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THE HYDROLYSIS OF AGRO-INDUSTRIAL RESIDUES BY HOLOCELLULOSE-DEGRADING ENZYMES

Leonora Rios de Souza Moreira*, Gaspar Virgilio Ferreira, Sheila Sousa Thurler Santos, Ana Paula Souza Ribeiro, Félix Gonçalves Siqueira, Edivaldo Ximenes Ferreira Filho

Laboratório de Enzimologia, Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, Brasil.

ABSTRACT

Holocellulose structures from agro-industrial residues rely on main and side chain attacking enzymes with different specificities for complete hydrolysis. Combinations of crude enzymatic extracts from different fungal species, including *Aspergillus terreus*, *Aspergillus oryzae*, *Aspergillus niger* and *Trichoderma longibrachiatum,* were applied to sugar cane bagasse, banana stem and dirty cotton residue to investigate the hydrolysis of holocellulose structures. *A. terreus* and *A. oryzae* were the best producers of FPase and xylanase activities. A combination of *A. terreus* and *A. oryzae* extracts in a 50% proportion provided optimal hydrolysis of dirty cotton residue and banana stem. For the hydrolysis of sugar cane bagasse, the best results were obtained with samples only containing *A. terreus* crude extract.

Key words: agricultural waste, enzymatic mixtures, hydrolysis.

INTRODUCTION

Agro-industrial residues are available for exploitation as sources of fuel, food and chemical feedstocks (12). Agro-industrial residues consist basically of lignocellulosic material containing mainly cellulose (40-50%), hemicellulose (30-40%), lignin (8-10%) and a lower percentage of pectin. (3,15). The use of agro-industrial residues as raw materials decreases the impact on the environment because it reuses these sub-products, reducing their accumulation in the environment and adding economic value to the waste. The production of ethanol from agro-industrial residues is a good alternative for improving energy availability. In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues for different applications, including biofuel

production (12). In addition, these agro-industrial residues can be used as a carbon source for the production of enzymes, especially holocellulases, by filamentous fungi. A broad range of hydrolytic enzymes are necessary for the degradation of the carbohydrate portion of lignocellulose (holocellulose) (1, 8, 14). Within this group of enzymes, xylanase, mannanase, polygalacturonase, endoglucanase and exoglucanase have important roles in the hydrolysis of holocellulose (10, 13).

The procedures to optimize the production of fungal holocellulases require only an inexpensive carbon source (13). Here, we investigate the potential use of dirty cotton residue (DCR), sugar cane bagasse (SCB) and banana stem residues (BS) as an inexpensive source of carbon. DCR is the fraction collected from the cotton spinning and yarn forming textile industries that is composed of very short fibers, husks and

*Corresponding Author. Mailing address: Laboratory of Enzymology, Department of Cellular Biology, University of Brasília, Brasília, DF, CEP 70910 900, Brazil.; E-mail: leonorarsm@gmail.com

other dark matter. BS, the grain stalk that supports the banana fruit, is normally discarded from the "packing houses" or delivering centers after harvesting the fruit because it is considered waste due to the great volume generated (10). SCB is the largest Brazilian agro-industrial waste, amounting to approximately 217-380 x 10^6 tons per year (5, 6). The bagasse piles have low economic value and represent an environmental problem due to the risk of spontaneous combustion. This study will investigate the quantity of reducing sugars liberated in the hydrolysis of SCB, DCR and BS using different combinations of enzymatic extracts from filamentous fungi.

MATERIALS AND METHODS

Organisms and enzyme production

The fungi (*Aspergillus flavus, Aspergillus niger, Aspergillus oryzae, Aspergillus terreus, Emericela nidulans, Monilia* sp., *Penicillium corylophilum* and *Trichoderma longibrachiatum*) were obtained from the fungus culture collection of the Enzymology Laboratory, University of Brasília, Brazil, and maintained in PDA medium (2.0% potato broth, 2.0% dextrose and 2.0% agar). An aliquot (2.5 mL) of a spore suspension (10^8 spores/mL) was inoculated in Erlenmeyer flasks containing 500 mL of liquid medium (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4·7H_2O$, 0.01% $(NH_4)_2SO_4$, and 0.06% yeast extract) at pH 7.0 with 1.0% (w/v) sugar cane bagasse or dirty cotton residue as the carbon source. Fungi were grown for eight days at 28°C under agitation at 100 rpm. The resulting supernatants, hereafter called crude extracts, were used for the determination of holocellulose-degrading enzyme activities and extracellular protein concentrations.

