Secretome analysis of Trichoderma reesei and Aspergillus niger cultivated by submerged and sequential fermentation processes: enzyme production for sugarcane bagasse hydrolysis

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1. Introduction

Hydrothermal, steam-explosion, and dilute acid pretreatments have been applied to enhance the digestibility of sugarcane bagasse by facilitating the access of the cellulose to enzymes or microorganisms [1, 2]. However, the enzymes for hydrolysis of pretreated bagasse and other types of biomass are costly. The use of lignocellulosic substrates as both carbon source and inducer provides an option for reducing costs [3, 4]. Cultivation of industrially relevant fungal strains such as *Aspergillus niger* and *Trichoderma reesei* on steam pretreated sugarcane bagasse has shown that, when solid state culture is transitioned to a submerged fermentation (i.e., sequential fermentation process), enzyme titers are increased, and results in showing cellulases and hemicellulases with relatively high specific activities [5-7]. Even though such studies indicate that the activity of enzymes from the sequential fermentation is higher than the conventional submerged fermentation, it remains unknown whether the profile of protein activities secreted upon a sequential fermentation process is responsible for the higher conversion of lignocellulose to monosaccharides.

Trichoderma reesei (teleomorph *Hypocrea jecorina*) is a mesophilic soft-rot ascomycete fungus that is widely used in industry as a source of cellulases and hemicellulases for the hydrolysis of plant cell wall polysaccharides. Although it has been shown to be the anamorph of the pantropical ascomycete *Hypocrea jecorina*, the organism remains most widely recognized by its former name. In addition to a long history of safe use for industrial enzyme production, *T. reesei* has also been an important model system for studying lignocellulose degradation [8-10].

Martinez et al. [10] assembled 89 scaffolds (sets of ordered and oriented contigs) to generate 34 Mbp of nearly contiguous *T. reesei* genome sequence comprising 9,129 predicted gene models. Interestingly, despite the industrial utility and effectiveness of the carbohydrate-active

1 enzymes of this fungus, its genome encodes fewer cellulases and hemicellulases than other
2 sequenced fungus able to hydrolyze plant cell wall polysaccharides. It was also observed that
3 many *T. reesei* genes encoding carbohydrate-active enzymes are distributed nonrandomly in
4 clusters that lie between regions of synteny with other Sordariomycetes. It was hypothesized
5 that numerous genes encoding biosynthetic pathways for secondary metabolites may promote
6 survival of *T. reesei* in its competitive soil habitat, but genome analysis provided little
7 mechanistic insight into its amazing capacity for protein secretion [10].

8 Aspergilli, of which *A. niger* is a member, contain a wide spectrum of enzymes for
9 polysaccharide, protein and lipid degradation [11]. Cellulases, hemicellulases, pectinases,
10 amylases, inulinases, lipases and proteases are used in a range of industrial applications. *A. niger*
11 has a long tradition of safe use in the production of enzymes and organic acids. Many of these
12 products have obtained GRAS (generally regarded as safe) status [12-15]. Pel et al [13]
13 sequenced the 33.9-megabase genome of *A. niger* CBS 513.88, the ancestor of currently used
14 enzyme production strains. A high level of synteny was observed with other aspergilli
15 sequenced, and strong function predictions were made for 6,506 of the 14,165 open reading
16 frames identified. They observed striking differences in the hydrolytic enzyme spectra of
17 aspergilli. Some specific differences include, in contrast to the other three aspergilla studied, *A.*
18 *niger* contains only one GH10 and four GH11 endoxylanases, from a larger oligosaccharide
19 liberating xylanase family.

20 Recent work [16] reported a detailed secretome analysis of *A. niger* and *T. reesei* grown
21 on sugarcane biomass under the conventional submerged fermentation method. Important
22 enzymes related to biomass degradation, including cellobiohydrolases, endoglucanases, β -
23 glucosidases, β -xylosidases, endoxylanases, xyloglucanases, and α -arabinofuranosidases were

identified in both fungi secretomes. Additional studies on the secretome analysis of *T. reesei* [17-20] or *A. niger* [21] strains cultivated on a variety of carbon sources have also been previously reported. However, a combined evaluation on how the fermentation process employed for enzyme production affects titers and the profile of proteins secreted, and in turn how this affects performance on lignocellulose hydrolysis is still not fully understood.

This work used proteomics as a tool to map characterize and compare the secretomes from *T. reesei* Rut C30 and *A. niger* A12 cultivated for 72h in submerged and sequential fermentation processes (summarized in Figure 1) and helped to assess how the type of process employed for enzyme production affected the profile of proteins secreted by two relevant strains. The performance of combined *T. reesei* and *A. niger* enzymatic extracts produced under both fermentation processes were evaluated based on hydrolysis of non-washed steam-exploded sugarcane bagasse.

2. Materials and methods

2.1 Fungal Strains

The *T. reesei* Rut-C30 strain was purchased from the Centre for Agricultural Bioscience International (CABI) culture collection in the United Kingdom (IMI number: 345108). The *A. niger* wild type A12 strain, originally isolated from black pepper [22], was obtained from Embrapa Food Technology collection (Rio de Janeiro, Brazil). The strains were maintained at 18°C in a 20% (w/w) glycerol/water solution and were activated in potato dextrose agar (PDA) for 7 days at 30°C (*T. reesei*) and for 4 days at 32°C (*A. niger*).

2.2 Cultivation Conditions

2.2.1 Submerged Fermentation Method

In the conventional submerged fermentation process, the pre-culture was initiated with a conidia suspension of 10^7 spores/mL in a total volume of 100 mL of nutrient medium enriched with 30g/L of glucose (Figure 1), as described initially from Mandels & Stenberg [23] and adapted by Cunha et al. [24]. The medium contained (% w/v): 0.14 $(\text{NH}_4)_2\text{SO}_4$, 0.20 KH_2PO_4 , 0.03 CaCl_2 , 0.02 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 peptone, 0.20 yeast extract, 0.03 urea, 0.10 Tween 80, and 0.10 of salt solution (5 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mg/L, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 mg/L CoCl_2). The incubation was carried out at an agitation rate of 200 rpm for 48 h at 30°C (for *T. reesei*) or at 32°C (for *A. niger*). For enzyme production, a volume of pre-culture suspension corresponding to 10% (v/v) was transferred to the culture medium and supplemented with 10 g/L of glucose and 1% (w/v) of steam-exploded non-washed sugarcane bagasse (donated by the Sugarcane Research Center (CTC, Brazil)). The steam explosion was conducted at 1667 kPa after the bagasse had been held at 205°C for 20 min. The cultivations were kept in an orbital incubator shaker at 200 rpm for 72 h at 30°C (for *T. reesei*) or 32°C (for *A. niger*) (Figure 1). The cultivation broth was filtered, centrifuged at 4°C and 10,000 rpm for 15 min, and stored at -18°C for further analysis. The cultivation experiments were carried out in triplicate, and the averaged data presented with standard deviations.

