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Research paper

Rice straw hydrolysis using secretomes from novel fungal isolates from Vietnam

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ABSTRACT

With a production of 39 million metric tons each year, rice is one of the main agricultural products of Vietnam. Thus, rice straw is a significant by-product, whose use in a biorefinery process would contribute to the bio-based transformation of the Vietnamese and South East Asian economy. In order to find novel efficient enzyme mixtures for the hydrolysis of rice straw and other agricultural residues, we took advantage of the rapidly evolving biodiversity of Vietnam and screened 1100 new fungal isolates from soil and decaying plant tissues for their CMCase activity. We selected 36 strains and evaluated them for their cellulases, xylanases, and accessory enzymes activities. Most of these isolates belonged to the genera *Aspergillus* and *Trichoderma*. We identified a few promising isolates, such as *A. brunneoviolaceus* FEC 156, *A. niger* FEC 130 and FEC 705, and *A. tubingensis* FEC 98, FEC 110 and FEC 644, whose produced enzyme mixtures released a mass fraction of the sugar content of alkali-treated rice straw higher than 20%, compared to 10% for *Trichoderma reesei* RUT C-30. We verified that the black Aspergilli are particularly efficient in their saccharification ability. We also identified strains that although they produced low amounts of cellulases and xylanases, their enzyme mixtures had high saccharification efficiencies, indicating the importance of the synergy effect, rather than the amount of enzymes available. Our results highlight the intra-species variation, especially in the *Trichoderma* genus, regarding the biomass degradation characteristics and the associated range of enzymatic activities.

1. Introduction

Lignocellulosic material will have a key role as a raw material in the bio-based economy concept, as it is *de facto* renewable and its use may well be sustainable. As a by-product of agriculture and forestry, lignocellulose could be used without ethical constraints for the production of biofuels and other biochemicals. In addition, and only if regulated properly, it could be a sustainable alternative to the dwindling forestry sector in Europe and North America [1]. Lignocellulose is recalcitrant, due to the interweaving of cellulose within the hemicellulose and lignin matrix [2].

According to the United Nations Environment Programme [3], five billion metric tons of lignocellulosic biomass is generated every year from agriculture. The energy that is stored in its chemical bonds has the thermal equivalent of 1.2 billion metric tons of oil, which is one fourth of the current production [3]. Rice, as an important part of human diet, contributes greatly to these amounts. Almost 700 million metric tons were produced in 2014 and a mass fraction of it higher than 90% originated from Asian countries [4]. This can be translated to 700–1050 million metric tons of rice straw [5]. Due to its high silicon concentration, rice straw has low digestibility compared to other straws [6] and is normally burnt or left to degrade naturally. Consequently, it is reasonable to assume that it could function as a readily available

Abbreviations: CMC, Carboxymethylcellulose; PDA, Potato Dextrose Agar; MFA, Methyl ferulate; MSP PCR, Micro Satellite primed PCR; ITS, Internal Transcribed Spacer; BLAST, Basic Local Alignment Search Tool; MCL, Maximum Composite Likelihood; HPAEC-PAD, High Performance Anion-Exchange Chromatography equipped with Pulsed Amperometric Detection; FAE, Feruloyl acid esterase.

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raw material in a biorefinery concept, provided an efficient degradation is achieved.

The hydrolysis of biomass to monosaccharides is still considered to be a major bottleneck from the point of view of efficiency and applicability [7–10]. Rice straw hydrolysis has been approached from various angles; the process has been optimized with dilute acid at high temperature and pressure [11], while successful efforts to improve the enzymatic hydrolysis efficiency with alkaline pretreatment have been reported [12]. Enzymatic hydrolysis, which is often presented as a sustainable approach for conversion of cellulose to glucose, must be preceded by other types of pretreatment in order to be efficient and it is burdened by the high enzyme cost [13]. Pretreatment modifies the structural characteristics of lignocellulose by decreasing crystallinity and by increasing the surface area and the volume of the pores [14], thus improving the enzymatic digestibility of the plant cell [15].

In nature, filamentous fungi are the main decomposers of lignocellulosic biomass. This ability has been attributed to the development of (a) growth through hyphae, which enabled them to colonise the soil and penetrate the bulky plant tissues, and of (b) new enzymatic pathways that allowed degradation of the inherently recalcitrant structural compounds of vascular plants [2]. *Trichoderma reesei* QM6a was isolated (and initially described as *T. viride*) during the World War II at the Solomon Islands. It was reported as a good producer of cellulases [16]. It was then subjected to several rounds of UV and chemical mutations and screening in order to enhance its cellulase yields and reduce its catabolite repression on enzyme production, resulting in the *T. reesei* RUT-C30 strain [17]. This strain is considered to be a model fungal one due to its biomass-degrading ability and it is commonly used for cellulase production. Even so, it may not be the ideal microorganism for the complete degradation of cellulose and hemicelluloses present in plant biomass. Actually, a mass fraction of 80–85% of the proteins secreted under cellulase-inducing conditions are considered to be cellobiohydrolases, and CBHI—the major cellobiohydrolase—accounts for a fraction of about 60% of all the proteins that are secreted [18]. In 2D-gel analysis of the secretome of *T. reesei* grown on xylose and lactose, the most abundant proteins have been also found to be cellobiohydrolases [19]. Moreover, MS/MS shotgun proteomics analysis of the secretome of RUT-C30 grown on corn stover revealed a clear predominance of CBHI in terms of spectral counts [20].

Considering the renewed interest in hemicellulose degradation, together with the ever-increasing interest in cellulose degradation for the use of plant biomass, it is important to screen for efficient degraders, which could be more specialized than *T. reesei* for the saccharification of common agricultural and forestry waste products. With the intention to identify enzymatic mixtures that could be applied in a biorefinery that utilises rice straw as raw material, we screened a large number of new isolates from northern Vietnam for cellulases and hemicellulases production. Being in the tropical zone, Vietnam has a rich microbial biodiversity. This has been recently attributed to an increased rate of formation of new species, colonisation by temperate species and a lower rate of extinction of species [21]. The biodiversity of fungi in Vietnam has not been studied extensively. Our work was a thorough investigation of 200 sampling points with a view to isolate strains with high biotechnological potential regarding biomass degradation. We aimed at the identification of strains that would be worth to investigate further for the hydrolysis of rice straw and other plant feedstocks in a biorefinery perspective.

We selected 36 strains that showed higher Carboxymethyl Cellulose (CMC) and xylan hydrolytic capacity in the initial screening and we also examined their respective enzyme activities when grown on two different carbon sources. We finally investigated their ability to hydrolyse alkali-treated and untreated rice straw from Vietnam.

2. Materials and methods

2.1. Sampling

Fungi were isolated from soil samples and decaying plant debris samples collected in northern Vietnam on modified Czapek with cellulose (cellulose filter paper 10 g L^{-1}) as the sole carbon source, agar (20 g L^{-1}) and mineral composition (g L^{-1}): $(NH4)_2SO_4$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01.

