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On-Site Enzyme Production by *Trichoderma asperellum* for the Degradation of Duckweed

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Abstract

The on-site production of cell wall degrading enzymes is an important strategy for the development of sustainable bio-refinery processes. This study concerns the optimization of production of plant cell wall-degrading enzymes produced by *Trichoderma asperellum*. A comparative secretome analysis was performed on *T. asperellum* growing on PDA agar, wheat bran and duckweed, respectively. *T. asperellum* proved to be able to produce a wide enzyme profile, including both depolymerization and debranching enzymes, mainly consisting of hemi-cellulases. The secretome analysis showed specific glycoside hydrolase-induction on duckweed compared with growth on wheat bran and PDA, including a GH62 α-L-arabinofuranosidase, a promising candidate enzyme for the degradation of duckweed. The enzyme cocktail from *T. asperellum* proved capable of degrading pretreated duckweed, obtaining up to 60% of the theoretical glucose yield, making it a potential candidate for on-site enzyme production.

Keywords: On-site enzyme production; Cazymes; Duckweed; *Trichoderma asperellum*

Introduction

Glycoside hydrolases (GHs) can enzymatically hydrolyze carbohydrate materials like plant cell walls, releasing building blocks, such as protein, bioactive polymers and simple sugars. These simple sugars can be fermented into a range of value-added bio-products, thereby maximizing the value derived from the biomass feedstock [1,2]. One of the major bottlenecks for the application of GHs in converting biomass in the bio-refinery platform is their high cost. This can be reduced by using a local on-site production, which decentralizes the process, making it overall more economically feasible [3]. The composition of the enzymatic blends for hydrolysis is shown to be dependent on the growth substrate [4,5]. It is therefore rational to produce on-site enzymatic blends, using the specific pretreated feedstock, which in turn will be enzymatically hydrolyzed by this enzymatic cocktail. This could as well reduce the production costs of downstream products, since both processes could share location, infrastructure and utilities.

For on-site GH production it is essential to select fungal strains that possess high expression and wide diversity of cell wall degrading enzymes with high activity [6]. The choice of substrate is important, as biomass induces a different fungal enzyme response depending on its structure and composition. Several *Trichoderma* species have been successfully cultivated on various lignocellulosic substrates, and their Carbohydrate Active enZymes (CAZymes) analyzed [7,8]. *Trichoderma asperellum* has shown its potential for secreting a range of GHs [9,10], growing on substrates such as cellulose [11] and sugar bagasse [10]. This paper will focus on the degradation of duckweed, a common name for the *Lemnaceae* family, is a small flowering plant, belonging to a family of five genera (*Lemna, Landoltia, Spirodela, Wolffia and Wolfiella*) and 38 species. The potential of using duckweed as a feedstock for biofuels mainly comes from its rapid growth and ability to store large amount of either starch or protein. Since the optimal conditions for duckweed growth and starch enrichment are quite different, growing high-starch duckweed for biofuel production is normally divided into two steps, biomass production and starch accumulation. By manipulating growth conditions, such as daily light integral and nutrient level [12], a starch content of 3-75% or a protein content of 15-45% of the dry weight, respectively, can be obtained [13,14]. These values place the protein content of dry duckweed biomass between alfalfa (20%) and soybean (41.7%) [15]. These are all traits that make duckweed an interesting feedstock for the bio-refinery platform.

The objective of the present work was to develop an on-site produced enzyme blend of cell wall degrading enzymes (CWDE) for the hydrolysis of pretreated duckweed. The production of CWDEs by a new strain of *T. asperellum* isolated from duckweed in Yunnan, China, was studied under different process and cultivation conditions. Enzyme blends were evaluated with azurine cross-linked (AZCL) substrates and used to hydrolyze pretreated duckweed. Furthermore, the degree of pretreatment was analyzed to evaluate the lightest pretreatment needed to promote enzyme production and effective hydrolysation of the duckweed. Subsequently, the secretome of *T. asperellum* grown on PDA, duckweed and wheat bran was analyzed by mass spectrometry to elucidate substrate specific GHs.

Materials and Methods

Duckweed species and growth

Two species of duckweed were used: the small *Lemna minor* and the larger *Spirodela polyrhiza*, both grown in Yunnan, China. An initial duckweed culture was maintained in a water bath containing tap water and nutrient soil. This was done on the roof top in Chengdu, where temperatures in that period varied from 15-28^{0}C during the day and 5-12^{0}C during the night. The duckweed was harvested and washed

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three times in a bucket with tap water, then placed in tap water in small plastic containers in a duckweed growth room. Here the duckweed was induced to build up the starch content for 5 days under fluorescent light 24h/day. High-starch duckweed was harvested and centrifuged for 2min at 1500g.

Pretreatment

The fresh duckweed was subject to four different levels of pretreatment: (1) not pretreated, (2) autoclaved for 20min at 120°C, (3) dried overnight at 60°C, then autoclaved for 20min at 120°C and (4) dried overnight at 60°C, then grinded to powder in a coffee grinder (SP-742, Buttler) and autoclaved for 20min at 120°C. On-site enzyme production was performed on duckweed that was dried overnight at 60°C, then autoclaved for 20min at 120°C. Samples of wheat straw were grinded in a coffee grinder (SP-742, Buttler) and either autoclaved 20min at 120°C or acid treated (0.75% (v/v) sulfuric acid at 100°C for 20min).

Hydrolysis by commercial enzymes

The recalcitrance of duckweed exposed to different pretreatment methods was evaluated by degrading the duckweed with the commercial enzyme blend GC220 from Genencor. The effectiveness of the pretreatment methods was evaluated based on the degree of released sugar. Triplicates of duckweed samples pretreated under different conditions was mixed with 0,2M sodium acetate buffer pH 5 and a variable volume of GC220 in a 250mL shake flask and hydrolyzed for two days at 60°C and 300rpm. The amount of sugar released, and hereby the level of hydroxylation, was evaluated by HPLC.

Fungal enzyme production

Trichoderma asperellum, strain number CBS 433.97 from the Centraalbureau, voor Schimmelcultures, CBS, The Netherlands, was used in this study. Stock cultures were maintained as agar plugs in 20% glycerol at -80°C. Agar plugs were sub-cultured on plates containing 39 g/l yeast extract potato dextrose (YPD) and incubated statically at 30°C for 7 days to obtain spores for inoculation. Solid state fermentation was done in 250mL Erlenmeyer flasks, containing 1g dried duckweed and either 10mL H20, 10mL Mandel's medium (1.87g/l (NH4)2SO4, 2.67g/l KH2PO4, 0.53g/l CaCl2·2H2O, 0.81g/l MgSO4·7H2O, 6.60mg/l FeSO4·7H2O, 2.10mg/l MnSO4·H2O, 1.90mg/l ZnSO4·7H2O, 26.70mg/l CoCl2·6H2O) or 10mL buffer solution (0,2M succinate buffer pH 5.5). The flasks were prepared by autoclaving (at 120°C, 20 min). The solid state fermentation was started by inoculating the prepared flasks with 2 agar plugs (0.5cm x 0,5cm), from the YPD plate described above. Liquid fermentation was carried out by growing a pre culture in 10% (w/v) glucose and Mandel´s medium for 3 days, followed by a transfer of fungal biomass to a 2% (w/v) solution of powdered duckweed and incubation at 30°C and 250rpm for 7 days. All cultivation experiments were carried out in triplicate, and the data calculated as mean ± standard deviation.

