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Comparative Hydrolytic Properties of Culture Filtrate of *Trichoderma viride Fd18* and *Aspergillus ustus* Fd12 on Lignocellulosic Substrates

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This paper reports the enzymatic hydrolysis of alkali-treated agri-waste with xylanase from *Trichoderma viride* Fd18 and *Aspergillus ustus* Fd12 isolated from saw dust. Alkali-treated wheat bran had the highest hydrolysis rate of 35% (*A. ustus* Fd12 xylanase) and 48.6% (*T. viride* Fd18 xylanase). Saw dust from different plants have a low rate of hydrolysis. The optimal pH and temperature for hydrolysis of wheat bran by xylanase from the 2 fungi were pH 4.0 – 4.5 and 35°C. A wheat bran concentration of 6% and incubation time of 24hr increased rate of hydrolysis. The enzyme can be used to hydrolyze xylan in agricultural residues for commercial applications.

Key words: Wheat bran, *Trichoderma viride*, *Aspergillus ustus*, hydrolysis, xylan, xylanase.

The focus in recent years is on the use of microbial enzymes for fuel production and industrial applications through the use of renewable resources such as agricultural wastes. Since hemicellulose is the second most abundant fraction available in nature, hemicellulases have become important in the enzymatic hydrolysis of lignocellulose in agricultural wastes[1]. Xylan is the most abundant of the hemicelluloses in plant cell walls. It has a linear backbone structure consisting of β-1,4-linked D-xylopyranose units that may contain branches of L-arabinofuranosyl, acetyl and glucuronosyl residues. Xylan degrading enzymes include xylanase, β-xylosidases and various side chain cleaving enzymes[2].

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There is great interest in the enzymatic hydrolysis of xylan due to possible applications in feedstock, chemical production, paper manufacturing and textiles industries. The structure of hemicellulose sets barriers for chemical and enzymatic degradation. Even in the enzymatic hydrolysis of pure xylan, a gradual drop in the reaction rate is observed due to end-product inhibition[3], and inactivation and unproductive binding or entrapment of the enzymes in the small pores of the lignocellulose[4,5]. Factors having significant effect on the rate of hydrolysis of lignocellulosic materials can be divided into two parts: those related to the structure of the substrates (degree of polymerization, crystallinity, structural composition, particle size, available surface area, degree of fibre swelling, pore structure and distribution) and those related to the mechanisms and interactions of the lignocellulolytic enzymes[4,6].

Many microorganisms, particularly fungi, display xylan degrading enzyme activities.

Among the fungi, strains of *Aspergillus, Trichoderma* and *Penicillium* are sources of xylanolytic enzymes[7].

In this paper, we report the results of comparative studies of hydrolysis of a number of agricultural wastes with culture filtrate of *Trichoderma viride* Fd18 and *Aspergillus ustus* Fd12 and the conditions affecting the hydrolysis.

MATERIAL AND METHODS

Lignocellulosic Substrates and pretreatment

Agricultural wastes, namely sugarcane bagasse, sawdust and wheat bran, were used as substrates for this work. Saw dust from timber of *Mallotus oppositifolius* ("Orokoro"), *Daniella* sp ("Iya"), *Boscia angustifolia* ("Laoro"), *Celtis* sp ("Ita") and *Terminalia superba* ("Afara") was obtained from the Bodija saw mill, while wheat bran was obtained from the Bodija market, Ibadan. Sugarcane bagasse was obtained from heap of dries remains of spent sugarcane in the same market. The bagasse obtained was dried at 65°C for 48hr. All the 3 types of substrates were ground (40 mesh), washed several times in hot water to remove free reducing sugars remaining in them and dried as earlier described[8]. Weighted amount of each sample was then alkali-treated by autoclaving at 121°C for 30 min with 0.25M NaOH (20 ml g substrate^{-1}). The substrates recovered by filtration through muslin cloth were thoroughly washed with deionised water and neutralized with 0.25M HCl. The substrates were finally washed with many changes of deionised water and dried at 65°C to constant weight [9].

Enzyme source

Trichoderma viride Fd18 and *Aspergillus ustus* Fd12 were isolated from saw dust collected from the Bodija saw mill, Ibadan, Nigeria. The isolate were maintained on potato dextrose agar slants at 4°C. The isolates were selected based on screening for high extracellular xylanase production in Mandels and Weber[10] medium composed of (g l^{-1}): 1.4 $(NH_4)_2SO_4$, 2 KH_2PO_4, 0.3 $CaCl_2$, 0.3 $MgSO_4.7H_2O$, $2CoCl_2$, oat-spelt xylan (Sigma Chemical Co.), 1% and 1ml trace elements. The composition of the trace element solution was (g l^{-1}) $MnSO_4.H_2O$, 1.56; $FeSO_4.7H_2O$, 5; $ZnSO_4.7H_2O$, 1.4. The culture filtrate of the isolate grown in Mandels and Weber[10] medium with 2% wheat bran as carbon source at 30^{0}C for 6 days served as the source of enzyme.

