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ORIGINAL PAPER

Evaluation of holocellulase production by plant-degrading fungi grown on agro-industrial residues

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Abstract *Agaricus brasiliensis* CS1, *Pleurotus ostreatus* H1 and *Aspergillus flavus* produced holocellulases when grown in solid and submerged liquid cultures containing agro-industrial residues, including sugar cane bagasse and dirty cotton residue, as substrates. These isolates proved to be efficient producers of holocellulases under the conditions used in this screening. Bromatological analysis of agro-industrial residues showed differences in protein, fiber, hemicellulose, cellulose and lignin content. Maximal holocellulase activity (hemicellulase, cellulase and pectinase) was obtained using solid-state cultivation with 10% substrate concentration. In this case, remarkably high levels of xylanase and polygalacturonase activity (4,008 and 4,548 IU/l, respectively) were produced by *A. flavus* when grown in media containing corn residue, followed by *P. ostreatus* H1 with IU/l values of 1,900 and 3,965 when cultivated on 5% and 10% sugar cane bagasse, respectively. *A. brasiliensis* CS1 showed the highest reducing sugar yield (11.640 mg/ml) when grown on medium containing sugar cane bagasse. *A. brasiliensis* was also the most efficient producer of protein, except when cultivated on dirty cotton residue, which induced maximal production in *A. flavus*. Comparison of enzymatic hydrolysis of sugar cane bagasse and dirty cotton residue by crude extracts of *A. brasiliensis* CS1, *P. ostreatus* H1 and *A. flavus* showed that the best reducing sugar yield was achieved using sugar cane bagasse as a substrate.

Keywords *Agaricus brasiliensis* CS1 · Agro-industrial residue · Holocellulose · Holocellulase · Xylanase · Pectinase

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Introduction

The capacity of a particular microorganism to grow in lignocellulosic substrates is directly related to its production of a spectrum of enzyme systems that act synergistically to deconstruct plant cell walls by depolymerizing substrates of different complexities. Within this context, a broad range of enzymes is necessary for the degradation of the carbohydrate

portion of lignocellulose (holocellulose) (Andreaus et al. 2008; Kumar et al. 2008). Among the enzymes showing activity against holocellulose, xylanase, mannanase, polygalacturonase, endoglucanase and exoglucanase play important roles in cleaving its polysaccharide backbone (Salles et al. 2007). Consequently, a great deal of effort is being devoted to the characterization of enzymes that break down holocellulose. Agro-industrial residues, including sugar cane bagasse and dirty cotton residues, contain lignocellulose material available for use as sources of fuels, chemical feedstocks, foods and livestock feeds (Kumar et al. 2008). Dirty cotton residue is a fraction collected from different cotton spinning and yarn forming textile industries that contains very short fibers, husks and other dark matter (Siqueira et al. 2010). In recent years, the use of such materials has also become an alternative approach for the production of holocellulases. Solid-state cultivation (SSC) is defined as the controlled growth of microorganisms on a moist solid substrate in the absence of free water (Cen and Xia 1999). It has shown some advantages over submerged liquid cultivation (SLC), including lower costs (simpler equipment, lower energy consumption and capital investment), improved enzyme stability and the production of enzymes with higher specific activities (Tuohy et al. 1989). On the other hand, SLC offers reproducible enzyme activities from batch to batch, ease of contamination control and is less labor-intensive (Cen and Xia 1999). However, the production costs of SLC are relatively high due to an inherently greater energy requirement, expensive medium composition and low enzyme concentrations. Basidiomycetes, the most conspicuous group of fungi in the environment, contains a number of edible and/or medicinal varieties (Erden et al. 2009). *Agaricus brasiliensis* CS1 is a medicinal mushroom native to Brazil, while *Pleurotus ostreatus* H1, commonly known as the oyster mushroom, is cultivated around the world for food. *Aspergillus flavus* is an imperfect ascomycete that does not produce ascospores. It grows rapidly as a haploid filamentous fungus on solid or liquid media under a variety of nutritional conditions. The focus of the present work was to compare the production of holocellulose-degrading enzymes by three fungus species with different anatomical and physiological characteristics when grown on different carbon sources (agro-industrial residues) using SSC and SLC as well as to evaluate the enzymatic hydrolysis of sugar cane bagasse and dirty cotton.

Materials and methods

Chemicals

All substrates were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dirty cotton residues were received from Hantex Resíduos Têxteis Ltda (Gaspar, SC, Brazil), a company that collects cotton residues from different spinning and yarn forming textile industries, mixes these residues and subjects them to further purification. Banana stems, sugar cane bagasse and corn and soybean residues were from a local source.

All experiments included five replicates and data are reported as averages with standard deviations indicated.