Enzyme blend

After the cultivation period, the enzymes from the solid state fermentation were extracted by adding 15mL of a 0.05M sodium citrate buffer pH 5.2. The suspension was stirred at 120 rpm for 5 hours at room temperature, and the crude enzyme solution recovered by centrifugation at 6,000g and 20°C for 10min, followed by filtration through an 8μm filter. For extracting the enzymes from the liquid fermentation, the whole culture, containing enzymes, mycelium and residual solid substrate, was centrifuged at 6,000g and 20°C for 10min and the supernatant used for enzymatic analysis and hydrolyze.

Enzymatic assay

1% Agarose plates were prepared with 20mL buffer (0,08M Phosphoric acid, acetic acid, boric acid, respectively) pH 6 containing 0.1% azurine cross-linked (AZCL) substrate and 15μL enzyme blend pipetted into holes punched in the plates. Enzyme activity was seen as blue color zones surrounding the holes, as a result of the hydrolysis of the substrate. The size of the blue zones was measured after 24 hours at 30°C. The area of a blue zone measured in cm^2 is proportional to the enzyme activity, and a range of AZCL substrates can thus be used to categorize the enzyme activity profile under the given conditions. The AZCL activity study was carried out in duplicate.

Enzymatic hydrolysis

For 1,5mL-experiments, 1,5mL of the crude enzyme blend was transferred to a 2mL Eppendorf tube containing 300mg autoclaved duckweed. The Eppendorf tubes were placed in Eppendorf thermo mixers at 50°C and 1100rpm for 2 days. For 15mL-experiments, 15mL enzyme blend was mixed with 3g duckweed in 250mL shake flasks and incubated at 50°C and 250rpm for two days.

Sugar Analysis

The content of simple sugars were analyzed by HPLC (Hewlet Packard 1100 series) on a 300 7.8 mm Aminex HPX-87H Column (BioRad) at 60°C with sulfuric acid as eluent at a flow rate of 0.6 ml/min and an injection volume of 10 μl. The components were detected by refractometrically on a RI detector. Theoretical yield of glucose was calculated as released amount of cellobiose and glucose measured with HPLC in relation to the total glucose content of the biomass.

Secretome

5 samples of 40mL supernatants for each growth condition were harvested by centrigugation (4°C, 10min, 10.000g) and 4g of crystalline TCA (Trichloroacetic acid) was added to each 40mL supernatant and stored in freezer overnight. After thawing, protein pellets were harvested by centrifugation (4°C, 30min, 10.000g) and washed with ice-cold acetone two times. Proteins were digested using trypsin and an in-solution protocol. For protein identification and quantification MaxQuant (v. 1.5.0.25) [16] was used. The label-free quantification (LFQ) algorithm [17], the *Match between runs* feature, and iBAQ (intensity based absolute quantification) [18] calculation were activated. Carbamidomethylation of cysteines was defined as fixed modification and oxidation of methionines as variable modification. The remaining settings were kept on default. This includes a maximum peptide and protein false discovery rate of 1%. Proteins had to be found in at least 3 out of 5 replicates to be reported as identified or quantified. For relative abundance changes between conditions, ratios of the mean LFQ values (at least 2 peptides) per condition were formed. For comparison between different proteins within one condition, the median iBAQ value (at least 1 peptide) was used. *Trichoderma* sequences were obtained by partly the sequenced transcriptome of *T. asperellum* from the Transcriptome Shotgun Assembly (TSA) database in Genbank at the NCBI with the accession number SRR1575447 and the sequenced genome of *Trichoderma atroviride* (Genbank accession number GCA_000171015.2) [19]. The reason for not using the published genome of *T. asperellum* in the bioinformatic work is that it is only published and not released and therefore cannot be used in a publication. For protein annotation KOG [20], KEGG [21], Pfam [22] and TIGRFAM [23] information as well as the NCBInr database were

used. KOG and KEGG data were obtained from JGI, Pfam using the webMGA server [24], and blast [25] against the NCB Inr database was done using CLC Main Workbench (v. 7.0.3, Aarhus, Denmark). Peptide Pattern Recognition (PPR) was used for classification of the identified GHs [26].

Results

Pretreatment

Four differently pretreated duckweed samples and two pretreated wheat straw samples were hydrolysed with commercial enzymes to find the least severe pretreatment necessary to ease the sugar release of the enzymatic hydrolysis step: (1) Fresh duckweed (not pretreated), (2) autoclaved duckweed (20min at 120^{0}C), (3) dried and autoclaved duckweed (overnight at 60^{0}C, 20min at 120 ^{0}C) and (4) dried, grinded, and autoclaved duckweed. Wheat straw samples were grinded and either autoclaved or treated with acid as stated in materials and methods. By hydrolyzing 33g/l of each sample with increasing volumes of added commercial enzymes, the degree of pretreatment needed for hydrolyzing duckweed was tested. Fresh duckweed exhibited almost no degradation (Figure 1). Autoclaved (2) and dried and autoclaved duckweed (3) resulted in a much higher release of sugars compared to fresh duckweed. Powdered duckweed, which was hypothesized to show the highest release of sugars in response to enzymatic treatment, showed only a slightly improved sugar release compared to fresh duckweed (Figure 1).

On-site enzyme production

The ability of *T. asperellum* to produce cell wall degrading enzymes on pretreated duckweed (dried and autoclaved at 120^{0}C for 20min) was tested under different conditions, such as time, duckweed species, buffer addition and salt addition (Supplementary information Table S1). The activity of CAZymes and proteases present in the enzyme blend was measured using a range of AZCL substrates. *T. asperellum* had difficulties growing on pure *Lemna minor* and *Spirodela polyrhiza* without any supplements and would quickly form spores compared with the other conditions. A relatively broad range of enzymes produced on pure duckweed was still observed, compared with almost no production on a simple medium such as PDA. The addition of buffer at pH 5.5, salt addition and a growth time of 10 days resulted in the widest range as well as the highest activity of enzymes in terms of the largest areas of the blue halos (Supplementary information Table S1). Both depolymerization and debranching enzymes were produced. Figure 2 illustrates enzyme profiles obtained from growth on duckweed with 0,2M succinate buffer at pH 5.5.

Growth on *Lemna minor* and *Spirodela polyrhiza* induced similar enzyme profiles. Growth on *Spirodela polyrhiza* gave the overall highest enzyme activity, mostly due to higher activity on AZCL-arabinoxylan, AZCL-xylan and to some degree AZCL-amylose. The highest activity for both species were on AZCL-arabinoxylan and AZCL-xylan, indicating a high endo-xylanase activity, whereas only smaller halo areas were detected for cellulases and β-glucosidases on AZCL-cellulose and AZCL-β-glucan, respectively (Figure 2)

Enzymatic hydrolysis

The hydrolytic efficiency of the obtained crude enzyme blends were tested on pretreated duckweed. To quantify the degradation process and to measure the effect of the treatment, samples were spun down and the supernatant analyzed for a range of sugars via HPLC. A *T. asperellum* enzyme blend obtained by on-site production with an activity profile such as the one shown in Figure 2 was used to degrade autoclaved intact duckweed leaves grown to a glucose content of 3% and 34% dry weight, respectively. Hydrolyzing pretreated intact duckweed leaves of both *Lemna minor* and *Spirodela polyrhiza* resulted in a total degradation of the leave structure, generating a mix of insoluble plant leave residues and soluble sugars. The concentration of soluble sugars was measured by HPLC (Figure 3). The predominant sugars were cellobiose, glucose and xylose. Enzymatic hydrolysation of *Lemna minor* and *Spirodela polyrhiza* grown to contain around 34% DW glucose released around 4.5g/l and 4.7g/l glucose, followed by minor concentrations of cellobiose and xylose of 0.1-0.2g/l and around 1g/l, respectively, for both species (Figure 3). Hydrolyzing duckweed batches with a low glucose content of around 3% resulted in almost no sugar release, corresponding to 0.4g/l glucose, 0.1g/l cellobiose and 0.2g/l xylose. Around 45% and 60% of the theoretical glucose yield was obtained by hydrolyzing high-glucose and low glucose duckweed, respectively (Figure 4).

