolated from soil and rotten cassava respectively. They

were purified, characterized and identified by the Department of Microbiology, Ahmadu Bello University Zaria-Nigeria.

Cellulosic Substrates

Corn bran, corn cob, rice bran, wheatbran and sorghum pomace were obtained from harvest dump or households in Zaria, Kaduna State.

Culture Media

Aspergillus niger sl.1 inoculum was prepared in yeast peptone soluble starch (YPS) agar medium containing the following in g/l; yeast extract, 5; peptone 10; soluble starch, 10; and agar 10. The culture medium was incubated for 96 hours at 30°C. The medium for *S.cerevisiae* consisted of the following in g/l; sucrose, 50; yeast extract 10; peptone 5; KH_2PO_4 , 1; $(\text{NH}_4)_2\text{SO}_4$, 2 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1. The medium was incubated for 96 hours at 30°C.

Fermentation for enzyme production

The fermentation medium comprised the following in g/l; yeast extract, 0.5; $(\text{NH}_4)_2\text{SO}_4$, 10.5; KH_2PO_4 , 10, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; CaCl_2 , 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.013; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0067 and carboxymethyl cellulose, 40. For studies on the use of sorghum pomace and mineral supplementation, carboxymethylcellulose was substituted with sorghum pomace (40g) in mineral salt media while whole pomace was used as the sole nutrient source in non-mineral supplemented media.

The media were sterilized for 15 minutes at 121°C in an autoclave and the pH was adjusted to 5.0. with 0.1M hydrochloric acid or sodium hydroxide. Monoculture media (including

(including that of carboxymethylcellulose CMC) were inoculated with a spore suspension (3.62×10^5 spores) of *A.niger* sl.1 while those for mixed culture were inoculated with 1.81×10^5 spores each of *A.niger* sl.1 and *S.cerevisiae*. The media were incubated at 30°C in an orbital shaker (CAT No. 14460; APP No. IB, 2621 Cuo Gallen KAMP) set at 100 rpm for 72 hours. Three replicate fermentation was carried out for each culture.

Enzyme Assay

Cellulase activity was assayed according to the method described by Ali *et al.*, 1991). The reaction mixture consisted of 1ml each of 0.1M acetate buffer (pH 5.0), crude enzyme and 1% CMC solution. The mixture was incubated at 30°C in a test-tube for 10 minutes and total reducing sugar was determined by the Dinitrosalicylate (DNS) method (Miller, 1959).

One unit of cellulase activity was defined as the amount of enzyme which released 1µmole glucose min⁻¹.

Saccharification of Cellulosic Substrates

Evaluation of substrates for enzymatic hydrolysis was carried out with 5g (dry weight) of substrate, 35ml 0.1M acetate buffer pH, 5.0 and 60ml of crude enzyme in 250ml conical flasks and incubated in a water bath at 30°C for 1 hour. The reducing sugar was measured by the dinitrosalicylate (DNS) procedure in aliquots of the suspension. Percentage saccharification of this 5% slurry was calculated as the product of milligram glucose and 1.8 (Mandels *et al.*, 1974).

Effect of pH on crude enzyme activity

Acetate buffer of 0.1M was used for pH 3.0-7.0 while 0.1M phosphate buffer was used for pH of 8.0 and 9.0 to study the effect of pH on cellulase activity.

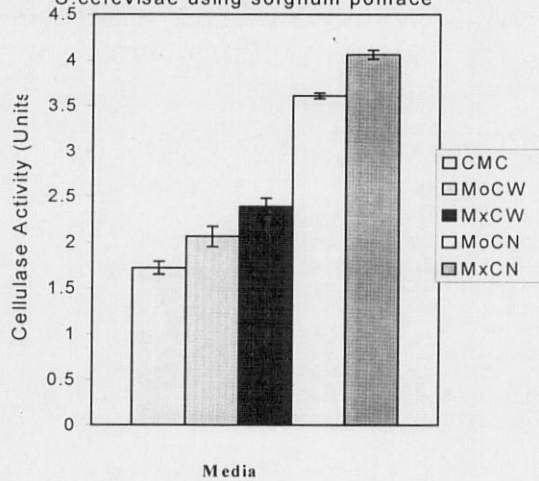
Statistical Analysis

Statistical analyses were by analysis of variance (ANOVA). Turkey test was used to identify means that differed significantly.

