Co-Cultivation of *Aspergillus niger* and *Trichoderma reesei* Enables Efficient Production of Enzymes for the Hydrolysis of Wheat Bran

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Dedicated to Prof. Dr. Christian Wandrey on the occasion of his 80th birthday

Agro-residues like wheat bran may be a useful carbon source for fermentation processes. However, due to their composition, hydrolysis appears complex. A co-cultivation process was established with the two fungi *A. niger* and *T. reesei* for the production of an adapted mixture of enzymes for hydrolysis. This enzyme solution reached a higher space-time yield of sugars during the hydrolysis of wheat bran compared to enzymes from respective monocultures, demonstrating the potential of this co-culture for the production of complementary enzyme mixtures for the hydrolysis of wheat bran.

Keywords: Co-cultivation, Enzymatic hydrolysis, Filamentous fungi, Wheat bran

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1 Introduction

A major challenge of current biotechnology is finding suitable alternatives to fossil raw materials for the production of chemicals and fuels. An environmentally-friendly and economical alternative can be provided by the vast amounts of residual organic materials that are produced by the agricultural and food industry, which until now have been incinerated or used uneconomically as animal feed [1–3]. One of the most important and abundant resources of the food industry is wheat, with a yearly global production of over 750 million tons. While most of the grain is typically milled into flour for a wide range of foods, the outermost layers are being left. This residue, the so-called wheat bran, amounts for ~ 150 million tons every year [4] and therefore poses a great and mostly untapped substrate for biotechnological utilization and valorization without competing with important food resources. However, its complex and varying composition of the polysaccharides arabinoxylan, starch and cellulose as well as proteins [4–6] hampers its degradation into easily convertible sugar monomers.

Most microbial enzyme producers are naturally specialized in the secretion of a limited set of hydrolases, whereas the enzymatic degradation of wheat bran demands the presence of a number of enzymes with a broad variety of activities due to the composition of several, heterogeneous polymers, with the most abundant one being the hemicellulose arabinoxylan [7]. It consists of a backbone of α-1,4-linked β-D-xylose units, often substituted with arabinose, glucuronic acid or acetyl side chains, and the arabinose side chains on their part can be linked with ferulic or coumaric acid [8–10]. Xylan-degrading enzymes, or xylanases, are therefore a heterogeneous group of enzymes with either hydrolase or esterase activity [11]. Endoxylanases catalyze the degradation of intact xylan polymer chains and can be classified depending on their ability to act on branch points of the xylan backbone or not [12]. The products released are subsequently further hydrolyzed by β-xylosidases, which prefer xylobiose or smaller oligomers [13]. Debranching enzymes like arabinofuranosidase and glucuronidase are responsible for cleaving off the respective side chains. Acetyl xylan esterases support the hydrolysis of xylan by removing acetyl side groups that could otherwise sterically hinder endoxylanase activity [11, 14].

Starch is a glucose polymer and can be found in unbranched, helical or linear, α-1,4-linked chains as amylose or as branched amylopectin, where some glucose units in the α-1,4-backbone bear α-1,6-branched side chains. Starch in wheat bran contains about 27 % non-branched amylose [15]. Three different types of amylolytic enzymes are needed for starch degradation, whereof fungi mainly produce two, α-amylases and γ-amylases [16]. Enzymes of the former group are able to randomly cleave glycosidic bonds in the glucose chains, releasing maltose and oligosaccharides. A special α-amylase is the maltase (or α-glucosi-

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dase), specifically decomposing the dimeric maltose [17]. Γ-amylases (glucoamylases) form glucose molecules by hydrolyzing the final glycosidic bonds at non-reducing ends of amylose and amylopectin chains. The amylase production with filamentous fungi like *A. niger* is well-studied and the produced enzymes are widely used in various industrial branches [18–21].

The third major polysaccharide in wheat bran is cellulose, a recalcitrant polymer based on β-1,4-linked glucose molecules. Linked with hydrogen bonds, these chains form microfibrils. This crystalline character, though interspersed with amorphous regions, makes cellulose degradation difficult. Nevertheless, many organisms manage to degrade cellulose, with three types of cellulolytic enzymes being required: endo-glucanases, exo-glucanases or cellobiohydrolases, and β-glucosidases [22, 23]. Endo-glucanases are able to cleave glycosidic bonds within the amorphous regions of cellulose, resulting in shorter β-1,4-linked glucan chains. The exo-glucanases subsequently remove cellobiose units from the non-reducing ends of these chains [23–25]. The resulting dimer is eventually degraded into single glucose molecules by the β–glucosidases [23].

As most microbial enzyme producers only produce a restricted set of enzymes, the total degradation of agricultural wastes like wheat bran using enzymes from only one microorganism is hardly feasible. Producing enzyme solutions from different microorganisms separately in order to synthetically create a hydrolytic mixture massively intensifies the input of labor and equipment [26]. Hence, the co-cultivation of complementary enzyme-producing organisms appears as a promising alternative to manufacture adapted enzyme solutions on-site [27].

The filamentous fungi *Aspergillus niger* and *Trichoderma reesei* are two microorganisms known for their great capability for secreting hydrolytic enzymes. *Aspergillus niger* is a ubiquitous, well-researched ascomycete that has been used for the production of citric acid for over a century [28]. Nowadays *A. niger* is one of the most important microorganisms in biotechnological processes and is applied for the production of organic acids as well as for enzymes [29, 30]. It offers a broad spectrum of hydrolytically active enzymes able to degrade biomass from various resources, among them pectinases, xylanases and amylases, of which the latter two are of great interest in the saccharification of wheat bran [31]. The strain used in this work is *A. niger* NRRL 2270 (ATCC 11414). Originating from citric acid-producing ATCC 1015 [32] and isolated due to even improved characteristics in citric acid production [33, 34], it was also investigated for its potential in the production of hydrolytic enzymes for biomass degradation like pectinases [35], and xylanases and amylases (pre-studies, not published). Its contemplated partner within this co-culture is the fungus *Trichoderma reesei*, which was discovered in the Solomon Islands during World War II [36] and first described as a good producer of cellulases in 1956 [37, 38]. The strain in this study, RUT-C30, which is the result of a three-step mutagenesis process using UV mutagenesis, N-nitrosoguanidine and again UV mutagenesis, each followed by screening and selection of the best candidates [39, 40], is catabolite-de-repressed and therefore capable of hypersecreting cellulases independently from the surrounding medium [40, 41]. Due to this feature, this strain has been the subject of numerous investigations regarding process characterization and optimization [42–45]. However, these several rounds of mutations led to various side effects affecting the fungus' growth, morphology and enzyme formation, e.g., low amylase and β-glucosidase production [36, 46, 47]. As *A. niger* or other *Aspergillus* species are capable of compensating for these deficiencies, co-cultures of *Aspergillus sp.* and *T. reesei* appear as a suitable combination of complementary enzyme producers for the degradation of agricultural residues such as wheat bran [27, 48–52].

Other studies reported unfavorable behavior between *A. niger* and *T. reesei*, where the strains interact in competition for substrates and even actively antagonize each other by forming secondary metabolites or cell wall-degrading enzymes [53]. Thus, a precise adjustment of process variables and conditions is essential for facilitating a stable co-culture in order to enable both fungi to secrete their respective set of enzymes.

In this study, the production of the adapted enzyme solution with the co-cultivation and the hydrolysis of the wheat bran was performed in a two-step approach with separation of fungal biomass in between. A defined synthetic wheat bran medium was used to imitate real wheat bran and induce secretion of all necessary enzymes while maintaining a high grade of definition and comparability. At first we focused on establishing a stable co-culture in a stirred-tank bioreactor with metabolic activity of *A. niger* and *T. reesei*, so both strains are able to grow and produce their enzymes in a batch process. Afterwards, the process variables agitation and aeration were varied to find a suitable point of operation for the batch process in a small-scale stirred-tank bioreactor. This process was subsequently transferred to a stirred-tank bioreactor with increased working volume to identify suitable scale-up criteria for this co-culture. A concluding hydrolysis experiment with grinded wheat bran comparing mono- and co-culture enzyme cocktails at optimized reaction conditions demonstrated the potential of the co-culture for the production of an adapted enzyme mixture for the hydrolysis of the agricultural residue wheat bran.