Substrates

Xylan preparation was carried out as described elsewhere (12). The filter paper activity (FPase) assay was carried out with a strip of paper of 1 x 6 cm, Whatman number 1.

Residue Pretreatment

SCB, BS and DCR were thoroughly washed with tap water and autoclaved at 121°C for 2 h. After autoclaving, they were dried at 65°C for 48 h and ground to form a homogeneous blend. A fine powder was obtained and used as a substrate for enzymatic hydrolysis experiments.

Enzyme assays

Xylanase activity was determined by mixing 50 μL of enzyme sample with 100 μL of 1% w/v substrate (oat spelt xylan) at 50°C for 30 min. FPase activity (9) was determined using 150 μL of enzyme with filter paper (Whatman N°1) as a substrate at 50°C for 1 h. The amount of reducing sugar released was measured using acid 3,5 dinitrosalicylic acid (DNS) (10). Activity was expressed as μmol reducing sugar formed per min per liter of enzyme solution, or IU/L. Glucose and xylose were used as standards. Protein concentration was measured by Bradford assay (2) using bovine serum albumin as the standard. Glucose content was measured by the glucose oxidase method (16).

Hydrolysis Assays

The degradation of agro-industrial residues by holocellulose-degrading enzyme crude extracts was determined by quantifying the reducing sugars that were released during the BS, SCB and DCR residue breakdown. The degradation of agro-industrial residues was determined by incubating different proportions (30% – 70%, 50% – 50% and 70% – 30%) of crude enzyme extracts named combinations A, B and C, respectively (comb A, B and C). The crude extracts from *A. terreus*, *A. oryzae* and *A. niger* were obtained from growth cultures containing SCB, while the crude extract from *T. longibrachiatum* was from a growth culture containing DCR. Hydrolysis of SCB, DCR and BS was performed as follows: 10 mL of sodium acetate buffer (50 mM, pH 5.0) was added to 0.5 g of pretreated substrate in 125 mL Erlenmeyer flasks. The mixture was then autoclaved at 121°C for 1 h, and the contents of the flasks were incubated with 10 mL of enzyme solutions for 168 h at 50°C and 120 rpm. At various time points, aliquots (2 mL) were withdrawn to quantify reducing sugars released

and glucose concentration. All experiments were performed in triplicate, and the data are reported as the average of those experiments, with the standard deviations indicated.

Statistical Analysis

The effect of different mixtures were statistically tested with the program PAST (Palaentological Statistics) (4), available in http://folk.uio.no/ohammer/past/. Data were submitted to normality test Shapiro-Wilk and than, One Way analysis of variance (ANOVA) and Tukey's Parwise Comparisons, with significance $P<0.05$.

RESULTS AND DISCUSSION

In this study, fungi were grown in liquid cultures supplemented with SCB or DCR. Crude extracts from *A. niger, A. oryzae, A. terreus, E. nidulans, Monilia* sp., *P. corylophilum* and *T. longibrachiatum* were prepared to investigate their ability to degrade lignocellulosic substrates. Previous research (13) has shown that SCB, DCR and BS represent a rich source of lignocellulose. Bromatological analysis revealed that DCR was found to be particularly rich in cellulose, followed by BS and SCB. The highest hemicellulose contents were present in BS and SCB. The lowest level of lignin was detected in SCB, while BS contained the highest lignin content. All crude extracts were screened for the production of holocellulose-degrading enzyme activities (β-xylanase, β-mannanase, pectinase, β-glucosidase, avicelase, FPase and CMCase). Although fungal growth was abundant on both carbon sources, there were differences in the production of holocellulose-degrading enzyme activities (data not shown). Compared to the other fungi, *A. terreus* and *A. oryzae* were responsible for the greatest production of holocellulose-degrading enzymes (13).

Combinations of two crude extract samples (286 mixtures) were tested for xylanase and FPase activities and selected for hydrolysis experiments. Combination B of *A. terreus* crude extract grown on SCB and DCR exhibited the highest yield of FPase activity, followed by combination B of *A. terreus* and *Monilia* sp. (Table 1). The best yield of FPase activity was obtained from *A. terreus* crude extract grown on SCB, which also has low protein content, while combination B of the *A. niger* and *E. nidulans* crude extracts was the most active, with a 25% increase in xylanolytic activity (Table 2). Combination B of *A. niger* and *E. nidulans* showed the highest protein content.

