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ORIGINAL PAPER

Cellulase production using different streams of wheat grain- and wheat straw-based ethanol processes

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Abstract Pretreatment is a necessary step in the biomass-to-ethanol conversion process. The side stream of the pretreatment step is the liquid fraction, also referred to as the hydrolyzate, which arises after the separation of the pretreated solid and is composed of valuable carbohydrates along with compounds that are potentially toxic to microbes (mainly furfural, acetic acid, and formic acid). The aim of our study was to utilize the liquid fraction from steam-exploded wheat straw as a carbon source for cellulase production by *Trichoderma reesei* RUT C30. Results showed that without detoxification, the fungus failed to utilize any dilution of the hydrolyzate; however, after a two-step detoxification process, it was able to grow on a fourfold dilution of the treated liquid fraction. Supplementation of the fourfold-diluted, treated liquid fraction with washed pretreated wheat straw or ground wheat grain led to enhanced cellulase (filter paper) activity. Produced enzymes were tested in hydrolysis of washed pretreated wheat straw. Supplementation with ground wheat grain provided a more efficient enzyme mixture for the hydrolysis by means of the near-doubled β-glucosidase activity obtained.

Keywords *Trichoderma reesei* RUT C30 · Wheat straw · Steam pretreatment · Cellulase fermentation · Hydrolyzate

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Introduction

Lignocellulosic materials, in the form of agricultural and forestry residues, are produced in abundant amounts worldwide. To utilize their carbohydrate content for ethanol fermentation, the feedstock needs to be pretreated and hydrolyzed by cellulase enzymes to liberate sugars. One of the drawbacks of the industrial-scale spread of the process is the high price of cellulase enzymes, which accounts for approximately 15% of the total ethanol production costs [21]. One possibility for reducing this cost is on-site enzyme production using the liquid stream of pretreatment as the carbon source.

Pretreatment is necessary to disrupt the resistant lignocellulose matrix consisting of cellulose, hemicellulose, and lignin. The main aim of the pretreatment is to make the cellulose surface accessible to cellulase enzymes during the subsequent hydrolysis [2]. However, because of the harsh conditions, some components are removed from the matrix and even degraded. In the case of steam explosion, partial hydrolysis and solubilization of the hemicellulose fraction occur [16], and therefore these substances remain in the liquid fraction after separation of the pretreated slurry. This liquid fraction, also referred to as the hydrolyzate, contains valuable sugars, mostly oligomeric forms of pentoses, along with degradation products that have possible inhibitory effects on the growth of microbes. This inhibitory property makes further utilization of the hydrolyzate difficult. The predominant degradation products present in the liquid fraction of steam explosion are hydroxymethylfurfural (HMF) and furfural, derived from glucose and xylose,

respectively [1]. Further breakdown of these inhibitors also occurs, leading to formic acid formation. Another aliphatic acid, acetic acid, originates from the acetyl groups on the xylan and mannan backbone of the hemicellulose. Besides these inhibitors, phenolic compounds derived from lignin degradation are also detected. Importantly, levels of these inhibitors depend on substrate composition and pretreatment conditions [31].

Although there are several options for utilizing the hydrolyzate from different raw materials and pretreatments, a preliminary detoxification is often required, regardless of the raw material and the type of pretreatment. The most investigated alternative involves producing ethanol from the sugar content of the liquid fraction using naturally occurring yeast strains (e.g., *Pichia stipitis*) or engineered bacteria or yeast strains (e.g., *Escherichia coli* or *Saccharomyces cerevisiae*) that are able to convert both hexoses and pentoses to ethanol [29, 40]. Xylitol production by different yeast strains has also been examined by using xylose-rich acid hydrolyzates as substrates [32]. Hydrolyzate of hydrothermally treated wheat straw was also used for production of biogas [19] and biohydrogen [18]. The liquid fraction of steam-pretreated willow was investigated as carbon source for β-glucosidase production by different *Aspergillus* and *Penicillium* strains [33]. Some attempts were also made to employ this fraction for cultivating *Trichoderma reesei*. One option for utilizing this fraction is detoxification; *T. reesei* is able to consume some of the inhibitors in low concentrations under certain conditions [22, 30]. The second possibility is using it as carbon source for cellulase production [7, 12, 23, 33, 36].

The filamentous fungus *T. reesei* (anamorph of *Hypocrea jecorina*) is one of the most studied cellulase-producing organisms. Cellulase secretion in *Trichoderma* is an inducible phenomenon, but the mechanism of cellulose sensing is still unclear, mostly due to its water insolubility. Several small compounds, like sophorose [35] and sorbose [28], possess the capability to induce cellulase secretion, but an industrial application of these compounds, as carbon sources, is unfeasible because they are expensive to purchase. Lactose, another inducer, could be a preferable choice for industry because whey, a by-product of cheese processing, contains lactose. However, whey is not readily available on-site as it is not biomass related [10, 42]. The liquid fraction obtained from steam pretreatment contains both glucose and xylose oligomers which may induce cellulase secretion in *Trichoderma*; owing to its availability, this liquid fraction can be a good substrate for on-site enzyme production.

Although starch is usually not recognized as an inducer for cellulase production in *Trichoderma* species, an inductive effect was reported for starch-derived compounds in *T. reesei* RUT C30 [10, 43]. One possible explanation for this finding is that during the acidic or enzymatic hydrolysis of starch, reversion products, such as sophorose, are also formed, and these products can act as inducers of cellulase transcription [10]. However, the same positive effect was observed in the case of utilizing native starch as partial carbon source supplemented with cellulosic materials [38, 43]. Using starchy materials like wheat grain for on-site enzyme production by *T. reesei* RUT C30 can be a good integration opportunity for the biomass-to-ethanol process in first-generation factories.

In our study, the potential utilization of the liquid fraction of steam-exploded wheat straw, a possible raw material for second-generation ethanol [39], was tested in shake flask cultures of *T. reesei* RUT C30. Additionally, the medium was supplemented with dried distiller's grain as nitrogen source and either with washed pretreated wheat straw or wheat grain as extra carbon source to examine their effect on specific activities. Produced fermentation broths were tested in enzymatic hydrolysis of washed pretreated wheat straw.

Materials and methods

Strain and raw materials

For enzyme production experiments, *T. reesei* RUT C30 (ATCC56765) strain obtained from the American Type Culture Collection was used. It was maintained at 30°C on malt agar slants composed of 20 g/l malt extract, 20 g/l agar, 5 g/l glucose, and 1 g/l peptone. Slants were subcultured biweekly.

The following materials were utilized as carbon sources for enzyme production alone or in combination: pretreated wheat straw separated into liquid and water-insoluble fractions, ground wheat grain with or without α-amylase treatment, wheat distiller's grain, and Solka Floc 200 FCC commercial cellulose product (International Fiber Corporation, North Tonawanda, NY, USA).

The wheat straw was harvested in 2008 in Soria, Spain. It was milled using a laboratory hammer mill to obtain a chip size of 10 mm, and it was stored at room temperature until use.