2 Material and Methods

2.1 Strains, Inoculum Preparation, and Cultivation Medium

A. niger NRRL 2270 was obtained from the NRRL collection, and *T. reesei* RUT-C30 was kindly provided by the group of Prof. Thomas Brück (Werner Siemens Chair of

Synthetic Biotechnology, Technical University of Munich, Germany). Spores of *A. niger* were grown on 39 g L^{-1} potato extract dextrose agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) supplemented with 10 g L^{-1} yeast extract and 1 mL L^{-1} trace elements solution. *T. reesei* spores were grown on potato extract dextrose agar without any supplements. Spores were harvested after 5 d and 7 d for *A. niger*, and *T. reesei*, respectively, with sterile 0.89 % NaCl solution with 0.05 % Tween 80. Spore suspensions were filtered through sterile cotton wool and diluted to the desired spore concentration.

All fungal cultivations were conducted using synthetic wheat bran medium, whose defined carbon composition of (L^{-1}) 10 g starch, 7 g xylan, 3 g microcrystalline cellulose (Avicel, Sigma Aldrich, St. Louis, USA) and 1 g glucose imitated the composition of real wheat bran. The other components of the medium were (L^{-1}) 23.1 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7\,H_2O$, 1 mL L^{-1} trace element solution and 0.1 % (v/v) PPG P2000 as antifoaming agent. The trace element solution consisted of (L^{-1}) 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7\,H_2O$, 1.01 g $MnCl_2 \cdot 4\,H_2O$, 0.32 g $CoCl_2 \cdot 6\,H_2O$, 0.32 g $CuSO_4 \cdot 5\,H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, 1.47 g $CaCl_2 \cdot 2\,H_2O$, and 1 g $FeSO_4 \cdot 7\,H_2O$ [54].

2.2 Batch Cultivations in Stirred-Tank Bioreactors on a 0.7 L-Scale

Cultivations on the 0.7 L-scale were conducted in a parallel bioreactor system consisting of four stirred-tank bioreactors with maximum volume of 1 L each (DASGIP, Eppendorf AG, Hamburg, Germany). The vessels were equipped with one elephant's ear turbine (80 mm above vessel bottom) and one Rushton turbine (30 mm above vessel bottom; both stirrers: DASGIP, Eppendorf AG, Hamburg, Germany). Each reactor was filled with 0.7 L of synthetic wheat bran medium and inoculated with 0.2×10^9 spores L^{-1} of *T. reesei*. 10^9 spores L^{-1} of *A. niger* were added either simultaneously or after 16.5 h or 24 h process time, respectively. In monocultures, each strain was employed individually with the same spore concentrations. Temperature was kept constant at 30 °C. The pH was controlled at a constant value of pH 4.5 by the automated addition of 1 M H_2SO_4 or 3 M KOH. Process times were 72–96 h. To prevent loss of spores in the beginning of the batch processes, the reactors were not actively aerated and stirred at a low agitation rate of 250 min^{-1} during the first 6 h. Afterwards, aeration and agitation rates were set to their respective set-points. The aeration rate was subsequently gradually increased to keep the dissolved oxygen concentration (DO) above 30 %. To indirectly observe the metabolic activity of the cultures, the composition of the exhaust gas (O_2, CO_2) was monitored (BlueVary, BlueSens gas sensors GmbH, Herten, Germany).

2.3 Batch Cultivations in Stirred-Tank Bioreactors on a 3.0 L-Scale

A 7 L stirred-tank bioreactor equipped with three baffles (Labfors, Infors-HT, Bottmingen, Switzerland) was used for the cultivation experiments on the 3.0 L-scale. Homogenization was achieved by a stirrer configuration of (from top to bottom) one downwards pumping elephant's ear stirrer (160 mm above vessel bottom), one upwards pumping elephant's ear stirrer (90 mm above vessel bottom) and one six-blade Rushton turbine (30 mm above vessel bottom; all stirrers: Infors-HT, Bottmingen, Switzerland). Three liters of synthetic wheat bran medium were initially inoculated with 0.2×10^8 spores L^{-1} of *T. reesei*, and 10^9 spores L^{-1} of *A. niger* were added after 16.5 h process time. The temperature was kept constant at 30 °C; the pH was controlled to a set point of pH 4.5 by adding either 1 M H_2SO_4 or 3 M KOH. The batch processes were carried out for 86 h. The stirred-tank bioreactor was not aerated and only mixed slowly at 250 min^{-1} during the first 6 h to prevent initial spore loss; afterwards, the stirrer speed was increased to 660 min^{-1} and aeration was set to the initial value of 0.2 vvm. Oxygen limitation was avoided by increasing the aeration rate by steps of 0.2 vvm when the DO dropped below 30 %, up to maximum of 2 vvm. The composition of the exhaust gas (O_2, CO_2) was monitored and recorded (BlueVary, BlueSens gas sensors GmbH, Herten, Germany).

2.4 Sample Treatment and Harvest of Enzyme Solutions

Samples were drawn aseptically from the reactors at various time points, aliquoted into 2 mL-reaction tubes and centrifuged at 21 000 × g for 20 min at 4 °C to separate fungal biomass as well as solid media components from the supernatant, which was subsequently filtrated (0.2 µm) and stored at –20 °C for further analysis.

The culture supernatants containing the produced enzymes were harvested after fermentation by centrifugation and sterile-filtration and stored at –20 °C until usage for the hydrolysis experiments.

2.5 Characterization of the Stirred-Tank Bioreactors

Both stirred-tank bioreactors used were characterized regarding their stirrer tip speeds and volumetric oxygen transport coefficient (k_La). These process variables were kept constant on both scales in order to achieve comparable shear forces and oxygen supply on both scales.

The stirrer tip speeds were kept constant by following

$$\pi n_1 d_{R,1} = \pi n_2 d_{R,2} \tag{1}$$

with n_i as the agitation rate and $d_{R,i}$ being the diameter of the respective stirrer.

The k_La was determined at varying agitation and aeration rates by using the nitrogen desorption method. A 0.2 % xanthan solution was used to emulate the rheological behavior of the fungal cultivation broth. The dissolved oxygen was completely displaced by gassing the reactor with nitrogen in order to record the oxygen desorption rate (representing the oxygen uptake rate (*OUR*)), before switching back to pressurized air for monitoring the oxygen saturation kinetics of the model fluid. The k_La was subsequently determined by plotting $dc_{O2}/dt - OUR$ against $c_{O2}^* - c_{O2}$ and performing linear regression with the k_La as the slope. A Python-based spline function was used for smoothing the noise of the recorded data. All measurements were performed in triplicate, and the means with respective standard deviations were used as the k_La values.

2.6 Enzymatic Hydrolysis of Wheat bran

Hydrolysis experiments were performed in 1 L stirred-tank bioreactors (DASGIP, Eppendorf AG, Hamburg, Germany) with pre-dried wheat bran, provided by Bayerischer Müllerbund (Munich, Germany). 50 g of wheat bran were mixed with 200 mL of 62.5 mM sodium citrate buffer (pH 4.5), autoclaved, and washed into the reactor with additional 200 mL of buffer. At process start, 100 mL of sterile enzyme solution from an *A. niger* monoculture, a *T. reesei* monoculture or the co-cultivation process, or a mixture of enzyme solutions from a *A. niger* monoculture and a *T. reesei* monoculture (50 mL each) were added aseptically into the stirred-tank reactor, resulting in a total working volume of 500 mL. The hydrolysis processes were conducted at 50 °C and 420 min^{-1} stirring for 45 h. Samples were drawn regularly and stored at –20 °C until further analysis. Data regarding the composition of the wheat bran for yield calculations were generated and kindly provided by the Werner Siemens Chair of Synthetic Biotechnology, TUM, Germany.