Table 1. Crude extract combinations with higher FPase activity and correspondent protein quantification.

Combinations	FPase activity (IU/mL)	± SD	Protein Concentration μg/mL
At[1]	0.507	0.001	5.50
At[1]/At[2] (Comb. B)	0.371	0.005	27.79
At[1]/Mo[1] (Comb. B)	0.322	0.020	24.56
Ao[1]/Tl[1] (Comb. A)	0.318	0.004	35.22
Ao[1]/Tl[1] (Comb. B)	0.312	0.015	46.05
At[1]/At[2] (Comb. A)	0.294	0.010	33.60
At[1]/Af[1] (Comb. A)	0,284	0.011	53.73
Ao[1]/Af[1] (Comb. A)	0.284	0.011	50.60
Ao[1]/Ao[2] (Comb. A)	0.278	0.007	63.24
Ao[1]/Mo[2] (Comb. A)	0.275	0.015	20.24

[1]- Crude extracts from the growth on sugar cane bagasse, [2]- Crude extracts from the growth on dirty cotton residue.
At: *A. terreus*; Mo*: Monilia* sp.; Ao: *A. oryzae*; Tl: *T. longibrachiatum*; Af: *A. flavus.*

Table 2. Crude extract combinations with higher xylanase activity and correspondent protein quantification.

Combinations	Xylanase activity (IU/mL)	± SD	Protein Concentration (µg/mL)
An[1]/En[2] (Comb. B)	0.978	0.013	94.16
Af[1]/Mo[1] (Comb. B)	0.778	0.015	74.03
An[1]/En[2] (Comb. A)	0.756	0.010	42.87
Af[1]/Mo[1] (Comb. C)	0.736	0.012	58.09
Af[2]/En[1] (Comb. C)	0.733	0.015	55.11
Ao[1]/An[1] (Comb. A)	0.720	0.011	51.69
Af[2]/An[1] (Comb. B)	0.716	0.020	90.47
Af[2]/An[1] (Comb. C)	0.703	0.013	86.13
Ao[1]/An[1] (Comb. B)	0.702	0.008	76.35
An[1]/En[1] (Comb. C)	0.696	0.012	60.83

[1]- Crude extracts from the growth on sugar cane bagasse, [2]- Crude extracts from the growth on dirty cotton residue.
An: *A. niger*; En: *E. nidulans*; Af: *A. flavus;* Mo: *Monilia* sp.; Ao: *A.oryzae*

The release of reducing sugars from pretreated SCB, BS and DCR by crude enzyme samples was measured by DNS and glucose oxidase assays (Figs. 1-6). Figures 1 and 2 indicate that the highest amount of reducing sugars released from DCR occurred after an incubation of 72 h with the crude extract combination B of *A. terreus* and *A. oryzae*, followed by the crude extract combination A. In addition, the highest release of reducing sugars after 96 h of incubation was observed with *T. longibrachiatum*. The glucose concentration decreased significantly after 72 and 96 h of incubation in comparison to the results shown in Figure 1 (Fig. 2). The release of reducing sugars from SCB increased steadily with the highest concentration detected after 168 h of incubation (Figs. 3 and 4). It is noteworthy that the release of reducing sugars continued to increase even after 168 h of incubation (Figs. 3 and 4). The best results for reducing sugar release were achieved with *A. terreus* crude extract alone or in combination A (Fig. 3). The maximal glucose release was detected in mixtures containing *A. terreus* crude extract alone and in combination C. A similar pattern was observed when BS was used as the substrate (Figs. 5 and 6).

Shapiro-Wilk test demonstrated that all data have normal distribution. ANOVA and Tukey's Pairwise comparisons showed that P values are less then 5% (P<0.05) in almost all the times and substrates used, demonstrating that mixtures have statistical differences among them.

A number of fungi species were found to secrete a group of enzyme activities able to breakdown holocellulose when grown on DCR and SCB. It appears that the activity yield of holocellulose-degrading enzymes is influenced by the agro-industrial residue used as the carbon source. These materials have complex structures composed of different polymers and components that interact with one another in ways that are not fully understood. The biological conversion of these structures requires a consortium of enzymes that interacts synergistically to release products, such as monomers and oligomers, that might serve as fuel precursors and other chemicals. Some isolates, notably *A. terreus,* produced a significant amount of holocellulose-degrading enzymes. Thus, the mechanism of enzymatic hydrolysis of holocellulose is an important factor to be considered for improving the enzymatic conversion in bioprocesses based on lignocelluloses. Furthermore, the phenomenon of enzyme adsorption should be taken into account when agro-industrial residues are used as substrates.