Mazurka-type wheat grain was harvested in the fall of 2009 in Somodorpuszta, Hungary. Dried grain was ground, and the following size distribution was obtained: 49.8% (related to total weight) was between 1.00 and 0.32 mm, while 82.2% was between 1.40 and 0.10 mm. For the α-amylase treatment, ground wheat grain was suspended in 0.1 M citrate–phosphate buffer (pH 5.6) to make a 30% solution. Liquefaction was performed for 1 h at 85°C using Termamyl SC DC (Novozymes A/S, Bagsvaerd, Denmark) at an enzyme dosage of 0.2 g/kg dry solid.

Table 1 Composition of wheat straw before and after steam pretreatment (210°C, 2.5 min)

	Glucan, %	Xylan, %	Lignin, %
Wheat straw	40.7 ± 2.7	27.6 ± 2.4	17.0 ± 0.8
Pretreated whole slurry	42.4 ± 1.3[a]	18.6 ± 0.5[a]	25.5 ± 2.2
WIS (washed solid)	57.5 ± 0.8	6.0 ± 0.1	30.1 ± 0.1

Data are presented as mean values of triplicates with standard deviations

[a] Monomers, oligomers, and polymers are presented together as polymers

Before composition analyses occurred, all samples were ground into a fine powder.

Pretreatment

Steam explosion pretreatment was performed in a 10-l reactor at 210°C for 2.5 min using Masonite technology [24]. These conditions were established according to previous experiments based on optimal sugar recovery and hydrolysis yields [3]. Under these circumstances, most of the xylan content in the wheat straw was solubilized (Table 1). Following the pretreatment, a slurry with 31.0% total solid was recovered. One portion of the slurry was vacuum-filtered to separate water-insoluble solids and the liquid fraction. The solidfraction was thoroughly washed with tap water resulting in washed pretreated wheat straw (WIS), and both fractions were stored at −20°C until further use. The ratio of WIS and soluble solid content was 21.1 and 9.9%, respectively, related to the whole slurry. The obvious lack of xylan content in the slurry is due to some loss during the recovery process after pretreatment and to degradation into furfural, which was present in the liquid fraction.

Detoxification

The liquid fraction was vacuum-evaporated at 50°C for 10 min, during which the starting volume of 250 ml decreased by 60 ml. This loss was replaced by distilled water to obtain the initial volume again. The solution was then overlimed at 35°C by adding $Ca(OH)_2$ powder to pH 10 with continuous stirring. Once a pH of 10 was achieved, the solution was stirred for an additional 1 h. To remove any precipitate, the overlimed liquid was centrifuged (3,400*g*, 5 min); the supernatant was stored at 4°C until use.

Inoculum preparation

Conidia from 14-day-old slants were harvested with sterile distilled water. This suspension was used to inoculate Erlenmeyer flasks containing 200 ml of sterile modified Mandels' medium to obtain a final concentration of 10^8 conidia/ml. This medium contained 1.87 g/l $(NH_4)_2SO_4$, 2.67 g/l KH_2PO_4, 0.53 g/l $CaCl_2{\cdot}2H_2O$, 0.81 g/l $MgSO_4{\cdot}7H_2O$, 0.40 g/l urea, 5.0 mg/l $FeSO_4{\cdot}7H_2O$, 1.7 mg/l $MnSO_4{\cdot}H_2O$, 1.4 mg/l $ZnSO_4{\cdot}7H_2O$, and 2.0 mg/l $CoCl_2{\cdot}6H_2O$; it was supplemented with peptone to 1.00 g/l, yeast extract to 0.33 g/l, and Solka Floc as carbon source to 10 g/l. Inoculated flasks were closed with cotton plugs and incubated at 30°C and 300 rpm on a rotary shaker for 4 days.

Shake flask cultivation

The medium for cellulase production was composed of $(NH_4)_2SO_4$, KH_2PO_4, and carbon sources at different concentrations. Concentrations of the two salts were equal and were based on the amount of the carbon source. When the carbohydrate content was 15 g/l or lower, 0.83 g/l of both salts were used; this amount was doubled (1.66 g/l) when the carbohydrate content was above 15 g/l. Each medium contained an additional 5 g/l wheat distiller's grain, which also included lignocellulosic carbohydrates (18.6% glucan and 14.6% xylan); although it was added to the medium as a nitrogen source, it contained 32.2% protein. To avoid pH changes 0.1 M TRIS and maleic acid were also added; it had been previously discovered that TRIS–maleic acid buffer can stabilize the pH during cultivation, and, under these conditions, some positive effects on cellulase production were also observed [17]. All of the substances were suspended either in tap water or in the liquid fraction of steam-pretreated wheat straw that had been undiluted or diluted with tap water (1:1 and 1:3). The pH was set to 5.8 with NaOH, resulting in a 0.1 M TRIS–maleic acid buffer system (pH 5.8).

Cellulase-producing media were inoculated with an aliquot of 4-day-old preculture at 10% (v/v), and cultures were propagated at 30°C and 300 rpm on a rotary shaker. Samples were withdrawn regularly and centrifuged (3,400*g*, 5 min) to separate supernatants for further analysis. Fermentations were terminated after 7 or 11 days, and whole fermentation broths were stored at −20°C to test some of them in hydrolysis assays.

Reducing sugar, enzyme activity, and protein assays

For measurement of reducing sugars (RS), 3 ml of the dinitrosalicylic acid (DNS) reagent was added to 1.5 ml of an appropriately diluted sample, and the mixture was placed in boiling water for 5 min. After cooling and adding 16 ml of distilled water, the absorbance was measured at 550 nm to determine the concentration of the RS. Glucose was used to prepare the standard curve.

Filter paper activity (FPA) measurements were performed to describe the overall potential for saccharification. Appropriately diluted supernatant was used to liberate 1 mg of glucose under assay conditions; the volume was increased to 1.5 ml by adding 0.05 M acetate buffer (pH 4.8). A strip of Whatman No. 1 filter paper (1 × 6 cm), equal to 50 mg of substrate, was added and incubated for 1 h at 50°C. Three milliliters of DNS reagent was added to each tube to stop the reaction. The amount of released RS was measured as discussed above. FPA was expressed as FPU/ml, where FPU was defined as the amount of liberated glucose given in micromoles per minute.

To measure xylanase activity, 0.1 ml of the appropriately diluted supernatant, to liberate approximately 0.25 μg xylose, was added to the mixture of 0.4 ml 0.05 M citrate buffer (pH 5.3) and 0.5 ml of 1% (w/v) birchwood xylan (Sigma–Aldrich, St. Louis, MO, USA) solution prepared in citrate buffer pH 5.3. After incubating at 50°C for 10 min, the reaction was terminated by adding 1.5 ml of the DNS reagent, and the mixture was placed in boiling water for 5 min. After cooling, the absorbance was read at 550 nm. A xylose calibration curve was used to calculate the activity unit, which was defined as the amount of xylose released, given in micromoles per minute.