2.7 Analytical Methods

2.7.1 Respirational rates Carbon Dioxide Evolution Rate and Oxygen Uptake Rate

The carbon dioxide evolution rate (*CER*) was calculated as follows:

$$CER = -\frac{\dot{V}_{g,in}}{V_L V_m}\left(x_{CO_2,in} - \frac{1 - x_{CO_2,in} - x_{O_2,in}}{1 - x_{CO_2,out} - x_{O_2,out}} x_{CO_2,out}\right) \quad (2)$$

with $\dot{V}_{g,in}$ as the gas flow rate into the reactor, V_L as the reaction volume, V_m as the molar volume of an ideal gas (22.4 L mol^{-1}), and $x_{CO2,in}$, $x_{O2,in}$, $x_{CO2,out}$, and $x_{O2,out}$ being the concentrations of carbon dioxide and oxygen in the inlet gas (in) and the exhaust gas (out), respectively [55].

The oxygen uptake rate (*OUR*) was determined analogously:

$$OUR = -\frac{\dot{V}_{g,in}}{V_L V_m}\left(x_{O_2,in} - \frac{1 - x_{CO_2,in} - x_{O_2,in}}{1 - x_{CO_2,out} - x_{O_2,out}} x_{O_2,out}\right) \quad (3)$$

Concentrations of carbon dioxide and oxygen were measured and recorded via exhaust gas analysis. The *CER* and *OUR* were calculated for time points every 10 min and plotted against the process time. While direct the measurement of biomass concentration or optical density is not feasible here, the *CER* is taken as an indirect measure for growth and metabolic activity [56].

2.7.2 Total protein concentration

Total protein concentration in the culture supernatant was determined using the Coomassie Plus (Bradford) Assay Kit (Thermo Scientific) following the manufacturer's instructions in the Micro Microplate Protocol. The samples were diluted with 0.1 M sodium acetate buffer (pH 4.5) before mixing with the Bradford reagent and incubation for 10 min at room temperature. Absorbance was measured at 595 nm in a microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, USA), and protein concentration was calculated with bovine serum albumin as the standard. Each sample was measured in triplicate and the concentrations are displayed as the mean with respective standard deviation.

2.7.3 Hydrolytic Enzyme Activities

Total xylanase, amylase and cellulase activities were determined following an adapted version of the Fructan Assay Kit protocol (Megazyme, Bray, Ireland) using xylan, starch, and carboxymethyl cellulose (CMC) as substrates, respectively. 100 µL of culture supernatant diluted with 0.1 M sodium acetate (pH 4.5) and 100 µL of the respective substrate solution (10 g L^{-1} xylan, starch or CMC, in 0.1 M sodium acetate, pH 4.5) were mixed in micro reaction tubes and incubated at 50 °C for 30 min (amylase, cellulase) or 50 min (xylanase). Samples of 20 µL were taken from the tubes regularly and pipetted into a PCR plate on ice immediately. The released reducing sugar ends were determined using a 4-hydroxybenzhydrazide solution as described in the Megazyme protocol and measured with a microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, USA). Samples for the blank values were drawn from the tubes before incubation, and enzyme activities were calculated via the slope of sugar concentration over reaction time. One unit (U) was defined as the amount of enzymatic activity releasing 1 µmol of xylose (xylanase) or glucose (amylase, cellulase) equivalents per minute under assay conditions. Activities of each sample were determined in triplicate and results were plotted as means with respective standard deviations. For the characterization of the

enzymes produced in the co-culture, the assay described above was performed at temperatures between 20–70 °C and pH 3.0–pH 7.0.

2.7.4 Quantification of Sugars and Organic Acids in Culture and Hydrolysis Supernatants

Concentrations of monosaccharides, disaccharides and organic acids in the culture and hydrolysis supernatants were determined by high-performance liquid chromatography (HPLC, 1100 Series, Agilent Technologies, Santa Clara, USA) using a Rezex ROA-Organic Acid H+ (8%) LC Column (300×7.8 mm) cation exchange column with a SecurityGuard Cartridge Carbo-H (4×3.0 mm) pre-column (both Phenomenex Ltd., Aschaffenburg, Germany). The samples were sterile-filtered beforehand, and the hydrolysis samples were diluted with deionized water. Volumes of 20 µL were injected into 5 mM H_2SO_4 as eluent at a flow rate of 0.5 mL min^{-1} and separated in the column at a constant temperature of 65 °C. The components were detected in a refractive index (RI) detector (1200 Series G1362A, Agilent Technologies, Santa Clara, USA) and quantified using respective standards.

2.7.5 Secretome Analysis via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The secretomes of the mono- and co-cultures were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 40 µL of sample were mixed with 10 µL 5x Laemmli sample buffer [57] and denatured (95 °C for 10 min) before being loaded onto a 12.5% polyacrylamide gel (pH 8.8). After electrophoresis (35 mA, 60 min), the gel was stained using the method of Fairbanks et al. [58]. Results regarding strain dominance within the co-cultures were concluded by comparing protein band patterns of co-culture samples with those of monocultures.

3 Results

3.1 Effects of Strain Combination and Delayed Inoculation

In order to efficiently produce hydrolytic enzymes in a co-culture of *A. niger* and *T. reesei*, it had to be ensured that both strains were able to grow and secrete their respective set of enzymes in the cultivation.

It became visible that both strains in monoculture had a characteristic course of the CO_2 evolution rate (*CER*, Fig. 1). Germination and growth of *A. niger* appears to begin after only a few hours of process time, and the metabolic activity reaches its maximum after around 20 h with formation of a characteristic double peak at 24 h process time, indicating a shift in metabolism between different substrates. *T. reesei* germinates slower and has its maximum activity at a process time of 40 h.

The simplest way to inoculate a co-cultivation process is to add both strains into the reactor simultaneously. However, the course of the *CER* in this co-cultivation appeared identical to the *CER* curve from the *A. niger* monoculture (Fig. 1A), with the characteristic double peak at 24 h process time (Fig. 2A). This indicates a strong dominance of *A. niger* in this batch cultivation, while the germination and growth of *T. reesei* seems to be repressed by the faster-growing *Aspergillus*. Thus, the delayed addition of the faster-growing strain was considered as a promising approach to evade such one-sided dominance within the co-culture [27, 59]. In a first experiment, *A. niger* spores were added 24 h after the inoculation with *T. reesei*. Two distinct metabolic maxima were observed, with the first one at 40 h being *T. reesei* and the second one at 64 h being *A. niger*. The latter one is, however, significantly lower than in the *A. niger* monoculture, pointing at inhibited growth of *A. niger* (Fig. 2B). These results are both supported by band pattern comparison from SDS-PAGE gels and could be caused by competitive or even antagonistic behavior of the strains against one another [53]. By reducing the delay of *A. niger* addition to 16.5 h, two clear distinct and comparably high metabolic maxima were achieved, which indicated that both fungi were metabolically active and can produce their respective set of enzymes (Fig. 2C). Therefore, this setup was regarded as the most promising one for co-cultivation processes, and a delay of 16.5 h was chosen for following batch fermentations.

