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# Sustainable Cultivation of Ascomycete Fungi on Wheat Bran for Hydrolytic Enzyme Production

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***Abstract*** **– To increase its product sustainability portfolio the detergent and personal care industry seeks bio-based alternatives to chemical performance additives in their products. Currently, enzymatic systems for these applications are mainly obtained from genetically modified organisms (GMO). However, these GMO products cannot be included in eco-certified household care formulations. Hence, this study evaluates alternative non-GMO enzymes systems that could be sustainable performance additives. Seven strain variants of *Ceratocystis paradoxa* and one strain of *Aspergillus niger van Tieghem* were cultivated in different liquid media with 3 % glucose (0.3 L cultivation volume, 120 rpm, 28 °C, pH 5.5, 336 hours). Specifically, the enzymatic activities were assessed initially qualitatively via 24 chromogenically labeled sugar polymer-substrates targeting various cellulolytic and hemicellulolytic activities. Quantitative assessment via spectrophotometric based on the same set of chromogenic substrates, was limited to cellulose, xylan, mannan, starch, galactan, rhamnogalacturonan and casein substrates. Using these assays, the *C. paradoxa* strains showed dominant cellulase, xylanase, amylase and galactanase activities, while the *A. niger* strain showed amylase and protease activities among other minor hydrolytic enzyme activities. To evaluate the efficiency of the respective hydrolase enzyme systems were concentrated and applied in the hydrolysis of wheat bran. Wheat bran a cereal milling by-product, which is available at low cost in high quantities, provides an ecologically and economically relevant carbon source for fungal cultivation. Hence, the ability of the new enzyme systems to liberate fermentable sugars was identified as a measure of efficiency. The best performing enzyme systems were identified in terms of total sugar released.**

***Keywords*** **– Biomass hydrolysate; biorefinery; bioremediation; cleaning agents; enzymatic hydrolysis; fungal strains; laundry detergents; wheat bran hydrolysis.**

**Nomenclature**

| | |
|---|---|
| GMO | Genetically modified organism |
| LB | Luria-Bertani medium |
| PDA | Potato Dextrose Agar |
| PM | Potato Medium |
| SFU | Spore Forming Units |
| TM | Trichoderma Medium |

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| WB | Wheat bran |
|---|---|
| YPD | Yeast Extract Peptone Dextrose |

## 1. INTRODUCTION

Many filamentous fungi, such as the well-researched *Aspergillus* sp., *Trichoderma* sp. and *Penicillium* sp., secrete hydrolytic enzyme systems to access complex biomass sources, such as wood and straw as carbon sources [1]. Secretory enzyme systems, often containing an array of different hydrolytic activities can therefore be easily recovered without the need for genetic manipulation and formulated for various industrial applications by isolating and concentrating the cultivation medium [2], [3]. Hence, hydrolytic enzymes derived from filamentous fungi cultivation are now bio-based performance additive in various household and personal care products with the detergent industry being one of the largest markets for these products [4], [5]. While fungal enzymes are important performance additives specifically in detergent industry, almost exclusively these enzymes and their corresponding production hosts are genetically engineered to meet industrial cost and activity boundary conditions [6], [7]. However, consumer awareness for natural, biological products has seen a rise within the last decades. Hence there is increasing demand for non-GMO products, such as eco-certified laundry detergent formulations, which are not allowed to include GMO derived enzymes as performance additives [8]–[10]. These non-GMO enzyme systems should provide the same enzyme activity and cleaning effectivity as their industrially available GMO counterparts. Moreover, the natural production hosts, being the filamentous fungi need to generate high total and space-time yields of these enzyme systems to allow for cost efficient production.

Non-GMO fungal enzymes have been successfully utilized in industries. However, several challenges are related to enzyme stability and activity under extreme conditions, such as high temperatures, varying pH levels, and the presence of inhibitors. Research is needed to identify and optimize fungal strains that can naturally produce more robust enzymes. Additionally, through fermentation technology and controlled fermentation the yield and bioactivity of these fungal enzymes can be optimized towards those seen in select recombinant enzymes.

To generate these non-GMO enzymes for the eco-certified detergent industry also requires a cost efficient and eco-certified carbon source for their fermentative production. One such fermentation substrate is eco-certified wheat-bran, a side stream of the milling industry. With wheat as a major global food crop [11], wheat bran (WB) makes up the highest part its waste fraction after processing [12]. WB is composed of three general layers: the pericarp (fruit coat), the testa (seed coat) and the endosperm containing the aleurone layer and the starchy endosperm. WB contains fibres and a broad spectrum of vitamins and minerals [13]. Its composition is highly dependent on the crop variety and the harvesting method. With a dry biomass content between 33.4 and 63.0 wt%, fibre content is one of the major constituents of the substrate. Protein content lies between 9.6 and 18.6 wt%, while starch makes up 9.10–38.9 wt%, cellulose 10–15 wt%, hemicellulose around 30 wt% and lignin 4–8 wt% [11], [14]–[16]. The polymeric sugars can selectively be hydrolysed using cellulases, hemicellulases and amlylases to serve as a carbon source in fermentative enzyme production using filamentous fungi [17]. Resulting side streams of these processes are the protein rich fraction left after WB hydrolysis and the solid fungal biomass, which is a residue of enzyme production. In order to add value to these side streams, these products could be used in the food and feed industry within a mass efficient circular bioeconomy concept [18]–[21].

The aim of this study was to identify new, non-GMO filamentous fungi strains, that secrete hydrolase enzyme system as potential performance additives in eco-certified detergent formulations.

In comparison to chemical hydrolysis, enzymatic hydrolysis offers various advantages, such as lower energy consumption and milder reaction conditions yielding a more efficient and sustainable approach [22]. Among hydrolases, glycosidases belong to an enzyme class, which degrades complex carbohydrates into polysaccharides, and further into mono- and disaccharides [23]. The saccharification process turns e.g., lignocellulose into sugar-rich hydrolysates [24] with the combined effort of various enzymes, such as lignin oxidases, cellulases, xylanases, amylases and mannanases, among others. Cellulose is a D-glucose polysaccharide connected via ß-1.4 glycosidic bonds [25] and its crystalline structure being stabilized through van-der-Waals forces and hydrogen bonds [26]. Cellulases are either Exoglucanases - working on terminal cellobiose units, Endoglucanases - cleaving internal ß-1.4-glycosidic bonds, [24], [27] and lastly, ß-glucosidase – degrading the cellobiose disaccharide [28]. Molecules of D-xylan, D-mannose or D-arabinose branching out of the cellulose polysaccharide constitute the hemicellulose polymer, among which the ß-1.4 linked D-xylose units are the most common branches. Combinations of e.g., D-xylose and D-arabinose units are present within arabinoxylan [29]. The main enzyme degrading D-xylan-rich polymers are endo ß-1.4 xylanase and exo-ß-1.4 xylosidase, degrading internal β-1.4-xylan chains into oligosaccharide and the oligo- and disaccharides (xylobiose) into monosaccharides, respectively [26]. Mannan polymers can be degraded by endo-ß-1.4-mannanases and exo-ß-1.4-mannanases, while glucomannan with ß-1.4-linked D-mannose and D-glucose units can be cleaved by ß-glucosidases [30].