Residue pretreatment

Sugar cane bagasse and dirty cotton residue 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 production

A. brasiliensis CS1 and *P. ostreatus* H1 were kindly provided by the Edible and Medicinal Mushroom Laboratory, Federal University of Lavras, Brazil and Dr. Arailde Urben, National Research Centre for Genetic Resources and Biotechnology, Cenargen, Brazil, respectively. *A. flavus* was obtained from the fungus culture collection of the Enzymology Laboratory, University of Brasília, Brazil. All fungi were maintained in PDA medium (2.0% potato broth, 2.0% dextrose and 2.0% agar) at 28°C and cultured on five sources of lignocellulosic substrates (sugar cane bagasse, banana stems, dirty cotton, corn and soybean residues) at three concentrations. The substrate concentrations for SLC were 1% (w/v) and 5% (w/v),

while SSC was carried out at a substrate concentration of 10% (w/v) (Tuohy et al. 1989). The basal culture medium was as follows: 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.05% $CaCl_2$, 0.001% $FeCl_2 \cdot 6H_2O$, 0.0007% $MnCl_2 \cdot 4H_2O$, 0.0004% $ZnCl_2$, 0.0001% $CuSO_4 \cdot 5H_2O$, 0.1% yeast extract, 0.1% peptone at pH 5.5. An aliquot (2.5 ml) of an *A. flavus* spore suspension (10^8 spores/ml) was inoculated in Erlenmeyer flasks containing 100 ml of liquid medium and agro-industrial residue as a carbon source. Cultures were grown for 5 days at 28°C without agitation. Cultures of *A. brasiliensis* CS1 and *P. ostreatus* H1 were grown for during 15 days using the conditions described above. Three cylindrical pieces (7 mm) of mycellium were placed in the submerged liquid and solid-state media. After the growth procedure, 100 ml of 50 mM sodium acetate buffer (pH, 5.0) was added for 1 h at 28°C and the media were then passed through filter paper (Whatman No. 1). The resulting supernatants, hereafter called crude extracts, were used for determination of holocellulase activities and extracellular protein concentration.

Enzyme assays

Endoglucanase, xylanase, polygalacturonase and mannanase activities were determined by mixing 50 µl of enzyme sample with 100 µl of 1% w/v substrate (carboxymethyl cellulose, oat spelt xylan and pectin respectively) or 0.5% w/v substrate (galactomannan) at 50°C for 30 min. FPase activity (Mandels et al. 1976) was determined using 150 µl of enzyme with filter paper (Whatman No. 1) as a substrate at 50°C for 1 h. Avicelase activity was determined by mixing a microcrystalline cellulose suspension substrate (1% w/v) (50 µl) and 100 µl of enzyme at 50°C for 2 h. The amount of reducing sugar released was measured using dinitrosalicylic reagent (Miller 1959). Activity was expressed as µmol reducing sugar formed per min per liter of enzyme solution, i.e., as IU/l. Glucose, xylose, mannose and galacturonic acid were used as standards. Protein concentration was determined by Bradford assay (1976) using bovine serum albumin as a standard after trichloroacetate (final concentration 5%) precipitation and redissolution. Glucose content was measured by the glucose oxidase method (Trinder 1969).

Bromatological analysis of agro-industrial residues

All residues were dried at 60°C for 48 h for bromatological analysis. Samples were then ground, kept in polyethylene bags, tied and stored at 20–25°C. Total protein content was determined using the micro Kjeldahl method (AOAC 1995). A factor of 6.25 was used to convert total nitrogen to crude protein. Fat content was measured by Soxhlet extraction with ethylic ether (AOAC 1995) and ash content was measured by the gravimetric method at 550°C (AOAC 1995). Crude fiber was content was evaluated by AOAC methodology (1995). Lignin, insoluble fiber in acid detergent, insoluble fiber in neutral detergent, humidity, hemicellulose, cellulose and dry matter contents were determined as described elsewhere (Gomide and Demuner 1986; Silva and Queiroz 2002; van Soest 1963; van Soest and Wine 1967). Calcium determination was performed by atomic absorption spectrophotometry at 422.7 nm (Cali et al. 1973). Phosphorus quantification was performed using a UV-visible spectrophotometer at 420 nm (Roig et al. 1999).

Enzymatic hydrolysis

Hydrolysis of sugar cane bagasse and dirty cotton residues was performed as follows: 25 ml of distilled water was added to 1 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 25 ml of enzyme solutions (crude extract samples of *A. brasiliensis* CS1, *P. ostreatus* H1 and *A. flavus* grown using solid state cultivation) for 72 h at 50°C. At various time points, aliquots (1 ml) were withdrawn for reducing sugar, total protein and xylanase and endoglucanase activity assays.

Results and discussion

In this study, fungi were grown in solid state and submerged liquid cultures supplemented with five agro-industrial residues and were subjected to experiments including bromatological analysis. Previous work (Siqueira et al. 2010) has shown that cotton residues and banana stems represent a rich source of

macro- and micronutrients. In this study, corn and dirty cotton residues were found to be particularly rich in cellulose, followed by banana stems, soybean residue and sugar cane bagasse (Table 1). Bromatological analysis also revealed that the highest hemicellulose contents were present in banana stems and sugar cane bagasse. Soybean residue and banana stems contained the highest lignin content. On the other hand, the lowest levels of lignin were detected in corn residue and sugar cane bagasse, respectively. Micro-nutrient and fiber contents were higher in dirty cotton residue. When comparing Kjeldahl nitrogen contents, we made the simplifying assumption that all nitrogen is protein-derived. On this basis, we noted that protein and fat contents were superior in soybean residue and banana stems, respectively. In addition, we also noted a higher amount of soluble carbohydrate in sugar cane bagasse.