2.2. Cultures

For initial screening, the isolated strains were cultured for seven days at 30 °C on solid medium containing rice bran (1.5 g, Hadong market, Hanoi, Vietnam), brewer's spent grain (1.5 g, from a pilot beer brewery at the Food Industries Research Institute (Hanoi, Vietnam), sugarcane bagasse (1.5 g, Lam Son Sugar JSC, Thanh Hoa, Vietnam), and 10 mL of the salt solution described above. Crude enzyme mixtures were extracted by addition of 0.1 mol L^{-1} sodium acetate, pH 5.0, incubation for 2 h at 30 °C with shaking at 3.3 Hz, and centrifugation at 10,000×*g* for 10 min at 4 °C.

Each selected strain was used to inoculate potato dextrose agar (PDA) plates and was incubated for 3–5 d at 30 °C. Spores suspensions were prepared by adding 10 mL of Tween 80 (0.5 mL L^{-1}) and by filtration through sterile Miracloth (Millipore, Darmstadt, Germany). The liquid cultures were performed for the selected strains, as well as for *Trichoderma reesei* RUT-C30 for comparison. Two different substrates were used: Avicel PH-101 (11365; Sigma-Aldrich, St. Louis, MO, USA) and wheat bran (Vetekli, Kungsörnen, Stockholm, Sweden). The carbon source was added in the medium at a concentration of 20 g L^{-1}. The composition of the medium was as follows (in g L^{-1}): KH_2PO_4, 4; $(NH_4)_2SO_4$, 13.6; $CaCl_2.2H_2O$, 0.8; $MgSO_4.7H_2O$, 0.6; and Bacto Peptone (Becton, Dickinson and Company; NJ, USA), 6. The concentrations of trace elements were (in mg L^{-1}): $FeSO_4.7H_2O$, 10; $MnSO_4.H_2O$, 3.2; $ZnSO_4.7H_2O$, 2.8; and $CoCl_2.6H_2O$, 4. Tween 80 was added to the medium at a final concentration of 200 μL L^{-1}.

All cultures were performed in duplicates in 100 mL flasks with 20 mL medium at 30 °C and shaking at 3 Hz. The initial spore concentration was approximately 5×10^6 spores mL^{-1}. Samples of 1 mL were collected at 24, 72, 120, and 168 h. The samples were centrifuged at 12,000×*g* for 5 min at 4 °C and the supernatant was filtered and maintained in aliquots at −20 °C for further use.

2.3. Enzymatic assays

For initial screening, 50 μL of the crude enzyme solution collected from the solid cultures was placed in 8-mm diameter wells on agar plates containing 20 g L^{-1} agar (LP0028B; Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) and 5 g L^{-1} low viscosity CMC (C5678; Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mol L^{-1} sodium acetate buffer, pH 5.0, and incubated for 16 h at 50 °C. The plate was then stained with a solution of 1 g L^{-1} Congo Red (C6277; Sigma Aldrich), for 30 min and destained with 1 mol L^{-1} NaCl for 10 min. The diameter of the clearing zone was measured as an indication of the corresponding enzymatic activity. Celluclast (10 g L^{-1}; Novozymes, Denmark) was used as a control.

Cellulase and xylanase activities of the liquid culture crude extracts were determined using dinitrosalicylic acid assay [22], by measuring the release of reducing sugars from filter paper (Whatman Chromatography paper 3001–931; GE Healthcare, Little Chalfont, UK) and xylan from birchwood (X0502; Sigma-Aldrich).

The assays were performed in 96-well microtitre plates using the FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The reaction suspension contained 3.4 mg filter paper (38.5 mm^2) suspended in 40 μL of 50 mmol L^{-1} citrate buffer, pH 4.8, for cellulase activity [23], or 40 μL of a 10 g L^{-1} suspension of xylan from birch wood (Sigma-Aldrich, Germany) in 50 mmol L^{-1} citrate buffer, pH 4.8. Twenty μL of enzyme solution, appropriately diluted, was added. Twenty μL of enzyme mixture, heat-treated at 100 °C for 15 min, was used instead as a control and buffer was used instead as a blank. The suspension was incubated at 50 °C for 60 min, followed by the addition of 120 μL DNS solution (3,5-dinitrosalicylic acid, 5.3 g L^{-1}; NaOH, 9.9 g L^{-1}; potassium sodium tartrate tetrahydrate, 153 g L^{-1}; disodium sulphite, 4.15 g L^{-1}; and phenol, 3.8 mL L^{-1}), and incubated at 100 °C for 5 min. Distilled water (160 μL) was added to 36 μL of the reaction mixture and the absorbance was measured at 540 nm. A calibration curve of absorbance versus glucose or xylose respectively was used to determine the enzymatic activity.

Alpha-glucuronidase activity was determined with the α-D-glucuronidase Assay Kit (K-AGLUA; Megazyme, Bray, Ireland), with modification of the microplate assay procedure to half volumes of all reagents. Citrate buffer (100 mmol L^{-1}), pH 5.8, was used as reaction buffer. Reaction time was 30 min at 40 °C.

Esterase activity was determined by measuring the p-N-phenol released from p-NP-acetate, after incubation with the enzyme solution. p-NP-acetate (Sigma-Aldrich) (2.5 μL from a stock solution of 34 mmol L^{-1}) was added in 197.5 μL of 100 mmol L^{-1} phosphate buffer, pH 6. Enzyme solution, appropriately diluted, was added (2.5 μL) and the difference in absorbance at 410 nm was determined after 10 min at 37 °C. The concentration of the nitrophenol released was calculated based on a standard curve, in the same buffer. Heat-deactivated enzyme mixtures were used as control.

Feruloyl esterase activity was determined according to Yue et al. [24], using methyl-ferulate (MFA) as substrate. The reaction mixture contained 150 μL of 100 μmol L^{-1} MFA in citrate buffer (50 mmol L^{-1}), pH 6, and 50 μL enzyme solution appropriately diluted in the same buffer. The reaction was performed for 10 min at 37 °C. The apparent absorbance at 340 nm is the cumulative absorbance of the ferulic acid and the remaining MFA, while the initial absorbance is only due to the initial MFA. All accessory enzyme activities (α-glucuronidase, esterase, feruloyl esterase) are presented qualitatively in the Results.

2.4. Molecular identification

Genomic DNA was isolated from mycelia of the fungal strains selected from the initial screening, using routine molecular techniques [25]. Microsatellite-primed (MSP) PCR [26] with the primer $(GAC)_5$ was used for fingerprinting of the strains, as previously described [27].