Addressing challenges related to enzyme stability, substrate specificity, and cost-effective production will be crucial for advancing the field of non-GMO enzyme technology. Additionally, exploring the untapped diversity of fungi could lead to the discovery of new enzymes with enhanced functionalities, further expanding the potential of non-GMO fungal enzymatic systems as performance additives in eco-certified detergent formulations.

## 2. MATERIAL AND METHODS

### *2.1. Characteristics of the Investigated Ascomycete Fungi*

A total of eight ascomycete fungal strains were investigated: seven *Ceratocystis paradoxa* strains and one *Aspergillus niger van Tieghem* strain. Six *C. paradoxa* strains, identified with the code CBS 374.83, CBS 128.32, CBS 453.66, CBS 601.70, CBS 101054, and CBS 116770 were purchased from the Westerdijk Fungal Biodiversity Institute culture collection (WI-KNAW), while the seventh one (*C. paradoxa* DSM 63054) was purchased from the German collection of Microorganisms and Cell cultures GmbH (DSMZ). The *A. niger van Tieghem* strain (ATCC 10535) was purchased from the American Type Culture Collection (ATCC).

### *2.2. Cultivation Media, Spore Production and Spore Quantification*

Potato Dextrose Agar (PDA) with 4 $gL^{-1}$ potato extract (foremedium, CAS Number 73049-73-7), 3 % glucose and 20 g $L^{-1}$ agar was utilized as solid cultivation medium for strain propagation and spore production. Four media were used to investigate the strains' enzyme activity: (i) Luria–Bertani medium (LB) with 5 $gL^{-1}$ yeast extract, 10 $gL^{-1}$ tryptone/peptone and 10 $gL^{-1}$ NaCl. (ii) Potato Medium (PM), which was prepared using 8 $gL^{-1}$ potato extract, instead of the usual preparation containing 4 $gL^{-1}$. (iii) Yeast Extract Peptone Dextrose Medium (YPD), which was prepared with 10 $gL^{-1}$ of yeast extract and 20 $gL^{-1}$

tryptone/peptone. (iv) Trichoderma Medium (TM), which was comprised of 5 $gL^{-1}$ $(NH_4)_2SO_4$, 15 $gL^{-1}$ $KH_2PO_4$, 0.6 $gL^{-1}$ $MgSO_4$, 0.6 $gL^{-1}$ $CaCl_2$, 5 $mgL^{-1}$ $FeSO_4 \cdot 7H_2O$, 1.6 $mgL^{-1}$ $MnSO_4 \cdot H_2O$, 1.4 $mgL^{-1}$ $ZnSO_4 \cdot 7H_2O$, and 2 $mgL^{-1}$ $CoCI_2$. A solution of 3 % glucose was added to each of the four liquid media. For spore production, the fungi were cultivated for seven days at 28 °C and under stirring at 120 rpm on PDA Agar with 3 % glucose. Then, the spores were collected via harvesting solution (phosphate-buffered saline (PBS) solution at pH 7.4, containing 80 $gL^{-1}$ NaCl, 2 $gL^{-1}$ KCl, 14.4 $gL^{-1}$ $Na_2HPO_4$, 2.4 $gL^{-1}$ $KH_2PO_4$ and 10 % glycerol) and filtered through gauze to remove mycelium debris and agar. 15 % glycerol was added for preservation of the spore working cultures, which were stored at –20 °C. For spore quantification, a dilution series from $10^{-1}$ $molL^{-1}$ to $10^{-6}$ $molL^{-1}$ of the spores was prepared and plated on 135 mm diameter PDA agar plates containing 0.1 % Triton-x100 to prevent the formation of mycelial networks. The plates were incubated at 28 °C for 24 to 48 hours until the formation of spore forming units. For the evaluation, plates containing between 100 and 300 colonies were considered. The calculated Spore Forming Units per mL ($SFUmL^{-1}$) for all eight strains are summarized in Table 1. After initial experiments concerning inoculation, the necessary concentration was deemed to be 1.0E+04 $SFUmL^{-1}$.

TABLE 1. FUNGAL STRAINS AND CORRESPONDING SPORE FORMING UNITS PER ML (SFU $mL^{-1}$)

| **Strain** | **Identifier** | **Institute** | **Original Host** | **Original Location** | **SFU $mL^{-1}$** |
|---|---|---|---|---|---|
| *C. paradoxa* | CBS 374.83 | WI-KNAW | *Phoenix canariensis* | Maspalomas, Gran Canaria | 1.04 E+07 |
| *C. paradoxa* | DSM 63054 | DSMZ | unknown | unknown | 2.97 E+06 |
| *C. paradoxa* | CBS 128.32 | WI-KNAW | *Elaeis guineensis* | unknown | 3.00 E+05 |
| *C. paradoxa* | CBS 453.66 | WI-KNAW | Wood | Yunnan, China | 1.83 E+06 |
| *C. paradoxa* | CBS 601.70 | WI-KNAW | *Ananas comosus* | Brazil | 2.38 E+05 |
| *C. paradoxa* | CBS 101054 | WI-KNAW | *Rosa* sp. | De Zier, Netherlands | 1.68 E+06 |
| *C. paradoxa* | CBS 116770 | WI-KNAW | Decaying palm | Bilsa Reserve, Ecuador | 1.27 E+06 |
| *A. niger van Tieghem* | ATCC 10535 | ATCC | Painted pine board | Virginia, United States | 1.62 E+07 |

### *2.3. Qualitative Enzyme Assay Based on Chromogenic Substrates*

A qualitative assay format containing 24 chromogenic substrates (Megazyme Ltd.) was developed in-house. The assay covers a broad range of hydrolytic enzyme substrates. The set of four plates contains three soluble and four insoluble cellulase substrates, two insoluble xylanase substrates, one insoluble and one soluble dextranase, arabinase, amylase, pullulanase, and one insoluble galactan, mannan and rhamnogalacturonan substrate among others (Table 2). 100 µL cell-free supernatant was then filled into a cavity within each agar substrate and the plates were incubated first at 28 °C and then at 37 °C for 24 hours each. The activity was qualitatively assessed based on the diameter of the halo forming around the cavity and categorized as in Table 2.

TABLE 2. HYDROLYTIC ENZYME ACTIVITY MEASURED BASED ON THE CHROMOGENIC SUBSTRATES OF THE QUALITATIVE PLATE ASSAY

| Strain ID | Cel | Xyl | Amy | Ara | Man | Gal | Rha | Prot |
|---|---|---|---|---|---|---|---|---|
| CBS 374.83 | H | H | H | H | H | H | H | − |
| DSM 63054 | H | H | H | H | L | H | L | − |
| CBS 128.32 | L | L | H | M | L | H | L | − |
| CBS 453.66 | M | H | H | M | L | L | M | − |
| CBS 601.70 | L | L | H | L | − | H | L | − |
| CBS 101054 | L | L | H | M | M | H | − | − |
| CBS 116770 | L | − | M | M | L | H | − | − |
| ATCC 10535 | H | M | H | L | L | L | − | M |
| AD mix | M | H | H | H | L | H | M | M |
| CD mix | H | H | H | H | M | H | M | − |

*Note:* ID: Identifyer used within the specific strain collection. Cel: Cellulase activity. Xyl: Xylanase activity. Amy: Amylase activity. Ara: Arabinase activity. Man: Mannanase activity. Gal: Galactanase activity. Rha: Rhamnogalacturonase activity. Prot: Protease activity. All activities, high (H), medium (M) low (L), none (−) are qualitative measures based on the diameter of the halo on the chromogenic agar substrate plates.