2.2.2 Sequential Fermentation Method

Sequential fermentation was performed in triplicate according to the methodology described previously [5]. Briefly, pre-culture was initiated as a two-step solid state fermentation using 5 g of dry sugarcane bagasse as solid substrate in 500 mL Erlenmeyer flasks. Substrate moisture was adjusted through the addition of 12 mL of nutrient medium (Figure 1). First the bagasse was inoculated with 10^7 spores/g of dry bagasse, and the culture was maintained under static conditions for 24 h at 30°C for *T. reesei* and 32°C for *A. niger*. The pre-culture step was

continued as a submerged fermentation after addition of 100 mL of nutrient medium containing 30 g/L of glucose per 5 grams of dry bagasse in an orbital incubator shaker for 48 h, 200 rpm and 30°C and 32°C, respectively, followed by enzyme production carried out as described in section 2.2.1 (Figure 1). Both fermentations were carried out with non-washed sugarcane bagasse as the carbon source.

2.3 Enzyme Activity Assays

Endoglucanase (EGase), xylanase, and β -xylosidase activities were determined following standard methods [25] with filter paper (FPase) and β -glucosidase activities based on protocols of the International Union of Pure and Applied Chemistry (IUPAC) [26]. Release of reducing sugar was measured using the DNS method [27]. Protein concentration was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL) using bovine serum albumin (BSA) as the standard. All enzymatic analyses were carried out in duplicate.

2.4 Secretome Analysis

T. reesei and *A. niger* protein extracts were prepared for proteomic analysis by vacuum filtration followed by centrifugation at 4°C and 10,000 rpm for 15 min. The supernatant was concentrated by about 10 times through ultrafiltration (Amicon ultrafiltration system/Millipore) using a 76 mm diameter, 10 kDa cut-off membrane. Two samples from each type of fermentation were subjected to duplicate proteomic analysis using high resolution LTQ-Orbitrap XL LC-MS/MS. A detailed description of procedures for determining the secretome, together with results in Tables 1 and 2 are given in the companion Data in Brief article submitted concomitantly to this paper [28].

2.5 Enzymatic hydrolysis of sugarcane bagasse by fungal combined extracts

Enzymatic hydrolysis was carried out in 250 mL Erlenmeyer flasks with 50 mM citrate buffer at pH 4.8, in an orbital incubator shaker operated at an agitation speed of 200 rpm at 50°C for 96 h, using a combination of enzymes from *T. reesei* and *A. niger* that were produced by either submerged or sequential fermentations. The non-washed steam-exploded sugarcane bagasse was applied at a concentration of 1% (w/v) of total solids. Composition of the pretreated bagasse on a w/w basis was 71% glucan, 0.5% pentosan, 27.8 % lignin and 3% ash [29]. The final reaction volume of 100 mL consisted of enzymes, bagasse solids and buffer.

Saccharification was performed using combined extracts from *T. reesei* and *A. niger* (enzyme composition in Tables 2 of this manuscript and Data in Brief article [28]). Enzyme loadings of 0.7 filter paper cellulase unit (FPU) or 71 mg protein (*T. reesei* cocktails) were mixed with 3.6 cellobiase unit (CBU) or 44.3 mg protein (*A. niger* cocktails) per gram of glucan [21, 30]. As control experiments, reaction blanks for both the substrate and enzymes were carried out. Samples were taken at 0, 24, 48, 72 and 96 h. All hydrolysis experiments were performed in triplicate.

2.6 Chemical analysis of hydrolyzed samples

Collected samples (0, 24, 48, 72 and 96 h) were filtered using 0.2 µm Syringe Filters with nylon membrane (Pall Corporation, Port Washington, NY) and diluted accordingly. The released glucose was measured by D-glucose assay procedure kit (Megazyme International, Ireland). HPLC analysis methodology used was previously described by Ximenes et al. [31].

3. Results and Discussion

3.1 Enzymatic profile of extracts produced by *T. reesei* and *A. niger*

Cellulase and hemicellulase production was measured for *A. niger* and *T. reesei* grown through conventional submerged and the sequential solid-state cultures followed by submerged fermentations as shown in Table 1. The highest values of activity for most of the quantified

enzymes were observed when the fungi were cultivated upon the sequential fermentation method. The cultivation of *A. niger* upon this method resulted in 8 and 3.4 times higher β -glucosidase and xylanase production, respectively, compared to results from submerged fermentation. For *T. reesei*, xylanase and β -xylosidase were 2.3 and 2.2-fold higher under the sequential method. Additionally, the sequential method resulted in higher values of endoglucanase activities, protein concentrations and specific activities for both strains (Table 1). These findings are in agreement with previous reports in the literature showing that the sequential solid-state and submerged cultivation method for enzyme production was superior to the conventional submerged fermentation for both *Aspergillus* and *Trichoderma* fungal strains [5-7].

3.2 Secretome Analysis

A proteomic analysis of the proteins expressed and secreted by *A. niger* and *T. reesei* grown under the conventional submerged and sequential fermentation methods was carried out in order to identify their differential glycoside hydrolase profiles (Table 1, Data in Brief article [28]). These analyses revealed 97 and 79 proteins for *T. reesei* and 99 and 112 proteins for *A. niger* extracts from the submerged and sequential fermentations, respectively. In previous studies [16] 45 different enzymes from CAZy database (CAZymes) in *T. reesei* and 89 in *A. niger* were identified. A longer cultivation time in our study (72 versus 24 h) and/or differences between the medium composition, could have resulted in a higher number of proteins. Some proteins in common were only found in secretomes from the same fungus cultivated upon different methods (Table 1, Data in Brief article [28]). In a previous study was reported that secretome profiles of *T. reesei* and *A. niger* grown in steam exploded bagasse and culm (the stem of sugarcane) *in natura* upon submerged fermentation indicated that they differed considerable, but the pattern of

1 *A. niger* enzyme production was similar in the presence of both substrates. Even though there
2 were no differences in fungi growth between these 2 substrates, it was observed that *A. niger* and
3 *T. reesei* secreted more enzymes when grown on culm, which was argued could be related to
4 differences in the recalcitrance of substrates.