The basidiomycetes *A. brasiliensis* CS1 and *P. ostreatus* H1 and the ascomycete *A. flavus* were subjected to studies involving holocellulase production under SSC and SLC. For comparative purposes, SLC was performed without shaking to simulate SSC conditions. All fungus strains produced holocellulases with variable activity levels. Holocellulase production was more relevant when the fungi were grown under SSC using different agro-industrial residues (Tables 2, 3, 4). The performance of *A. brasiliensis* CS1 is displayed in Table 2. Dirty cotton residue (10%) was the best carbon source for most holocellulase activities, including xylanase, mannanase, endoglucanase and FPAse, with the exception of pectinase activity (polygalacturonase), which was higher when grown with sugar cane bagasse (5%). It should be noted that the highest activity, 1,349 IU/l, was observed for xylanase. Avicelase activity was detected at low levels in all carbon sources, corn residue being the best inducer. The influence of carbon source on holocellulase production by *P. ostreatus* H1 was also investigated (Table 3). Maximal xylanase activity, 1,900 and 1,220 IU/l, was observed when *P. ostreatus* H1 was grown on sugar cane bagasse at concentrations of 5% and 10%, respectively. Mannanase activity reached its highest value (197 IU/l) using sugar cane bagasse (10%) as a carbon source. It is important to note that sugar cane bagasse induced the highest levels of polygalacturonase activity, while banana stems were the best inducer of endoglucanase and FPAse activities. The highest avicelase activity was achieved by cultivating the fungus on dirty cotton residue. *A. flavus* showed markedly elevated levels of polygalacturonase activity (Table 4). In this case, the highest activity level was found to be 4,548 IU/l following cultivation on corn residue (10%). According to Mellon et al. (2007), *A. flavus* produces high yields of pectinase when grown in different carbon sources. Banana stems (10%) induced the highest xylanase, endoglucanase and FPAse activities,

Table 1 Bromatological analysis of agro-industrial residues

Bromatological analysis	Sugar cane bagasse	Banana stem	Corn residue	Dirty cotton residue	Soybean residue
Ash (%)	0.8	7.8	3.4	4.6	3.6
Dry matter (%)	92.4	91.6	90.8	91.5	90.8
Crude protein (%)	3.1	8.6	3.6	6.9	7.5
Fat (%)	1.2	2.3	1.1	1.8	2.5
FDA (%)	39.7	62.3	80.2	72.0	61.3
FDN (%)	55.9	79.8	80.8	81.8	71.7
Soluble carbohydrate (%)	64.7	36.7	50.7	30.0	41.5
kcalorie (kcal g*100 g)	281.7	202.0	226.7	163.7	218.5
Cellulose (%)	34.6	52.9	75.9	65.7	51.9
Hemicellulose (%)	16.2	17.4	0.6	9.8	10.4
Lignin (%)	5.1	9.4	4.3	6.3	9.4
Calcium (%)	0.02	0.98	0.16	1.01	0.78
Phosphorous (%)	0.03	0.09	0.06	0.14	0.08

FDA Insoluble fiber in acid detergent, *FDN* Insoluble fiber in neutral detergent

All the experiments included five replicates

Table 2 Determination of holocellulase activities in crude extracts of *Agaricus brasiliensis* CS1 grown in different concentrations of agro-industrial residues