### *2.4. Quantitative Spectrophotometric Enzyme Assays Based on Chromogenic Substrates*

For the quantitative enzyme assays, the eight fungal strains were cultivated for 14 days with a sampling period every 24 hours. The strains were cultivated in parallel in LB and PM medium. The two most promising strains were further cultivated in YPD and TM medium. The high-throughput assay in microtiter plate format was developed around the same chromogenic substrates (Megazyme Ltd.) and used in the qualitative analysis with the Genesis 10S UV-Vis spectrophotometer (Thermo Scientific). The enzyme activity for the cellulase, xylanase, amylase, mannanase and galactanase assay was calculated in $mUmL^{-1}$ relative to the calibration curve with a commercial enzyme. The activity for the rhamnogalacturonase assay was measured as in one $mUmL^{-1}$ is the increase of fluorescence by OD 0.01. The protease activity for ATCC 10535 was measured as in one $mUmL^{-1}$ is the increase of fluorescence by OD 0.01.

### *2.5. Wheat Bran Composition and Hydrolysis*

Wheat bran composition was assessed via various methods. The carbohydrate composition was determined by thermal treatment and sequential hydrolysis with 2 % and 72 % $H_2SO_4$. Sugar content was measured by HPLC. The remaining solids were dried and deemed as not acid-insoluble (e. g. lignin). These solids were burned at 650 °C for 3 h to determine the total ash content. Protein content was analyzed with the Kjeldahl-Method [22]. For the hydrolysis experiment, 100 $gL^{-1}$ WB was prepared in a 50 mM sodium acetate buffer at pH 5.0. Three fungal strains, DSM 63054, CBS 374.83 and ATCC 10535 were cultivated for twelve days at 28 °C and 120 rpm. The cell-free supernatant of CBS 374.83 and DSM 63054 (CD mix), as well as DSM 63054 and ATCC 10535 (AD mix) were combined, respectively. The total incubation for the experiment period lasted 96 hours and the analysis was conducted at three temperatures (28 °C, 40 °C and 50 °C). The cell-free supernatant of mixtures AD and CD were added to the WB in mass concentrations of 1 %, 3 % and 6 %. The total protein content of the mixtures was assessed via Bradford assay. The release of reducing sugars was

quantified via 3.5-dinitrosalicylic acid (DNS) assay with glucose as a standard, as well as HPLC.

### *2.6. Quantification of Released sugars via DNS assay and HPLC*

DNS solution was prepared with 10 g $L^{-1}$ 3.5-dinitrosalicylic acid, 500 $mgL^{-1}$ of $Na_2SO_3$ and 10 $gL^{-1}$ NaOH, dissolved in 20 ml $ddH_2O$. 400 $gL^{-1}$ potassium sodium tartrate solution was prepared. The glucose standard curve was set between 0 and 4 $mgmL^{-1}$. The samples were centrifuged after sampling. 150 µL of either sample or standard solution were added to 150 µl DNS solution. The mixture was heated at 95 °C for 10 minutes. 50 µl of potassium sodium tartrate was added and the samples were cooled down on ice. 200 µl from this solution was measured within a 96-well plate at 575 nm. Sugar analysis via HPLC was performed according to [23] on an Agilent 1260 Infinity II LC system with quintenary pump and equipped with Diode Array (DA) and Refractive Index (RI) detectors, a Rezex ROA-organic H + 8 % column from Phenomenex (300×7.8 mm).

## 3. Results and Discussion

Although seven of the eight analysed strains are *C. paradoxa* isolates, they exhibit different morphological features, distinctive smells and varying enzyme activities and secretion profiles. Fig. 1 presents top views of the Petri dishes containing the cultivation of the eight strains. As the isolation of the strains was performed on various continents, there may be large genetic differences between the isolates of the same strain, as they adapt to their host organism and environment, as well as climate conditions. While the isolates CBS 101054 and CBS 116770 did not show any of the blueish-black colour that was present in the cultures of CBS 453.66, DSM 63054 and CBS 374.83, they also lacked the characteristic fruity and tropical smell present in the later strains.

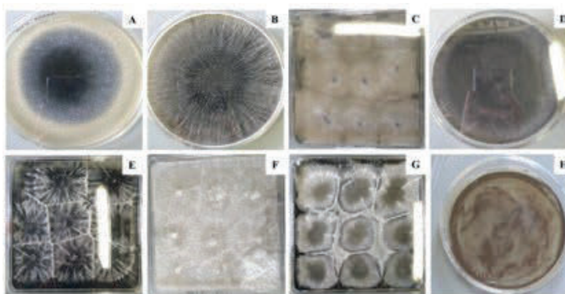


Fig. 1. Strains of *C. paradoxa* (A) DSM 63054; (B) CBS 374.83; (C) CBS 101054; (D) CBS 453.66; (E) CBS 128.32; (F) CBS 116770; (G) CBS 601.70 and (H) strain of *A. niger van Tieghem* ATCC 10535.

### *3.1. Qualitative Analysis of the A. Niger Strain and the Seven Isolates of C. Paradoxa*

The qualitative analysis of the hydrolytic enzyme profile of the chosen seven *C. paradoxa* isolates, as well as the *A. niger* strain is summarized in Table 2. Overall, CBS 374.83 and DSM 63054 have shown the most promising enzyme activities. CBS 374.83 has shown high activity on all tested substrates, apart from protease activity on casein agar. DSM 63054 has exhibited a similar enzyme profile to CBS 374.83, but less mannanase and rhamnogalacturonase activity than the previous strain. CBS 453.66 shows a high xylanase and amylase activity, but only a medium cellulose, arabinase and rhamnogalacturonase

activity and a low mannanase and galactanase activity, compared to the first two *C. paradoxa* strains. ATCC 10535 was found to have the only protease activity of the eight tested strains. Additionally, it presented a high cellulase and amylase activity, but only medium xylanase, low arabinose, mananase, galactanase and no rhamnogalacturonase activity. Among the other strains, CBS 128.32 exhibited a high amylase and galactanase activity, but only medium arabinase and low cellulase, xylanase, mannanase and rhamnogalacturonase activity. CBS 601.70 has shown a similar hydrolase activity as CBS 128.32, but with only low arabinase and no mannanase activity. Lastly, CBS 101054 and CBS 116770 exhibited the lowest overall activities. CBS 101054 only showed high amylase and galactanase activities, medium arabinose and mannanase activity, low cellulose, xylanase and no rhamnogalacturonase activity. Compared to the former, CBS 116770 displayed only medium amylase activity, low mannanase and no xylanase activity.

### *3.2. Quantification of Hydrolytic Enzyme Production of the Eight Strains on LB and PM Media*

The quantification of selected enzymatic activities was performed via a high-throughput spectrophotometric method based on chromogenic substrates. All eight filamentous fungal strains were cultivated in liquid LB and PM media. The results of the enzymatic activity expressed in $mUmL^{-1}$ are listed in Table 3.