5 The *T. reesei* secretomes from submerged and sequential fermentations showed 27% of the
6 total 176 identified proteins to be in common to both cultivation methods. They included 3
7 endoglucanases, 2 cellobiohydrolases (CBH), 8 hemicellulases, 10 families of glycoside
8 hydrolases, 4 proteases, 6 predicted proteins, 3 hypothetical proteins and 10 other proteins,
9 including some esterases and cell-wall proteins, resulting in a total of 46 common proteins in
10 both cultivation methods (Table 1, Data in Brief article) [28]. Hypothetical proteins are those
11 which may have the same profile of another protein, but no assigned function. Together with the
12 predicted proteins, these are representative of the secretomes of different fungi [21, 32],
13 including *T. reesei* [20]. It is noteworthy that some relevant biomass-degrading enzymes such as
14 α -glucuronidases (GH67) were only present in the sequential cultivation extract. Such enzymes
15 remove uronic acid from the non-reducing end of glucurono-xylooligosaccharides, and thus
16 contribute to the complete hydrolysis of xylan chains [33].

17 The secretomes from *A. niger* grown under submerged and sequential fermentations showed
18 29% of the total 211 proteins to be common between the two methods. These common proteins
19 included 2 endoglucanases, 1 CBH, 3 β -glucosidases, 14 hemicellulases, 5 proteases, 6
20 hypothetical proteins, 14 unnamed proteins, 3 proteins associated with fungal cell-wall, and 14
21 additional proteins including amylases, esterases and others (Table 1, Data in Brief article) [28].
22 It is interesting to note that, even though the sequential fermentation method also has a
23 submerged fermentation step, many enzymes secreted in submerged fermentation were not found

1 in the sequential fermentation cocktails. These results may indicate that, in the sequential
2 process, the germination of the spores in the solid-state fermentation step in the presence of
3 sugarcane bagasse as inducer substrate could direct the fungus metabolism towards the secretion
4 of some distinct proteins from those produced when the fungal spores germinated during the
5 submerged fermentation process. A more detailed comparative analysis on how the cultivation
6 method affected the profile of proteins in the individual secretomes from each of these two
7 relevant industrial strains is presented as follows.

8 3.2.1 *T. reesei* secretome

9 The enzymatic proteins produced by *T. reesei* under sequential fermentation contained the
10 highest levels of cellulolytic and hemicellulolytic activities, while those from submerged
11 fermentation showed higher protein diversity according to the LC-MS/MS data (Table 1, Data in
12 Brief article) [28]. The percentages of the secreted proteins involved in cellulose and
13 hemicellulose degradation were similar for submerged (20.6%) and sequential (19%)
14 fermentation methods. In both cultivation methods, expansion-like proteins (such as swollenin,
15 which plays a non-hydrolytic role in the disruption of lignocellulose) [18], and CIP1 protein
16 (which acts as cellulase enhancing protein) were also identified.

17 The α - and β -glycoside hydrolase (GH) families in the secretome of *T. reesei* made up
18 25.7% of the 97 proteins in submerged fermentation and 17.7% of the 79 proteins in the
19 sequential fermentation, according to the carbohydrate-active enzyme database (CAZy,
20 www.cazy.org) (Figure 2). Grouping and distribution analysis of the secreted proteins identified
21 46 different GH families in the total pool of proteins from secretome extracts of both cultivation
22 methods. Potential representatives of the GH6 and GH7 (cellobiohydrolases), GH11
23 (endoxylanases), GH54 (α -L/N-arabinofuranosidases), GH74 (xyloglucanases), GH61 (cooper-

1 dependent lytic polysaccharide monooxygenases now reclassified in family AA9) families were
2 detected in extracts from both cultivation methods. However, similar to what has been reported
3 in other studies [18], not all of the predicted GHs were identified.

4 Proteins belonging to GH families 5 (endo- β -1,4-glucanase, β -mannosidase, among others),
5 16 (glucanosyltransferase, endoglucanase, galactosidase, among others), 28 (polygalacturonase),
6 72 (glucanosyltransglycosylase), 92 (mannosidase) were detected in extracts from the submerged
7 fermentations. Representatives of GH families 17 (glucanosyltransglycosylase), 55
8 (endoglucanase), 74 (endoglucanase and xyloglucanase) were unique to submerged
9 fermentations while only representative of GH families 3 (β -glucosidases and β -xylosidases), 38
10 (mannosidase), 67 (glucuronidase) were found for extracts produced upon sequential
11 fermentation.

12 The presence of GH families 16 (endo-1,4- β -D-glucanase) and 55 (endo-1,3- β -D-glucanase)
13 in the submerged fermentation secretome is not commonly reported in the literature. A
14 comparative secretome analyses study of two *T. reesei* strains (Rut-C30 and CL847) using
15 lactose as carbon source and inducer of cellulases has shown the expression of a new putative
16 endoglucanase, in addition to an arabinofuranosidase, by the reference map of the secreted
17 proteins and experimental evidence [34]. The more diversified secretome of CL847 (an
18 industrial mutant strain) suggests a more general hypersecretory strain, while Rut-C30 may be
19 more cellulase production oriented. This difference in the secretome of these 2 strains may have
20 occurred due to the completely different “mutation route” followed towards becoming efficient
21 enzyme producers.

22 The extracellular cellulolytic system of *T. reesei* is composed of 60–80% cellobiohydrolases
23 or exoglucanases (EC3.2.1.74), 20–36% endoglucanases (EC3.2.1.4) and 1% β -glucosidases

(EC3.2.1.21), which act synergistically in the conversion of cellulose into glucose [35, 36]. The cellulolytic system includes eight endo-1,4- β -D-glucanase (Cel7B, Cel12, Cel45, two Cel5 and three Cel61) responsible for primary hydrolysis that cleaves accessible intramolecular β -1,4-glucosidic bonds of cellulose chains randomly in a non-processive manner with formation of new chain ends [37]. Our data showed that only endoglucanase Cel45 was not secreted by *T. reesei* upon submerged fermentation conditions. Three endoglucanases (Cel7, Cel12 and Cel45) were secreted during the sequential fermentation. Cellobiohydrolases I and II, belonging to the GH7 and GH6 families, respectively [20], were identified in secretomes of both cultivation methods. Only two of the seven β -glucosidases that are part of the cellulolytic system of *T. reesei* strain [37] were observed for the submerged fermentation. None accumulated in the sequential fermentation secretome, although GH3 proteins [38, 39] were present.

