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1 To: International Biodeterioration & Biodegradation

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4 **Cellulolytic and xylanolytic activities of common indoor fungi**

5

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15 **Keywords:** AZCL enzyme assay, endo-enzymes, wheat bran/sphagnum peat medium

16

17

18 **ABSTRACT**

19 Mouldy building materials, such as chip wood and gypsum, should be a good source for fungal strains with
20 high production of lignocellulolytic enzymes. Screening of 21 common indoor fungal strains showed,
21 contrary to the expected, that the *Chaetomium* and *Stachybotrys* strains had little or no cellulolytic and
22 xylanolytic activities using AZCL-assays. On the other hand, both *Cladosporium sphaerospermum* and
23 *Penicillium chrysogenum* showed the highest cellulase, β -glucosidase, mannase, β -galactanase and
24 arabinanase activities and would be good candidates for over-producers of enzymes needed to supplement
25 or boost the bioconversion of lignocellulose-rich biomass.

26 **1. Introduction**

27 Filamentous fungi are among the most efficient degraders of plant biomass, whether it is undesired, as in
28 the deterioration of Army Cotton Canvas, or by design, as in the bioconversion of organic waste material.
29 They are, therefore, the main source of commercial lignocellulase production (Glass et al., 2013) and high
30 yielding fungal strains are always in demand in the biotech industry (Pedersen et al., 2009; Hansen et al.,
31 2015). The most commonly used organism for commercial enzyme production is *Trichoderma reesei* (Glass
32 et al., 2013), but it has its limitations. *Trichoderma reesei* produces a high amount of exo-cellulases, but is a
33 poorer producer of e.g. β -glucosidase (Okeke, 2014).

34
35 Research and screening of filamentous fungi for new high-yielding strains have shown that the original
36 habitat of a strain is important in order to get as specific an enzyme profile as possible. Most of the
37 commercial enzyme producers originate from habitats and substrates rich in lignocellulose, such as
38 compost or agricultural soil (Hansen et al., 2015). Pedersen et al. (2008) showed that *Ulocladium* strains
39 originating from cereal grain (starch) had the highest production of amylase compared to strains from the
40 indoor environment (lignocellulose), which in return had the highest production of arabinanase.

41
42 Incidences of fungal deterioration of indoor environments are increasing and can now be seen in most of
43 the western world (WHO, 2010). It is most obvious on lignocellulose rich materials, such as chip wood or
44 plywood, where the fungal growth can be substantial. It has been shown that there are particular fungal
45 associations between 1) *Cladosporium sphaerospermum* and plywood 2) *Stachybotrys chartarum* and
46 gypsum board and 3) *Ulocladium alternariae* and wallpaper (Andersen et al., 2011). Mouldy building
47 materials should therefore be a good source for discovery of fungal strains that either produce novel
48 enzyme profiles or over-produce known desirable enzymes.

49

50 The purpose with this study was to screen and compare the ten most common fungal species from water-
51 damaged buildings for their production of lignocellulases using *T. reesei* RUT C30 as a reference strain.

52

53 **2. Materials and Methods**

54 2.1. Fungal strains, media, growth conditions and enzyme extraction

55 Twenty-one common indoor strains and one reference strain were used in the study. The identity, source
56 and geographic origin of all fungi are listed in Table 1. All fungal strains are held at the IBT culture collection
57 at Department for Systems Biology, DTU, Denmark. To generate inoculum and check the identity, each
58 strain was inoculated on Campbell's V8 juice agar (V8, Samson et al., 2010) and incubated for 7 days at 25
59 °C. For enzyme production a semi-solid wheat bran/sphagnum peat (WB/SP) medium was made containing
60 (per kg): 256.25 g wheat bran (Finax, Denmark), 153.75 g sphagnum peat (Mosebrug, Denmark) and 590 g
61 water. 40.0 g of WB/SP medium was placed in each 250 ml shake flask and autoclaved. Each fungal strain
62 was inoculated by cutting 3 agar plugs (10 mm in diameter) with spores and mycelium from the V8 plate
63 and transferring these to the shake flask with the WB/SP medium. The shake flasks were incubated at 25 °C
64 and shaken twice a day manually for one week. The experiment was performed in triplicates (66 shake
65 flasks in total).

66

67 Each shake flask was added 50 ml double distilled autoclaved water and shaken at 175 rpm overnight at 4
68 °C. Each extract was filtered through Miracloth into a 50 ml falcon tube and centrifuged at 10,000 g at 5 °C
69 for 15 min. The supernatant (enzyme extract) was transferred to a clean 50 ml falcon tube and stored at
70 4°C prior to screening.

71

72 2.2. AZCL assay preparation and screening

73

74 Six different Azurine cross-linked (AZCL) substrates were used for screening: arabinan, arabinoxylan (wheat
75 and birchwood), HE-cellulose, galactan and β -galactomannan (Megazyme, Bray, Ireland). For each AZCL
76 assay plates were made containing (per 500 ml): 144 ml stock solution, 356 ml double distilled water, 7.5 g
77 agarose (Litex, HSB 200 Protein grade) and 0.5 g AZCL substrate. The stock solution consisted of phosphoric
78 acid (0.08 M) (Merck, Ortho-Phosphorsäure, 85 %), glacial acetic acid (0.08 M) (Merck, 100 %) and boric
79 acid (0.08 M) (Merck) in double distilled water. To prepare the different AZCL assay plates, 200 ml double
80 distilled water was added to the 144 ml stock solution. The pH was adjusted to 6 and double distilled water
81 was added again to give a total volume of 500 ml. Agarose was added and the solution was autoclaved at
82 120 °C. The AZCL substrate was pre-soaked in 96 % ethanol for 10 min before use. When the agarose
83 solution had cooled to approximately 65 °C, the AZCL substrate suspension was added while stirring. The
84 agarose solution was poured into Petri dishes (90 mm in diam.) and when solidified, 8 wells (5 mm in diam.)
85 were cut in the plates and stored at 4 °C.