TABLE 3. QUANTIFICATION OF ENZYMATIC ACTIVITIES OF THE EIGHT STUDIED FUNGAL STRAINS VIA SPECTROPHOTOMETRIC METHOD BASED ON CHROMOGENIC SUBSTRATES.

| Hours | | 48 | 96 | 144 | 192 | 240 | 288 | 336 | 48 | 96 | 144 | 192 | 240 | 288 | 336 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **CBS 374.83** | | | | | | | **DSM 63054** | | | | | | |
| C | **LB** | - | 32 | 80 | 162 | 218 | 717 | 1106 | 116 | 418 | 454 | 851 | 990 | 1983 | 1928 |
| | **PM** | 30 | 134 | 421 | 597 | 851 | 911 | 1025 | 46 | 302 | 570 | 733 | 700 | 682 | 614 |
| | **YPD** | 69 | 46 | 45 | 74 | 110 | 385 | 507 | 93 | 90 | 190 | 338 | 496 | 1184 | 1236 |
| | **TM** | 70 | 70 | 97 | 214 | 506 | 761 | 795 | 214 | 272 | 490 | 518 | 543 | 974 | 1132 |
| X | **LB** | 3 | 73 | 484 | 712 | 817 | 1139 | 1192 | – | – | – | – | – | 27 | 133 |
| | **PM** | 11 | 2 | 13 | 37 | 265 | 771 | 1173 | – | – | – | 177 | 537 | 806 | 1023 |
| | **YPD** | – | – | 73 | 69 | 4 | 118 | 116 | – | – | – | – | – | 557 | 561 |
| | **TM** | 78 | – | – | 29 | 44 | 44 | 42 | – | – | – | – | – | 1 | 13 |
| A | **LB** | 12 | 50 | 55 | 128 | 53 | 257 | 270 | 14 | 748 | 1188 | 1433 | 1600 | 1758 | 1570 |
| | **PM** | 95 | 193 | 747 | 1352 | 1457 | 1428 | 1321 | 20 | 16 | 65 | 100 | 125 | 81 | 86 |
| | **YPD** | 53 | 145 | 265 | 478 | 800 | 721 | 766 | 25 | 844 | 1340 | 1586 | 1574 | 1496 | 1476 |
| | **TM** | 8 | 99 | 276 | 672 | 1204 | 1254 | 1224 | 32 | 897 | 1141 | 1294 | 1431 | 1463 | 1565 |
| M | **LB** | 0 | – | 17 | 39 | 84 | 269 | 748 | – | 52 | 219 | 539 | 798 | 1222 | 1298 |
| | **PM** | 4 | 37 | 167 | 309 | 569 | 728 | 745 | – | 46 | 179 | 279 | 327 | 457 | 396 |
| | **YPD** | 5 | 21 | 29 | 46 | 64 | 100 | 189 | 4 | 64 | 116 | 281 | 403 | 727 | 832 |
| | **TM** | 17 | 19 | 26 | 56 | 180 | 268 | 309 | 23 | 39 | 117 | 143 | 175 | 560 | 776 |
| G | **LB** | 1 | – | – | 4 | 6 | 147 | 261 | 10 | 42 | 257 | 423 | 620 | 1671 | 1730 |
| | **PM** | 3 | 6 | 10 | 15 | 43 | 53 | 2 | 1 | – | – | – | – | 85 | 85 |
| | **YPD** | – | – | 25 | 53 | 82 | 278 | 201 | 43 | 321 | 642 | 786 | 799 | 1154 | 1395 |
| | **TM** | 10 | 21 | 27 | 28 | 34 | 53 | 47 | 14 | 53 | 61 | 6 | 2 | 149 | 313 |
| R | **LB** | 1 | – | 3 | 10 | 8 | 21 | 13 | – | – | 19 | 5 | 12 | 115 | 104 |
| | **PM** | – | – | 5 | 5 | 5 | 5 | 6 | – | – | – | – | – | – | – |
| | **YPD** | – | 5 | – | 13 | 10 | 10 | 24 | 4 | 11 | 9 | 5 | 3 | 69 | 75 |
| | **TM** | 7 | 2 | 2 | 9 | 13 | 19 | 19 | – | 5 | – | – | – | – | – |

Continue next page

| | | **CBS 128.32** | | | | | | | **BS 453.66** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **C** | **LB** | – | 87 | 86 | 65 | 81 | 70 | 81 | – | 87 | 86 | 65 | 81 | 70 | 81 |
| | **PM** | 16 | 37 | 73 | 77 | 74 | 81 | 140 | 16 | 37 | 73 | 77 | 74 | 81 | 140 |
| **X** | **LB** | 19 | 4 | 68 | 41 | 48 | 74 | 80 | 19 | 4 | 68 | 41 | 48 | 74 | 80 |
| | **PM** | – | 90 | 14 | 81 | 27 | 192 | 7 | – | 90 | 14 | 81 | 27 | 192 | 7 |
| **A** | **LB** | – | 36 | 83 | 99 | 131 | 113 | 163 | – | 36 | 83 | 99 | 131 | 113 | 163 |
| | **PM** | – | – | – | 2 | 4 | 10 | 23 | – | – | – | 2 | 4 | 10 | 23 |
| **M** | **LB** | – | – | – | – | – | – | – | 4 | 37 | 30 | 30 | 29 | 36 | 37 |
| | **PM** | – | – | – | – | – | – | – | 6 | 56 | 152 | 508 | 899 | 1113 | 1220 |
| **G** | **LB** | 3 | 26 | 55 | 97 | 139 | 170 | 199 | 5 | 22 | 39 | 51 | 64 | 70 | 90 |
| | **PM** | 6 | 39 | 73 | 96 | 104 | 108 | 71 | 7 | 39 | 62 | 76 | 63 | 69 | 89 |
| **R** | **LB** | 2 | 22 | 8 | 7 | 18 | 28 | 50 | – | – | – | – | – | 4 | 4 |
| | **PM** | – | – | – | – | – | – | 5 | – | 6 | 7 | 12 | 16 | 15 | 16 |
| | | **CBS 601.70** | | | | | | | **CBS 101054** | | | | | | |
| **C** | **LB** | – | 87 | 86 | 65 | 81 | 70 | 81 | – | 87 | 86 | 65 | 81 | 70 | 81 |
| | **PM** | 16 | 37 | 73 | 77 | 74 | 81 | 140 | 16 | 37 | 73 | 77 | 74 | 81 | 140 |
| **X** | **LB** | 19 | 4 | 68 | 41 | 48 | 74 | 80 | 19 | 4 | 68 | 41 | 48 | 74 | 80 |
| | **PM** | – | 90 | 14 | 81 | 27 | 192 | 7 | – | 90 | 14 | 81 | 27 | 192 | 7 |
| **A** | **LB** | – | 36 | 83 | 99 | 131 | 113 | 163 | – | 36 | 83 | 99 | 131 | 113 | 163 |
| | **PM** | – | – | – | 2 | 4 | 10 | 23 | – | – | – | 2 | 4 | 10 | 23 |
| **M** | **LB** | – | – | – | – | – | – | – | 4 | 37 | 30 | 30 | 29 | 36 | 37 |
| | **PM** | – | – | – | – | – | – | – | 6 | 56 | 152 | 508 | 899 | 1113 | 1220 |
| **G** | **LB** | 3 | 26 | 55 | 97 | 139 | 170 | 199 | 5 | 22 | 39 | 51 | 64 | 70 | 90 |
| | **PM** | 6 | 39 | 73 | 96 | 104 | 108 | 71 | 7 | 39 | 62 | 76 | 63 | 69 | 89 |
| **R** | **LB** | 2 | 22 | 8 | 7 | 18 | 28 | 50 | – | – | – | – | – | 4 | 4 |
| | **PM** | – | – | – | – | – | – | 5 | – | 6 | 7 | 12 | 16 | 15 | 16 |
| | | **CBS 116770** | | | | | | | **ATCC 10535** | | | | | | |
| **C** | **LB** | – | – | 1 | – | – | 98 | 99 | 4 | 19 | 39 | 47 | 62 | 67 | 90 |
| | **PM** | 3 | 1 | 5 | 6 | 4 | 56 | 6 | 49 | 83 | 159 | 83 | 4 | 5 | 5 |
| **X** | **LB** | – | – | – | – | – | 185 | 241 | – | 4 | 40 | 105 | 214 | 251 | 223 |
| | **PM** | 7 | – | 12 | 12 | 22 | 146 | 132 | 5 | 21 | 19 | 15 | 16 | 23 | 24 |
| **A** | **LB** | – | – | – | – | – | – | – | 18 | 13 | 29 | 46 | 51 | 124 | 127 |
| | **PM** | – | – | – | – | – | – | – | 40 | 29 | 59 | 141 | 213 | 285 | 322 |
| **M** | **LB** | – | – | – | – | – | – | – | – | – | 9 | 1 | – | – | – |
| | **PM** | – | – | – | – | – | – | – | 10 | 20 | 33 | 24 | 9 | 6 | 10 |
| **G** | **LB** | – | 1 | 0 | 0 | n. | 161 | 175 | 0 | 3 | 3 | 9 | 8 | 2 | 3 |
| | **PM** | 1 | 6 | 4 | 3 | 4 | 64 | 2 | – | – | – | – | – | 1 | 1 |
| **R** | **LB** | – | – | – | – | – | – | – | 1 | – | 4 | 7 | 8 | 9 | 6 |
| | **PM** | – | – | – | – | – | – | – | – | 1 | 8 | 37 | 38 | 44 | 79 |
| **P** | **LB** | – | – | – | – | – | – | – | 1 | 1 | 1 | 2 | 3 | 4 | 8 |
| | **PM** | – | – | – | – | – | – | – | 1 | 1 | 8 | 15 | 21 | 28 | 36 |