The predicted proteins corresponded to 14.4% and 11.4% in the submerged and sequential fermentation methods, respectively; while hypothetical proteins were 14.4% and 17.7% (Figure 2). Similar levels of proteases and peptidases (8%) and lignocellulolytic-related enzymes (9%) were identified in secretomes of both cultivation methods. Other proteins in the submerged (16.5%) and sequential fermentation (25.3%) secretomes included those involved in lipid transportation and metabolism, pectin degradation, cell-wall biosynthesis and morphogenesis, and chitin degradation. The *T. reesei* submerged fermentation secretome also contained one bifunctional catalase-peroxidase involved in the removal of H₂O₂ from the cells, which may also contribute to lignin degradation [17, 40].

According to some studies, the enzyme production by *T. reesei* is transcriptionally regulated and dependent on the carbon source [41-44]. The range of potential substrates is still limited because most carbon sources are too expensive for industrial scale fermentations [19, 45]. The

1 effective use of sugarcane bagasse as carbon source for fungal growth may address this
2 limitation. Our results revealed that the enzymes induced in the presence of sugarcane bagasse
3 under different cultivation methods display a distinctive profile, suggesting that the cultivation
4 conditions (reported in this work) enable production of tailored enzymatic compositions that
5 efficiently hydrolyze pretreated biomass.

6 3.2.2 *A. niger* secretome

7 In the submerged and sequential fermentation secretomes obtained from *A. niger* (Figure 3)
8 the proteins involved in cellulose and hemicellulose degradation corresponded to 26% and 29%,
9 respectively, of the total 99 secreted proteins in submerged and 112 in sequential fermentation.
10 The number of proteins acting in the cellulose and hemicellulose degradation pathways was
11 almost 1.5-fold higher in the extract from the sequential fermentation. This is in agreement with
12 the higher enzymatic activity (cellulases and hemicellulases) observed in the enzyme mixture
13 produced under these conditions (Table 1).

14 GH families acting in the cellulose degradation were observed in both cultivation methods,
15 including GH3, 12 and 16 (β -glucosidase), GH5 (endoglucanase), and GH7 (cellobiohydrolase).
16 Some identified proteins and one GH protein were exclusive to sequential fermentation extracts
17 (β -glucosidase M4, endo- β -1,4-glucanase A and GH31 (β -glucosidase)). GH proteins that
18 degrade hemicellulose were also observed in both cultivation methods, including GH2
19 (mannosidase), GH10 and GH11 (endoxylanase), and GH62 (α -arabiofuranosidase). GH38
20 (mannosidase) and GH43 (endo-arabinase) were observed only in the submerged and sequential
21 fermentation secretomes, respectively.

22 The number of identified proteins in hypothetical and unnamed categories represents 35%
23 and 31% for submerged and sequential fermentation methods, respectively. Some hypothetical

1 proteins belonging to GH families were observed in secretome of both cultivation methods,
2 including GH72 (glucanotransglycosylase) and 75 (chitosanase). The sequential fermentation
3 secretome showed 2 GH families as hypothetical proteins: GH3 (β -glucosidase and β -
4 xylosidase) and GH43 (arabinofuranosidase and β -xylosidase). Hypothetical proteins belonging
5 to GH family 1 (β -glucosidase) and 31 (β -glucosidase and β -xylosidase) were also observed, as
6 well as proteins of unknown function. Some of them may encode putative proteins important for
7 sugarcane bagasse hydrolysis, such as non-hydrolytic accessory proteins that increase or favor
8 enzymatic efficiency [46]. The percentage of the identified peptidases and proteases in the *A.*
9 *niger* secretome under both cultivation methods were similar (9%).

10 *A. niger* also produces cell-wall proteins including chitinases. This study shows that these
11 proteins represent 5% and 3.5% of the total secreted proteins in submerged and sequential
12 fermentations, respectively. Two (B and C) and three (A, B1 and C) feruloyl esterases were
13 observed in submerged and sequential fermentation secretomes, respectively. Feruloyl esterases
14 hydrolyze diferulate cross-links in plant cell-walls and facilitate access to the polysaccharide
15 backbone [11]. Five lipases were observed only in the sequential fermentation secretome.

16 Lignocellulolytic-related proteins (in the combined hemicellulase and cellulase fraction)
17 corresponded to 24.2% and 23.2% in the submerged and sequential fermentation secretomes,
18 respectively, and other enzymes components, in addition to the hydrolases, were identified as
19 dehydrogenases with a suggested role to dehydrogenate or oxidize cellobiose and other
20 cellodextrins [47, 48]. Oxygenases, belonging to the class of copper-dependent metalloenzymes,
21 which oxidatively cleave glycosidic bonds on the surface of cellulose without requiring a
22 separation of the glucan chain, were also identified [49, 50].

Comparison of the *T. reesei* and *A. niger* proteomic results shows that *A. niger* expresses a more versatile range of cellulases, hemicellulases and esterases than *T. reesei*. The latter two groups of enzymes become even more important for pretreatment steps that remove xylans [46]. The observation that the secretome of the 2 fungal strains studied here do not share proteins with the same amino acid sequence is consistent with their effective complementary use in lignocellulose hydrolysis, as pointed out by the work of Borin et al. [16] and the results presented in this paper. In the work of Borin et al [16], it was observed GHs involved in hemicellulose degradation that were exclusively found in *A. niger* secretome. *T. reesei* produces more efficient cellulases, while *A. niger* produces more effective hemicellulases and β -glucosidases [51], with enzymes from these two sources showing different patterns of resistance to lignin-derived inhibitors and deactivators [31, 52, 53]. Hence, formulations involving enzymes from both microorganisms are expected to result in more effective enzyme hydrolysis.