The mechanism of holocellulose-degrading enzyme adsorption has to do with the presence of highly ordered structures that represent the rate-limiting step in the conversion of holocellulose to soluble products (17). Thus, there appears to be a strong correlation between holocellulose accessibility and the degree of enzyme adsorption. This might contribute to the conversion rates and yields of lignocellulosic structures in plant cell walls (7).
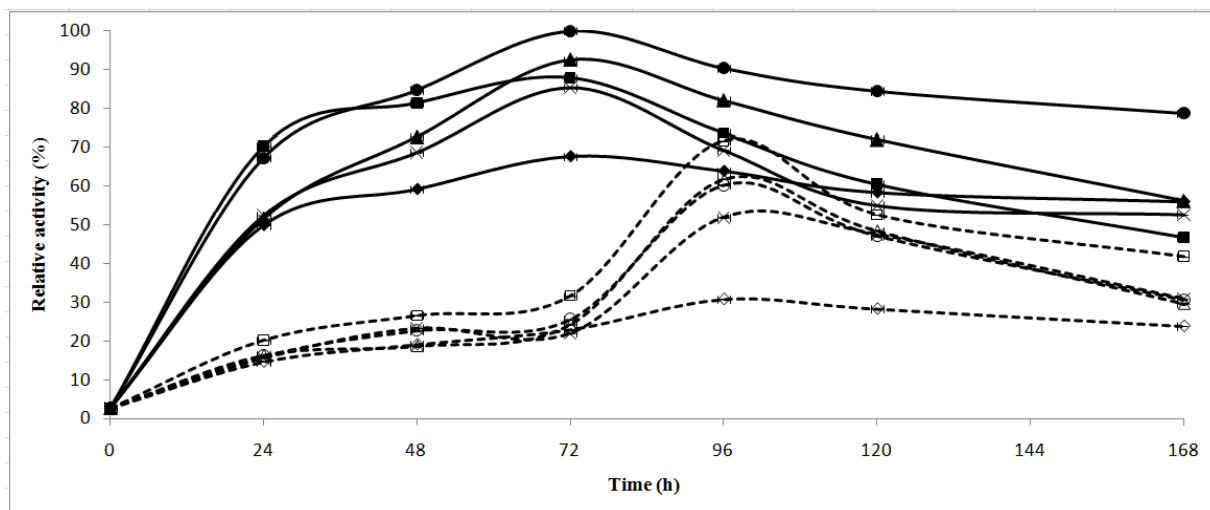
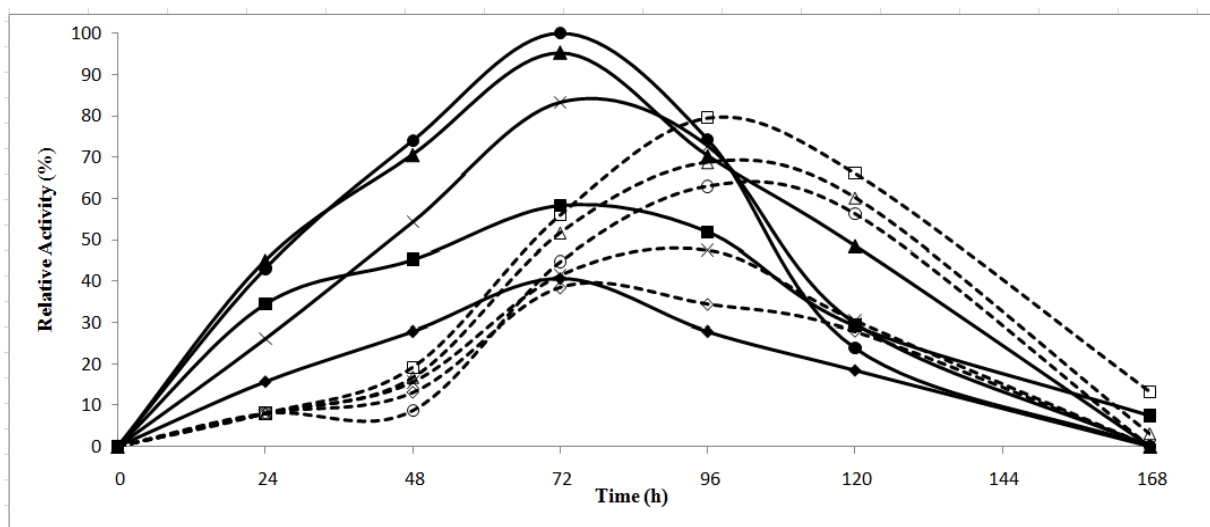**Figure 1.** The production of reducing sugars by enzymatic hydrolysis of dirty cotton residue measured by DNS. *A. terreus* crude extract (━■━), *A. oryzae* crude extract (━◆━), *A. terreus/A. oryzae* (Comb. A) (━▲━), *A. terreus/A. oryzae* (Comb. B) (━●━), *A. terreus/A. oryzae* (Comb. C) (━×━), *T. longibrachiatum* (⸗▱), *A. niger* (‑◇‑), *T. longibrachiatum/A. niger* (Comb A.) (‑△‑),*T. longibrachiatum/A. niger* (Comb B.) (‑○‑), and *T. longibrachiatum/A. niger* (Comb C.) (•✳•).