Secretome analysis

A proteomic approach using mass spectrometry (LC-MS/MS) was used in this study to compare the secretomes of *T. asperellum* grown on PDA, wheat bran and duckweed, using a single time point and temperature. The secretomes were analyzed *in silico* for the presence of GHs. Overall 78 GHs from 35 families were identified, 73 GHs from 33 families were found in the secretome derived from growth on wheat bran and 46 GHs from 26 families were found in the secretome derived from growth on duckweed (Figure 5). Nearly half of the enzymes (37 out of the 78 identified GHs) were in common among the three secretomes. 7 GHs were uniquely induced on duckweed compared with PDA, and, besides these, ten were furthermore up-regulated (Table 1). Only two enzymes were uniquely induced on duckweed: a GH36

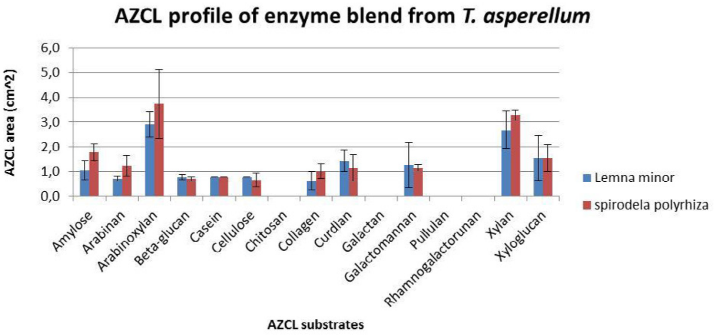

Figure 1: AZCL profiles of the enzyme blend obtained from *T. asperellum* grown on the pretreated duckweed species *Lemna minor* and *Spirodela polyrhiza*, respectively. The AZCL area is the area of a blue zone measured in cm^2.

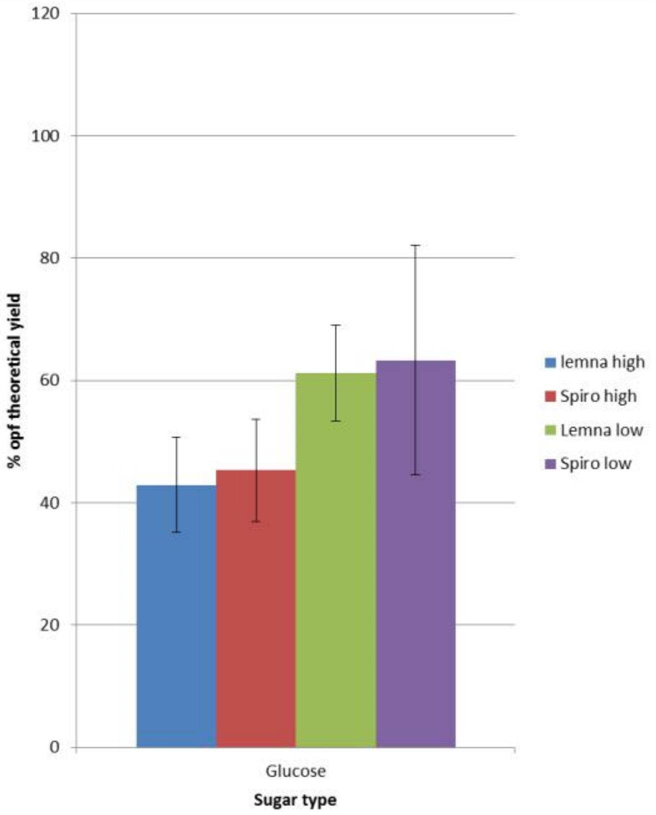

Figure 2: Sugars released after enzymatic hydrolysis of intact *Lemna minor* and *Spirodela polyrhiza* leaves. The enzyme blend from *T. asperellum* grown on pretreated duckweed was used for the hydrolysis, incubating for 2 days at 50°C and 1000rpm. The duckweed was grown to achieve either high or low glucose content (35% and 3% DW glucose).

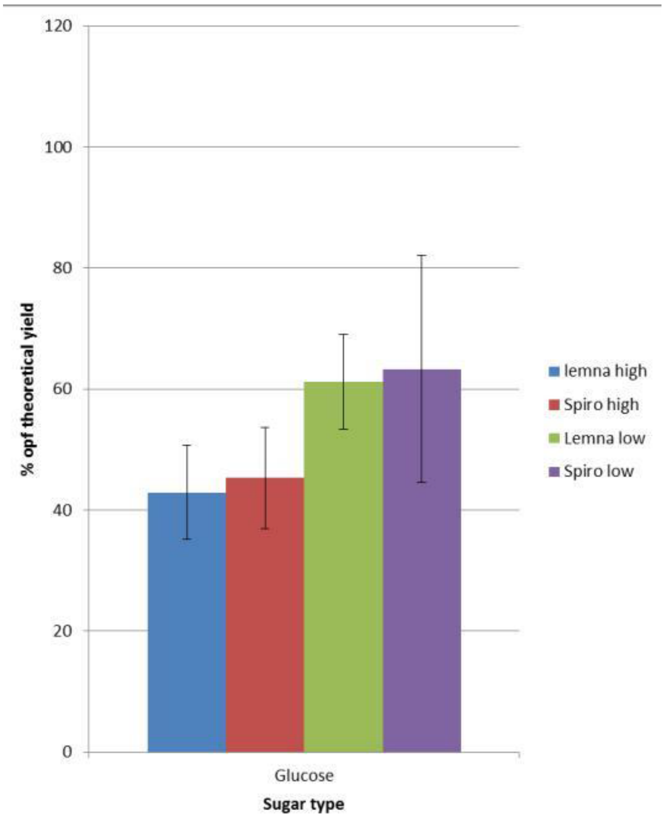

Figure 3: Percent theoretical glucose yield after enzymatic hydrolysis of intact *Lemna minor* and *Spirodela polyrhiza* leaves. The glucose yield from the hydrolysis step (Figure 3) was compared to the theoretical glucose content of the duckweed hydrolyzed. The duckweed was grown to achieve either high or low glucose content (35% and 3% DW glucose).

α-galactosidase (ID 20409) and an endo-1,4-β-xylanase (ID 12950). In comparison, 21 GHs were induced on wheat bran and not on PDA, and 24 were furthermore up-regulated (Table 2). Hence, growth on wheat bran induced a much larger and more diverse GH profile than growth on PDA and duckweed, including 16 GHs not induced on either of these substrates.