3.3 Enzymatic hydrolysis of sugarcane bagasse using the combined *T. reesei* and *A. niger* enzymes

Enzymes from the sequential fermentation at 0.7 FPU/g glucan gave 30% conversion to glucose while enzyme extracts from the submerged fermentation gave 10% for hydrolysis of steam exploded sugarcane bagasse (Figure 4). The better performance of enzymes produced by the sequential fermentation may be explained by the higher xylanase titers (Table 2) and a balance of cellulolytic/hemicellulolytic enzymes for the hydrolysis of the pretreated sugarcane bagasse (Table 2, Data in Brief article) [28]. Table 2 showed that fixing the enzyme loadings for the steam-exploded sugarcane bagasse hydrolysis experiments at 0.7 FPU and 3.6 CBU for *T. reesei* and *A. niger* extracts, respectively, did not result in major differences in the final enzyme activities measured as filter paper units, endoglucanase and cellobiase activities for the

1 submerged and sequential fermentation enzyme extracts. However, higher levels of xylanase
2 activities were present in the latter (Table 1). Additionally, the overall amount of proteins
3 identified by LC-MS/MS as hemicellulases were higher for the sequential fermentation
4 compared to the submerged fermentation (52% versus 41%, respectively, Table 2, Data in Brief
5 article) [28].

6 The proteomic analysis shown in Table 2 (Data in Brief article) [28] is for extracts of *T.*
7 *reesei* and *A. niger* that were combined in a ratio of 1:5 filter paper to cellobiase activities giving
8 the resulting enzyme profiles. Even though these runs were performed with combined extracts at
9 relatively low enzyme loading to hydrolyze non-washed steam exploded pretreated bagasse,
10 which is known to contain enzyme inhibitors [31, 54], significant hydrolysis of 30% occurred.
11 This indicates important role of the non-cellulolytic proteins in the combined extract. While the
12 cellulase loading of 0.7 FPU/g glucan was low, the total protein was still 70 mg/g which is
13 consistent with lignin blocking role of protein [55].

14 Wang et al. [56] and Yu et al. [57], respectively, reported 60% and 52% cellulose
15 conversion of liquid hot water pretreated sugarcane bagasse (5% w/v) using commercial enzyme
16 preparations at loadings of 20 and 15 FPU/g of cellulose. We used 0.7 FPU/g glucan, or about
17 20 to 30 times lower loading for hydrolysis of 1% w/v non-washed steam exploded sugarcane
18 bagasse. While the hydrolysis conditions are not directly comparable, the significantly lower
19 cellulolytic enzyme loadings of extracts, where only 15 to 20% of the protein was cellulase, was
20 notable, and may reflect the diversity of other proteins in the secretome.

21 A higher diversity of feruloyl esterases, xylanases and other auxiliary hemicellulolytic
22 enzymes was observed in the enzyme mixtures from the sequential fermentation (Data in Brief
23 article, Table 2) [28]. In lignocellulose hydrolysis, xylanases and β -xylosidases play a key role

1 in minimizing inhibitory effects of xylo-oligosaccharides on cellulases [31, 58]. The auxiliary
2 hemicellulolytic enzymes including ferulic acid esterases, acetylxylan esterase and α -
3 arabinofuranosidase further enhance the hydrolysis of lignocellulose by cleaving ester bonds on
4 xylan, removing α -arabinose and O-acetyl groups, and allowing the xylan/xylooligosaccharides
5 to be hydrolyzed to monomeric sugars that are much less inhibitory [59].

6 The higher saccharification efficiency obtained by using a combination of enzymes from *T.*
7 *reesei* and *A. niger* has been previously observed [16, 60]. The proteins identified in the
8 secretomes of the two fermentation methods studied here represented a broad range of hydrolase
9 activities, where endoglucanase, cellobiohydrolase, and β -glucosidase activities accounted for 15
10 to 20% of the total protein. The additional proteins secreted by *A. niger* and *T. reesei* may have
11 the potential to enable a low cellulase loading through interactions with the lignocellulosic
12 biomass, by blocking adsorption of cellulases on lignin, and providing enzyme activities that
13 cleave inhibitory oligosaccharides. This is consistent with prior reports that document the effects
14 of added, non-cellulolytic enzyme proteins in blocking cellulase adsorption on lignin, removing
15 product inhibitors, and reducing the amount of cellulases and β -glucosidase needed for
16 hydrolysis of pretreated biomass [31, 52, 53, 55, 61-65].

17 **4. Conclusion**

18 A remarkable variation in the secretome of *T. reesei* and *A. niger* occurred as function of
19 cultivation methods. Higher cellulolytic and hemicellulolytic production occurred for sequential
20 (solid state followed by submerged) fermentation compared to submerged fermentation for
21 extracts of both fungi when non-washed steam-exploded sugarcane bagasse was used as carbon
22 source. Proteomic analysis for the *A. niger* strain showed that sequential fermentation gave the
23 secretome with highest number of identified proteins and titers of enzyme activity. Higher

enzymatic activities and/or a better balance of secretome composition from the sequential fermentation in key cellulolytic and hemicellulolytic activities was reflected by 3-fold higher saccharification of the pretreated sugarcane bagasse for the combined enzyme mixtures from the sequential fermentations of *A. niger* and *T. reesei*. This new knowledge about the secretomes provides additional rationale for combining enzyme extracts [16, 66, 67] or culture broths from *T. reesei* and *A. niger* to achieve enhanced lignocellulose hydrolysis as well as demonstrating the potential benefits of extended fermentation times in producing these fungal enzymes.

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References

- [1] C.R. Soccol, L.P.S. Vandenberghe, A.B.P Medeiros, S.G. Karp, M. Buckeridge, L.P. Ramos, et al., Bioethanol from lignocelluloses: Status and perspectives in Brazil, *Bioresour. Technol.* 101 (2010) 4820-4825.
- [2] S.C. Pereira, L. Maehara, C.M.M. Machado, C.S. Farinas. 2G ethanol from the whole sugarcane lignocellulosic biomass. *Biotechnol. Biofuels* 8 (2015) 1-16.
- [3] A. Sorensen, P.J. Teller, P.S. Lubeck, B.K. Ahring, Onsite Enzyme Production During Bioethanol Production from Biomass: Screening for Suitable Fungal Strains. *Appl. Biochem. and Biotechnol.* 164 (2011) 1058-1070.