Hydrolysis of Substrates

A suspension of substrate (10 mg/ml) was prepared by adding 100 ml of 50 mM acetate buffer (pH 5.0) to 1 gram of the substrate. Fifteen millilitre (15 ml) of each of the substrate suspension was sterilized at 121°C for 20 min in 50 ml conical flasks. Five ml of culture filtrate obtained as described previously from *T. viride* Fd18 and *A. ustus* Fd12 was added to the sterilized substrates. Hydrolysis was performed at 30°C for 48 hr and samples were withdrawn at 1, 4, 24 and 48 hr for analysis of reducing sugar. The resultant supernatant following centrifugation (2500g, 15 min) was assayed for total reducing sugars using DNSA method[11]. The release of sugar is expressed as equivalent of xylose. The percentage hydrolysis was calculated as given by[12].

$$\% \text{ Hydrolysis} = \frac{Xylose(mg/ml) \times 100}{Substrate(mg/ml)}$$

Effect of pH on hydrolysis

The effect of pH on rate of hydrolysis was studied using *A. ustus* Fd12 and *T. viride* Fd18 xylanases by preparing a suspension of 2% wheat bran in 50 mM acetate buffer at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5. The hydrolysis was carried out as earlier described. Total reducing sugar produced was determined by the DNSA method[11].

Effect of temperature on hydrolysis

The optimum temperature for hydrolysis of wheat bran by *T. viride* Fd18 and *A. ustus* Fd12 xylanases was determined by incubating the hydrolysis reaction mixtures earlier described at different temperatures ranging between 30°C and 55°C for 48 hr.

Effect of time on hydrolysis

The optimum time for hydrolysis was obtained by withdrawing aliquot from the reaction of wheat bran and *A. ustus* Fd12 and *T. viride* Fd18 xylanase after 4, 8, 12, 16, 20 and 24 hr and determining the level of reducing sugar released.

Effect of substrate concentration on hydrolysis

The effect of substrate concentration on extent of hydrolysis was determined by varying the concentration of the wheat bran in the reaction mixture with *Aspergillus ustus* Fd12 and *T. viride* Fd18 culture filtrate to 5, 10 and 15%.

Table 1. Hydrolysis (%) of different lignocellulosic substrates by xylanase from *Aspergillus ustus* Fd12

Substrate	Treatment / Time of Hydrolysis (hr) / Percentage hydrolysis							
	Untreated				Alkali treated			
	1	4	24	48	1	4	24	48
Wheat bran	*8.9±1.56[a]	10.4±0.42[a]	20.4±0.57[a]	32.8±0.28[a]	5.5±0.71[a]	16.5±0.00[a]	21±1.41[a]	35±2.83[a]
Sugarcane bagasse	0.6±0.00[d]	1.4±0.00[d]	2.1±0.14[d]	3.5±0.28[de]	3.4±0.00[b]	7.6±0.00[b]	8.2±1.13[b]	15.3±0.42[b]
Mallotus oppositifolus Saw dust	0.2±0.14[d]	0.4±0.14[f]	1.0±0.28[e]	2.7±0.00[de]	0.4±0.00[c]	0.6±0.00[d]	1.2±0.00[e]	4.4±0.57[c]
Daniellia sp	0.3±0.00[d]	1.2±0.28[de]	2.1±0.14[d]	3.0±1.13[e]	0.6±0.00[c]	1.0±0.00[cd]	3.0±0.71[cd]	4.4±0.14[c]
Boscia angustifolia Saw dust	0.3±0.00[d]	0.6±0.00[f]	0.9±0.00[e]	1.8±0.28[e]	0.3±0.00[c]	0.9±0.14[cd]	2.7±0.28[cde]	5.4±0.42[c]
Celtis sp Saw dust	0.6±0.00[d]	0.9±0.00[def]	1.2±1.20[e]	3.6±0.00[de]	0.8±0.00[c]	1.7±0.00[cd]	2.1±0.28[c]	5.2±1.70[c]
Terminalia superba Saw dust	0.4±0.14[d]	0.9±0.90[def]	2.7±0.28[d]	4.7±1.84[d]	0.3±0.00[c]	2.1±1.56[c]	3.0±0.71[de]	5.6±0.42[c]
Xylan	6.9±0.00[b]	9.6±0.71[b]	10.0±0.28[b]	20.6±0.42[b]				
CMC	4.3±0.00[c]	5.0±0.00[c]	8.1±0.14[c]	15.6±1.13[c]				

*Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different superscript within each column differ significantly ($p \leq 0.05$).