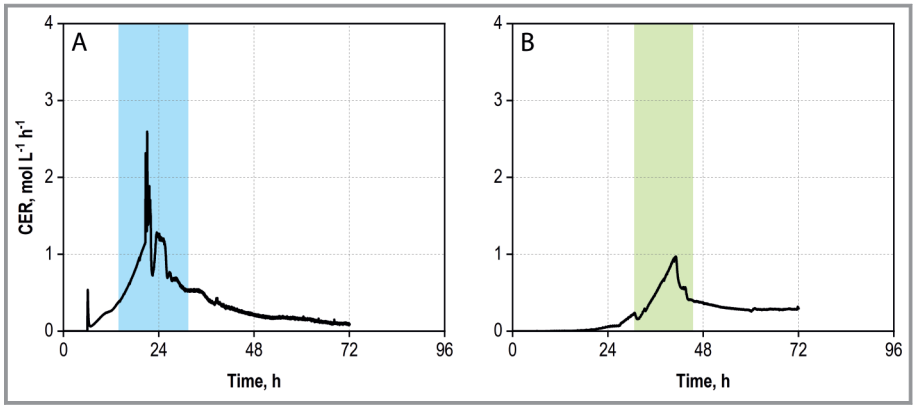

Figure 1. Carbon dioxide evolution rates (CER, mol $L^{-1}h^{-1}$) of *A. niger* (A) and *T. reesei* (B) monocultures during batch cultivation in a 0.7 L stirred-tank bioreactor using synthetic wheat bran medium. *A. niger* was inoculated with an initial spore concentration of 10^9 spores L^{-1}. *T. reesei* was inoculated with an initial spore concentration of 2×10^8 spores L^{-1}. Other process variables were agitation rate 600 min^{-1}, aeration rate 0.2–2 vvm, temperature 30 °C, pH 4.5. Colored areas mark the maximum metabolic activity of *A. niger* (blue) and *T. reesei* (green) in the respective process.

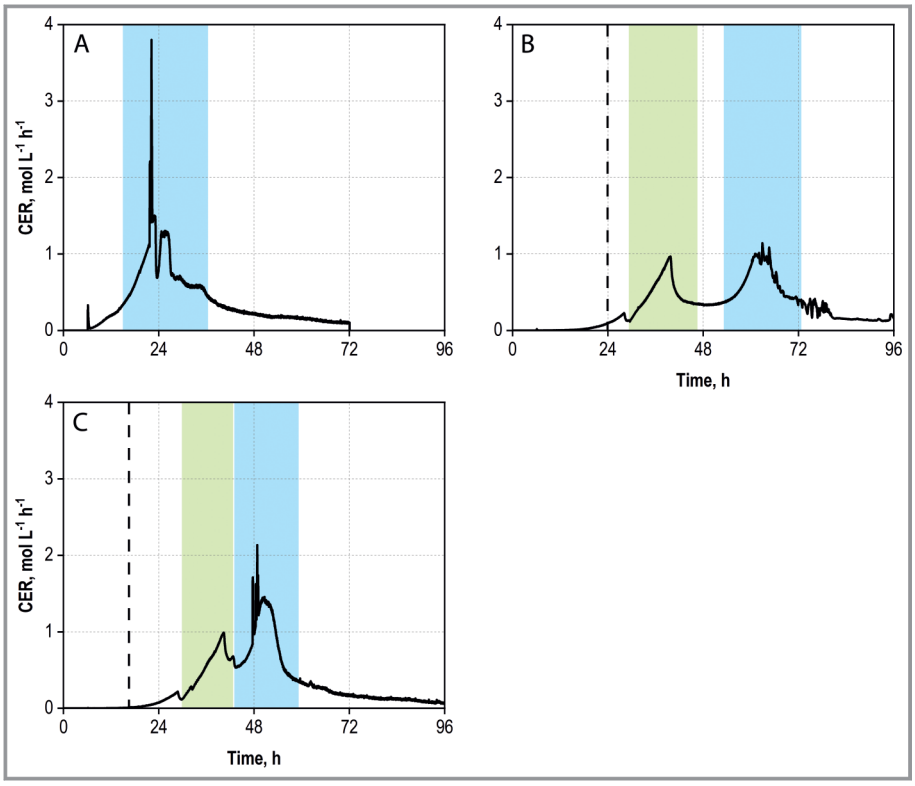

Figure 2. Carbon dioxide evolution rates (CER, mol $L^{-1}h^{-1}$) of co-cultures of *A. niger* and *T. reesei* during cultivation in a 0.7 L stirred-tank bioreactor using synthetic wheat bran medium. *T. reesei* was inoculated with an initial spore concentration of 2×10^8 spores L^{-1}. *A. niger* was added simultaneously (A), after 24 h process time (B), or after 16.5 h process time (C) with an initial spore concentration of 10^9 spores L^{-1}. The black dashed line marks the time of addition of *A. niger* spores. Other process variables were agitation rate 600 min^{-1}, aeration rate 0.2–2 vvm, temperature 30 °C, pH 4.5. Colored areas mark the maximum metabolic activity of *A. niger* (blue) and *T. reesei* (green) in the respective process.

3.2 Variation of Agitation Rate and Aeration in Co-Cultivation Processes

The mechanical power input into the stirred-tank bioreactor has been reported to have a great influence on morphology and product formation of *A. niger* as well as *T. reesei* [19, 60, 61]. Therefore, an ideal combination of these process variables had to be identified for the co-cultivation of those strains for enzyme production. Various combinations of agitation rates between 400–1000 min^{-1} and initial aeration rates of 0.1–0.3 vvm were tested for the production of xylanolytic, amylolytic and cellulolytic activity. All enzyme activities were normalized on the respective maximum value for comparability reasons.

A low aeration rate as well as high stirrer speeds seem to be disadvantageous for xylanase and cellulase formation as well as overall protein secretion (Figs 3A, C, and D). Low gassing rates led to oxygen limitations in the fermentation broth, which could be observed through flattened curves of the respirational rates *OUR* and *CER*, whereas intense stirring resulted in high shear forces and consequently mechanical disruption of the mycelium, visible in microscopy. Maximum values for total protein concentration, xylanase activity and cellulase activity were achieved with an agitation rate of 800 min^{-1} and an initial gassing rate of 0.2 vvm with an increase to 2 vvm during the process. In contrast to those results, amylase activity reached its maximum values when low aeration rates were combined with intense stirring (Fig. 3B). While other studies mostly suggest a negative influence of high agitation on enzyme production, this could rather be caused by oxygen limitation of *T. reesei*, visible through flattened *CER* curves, and hence reduced antagonism rather than advantageous conditions for the amylase-producing *A. niger* [19, 60, 61]. The ideal point of operation was determined by calculating the means of the normalized activities and was set at an agitation rate of 800 min^{-1} and an initial aeration rate of 0.2 vvm.

The enzymatic activities from this improved co-cultivation process were subsequently compared with results from monocultures under the same conditions to evaluate the potential of the co-culture for enzyme production. All three desired enzyme activities were only produced in the co-culture supernatant, with the maximum amylase activity and equivalent xylanase and cellulase activities (Fig. 4). *A. niger* was only able to produce reduced cellulase activity in monoculture and ~ 70 % of the amylase activity of the co-culture, and the latter one was missing completely in the *T. reesei* monoculture. This demonstrates the potential advantages of this fungal co-culture for the production of an adapted enzyme set for the degradation of wheat bran.