The Internal Transcribed Spacer (ITS) region was isolated by PCR, using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) as described previously [28]. The DNA fragments were separated by gel electrophoresis and the corresponding bands were purified using QIAEX II (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, and sequenced by Macrogen Inc. (Seoul, Korea). Sequences were compared with the available databases, using BLAST at the National Center for Biotechnology Information (NCBI). Strains FEC 156 and FEC 385 were identified by the CBS-KNAW Fungal Biodiversity Centre. All strains are deposited in the Vietnam Collection of Industrial Microorganisms which is hosted by Food Industries Research Institute and they have been given the prefix FEC in the name of the strains.

For the phylogenetic association between the selected strains, their ITS regions were aligned using MUSCLE [29] and manually checked for discrepancies.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [30]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are also reported [31]. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method [32] to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There was a total of 1275 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [33].

2.5. Hydrolysis analysis

Rice straw (variety C70-2043) was used to investigate the hydrolysing efficiency of the crude enzyme mixtures from the selected fungal strains. Rice straw was hammermilled and passed through an 18-mesh sieve. Alkaline treatment was performed using 100 g L^{-1} NaOH at a liquid-to-solid ratio of 8, at 121 °C for 60 min [34]. It was then cooled to room temperature and the pH was adjusted to 7.0 with HCl before being freeze-dried. Hydrolysis tests were performed in a final volume of 1 mL of 50 mmol L^{-1} citrate buffer, pH 5.0, complemented with 0.2 g L^{-1} sodium azide, using 10 mg of untreated rice straw (9 mg of dry matter) as substrate. Hydrolysis tests on alkali-treated rice straw were performed in a final volume of 1.5 mL with 100 mg dry matter of rice straw. Alkali-pretreated rice straw is more easily hydrolysed than untreated rice straw, so higher concentrations were used in the hydrolysis tests, to detect differences in strain performance. All reactions were performed in 1.5-mL and 2-mL Eppendorf tubes (Eppendorf, Hamburg, Germany) to allow for adequate mixing and prevent accumulation of the substrate at the bottom of the tube.

The reaction mixture was brought to the incubation temperature, 50 °C, for 15 min before addition of the enzyme mixture. As separate controls, heat-deactivated enzyme mixture or buffer was added instead of the enzyme mixture.

Fifty μg of total protein, determined with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) based on the Bradford assay, according to the manufacturer's instructions, was used from the last sample (168 h) of the cultures grown on wheat bran. If necessary, enzyme mixtures were concentrated with Amicon Ultra 0.5 mL centrifugal filters for protein purification and concentration (Millipore, Darmstadt, Germany), according to the instructions of the manufacturer.

The reaction mixture, buffered to pH 6.0 with 50 mmol L^{-1} citrate and supplemented with 0.2 g L^{-1} sodium azide, was incubated at 50 °C and 23.3 Hz in a Thermomixer Comfort (Eppendorf, Hamburg, Germany). Samples of 100 μL were collected after 1, 6, 24, 48, and 72 h. Each sample was boiled at 100 °C for 10 min to stop the reaction and to precipitate the proteins. It was then centrifuged at 12,000×*g* for 10 min and the supernatant was kept at −20 °C until analysis. All reactions were performed in duplicates (technical duplicates) with enzyme extracts from two cultures (biological duplicates).

2.6. Analytics

Carbohydrates released from rice straw after enzymatic hydrolysis were analysed with a high-performance anion-exchange chromatography (HPAEC) system equipped with pulsed amperometric detection, a Carbopac PA-1 column (4 mm × 250 mm i.d.; Dionex; Thermo Scientific, Waltham, MA, USA) and a Carbopac PA-1 guard column (4 mm × 50 mm i.d.; Dionex) maintained at 25 °C

throughout the analysis. The column was equilibrated with 160 mmol L^{-1} NaOH and 34 mmol L^{-1} sodium acetate for 6 min and then with MilliQ water for 5 min. Then 20 μL samples, prefiltered through nylon filters of 0.22 μm pore size (514–0066; VWR, Darmstadt, Germany) were injected in the column using an autosampler, and eluted at 1 mL min^{-1}—initially with an isocratic elution of MilliQ water for 20 min, followed by a linear gradient of NaOH from 0 to 100 mmol L^{-1} for 5 min and then by a linear gradient of sodium acetate from 0 to 85 mmol L^{-1} for 5 min. Identification and quantification of hydrolysis products was done based on glucose, xylose, mannose, arabinose, rhamnose, galactose and cellobiose standards.

Rice straw monosaccharide composition was determined according to the Laboratory Analytical Procedures of the National Renewable Energy Laboratory [35].

3. Results and discussion

3.1. Strain isolation and initial screening

During the period 2010–2012, about 1100 fungal strains were isolated from soil and rotting wood from northern Vietnam. With a view to identifying novel strains with biomass degrading capabilities and biotechnological potential in the context of a biorefinery for the utilization of agricultural waste, we worked on narrowing down the isolated strains, using various culture conditions and enzymatic assays.

In an initial screening, all the strains isolated were cultured in solid-state cultures with rice bran and brewer's spent grain as carbon source. The crude enzyme extracts of the solid-state cultures were evaluated for their cellulase potential on agar plates with CMC. After staining with Congo red, the formed halo was used as a marker of the activity of the respective enzyme (data not shown). Isolates with cellulase activity were selected and grouped based on morphological appearances. The isolates demonstrating similar appearance were compared by microsatellite PCR fingerprinting to confirm genetic homogeneity. Selected isolates from each genetic group were sequenced the ITS region for species identification. In total, approximately 500 isolates were subjected to PCR fingerprinting.

3.2. Species identification

From the strains tested, 36 strains demonstrating high hydrolytic activities and being genetically distinctive as assessed by micro-satellite PCR fingerprinting were selected for species identification by sequencing of the ITS region. Species assignment was based on similarity to the reference sequences of corresponding type strains. In some cases, where ITS sequences were not available for type strain, corresponding sequences of other well-studied strains were used as references. Table 1 is a list of all 36 strains, with the strain name and the respective species giving the highest identity.

The species assignment was fairly reliable since all 36 strains

Table 1
Identification of strains selected in this study, based on ITS sequencing.