Carbon source and concentration	Hemicellulase and pectinase activities			Cellulase activity		
	IU/l					
	Xylanase	Mannanase	Polygalacturonase	Endoglucanase	FPase	Avicelase
Banana stem (1%)	11.3 ± 6.8	9.0 ± 2.4	25.9 ± 3.1	12.4 ± 3.7	77.7 ± 3.8	1.0 ± 0.3
Banana stem (5%)	8.6 ± 3.0	3.0 ± 1.6	35.4 ± 5.0	45.5 ± 4.9	91.4 ± 3.5	1.0 ± 0.7
Banana stem (10%)	29.2 ± 9.4	6.0 ± 1.6	37.0 ± 3.6	97.9 ± 7.4	65.9 ± 4.2	1.0 ± 0.4
Corn residue (1%)	35.4 ± 22.8	2.0 ± 1.7	8.4 ± 38.7	37.5 ± 18.6	103.9 ± 10.8	1.0 ± 0.8
Corn residue (5%)	31.0 ± 15.5	6.0 ± 1.5	52.2 ± 26.6	86.0 ± 25.8	63.3 ± 7.2	1.0 ± 0.5
Corn residue (10%)	562.7 ± 42.6	7.0 ± 2.4	67.3 ± 43.9	190.8 ± 11.5	107.0 ± 9.2	26.0 ± 2.6
Soybean residue (1%)	9.5 ± 11.6	40.5 ± 8.5	7 ± 2.7	62.0 ± 15.4	80.7 ± 13.1	2.1 ± 1.0
Soybean residue (5%)	17.5 ± 14.6	195.8 ± 18.5	6 ± 3.1	93.3 ± 12.2	61.9 ± 3.2	0 ± 0.9
Soybean residue (10%)	377.7 ± 17.8	7.0 ± 1.2	7.0 ± 1.9	115.4 ± 28.1	59.8 ± 7.0	0 ± 0.7
Sugar cane bagasse (1%)	109.4 ± 9.0	68.6 ± 5.9	283.3 ± 13.7	64.4 ± 3.7	5.0 ± 2.6	5.7 ± 0.7
Sugar cane bagasse (5%)	148.6 ± 13.8	60.7 ± 9.7	451.3 ± 13.1	110.8 ± 15.0	13.4 ± 1.4	19.1 ± 1.4
Sugar cane bagasse (10%)	183.6 ± 19.7	86.6 ± 10.9	155.6 ± 14.1	101.6 ± 11.1	15.1 ± 4.9	15.7 ± 2.5
Dirty cotton residue (1%)	38.1 ± 21.3	8 ± 2.7	7.0 ± 4.4	6.9 ± 2.0	46.1 ± 5.9	1.4 ± 0.5
Dirty cotton residue (5%)	304.0 ± 37.8	196.3 ± 19.7	73.8 ± 60.3	96.0 ± 11.6	111.1 ± 6.4	1.2 ± 0.5
Dirty cotton residue (10%)	1,348.8 ± 99.9	206.4 ± 53.9	219.0 ± 78.4	315.3 ± 30.7	180.4 ± 8.1	5.7 ± 0.9

Table 3 Holocellulase activities in crude extracts of *Pleurotus ostreatus* H1 grown in different concentrations of agro-industrial residues

Carbon source and concentration	Hemicellulase and pectinase activities			Cellulase activity		
	IU/l					
	Xylanase	Mannanase	Polygalacturonase	Endoglucanase	FPase	Avicelase
Banana stem (1%)	14.4 ± 1.4	9.0 ± 3.0	4.0 ± 1.4	22.6 ± 0.0	1.6 ± 4.2	4.0 ± 1.6
Banana stem (5%)	228.4 ± 5.0	30.2 ± 5.2	148.4 ± 35.8	62.1 ± 7.6	73.2 ± 7.6	50.2 ± 5.9
Banana stem (10%)	1,072.3 ± 8.4	67.5 ± 8.5	161.9 ± 15.5	325.3 ± 11.5	142.2 ± 3.0	56.7 ± 2.0
Corn residue (1%)	40.1 ± 1.2	38.4 ± 2.8	8.3 ± 3.6	29.0 ± 5.4	4.4 ± 2.3	5.1 ± 1.3
Corn residue (5%)	118.1 ± 6.7	27.1 ± 5.2	7.9 ± 2.2	40.5 ± 2.7	16.8 ± 2.6	7.0 ± 2.3
Corn residue (10%)	21.6 ± 2.8	24.0 ± 2.1	6.3 ± 1.6	24.8 ± 2.0	2.0 ± 5.2	5.3 ± 0.3
Soybean residue (1%)	49.6 ± 4.9	16.7 ± 0.1	1.8 ± 1.2	14.0 ± 1.2	11.1 ± 7.6	4.2 ± 0.6
Soybean residue (5%)	85.8 ± 6.7	17.5 ± 2.1	8.8 ± 3.2	22.4 ± 2.9	31.7 ± 1.9	7.1 ± 0.7
Soybean residue (10%)	170.8 ± 2.7	45.8 ± 4.2	119.4 ± 16.2	40.4 ± 3.1	23.3 ± 3.7	6.7 ± 0.6
Sugar cane bagasse (1%)	721.4 ± 11.4	26.0 ± 6.2	801.5 ± 20.1	17.1 ± 2.0	3.2 ± 3.6	4.0 ± 1.2
Sugar cane bagasse (5%)	1,900 ± 42.5	79.0 ± 6.1	3,035.6 ± 42.3	142.0 ± 13.2	94.3 ± 6.4	55.2 ± 8.4
Sugar cane bagasse (10%)	1,226.0 ± 80.2	196.5 ± 25.0	3,965.4 ± 105.3	138.6 ± 9.6	54.3 ± 1.3	74.9 ± 4.5
Dirty cotton residue (1%)	252.5 ± 13.1	30.7 ± 3.5	172.0 ± 8.2	82.6 ± 4.9	1.5 ± 0.8	20.1 ± 1.2
Dirty cotton residue (5%)	275.9 ± 9.1	40.8 ± 4.4	243.1 ± 19.8	129.1 ± 7.4	94.5 ± 5.2	105.9 ± 12.0
Dirty cotton residue (10%)	684.5 ± 8.1	54.1 ± 5.0	216.4 ± 17.7	240. ± 6.4	102.35.1	94.6 ± 5.9