Glycoside hydrolases in the secretome of *T. asperellum* grown on duckweed

Overall, a large amount of GHs were identified in the secretome of *T. asperellum* grown on duckweed. The secretome covered a wide range of GH families and all the major plant cell wall degrading enzyme functions: cellulases, hemicellulases, α-glucosidases and pectinases (Table 1). The enzyme abundance was quantified either by arbitrary absolute abundances within sample conditions, or as a relative log2-fold changes compared with growth on PDA. The 20 most abundant enzymes included mainly hemicellulases (ID 8432, 10314, 15847, 15652, 9036), but also starch-degrading α-glucosidases (ID 2969, 396, 20886) and pectinases (ID 4031). Beside these, β-glucosidases (ID 2199, 11046, 958) are included in the top 20 most secreted enzymes, while cellulases (ID 4405, 774, 7861, 6619, 13540) are not among the most abundant enzymes. The most up regulated enzyme on duckweed compared with growth on PDA was a β-glucosidase (ID 2199), followed by an α-mannosidase (ID 16922) and a xylan 1,4-β-xylosidase (ID 10314). The most down regulated enzymes were an α-L-arabinofuranosidase

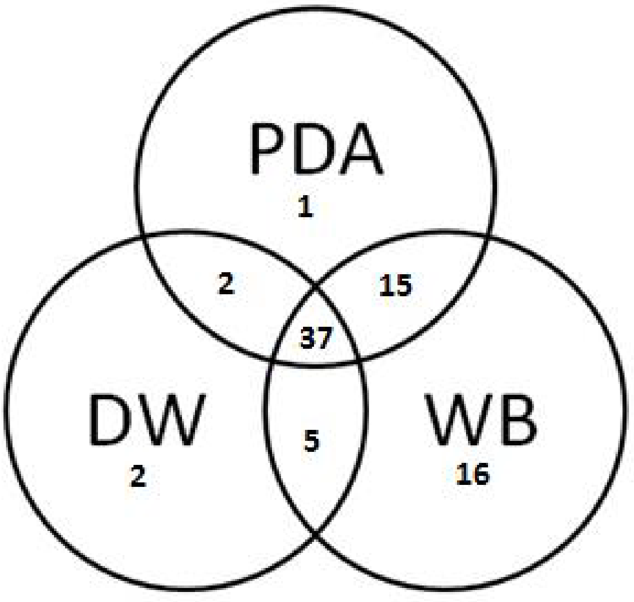

Figure 4: Venn diagram of the number of glycoside hydrolases present in the secretome derived from *T. asperellum* grown on potato dextrose agar (PDA), wheat bran (WB) and duckweed (DW), respectively.

ProteinID	GH family	EC Number	Annotation	IBAQ	log2(DW/PD)
2199	GH55	3.2.1.58	glucan 1,3-β-glucosidase	2,23E + 09	5,0
8432	GH62	3.2.1.55	α-L-arabinofuranosidase	3,39E + 08	on/off
2969	GH31	3.2.1.20	α-glucosidase	1,91E + 08	1,3
10314	GH43	3.2.1.37	xylan 1,4-β-xylosidase	1,65E + 08	4,6
3042	GH38	3.2.1.24	α-mannosidase	9,19E + 07	4,3
15847	GH35	3.2.1.23	β-galactosidase	7,94E + 07	4,3
6963	GH18			7,52E + 07	0,5
396	GH15	3.2.1.3	glucan 1,4-α-glucosidase	3,89E + 07	-0,5
4031	GH28	3.2.1.67	galacturan 1,4-α-galacturonidase	2,89E + 07	-1,4
15652	GH92			2,34E + 07	on/off
19505	GH37	3.2.1.28	trehalase	2,13E + 07	on/off
11046	GH17	3.2.1.39*	endo-β-1,3-glucanase	1,79E + 07	-3,9
16922	GH92			1,68E + 07	4,6
9036	GH03	3.2.1.37	xylan 1,4-β-xylosidase	8,98E + 06	2,2
5992	GH76		*mannan endo-1,6-α-mannosidase	8,54E + 06	-2,0
3977	GH76		*mannan endo-1,6-α-mannosidase	6,70E + 06	-3,4
29229	GH79			6,43E + 06	2,1
958	GH17	3.2.1.39*	exo-1,3-glucanase	6,15E + 06	-1,3
20886	GH15	3.2.1.3	glucan 1,4-α-glucosidase	5,80E + 06	-4,1
24	GH92			5,18E + 06	on/off
20409	GH36	3.2.1.22	α-galactosidase	5,05E + 06	on/off
8089	GH18			5,04E + 06	1,8
1796	GH16	3.2.1.39	glucan endo-1,3-β-D-glucosidase	4,77E + 06	on/off
32409	GH55	3.2.1.58	glucan 1,3-β-glucosidase.	4,16E + 06	on/off
14720	GH13	3.2.1.1	α-amylase	4,01E + 06	on/off
23911	GH03	3.2.1.37	β-xylosidase	3,62E + 06	on/off
32658	GH36	3.2.1.22	α-galactosidase	3,48E + 06	on/off
2441	GH11	3.2.1.8	endo-1,4-β-xylanase	3,34E + 06	on/off
4405	GH03	3.2.1.21	β-glucosidase	3,34E + 06	-1,1
4138	GH54	3.2.1.55	α-L-arabinofuranosidase	3,02E + 06	-5,2
10624	GH02	3.2.1.25	β-mannosidase	2,67E + 06	0,7
29722	GH43	3.2.1.55	β-xylosidase/α-L-arabinofuranosidase	2,67E + 06	on/off
1093	GH17	3.2.1.*	glucan 1,3-β-glucosidase	2,63E + 06	on/off
774	GH16	3.2.1.6	β-1,3-1,4-glucanase	2,14E + 06	on/off
24619	GH02	3.2.1.165	exo-β-D-glucosaminidase	1,97E + 06	on/off
7861	GH03	3.2.1.21	β-glucosidase	1,43E + 06	-2,1
6619	GH07	3.2.1.176	cellulose 1,4-β-cellobiosidase (reducing end)	1,33E + 06	on/off
17015	GH02	3.2.1.25	β-mannosidase	1,23E + 06	on/off
21431	GH18			1,15E + 06	on/off
12950	GH10	3.2.1.8	endo-1,4-β-xylanase	8,33E + 05	on/off
9240	GH78	3.2.1.40	α-L-rhamnosidase	7,54E + 05	3,8
543	GH65	3.2.1.28	α trehalase	5,90E + 05	on/off
1731	GH64	3.2.1.39*	β-1,3-glucanase	5,46E + 05	on/off
5138	GH76			3,70E + 05	on/off
5396	GH31	3.2.1.20	α-glucosidase	2,95E + 05	on/off
13540	GH03	3.2.1.21	β-glucosidase	1,47E + 05	on/off

Table 1: Glycoside hydrolases in the secretome of *T. asperellum* grown on *Spirodela polyrhiza*. Proteins are ordered by decreasing abundance measured by relative intensity (IBAQ). The up or down regulation of specific enzymes compared with growing *T. asperellum* on PDA is calculated as the log2 change. Enzymes that were not identified in the PDA derived secretome do not have a calculated log2 change and are instead marked as on/off, meaning they were induced on duckweed, but not on PDA. EC numbers with "*" are of uncertain function, meaning that only a few identification peptides were found.

(ID 4138), a 1,4-α-glucosidase (ID 20886) and a 1,3-β-glucosidase (ID 11046), where the two latter were also among the most abundant enzymes as well. No enzyme types were exclusively up or down regulated, as can be seen for e.g. α-glucosidases (ID 2969, 396, 20886), α-L-arabinofuranosidases (ID 8432, 4138) and 1,3-β-glucosidases (ID 2199, 11046, 958), though hemicellulases were in general upregulated (Table 1).