Strain	Species name	Genbank	Reference	Similarity
FEC 40	*Trichoderma harzianum*	KT824792	AF414297$^{(T)}$	98.4% (493/501)
FEC 42	*Trichoderma harzianum*	KM386415	AF414297$^{(T)}$	99.2% (497/501)
FEC 65	*Trichoderma harzianum*	KM386418	AF414297$^{(T)}$	98.4% (493/501)
FEC 67	*Trichoderma atroviride*	KM386431	FN396554	99.8% (499/500)
FEC 70	*Trichoderma brevicompactum*	KM386430	EU338330	100.0% (501/501)
FEC 83	*Trichoderma harzianum*	KM386417	AF414297$^{(T)}$	98.8% (494/500)
FEC 93	*Penicillium oxalicum*	KM386408	AF033438$^{(T)}$	99.8% (677/678)
FEC 98	*Aspergillus tubingensis*	KM386437	AJ223853$^{(T)}$	100.0% (428/428)
FEC 108	*Trichoderma virens*	KM386421	EU280073$^{(T)}$	99.8% (500/501)
FEC 110	*Aspergillus tubingensis*	KM386436	AJ223853$^{(T)}$	99.8% (466/467)
FEC 112	*Trichoderma virens*	KM386422	EU280073$^{(T)}$	99.6% (499/501)
FEC 115	*Trichoderma harzianum*	KM386420	AF414297$^{(T)}$	98.6% (494/501)
FEC 118	*Trichoderma virens*	KM386423	EU280073$^{(T)}$	100.0% (501/501)
FEC 128	*Penicillium oxalicum*	KM386409	AF033438$^{(T)}$	100.0% (582/582)
FEC 130	*Aspergillus niger*	KM386439	AJ223852$^{(T)}$	99.6% (529/531)
FEC 142	*Trichoderma harzianum*	KM386419	AF414297$^{(T)}$	99.4% (498/501)
FEC 147	*Trichoderma harzianum*	KM386412	AF414297$^{(T)}$	98.8% (484/490)
FEC 156	*Aspergillus brunneoviolaceus*[a]	KM386435	AJ280003$^{(T)}$	99.8% (507/508)
FEC 158	*Trichoderma asperellum*	KM386429	AY380912$^{(T)}$	99.7% (490/491)
FEC 161	*Trichoderma virens*	KM386424	EU280073$^{(T)}$	99.8% (500/501)
FEC 162	*Trichoderma virens*	KM386425	EU280073$^{(T)}$	99.8% (500/501)
FEC 258	*Trichoderma harzianum*	KM386413	AF414297$^{(T)}$	98.8% (495/501)
FEC 383	*Trichoderma harzianum*	KM386414	AF414297$^{(T)}$	99.4% (498/501)
FEC 385	*Penicillium oxalicum*[a]	KM386410	AF033438$^{(T)}$	99.1% (345/348)
FEC 600	*Penicillium simplicissimum*	KM386411	GU981601$^{(T)}$	98.0% (493/503)
FEC 616	*Trichoderma asperellum*	KM386426	AY380912$^{(T)}$	99.6% (473/475)
FEC 629	*Trichoderma asperellum*	KM386427	AY380912$^{(T)}$	99.3% (426/429)
FEC 644	*Aspergillus tubingensis*	KM386438	AJ223853$^{(T)}$	99.6% (476/478)
FEC 645	*Trichoderma asperellum*	KM386428	AY380912$^{(T)}$	100.0% (424/424)
FEC 704	*Curvularia affinis*	KM386433	HM770741	100.0% (512/512)
FEC 705	*Aspergillus niger*	KM386440	AJ223852$^{(T)}$	99.6% (525/527)
FEC 714	*Epicoccum sorghi*	KM386432	FJ427067	100.0% (452/452)
FEC 723	*Aspergillus oryzae*	KM386434	EF661560$^{(T)}$	100.0% (477/477)
FEC 730	*Aspergillus niger*	KM386441	AJ223852$^{(T)}$	99.0% (518/523)
FEC 745	*Trichoderma harzianum*	KM386416	AF414297$^{(T)}$	98.3% (482/490)
FEC 755	*Trichoderma harzianum*	KM569672	AF414297$^{(T)}$	98.9% (526/532)

The name and number of the strain refer to its code in the Food Industries Research Institute strain collection. Reference accession no. is the accession no. of the strain giving the higher similarity after BLAST with GenBank. $^{(T)}$ Sequence of the type strain.

[a] Identification confirmed by the CBS.

demonstrated greater than 98% sequence similarity to the references. Among them, 28 strains showed more than 99% similarity. The 36 strains could be placed under 13 species belonging to 5 fungal genera. Strains belonging to the same species were often not identical and showed some slight variation in the ITS region. The most diverse was genus *Trichoderma* with 22 strains and 5 species detected, followed by *Aspergillus* (8 strains and 4 species), *Penicillium* (4 strains, 2 species), and *Curvularia* and *Epicoccum* (1 strain, 1 species each). The most common species was *Trichoderma harzianum* with 11 strains recorded. *Trichoderma harzianum*, with the sexual state known as *Hypocrea lixii*, is a cellulase producer and widely used in agriculture as biocontrol against fungal pathogens [36,37]. Most of the identified species are saprotrophic and known as strong lignocellulolytic enzyme producers. We also identified one strain of *Epiccocum sorghi* and a strain of *Curvularia affinis*, both of which have been reported as plant pathogens [38,39]. *E. sorghi* is a Pleosporales ubiquitous fungus in tropical and subtropical regions and it is a weak parasite on *Poaceae*, such as rice, sugar cane, wheat,