Table 4 Holocellulase activities in crude extracts of *Aspergillus flavus grown* in different concentrations of agro-industrial residues

Carbon source and concentration	Hemicellulase and pectinase activities			Cellulase activity		
	IU/l					
	Xylanase	Mannanase	Polygalacturonase	Endoglucanase	FPase	Avicelases
Banana stem (1%)	218.1 ± 4.1	14.2 ± 0.6	263.0 ± 21.0	22.1 ± 1.9	11.2 ± 1.9	3.1 ± 1.5
Banana stem (5%)	279.6 ± 18.0	63.5 ± 2.5	295.1 ± 13.7	34.0 ± 2.5	119.7 ± 9.7	108.3 ± 7.5
Banana stem (10%)	3,346.9 ± 37.2	497.2 ± 13.6	2,547.7 ± 75.7	439.9 ± 4.4	197.7 ± 16.2	52.7 ± 6.2
Corn residue (1%)	585.3 ± 12.1	23.9 ± 4.6	257.9 ± 13.5	37.1 ± 3.5	30.0 ± 2.3	5.7 ± 1.4
Corn residue (5%)	707.1 ± 27.0	67.7 ± 10.1	576.1 ± 31.5	73.5 ± 7.7	91.4 ± 8.9	62.6 ± 7.3
Corn residue (10%)	4,008.0 ± 139.5	134.2 ± 12.8	4,547.6 ± 158.3	432.7 ± 11.7	199.2 ± 8.5	11.4 ± 4.9
Soybean residue (1%)	341.4 ± 14.0	102.8 ± 3.0	233.3 ± 9.5	42.8 ± 3.6	21.9 ± 6.9	4.6 ± 2.2
Soybean residue (5%)	588.4 ± 10.6	137.1 ± 14.5	249.3 ± 22.3	65.5 ± 7.8	96.2 ± 14.0	62.0 ± 4.4
Soybean residue (10%)	1,823.5 ± 68.8	228.8 ± 11.2	2,029.3 ± 44.4	91.9 ± 11.2	103.3 ± 17.0	32.7 ± 7.7
Sugar cane bagasse (1%)	402.7 ± 18.3	37.1 ± 2.6	943.1 ± 26.2	33.2 ± 5.3	1.7 ± 0.2	2.8 ± 1.9
Sugar cane bagasse (5%)	383.4 ± 16.7	42.8 ± 13.2	565.2 ± 22.4	47.4 ± 16.9	104.3 ± 11.2	2.1 ± 1.2
Sugar cane bagasse (10%)	758.3 ± 71.9	27.4 ± 12.8	3,357.6 ± 103.1	17.5 ± 8.0	36.3 ± 8.0	19.0 ± 4.2
Dirty cotton residue (1%)	276.5 ± 20.8	20.2 ± 9.1	54.5 ± 7.0	21.3 ± 8.2	27.3 ± 7.1	2.0 ± 0.8
Dirty cotton residue (5%)	538.2 ± 8.3	47.7 ± 4.2	118.1 ± 5.9	11.0 ± 6.8	65.7 ± 10.7	14.8 ± 5.4
Dirty cotton residue (10%)	1,983.2 ± 86.3	154.9 ± 19.3	2,300.2 ± 55.3	58.2 ± 8.8	103.7 ± 7.3	45.2 ± 12.6

whereas maximum mannanase and avicelase activities were obtained following cultivation on soybean residue and banana stems, respectively. Based on these results, it appears that the enzyme induction pattern depends on the nature of the substrate. *A. flavus* was the overall best producer of holocellulase activities. In comparison to *A. brasiliensis* CS1, *P. ostreatus* H1 was a more efficient producer of holocellulase activity, producing high levels of xylanase and polygalacturonase activities. From the above results, it is clear that SSC is a more efficient procedure for production of holocellulase activities than SLC. *A. brasiliensis* CS1 showed the best reducing sugar yield (11.640 mg/ml) when cultivated with 10% sugar cane bagasse (Table 5). The highest amount of protein was observed when *A. brasiliensis* CS1 was grown with 10% banana stems.

The ability of crude extracts from *A. flavus*, *A. brasiliensis* CS1 and *P. ostreatus* H1 to degrade lignocellulosic substrates was examined using pretreated sugar cane bagasse and dirty cotton residue. The crude extracts were selected according to their highest xylanase, endoglucanase and FPase activities (Tables 2, 3, 4). Therefore, six crude extracts obtained after SSC were used to determine their enzymatic potential to degrade lignocellulosic residues. In the case of *A. brasiliensis* CS1, crude extracts were from fungi grown in media containing dirty cotton and corn residues. With respect to *P. ostreatus* H1 extracts, extracts were obtained after cultivation using banana stems and dirty cotton residue as substrates, while *A. flavus* extracts were derived from cultures using corn residue and banana stems as sources of xylanase, endoglucanase and FPase activities.