Figure 2. The production of reducing sugars by enzymatic hydrolysis of dirty cotton residue measured by the glucose oxidase method. *A. terreus* crude extract (━■━), *A. oryzae* crude extract (━◆━), *A. terreus/A. oryzae* (Comb. A) (━▲━), *A. terreus/A. oryzae* (Comb. B) (━●━), *A. terreus/A. oryzae* (Comb. C) (━×━), *T. longibrachiatum* (⸗▱), *A. niger* (‑◇‑), *T. longibrachiatum/A. niger* (Comb A.) (‑△‑), *T. longibrachiatum/A. niger* (Comb B.) (‑○‑), and *T. longibrachiatum/A. niger* (Comb C.) (•✳•).
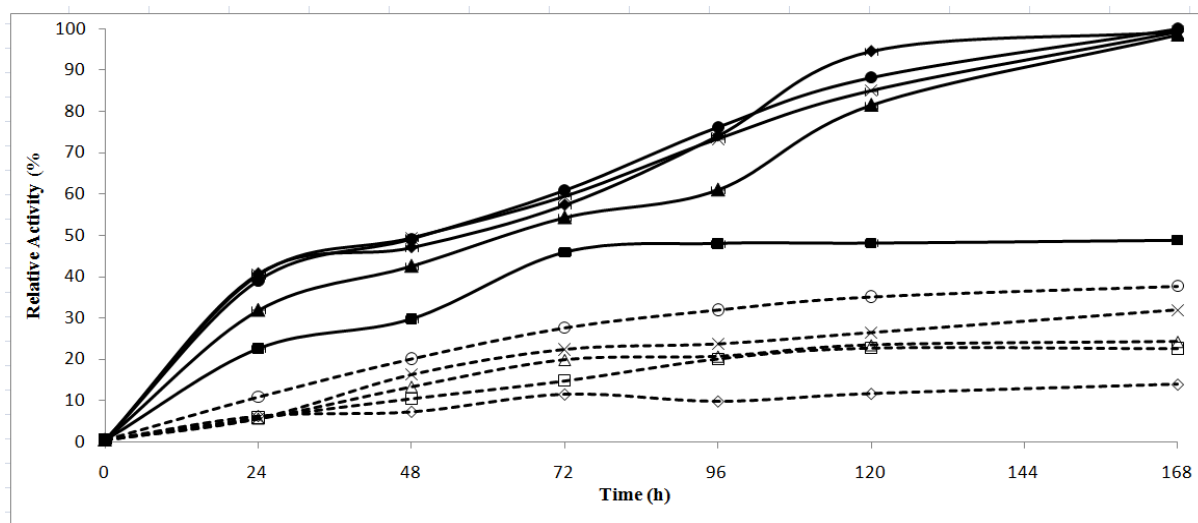
Figure 3. The production of reducing sugars by enzymatic hydrolysis of sugar cane bagasse measured by DNS. *A. terreus* crude extract (▬■▬), *A. oryzae* crude extract (▬◆▬), *A. terreus/A. oryzae* (Comb. A) (▬▲▬), *A. terreus/A. oryzae* (Comb. B) (▬●▬), *A. terreus/A. oryzae* (Comb. C) (▬✕▬), *T. longibrachiatum* (⌑), *A. niger* (-◇-), *T. longibrachiatum/A. niger* (Comb A.) (-△-), *T. longibrachiatum/A. niger* (Comb B.) (-○-), and *T. longibrachiatum/A. niger* (Comb C.) (✳).
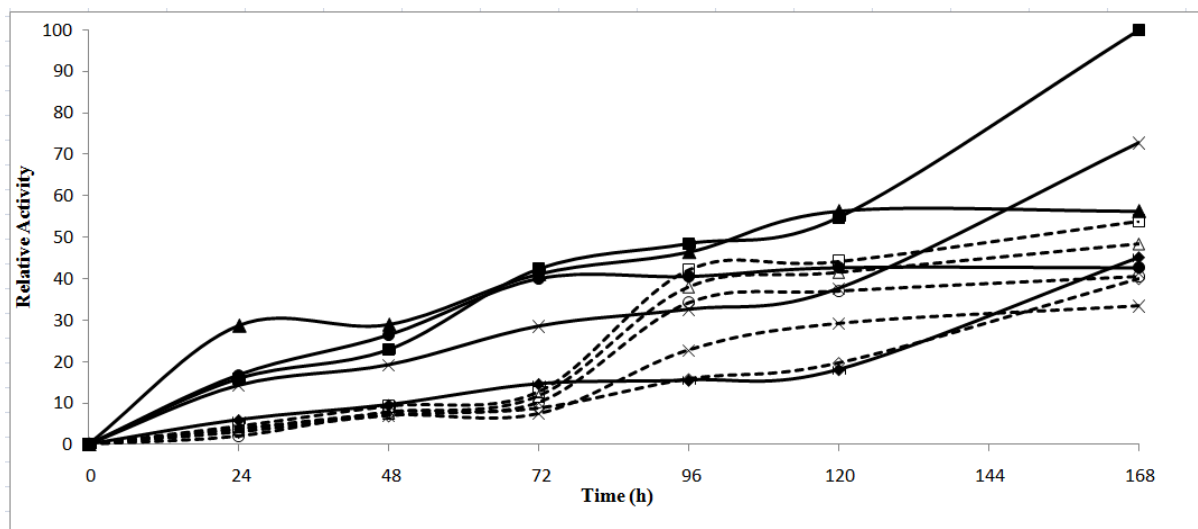