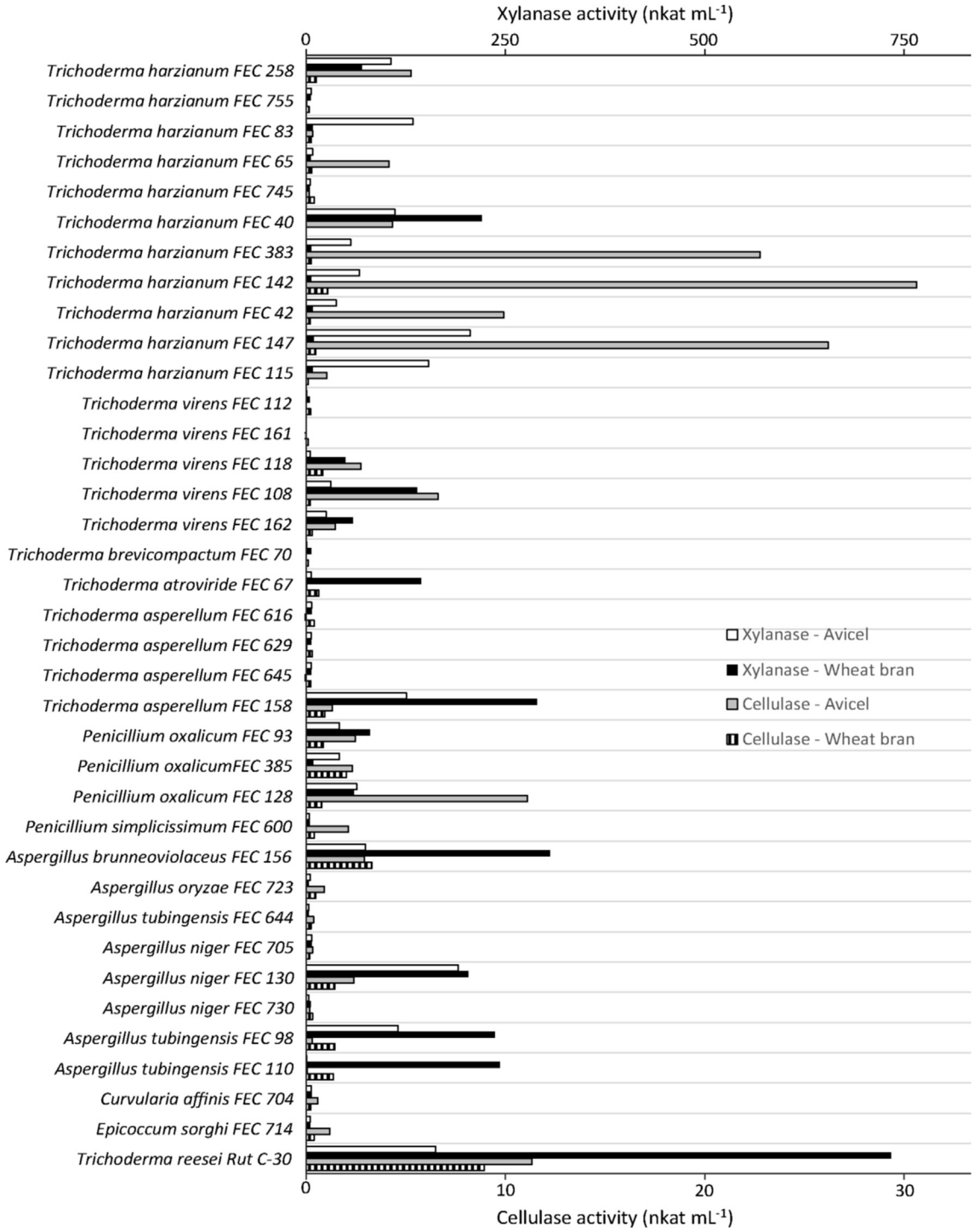


Fig. 1. Cellulase activity on filter paper and xylanase activity on xylan from beechwood after seven days of growth in liquid cultures on Avicel® or wheat bran as carbon source. Xylanase when grown on Avicel (white), xylanase when grown on wheat bran (black), cellulase when grown on Avicel (grey), cellulase when grown on wheat bran (striped).

sorghum, and maize [40]. *C. affinis* is a Pleosporales soil fungus, found frequently in healthy leaf tissues of oil palm trees [41] and on the stem of the red ginger plant [42].

3.3. Production of enzyme mixtures and determination of their activities

The cellulolytic and hemicellulolytic capacity of these 36 strains was assayed after culturing them in liquid medium with Avicel® and wheat bran as carbon source for 168 h. Wheat bran has been shown to promote the production of hydrolytic enzymes in *Aspergillus oryzae* [43] and *T. reesei* [44] and its saccharide content includes mostly starch, arabinoxylan, and cellulose [45,46]. Fig. A1 in the supplementary material shows the cellulase activity detected after 24, 72, 120, and 168 h with growth on Avicel® (Fig. A1-a) or wheat bran (Fig. A1-b). The corresponding xylanase activity detected after 24, 72, 120, and 168 h is shown in the supplementary material in Fig. A2-a and b. The phylogenetic distance of the different strains is also presented, pinpointing the variation in the assayed activities within similar groups of microorganisms. The activities only for the time point of 168 h are summarized in Fig. 1.

There was significant variation in the cellulase and xylanase activities detected even among strains of the same species. For example, *Trichoderma harzianum* FEC 755 had significantly lower cellulase and xylanase activity than *T. harzianum* FEC 40 when grown in either Avicel® or wheat bran. It was not surprising that *T. reesei* RUT C-30, a strain selected for its hydrolytic efficiency, outperformed the rest in both cellulase activity and xylanase activity when grown on wheat bran. It was quite unexpected, though, that in Avicel®, several strains—such as *T. harzianum* FEC 142, and *T. harzianum* FEC 147—showed higher cellulase activity than *T. reesei* RUT C-30. Even so, in these cultures, cellulase activity did not exceed 33 nkat mL^{-1}.

Accessory enzymes contribute to the complete degradation of hemicellulose in a synergistic way [47] by removing the different side-residues that are present in the native hemicelluloses. They are expected to have biotechnological application by increasing the hydrolysing efficiency of the crude enzyme extract [48]. Especially feruloyl esterases can have applications as feed additives to improve nutrient assimilation or for the extraction of ferulic acid from agro-industrial waste materials [49]. Ferulic acid is a precursor for vanillin and a compound with reported antimicrobial activity [49]. Alpha-glucuronidases can have a significant role in xylan degradation [50]. A number of assays were performed to evaluate the production of such accessory enzymes. Table 2 shows the strains in whose crude enzyme extracts we detected esterase, feruloyl esterase, and α-glucuronidase activities, as accessory activities that contribute to the general hydrolytic efficiency of the crude enzyme extract. Members of the Aspergilli showed high esterase and feruloyl esterase activity, while α-glucuronidase was detected in both *Aspergillus* and *Trichoderma* genera. *A. brunneoviolaceus* FEC 156, a member of the black Aspergilli, showed the highest α-glucuronidase activity. It is worth mentioning that for all strains whose enzyme cocktails showed high saccharification potential (final saccharification of a mass fraction higher than 20%; Fig. 2), we also detected all three of the assayed accessory activities. Nevertheless, the opposite cannot be supported. Although esterase activity can be exhibited by a wide range of enzymes, the genome of *A. niger* has been reported to contain at least one acetyl-esterase, similar with an acetyl-esterase from *A. aculeatus* and similar with other esterases and lipases [51], while similar genes have been reported for *T. harzianum*, *T. reesei,* and *A. oryzae* [52,53]. Feruloyl esterases have been characterised from *A. niger* [54] and *A. tubingensis* [55], while α-glucuronidases have been reported on *A. niger* [56], *A. tubingensis* [57] and *T. reesei* [58].