The release of reducing sugars from pretreated sugar cane bagasse and dirty cotton residues by crude enzyme samples from *A. brasiliensis* CS1, *P. ostreatus* H1 and *A. flavus* was measured by DNS and glucose oxidase methods (Figs. 1, 2, 3, 4). Our results indicate that the highest amount of reducing sugar was released following culture with sugar cane bagasse, peaking at 72 h of incubation. At 12 h of incubation, the amount of reducing sugar detected was approximately double that of reaction mixtures containing dirty cotton residue as a substrate (Figs. 1 and 2). Figure 1 indicates that, at 12 h of incubation, the highest level of reducing sugar was observed in *A. flavus* (corn residue crude extract), while after 48 h of incubation, *A. brasiliensis* CS1 (dirty cotton residue crude extract) and *A. flavus* (banana stem crude extract) showed an increase in reducing sugar release. A similar pattern was observed when dirty cotton

Table 5 Reducing sugar and protein content in crude extracts of *Agaricus brasiliensis* CS1, *Pleurotus ostreatus* H1 and *Aspergillus flavus* grown in different concentrations of agro-industrial residues

Carbon source and concentration	Reducing sugar (mg/ml)			Protein (μg/ml)		
	A. brasiliensis CS1	*P. ostreatus*	*A. flavus*	*A. brasiliensis CS1*	*P. ostreatus*	*A. flavus*
Banana stem (1%)	0.697 ± 0.069	0.028 ± 0.003	0.119 ± 0.013	40.2 ± 1.0	42.3 ± 1.1	36.0 ± 2.9
Banana stem (5%)	2.553 ± 0.172	0.091 ± 0.007	0.200 ± 0.015	200.5 ± 6.0	89.4 ± 4.1	68.9 ± 1.3
Banana stem (10%)	4.997 ± 0.099	0.579 ± 0.010	0.830 ± 0.042	344.4 ± 9.0	183.8 ± 17.4	170.7 ± 2.6
Corn residue (1%)	0.620 ± 0.021	0.024 ± 0.007	0.097 ± 0.012	10.1 ± 1.6	57.0 ± 4.1	31.6 ± 1.2
Corn residue (5%)	2.148 ± 0.009	0.454 ± 0.013	0.683 ± 0.020	41.4 ± 3.8	61.7 ± 1.4	50.5 ± 2.1
Corn residue (10%)	6.571 ± 0.386	0.337 ± 0.010	0.688 ± 0.044	84.2 ± 1.7	17.9 ± 8.4	85.9 ± 2.7
Sugar cane bagasse (1%)	2.080 ± 0.221	0.165 ± 0.013	0.325 ± 0.015	5.4 ± 0.6	36.0 ± 2.5	24.6 ± 1.9
Sugar cane bagasse (5%)	9.396 ± 0.337	2.129 ± 0.034	1.883 ± 0.050	34.1 ± 3.1	38.2 ± 1.7	12.5 ± 0.8
Sugar cane bagasse (10%)	11.640 ± 0.199	2.330 ± 0.034	2.642 ± 0.036	54.3 ± 8.8	47.5 ± 6.4	11.0 ± 3.2
Soybean residue (1%)	0.537 ± 0.027	0.021 ± 0.003	0.108 ± 0.002	7.2 ± 1.0	34.6 ± 6.9	35.4 ± 4.0
Soybean residue (5%)	1.817 ± 0.166	0.169 ± 0.010	0.425 ± 0.044	56.7 ± 2.7	60.4 ± 2.0	53.1 ± 6.4
Soybean residue (10%)	4.670 ± 0.284	0.428 ± 0.027	0.763 ± 0.032	104.6 ± 8.6	61.0 ± 2.1	90.6 ± 10.1
Dirty cotton residue (1%)	0.433 ± 0.045	0.078 ± 0.000	0.131 ± 0.016	4.8 ± 0.3	44.0 ± 4.0	34.1 ± 3.6
Dirty cotton residue (5%)	1.989 ± 0.229	0.196 ± 0.017	0.420 ± 0.027	45.7 ± 1.6	73.1 ± 5.8	53.9 ± 1.9
Dirty cotton residue (10%)	4.622 ± 0.540	0.347 ± 0.013	0.592 ± 0.041	56.6 ± 1.7	74.5 ± 3.6	95.5 ± 8.5