Glycoside hydrolases in the secretome of *T. asperellum* grown on wheat bran

Growth of *T. asperellum* on wheat bran induced the widest range of enzymes (73 out of the 78 identified GHs), compared to growth on duckweed and PDA. The most abundant enzymes in the wheat bran-derived *T. asperellum* secretome covered, like the duckweed-derived

ProteinID	GH family	EC Number	Annotation	IBAQ(WB)	log2 (WB/PD)
9036	GH03	3.2.1.37	xylan 1,4-β-xylosidase	5,83E + 09	17,2
2441	GH11	3.2.1.8	endo-1,4-β-xylanase	4,50E + 09	on/off
20886	GH15	3.2.1.3	glucoamylase	2,96E + 09	1,7
6619	GH07	3.2.1.176	cellulose 1,4-β-cellobiosidase (reducing end)	2,03E + 09	on/off
18901	GH27	3.2.1.22	α-galactosidase	1,66E + 09	5,2
396	GH15	3.2.1.3	glucan 1,4-α-glucosidase	9,29E + 08	3,2
4031	GH28	3.2.1.67	galacturan 1,4-α-galacturonidase	5,11E + 08	2,5
21474	GH54	3.2.1.55	α-L-arabinofuranosidase	4,19E + 08	on/off
2969	GH31	3.2.1.20	α-glucosidase	3,93E + 08	2,4
4138	GH54	3.2.1.55	α-L-arabinofuranosidase	3,86E + 08	1,1
11046	GH17	3.2.1.39*	endo-β-1,3-glucanase	3,78E + 08	0,1
2199	GH55	3.2.1.58	glucan 1,3-β-glucosidase	2,93E + 08	3,0
16973	GH07	3.2.1.4	endo-1,4-glucanase	2,36E + 08	on/off
8089	GH18			2,09E + 08	4,1
1731	GH64	3.2.1.39*	β-1,3-glucanase	1,17E + 08	5,9
4405	GH03	3.2.1.21	β-glucosidase	1,03E + 08	1,7
10624	GH02	3.2.1.25	β-mannosidase	9,67E + 07	6,5
15011	GH03	3.2.1.21	β-glucosidase	6,95E + 07	1,0
10314	GH43	3.2.1.37	xylan 1,4-β-xylosidase	6,12E + 07	3,2
4404	GH3	3.2.1.21	β-glucosidase	5,88E + 07	1,1
543	GH65	3.2.1.28	α trehalase	5,30E + 07	1,8
1796	GH16	3.2.1.39	glucan endo-1,3-β-D-glucosidase	5,26E + 07	-2,3
16922	GH92			4,73E + 07	7,4
6963	GH18			4,58E + 07	-1,9
32256	GH13	3.2.1.1	α-amylase	4,12E + 07	1,2
29570	GH05	3.2.1.78	β-mannanase	3,93E + 07	on/off
7861	GH03	3.2.1.21	β-glucosidase	3,87E + 07	2,3
15847	GH35	3.2.1.23	β-galactosidase	3,28E + 07	2,8
1093	GH17	3.2.1.*	glucan 1,3-β-glucosidase	2,74E + 07	1,2
19505	GH37	3.2.1.28	trehalase	2,62E + 07	on/off
21030	GH05	3.2.1.78	β-mannanase	2,52E + 07	on/off
3042	GH38	3.2.124	α-mannosidase	2,40E + 07	2,0
13870	GH18			2,36E + 07	on/off
6759	GH16	3.2.1.39	glucan endo-1,3-β-D-glucosidase	2,35E + 07	on/off
10579	GH11	3.2.1.8	xylanase	2,12E + 07	on/off
22908	GH71	3.2.1.59	endo-1,3-α-glucosidase	2,05E + 07	on/off
9622	GH06	AA9	AA9	1,64E + 07	on/off
6388	GH16			1,62E + 07	-3,5
20252	GH18			1,55E + 07	on/off
7664	GH02	3.2.1.23	β-galactosidase	1,41E + 07	on/off
24	GH92			1,37E + 07	on/off
5992	GH76		*mannan endo-1,6-α-mannosidase	1,27E + 07	-1,8
774	GH16	3.2.1.6	β-1,3-1,4-glucanase	1,12E + 07	on/off
24000	GH28	3.2.1.15	exopolygalacturonase	9,91E + 06	on/off
29229	GH79		β-glucuronidase	9,69E + 06	2,1
29722	GH43	3.2.1.55	β-xylosidase/α-L-arabinofuranosidase	8,98E + 06	on/off
32409	GH55	3.2.1.58	glucan 1,3-β-glucosidase.	8,68E + 06	on/off
15652	GH92			7,84E + 06	on/off
8432	GH62	3.2.1.55	α-L-arabinofuranosidase	6,04E + 06	on/off
9073	GH78	3.2.1.40	α-l-rhamnosidase	5,56E + 06	on/off
21431	GH18			4,42E + 06	on/off
24619	GH02	3.2.1.165	exo-β-D-glucosaminidase	3,84E + 06	on/off
12037	GH92			3,78E + 06	-0,7
28273	GH71	3.2.1.59	α-1,3-glucanase	3,61E + 06	on/off
21751	GH31	3.2.1.20	α-glucosidase	3,32E + 06	on/off
27277	GH27	3.2.1.22	α-galactosidase	2,86E + 06	on/off
9240	GH78	3.2.1.40	α-L-rhamnosidase	2,38E + 06	2,1

958	GH17	3.2.1.39*	exo-1,3-glucanase	2,32E + 06	-3,2
3977	GH76			2,32E + 06	-4,0
27258	GH67	3.2.1.131	α-glucuronidase	2,16E + 06	on/off
23911	GH03	3.2.1.37	β-xylosidase	1,95E + 06	on/off
17015	GH02	3.2.1.25	β-mannosidase	1,93E + 06	on/off
29207	GH05			1,88E + 06	on/off
4419	GH05		*endo-1,4-β-glucanase	1,15E + 06	-4,9
32658	GH36	3.2.1.22	α-galactosidase	1,14E + 06	on/off
23777	GH89	3.2.1.50	α-N-acetylglucosaminidase	9,79E + 05	-1,9
5396	GH31	3.2.1.20	α-glucosidase	9,63E + 05	-0,7
1711	GH47	3.2.1.113	α-1,2-mannosidase	9,37E + 05	on/off
14720	GH13	3.2.1.1	α-amylase	8,83E + 05	on/off
15196	GH43	3.2.1.*	β-1,3-glucanase	5,41E + 05	on/off
17246	GH16	3.2.1.6	β-1,3-1,4-glucanase	3,52E + 05	on/off
4496	GH63	3.2.1.106	mannosyl-oligosaccharide glucosidase	3,14E + 05	on/off
3741	GH18			4,49E + 04	on/off

Table 2: Glycoside hydrolases in the secretome of *T. asperellum* grown on wheat bran. Proteins are ordered by decreasing abundance measured by relative intensity (IBAQ). The up or down regulation of specific enzymes compared with growing *T. asperellum* on PDA is calculated as the log2 change. Enzymes that were not identified in the PDA derived secretome do not have a calculated log2 change and are instead marked as on/off, meaning they were induced on wheat bran, but not on PDA. EC numbers with "*" are of uncertain function, meaning that only a few identification peptides were found.

secretome, all the major plant cell wall degrading functions (Table 2), including main chain hemicellulases, such as xylan 1,4-β-xylosidases (ID 9036. 10314) and endo-1,4-β-xylanases (ID2441), cellulases (ID 6619, 16973, 4405, 15011, 4404) and also a relatively high abundance of α-glucosidases (ID 20886, 396, 2969). Among the 20 most abundant enzymes, five are not induced on PDA: the endo-1,4-β-xylanase (ID 2441), cellulose 1,4-β-cellobiosidase (ID 6619), an α-L-arabinofuranosidase (ID 21474), an endo-1,4-glucanase (ID 16973). The rest of the 20 most abundant GHs are all up regulated compared with growth on PDA. The most up regulated enzymes are hemicellulases: a xylan 1,4-β-xylosidase (ID 9036), an α-mannosidase (ID 16922), a β-mannosidase (ID 10624) and an β-1,3-glucanase (ID 1731). In general, only a few GHs were down regulated on wheat bran compared to PDA, whereof most have not been annotated, except a glucan endo-1,3-β-D-glucosidase (ID 1796) α-N-acetylglucoaminidase (ID 23777) and a potential mannan endo-1,6-α-mannosidase identified with low probability (Table 2).