3. Results and Discussion

Cellulase production using different sorghum pomace media compared to carboxymethyl cellulose medium is shown in Figure 1. All the sorghum pomace media recorded significantly ($P < 0.05$) higher level of cellulase activity than that of CMC medium. This result is encouraging because until now carboxymethyl cellulose has been regarded as the conventional substrate for cellulase production. Cellulase production in mixed culture media (with or without mineral supplementation) was significantly ($P < 0.05$) higher than the corresponding monoculture media (Figure 1). This could be due to kinetic advantage of symbiotic relationship between fungus and yeast in the mixed culture medium. End-product inhibition of cellulases produced by fungus is well-known (Ladish *et al.*, 1983; Holzapple *et al.*, 1990) but periodic removal of product sugars were found to be

Figure 1: Cellulase Production (Units/ml) by mixed culture of *A.niger* and *S.cerevisiae* using sorghum pomace



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- CMC = Carboxymethylcellulose
- MoCW = Monoculture of *A.niger* with mineral supplementation
- MxCW = Mixed culture of *A.niger* and *S.cerevisiae* with mineral supplementation
- MoCN = Monoculture of *A.niger* without mineral supplementation
- MxCN = Mixed culture of *A.niger* and *S.cerevisiae* without mineral supplier

effective at reducing end product inhibition (Gregg and Saddler, 1996). The fermentation of sugars to ethanol by *S.cerevisiae* reduces the end-product inhibition of cellulases produced by *A.niger*. This could be responsible for increased cellulase production in the mixed culture media. (Szczo drak and Targonski, 1989; Phillipidis, 1994).

Supplementation of sorghum pomace with mineral elements was found to suppress cellulase production by over 40% in both monoculture and mixed culture media as shown in Figure 1. This implies that sorghum pomace has adequate minerals nutrients required for growth of the organism as well as enzyme production. Excess levels of some mineral ions in the medium has been reported to have inhibitory effect on growth and activity of micro-organisms (Mbanefo, 1991).

The results of the effect of crude enzymes on enzymatic hydrolysis of the cellulosic substrates are illustrated in Table 1. It can be observed that wheatbran and ricebran are generally more susceptible to enzymatic hydrolysis than sorghum pomace, corn bran or corn cob. Even though sorghum pomace was used as substrate for crude cellulase pro-

Figure 2: Total Saccharification of Cellulose by Crude Enzyme Extract

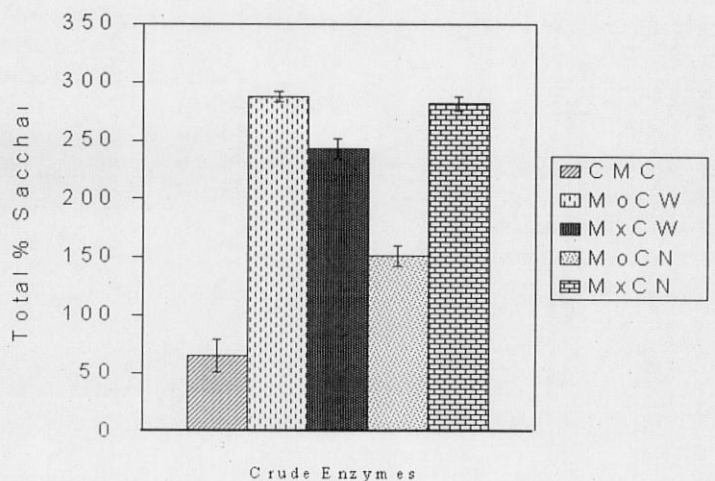


Table 1: Effect of Crude enzyme Extract on the Saccharification (%) of Cellulosic Substrates

SUBSTRATES	CMC	M ₀ CW	M _x CW	M ₀ CN	M _x CN	Total % Saccharification
Corn bran	4.05 ± 0.65 ^c	52.65±4.07 ^a	52.65±2.71 ^a	25.65±2.05 ^b	49.95±2.61 ^a	184.95 ± 21.63
Corn cob	4.05 ± 0.76 ^e	58.05±3.89 ^a	47.25 ±3.77 ^c	22.95±3.91 ^d	52.65±2.82 ^b	184.95 ± 22.78
Rice bran	17.55± 1.66 ^c	54.00±2.11 ^a	55.35 ±2.11 ^a	39.15±2.67 ^b	58.05±3.33 ^a	224.10 ± 16.93
Wheat bran	36.45±2.89 ^c	62.10±1.04 ^b	33.75±4.01 ^d	40.50±3.62 ^c	66.15±2.69 ^a	238.950± 15.17
Sorghum pomace	2.70 ±0.71 ^d	60.75±2.06 ^a	54.00±3.92 ^b	22.95±2.74 ^c	55.35±2.14 ^b	195.75 ±25.20

Values are means ± SD of three replicate cultures

Values not followed by the same superscript in a row are significantly different (P<0.05).