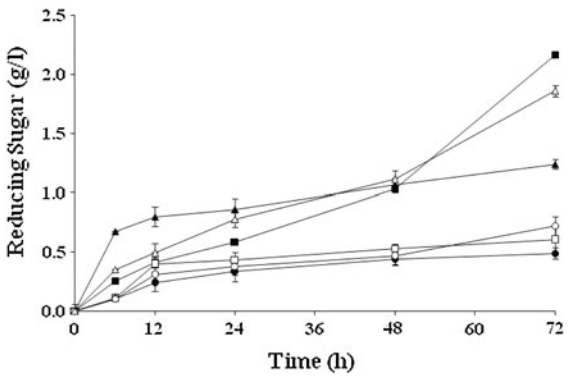

Fig. 1 Production of reducing sugar by enzymatic hydrolysis of sugar cane bagasse measured by DNS. Dirty cotton residue crude extract of *A. brasiliensis* CS1 (*filled square*); corn residue crude extract *of A. brasiliensis* (*open square*); banana stem crude extract of *P. ostreatus* H1 (*filled circle*); dirty cotton residue crude extract of *P. ostreatus* H1 (*open circle*); corn residue crude extract of *A. flavus* (*filled triangle*); banana stem crude extract of *A. flavus* (*open triangle*)

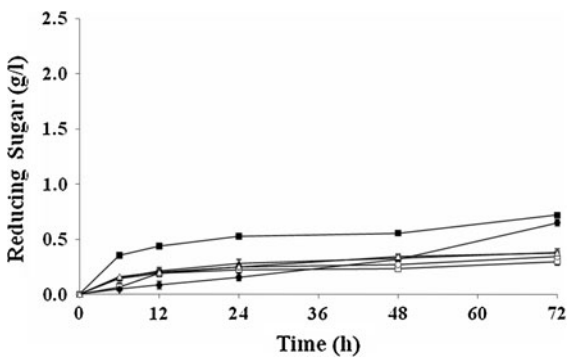

Fig. 2 Production of reducing sugar by enzymatic hydrolysis of dirty cotton residue measured by DNS. Dirty cotton residue crude extract of *A. brasiliensis* CS1 (*filled square*); corn residue crude extract of *A. brasiliensis* CS1 (*open square*); banana stem crude extract of *P. ostreatus* H1 (*filled circle*); dirty cotton residue crude extract of *P. ostreatus* H1 (*open circle*); corn residue crude extract of *A. flavus* (*filled triangle*); banana stem crude extract of *A. flavus* (*open triangle*)

residue was used as the substrate (Fig. 2). However, in this case *A. flavus* (banana stem crude extract) was replaced by *P. ostreatus* H1 (banana stem crude extract). The results displayed in Figs. 3 and 4 show that maximal glucose release was detected in reaction mixtures containing *A. brasiliensis* CS1 (dirty cotton residue crude extract) and *A. flavus* (banana stem crude extract), followed by *A. flavus* (corn residue crude extract). The best results for dirty cotton residue hydrolysis were achieved with *A. brasiliensis* CS1 (dirty cotton and corn residue crude extracts) (Fig. 4). In addition to hydrolysis experiments, at three time periods, aliquots were withdrawn and

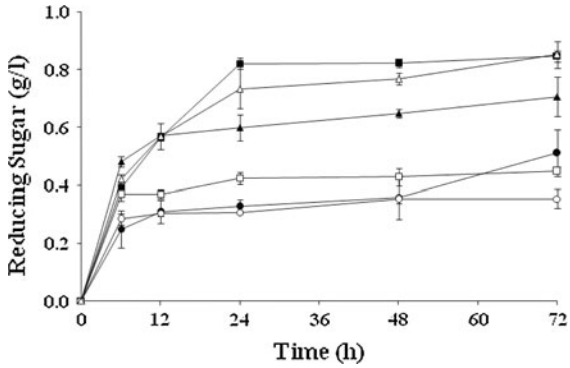

Fig. 3 Production of reducing sugar by enzymatic hydrolysis of sugar cane bagasse measured by the glucose oxidase method. Dirty cotton residue crude extract of *A. brasiliensis* (*filled square*); corn residue crude extract of *A. brasiliensis* CS1 (*open square*); banana stem crude extract of *P. ostreatus* H1 (*filled circle*); dirty cotton residue crude extract of *P. ostreatus* H1 (*open circle*); corn residue crude extract of *A. flavus* (*filled triangle*); banana stem crude extract of *A. flavus* (*open triangle*)

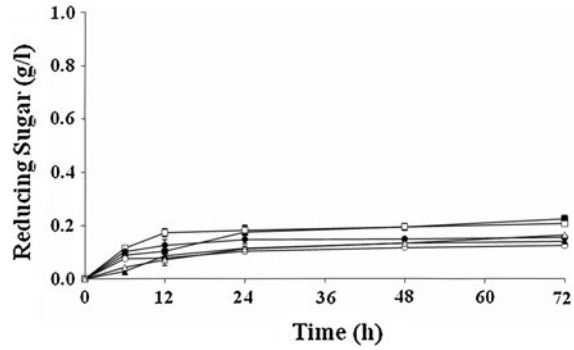

Fig. 4 Production of reducing sugar by enzymatic hydrolysis of dirty cotton residue measured by the glucose oxidase method. Dirty cotton residue crude extract of *A. brasiliensis* CS1 (*filled square*); corn residue crude extract of *A. brasiliensis* CS1 (*open square*); banana stem crude extract of *P. ostreatus* H1 (*filled circle*); dirty cotton residue crude extract of *P. ostreatus* H1 (*open circle*); corn residue crude extract of *A. flavus* (*filled triangle*); banana stem crude extract of *A. flavus* (*open triangle*)