- [4] P.D. Delabona, J. Cota, Z.B. Hoffmam, D.A.A. Paixao, C.S. Farinas, J. Cairo, et al. Understanding the cellulolytic system of *Trichoderma harzianum* P49P11 and enhancing saccharification of pretreated sugarcane bagasse by supplementation with pectinase and alpha-L-arabinofuranosidase. *Bioresour. Technol.* 131 (2013) 500-507.
- [5] F.M. Cunha, M.N. Esperanca, T.C. Zangirolami, A.C. Badino, C.S. Farinas, Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulase, *Bioresour. Technol.* 112 (2012) 270-274.
- [6] F.M. Cunha, M.N. Esperanca, C. Florencio, V.M. Vasconcellos, C.S. Farinas, A.C. Badino, Three-phasic fermentation systems for enzyme production with sugarcane bagasse in stirred tank bioreactors: Effects of operational variables and cultivation method, *Biochem. Eng. J* 97 (2015) 32-39.
- [7] C. Florencio, F.M. Cunha, A.C. Badino, C.S. Farinas, Validation of a Novel Sequential Cultivation Method for the Production of Enzymatic Cocktails from *Trichoderma* Strains, *Appl. Biochem. and Biotechnol.* 175 (2015) 1389-1402.
- [8] K. Kuhls, E. Lieckfeldt, G.J. Samuels, W. Kovacs, W. Meyer, O. Petrini, et al. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc. Natl. Acad. Sci. USA* 93 (1996) 7755-7760.
- [9] H. Nevalainen, P. Suominen, K. Taimisto. On the safety of *Trichoderma reesei*. *J. Biotechnol.* 37 (1994) 193.
- [10] D. Martinez, R.M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S.E. Baker, et al. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nature Biotechnol.* 26 (2008) 553-560.
- [11] R.P. de Vries, J. Visser, *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides, *Microbiol. Mol. Biol. Rev.* 65 (2001) 497-522.
- [12] A. Pandey, P. Selvakumar, C.R. Soccol, P. Nigam. Solid state fermentation for the production of industrial enzymes. *Cur. Science* 77 (1999) 149-162.
- [13] E. Schuster, N. Dunn-Coleman, J.C. Frisvad, P.W.M. van Dijck. On the safety of *Aspergillus niger* - a review. *Appl. Microbiol. Biotechnol* 59 (2002) 426-435.
- [14] H.J. Pel, J.H. de Winde, D.B. Archer, P.S. Dyer, G. Hofmann, P.J. Schaap, et al. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* 25 (2007) 221-231.

- [15] R.M. Berka, N. Dunn-Coleman, M. Ward. Industrial Enzymes from *Aspergillus* species. Biotechnology 23 (1992) 155-202.
- [16] G.P. Borin, C.C. Sanchez, A.P. de Souza, E.S. de Santana, A.T. de Souza, A.F. Paes Leme, et al., Comparative Secretome Analysis of *Trichoderma reesei* and *Aspergillus niger* during Growth on Sugarcane Biomass, Plos One 10 (2015) 1-20.
- [17] A.J. da Silva, D.P. Gomez-Mendoza, M. Junqueira, G.B. Domont, R.M.L. Queiroz, M.V. de Sousa, et al. Secretomic Analysis Reveals Multi-Enzymatic Complexes in *Trichoderma reesei* Grown in Media Containing Lactose or Galactose. Bioenergy Res. 8 (2015) 1906-1911.
- [18] I.J. Marx, N. van Wyk, S. Smit, D. Jacobson, M. Viljoen-Bloom, H. Volschenk, Comparative secretome analysis of *Trichoderma asperellum* S4F8 and *Trichoderma reesei* Rut C30 during solid-state fermentation on sugarcane bagasse, Biotechnol. Biofuels 6 (2013) 172-185.
- [19] H. Jun, H. Guangye, C. Daiwen, Insights into enzyme secretion by filamentous fungi: Comparative proteome analysis of *Trichoderma reesei* grown on different carbon sources, J. Proteomics 89 (2013) 191-201.
- [20] S.S. Adav, L.T. Chao, S.K. Sze, Quantitative Secretomic Analysis of *Trichoderma reesei* Strains Reveals Enzymatic Composition for Lignocellulosic Biomass Degradation, Mol. Cell Proteomics 11 (2012) 1-15.
- [21] S.S. Adav, A.A. Li, A. Manavalan, P. Punt, S.K. Sze, Quantitative iTRAQ Secretome Analysis of *Aspergillus niger* Reveals Novel Hydrolytic Enzymes, J. Proteome. Res. 9 (2010) 3932-3940.
- [22] S. Couri, A.X. de Farias, Genetic manipulation of *Aspergillus niger* for increased synthesis of pectinolytic enzymes, Rev. Microbiol. 26 (1995) 314-317.
- [23] M. Mandels, D. Sternberg, Recent advances in cellulase technology, J. Ferment. Technol. 54 (1976) 267-286.
- [24] F.M. Cunha, A.L.G. Bacchin, A.C.L. Horta, T.C. Zangirolami, A.C. Badino, C.S. Farinas, Indirect method for quantification of cellular biomass in a solids containing medium used as pre-culture for cellulase production, Biotechnol. Bioprocess. Eng. 17 (2012) 100-108.
- [25] B.S. Dien, E.A. Ximenes, P.J. O'Bryan, M. Moniruzzaman, X.L. Li, V. Balan, et al., Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol, Bioresour. Technol. 99 (2008) 5216-5225.