Note: Enzyme activities in mU $mL^{-1}$. LB: LB medium. PM: PM medium. YPD: Yeast extract peptone medium. TM: Trichoderma medium. All media containing 3 % Glucose. Symbol – indicates that no significant activity was detected. C: Cellulase activity. X: Xylanase activity. A: Amylase activity. M: Mannanase activity. G: Galactanase activity. R: Rhamnogalacturonase activity. P: Protease activity.

### *3.3. Hydrolytic Enzyme Activity of CBS 374.83 and DSM 63054 on YPD and TM*

For further insight into the enzyme secretion, the two leading production strains (CBS 374.83 and DSM 63054) were cultivated on two other complex media (YPD and TM). Results of the enzymatic activity are shown in Fig. 2.

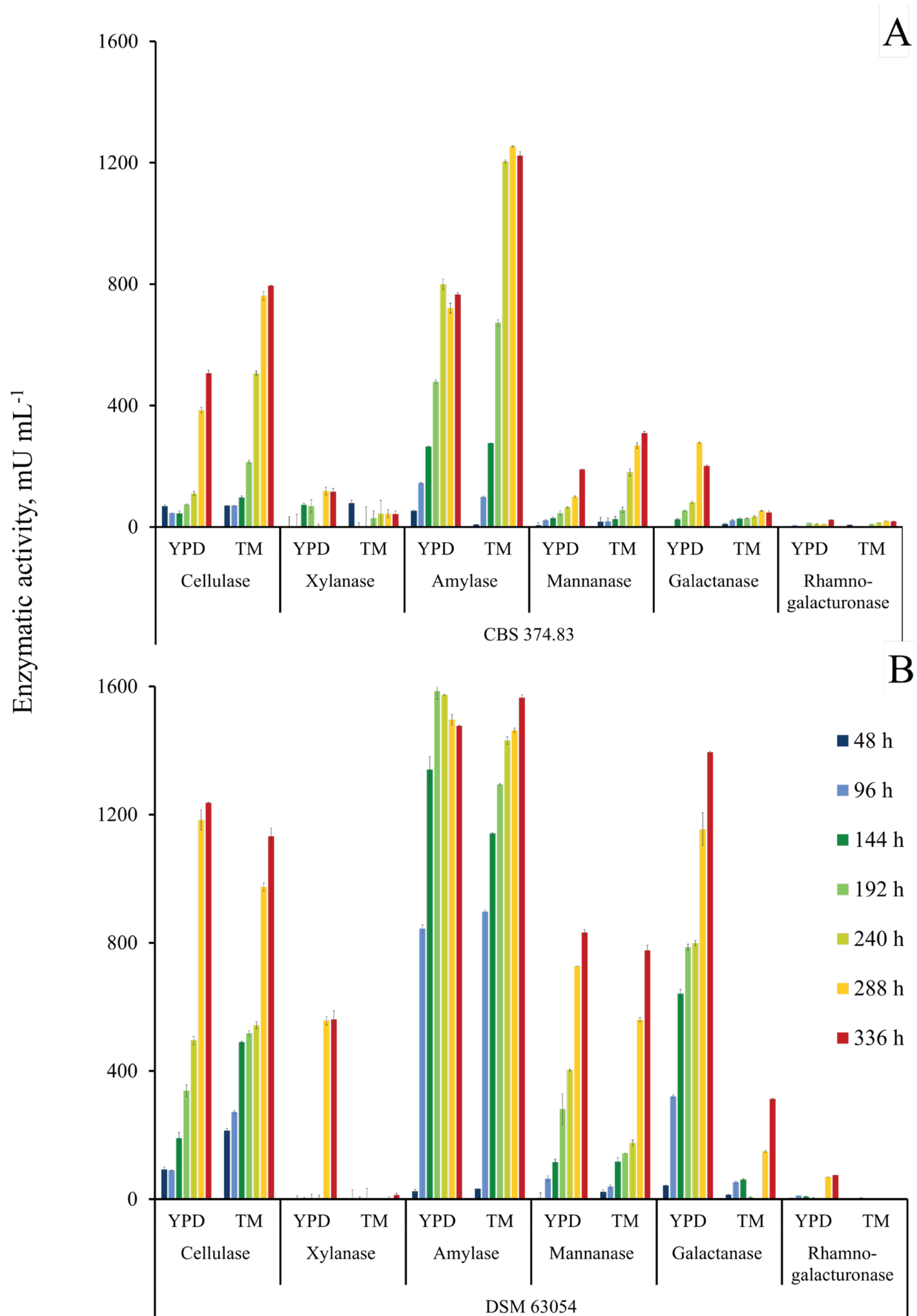


Fig. 2. Quantification of enzymatic activity (in $mUmL^{-1}$) of (A) CBS 374.83 and (B) DSM 63054 strains cultivated on YPD and TM at different time periods (in hours). Measured activities include: cellulase, xylanase, amylase, mannanase, galactanase and rhamnogalacturonase.