Table 6 Xylanase and endoglucanase activities in crude extracts of *Agaricus brasiliensis* CS1, *Pleurotus ostreatus* H1 and *Aspergillus flavus* after incubation with pretreated sugar cane bagasse and dirty cotton residue

Incubation mixture	Xylanase			Endoglucanase		
	IU/l					
	0 h	24 h	72 h	0 h	24 h	72 h
A. brasiliensis[a] + SCB	1,339.2 ± 12.1	618.2 ± 9.4	494.5 ± 25.0	339.5 ± 1.5	80.8 ± 16.7	44.4 ± 8.3
A . brasiliensis[a] + DCR	961.3 ± 25.2	457.2 ± 1.5	373.4 ± 7.2	102.2 ± 5.2	73.3 ± 10.2	64.3 ± 17.2
A. brasiliensis[b] + SCB	985.5 ± 38.8	607.3 ± 29.9	310.6 ± 38.8	169.2 ± 18.3	120.1 ± 7.1	40.4 ± 33.3
A. brasiliensis[b] + DCR	699.7 ± 29.5	615.5 ± 23.2	473.9 ± 43.4	152.4 ± 18.1	85.2 ± 10.3	65.2 ± 33.9
P.ostreatus[c] + SCB	1,048.4 ± 33.4	573.5 ± 29.4	504.3 ± 58.9	329.6 ± 17.4	75.1 ± 19.4	10.1 ± 13.5
P.ostreatus[c] + DCR	564.2 ± 47.5	381.4 ± 16.1	379.4 ± 26.5	139.8 ± 29.9	50.3 ± 8.2	35.4 ± 12.1
P.ostreatus[a] + SCB	627.5 ± 35.2	391.4 ± 41.3	396.0 ± 51.3	252.1 ± 21.1	93.4 ± 14.2	40.4 ± 9.4
P.ostreatus[a] + DCR	333.6 ± 40.3	432.7 ± 22.4	324.3 ± 11.7	61.8 ± 14.2	5.4 ± 3.1	3.3 ± 13.2
A.flavus[b] + SCB	3,320.9 ± 73.5	2,192.3 ± 53.4	1,803.6 ± 106.7	427.7 ± 31.4	221.8 ± 36.5	100.9 ± 9.9
A.flavus[b] + DCR	3,136.3 ± 59.5	1,974.4 ± 39.9	1,731.4 ± 29.5	119.2 ± 28.8	138.4 ± 11.4	56.4 ± 3.3
A.flavus[c] + SCB	3,970.7 ± 59.5	1,841.1 ± 131.4	1,762.0 ± 121.3	450.1 ± 26.1	184.5 ± 40.9	147.3 ± 35.7
A. flavus[c] + DCR	2,515.6 ± 56.1	1,986.5 ± 28.7	1,867.3 ± 86.1	186.0 ± 6.3	188.0 ± 11.2	152.2 ± 13.1

SCB Sugar cane bagasse, *DCR* Dirty cotton residue

[a] Dirty cotton residue crude extract

[b] Corn residue crude extract

[c] Banana stem crude extract

residual xylanase and endoglucanase activities were determined under standard conditions (Table 6). The incubation mixture containing *A. flavus* crude extract showed the highest yields of xylanase and endoglucanase activities over an interval of 0-72 h. Another line of evidence for enzyme adsorption was the observed decrease in protein content along the hydrolysis time course. However, we cannot discard

the fact that increased reducing sugar concentration could be the primary cause of inhibition. We observed a decrease in enzyme activities along the incubation period, suggestive of enzyme adsorption on both lignocellulosic substrates. The exact extent and mechanism of holocellulase 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 (Zhang et al. 2006). Thus, there appears to be a strong correlation between holocellulose accessibility and the degree of holocellulase adsorption. Indeed, both parameters are known to contribute to conversion rates and yields of lignocellulosic structures in plant cell walls (Kristensen et al. 2009).

Conclusions

Intensive research over the last 30 years has led to the identification, isolation and growth of filamentous fungi that are able of producing a consortium of enzyme activities, including cellulases, hemicellulases and pectinases (Sanchez 2009). *A. brasiliensis* CS1, *P. ostreatus* H1 and *A. flavus* produced several holocellulase enzymes when grown in solid and submerged liquid cultures on agro-industrial residue-based media. It appears that holocellulase production was influenced by the type of substrate used. Of the holocellulases examined, remarkably high levels of xylanase and pectinase activities were produced by *A. flavus*, followed by *P. ostreatus* H1. Cultivation by SSC produced the best holocellulase activity yields. Further studies are necessary to determine the role of the purified enzyme systems on the hydrolysis of holocellulose, particularly the roles of xylanase and pectinase.

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