3.3 Transfer of the Co-Cultivation Process to the 3.0 L Scale

Transferring a fungal cultivation process from one reactor scale to another is not as simple as with bacterial processes due to the influence of agitation and aeration on fermentations with filamentous microorganisms. Conducting this transfer empirically by, e.g., comparing the morphology of the organisms comes with a tremendous effort of labor and thus does not seem practicable. Therefore, a rational method based on two scale-up criteria was applied for the transfer of

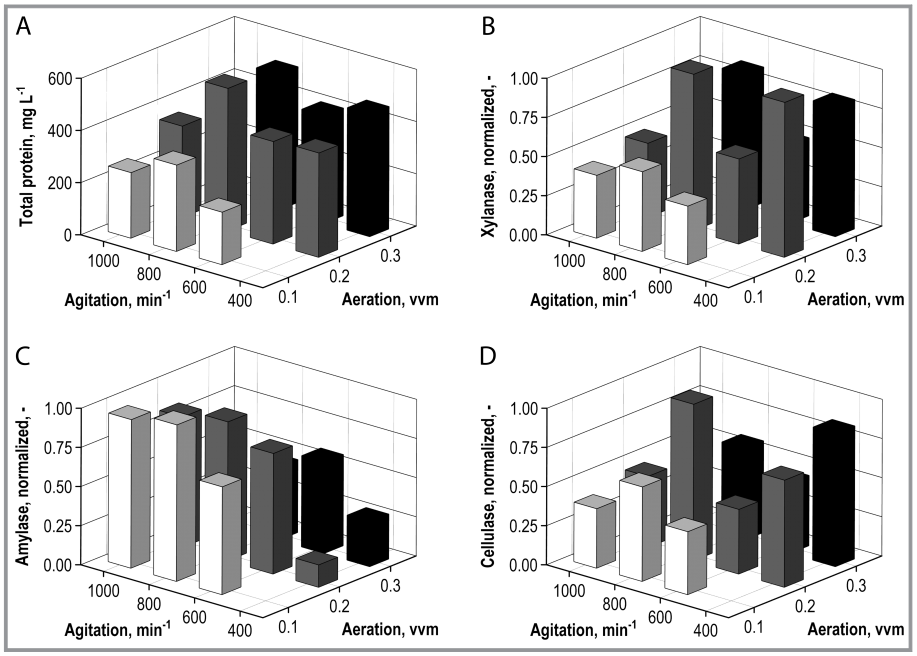

Figure 3. A) Total protein concentration [mg L^{-1}], B) normalized xylanase activity [–], C) normalized amylase activity [–], D) normalized cellulase activity [–], in the supernatant of the co-cultivation of *A. niger* and *T. reesei* in a 0.7 L stirred-tank bioreactor in batch processes after 86 h process time using synthetic wheat bran medium at different operating points (agitation rates 400–1000 min^{-1}, initial aeration rates 0.1–0.3 vvm). *T. reesei* was inoculated with an initial spore concentration of 2×10^8 spores L^{-1}, *A. niger* was added after 16.5 h process time with an initial spore concentration of 10^9 spores L^{-1}. One unit (U) was defined as the amount of enzymatic activity releasing 1 μmol of xylose (B) or glucose (C, D) equivalents per minute under assay conditions.

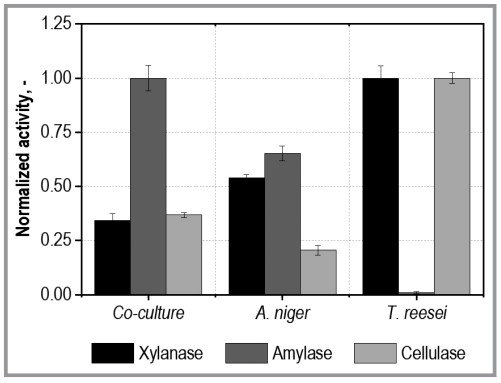

Figure 4. Normalized enzyme activities [–], in the culture supernatants of a co-culture of *A. niger* and *T. reesei*, an *A. niger* monoculture and a *T. reesei* monoculture in a 0.7 L stirred-tank bioreactor using synthetic wheat bran medium at the selected point of operation (800 min^{-1}, 0.2–2 vvm) after 86 h process time. The co-culture was started with 2×10^8 spores L^{-1} of *T. reesei* and 10^9 spores L^{-1} of *A. niger* were added after 16.5 h. Monocultures were inoculated with the same respective spore concentrations. One unit was defined as the amount if enzymatic activity releasing 1 μmol of xylose (xylanase) or glucose (amylase, cellulase) equivalents per minute under assay conditions.

the co-cultivation process from the 0.7 L to a 3.0 L stirred-tank bioreactor. Constant stirrer tip speed aims to keep the maximum local shear forces introduced into the reactor on the same level, whereas a constant volumetric oxygen transport coefficient k_La serves for a comparable oxygen supply [62, 63]. Both stirred-tank reactors were characterized regarding those two process variables, and an agitation rate of 660 min^{-1} in combination with an initial aeration rate of 0.2 vvm was selected as a suitable point of operation for the 3.0 L-reactor (Tab. 1).

A co-cultivation process conducted in the 3.0 L-reactor at this point of operation with all other process variables kept constant resulted in very similar enzyme activities and protein secretion compared to a batch process in the smaller stirred-tank reactors, demonstrating a successful process transfer between those two reactors of different sizes (Fig. 5).

3.4 Characterization of Enzymes Produced in the Co-Culture

The enzymes produced in the co-culture were characterized regarding their temperature and pH optima in order to find suitable reaction conditions for the hydrolysis of wheat bran.

The xylanolytic activity showed a clear maximum at a temperature of 60 °C and pH 4.5. Lower temperatures of 30–50 °C led to reduced activity, and at 70 °C the enzymes were obviously inactivated (Fig. 6A). The amylase activity in the culture supernatant had a tendency towards higher temperatures and a pH below pH 5.5 (Fig. 6B). The maximum cellulase activity could be observed at 50 °C and around pH 5.0 (Fig. 6C). Consequently, a temperature of 50 °C and pH 4.5 were chosen as the process variables for the enzymatic hydrolysis of wheat bran.

3.5 Enzymatic Hydrolysis of Wheat Bran

The agricultural residue wheat bran mainly consists of hemicellulose, cellulose and starch. Aside from the co-culture enzymes, enzyme solutions produced by *A. niger* and *T. reesei* monocultures were applied for the enzymatic hydrolysis of grinded wheat bran separately as well as in a 1:1 mixture.

The courses of sugar release with enzymes from *A. niger*, the enzyme mix and the co-culture enzymes resemble most of the release happening in the first few hours. Enzymatically released sugar concentrations of 19.5 g L^{-1} and 20.3 g L^{-1} were already measured within 6 h process time

Table 1. Process characteristics for the transfer of the co-cultivation process of *A. niger* and *T. reesei* from the 0.7 L to the 3.0 L stirred-tank bioreactor based on constant stirrer tip speed and k_La.

Bioreactor volume [L]	Agitation rate [min^{-1}]	Stirrer tip speed [$m\,s^{-1}$]		k_La [s^{-1}]
		Inclined blade stirrer	Rushton turbine	
0.7	800	2.09	1.88	0.0124
3.0	660	2.07	1.90	0.0124