Overall, the enzyme activities of DSM 63054 exceeded those of CBS 374.83, though it has a longer lag phase during the first 48 hours of the cultivation compared to the later. Direct comparison of CBS 374.83 and DSM 63054 cultivated on YPD and TM yielded an overall higher enzymatic activity of the supernatant. Among the maximum activities, amylase activity was the highest with 1586 $mUmL^{-1}$ after 192 hours in YPD for DSM 63054, followed by galactanase activity of 1395 $mUmL^{-1}$ after 336 hours in YPD. Cellulase activity was slightly lower at 1236 $mUmL^{-1}$ after 336 hours in YPD. Mannanase activity was the second lowest activity at 832 $mUmL^{-1}$ after 336 hours in YPD and rhamnogalacturonase, the lowest one at 75 $mUmL^{-1}$ after 336 hours in YPD. Among the tested substrates for CBS 374.83, the strain exhibited the highest amylase activity within the TM supernatant after 288 hours cultivation (1254 $mUmL^{-1}$). Other maximal activities include 795 $mUmL^{-1}$ cellulase activity and 309 $mUmL^{-1}$ mannanase after 336 hours in TM, as well as 118 $mUmL^{-1}$ xylanase activity and 278 $mUmL^{-1}$ galactanase activity after 288 hours in YPD and lastly 24 $mUmL^{-1}$ rhamnogalacturonase activity after 336 hours in YPD. Though the two strains stem from the same genetic background, they exhibit a different macromorphology on solid agar, as well as liquid medium. Alongside morphological differences, these two isolates also produce slightly different smells during cultivation. For DSM 63054 YPD has proven more successful than TM as a cultivation medium. On the other hand, for CBS 374.83 TM has shown a better source for enzyme production.

### *3.4. Hydrolytic Enzyme Activity of CBS 374.83, DSM 63054 and ATCC 10535 on LB and PM*

The last two rows of Table 2 provide the values of the qualitative selected hydrolase activities of the mixture of CBS 374.83 and DSM 63054 (CD mix), as well as of ATCC 10535 and DSM 63054 (AD mix).

Quantification of the enzyme activities of the strains cultivated on LB and PM media yielded an insight into their secretion profiles, similarly to the comparison of the two *C. paradoxa* strains cultivated in YPD and TM presented in the previous section 3.4. Results of the enzymatic activity of the three strains are shown in Fig. 3. The overall highest activities among the strains CBS 374.83, DSM 63054 and ATCC 10535 cultivated on LB and PM media include: cellulase, xylanase, galactanase, rhamnogalacturonase and mannanase activity of DSM 63054, amylase activity of CBS 374.83, and protease activity of ATCC 10535, as the later was the only strain exhibiting this specific activity. DSM 63054 shows an overall higher activity in LB than in PM, except for Amylase activity, which is higher in PM. This behavior is attributed to the higher amounts of starch, which is contained. While the averaged activity of the hydrolases secreted by CBS 374.83, in both LB and PM, is lower than that secreted by DSM 63054, amylase activity in PM exceeds that of DSM 63054. Protease activity was only exhibited by *A. niger* ATCC 10535 out of all eight analysed strains. The activity of this enzyme was also heightened during cultivation in PM. This result was explained by the characteristic of PM to contain comparably more protein than LB. The largest discrepancies between the two *C. paradoxa* strains are present considering the galactanase and rhamnogalcturonase activity, which is insignificant in CBS 374.83 compared to DSM 63054. For DSM 63054 LB medium performed best in the enzyme production process. Highest cellulase activity was measured at 1983 $mUmL^{-1}$ after 288 hours in LB, followed by 1730 $mUmL^{-1}$ galactanse and 1298 $mUmL^{-1}$ mannanase after 336 hours in LB, 1570 $mUmL^{-1}$ amylase after 336 hours and 104 $mUmL^{-1}$ rhamnogalacturonase after 288 hours in LB. For CBS 374.83 LB medium performed better than PM as production medium, except for amylase production, as PM is rich in starch. Highest activity was amylase, measured at 1457 $mUmL^{-1}$ after 240 hours in PM, followed by 1192 $mUmL^{-1}$ xylanase after 336 hours in LB, 1106 $mUmL^{-1}$ cellulase and 748 $mUmL^{-1}$ mannanase after 336 hours in LB, 261

mUmL$^{-1}$ galactanase after 336 hours and 13 mUmL$^{-1}$ rhamnogalacturonase after 288 hours in LB. For ATCC 10535 PM medium performed better than LB as production medium, except for xylanase production. Highest activity was amylase, measured at 285 mUmL$^{-1}$ after 288 hours in PM, followed by 251 mUmL$^{-1}$ xylanase after 288 hours in LB, 159 mUmL$^{-1}$ cellulase after 144 hours in PM, 79 mUmL$^{-1}$ rhamnogalacturonase and 36 mUmL$^{-1}$ protease activity after 336 hours in PM.

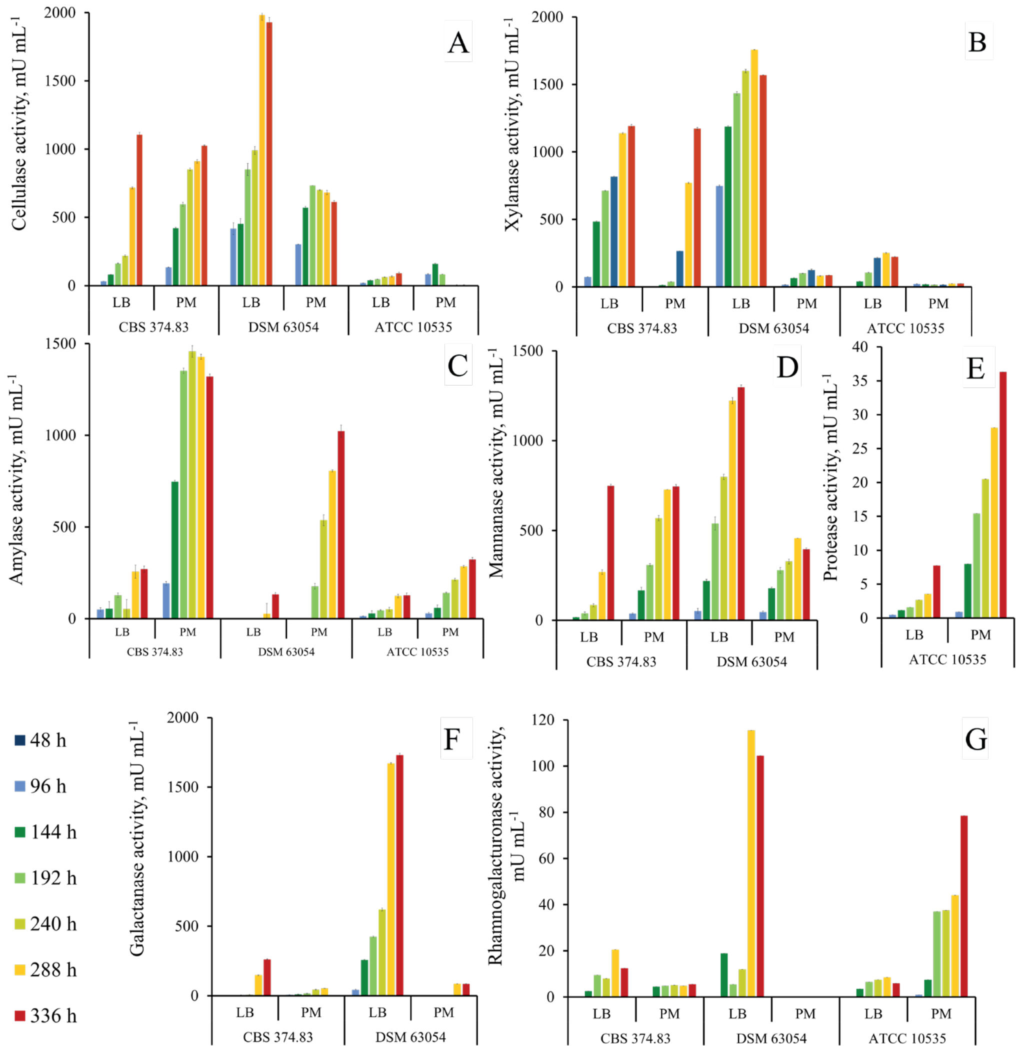


Fig. 3. Quantification of enzymatic activity (in mU mL$^{-1}$) of CBS 374.83, DSM 63054 and ATCC 10535 strains cultivated on LB and PM at different time periods (in hours). Measured activities include: (A) cellulase, (B) xylanase, (C) amylase, (D) mannanase, (E) protease, (F) galactanase, and (G) rhamnogalacturonase.