- [26] Ghose T.K., Measurement of cellulase activities, *Pure & Appl. Chem.* 59 (1987) 257-268.
- [27] Miller G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* 31 (1959) 426-428.
- [28] C. Florencio, F.M. Cunha, A.C. Badino, C.S. Farinas, E. Ximenes, M.R. Ladisch. Secretome data from *Trichoderma reesei* and *Aspergillus niger* cultivated upon submerged and sequential fermentation methods. Data in Brief (concomitantly submitted with this manuscript).
- [29] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, et al., Determination of Structural Carbohydrates and Lignin, in *Biomass*, Golden, CO, USA.: National Renewable Energy Laboratory 2008, pp. 15.
- [30] D. Sternberg, P. Vijayakumar, E.T. Reese, Beta-Glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose, *Can. J Microbiol.* 23 (1977) 139-147.
- [31] E. Ximenes, Y. Kim, N. Mosier, B. Dien, M. Ladisch, Inhibition of cellulases by phenols, *Enzyme Microb. Tech.* 46 (2010) 170-176.
- [32] S.S. Adav, E.S.H. Cheow, A. Ravindran, B. Dutta, S.K. Sze. Label free quantitative proteomic analysis of secretome by *Thermobifida fusca* on different lignocellulosic biomass. *Journal of Proteomics* 75 (2012) 3694-3706.
- [33] A. Rogowski, A. Basle, C.S. Farinas, A. Solovyova, J.C. Mortimer, P. Dupree, et al., Evidence That GH115 alpha-Glucuronidase Activity, Which Is Required to Degrade Plant Biomass, Is Dependent on Conformational Flexibility, *J. Biol. Chem.* 289 (2014) 53-64.
- [34] I. Herpoel-Gimbert, A. Margeot, A. Dolla, G. Jan, D. Molle, S. Lignon, et al. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. *Biotechnol. Biofuels* (2008);1-18.
- [35] J. Zaldivar, J. Nielsen, L. Olsson, Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration, *Appl. Microbiol. Biotechnol.* 56 (2001) 17-34.
- [36] R. Muthuvelayudham, T. Viruthagiri, Fermentative production and kinetics of cellulase protein on *Trichoderma reesei* using sugarcane bagasse and rice straw, *Afr. J. Biotechnol.* 5 (2006) 1873-1881.
- [37] L.H.F. Do Vale, E.X.F. Filho, R.N.G. Miller, C.A.O. Ricart, M.V. Sousa, Cellulase Systems in *Trichoderma*: An Overview, in: V. Gupta, M. Schmoll, A. Herrera-Estrella, R. Upadhyay, I. Druzhinina, M. Tuohy (Eds.), *Biotechnology and Biology of Trichoderma*, Elsevier B.V., Amsterdam, 2014, pp. 229-244.

- [38] B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics, *Nucleic Acids Res.* 37 (2009) 233-238.
- [39] A.J. Harvey, M. Hrmova, R. De Gori, J.N. Varghese, G.B. Fincher, Comparative modeling of the three-dimensional structures of family 3 glycoside hydrolases, *Proteins: Struct. Funct. Genet.* 41 (2000) 257-269.
- [40] P. Chelikani, I. Fita, P.C. Loewen, Diversity of structures and properties among catalases, *Cell Mol. Life Sci.* 61 (2004) 192-208.
- [41] T. Juhasz, Z. Szengyel, K. Reczey, M. Siika-Aho, L. Viikari, Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources, *Process Biochem.* 40 (2005) 3519-3525.
- [42] P.K. Foreman, D. Brown, L. Dankmeyer, R. Dean, S. Diener, N.S. Dunn-Coleman, et al., Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*, *J. Biol. Chem.* 278 (2003) 31988-31997.
- [43] A.R. Stricker, R.L. Mach, L.H. de Graaff, Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*), *Appl. Microbiol. Biotechnol.* 78 (2008) 211-220.
- [44] C.P. Kubicek, Systems biological approaches towards understanding cellulase production by *Trichoderma reesei*, *J. Biotechnol.* 163 (2013) 133-142.
- [45] L. Olsson, T. Christensen, K.P. Hansen, E.A. Palmqvist, Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30, *Enzyme Microb. Tech.* 33 (2003) 612-619.
- [46] W.R. de Souza, P.F. de Gouvea, M. Savoldi, I. Malavazi, L.A.D. Bernardes, M.H.S. Goldman et al., Transcriptome analysis of *Aspergillus niger* grown on sugarcane bagasse, *Biotechnol. Biofuels* 4 (2011) 1-16.
- [47] J.A. Langston, T. Shaghasi, E. Abbate, F. Xu, E. Vlasenko, M.D. Sweeney, Oxidoreductive Cellulose Depolymerization by the Enzymes Cellobiose Dehydrogenase and Glycoside Hydrolase 61, *Appl. Environ. Microbiol.* 77 (2011) 7007-7015.
- [48] M.D. Sweeney, F. Xu, Biomass Converting Enzymes as Industrial Biocatalysts for Fuels and Chemicals: Recent Developments, *Catalysts* 2 (2012) 244-263.

- [49] W.T. Beeson, C.M. Phillips, J.H.D. Cate, M.A. Marletta, Oxidative Cleavage of Cellulose by Fungal Copper-Dependent Polysaccharide Monooxygenases, *J. Am. Chem. Soc.* 134 (2012) 90-92.
- [50] R.J. Quinlan, M.D. Sweeney, L. Lo Leggio, H. Otten, J.-CN. Poulsen, K.S. Johansen, et al., Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components, *Proc. Natl. Acad. Sci. USA* 108 (2011) 15079-15084.
- [51] L. Rosgaard, S. Pedersen, J.R. Cherry, P. Harris, A.S. Meyer, Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose, *Biotechnol. Prog.* 22 (2006) 493-498.
- [52] Y. Kim, E. Ximenes, N.S. Mosier, M.R. Ladisch, Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass, *Enzyme Microb. Tech.* 48 (2011) 408-415.
- [53] S.I. Mhlomo, R. den Haan, M. Viljoen-Bloom, W.H. van Zyl, Lignocellulosic hydrolysate inhibitors selectively inhibit/deactivate cellulase performance, *Enzyme Microb. Tech.* 81 (2015) 16-22.
- [54] E. Ximenes, Y. Kim, N. Mosier, B. Dien, M. Ladisch, Deactivation of Cellulases by Phenols, *Enzyme Microb. Tech.* 48 (2011) 54-60.
- [55] J.K. Ko, E. Ximenes, Y. Kim, M.R. Ladisch, Adsorption of Enzyme Onto Lignins of Liquid Hot Water Pretreated Hardwoods, *Biotechnol. Bioeng.* 112 (2015) 447-456.
- [56] W. Wang, X. Zhuang, Z. Yuan, Q. Yu, W. Qi, Investigation of the pellets produced from sugarcane bagasse during liquid hot water pretreatment and their impact on the enzymatic hydrolysis, *Bioresour. Technol.* 190 (2015) 7-12.
- [57] Q. Yu, X. Zhuang, S. Lv, M. He, Y. Zhang, Z. Yuan, et al., Liquid hot water pretreatment of sugarcane bagasse and its comparison with chemical pretreatment methods for the sugar recovery and structural changes, *Bioresour. Technol.* 129 (2013) 592-598.
- [58] Q. Qing, B. Yang, C.E. Wyman, Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes, *Bioresour. Technol.* 101 (2010) 9624-9630.
- [59] M. Polizeli, A.C.S. Rizzatti, R. Monti, H.F. Terenzi, J.A. Jorge, D.S. Amorim, Xylanases from fungi: properties and industrial applications, *Appl. Microbiol. Biotechnol.* 67 (2005) 577-591.