Figure 4. The production of reducing sugars by enzymatic hydrolysis of sugar cane bagasse measured by the glucose oxidase method. *A. terreus* crude extract (▬■▬), *A. oryzae* crude extract (▬◆▬), *A. terreus/A. oryzae* (Comb. A) (▬▲▬), *A. terreus/A. oryzae* (Comb. B) (▬●▬), *A. terreus/A. oryzae* (Comb. C) (▬✕▬), *T. longibrachiatum* (⌑), *A. niger* (-◇-), *T. longibrachiatum/A. niger* (Comb A.) (-△-), *T. longibrachiatum/A. niger* (Comb B.) (-○-), and *T. longibrachiatum/A. niger* (Comb C.) (✳).
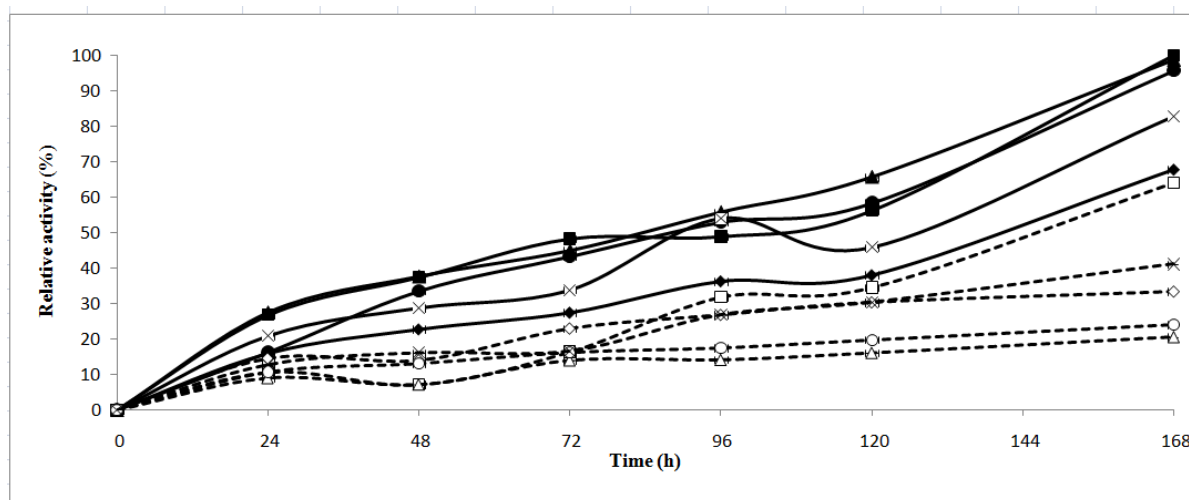
Figure 5. The production of reducing sugars by enzymatic hydrolysis of banana stem by DNS. *A. terreus* crude extract (━┿━), *A. oryzae* crude extract (━╈━), *A. terreus/A. oryzae* (Comb. A) (━╈━), *A. terreus/A. oryzae* (Comb. B) (━●━), *A. terreus/A. oryzae* (Comb. C) (─×─), *T. longibrachiatum* (⋅□⋅), *A. niger* (-◇-), *T. longibrachiatum/A. niger* (Comb A.) (-△-), *T. longibrachiatum/A. niger* (Comb B.) (-○-), and *T. longibrachiatum/A. niger* (Comb C.) (⋅✳⋅).