### *3.5. Determination of Sugar Release via DNS*

The DNS assay was used as preliminary quantification method for the AD (DSM 63054, ATCC 10535) and CD (CBS 374.83, DSM 63054) enzyme mixtures. However, this method is not very accurate, due to the reagent being not more sensitive to monosaccharides, than other reducing substances [24]. Therefore, other quantitative analysis methods, such as HPLC, are preferable, as discussed in the next section. Table 4 presents the main results obtained with DNS. Generally, it can be concluded that more samples from the CD mix reached a concentration over 6.0 mgmL$^{-1}$ (11 samples) compared to the AD mix (4 samples).

TABLE 4. SUGAR RELEASE OF WB SUBSTRATE DURING HYDROLYSIS ASSESSED VIA DNS ASSAY. FOR PARAMETRIC DETAILS, SEE THE NOTE BELOW THE TABLE

| **T °C** | **28° C** | | | | | | **40 °C** | | | | | | **50 °C** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **%** | **1 %** | | **3 %** | | **6 %** | | **1 %** | | **3 %** | | **6 %** | | **1 %** | | **3 %** | | **6 %** | |
| **Mix** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** | **CD** | **AD** |
| **24h** | 1.8 | 2.2 | 3.1 | 2.4 | 4.5 | 1.8 | 2.4 | 3.0 | 3.5 | 2.5 | 3.4 | 2.2 | 2.3 | 2.8 | 2.6 | 3.3 | 2.1 | 3.3 |
| **48h** | 4.1 | 3.1 | 6.7 | 5.2 | 7.9 | 4.0 | 4.7 | 5.1 | 6.8 | 4.2 | 6.2 | 4.7 | 5.2 | 5.1 | 5.2 | 5.9 | 6.1 | 5.9 |
| **72h** | 3.9 | 4.4 | 6.0 | 5.9 | 8.4 | 4.3 | 4.8 | 5.7 | 6.8 | 4.8 | 6.0 | 5.8 | 4.6 | 6.3 | 5.0 | 5.8 | 7.1 | 5.8 |
| **96h** | 3.1 | 4.0 | 4.2 | 6.4 | 6.0 | 3.7 | 3.5 | 7.5 | 4.4 | 5.4 | 4.6 | 5.6 | 3.1 | 6.4 | 3.7 | 5.4 | 4.0 | 5.4 |

*Note:* Sugar release based on glucose calibration curve in mgmL$^{-1}$. Incubation temperatures of 28 °C, 40 °C and 50 °C on 10 % WB. Concentration of fungal enzyme mixture (AD or CD) 1 %, 3 % or 6 %. Measurements taken after 24h, 48h, 72h and 96h.

On average the highest release was found in the samples incubated at 28 °C, followed by those at 40 °C and last those at 50 °C. Literature data indicate, that a higher hydrolysis temperature promotes the reaction [25]–[28]. However, natural enzyme secreting fungi tend to have heightened activities at lower reaction temperatures, making them favorable for industrial processes [29]–[31]. The maximum concentration of released sugars at 8.4 mgmL$^{-1}$, relative to the glucose calibration curve, was reached at 28 °C incubation temperature with 6 % AD mix enzyme solution. The hydrolysis experiment was conducted over a span of 96 hours. Overall, maximum concentrations were reached after 72 hours incubation, decreasing at 96 hours for most. The exception being 1 % AD mix incubated at 40 °C and 50 °C and 3 % AD mix incubated at 28 °C with a slight increase at 96 hours. Some samples indicate a peak before 72 hours, such as 1 %, 3 % CD mix at 28 °C, 6 % CD mix at 40 °C, 1 %, 3 % CD mix at 50 °C and 3 %, 6 % AD mix at 50 °C.

### *3.6. Quantification of Released Monosaccharides via HPLC Analysis*

After the first quantification via DNS assay presented in section 3.6, a more accurate quantification via HPLC was performed on the WB hydrolysate resulting from the hydrolysis with AD (DSM 63054, ATCC 10535) and CD (CBS 374.83, DSM 63054) enzyme mixtures. Fig. 4 presents the HPLC results of three produced enzymes combined in two enzyme mixtures, incubated at three different thermal conditions (28, 40 and 50 °C) with three concentrations of enzyme mixture (1 %, 3 %, and 6 %). The results indicate the highest glucose concentration after 96 hours at 50 °C incubation temperature for the AD enzyme mixture at 3 %. The lowest concentration was found for the 1 % CD enzyme mix incubated at 28 °C for 96 hours. The overall performance of the AD mix was higher than the CD mix. For the arabinose content after hydrolysis the samples showed little significance between the different incubation parameters and enzyme mixtures. The mixed peak for xylose, fructose,

mannose and galactose showed similar trends for the AD mix, albeit at a much lower maximal concentration than glucose.