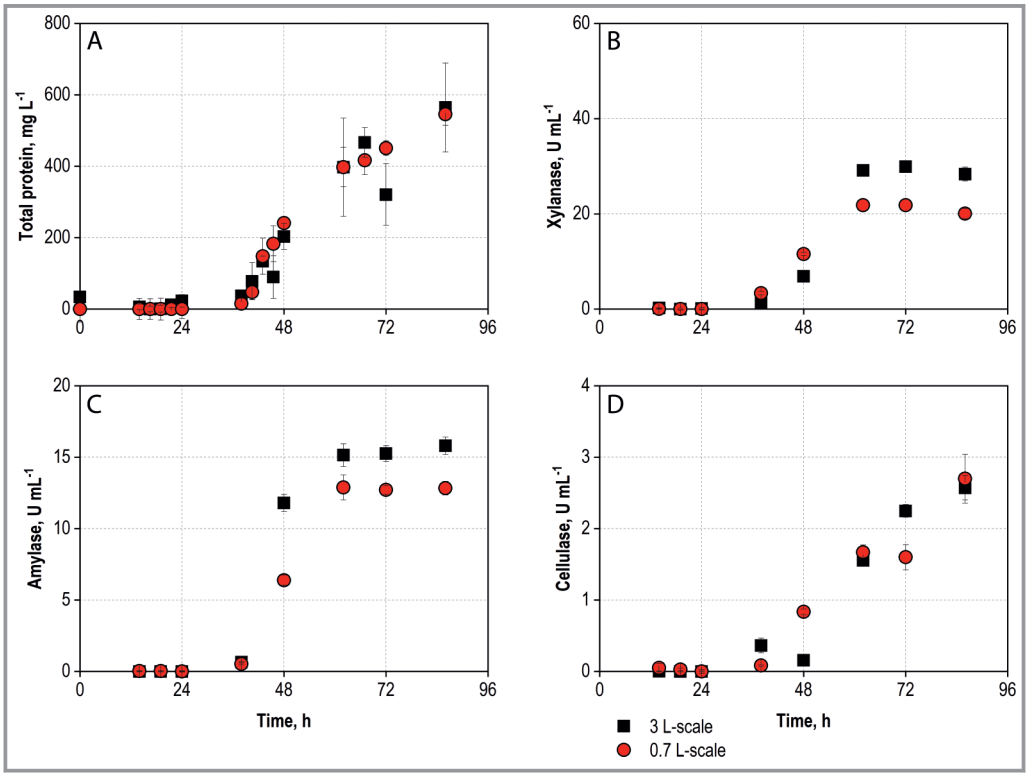

Figure 5. A) Total protein concentration [$mg\,L^{-1}$], B) total xylanase activity [$U\,mL^{-1}$], C) total amylase activity [$U\,mL^{-1}$], D) total cellulase activity [$U\,mL^{-1}$], in the supernatant during the co-cultivation of *A. niger* and *T. reesei* in a 3.0 L stirred-tank bioreactor (agitation 660 min^{-1}, aeration 0.2–2 vvm, black squares) and a 0.7 L stirred-tank bioreactor (agitation 800 min^{-1}, aeration 0.2–2 vvm, red circles) using synthetic wheat bran medium. *T. reesei* was inoculated with an initial spore concentration of 2×10^8 spores L^{-1}, *A. niger* was added after 16.5 h process time with an initial spore concentration of 10^9 spores L^{-1}. One unit (U) was defined as the amount of enzymatic activity releasing 1 μmol of xylose (B) or glucose (C, D) equivalents per minute under assay conditions.

(Fig. 7). The co-culture enzymes had the highest space-time yield in the first hours (4.8 $g\,L^{-1}h^{-1}$ in 4 h, *A. niger* monoculture and enzyme mix 4.5 $g\,L^{-1}h^{-1}$) and showed improved cellulose utilization, as the highest cellobiose concentration was measured after 2 h, which was subsequently degraded within 25 h. *T. reesei* enzymes released significantly less sugar, with a higher share of hemicellulosic sugars (xylose, mannose, galactose) and a noteworthy accumulation of cellobiose up to 3.6 $g\,L^{-1}$, caused by cellulase activity and a known deficiency of *Trichoderma* β-glucosidases [64]. Due to the sterilization process (121 °C for 20 min), a total of 8.7–9.6 $g\,L^{-1}$ sugars were already present before enzyme addition (not shown in Fig. 7).

The hydrolysates from *A. niger* monoculture enzymes, the enzyme mix and the enzymes produced in a co-culture clearly resemble each other. After 45 h, final sugar concentrations of 30.2 $g\,L^{-1}$ (*A. niger*), 32.3 $g\,L^{-1}$ (co-culture), and 33.9 $g\,L^{-1}$ (enzyme mix), respectively, were measured with 57.2–58.2 % glucose, 34.2–34.6 % xylose, mannose, galactose or fructose and 5.0–5.4 % arabinose (Fig. 8). These concentrations correspond to ~ 50 % of the theoretical yield. The low yield of sugars is presumably caused by the high recalcitrance and the particle size of the wheat bran, as enzyme activities were preserved over the time of hydrolysis in previous experiments (data not shown). However, these yields are similar or even higher than those achieved in compara-

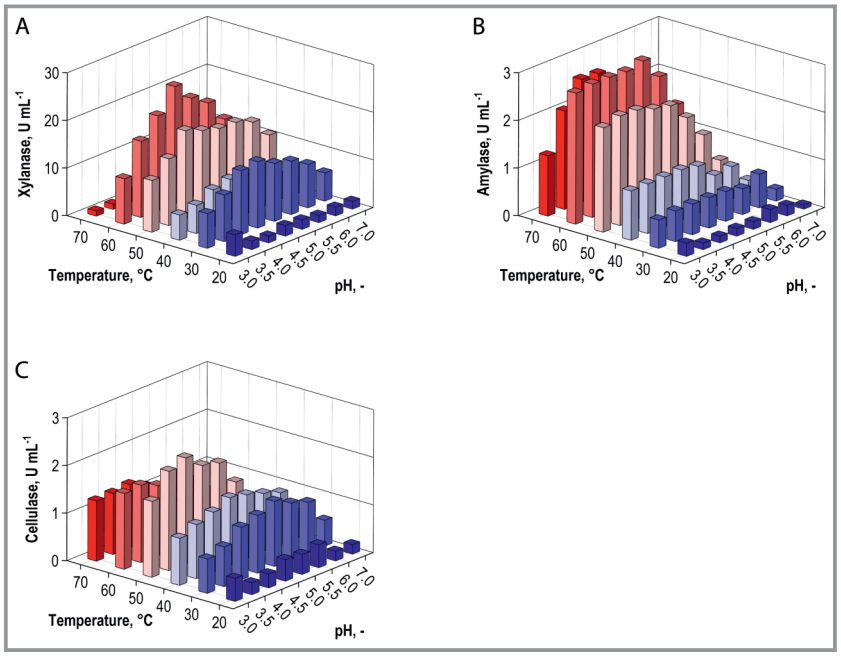

Figure 6. A) Total xylanase activity [U mL^{-1}], B) total amylase activity [U mL^{-1}], C) total cellulase activity [U mL^{-1}] at varying temperatures and pH of the enzyme solution produced by a co-culture of *A. niger* and *T. reesei* in synthetic wheat bran medium. *T. reesei* was inoculated with an initial spore concentration of 2×10^8 spores L^{-1}, *A. niger* was added after 16.5 h process time with an initial spore concentration of 10^9 spores L^{-1}. One unit (U) was defined as the amount of enzymatic activity releasing 1 µmol of xylose (A) or glucose (B, C) equivalents per minute under assay conditions.

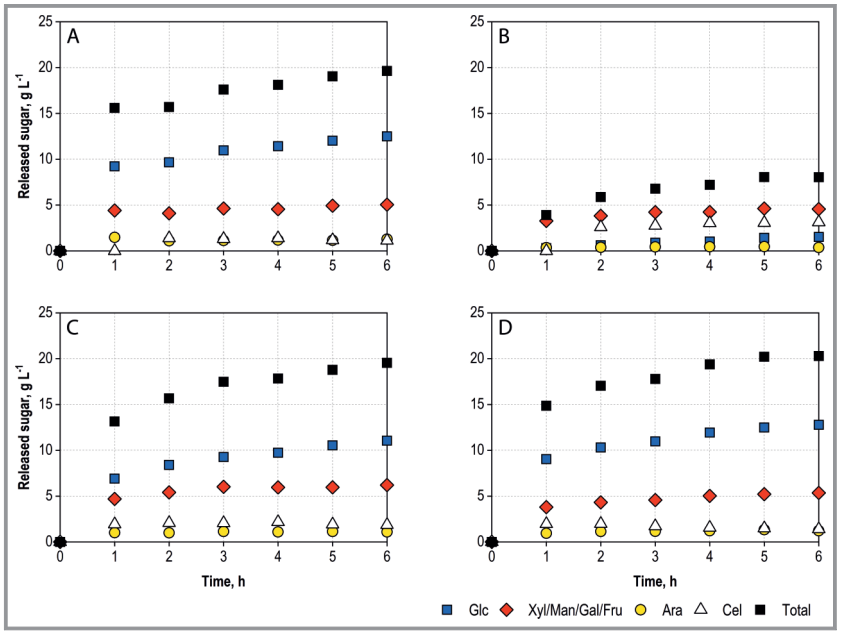

Figure 7. Sugar concentrations released enzymatically from 100 g L^{-1} wheat bran during hydrolysis with enzymes from *A. niger* (A) and *T. reesei* (B) monocultures, a 1:1 mixture of these enzyme solutions (C) and enzymes produced in a co-culture of *A. niger* and *T. reesei* (D). Hydrolysis was performed in 0.5 L-bioreactors at 50 °C and pH 4.5, buffered with 50 mM sodium citrate. 10 % (v/v) enzyme solution was applied in each stirred-tank bioreactor. Glc: glucose, Xyl/Man/Gal/Fru: xylose/mannose/galactose/fructose, Ara: arabinose, Cel: cellobiose.