Discussion

Enzyme production by *Trichoderma* has been reported for a range of different conditions, indicating that several experimental parameters influence enzyme yield, including incubation time, extraction method and substrate loading [27]. Other factors that improve cellulase production by *T. reesei* include relative humidity and temperature [28,29], continuous light exposure [27] and aeration and higher substrate concentration (Pandey, 2003)[30]. In a study performed on *T. asperellum*, a marginally higher endoglucanase and β-xylosidase activity was measured when it was incubated in darkness under a controlled relative humidity of 90%, compared with the standard culture without relative humidity control [10]. In the contrary, exposure to continuous light generally had a negative effect on the activity of endoxylanases and side chain hemicellulases [10]. This study focused on the substrate composition by changing the growth substrate to correlate it to enzyme production and enzyme diversity.

Enzymatic hydrolysis

Enzyme blends are typically produced in two ways, either in liquid fermentation or solid state fermentation, where microorganisms are cultured on a moist solid bed [7]. Most research on enzymatic hydrolysis has been done using submerged liquid fermentation in either shake flasks or larger fermenters. This method includes several advantages, such as increased control of process parameters, but can in contrast be more complicated than solid state fermentation. Solid state fermentation is simpler, requires less energy and may utilize more insoluble carbohydrate materials with lower quality [31] and has furthermore been demonstrated to produce potent enzyme blends with a high potential for hydrolyzing lingo cellulosic material [32,33].

On-site production of enzymes by *Trichoderma* has been investigated for the hydrolysis of a range of substrates, such as: wheat straw [34-36], wet-exploded corn stover [37], sugar cane bagasse [38-40], steam-treated spruce [41], rice straw [42,43], wet-exploded loblolly pine [37] as well as newspaper sludge and steam-exploded wood al., [44]. Compared with these substrates, the duckweed species *Lemna minor* and *Spirodela polyrhiza* should in theory exhibit lower recalcitrance, due to their simpler structure and low lignin content. The enzyme extract that we obtained from *T. asperellum* grown on duckweed proved to significantly degrade duckweed. This was largely affected by the substrate loading for the hydrolysis, showing that above a certain load, the ability to degrade the plant cell wall structure, as well as the sugar release, significantly decreased. This was also seen by Kristensen et al [44]. Kristensen et al. [44], who found that the enzymatic conversion decreased linearly with an increase in the concentration of the solid loading. The authors argued that this can be an effect of the substrate inhibiting the cellulases. In studies on the inhibition of enzyme hydrolysis by sugar structures, Varnai et al. [46] concluded that enzymatic hydrolysis was limited by the structure and recalcitrance of the plant cell walls.

Ultimately, our experiment resulted in a glucose release for duckweed with 34% glucose content of around 4.5 g/l, corresponding to 45% of the theoretical yield with a substrate loading of 30 g/l. In comparison, enzyme blends from *T. reesei* and *T. atroviride* grown on steam-pretreated sugarcane bagasse at 20 g/l substrate loading showed glucose yields of 8.3g/l and 6.7g/l, respectively [41]. Benoliel et al. [38] reported a release of 2.28g/l from sugarcane bagasse and 4.48g/l from partially delignified celluignin after 18 h of substrate hydrolysis, corresponding to a theoretical glucose yield of 60.32% and 59.35%, respectively. Delabona et al. [39] reported that a *T. harzianum* enzyme blend is able to release 5.5 g/l glucose from sugar cane bagasse and

8.18g/l from delignified sugar cane bagasse, while a *T. reesei* RUT C30 enzyme blend released 5.8g/l and 9.7g/l, respectively. That corresponded to yields of 19.4% and 16.6%, respectively, for *T. harzianum* and 20.6% and 19.9%, respectively, for *T. reesei* RUT C30 [39]. Shaibani Nasim and coworkers [39] made a comparison of enzyme blends produced by several *Trichoderma* species by hydrolyzing pretreated sugarcane bagasse, resulting in glucose releases ranging from 5g/l to 10g/l, corresponding to an efficiency rate of 20-30%. Comparison with these previous studies puts the glucose yield of the *T. asperellum* enzyme blend of our study in the lower end. To be able to handle larger solid loadings and thus to increase the yield, the process needs further optimization, by e.g. using harsher pre-treatment or identifying rate-limiting enzymes.

Secretome comparison

Wheat bran has been reported to induce a wide range of CWDEs in several fungi [47-49], and the production of CWDEs on wheat bran was included in the current study for comparison to the production of CWDEs on duckweed. In general, we detected a diverse spectrum of de-polymerization and de-branching enzymes in the secretomes, confirming the consensus that more complex substrates, such as duckweed and wheat bran, will lead to induction of more complex CAZyme cocktails. The profile of secreted CAZymes is known to be dependent on the type and composition of the carbon source used, and it was, thus, to be expected that the *T. asperellum* secretome would vary depending on the substrate. Zhao and co-workers [50] performed a comprehensive dry matter compositional analysis of *Lemna minor*, showing a carbohydrate content of 51.2%, of which 33.1% was glucose, whereof 19.9% was from starch and the rest mainly from cellulose. Additionally, 20% was pectin, mainly consisting of galacturonan and with only small amounts of xylogalacturonan and rhamnogalacturonan, 3.5% was hemicellulose, mainly consisting of xyloglucan and xylan, and only 3% was lignin [50]. As the major part of the carbohydrate is glucose in the form of starch and cellulose, an enzyme blend capable of fully degrading duckweed must include cellulases and amylases. Both cellulases (ID 4405, 774, 7861, 6619, 13540) and α-glucosidases (ID 2969, 369, 20886, 14720) were present in the *T. asperellum* enzyme blend (Table 1). However, the cellulases were not among the most abundant GHs and the several of the α-glucosidases were down regulated compared with growth on PDA, indicating that there is no direct link between carbohydrate composition and abundance of corresponding polysaccharide-specific enzymes. Furthermore, hemicellulases were among the most abundant enzymes (ID 8432, 10314, 15847, 15652, 9036), despite the fact that the amount of hemicellulose present in *Lemna minor* is relatively low. An explanation could be that *T. asperellum* cellulases and amylases have a much higher specific activity than the hemicellulases and therefore are needed in much lower amount. The hydrolytic pectinases present in the enzyme blend, a 1,4-α-galacturonidase (ID 4031) and the relatively high upregulated α-L-rhamnosidase (ID 9240), corresponds well with the composition of pectin in duckweed, consisting of mainly galacturonan and, to a smaller degree, rhamnogalacturonan.