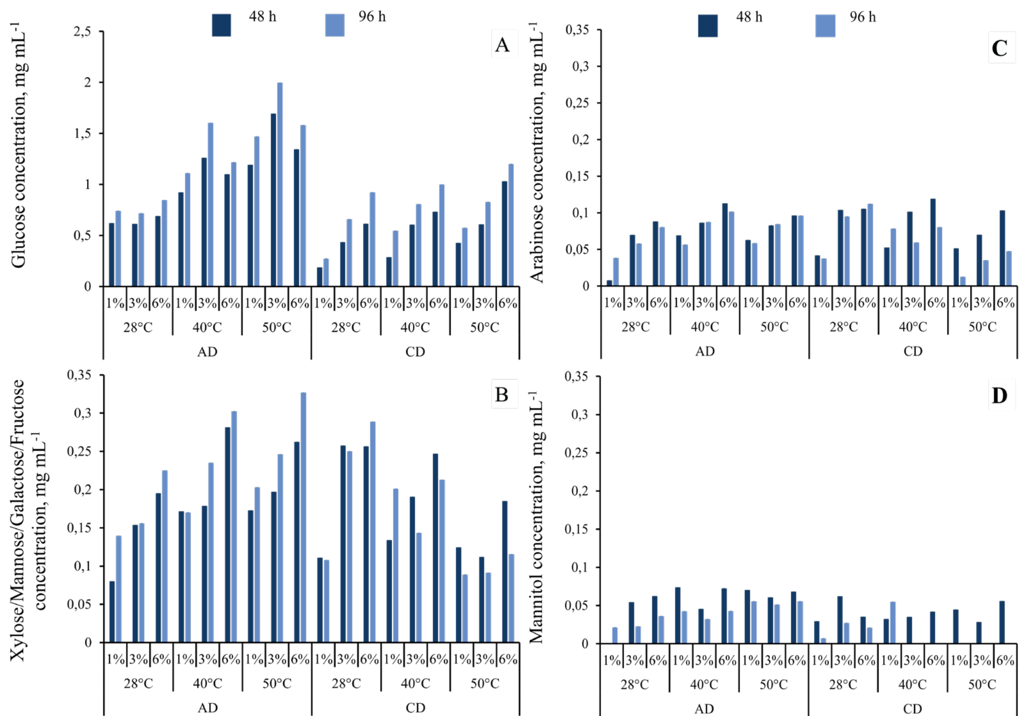


Fig. 4. HPLC analysis data of cell–free supernatant collected during hydrolysis of 10 % WB hydrolysed for AD (ATCC 10535, DSM 63054) and CD (CBS 374.83, DSM 63054) enzyme mixtures at three temperature and three enzyme concentrations. Enzyme concentration is in mgmL$^{-1}$. Quantification of (A) glucose, (B) arabinose, (C) xylose/mannose/galactose/fructose mix and (D) mannitol are reported.

The CD mix showed higher response than Fig. 4(A), relative to the concentration within the samples containing AD mix. Mannitol concentration and distribution among the measured samples was not quite significant. Contrary to the DNS assay the CD samples did not perform nearly as good the AD mix. However, the DNS assay is only to be used as an estimation, as other carbonyl groups influence the results, e.g., furfural or 5-hydroxymethylfurfural [24], [32]. The HPLC data is therefore more reliable.

## 4. Conclusions and Outlook

General purpose for the secretion of glycoside hydrolases by fungi is biomass maintenance and growth, as well as the entry into new substrates and hosts to support that venture [42]. Ascomycete fungus *Trichoderma reesei* is well known as an industry standard within non-GMO enzyme production. Due to its high hydrolase secretion, it has been used widely for e. g. degradation of lignocellulolytic plant material [43], [44]. Whole genetic modification of fungal strains is possible to increase enzyme secretion, e. g. by overexpression of spt1 a gene encoding a serine protease in *T. reesei* increasing the endoglucanase, cellobiohydrolase

and β-glucosidase activity by 63.3 %, 302.0 % and 260.0 %, respectively, compared to the initial strain [45].

Challenges remain when using recombinant hydrolase production, such as the possibility of faulty protein folding, degradation of secreted targets through host proteases or the formation of inclusion bodies [46]. The underlying causes are sub-optimal expression conditions e.g., temperature, pH, insufficient chaperone presence, nutrient deficiency. Resulting side effects include a decrease in bioactivity, solubility and stability. Lengthy cultivation periods necessary for a great number of eukaryotic expression systems can be circumvented by using prokaryotes, though they bring their own drawbacks. Problems arising from the use of prokaryotic organisms for protein expression include accumulation of possibly toxic compounds from the expression organism, as well as missing post-translational modifications often necessary for eukaryotic proteins [47]. Targeted genome engineering is a staple approach in current research. However, the precise integration of large fragments into the vector with e.g., CRISPR/Cas9-mediated genome editing remains complex even with the benefits of yeast expression systems [48]. The later pose an alternative to bacterial hosts, as they have the potential for higher yields of bioactive, correctly folded and soluble recombinant proteins via the additional overexpression of chaperones and other folding-related support molecules [49], [50].

The enhanced expression of non-recombinant proteins in non-GMO fungal strains is possible via multiple approaches, such as the optimization of cultivation media composition. Basic cultivation parameters, e.g., temperature, pH, cultivation period and aeration can influence expression levels. Further, the use of lignocellulosic biomass as substrate may lead to upregulated lignocellulolytic enzyme production in ascomycetes e.g., *T. reesei* [51], *Aspergillus terreus* and *Neurospora crassa* [52], as well as white-rot basidiomycetes e. g. *Phanerochaete chrysosporium*, which possess a broad spectrum of hydrolytic enzymes utilized in wood decay [53]. Further, the use of thermophilic fungi [54], [55] enables the production of hydrolases, which are highly active at low temperatures with possible applications in washing detergents and reducing productions costs in food and feed industry applications, such as the juice industry [56]. Another strategy is the enhancement of hydrolase enzyme systems from thoroughly researched strain, such as *T. reesei* with the supplementation of new highly active enzyme systems [57]. In the former the synergistic effect of thermophilic strains e.g., *Chaetomium thermophilum*, *Thielavia terrestris* or mesophilic strain *Penicillum funiculosum* with commercially available *T. reesei* (Celluclast) and *A. niger* (*Novozym* 188) enzymes is critical.

In the present study, highly different enzyme expression profiles and secretion concentrations was found for 7 different *C. paradoxa* isolates, as well as in comparison to the *A. niger* ATCC 10535 strain. Quantitative analysis of *C. paradoxa* DSM 63054 yielded an overall higher activity on both YPD and TM in comparison to CBS 374.83. For DSM 63054 the highest enzyme activities were 1586 $mUmL^{-1}$ amylase, 1395 $mUmL^{-1}$ galactanase, 1236 $mUmL^{-1}$ cellulase, 832 $mUmL^{-1}$ mannanase, 561 $mUmL^{-1}$ xylanase and 75 $mUmL^{-1}$ rhamnogalacturonase. For CBS 374.83 the highest enzyme activities were 1254 $mUmL^{-1}$ amylase, 795 $mUmL^{-1}$ cellulase, 309 $mUmL^{-1}$ mannanase, 118 $mUmL^{-1}$ xylanase, 278 $mUmL^{-1}$ galactanase, 24 $mUmL^{-1}$ rhamnogalacturonase. For CBS 374.83, DSM 63054 and ATCC cultivated in PM and LB, DSM 63054 showed the highest overall activity. For DSM the highest activity measured was cellulase at 1983 $mUmL^{-1}$, followed by 1730 $mUmL^{-1}$ galactanase, 1298 $mUmL^{-1}$ mannanase, 1570 $mUmL^{-1}$ amylase and 104 $mUmL^{-1}$ rhamnogalacturonase. For CBS 374.83 the highest activity was amylase, measured at 1457 $mUmL^{-1}$. For ATCC 10535 the highest activity was amylase, measured at 285

mUmL$^{-1}$, followed by 251 mUmL$^{-1}$ xylanase, 159 mUmL$^{-1}$ cellulase, 79 mUmL$^{-1}$ rhamnogalacturonase and 36 mUmL$^{-1}$ protease activity.

Directly comparing enzyme activities obtained from one cultivation to another or utilizing different quantification methods within the same batch is not easily feasible, as it leads to mostly incomparable results. However, by using proteomic analysis of the secreted fermentation broth it would be possible to provide a qualitative assessment of the present enzymes, as well as comparative expression levels for all tested fungal samples. With a comparative genomic analysis, it would be further possible to determine the evolutionary relationship between the tested isolates and a basis for biosynthetic gene cluster analysis.

Research following this publication is being conducted on the cultivation of two *C. paradoxa* leading isolates on WB and other sustainable sources for a one-pot approach in enzyme mixture and sugar-rich hydrolysate production, as well as whole genome sequencing and proteomic analysis under selected conditions.

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