ble studies, without using harsh pretreatment methods such as steam explosion, extrusion or chemical pretreatment [65, 66]. The *T. reesei* hydrolysate contained a total sugar concentration of only 21.9 g L^{-1}, composed of 40.4 % glucose, 40.1 % xylose/mannose/galactose/fructose, 3.1 % arabinose, and 16.4 % cellobiose.

4 Conclusion

The co-cultivation of the filamentous fungi *A. niger* and *T. reesei* demands additional effort in process development due to the antagonistic and complex interactions between the two microorganisms if the batch processes are inoculated by spore additions. However, after introducing time-delayed inoculation with the faster growing *A. niger* as well as tuning agitation and aeration in stirred-tank bioreactors, it offers an opportunity to efficiently produce an enzyme mix that is adapted to the hydrolysis of wheat bran. This enzyme solution was proven to hydrolyze wheat bran without extensive pre-treatment and under mild reaction conditions. A higher initial space-time yield was achieved compared to mixed monoculture enzymes while having the advantage of being produced in a single fermentation process. Additionally, a rational approach for the process transfer of filamentous co-cultivations was demonstrated, which enables a further scale-up of the co-cultivation process while tremendously reducing the experimental workload.

By lowering the enzyme concentration applied in the hydrolysis, the overall sugar yield achievable after one co-cultivation process should be increased further, leading to an increased economically and ecologically advantageous utilization of an agricultural residue that would have been burned otherwise. An additional promising approach to increase the sugar yield in the enzymatic hydrolysis could be a decrease in the particle size of the wheat bran, resulting in a greater surface area to be attacked by the enzymes.

Overall, the combination of adapted on-site enzyme production and saccharification opens a way towards sustainable

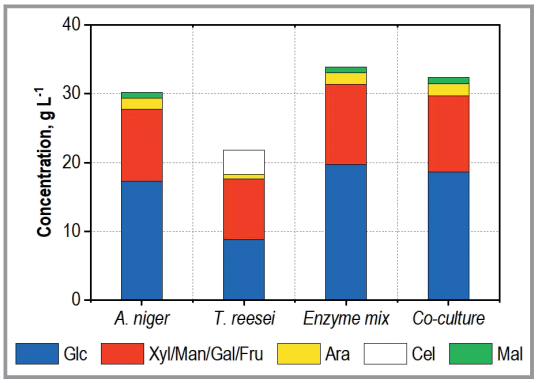

Figure 8. Hydrolysate composition after 45 h hydrolysis of 100 g L^{-1} autoclaved wheat bran using enzymes from *A. niger* and *T. reesei* monocultures, a 1:1 mixture of these enzyme solutions and enzymes produced in a co-culture of *A. niger* and *T. reesei*. Hydrolysis was performed in 0.5 L stirred-tank bioreactors at 50 °C and pH 4.5, buffered with 50 mM sodium citrate. Wheat bran was sterilized at 121 °C for 20 min before 10 % (v/v) enzyme solution was added into each reactor. Sugars released after sterilization and after enzymatic hydrolyses are summed up. Glc: glucose, Xyl/Man/Gal/Fru: xylose/mannose/galactose/fructose, Ara: arabinose, Cel: cellobiose, Mal: maltose.

production of a broad variety of potential products, e.g., platform chemicals or detergents, from agricultural residues and other renewable resources.

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Authors' contributions

Konstantina Xypolia Vasila and Fabian Mittermeier planned and conducted the experiments on the effects of strain combination and delayed inoculation. Nathalie Hafner and Fabian Mittermeier planned and conducted the experiments on the variation of agitation and aeration rate as well as the process transfer. All other experiments were performed by Fabian Mittermeier. Fabian Mittermeier and Dirk Weuster-Botz contributed to the conception of the study. Fabian Mittermeier designed the figures; Fabian Mittermeier and Dirk Weuster-Botz wrote the manuscript. All authors contributed to the critical revision and final approval of the manuscript.

Conflict of interest

The authors have declared no conflicts of interest.

Symbols used

CER	[mol $L^{-1}h^{-1}$]	carbon dioxide evolution rate
c_{O2}	[mol L^{-1}]	oxygen concentration
c_{O2}^*	[mol L^{-1}]	oxygen concentration on the boundary layer in the liquid phase
$d_{R,i}$	[m]	diameter of stirrer i
k_La	[s^{-1}]	volumetric oxygen transport coefficient
n_i	[min^{-1}]	agitation rate of stirrer i
OUR	[mol $L^{-1}h^{-1}$]	oxygen uptake rate
t	[s]	time
$x_{CO2,in}$	[–]	fraction of carbon dioxide at the inlet
$x_{CO2,out}$	[–]	fraction of carbon dioxide at the outlet
$x_{O2,in}$	[–]	fraction of oxygen at the inlet
$x_{O2,out}$	[–]	fraction of oxygen at the outlet
$\dot{V}_{g,in}$	[L h^{-1}]	gas flow rate at the inlet
V_L	[L]	reaction volume
V_m	[L mol^{-1}]	molar volume of an ideal gas (22.414 L mol^{-1})

Abbreviations

A. niger	*Aspergillus niger*
CMC	carboxymethyl cellulose
DO	dissolved oxygen concentration
HPLC	high-performance liquid chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
U	unit of enzymatic activity

References

[1] E. Bonnin, M.-C. Ralet-Renard, H. A. Schols, J.-F. Thibault, in *Handbook of waste management and co-product recovery in food processing Vol. 2* (Ed: K. Waldron), Woodhead Publishing, Sawston, UK **2009**, 257–285.