3.4. Rice straw hydrolysis

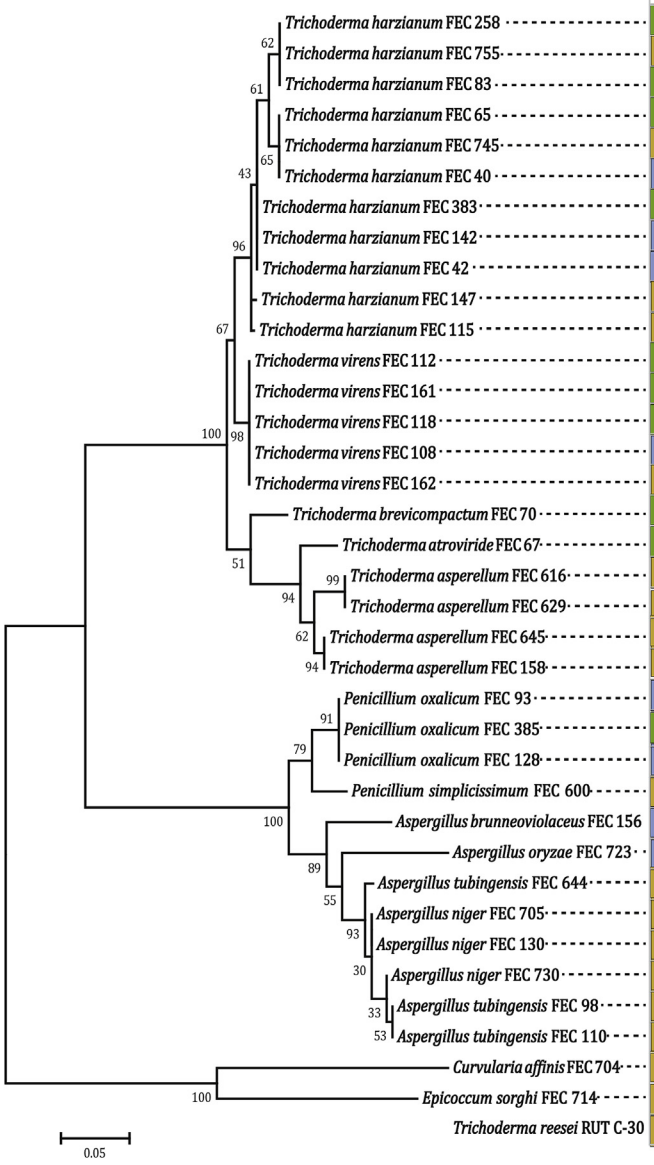
Fungal rice straw hydrolysis has been studied previously from different perspectives. Zhang and Cai studied the fungal treatment of alkali-pretreated rice straw using a strain of *T. reesei* originated through random mutagenesis [59]. Although it is not clear on what basis the mutant was selected, the optimal conditions that yielded the highest amount of (soluble) sugars were selected. Still, as the decomposition of rice straw was measured gravimetrically, it is not clear what percentage of rice straw was used for fungal growth and how applicable this approach would be in an industrial perspective. In a more extensive study, Lee et al. evaluated a number of fungal strains isolated from rice straw for their cellulolytic and xylanolytic enzyme production [60]. Although their efficiency on rice straw saccharification was not determined, the fungi with the highest relevant enzyme activities included an *A. niger* and a *T. harzianum* strain. A strain of *E. sorghi* was also isolated from rice straw [60].

In our study, in order to evaluate the applicability of the crude enzyme mixtures for their hydrolytic capabilities, 50 μg of crude protein from the last day of each culture on wheat bran was used for the hydrolysis of untreated and alkaline-treated rice straw. While using wheat bran instead of rice straw as substrate for the enzyme production could have affected the composition of the enzyme mixture as it has been previously demonstrated for *T. reesei* on different substrates [61], there are several reports downplaying the importance of this effect [62,63].

The sugar composition of rice straw presented as a mass fraction of its dry weight was determined as 3.4% arabinose, 0.1% rhamnose, 1.5% galactose, 40.9% glucose, 19.4% xylose, and 0.7% mannose. The

Table 2
Esterase, α-glucuronidase and feruloyl esterase activity detected in the investigated strains.

Strain	GlcAse	Est	FAE
Trichoderma harzianum FEC 258	−	−	++
Trichoderma harzianum FEC 755	−	−	−
Trichoderma harzianum FEC 83	−	−	−
Trichoderma harzianum FEC 65	−	−	−
Trichoderma harzianum FEC 745	−	−	−
Trichoderma harzianum FEC 40	−	−	+
Trichoderma harzianum FEC 383	−	+	−
Trichoderma harzianum FEC 142	−	−	+
Trichoderma harzianum FEC 42	++	−	+
Trichoderma harzianum FEC 147	++	−	−
Trichoderma harzianum FEC 115	−	−	−
Trichoderma virens FEC 112	−	−	−
Trichoderma virens FEC 161	−	−	+
Trichoderma virens FEC 118	−	−	−
Trichoderma virens FEC 108	−	+	+
Trichoderma virens FEC 162	−	−	+
Trichoderma brevicompactum FEC 70	−	−	−
Trichoderma atroviride FEC 67	++	−	−
Trichoderma asperellum FEC 616	−	−	−
Trichoderma asperellum FEC 629	−	−	−
Trichoderma asperellum FEC 645	−	−	−
Trichoderma asperellum FEC 158	−	+	+
Penicillium oxalicum FEC 93	−	+	+++
Penicillium oxalicum FEC 385	−	+	++
Penicillium oxalicum FEC 128	−	+	+++
Penicillium simplicissimum FEC 600	−	−	−
Aspergillus brunneoviolaceus FEC 156	+++	++	+
Aspergillus oryzae FEC 723	−	−	−
Aspergillus tubingensis FEC 644	−	−	−
Aspergillus niger FEC 705	−	−	−
Aspergillus niger FEC 130	++	+++	++
Aspergillus niger FEC 730	−	−	−
Aspergillus tubingensis FEC 98	+	++	−
Aspergillus tubingensis FEC 110	++	+++	+
Curvularia affinis FEC 704	−	−	−
Epicoccum sorghi FEC 714	−	−	−



	Hydrolysis rate[1] (mg h^{-1})	Initial Saccharification[2]	Total monosaccharides[3] (mg)	Final saccharification[4]
Trichoderma harzianum FEC 258	1.2	1.8	4.5	6.8
Trichoderma harzianum FEC 755	2.6	4.0	13.3	20.1
Trichoderma harzianum FEC 83	0.8	1.2	1.6	2.5
Trichoderma harzianum FEC 65	0.8	1.2	1.5	2.3
Trichoderma harzianum FEC 745	1.0	1.6	4.4	6.6
Trichoderma harzianum FEC 40	0.8	1.3	2.2	3.3
Trichoderma harzianum FEC 383	0.8	1.3	2.1	3.1
Trichoderma harzianum FEC 142	0.9	1.3	2.4	3.7
Trichoderma harzianum FEC 42	0.8	1.2	2.3	3.5
Trichoderma harzianum FEC 147	1.0	1.6	5.0	7.6
Trichoderma harzianum FEC 115	1.0	1.5	3.7	5.6
Trichoderma virens FEC 112	0.7	1.1	1.2	1.8
Trichoderma virens FEC 161	5.3	8.1	8.6	13.0
Trichoderma virens FEC 118	0.8	1.2	2.1	3.2
Trichoderma virens FEC 108	1.1	1.6	3.0	4.6
Trichoderma virens FEC 162	1.4	2.2	6.6	10.0
Trichoderma brevicompactum FEC 70	0.9	1.3	1.6	2.4
Trichoderma atroviride FEC 67	0.4	0.6	3.1	4.7
Trichoderma asperellum FEC 616	2.8	4.3	8.3	12.6
Trichoderma asperellum FEC 629	3.1	4.6	10.0	15.2
Trichoderma asperellum FEC 645	2.2	3.3	9.9	15.0
Trichoderma asperellum FEC 158	2.1	3.3	9.3	14.0
Penicillium oxalicum FEC 93	1.5	2.3	6.0	9.1
Penicillium oxalicum FEC 385	1.4	2.2	7.9	11.9
Penicillium oxalicum FEC 128	1.6	2.4	6.2	9.4
Penicillium simplicissimum FEC 600	2.0	3.0	7.2	10.9
Aspergillus brunneoviolaceus FEC 156	2.3	3.5	14.9	22.6
Aspergillus oryzae FEC 723	2.0	3.0	7.2	10.8
Aspergillus tubingensis FEC 644	3.6	5.5	14.6	22.2
Aspergillus niger FEC 705	3.7	5.6	14.2	21.5
Aspergillus niger FEC 130	3.2	4.9	16.3	24.7
Aspergillus niger FEC 730	2.7	4.0	11.6	17.6
Aspergillus tubingensis FEC 98	2.8	4.2	14.4	21.8
Aspergillus tubingensis FEC 110	2.5	3.8	14.5	22.0
Curvularia affinis FEC 704	0.4	0.7	3.8	5.8
Epicoccum sorghi FEC 714	0.7	1.0	4.1	6.2
Trichoderma reesei RUT C-30	0.6	0.9	6.6	10.0