β-Glucosidase activity was assayed according to Berghem and Pettersson with a 5 mM 4-nitrophenyl-β-D-glucopyranoside solution as the substrate [5].

The concentration of the extracellular protein in the fermentation supernatants was determined by the Bradford method using Coomassie Blue G250 reagent [8]. A calibration curve was obtained by using bovine serum albumin as the standard.

Hydrolysis

Hydrolysis experiments were carried out in a 5-ml reaction volume in test tubes with magnetic stirring at 50°C. Dry WIS content was 2% suspended in 0.05 M acetate buffer (pH 4.8). The enzyme to substrate ratio used achieved a glucan dosage of 20 FPU/g. As a control, the substrate was hydrolyzed by commercial enzyme preparation, Celluclast 1.5L (Novozymes A/S, Bagsvaerd, Denmark) supplemented with Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark) in 20 FPU/g glucan cellulase and 20 IU/g glucan total β-glucosidase dosages. Hydrolysis was run for 72 h with withdrawals at 0, 3, 6, 9, 24, 48, and 72 h. At each time point, three tubes were taken (representing triplicates) and boiled for 5 min to inactivate the enzymes. They were then centrifuged, and the supernatant was prepared for high-performance liquid chromatography (HPLC) analysis to measure cellobiose, glucose, and xylose concentrations. The conversion values were calculated based on the initial glucan and xylan content of the WIS.

Analytical methods

The glucan and xylan contents of wheat straw before and after steam pretreatment were determined in triplicates using a two-step sulfuric acid hydrolysis, which was based on a method described by Hägglund [13]. In our analysis, a modified method was performed in which 0.5 g raw material was dispersed in 2.5 ml 72% sulfuric acid and maintained at room temperature for 2 h with occasional stirring. Next, 75 ml of distilled water was added and autoclaved for 1 h at 121°C. The reaction mixture was separated on preweighed, dried G4 glass filtering crucibles. The filtrate was analyzed for carbohydrates by HPLC. The filter cake was washed with hot distilled water and dried at 105°C. This residue was defined as the lignin content, and it was corrected for its ash content, which was determined after incineration at 550°C for 6 h.

To determine the starch content of wheat grain, a 0.5-g sample was suspended in a mixture of 25 ml distilled water and 10 ml 2 M NaOH. It was then incubated at 90°C for 20 min, followed by cooling to a temperature below 50°C. The sample was neutralized through the addition of 10 ml 2 M HCl. Then 10 ml of 0.1 M sodium acetate buffer (pH 4.2) and AMG 300L amyloglucosidase at 600 U/g solid were added for a 12-h hydrolysis at 40°C. This mixture was centrifuged at 3,400*g* for 5 min, and the supernatant was analyzed for glucose via HPLC. Measured glucose concentrations were reduced by the glucose content of the AMG 300L. The remaining solid was washed to remove residual glucose, dried, and analyzed for lignocellulose content as described above.

Samples for HPLC analysis were prepared by filtering through a regenerated cellulose syringe filter with 0.45-μm pore size (ProFill, Langerwehe, Germany). The glucose, cellobiose, and xylose contents of hydrolysis and the composition analysis samples were separated on an Aminex ion exclusion HPX-87H cation-exchange column (BioRad, Hercules, CA USA), which was run at 65°C with 5 mM sulfuric acid as mobile phase at a flow rate of 0.5 ml/min. After separation, compounds were detected by a Shimadzu RID-I0A refractive index detector (Shimadzu, Kyoto, Japan).

Sugars and degradation compounds in the liquid fraction were also measured. Total sugars (including both monomers and oligomers) were determined after mild acid hydrolysis (4% (v/v) H_2SO_4, 120°C and 30 min). Monomeric and total sugar concentrations in the liquid fraction were measured by HPLC (Waters, Mildford, MA, USA) with a 2414 refractive index detector (Waters, Mildford, MA, USA). A Transgenomic CarboSep CHO-682 carbohydrate analysis column was employed for the separation, and it operated at 80°C with deionized water as mobile phase (0.5 ml/min).

Furfural, HMF, vanillin, 4-hydroxybenzaldehyde, coumaric acid, and ferulic acid were analyzed on an Aminex HPX-87H column at 65°C. For the mobile phase, 89% 5 mM H_2SO_4 and 11% acetonitrile, at a flow rate of 0.7 ml/min, were used. For detection, a 1050 photodiode-array detector (Agilent, Waldbronn, Germany) was employed. Acetic and formic acid were also quantified by HPLC (Waters, Milford, MA, USA) with a 2414 refractive index detector and an Aminex HPX-87H column maintained at 65°C with a 5 mM sulfuric acid mobile phase at a flow rate of 0.6 ml/min.

Results and discussion

Effect of wheat grain addition on cellulase production

To investigate the effect of wheat grain on cellulase production by *T. reesei* RUT C30, the following experiment was performed. As a carbon source, 5 g/l Solka Floc was supplemented with 6.25 g/l ground wheat grain (80% carbohydrate content). As controls, 12.5 g/l ground wheat grain and 10 g/l Solka Floc were used as sole carbon sources.

After 7 days of cultivation, the highest overall cellulase activity, 1.03 FPU/ml, was measured from supernatant samples produced on Solka Floc plus ground wheat grain (SFWG). The cellulase activity of this sample was 20% higher than that in the Solka Floc control (Fig. 1). Broths growing on medium containing wheat grain afforded the lowest activity (0.63 FPU/ml). β-Glucosidase secretion (data not shown) was the most effective on the mixed carbon source (0.50 IU/ml), and, surprisingly, the next highest value was measured from broths on solely ground wheat grain (0.49 IU/ml). These samples had 25 and 23% higher β-glucosidase activity, respectively, than that of the Solka Floc control (0.40 IU/ml). Because the main component of wheat grain is starch (65.9%), it was assumed that the effect caused by the wheat grain addition was most likely related to its starch content. Nevertheless, a small amount of lignocellulosic carbohydrate from wheat distiller's grain and wheat bran was also present in each case. Wayman and Chen [43] also observed that untreated whole wheat flour was good at inducing cellulase synthesis in *T. reesei* RUT C30. They assumed that the reason for this induction involved some synergy between the effects of bran and starch in the whole wheat flour.

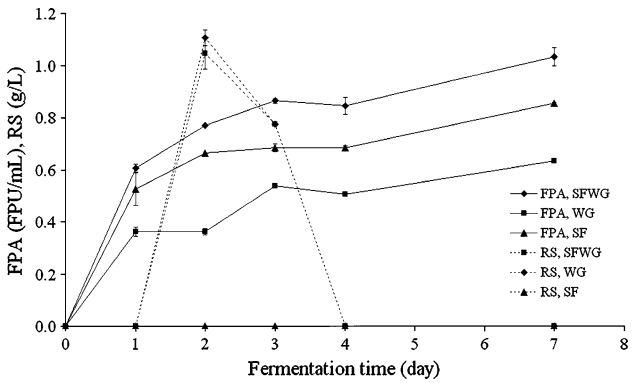

Fig. 1 FPA and RS profile on 10 g/l Solka Floc (SF), 12.5 g/l ground wheat grain (WG), and 5 g/l Solka Floc plus 6.25 g/l ground wheat grain (SFWG). Standard deviation values are calculated from two shake flasks run in parallel

In the broths containing ground wheat grain, accumulation of RS content was observed with peaking on day 2 (1.11 and 1.05 g/l for wheat grain with and without Solka Floc, respectively) and was consumed by day 4. Because RS liberation was not found in broths containing Solka Floc alone, and Solka Floc with wheat grain did not yield significantly more glucose, hydrolysis of starch and consumption of its products occurred. Production of amylolytic enzymes by the *Trichoderma* species (including *T. reesei*) has already been reported [4].