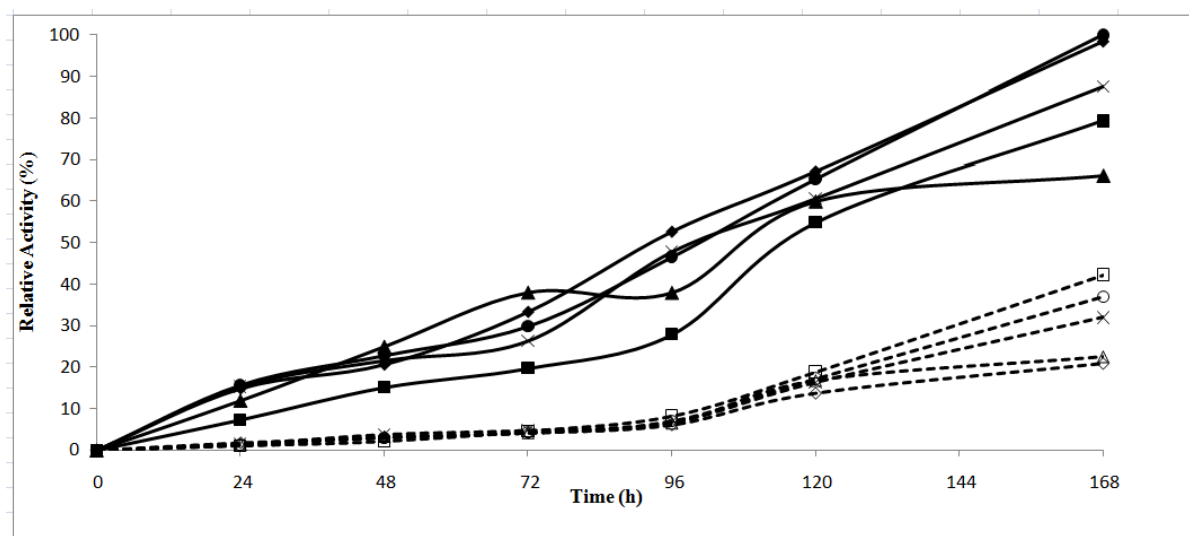

Figure 6. The production of reducing sugars by enzymatic hydrolysis of banana stem measured by the glucose oxidase method. *A. terreus* crude extract (━┿━), *A. oryzae* crude extract (━╈━), *A. terreus/A. oryzae* (Comb. A) (━╈━), *A. terreus/A. oryzae* (Comb. B) (━●━), *A. terreus/A. oryzae* (Comb. C) (─×─), *T. longibrachiatum* (⋅□⋅), *A. niger* (-◇-), *T. longibrachiatum/A. niger* (Comb A.) (-△-), *T. longibrachiatum/A. niger* (Comb B.) (-○-), and *T. longibrachiatum/A. niger* (Comb C.) (⋅✳⋅).

In conclusion, DCR, SCB and BS are inexpensive residues that can be used as substrates to reduce the cost of enzyme production while enzymatically converting the carbohydrate portion of DCR, SCB and BS into fermentable sugars. Further research will be required to study the enzyme mechanisms, particularly the role of xylanases.

ACKNOWLEDGEMENTS

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