When comparing the secretome from growth on duckweed to the secretome from growth on wheat bran, 8 enzymes were shared among the 20 most abundant enzymes (ID 9036, 20886, 369, 4031, 2969, 11046, 2199, 10314) (Table 1 and 2). An endo-1,4-β-xylanase (ID 2441) and a cellobiosidase (ID 6619) were highly abundant in the secretome produced on wheat bran, but not in the secretome produced on duckweed. In contrast, a GH62 α-L-arabinofuranosidase (ID 8432) was highly abundant in the secretome produced on duckweed, but not in the secretome produced on wheat bran. Interestingly, a GH54 α-L-arabinofuranosidase (ID 4138) was up regulated on wheat bran, but down regulated on duckweed, underlining substrate dependent regulation. A GH54 α-arabinofuranosidases belonging *Trichoderma koningii* is reported to contain β-D-xylosidase activity [51], while the ones from GH62 are more strictly α-arabinofuranosidases [52]. This indicates that we saw a specialized up and down regulation, where α-arabinofuranosidases with activity towards xylan were up regulated on wheat bran, having a higher xylan content than duckweed. A similar situation was seen for *T. harzianum*, which up regulated different GH5s dependent on the substrate [53].

As commercial *T. reesei* cellulase preparations are typically low in β-glucosidase activity, supplementing with exogenous β-glucosidases, either by homologous or heterologous expression of β-glucosidase genes, or co-cultivating

T. reesei with other high β-glucosidase-producing fungi, is often required for efficient hydrolysis of complex substrates [54]. Consequently, the relatively high proportion of β-glucosidases that we find in the *T. asperellum* enzyme blend (ID 4405, 15011, 4404) could make it suitable to meet this requirement [10]. The relatively high production of β-glucosidases by *T. asperellum* could be due to the presence of cellulose in the plant cell wall, but could also be the result of culture conditions near pH 6, as was described for enhanced β-glucosidase activity in *T. reesei* [55].

Besides the plant cell wall degrading enzymes, the secretome from growth on duckweed included a potential chitinase (ID 6963) and several endo-1,3-β-glucanases (ID 2199, 11046, 958, 1796), enzymes that have been associated with antimicrobial activities [56-60]. This correlates with the mycoparasitic nature previously described for *T. asperellum* [11,61,62].

Conclusion

In the current study we have shown that *T. asperellum* produces an enzyme blend with a wide range of CAZymes, when grown on different species of duckweed. The obtained enzyme blend was able to hydrolyze pretreated duckweed and resulted in a glucose release of 4.5g/l and 4.7g/l for two duckweed species grown to a high glucose theoretical yield for a substrate loading of 30g/l. The secretome of *T. asperellum* was analyzed when grown on PDA, wheat bran and duckweed. A total of 78 glycoside hydrolases were found in the secretomes. 73 GHs from 33 families were found in the secretome derived from growth on wheat bran, while 46 GHs from 26 families were found in the secretome derived from growth on duckweed. The most abundant GHs produced on duckweed, were a glucan 1,3-β-glucosidase and an α-L-arabinofuranosidase, whereas the most abundant GHs produced on wheat bran were a xylan 1,4-β-xylosidase and an endo-1,4-β-xylanase. Additionally, two enzymes were uniquely induced on duckweed: a GH36 α-galactosidase, which is probably involved in cell-wall bound polysaccharides and an endo-1,4-β-xylanase, which might be a key enzyme in the degradation of duckweed. Besides this, a GH62 α-L-arabinofuranosidase was up regulated on duckweed and down regulated on wheat bran, while a GH54 α-L-arabinofuranosidase was up regulated on wheat bran, but down regulated on duckweed, indicating some regulation, depending on the substrate and suggesting further possible key enzyme for the degradation of duckweed.

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References

1. Bozell JJ, Petersen GR (2010) Technology development for the production of biobased products from biorefinery carbohydrates-the US department of energy's "top 10" revisited. Green Chemistry 12(4): 539-554.
2. Ragauskas AJ1, Williams CK, Davison BH, Britovsek G, Cairney J, et al. (2006) The path forward for biofuels and biomaterials. Science 311: 484-489.
3. Percival Zhang YH1, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24: 452-481.
4. Jørgensen H, Olsson L (2006) Production of cellulases by Penicillium brasilianum IBT 20888—Effect of substrate on hydrolytic performance. Enzym. Microb. Technol 38: 381-390.
5. Sørensen A, Teller PJ, Lübeck PS, Ahring BK (2011) Onsite enzyme production during bioethanol production from biomass: Screening for suitable fungal strains. Appl. Biochem. Biotechnol 164: 1058-1070.
6. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev 66: 506-577.
7. Hölker U, Höfer M, Lenz J (2004a) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl. Microbiol. Biotechnol 64: 175-186.
8. Singhania RR, Patel AK, Soccol CR, Pandey A (2009) Recent advances in solid-state fermentation. Biochem Eng J 44: 13-18.
9. Aires RdS, Steindorff A S, Ramada SMH., Linhares de Siqueira SJ, Ulhoa CJ (2012) Biochemical characterization of a 27 kDa 1,3-ß-D-glucanase from Trichoderma asperellum induced by cell wall of Rhizoctonia solani. Carbohydrate Polymers 87: 1219-1223.
10. Marx IJ, van Wyk N, Smit S, Jacobson D, Viljoen-Bloom M, et al. (2013) Comparative secretome analysis of Trichoderma asperellum S4F8 and Trichoderma reesei rut C30 during solid-state fermentation on sugarcane bagasse. Biotechnol Biofuels 6: 172.
11. Raghuwanshi S, Deswal D, Karp M, Kuhad RC (2014) Bioprocessing of enhanced cellulase production from a mutant of Trichoderma asperellum RCK2011 and its application in hydrolysis of cellulose. Fuel 124: 183-189.
12. Xu J, Cui W, Cheng JJ, Stomp A (2011) Production of high-starch duckweed and its conversion to bioethanol. Biosyst Eng 110: 67-72.
13. Cheng J, Landesman L, Bergmann B, Classen J, Howard J, et al. (2002) Nutrient removal from swine lagoon liquid by Lemna minor 8627. Trans ASAE 45: 1003-1010.
14. Tillberg E, Holmvall M, Ericsson T (1979) Growth cycles in Lemna gibba cultures and their effects on growth rate and ultrastructure. Physiol. Plant 46: 5-12.
15. Xu J, Zhao H, Stomp A, Cheng JJ (2012) The production of duckweed as a source of biofuels. Biofuels 3: 589-601.
16. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide protein quantification. Nature Biotechnol 26: 1367-1372.
17. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, et al. (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics 13: 2513-2526.
18. Schwanhäusser B1, Busse D, Li N, Dittmar G, Schuchhardt J, et al. (2011) Global quantification of mammalian gene expression control. Nature 473: 337-342.
19. Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, et al. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. Genome Biol 12: R40.
20. Tatusov RL1, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, et al. (2003) The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4: 41.
21. Ogata H1, Goto S, Sato K, Fujibuchi W, Bono H, et al. (1999) KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 27: 29-34.
22. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, et al. (2014) Pfam: the protein families database. Nucleic Acids Res 42: D222-230.
23. Haft DH, Selengut JD, White O (2003) The TIGRFAMs database of protein families. Nucleic Acids Res 31: 371-373.
24. Wu S, Zhu Z, Fu L, Niu B, Li W (2011) WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics 12: 444.
25. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410.
26. Busk PK, Lange L (2013) Function-based classification of carbohydrate-active enzymes by recognition of short, conserved peptide motifs. Appl Environ Microb 79: 3380-3391.
27. Castellanos F, Schmoll M, Martínez P, Tisch D, Kubicek CP, et al. (2010) Crucial factors of the light perception machinery and their impact on growth and cellulase gene transcription in Trichoderma reesei. Fungal Genet Biol 47: 468-476.
28. Latifian M, Hamidi-Esfahani Z, Barzegar M (2007) Evaluation of culture conditions for cellulase production by two Trichoderma reesei mutants under solid-state fermentation conditions. Bioresour. Technol 98: 3634-3637.
29. Rezende MI, Barbosa AdM, Vasconcelos AFD, Endo AS (2002) Xylanase production by Trichoderma harzianum rifai by solid state fermentation on sugarcane bagasse. Braz. J. Microbiol 33: 67-72.
30. Pandey A (2003) Solid-state fermentation. Biochem. Eng. J 13: 81-84.
31. Lever M, Ho G, Cord-Ruwisch R (2010) Ethanol from lignocellulose using crude unprocessed cellulase from solid-state fermentation. Bioresour. Technol 101: 7083-7087.
32. Jørgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities. Biofuels, Bioprod. Biorefin 1: 119-134.
33. Nasim S, Soheila Y, Reza AM, Ghazvini S (2012) Ethanol production from sugarcane bagasse by means of on-site produced and commercial enzymes; A comparative study. Periodica Polytechnica-Chemical Engineering 56: 91-96.
34. Alvira P, Gyalai-Korpos M, Barta Z, Oliva MJ, Reczey K, et al. (2013) Production and hydrolytic efficiency of enzymes from Trichoderma reesei RUTC30 using steam pretreated wheat straw as carbon source. J Chem Technol Biot 88: 1150-1156.
35. Khokhar Z, Syed Q, Wu J, Athar MA (2014) On-site cellulase production by Trichoderma reesei 3ems(35) mutant and same vessel saccharification and fermentation of acid treated wheat straw for ethanol production. Excli Journal 13: 82-97.
36. Kolasa M, Ahring BK, Lubeck PS, Lubeck M (2014) Co-cultivation of Trichoderma reesei RutC30 with three black Aspergillus strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour. Technol 169: 143-8.
37. Rana V1, Eckard AD1, Teller P1, Ahring BK2 (2014) On-site enzymes produced from Trichoderma reesei RUT-C30 and Aspergillus saccharolyticus for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 154: 282-289.
38. Benoliel B, Torres FAG, de Moraes LMP (2013) A novel promising Trichoderma harzianum strain for the production of a cellulolytic complex using sugarcane bagasse in natura. SpringerPlus, 2: 656-656.
39. Delabona PdS, Farinas CS, da Silva MR, Azzoni SF, da Cruz Pradella JG (2012) Use of a new Trichoderma harzianum strain isolated from the amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. Bioresour. Technol 107: 517-521.
40. Mekala NK, Singhania RR, Sukumaran RK, Pandey A (2008) Cellulase production under solid -state fermentation by Trichoderma reesei RUT C30: Statistical optimization of process parameters. Appl. Biochem. Biotechnol 151: 122-131.
41. Kovacs K, Szakacs G, Zacchi G (2009) Enzymatic hydrolysis and simultaneous saccharification and fermentation of steam-pretreated spruce using crude Trichoderma reesei and Trichoderma atroviride enzymes. Process Biochem 44: 1323-1329.
42. Zhang Q, Cai W (2008) Enzymatic hydrolysis of alkali-pretreated rice straw by Trichoderma reesei ZM4-F3.Biomass Bioenerg 32: 1130-1135.
43. Zhang Q, He G, Wang J, Cai W, Xu Y (2009) Two-stage co-hydrolysis of rice straw by Trichoderma reesei ZM4-F3 and Pseudomonas aeruginosa BSZ-07. Biomass Bioenerg 33: 1464-1468.