[2] J. Amoah, P. Kahar, C. Ogino, A. Kondo, *Biotechnol. J.* **2019**, *14 (6)*, 1800494.
[3] R. Singhania, A. Patel, A. Pandey, in *Current Developments in Biotechnology and Bioengineering*, Elsevier, Amsterdam **2017**.
[4] M. Prueckler et al., *LWT Food Sci. Technol.* **2014**, *56 (2)*, 211–221.
[5] J. Hell, W. Kneifel, T. Rosenau, S. Boehmdorfer, *Trends Food Sci. Technol.* **2014**, *35 (2)*, 102–113.
[6] C. D. Nandini, P. V. Salimath, *Food Chem.* **2001**, *73 (2)*, 197–203.
[7] C. R. Babu, H. Ketanapalli, S. K. Beebi, V. C. Kolluru, *Adv. Biotechnol. Microbiol* **2018**, *9*, 1–7.
[8] M. Izydorczyk, J. Dexter, *Food Res. Int.* **2008**, *41 (9)*, 850–868.
[9] M. Schooneveld-Bergmans, G. Beldman, A. Voragen, *J. Cereal Sci.* **1999**, *29 (1)*, 63–75.
[10] R. C. Rudjito, A. C. Ruthes, A. JimÕnez-Quero, F. Vilaplana, *ACS Sustainable Chem. Eng.* **2019**, *7 (15)*, 13167–13177.
[11] M. L. T. M. Polizeli et al., *Applied Microbiol. Biotechnol.* **2005**, *67 (5)*, 577–591.
[12] K. Wong, L. Tan, J. N. Saddler, *Microbiol. reviews* **1988**, *52 (3)*, 305–317.
[13] N. N. van Peij et al., *Eur. J. Biochem.* **1997**, *245 (1)*, 164–173.
[14] Q. Beg, M. Kapoor, L. Mahajan, G. Hoondal, *Applied Microbiol. Biotechnol.* **2001**, *56 (3)*, 326–338.
[15] X. S. Xie, S. W. Cui, W. Li, R. Tsao, *Food Res. Int.* **2008**, *41 (9)*, 882–887.
[16] P. Saranraj, D. Stella, *Int. J. Microbiol. Res* **2013**, *4 (2)*, 203–211.
[17] S. Chiba, *Biosci. Biotechnol., Biochem.* **1997**, *61 (8)*, 1233–1239.
[18] M. S. Hernández, M. R. Rodríguez, N. P. Guerra, R. P. Rosés, *J. Food Eng.* **2006**, *73 (1)*, 93–100.
[19] S. Kelly et al., *Bioprocess. Biosyst. Eng.* **2004**, *26 (5)*, 315–323.
[20] R. P. Rosés, N. P. Guerra, *World J. Microbiol. Biotechnol.* **2009**, *25 (11)*, 1929–1939.
[21] R. Gupta et al., *Process Biochem.* **2003**, *38 (11)*, 1599–1616.
[22] M. Bhat, S. Bhat, *Biotechnol. Adv.* **1997**, *15 (3–4)*, 583–620.
[23] L. R. Lynd, P. J. Weimer, W. H. Van Zyl, I. S. Pretorius, *Microbiol. Mol. Biol. Rev.* **2002**, *66 (3)*, 506–577.
[24] K. M. Kleman-Leyer, M. SiiKa-Aho, T. T. Teeri, T. K. Kirk, *Appl. Environ. Microbiol.* **1996**, *62 (8)*, 2883–2887.
[25] S. Wu, S. Wu, *Applied Biochem. Biotechnol.* **2020**, *190 (2)*, 448–463.
[26] S. J. Duff, D. G. Cooper, O. M. Fuller, *Biotechnol. Lett* **1985**, *7 (3)*, 185–190.
[27] M. Kolasa, B. K. Ahring, P. S. Lübeck, M. Lübeck, *Bioresour. Technol.* **2014**, *169*, 143–148.
[28] T. C. Cairns, C. Nai, V. Meyer, *Fungal Biol. Biotechnol.* **2018**, *5 (1)*, 1–14.
[29] V. Meyer et al., *Fungal Biol. Biotechnol.* **2016**, *3 (1)*, 1–17.
[30] V. Meyer et al., *Fungal Biol. Biotechnol.* **2020**, *7 (1)*, 1–23.
[31] R. P. De Vries et al., *Genome Biol.* **2017**, *18 (1)*, 1–45.
[32] M. R. Andersen et al., *Genome Res.* **2011**, *21 (6)*, 885–897.
[33] S. E. Baker, *Med. Mycol.* **2006**, *44 (Supplement_1)*, S17–S21.
[34] D. Perlman, D. Kita, W. Peterson, *Arch. Biochem.* **1946**, *11*, 123–129.
[35] D. Schäfer, K. Schmitz, D. Weuster-Botz, J. P. Benz, *Bioprocess. Biosyst. Eng.* **2020**, *43 (9)*, 1549–1560.
[36] R. Peterson, H. Nevalainen, *Microbiol.* **2012**, *158 (1)*, 58–68.
[37] M. Mandels, E. T. Reese, *J. Bacteriol.* **1957**, *73 (2)*, 269–278.
[38] E. T. Reese, *Appl. Microbiol.* **1956**, *4 (1)*, 39–45.
[39] B. S. Montenecourt, D. Eveleigh, *Appl. Environ. Microbiol.* **1977**, *34 (6)*, 777–782.
[40] B. S. Montenecourt, D. E. Eveleigh, ACS, Wahington, DC **1979**.
[41] V. S. Bisaria, T. K. Ghose, *Enzyme Microb. Technol.* **1981**, *3 (2)*, 90–104.
[42] N. A. Hendy, C. R. Wilke, H. W. Blanch, *Enzyme Microb. Technol.* **1984**, *6 (2)*, 73–77.
[43] R. R. Singhania, R. K. Sukumaran, A. Pandey, *Appl. Biochem. Biotechnol.* **2007**, *142 (1)*, 60–70.
[44] C. Bendig, D. Weuster-Botz, *Bioprocess. Biosyst. Eng.* **2013**, *36 (7)*, 893–900.
[45] Y. Li, C. Liu, F. Bai, X. Zhao, *Bioresour. Technol.* **2016**, *216*, 503–510.
s[46] R. Peterson, J. Grinyer, H. Nevalainen, *Mycol. Progress* **2011**, *10 (2)*, 207–218.
[47] I. Herpoël-Gimbert et al., *Biotechnol. Biofuels* **2008**, *1 (1)*, 1–12.
[48] D. Maheshwari, S. Gohade, J. Paul, A. Varma, *Carbohydr. Polym.* **1994**, *23 (3)*, 161–163.
[49] A. Ahamed, P. Vermette, *Biochem. Eng. J.* **2008**, *42 (1)*, 41–46.
[50] M. Gutierrez-Correa, L. Portal, P. Moreno, R. P. Tengerdy, *Bioresour. Technol.* **1999**, *68 (2)*, 173–178.
[51] S. Deshpande, M. Bhotmange, T. Chakrabarti, P. Shastri, *Indian J. Chem. Technol.* **2008**, *15*, 449–456.
[52] C. Zhao, L. Deng, H. Fang, *Biomass Bioenergy* **2018**, *112*, 93–98.
[53] P. Daly et al., *Fungal Genet. Biol.* **2017**, *102*, 4–21.
[54] W. Vishniac, M. Santer, *Bacteriol. Rev.* **1957**, *21 (3)*, 195–213.
[55] H. Chmiel, R. Takors, D. Weuster-Botz, *Bioprozesstechnik*, Springer, Berlin **2018**.
[56] A. M. Musaalbakri, C. Webb, *Malays. J. Microbiol.* **2018**, *14 (1)*, 61–69.
[57] U. K. Laemmli, *Nature* **1970**, *227 (5259)*, 680–685.
[58] G. Fairbanks, T. L. Steck, D. Wallach, *Biochem.* **1971**, *10 (13)*, 2606–2617.
[59] I. Schlembach et al., *Biotechnol. Biofuels* **2020**, *13 (1)*, 1–18.
[60] P.-J. Lin, A. Scholz, R. Krull, *Biochem. Eng. J.* **2010**, *49 (2)*, 213–220.
[61] R. Lejeune, G. V. Baron, *Appl. Microbiol Biotechnol.* **1995**, *43 (2)*, 249–258. DOI: https://doi.org/10.1007/BF00172820
[62] D. Pollard et al., *Biotechnol. Bioeng.* **2007**, *96 (2)*, 307–317.
[63] W.-S. Shin et al., *J. Microbiol. Biotechnol.* **2013**, *23 (10)*, 1445–1453.
[64] D. D. Ryu, M. Mandels, *Enzyme Microb. Technol.* **1980**, *2 (2)*, 91–102.
[65] S.-T. Jiang, N. Guo, *Energy Sources, Part A* **2016**, *38 (2)*, 295–299.
[66] E. Aktas-Akyildiz, M. T. Masatcioglu, H. Köksel, *J. Cereal Sci.* **2020**, *93*, 102941.