Fermentations were also performed on the α-amylase-treated ground wheat grain in combination with Solka Floc. This carbon source mixture resulted in a higher FPA but a lower β-glucosidase activity, compared to the results with untreated ground grain (data not shown). Because β-glucosidase activity is a crucial factor in hydrolysis with *T. reesei* enzymes [9], untreated ground grain was used in the following experiments. Higher cellulase activity obtained on amylase-treated grain agreed with the theory of Chen and Wayman [10], who assumed that during enzymatic or acidic hydrolysis of starch, reversion products, such as sophorose, are formed under certain circumstances. These products can act to induce cellulase expression. Differences in the β-glucosidase activity can be explained by the mechanisms of bacterial amylases (Thermamyl SC from *Bacillus licheniformis*) or those of fungal (produced by *Trichoderma* itself) origin. While the amylases from bacteria randomly attack the α-1–4 bonds of starch to create dextrins, the fungal amylases, presumably also those of *T. reesei* RUT C30, liberate maltose and glucose from chain ends. β-Glucosidase triggered by lignocellulosic components in the medium can form cellulase inducers via transglycolysation from this starch-derived glucose. This ability of β-glucosidases from different species has already been proposed [10, 11]. It was hypothesized that some of these compounds can trigger even more β-glucosidase, creating a positive feedback loop.

On the other hand, *T. reesei* RUT C30 does not possess any functional genes encoding maltose permease, which is responsible for maltose uptake into the cell. As a consequence, its growth on media where the sole carbon source is starch, or other α-glucans, is strongly impaired [34]. This

phenomenon is supported by an earlier finding that starch itself was a poor inducer of cellulase enzyme production [10]. Furthermore, the catabolite derepressive property of *T. reesei* RUT C30 [15] also has an important role in helping to avoid repression by glucose from starch. These unique features of RUT C30 are crucial in cellulase secretion triggered by starch or its derivates because no cellulolytic enzyme production was observed in *T. reesei* QM9414 cultures growing on acid-hydrolyzed potato starch [25].

Liquid fraction as carbon source for cellulase production

The liquid fraction separated after steam pretreatment of wheat straw was tested in cellulase fermentations with no dilution, twofold or fourfold dilutions supplemented with other medium components at a pH of 5.8. The concentration of the RS remained constant during the 11-day cultivation (12.5 g/l for 1:3 dilution, 22.4 g/l for 1:1 dilution, and 36.5 g/l for undiluted liquid fraction), indicating that there was no utilization of the carbon source even at the highest dilution; this result also indicates that no growth occurred.

Based on these results, a two-step detoxification process of the liquid fraction was executed. Importantly, there was no optimization of the detoxification process; the primary aim of the current process was to make the liquid fraction useable. Originally, the liquid fraction contained HMF and furfural, derived from glucose and xylose, respectively. Additionally, a large amount of acetic acid was present, which demonstrates the high degree of hemicellulose solubilization. Further degradation of furfural and HMF occurred, resulting in formation of formic acid (Table 2). Besides the inhibitors listed in Table 2, other compounds, namely 4-hydroxybenzaldehyde, vanillin, syringaldehyde, coumaric and ferulic acids, were identified at minor concentrations (<0.1 g/l).

During evaporation, volatile compounds were partially eliminated from the mixture. Approximately 61, 10, and 4% of the initial amount of furfural, acetic acid, and formic acid, respectively, was found in the condensate (Table 2). With a prolonged boiling time, it is possible that more acids could be stripped out. For acetic acid and HMF, the mass balances are complete within error intervals; 89 and 100% were left in liquid fraction. In the case of furfural (24% left in liquid fraction) and formic acid (88%), the mass balances are only partial, and therefore it was assumed that even more evaporated from the liquid fraction, leaving the cooler without condensation. As a result of the two-step detoxification process, furan aldehydes were almost completely eliminated, but an increase in acetic acid concentration was observed. The results of repeated evaporation showed a good correspondence with the first test in terms of the concentrations of each compound. However, the results of repeated overliming were in line with the first experiment only for the furfural, HMF, and acetic acid. In contrast to the other compounds, the concentration of formic acid decreased during the first overliming, but it increased during the second; this difference can also be detected in the high standard deviation of the mean value. Effectiveness of overliming with $Ca(OH)_2$ depends on its severity, which can be described with three parameters: final pH, temperature, and treatment time. Aliphatic acids are usually unaffected, but under harsher conditions, their concentration can increase. Horváth et al. investigated the effect of these factors on the composition, and only one setup was found to decrease the concentration of formic acid, but even in this case the concentration of acetic acid increased; in all other investigated setups, increases in both concentrations were observed [14]. The parameters of that one exception (pH 10, 30°C, and 1 h) are quite similar to the overliming process applied in our experiments (pH 10, 35°C, and 1 h). However, some oscillations in temperature may result in an increased formic acid concentration as well.

Table 2 Effect of detoxification on inhibitor concentrations (g/l) in liquid fraction separated after steam pretreatment

Compound	Untreated	After evaporation[a]	Condensate	After overliming[b]
Furfural	2.1 ± 0.5	0.5 ± 0.0	5.3 ± 0.8	0.0
HMF	0.7 ± 0.0	0.7 ± 0.0	0.0	0.1 ± 0.1
Acetic acid	8.8 ± 0.8	7.8 ± 0.1	3.8 ± 0.1	12.4 ± 1.7
Formic acid	7.2 ± 0.9	6.3 ± 0.0	1.1 ± 0.1	6.8 ± 2.2[c]

Mean values of two detoxifications with standard deviations are presented. During evaporation from an initial volume of 250 ml, approximately 60 ml of volume was lost, and it was replaced by distilled water

[a] Refilled to original volume with distilled water

[b] Adjusted to pH 5.8 as used in fermentations

[c] The values of two detoxification trials showed different trends, for details see text

Table 3 Sugar concentrations in liquid fraction before and after detoxification (given in g/l)

	Untreated		Detoxified	
	Monomer	Total	Monomer	Total
Glucose	0.6 ± 0.1	7.2 ± 0.7	2.1 ± 0.2	7.1 ± 0.3
Xylose	5.5 ± 0.2	40.5 ± 3.1	6.4 ± 0.2	37.6 ± 3.3
Others[a]	2.6 ± 0.3	4.5 ± 0.6	2.4 ± 0.1	4.8 ± 0.1

Data are presented as mean values of triplicates with standard deviations

[a] Galactose, mannose, and arabinose

Interestingly, an elevated concentration of ferulic acid was detected, from the initial 0.04 to 0.49 g/l after overliming. One possible reason for this increase is that at the low pH of the original liquid (around 3.4), ferulic acid can form polymers, which were not determined. However, at higher pH values, such as those following overliming and the pH adjustment to 5.8, these polymers are degraded and can be measured as monomers.