44. Shin CS1, Lee JP, Lee JS, Park SC (2000) Enzyme production of Trichoderma reesei Rut C-30 on various lignocellulosic substrates. Appl Biochem Biotechnol 84-86: 237-45.
45. Kristensen JB, Börjesson J, Bruun MH, Tjerneld F, Jørgensen, H (2007) Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose. Enzym. Microb. Technol 40: 888-895.
46. Várnai A, Siika-aho M, Viikari L (2010) Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. Enzym. Microb. Technol 46: 185-193.
47. Lan TQ, Wei D, Yang ST, Liu X (2013) Enhanced cellulase production by Trichoderma viride in a rotating fibrous bed bioreactor. Bioresour Technol 133: 175-182.
48. Smits JP, Rinzema A, Tramper J, VanSonsbeek H, Knol W (1996) Solid-state fermentation of wheat bran by Trichoderma reesei QM9414: Substrate composition changes, C balance, enzyme production, growth and kinetics. Appl. Microbiol. Biotechnol 46: 489-496.
49. Sun X1, Liu Z, Qu Y, Li X (2008) The effects of wheat bran composition on the production of biomass-hydrolyzing enzymes by Penicillium decumbens. Appl Biochem Biotechnol 146: 119-128.
50. Zhao X1, Moates GK1, Wellner N1, Collins SR1, Coleman MJ2, et al. (2014) Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (Lemna minor). Carbohydr Polym 111: 410-418.
51. Wan C, Chen C, Li Y, Huang L (2007) Expression, purification and characterization of a bifunctional a-L- arabinofuranosidase/ß-D-Xylosidase from Trichoderma koningii G-39. J. Chin. Chem. Soc 54: 109-116.
52. Lagaert S, Pollet A, Courtin CM, Volckaert G (2014) ß-xylosidases and a-L-arabinofuranosidases: Accessory enzymes for arabinoxylan degradation. Biotechnol. Adv 32: 316-332.
53. Uversky VN, Horta MA, Vicentini R, Delabona Pda S, Laborda P, Crucello A, et al. (2014) Transcriptome profile of Trichoderma harzianum IOC-3844 induced by sugarcane bagasse. PLoS One 9: e88689.
54. Singhania RR1, Patel AK, Sukumaran RK, Larroche C, Pandey A (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 127: 500-507.
55. Tangnu SK, Blanch HW, Wilke CR (1981) Enhanced production of cellulase, hemicellulase, and ß- glucosidase by Trichoderma reesei (rut C-30). Biotechnol. Bioeng 23: 1837-1849.
56. Donzelli BG, Lorito M, Scala F, Harman GE (2001) Cloning, sequence and structure of a gene encoding an antifungal glucan 1,3-beta-glucosidase from Trichoderma atroviride (T. harzianum). Gene 277: 199-208.
57. Duo-Chuan L (2006) Review of fungal chitinases. Mycopathologia 161: 345-360.
58. Ike M, Nagamatsu K, Shioya A, Nogawa M, Ogasawara W, et al. (2006) Purification, characterization, and gene cloning of 46 kDa chitinase (Chi46) from Trichoderma reesei PC-3-7 and its expression in Escherichia coli. Appl. Microbiol. Biotechnol 71: 294-303.
59. Morant AV1, Jørgensen K, Jørgensen C, Paquette SM, Sánchez-Pérez R, et al. (2008) beta-Glucosidases as detonators of plant chemical defense. Phytochemistry 69: 1795-1813.
60. Garciduenas SV, Morales CAL, Estrella AH (1998) Analysis of the ß-1,3-glucanolytic system of the biocontrol agent Trichoderma harzianum. Appl Environ Microb 64: 1442-1446.
61. Kubicek PL, Peterbauer KM (2001) Trichoderma: From genes to biocontrol. Plant Pathol : 11.
62. Gradinger CK, Szakacs G, Kubicek C (2002) Phylogeny and evolution of the genus Trichoderma: A multigene approach. Mycol Res 106: 757-767.