Table 2. Hydrolysis (%) of different lignocellulosic substrates by xylanase from *Trichoderma viride* Fd18

Substrate	Treatment / Time of Hydrolysis (hr) / Percentage hydrolysis							
	Untreated				Alkali treated			
	1	4	24	48	1	4	24	48
Wheat bran	*9.8±0.14^{b}	24.0±1.41^{a}	29.0±0.71^{a}	45.8±0.07^{a}	14.5±0.71^{a}	20.4±0.57^{a}	31.8±0.80^{a}	48.6±2.83^{a}
Sugarcane bagasse	2.0±0.00^{d}	4.1±0.35^{c}	7.4±1.41^{c}	12.8±1.41^{d}	3.7±0.99^{b}	10.0±0.00^{b}	17.8±0.00^{b}	31.8±2.05^{b}
Mallotus oppositifolus Saw dust	0.8±0.14^{ef}	2.0±0.00^{cd}	3.3±0.99^{de}	5.1±0.07^{e}	0.7±0.00^{e}	3.2±0.20^{d}	4.0±0.00^{d}	5.7±0.00^{e}
Daniellia sp Saw dust	1.1±0.14^{e}	2.0±0.71^{cd}	3.5±0.71^{d}	5.2±0.28^{e}	1.6±0.00^{e}	4.1±0.00^{e}	5.8±0.14^{e}	7.4±0.57^{e}
Boscia angustifolia Saw dust	0.5±0.00^{g}	0.9±0.00^{d}	1.5±0.28^{ef}	2.8±1.13^{e}	1.5±0.00^{e}	2.1±0.00^{e}	2.7±0.42^{e}	6.9±0.14^{e}
Celtis sp Saw dust	0.6±0.00^{fg}	0.9±0.00^{d}	1.3±0.00^{f}	2.4±0.57^{e}	1.7±0.00^{e}	2.2±0.00^{e}	2.6±0.21^{e}	5.4±0.57^{e}
Terminalia superba Saw dust	1.2±0.00^{e}	2.1±0.07^{cd}	3.4±0.14^{d}	4.4±1.13^{e}	1.7±0,00^{e}	2.8±0.42^{e}	4.1±0.14^{d}	6.0±0.00^{e}
Xylan	10.3±0.35^{a}	14.8±0.99^{b}	30.4±0.28^{a}	48.4±0.64^{b}				
CMC	6.2±0.00^{e}	14.0 ±1.41^{b}	17.2±1.27^{b}	22.0±2.83^{c}				

*Each value is a mean of two replicates; ± stands for standard deviation among replicates; means followed by different superscript within each column differ significantly at $p \leq 0.05$.

respectively. The hydrolysis of wheat bran was significantly ($P \leq 0.05$) higher than of the other substrate at all the times treated for culture filtrates from both fungal isolates. The non-agricultural substrates, xylan and carboxy methyl cellulose (CMC) were hydrolyzed at 20.6% and 15.6% by *A. ustus* Fd12 and 48.4 and 22% by *T. viride* Fd18 after 48hr respectively. Saw dust from different woody plants were hydrolyzed to a lesser extent, i.e., were more resistant to enzymatic saccharification.

Culture filtrates from *A. ustus* Fd12 and *T. viride* Fd18 hydrolyzed untreated wheat bran, sugar cane bagasse and saw dust at very low rates compared to the alkali treated substrates. The hydrolysis value of sugar cane bagasse increased from 3.5% to 15.3% for *A. ustus* Fd12 culture filtrate upon alkali treatment (Table 1). Saw dust gave low hydrolysis values (1.8% – 4.7% for *A. ustus* Fd12 and 2.4% – 5.2% for *T. viride* Fd18) which increased slightly after alkali treatment. There was an increase in the rate of hydrolysis with time for all the substrates.

Effect of pH on hydrolysis

The enzymatic hydrolysis of alkali-treated wheat bran by *A. ustus* Fd12 and *T. viride* Fd18 xylanases at different values of pH from 3.5 to 5.5 is shown in Fig. 1. The best hydrolysis was obtained at pH 4 for *A. ustus* Fd12 (16.7%) and pH 4.5 for *T. viride* Fd18 (16.1%). There was a decrease in hydrolysis of wheat bran at pH values above 4 and 4.5 for the two fungi under study. Statistically, there was no significant difference in the rate of hydrolysis at the different pH values tested for the two organisms.

Effect of temperature on hydrolysis

The optimal temperature for hydrolysis of alkali-treated wheat bran by culture filtrates of *A. ustus* Fd12 and *T. viride* Fd18 was 35°C (Fig. 2) yielding 16.5% and 19.6% hydrolysis, respectively, after 24hrs of incubation. The rate of hydrolysis fell at temperatures above 35°C giving 8.3% and 11.4% hydrolysis for *A. ustus* Fd12 and *T. viride* Fd18 culture filtrates respectively at 55°C. The rate of hydrolysis at 35°C was significantly different from the rate at other temperatures.