As an effect of detoxification, the content of glucose and xylose increased but with slightly decreasing total sugar concentrations, which shows that during detoxification further degradation of oligomers occurred (Table 3). The concentrations of other carbohydrates remained constant, within error intervals.

Fermentations were performed with the detoxified liquid, and enzyme production was only observed in the case of the fourfold-diluted liquid fraction, which reached an activity of 1.18 FPU/ml after 11 days. At the other dilutions, the concentration of the RS was constant during fermentation. Therefore, in the subsequent parts of the study, the detoxified liquid fraction was applied at a 1:3 dilution with tap water.

In the case of this dilution, the initial acetic acid concentration of the treated liquid fraction was 3.1 g/l in the culture broths. The effect of acetic acid on cellulase production by *T. reesei* RUT C30 was investigated up to 3 g/l at a pH of 6.0 using Mandels' medium with washed steam-pretreated willow as carbon source and no influence was observed [37]. However, there is no information about the effect of acetic acid at higher concentrations. When furfural (0.4, 0.8, and 1.2 g/l) was also added into the same medium containing acetic acid at low concentrations (1.0 and 2.0 g/l), the acetic acid appeared to reduce the inhibitory effect of the furfural [37]. Both the acetic acid and the furfural concentrations were in these ranges in the fourfold-diluted, untreated liquid fraction (2.20 and 0.52 g/l, respectively), but no sugar consumption was observed during cultivation, demonstrating an effect of other inhibitors present in the liquid fraction. This synergy between inhibitors was also confirmed by the fact that the total amount of inhibitors in the treated liquid was higher than in the original one, but mostly contained only aliphatic acids and enzyme production occurred when it was diluted fourfold.

Inhibitors were also measured in fermentation broths at the end of cultivation. In the case of broths containing undiluted or twofold dilutions of the liquid fraction, the concentrations of the inhibitors did not change during cultivation. Neither aliphatic acids, nor the other inhibitors were detected in the broth with fourfold-diluted treated liquid fraction. Palmqvist et al. [30] and Szengyel et al. [37] also found that *T. reesei* was able to consume acetic acid in media containing low overall levels of inhibitors. In the current study, consumption of formic acid by *T. reesei* was also detected.

Integrated enzyme production

The fourfold-diluted, treated liquid fraction was supplemented with other carbon sources related to wheat processing to enhance cellulase productivity and to optimize the composition of the enzyme mixture for the hydrolysis. Media compositions are presented in Table 4. With these media, every compound, excluding salts and buffer, would be available in-house through an integrated wheat processing factory. As reference fermentation, WIS of steam-pretreated wheat straw was used as carbon source and suspended in 0.1 M TRIS–maleic acid buffer prepared with tap water.

The presence of inhibitory compounds in the liquid fraction prolonged the lag phase of enzyme production compared to the reference. Therefore, when the reference had already reached maximal FPA after 7 days, the other cultures still showed increasing activities (Fig. 2).

The prolonged lag phase could be perceived on the RS curves as well. RS concentration increased during the first 2 days of fermentation, which was due to the enzymes present in the inocula and the presence of inhibitors. No RS accumulation was observed in REF where cells were immediately able to utilize the released sugars from the WIS. When *T. reesei* started to grow, the concentration of RS dropped sharply. This event occurred at the same time in the media containing the liquid fraction, irrespective of the supplementing carbon source. Thus, the supplementary substrates did not decrease the length of the lag phase. During RS consumption, enzyme production had also started, possibly even in the presence of monomers. In this case, the catabolite derepressive feature of the RUT C30 strain became a crucial factor again because it can contribute largely to this observed enzyme production. It has been determined that in *T. reesei* RUT C30 cultures growing on a mixture of xylose and cellulose, xylose was consumed first to support growth [27]. However, when

Table 4 Main carbohydrate composition of fermentation media (g/l)

Abbr.	Carbon source	Glucan	Xylan	Others[a]	Monomers[b]	Total
LIQ	1:3 diluted liquid fraction	2.2	8.5	0.6	2.7	14.0
LIQWG	LIQ + 12.5 g/l wheat grain	11.2	9.5	0.6	2.7	24.0
LIQWIS	LIQ + 14.3 g/l WIS	10.4	9.4	0.6	2.7	23.1
REF	14.3 g/l WIS	8.2	0.9	–	–	9.1

Carbohydrate content of wheat distiller's grain supplementation (5 g/l) and non-starch carbohydrates from wheat grain are included

[a] Arabinose, galactose, and mannose oligomers

[b] Including glucose, xylose, arabinose, galactose, and mannose

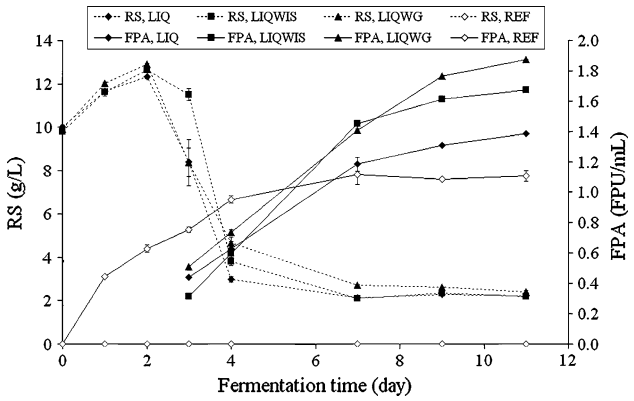

Fig. 2 FPA and RS profile for LIQ, LIQWG, LIQWIS, and REF (for abbreviations see Table 4). Mean values and standard deviations are shown for two flask cultures that were grown in parallel. FPA values of the liquid fraction containing media for the first and second days are not shown because they are highly affected by the amount of RS background

glucose (2.5 g/l) was present in the medium with xylose (7.5 g/l), in the form of a conditioned hydrolyzate and supplemented with 1% Solka Floc, the glucose was utilized first, resulting in its depletion by the second day, followed by depletion of xylose on the third day, and no increase was detected in either concentration [12]. Therefore, we hypothesized that these easily accessible sugars can promote growth of the fungus even in the presence of inhibitors.

An experiment was also carried out with the twofold dilution of the liquid fraction supplemented with 12.5 g/l ground wheat grain to determine whether additional substrates could decrease the inhibitory effect. However, no RS consumption occurred.