Fig. 2. Hydrolytic activity of crude enzyme extracts from different fungal strains on alkali-treated rice straw. [1] The hydrolysis rate refers to the mass in mg of all monosaccharides detected after 1 h of hydrolysis; [2] The hydrolysis rate as a mass fraction of the sugars that the substrate is composed of; [3] The total mass of monosaccharides released after 3 d of hydrolysis; [4] The total mass of the monosaccharides released after 3 d as a fraction of the monosaccharide composition of the substrate. The colour refers to the main monosaccharide released; green – glucose; orange – xylose; blue – no significant difference. The selected fungal strains' phylogenetic relationship is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

monosaccharides released were mainly arabinose, galactose, glucose, xylose, and mannose. Cellobiose was also quantified. Fig. 3 shows indicative sugar release profiles from treated rice straw for the crude enzyme extracts of *T. reesei* RUT C-30, *T. harzianum* FEC 755 and *T. virens* FEC 161 during three days of incubation. The sugar release profiles for all strains are presented in Fig. A3, panels a–c (Supplementary material).

The evaluation of the sugar release profiles of the crude enzyme extracts revealed that the enzyme cocktail of *T. reesei* RUT C-30 released significant amounts of cellobiose, while the main monosaccharide released was xylose (Fig. 3, panel A). On the other hand, although the enzyme cocktail of *A. brunneoviolaceus* FEC 156 released significant amounts of cellobiose (more than 0.75 g cellobiose per g of glucose, supplementary material, Fig. A3-C), it also released high amounts of xylose and glucose, indicating that the accumulation of cellobiose might be the result of its too efficient biomass degrading system, rather than its lack of β-glucosidase, as it is the case for *T. reesei* RUT C-30. As another example, the enzymatic mixture of *T. harzianum* FEC 755 performed much better than that of *T. reesei* RUT C-30 in terms of xylose and glucose released (Fig. 3, panel B). This can be partly attributed to the more efficient β-glucosidase, as the cellobiose detected was much lower. Low β-glucosidase has been reported for *T. reesei* RUT C-30 [64], which also results in an inhibitory effect from cellobiose on the other cellulases [65]. Considering that in the cultures of *T. harzianum* FEC 755 we did not detect significant cellulase and

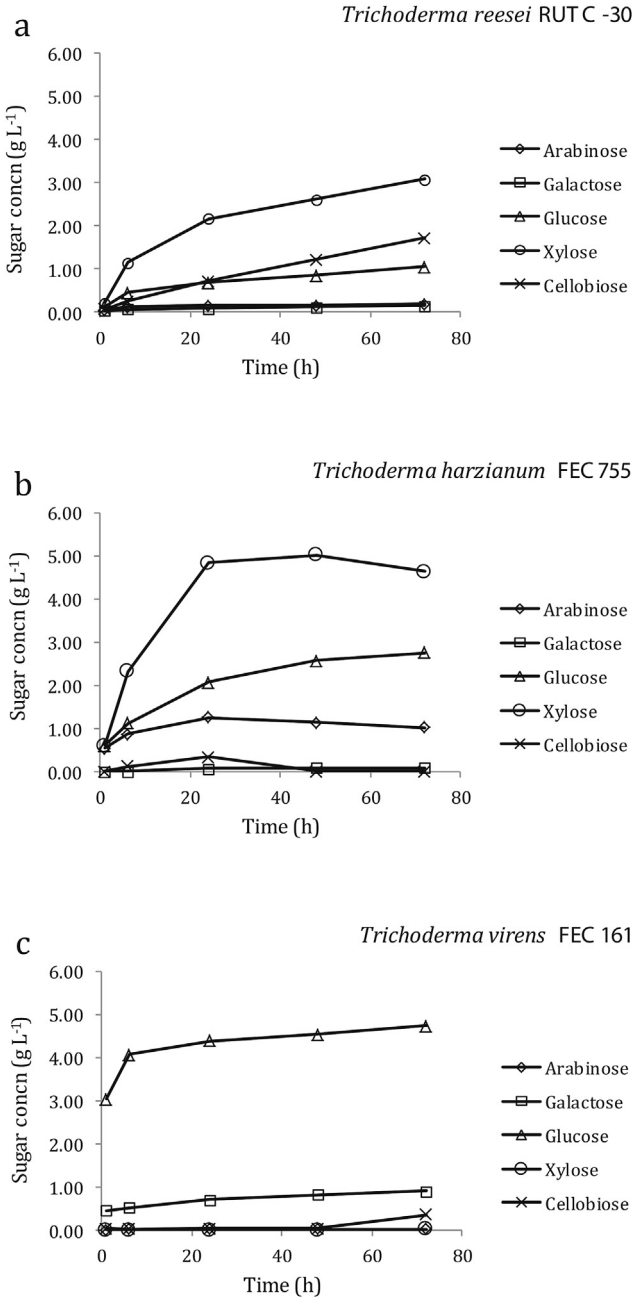


Fig. 3. Examples of the sugar release profiles acquired over three days of hydrolysis, using supernatants of the cultures of *T. reesei* RUT C-30 (A), *T. harzianum* FEC 755 (B), and *T. virens* FEC 161 (C). *T. harzianum* FEC 755 mainly released xylose and *T. virens* FEC 161 mainly released glucose. Both strains outperformed *T. reesei* RUT C-30 in the hydrolysis of alkali-treated rice straw.

xylanase activities, the saccharification efficiency of its enzymatic cocktail suggests that its qualitative composition and the putative synergy effects are more important than the amount of the enzymes produced, as it has been reported for other fungal species [66]. On the other hand, the enzymatic cocktail of *T. harzianum* FEC 40, from the culture of which we detected significant cellulase and xylanase activities (Fig. 1, Figs. A1 and A2), performed poorly in the saccharification of rice straw (Fig. 2). This also suggests that the detection of specific broad range enzymatic activities does not necessarily correlate with the saccharification potential of the microorganism in real substrates. The enzyme mixture of *T. virens* FEC 161 released higher amounts of glucose, but performed poorly on the hydrolysis of hemicellulose (Fig. 3, panel C).