Effect of substrate concentration on hydrolysis

The effect of alkali-treated wheat bran concentration on percentage of hydrolysis by *A. ustus* Fd12 and *T. viride* Fd18 culture filtrates is shown in Fig. 3. The percentage of hydrolysis increased with increase in concentration of wheat bran and then became steady. The most suitable wheat bran concentration of hydrolysis for both *A. ustus* Fd12 and *T. viride* Fd18 (19% hydrolysis) culture filtrate was 6%. Hydrolysis of alkali-treated wheat bran obtained at 1% concentration differ significantly ($p \leq 0.05$) to other conc.

Effect of incubation time on hydrolysis

The influence of length of incubation on the rate of hydrolysis of alkali-treated wheat bran by culture filtrates of *A. ustus* Fd12 and *T. viride* Fd18 is shown in Figure 4.0. An increase in rate of hydrolysis was observed with time. There was a significant increase ($p \leq 0.05$) in hydrolysis from 9.9% and 11% at 1 hr to 16.5% and 16.8% at 24 hr for *A. ustus* Fd12 and *T. viride* Fd18 respectively.

DISCUSSION

Xylan, the most abundant of the hemicelluloses, could be hydrolysed by mild alkali/acid or enzymatic action[13]. As the enzymatic reaction, by nature, is a more specific process, enzymatic hydrolysis of some lignocellulosic substrates was attempted. Of the lignocellulosic materials used for hydrolysis, wheat bran was found to be more susceptible to enzymatic hydrolysis (Tables 1 & 2). Treatment of alkali treated wheat bran with culture filtrates of *A. ustus* Fd12 and *A. viride* Fd18 produced 35% and 48.6% hydrolysis, respectively. This rate of hydrolysis by wheat ban was followed by alkali treated sugarcane bagasse, while alkali treated sawdust from different plants was found to be more resistant to enzymatic hydrolysis. The high rate of hydrolysis of wheat bran could be attributed to the high hemicellulose and low lignin content of wheat bran compared with the other lignocellulosic substrates. Lignin, which is less sensitive to chemical and enzymatic attack, may limit access of the hemicelluloses to hydrolytic enzymes. Some other important factors which affect relative enzyme susceptibility of lignocellulose are crystallinity, available surface, admixture with impurities and affinity of substrate with enzyme[14]. The different resistances of these agricultural residues to enzymatic hydrolysis have been attributed to their lignin content. Furthermore, the

content, position and structure of xylan are factors associated with different hydrolytic resistance[1]. However, the enzyme appeared to hydrolyse xylan in the cell wall of these plant materials and produced appreciable amount of hydrolysis from all the materials.

Various acid-, or alkali-pretreatment procedures have been reported to improve the susceptibility of lignocelluloses to enzymatic hydrolysis[9,15]. Alkali pretreatment of the agricultural wastes used in this study favoured higher rate of hydrolysis. This could be due to the fact that alkaline pretreatment of a natural plant cell wall residue is the last step leading to the exposure of the lignocellulose components and this aids degradation of such components[16].

Hydrolysis of alkali-treated wheat bran increased and reached a maximum at a concentration of 6.0% and then leveled up at higher concentrations. The leveling of the solubilization at higher substrate concentration might be due to adsorption of enzyme on to the wheat bran during hydrolysis. Hydrolysis of wheat bran was dependent on the concentration of enzyme in the reaction mixture, but decreased with increasing enzyme concentration. A major problem in optimizing hydrolysis reactions is the presence of lignin in lignocellulosic products, which may limit access of the enzyme to the desired substrate.

Results obtained by optimizing the conditions of hydrolysis of wheat bran by *A. ustus* Fd12 and *T. viride* Fd18 xylanases showed that the alkali-treated wheat bran was best hydrolysed at pH 4.0-4.5, 30°C and wheat bran concentration of 6% for 24 hrs. There was an initial faster rate of hydrolysis during the first hour of hydrolysis followed by a decreased rate. This could be due to the enzyme hydrolyzing the readily susceptible regions are hydrolysed early in the process leaving the more resistant substrates. Alternatively, it could result from the gradual accumulation of reducing sugars as the products of hydrolysis. Singh *et al*.[17] reported optimum hydrolysis of alkali-treated bagasse at pH 5.2 and a temperature range of 55 to 60°C, while the optimum hydrolysis of banana waste was at pH 6.0 and 45°C as reported[12]. Though much studies has been made with thermophilic fungi and thermostable enzymes for hydrolysis, fewer studies of enzymes from mesophilic fungi are available[12]. Okeke and Obi[18] obtained higher hydrolysis rate but recorded lower enzyme stability with thermophiles. This work presents a report on hydrolysis of alkali-treated wheat-bran within the mesophilic temperature range.

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