Beside the presence of inhibitors, a different matrix of carbohydrates in the media could also cause differences in FPA profiles compared to the reference. The liquid fraction only contained soluble xylose and glucose in addition to their oligomers derived from lignocellulosic material. Induction of cellulase secretion by water-soluble glucose oligomers ($n < 6$) in *T. koningii* [41] has been reported and also partially by xylose in *T. reesei* RUT C30 [27]. In the liquid fraction used in this study, these soluble inducing oligomers are readily available. However, in the case of WIS, only water-insoluble polymers were present, and they needed to be degraded before utilization.

After 11 days, the highest volumetric activities of the cultures were obtained in the medium supplemented with wheat grain, which was also reflected in the protein content (Table 5). Supplementation with wheat grain caused a 35% increase in FPA, which was 21% in the case of WIS compared to the liquid fraction alone. The highest FPA yield was obtained by the reference containing no inhibitors, followed by the unsupplemented liquid fraction (Table 5). In the case of supplementations, the enhanced carbohydrate content did not result in increased FPA yields. Xylanase activities were practically equal in the LIQ and LIQWIS samples, while 30% higher xylanase activity was measured by using wheat grain supplementation. Final xylanase activity of REF was 62% lower than that obtained in the LIQ sample. On one hand, the reason for this low activity may be the lower xylan content in the washed pretreated wheat straw (Table 4). On the other hand presence of D-xylose di- and trisaccharides in the liquid fraction may cause more powerful stimulation of xylanase secretion than the monomers alone [45]. Moreover, L-arabinose was found to be a more effective inducer of xylanase secretion than xylose [44]. In the liquid fraction used for this study, the presence of arabinose was significant because it was the third highest concentration among monomers (0.5 g/l) in the fourfold-diluted, treated liquid fraction.

No differences in β-glucosidase activity were observed between the LIQ and REF samples. The main improvement with the wheat grain addition (LIQWG) was the near-doubled β-glucosidase activity, leading to a more favorable BG/FPA ratio. With the WIS supplementation (LIQWIS), no significant increase could be obtained, at least extracellularly. This positive effect on β-glucosidase activity with the LIQWG may be related to the starch content of wheat grain because enhanced β-glucosidase activity has been observed when *T. reesei* RUT C30 was cultivated on Mandels' medium with different carbon sources supplemented with 1% starch [38].

Table 5 Final protein concentrations, volumetric activities, and yields after 11 days of cultures for the LIQ, LIQWG, LIQWIS, and REF samples

	Protein (g/l)	BGL (IU/ml)	XYL (IU/ml)	FPA (FPU/ml)	Yield (FPU/g CH)	BGL/FPA ratio (%)
LIQ	0.58 ± 0.03	0.88 ± 0.02	127 ± 4	1.38 ± 0.01	98.6	64
LIQWG	1.18 ± 0.05	1.74 ± 0.03	165 ± 4	1.87 ± 0.05	77.9	93
LIQWIS	0.69 ± 0.02	0.91 ± 0.06	130 ± 5	1.67 ± 0.04	72.3	55
REF	0.44 ± 0.06	0.88 ± 0.01	49 ± 6	1.11 ± 0.04	122.1	79

For abbreviations see Table 4. Data are presented as mean values and standard deviations from two parallel flask cultures

Fermentation was repeated to confirm the results, and the repetitions yielded good correspondence with the initial data.

Hydrolysis

For hydrolysis experiments, whole fermentation broths from 11-day cultures in LIQ, LIQWG, and LIQWIS media were used. Hereafter, these abbreviations refer to enzymes produced in the corresponding medium. Whole fermentation broth of *T. reesei* RUT C30 has been reported to be more effective than the supernatant in hydrolysis in terms of glucose liberation [20]. The reason for this finding is likely that β-glucosidase mostly appears in hyphae-bound form [26], and the presence of this enzyme component at a sufficient concentration is crucial for an elevated glucose yield.

Hydrolysis of the pretreated and washed wheat straw (WIS) was performed with equal enzyme doses, 20 FPU/g glucan of WIS for each broth. For a reference, a commercial enzyme preparation from *T. reesei*, Celluclast 1.5L, was used at the same dosage. However, it was supplemented with β-glucosidase from *Aspergillus niger* (Novozym 188) to obtain 20 BG IU/g glucan. This addition was necessary because the β-glucosidase level of Celluclast 1.5L is suboptimal [9]. Other activities of the culture broths (supernatants) in the volume containing 20 FPU are presented in Table 6. While the BGL/FPA ratio for the LIQ and LIQWIS samples was 64 and 55%, respectively, that of LIQWG was similar to the ratio of the reference (93 vs. 100%, respectively). The doses of xylanase were similar in the cases of LIQ and LIQWG, whereas LIQWIS presented the lowest xylanase and β-glucosidase activities. However, activities by the bound and intracellular enzymes were not included in the dosages because the activity values used for calculating the needed volume were measured from the supernatants.

Hydrolysis time curves for released glucose (cellobiose included) and xylose are presented in Fig. 3. Some cellobiose accumulation was observed with each fermentation broth but to a different extent. The highest cellobiose concentration, 3.3 g/l, was measured from samples hydrolyzed with LIQ broth after 24 h, while a decline was already occurring in the other samples at this time. Although less β-glucosidase activity was added with broth LIQWIS (Table 6), it showed less cellobiose accumulation than the aforementioned sample. This finding is somehow contrary to the measured amount of β-glucosidase, but these measurements were taken from the supernatant. Thus, it can be assumed that the LIQWIS broth had more enzymes in the hyphae or substrate-bound form (the LIQWIS broth contained a solid residue of the steam-pretreated substrate resulting in higher enzyme adsorption). Although LIQWG had the optimal BGL/FPA ratio (Table 5), cellobiose accumulation was much higher than in the reference. However, LIQWG was the most effective among the broths tested (Fig. 3), in terms of glucose liberation. By the end of the 72-h hydrolysis, differences between broths and the reference decreased. Even the LIQWG broth reached the level of the commercial enzymes.

In connection with the concentration of cellobiose, the glucose in the reference sample increased fastest, but at the

Table 6 Enzyme activities and protein content of produced and commercial enzymes representing 20 FPU cellulase activity

	Volume (ml)	FPA (FPU)	Protein (mg)	BGL (IU)	XYL (IU)
LIQ	14.49	20	8.40	12.75	1,840
LIQWG	10.70	20	12.63	18.62	1,766
LIQWIS	11.98	20	8.27	10.90	1,557
Celluclast + Novozym 188[a]	0.34	20	13.04	20	243

Although whole culture broths were used in hydrolysis, enzyme activities were measured from supernatants