- [60] R.D.P.B. Pirola, P.S. Delabona, C.S. Farinas, Enzymatic Hydrolysis of Sugarcane Bagasse Using Enzyme Extract and Whole Solid-state Fermentation Medium of Two Newly Isolated Strains of *Aspergillus Oryzae*, Chemical Eng. Trans. 38 (2014) 259-264.
- [61] J.K. Ko, Y. Kim, E. Ximenes, M.R. Ladisch, Effect of Liquid Hot Water Pretreatment Severity on Properties of Hardwood Lignin and Enzymatic Hydrolysis of Cellulose, Biotechnol. and Bioeng. 112 (2015) 252-262.
- [62] Y. Kim, T. Kreke, J.K. Ko, M.R. Ladisch, Hydrolysis-Determining Substrate Characteristics in Liquid Hot Water Pretreated Hardwood, Biotechnol. Bioeng. 112 (2015) 677-687.
- [63] Y. Kim, T. Kreke, R. Hendrickson, J. Parenti, M.R. Ladisch, Fractionation of cellulase and fermentation inhibitors from steam pretreated mixed hardwood, Bioresour. Technol. 135 (2013) 30-38.
- [64] M.R. Ladisch, C.S. Gong, G.T. Tsao, Cellobiose hydrolysis by endoglucanase (glucan glucanohydrolase) from *Trichoderma reesei* - kinetics and mechanism, Biotechnol. Bioeng. 22 (1980) 1107-1126.
- [65] C.S. Gong, M.R. Ladisch, G.T. Tsao, Cellobiase from *Trichoderma viride* - purification, properties, kinetics, and mechanism, Biotechnol. Bioeng. 19 (1977) 959-981.
- [66] R.D.P.B. Pirola, F.C.F. Baleeiro, C.S. Farinas, Saccharification of Biomass Using Whole Solid-State Fermentation Medium to Avoid Additional Separation Steps, Biotechnol. Prog. 29 (2013) 1430-1440.
- [67] R.D.P.B. Pirola, P.S. Delabona, C.S. Farinas, Simplification of the Biomass to Ethanol Conversion Process by Using the Whole Medium of Filamentous Fungi Cultivated Under Solid-State Fermentation, Bioenerg. Res. 7 (2014) 744-752.

Figure captions:

Figure 1. Experimental procedures for submerged and sequential cultivation methods.

Submerged fermentation consists of pre-culture and enzyme production steps, while sequential fermentation consists of solid phase pre-culture followed by submerged pre-culture, and then enzyme production. Conditions given in the figure are discussed in Materials and Methods.

Figure 2. Functional classification of proteins identified from *T. reesei* Rut C30 secretome. The proteins identified by LC – MS/MS analysis of the secretome produced from growth in submerged and sequential fermentation were classified according to their biological function.

Figure 3. Functional classification of proteins identified from *A. niger* A12 secretome. The proteins identified by LC – MS/MS analysis of the secretome produced from growth in submerged and sequential fermentation were classified according to their biological function.

Figure 4. Comparison of performance of submerged and sequential fermentation enzyme extracts based on glucose released during enzymatic hydrolysis (96 h) of sugarcane bagasse using the equivalent loading of both enzymatic extracts. Conditions for hydrolysis of sugarcane bagasse: 0.7 FPU/g glucan or 71 mg protein/g glucan (for *T. reesei*): 3.6 CBU/g glucan or 44.3 mg protein/g glucan (from *A. niger*) at a bagasse solids loading of 1% (w/v) based steam exploded sugarcane bagasse with composition given in section 2.6. Hydrolysis carried out at pH 4.8 and 50°C in an orbital shaker at 200 rpm for 96 hours.

Table 1. Comparison of enzyme activity profiles for *T. reesei* Rut C30 and *A. niger* A12 under submerged (A) and sequential fermentation (B).

			Enzyme Activity (IU.mL ⁻¹)			
Enzymes	Substrate	Method	<i>T. reesei</i> Rut C30		<i>A. niger</i> A12	
			A	B	A	B
Endoglucanase	carboxymethylcellulose	Dien et al, 2008	1.6	2.5±0.6	0.6	0.9
β-glucosidase	cellobiose	Ghose, 1977	0.02	0.02	0.1	0.8
FPase	filter paper	Ghose, 1977	0.02	0.02	0.01	0.01
Xylanase	xylan birchwood	Dien et al, 2008	7.9±0.6	18.3±0.3	7.8±0.6	26.4±2.1
β-xylosidase	p-nitrophenol	Dien et al, 2008	0.6	1.3	24.6	31.5
Total protein*	bovine serum albumin	Pierce BCA protein assay kit	3.0±0.2	3.7±0.1	2.9±0.1	3.2

*expressed in mg.mL⁻¹

Table 2. Activity profile for enzyme extracts combined for hydrolysis of non-washed sugarcane bagasse. Enzyme extracts were produced by *T. reesei* Rut C30 and *A. niger* A12 under (A) submerged and (B) sequential fermentation.

	Enzymes (g of glucan)	<i>T. reesei</i>	<i>A. niger</i>	Total
A	FPase (FPU.g ⁻¹)	0.7	0	0.7
	Endoglucanase (IU.g ⁻¹)	57.1	2.1	59.3
	Cellobiase (CBU.g ⁻¹)	1.0	3.6	4.6
	Xylanase (IU.g ⁻¹)	506.4	26.4	532.9
	Total protein (mg.g ⁻¹)	69.1	1.9	71.0
B	FPase (FPU.g ⁻¹)	0.7	0	0.7
	Endoglucanase (IU.g ⁻¹)	61.9	0.7	62.6
	Cellobiase (CBU.g ⁻¹)	0.7	3.6	4.3
	Xylanase (IU.g ⁻¹)	662.6	65.7	728.3
	Total protein (mg.g ⁻¹)	43.6	0.7	44.3

Fig 1.

Fig. 2

Fig. 3

Fig. 4