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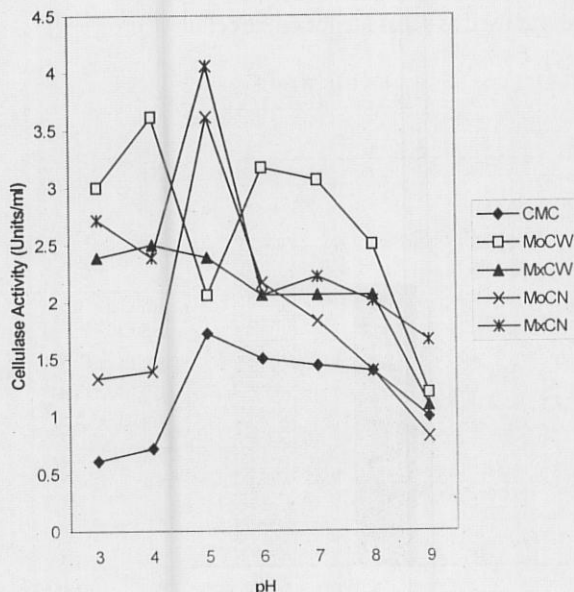
CMC	=	Carboxymethylcellulose
M ₀ CW	=	Monoculture of <i>A.niger</i> with mineral supplementation
M _x CW	=	Mixed culture of <i>A.niger</i> and <i>S.cerevisiae</i> with mineral supplementation
M ₀ CN	=	Monoculture of <i>A.niger</i> without mineral supplementation
M _x CN	=	Mixed culture of <i>A.niger</i> and <i>S.cerevisiae</i> without mineral supplementation

duction, no correlation existed between the capacity of sorghum pomace to induce cellulase enzyme and its susceptibility to enzymatic hydrolysis from its own crude enzyme (Okolo *et al.*, 1995). Hence cellulase produced using sorghum pomace could have a broad spectrum of substrate for hydrolysis. The results further show that the susceptibility of the cellulose substrates depends on the crude enzyme source (see Figure 2). Cellulosic substrates, based on Fig. 2, were more resistant to crude enzyme from CMC than those from sorghum pomace media. However mixed culture fermentation or mineral supplementation had no significant (P>0.05) influence on the hydrolytic capacity of crude enzyme extracts (Figure 2).

The activity of the crude enzyme extract was evaluated at various pH values (Figure 3). Optimum pH for enzyme activity, which varied from 4.0 to 6.0 depended on the media

source. The optimum pH values for cellulase activities from fungal species has been reported to vary from species ranging from 3.0 to 6.0 (Ali *et al.*, 1991; Shambe, 1999; Abu *et al.*, 2003). Two pH optima were discernable for MoCW and M_xCN enzymes. Okolo *et al.* (1995) reported similar pH optima for raw starch digesting amylase using 0.1M acetate buffer of pH 3.0-7.0 when *A.niger* was grown on native starch.

This presumably suggests two or three distinct cellulolytic activities in these media. Cellulase has been reported to be composed of at least three different components which act synergistically (Eveleigh, 1987; Coughlan, 1990). Single pH peak in some other media (CMC and MoCN in Fig. 3) shows the closeness of the optimal pH for different cellulase components (Ueda, 1984). pH insensitivity observed over a wide pH range 3.0 – 8.0 in these media could be a reflection of the pH relationship of the synergistic interaction of the various cellulases (Coughlan, 1990).

Figure 3: Effect of pH on Cellulase Activity (Units/ml)**4. Conclusion**

In conclusion, cellulase which demonstrates high level hydrolytic capacity can be produced by mixed culture of *A.niger* and *S.cerevisiae* using sorghum pomace. Mixed culture of *A.niger* and *S.cerevisiae* significantly (P<0.05) enhanced cellulase production but further supplementation of pomace with mineral nutrients suppressed enzyme production. Sorghum pomace is preferred to carboxymethyl cellulose for cellulase production. The most pressing requirement for further development and practical application of enzymatic saccharification of cellulose using crude enzyme from this source, is a thorough elucidation of the economics of the complete process through large scale studies. Nevertheless, the local availability and the status of sorghum pomace as food processing by-product could be of a great economic value to developing nations like Nigeria.

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