[a] BGL supplemented to 20 IU/g glucan

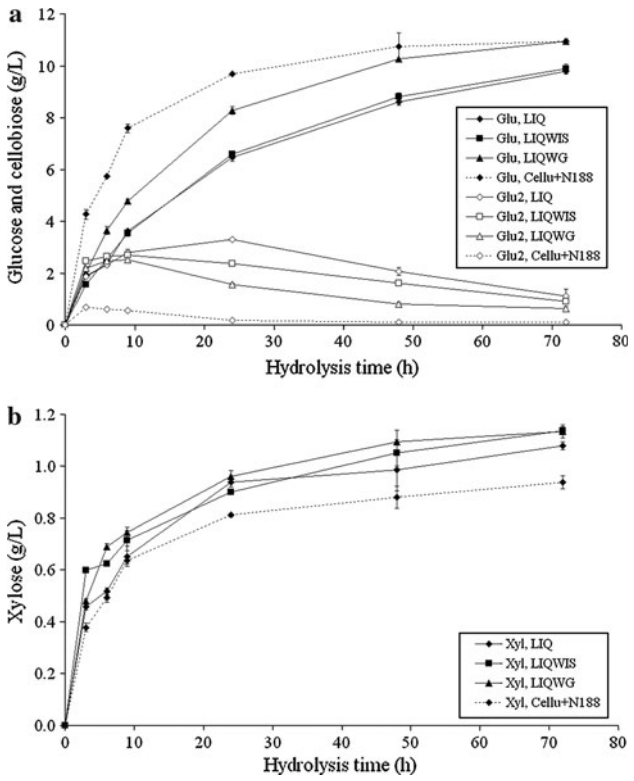

Fig. 3 Concentrations of released sugars during hydrolysis of pretreated and washed wheat straw by 20 FPU/g glucan dosages of LIQ, LIQWG, and LIQWIS broths. A reference hydrolysis was performed by using commercial enzyme preparations (Celluclast 1.5L supplemented with Novozym 188). Data are presented as mean values of three parallel tubes with standard deviations. **a** Glucose (Glu) and cellobiose (Glu2) concentrations. **b** Xylose concentration

end of the 72-h hydrolysis, glucose in samples with LIQWG broth also reached 11 g/l. Surprisingly, the liberated glucose in samples with broths LIQ and LIQWIS did not reflect the difference between their respective accumulation of cellobiose (approximately 1 g/l after 72 h). During the 72-h hydrolysis, both the reference sample and that from broth LIQWG attained 85% glucan conversion and LIQWIS and LIQ, 77 and 76%, respectively, compared to the theoretical maximum.

Liberation of xylose was similar in each case and more effective than with the Celluclast 1.5L-Novozym 188 combination, which had the lowest dose of xylanase. In terms of the dose of xylanase, there was no large difference between the broths. However, the xylan content of the substrate was rather low. On the other hand, higher xylanase activities may have contributed to the efficient glucose liberation achieved by increasing the accessibility of cellulose [6]. Final xylan conversion values were the following for each broth: 70% for the reference, and 81, 85, and 85% for LIQ, LIQWG, and LIQWIS, respectively, although LIQWIS contained the lowest xylanase activity among the broths.

Conclusions

In this study, we investigated the possibility of using the liquid fraction of steam pretreatment in on-site enzyme production by *T. reesei* RUT C30. As a result of the presence of inhibitors, no enzyme production was observed with an untreated liquid fraction, even when it was diluted fourfold. Successful fermentation could be performed only in a fourfold-diluted, detoxified liquid fraction. In this treated liquid furfural and HMF were eliminated, but concentrations of aliphatic acids increased. Therefore, the total amount of inhibitors remained unchanged. Our findings demonstrate that up to a specific level, the synergy of the inhibitors is more important than their discrete concentrations.

The main advantage of using the liquid fraction as carbon source was the highly enhanced xylanase activity compared to the reference (WIS in tap water). Wheat grain supplementation resulted in nearly doubled β-glucosidase activity compared to the original liquid fraction alone. However, FPA yields from supplemented media (LIQWIS and LIQWG) were considerably lower than those of the references.

Steam-pretreated and washed wheat straw was found to be a good substrate for enzymatic hydrolysis, for both commercial and in-house-produced enzymes. Contrary to expectations, the enzyme produced in medium supplemented with washed pretreated wheat straw (broth LIQWIS) did not yield any more glucose. However, adding wheat grain into the medium resulted in enhanced efficiency of hydrolysis, which is most likely due to the elevated β-glucosidase activity. This sample eventually reached the same final conversion as the commercial enzyme mixture supplemented with β-glucosidase.

With this study, we demonstrated that after detoxification, the liquid fraction of steam-pretreated wheat straw is a reasonable substrate for *T. reesei* RUT C30, which makes on-site enzyme production available using a less expensive carbon source. Moreover, supplementing this medium with ground wheat grain also carries apparent advantages and provides the possibility for the integration of second-generation ethanol technologies with on-site cellulase production in wheat grain processing ethanol factories.

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References

1. Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349. doi: 10.1002/jctb.1676

2. Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 101:4851–4861. doi:10.1016/j.biortech.2009.11.093
3. Ballesteros I, Negro MJ, Oliva JM, Cabanas A, Manzanares P, Ballesteros M (2006) Ethanol production from steam-explosion pretreated wheat straw. Appl Biochem Biotechnol 129–132: 496–508. doi:10.1385/ABAB:130:1:496
4. Benitez T, Limón C, Delgado-Jarana J, Rey M (1998) Glucanolytic and other enzymes and their genes. In: Harman GE, Kubicek CP (eds) Trichoderma and gliocladium, vol 2. Taylor & Francis, London, UK, pp 89–113
5. Berghem LE, Pettersson LG (1974) The mechanism of enzymatic cellulose degradation. Isolation and some properties of a β-glucosidase from *Trichoderma viride*. Eur J Biochem 46:295–305. doi:10.1111/j.1432-1033.1974.tb03621.x
6. Berlin A, Gilkes N, Kilburn D, Bura R, Markov A, Skomarovsky A, Okunev O, Gusakov A, Maximenko V, Gregg D, Sinitsyn A, Saddler J (2005) Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates—evidence for the role of accessory enzymes. Enzyme Microb Technol 37:175–184. doi:10.1016/j.enzmictec.2005.01.039
7. Bigelow M, Wyman C (2002) Cellulase production on bagasse pretreated with hot water. Appl Biochem Biotechnol 98–100:921–934. doi:10.1385/ABAB:98-100:1-9:921
8. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3
9. Breuil C, Chan M, Gilbert M, Saddler JN (1992) Influence of β-glucosidase on the filter paper activity and hydrolysis of lignocellulosic substrates. Bioresour Technol 39:139–142. doi:10.1016/0960-8524(92)90132-H
10. Chen S, Wayman M (1992) Novel inducers derived from starch for cellulase production by *Trichoderma reesei*. Process Biochem 27:327–334. doi:10.1016/0032-9592(92)87010-E
11. Fujimoto H, Nishida H, Ajisaka K (1988) Enzymatic syntheses of glucobioses by a condensation reaction with α-glucosidase, β-glucosidases and glucoamylase. Agric Biol Chem 52:1345–1351
12. Hayward T, Hamilton J, Templeton D, Jennings E, Ruth M, Tholudur A, McMillan J, Tucker M, Mohagheghi A (1999) Enzyme production, growth, and adaptation of *T. reesei* strains QM9414, L-27, RL-P37, and RUT C-30 to conditioned yellow poplar sawdust hydrolysate. Appl Biochem Biotechnol 77:293–309. doi:10.1385/ABAB:77:1-3:293
13. Hägglund E (1951) The wood components and their chemical properties. In: Hägglund E (ed) Chemistry of wood. Academic, New York, USA, pp 37–389
14. Horváth IS, Sjöde A, Alriksson B, Jönsson LJ, Nilvebrant N (2005) Critical conditions for improved fermentability during overliming of acid hydrolysates from spruce. Appl Biochem Biotechnol 121–124:1031–1044. doi:10.1007/978-1-59259-991-2_87
15. Ilmén M, Thrane C, Penttilä M (1996) The glucose repressor gene cre1 of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. Mol Gen Genet 251:451–460. doi:10.1007/BF02172374
16. Jorgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod Biorefin 1:119–134. doi:10.1002/bbb.4
17. Juhász T, Szengyel Z, Szijártó N, Réczey K (2004) Effect of pH on cellulase production of *Trichoderma reesei* RUT C30. Appl Biochem Biotechnol 113:201–211. doi:10.1385/ABAB:113:1-3:201
18. Kaparaju P, Serrano M, Thomsen AB, Kongjan P, Angelidaki I (2009) Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour Technol 100:2562–2568. doi:10.1016/j.biortech.2008.11.011
19. Kaparaju P, Serrano M, Angelidaki I (2009) Effect of reactor configuration on biogas production from wheat straw hydrolysate. Bioresour Technol 100:6317–6323. doi:10.1016/j.biortech.2009.06.101
20. Kovács K, Szakacs G, Zacchi G (2009) Comparative enzymatic hydrolysis of pretreated spruce by supernatants, whole fermentation broths and washed mycelia of *Trichoderma reesei* and *Trichoderma atroviride*. Bioresour Technol 100:1350–1357. doi:10.1016/j.biortech.2008.08.006
21. Kumar S, Singh SP, Mishra IM, Adhikari DK (2009) Recent advances in production of bioethanol from lignocellulosic biomass. Chem Eng Technol 32:517–526. doi:10.1002/ceat.200800442
22. Larsson S, Reimann A, Nilvebrant N, Jönsson L (1999) Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. Appl Biochem Biotechnol 77:91–103. doi:10.1385/ABAB:77:1-3:91
23. Lo C, Zhang Q, Lee P, Ju L (2005) Cellulase production by *Trichoderma reesei* using sawdust hydrolysate. Appl Biochem Biotechnol 121–124:561–573. doi:10.1385/ABAB:122:1-3:0561
24. Marchessault RH, Malhotra SL, Jones AY, Perovic A (1983) The wood explosion process: characterization and uses of lignin/cellulose products. In: Wood and agriculture residues. Academic, New York, pp 401–413
25. Marquina D, Flores M (1997) Use of potato starch extraction wastes to grow cellulolytic fungi. Adv Food Sci 19:75–80
26. Messner R, Kubicek CP (1990) Evidence for a single, specific β-glucosidase in cell walls from *Trichoderma reesei* QM9414. Enzyme Microb Technol 12:685–690. doi:10.1016/0141-0229(90)90008-E
27. Mohagheghi A, Grohmann K, Wyman C (1988) Production of cellulase on mixtures of xylose and cellulose. Appl Biochem Biotechnol 17:263–277. doi:10.1007/BF02779162
28. Nogawa M, Goto M, Okada H, Morikawa Y (2001) L-Sorbose induces cellulase gene transcription in the cellulolytic fungus *Trichoderma reesei*. Curr Genet 38:329–334. doi:10.1007/s002940000165
29. Olsson L, Hahn-Hägerdal B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Technol 18:312–331. doi:10.1016/0141-0229(95)00157-3
30. Palmqvist E, Hahn-Hägerdal B, Szengyel Z, Zacchi G, Réczey K (1997) Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. Enzyme Microb Technol 20:286–293. doi:10.1016/S0141-0229(96)00130-5
31. Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour Technol 74:17–24. doi:10.1016/S0960-8524(99)00160-1
32. Parajó JC, Domínguez H, Domínguez JM (1998) Biotechnological production of xylitol part. 3: Operation in culture media made from lignocellulose hydrolysates. Bioresour Technol 66:25–40. doi:10.1016/S0960-8524(98)00037-6
33. Réczey K, Brumbauer A, Bollók M, Szengyel Z, Zacchi G (1998) Use of hemicellulose hydrolysate for β-glucosidase fermentation. Appl Biochem Biotechnol 70–72:225–235. doi:10.1007/BF02920139
34. Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP (2008) The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. BMC Genomics 9:327. doi:10.1186/1471-2164-9-327
35. Sternberg D, Mandels GR (1979) Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. J Bacteriol 139:761–769
36. Szengyel Z, Zacchi G, Réczey K (1997) Cellulase production based on hemicellulose hydrolysate from steam-pretreated

willow. Appl Biochem Biotechnol 63–65:351–362. doi:10.1007/BF02920437
37. Szengyel Z, Zacchi G (2000) Effect of acetic acid and furfural on cellulase production of *Trichoderma reesei* RUT C30. Appl Biochem Biotechnol 89:31–42. doi:10.1385/ABAB:89:1:31
38. Taj-Aldeen SJ (1993) Effect of starch on the induction of β-glucosidase in *Trichoderma reesei*. Mycol Res 97:318–320. doi:10.1016/S0953-7562(09)81128-3
39. Talebnia F, Karakashev D, Angelidaki I (2010) Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation. Bioresour Technol 101:4744–4753. doi:10.1016/j.biortech.2009.11.080
40. van Maris AJA, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MAH, Wisselink HW, Scheffers WA, van Dijken JP, Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie van Leeuwenhoek 90:391–418. doi:10.1007/s10482-006-9085-7
41. Wang CH, Hseu TH, Huang CM (1988) Induction of cellulase by cello-oligosaccharides in *Trichoderma koningii* G-39. J Biotechnol 9:47–59. doi:10.1016/0168-1656(88)90014-4
42. Warzywoda M, Larbre E, Pourquié J (1992) Production and characterization of cellulolytic enzymes from *Trichoderma reesei* grown on various carbon sources. Bioresour Technol 39:125–130. doi:10.1016/0960-8524(92)90130-P
43. Wayman M, Chen S (1992) Cellulase production by *Trichoderma reesei* using whole wheat flour as a carbon source. Enzyme Microb Technol 14:825–831. doi:10.1016/0141-0229(92)90099-A
44. Xiong H, Turunen O, Pastinen O, Leisola M, von Weymarn N (2004) Improved xylanase production by *Trichoderma reesei* grown on L-arabinose and lactose or D-glucose mixtures. Appl Microbiol Biotechnol 64:353–358. doi:10.1007/s00253-003-1548-4
45. Xiong H, von Weymarn N, Turunen O, Leisola M, Pastinen O (2005) Xylanase production by *Trichoderma reesei* RUT C-30 grown on L-arabinose-rich plant hydrolysates. Bioresour Technol 96:753–759. doi:10.1016/j.biortech.2004.08.007