Even though *T. reesei* RUT C-30 produces a large variety of biomass-degrading enzymes, even in the presence of only cellulose as carbon source [67], our results indicate that it may not be efficient in the hydrolysis of rice straw and that other microorganisms may be more suitable. This could also be the case in the hydrolysis of other agricultural by-products [66].

The initial hydrolysis rate, the maximum amount of monosaccharides released and the initial and maximum amounts of sugars released relative to the composition of rice straw are presented in Fig. 2 for the alkali-treated rice straw and in Table A1 in the supplementary material for untreated rice straw.

The highest initial hydrolysis rate was observed for *T. virens* FEC 161, which released a mass fraction higher than 8% of the sugars present after 1 h of incubation, followed by *A. niger* FEC 130, *T. asperellum* FEC 629, *A. tubingensis* FEC 644, and *A. niger* FEC 705. The latter released a mass fraction of almost 5% of the total sugar composition of rice straw. *A. tubingensis* FEC 98, *A. tubingensis* FEC 110, *A. niger* FEC 130, *A. brunneoviolaceus* FEC 156, *A. tubingensis* FEC 644, and *A. niger* FEC 705 showed the highest total amount of saccharification achieved, three days after the start of the incubation, verifying the evolutionary dominance of the black aspergilli as biomass degraders [68].

When the enzyme mixtures were evaluated on untreated rice straw, the efficiency of the saccharification was lower, as expected, due to the recalcitrance of the material. The strains that showed high release of monosaccharides from the treated rice straw were also present here, in most cases achieving a release of a mass fraction higher than 7% of the total sugars. The variability observed between the duplicates, leading to a high deviation (data not shown) can be justified by the heterogeneity of the material when not subjected to any kind of pretreatment other than milling. It is also interesting that although some strains (such as *P. simplicissimum* FEC 600 and *T. asperellum* FEC 616) were among the most efficient in the hydrolysis of untreated rice straw—achieving more than 7% saccharification—they did not benefit from the alkaline treatment of rice straw as much as most other top degraders, since they achieved only 10.9% and 12.6% saccharification respectively. This might be an indication that although their hydrolytic toolbox is not as efficient as for the other strains, they might have other auxiliary enzymes that allow better penetration in the non-pretreated rice straw. Experiments on the synergistic action of these enzyme mixtures could shed light on the validity of such a hypothesis.

The low saccharification efficiency of most *T. harzianum* and *T. virens* strains could be related to the close association that these fungi can have with the plant root system, where their role is often beneficial for the plant growth, as they induce characteristic auxin-related phenotypes on *Arabidopsis thaliana* [69,70]. In a similar interaction, *T. asperellum* T203, which has been shown to penetrate the roots of cucumber seedlings and colonise the epidermis and the outer root cortex [71], was also reported to induce systemic resistance in plants, i.e. promote a state in the plant that enables it to be more resistant to subsequent pathogen infection [72].

The strains in this study could be categorized depending on whether their crude enzyme extract released mainly glucose during hydrolysis of alkaline-treated rice straw (*T. virens* FEC 112, FEC 118, FEC 161; *T. harzianum* FEC 65, FEC 83, FEC 258, FEC 383; *T. atroviride* FEC 67; *T. brevicompactum* FEC 70; *P. oxalicum* FEC 385), xylose (*T. harzianum* FEC 115, FEC 147, FEC 745, FEC 755; *A. tubingensis* FEC 98, FEC 110, FEC 644; *A. niger* FEC 130, FEC 705; FEC 730, *T. asperellum* FEC 158, FEC 616, FEC 629; FEC 645, *T. virens* FEC 162; *P. simplicissimum* FEC 600; *C. affinis* FEC 704; *E. sorghi* FEC 714) and those producing similar amounts of glucose and

xylose—less than 20% difference (*T. harzianum* FEC 40, FEC 42, FEC 142; *P. oxalicum* FEC 93, FEC 128; *T. virens* FEC 108; *A. brunneoviolaceus* FEC 156; *A. oryzae* FEC 723). Depending on the application in mind, different enzymatic cocktails could be used. For example, *T. virens* FEC 161 seems to favour the production of cellulases, regardless the presence of hemicellulose in the medium, and could be applied on occasions when low xylanase activities are desirable. A high intra-species variation was found in all cases, both in terms of the capacity of the produced enzyme mixtures to hydrolyse rice straw, and the profile and levels of enzymatic activities produced by the strains. High intra-species variation in biomass degradation pattern indicates rapid rate of differentiation and adaptation of fungi toward heterogeneous plant substrates in the tropics. In the search for novel biomass modifying enzymes, it also implies the immense diversity and potentials that tropical fungi may offer.

4. Conclusions

This is the first report of this scale on the identification of novel fungal strains from the tropical environment of Vietnam, based on their plant biomass degrading capability. From 1100 new fungal isolates, we selected 36 strains for which we evaluated the production of cellulolytic and hemicellulolytic enzymes. In view of the availability of rice straw as a feedstock in South-east Asia, we evaluated their efficiency in its hydrolysis. Most of the selected strains were from the genera *Trichoderma* and *Aspergillus*, verifying their predominant position as biomass degraders. The crude enzyme extract of the latter released the highest amount of monosaccharides from alkaline-treated rice straw. Several of the evaluated strains' enzyme mixtures performed better than *T. reesei* RUT C-30, raising the question whether this is due to the inherent traits of the selected strains or a peculiarity of rice straw, as feedstock. *A. niger* FEC 130 released a mass fraction of almost 25% of the total monosaccharides present in the rice straw cellulose and hemicellulose polymers. *A. brunneoviolaceus* FEC 156, *A. niger* FEC 705 and three strains of *A. tubingensis* (FEC 644, 98 and 110) released a mass fraction of around 22% of the total sugars as monosaccharides. The same strains had the highest hydrolysis rates among the strains studied. Moreover, our results highlight the intra-species variation regarding the biomass degradation characteristics, as demonstrated by their capacity to hydrolyse rice straw, as well as the associated enzymatic activities. Alkaline pretreatment of rice straw improved the hydrolytic efficiency of the enzyme mixtures produced, pinpointing the importance of the pretreatment method in the efficiency of this process. Optimisation of the pretreatment and enzymatic application could lead to further improvements on the yields and efficiencies of the whole process.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biombioe.2017.02.008.

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