86

87 For screening 35 μ l of enzyme extract was added to each well of the six different AZCL assay plates. The
88 plates were incubated for 24 h at 30 °C. The activity of each enzyme was measured as the radius of the
89 zone of released azurine dye (the blue halo) around each application well. The radius was recorded and
90 converted to area (mm²).

91

92 2.3. β -glucosidase assay and screening

93 Screening for β -glucosidase activity was done using para-nitrophenyl- β -D-glucopyranoside (pNPG) 5 mM
94 (Sigma Aldrich) as substrate in 50 mM sodium citrate (pH 4.8). The screening was carried out in a
95 microtiter-plate format according to (Sørensen et al., 2011). A 10 μ l volume of enzyme extract was added
96 to 100 μ l substrate in 1.5 ml Eppendorf tubes and incubated in a Thermomixer® comfort (Eppendorf) at 50
97 °C for 15 min. At the end of the reaction 60 μ l of the reaction volume was transferred to a microtiter plate
98 already containing 100 μ l 1 M Na₂CO₃ for termination of the reaction. Absorbance at 400 nm was measured

99 in a plate reader (BioTek, EL800). Para-nitrophenol was used for preparation of a standard curve. One unit
100 (U) of enzyme activity was defined as the volume of enzyme needed to hydrolyze 1 μ mol of pNPG in 1 min.
101 Background subtraction was prepared for each sample with 100 μ l substrate at reaction temperature.
102 Hereafter, 100 μ l stop reagent was added to the Eppendorf tube then 10 μ l enzyme for reaction time 15
103 min. 160 μ l of the reaction mixture was then transferred to the microtiter plate and the absorbance was
104 measured at 400 nm.

105

106 3. Results

107 The AZCL enzyme screenings of the 21 indoor strains were made from crude enzyme extracts from 7 day-
108 old wheat bran/sphagnum peat (WB/SP) medium in solid state fermentations. The analyses showed that
109 both *Chaetomium elatum* and *C. globosum* had low or no cellulase, mannase or galactanase activities (Fig.
110 1) with the exception of *C. globosum* (IBT 7029) that had an average mannase activity, compared to the
111 highest activities (Fig. 1b). None of the strains of *Stachybotrys chartarum* or *S. chlorohalonata* showed any
112 cellulase, mannase or β -galactanase activity (Fig. 1). *Penicillium chrysogenum* showed the highest cellulase
113 and mannase activities (Fig. 1a and b), while *Cladosporium sphaerospermum* showed the highest
114 galactanase activity (Fig. 1c). *Trichoderma*, including the reference strain (*T. reesei* RUT C30), had in general
115 good cellulase and mannase activities, but very low galactanase activity (Fig. 1).

116

117 The β -glucosidase screening again showed that all the *Chaetomium* and *Stachybotrys* strains had a very low
118 activity (0.008 - 0.154 U/ml) compared to the reference strain (*T. reesei* RUT C30) that had an activity of
119 0.513 U/ml (Fig. 2a). Compared to the high activities (7.823 - 7.653 U/ml) shown by *P. chrysogenum* and *C.*
120 *sphaerospermum*, both *T. harzianum* and *T. reesei* (RUT C30) showed low activities (Fig. 2b).

121

122 The screening for xylanase production was done on xylan from both wheat and birch and gave similar
123 results (Fig 3a and b). It showed all ten *Chaetomium* and *Stachybotrys* strains as the low producers, while
124 *Aspergillus versicolor* and *T. harzianum* were the high producer for both types of xylanases (Fig. 3a and b).

125

126 The arabinanase screening, however, showed that neither *T. harzianum* nor *A. versicolor* had any activity,
127 while *P. chrysogenum* had the highest (Fig. 3c). Some of the *Chaetomium* and *Stachybotrys* strains had
128 average activities (e.g. *C. globosum* (IBT 7029) and *S. chlorohalonata* (IBT 40285)), while others showed no
129 arabinanase activity after growth on the WB/SP medium.

130

131 A Principal Component Analysis in Figure 4 of all the enzyme screenings shows that *Chaetomium* and
132 *Stachybotrys* (to the left) had similar enzyme profiles, with a general low activity of all tested enzymes.
133 *Aspergillus* and *Trichoderma* (at the bottom) also had similar profiles, both showing high xylanase activities,
134 average cellulase, β -glucosidase and mannanase activities and low or no β -galactanase and arabinanase
135 activities. *Penicillium*, *Cladosporium* and to some extent *Ulocladium* (at the top) were similar in having
136 average to high activities of all enzymes tested.

137

138 Three strains, *C. globosum* (IBT 7029), *U. alternariae* (IBT 9058) and *T. harzianum* (41332) fell outside their
139 respective groups. *Chaetomium globosum* (IBT 7029) had, overall, a higher activity than the other
140 *Chaetomium* strains, while *U. alternariae* (IBT 9058) and *T. harzianum* (41332) had a lower activity
141 compared to their sibling strains.

142

143 **4. Discussion**

144 Wheat bran/sphagnum peat (WB/SP) medium was used since it has been shown to be superior in inducing
145 a broad variety of enzymes (Kolasa M. et al., 2014; Meijer M. et al., 2011). WB was also the medium of
146 choice in the study of Pedersen et al., (2009), where 50 *Ulocladium* strains were screened for enzyme

147 activity using the AZCL substrates. An attempt to grow the indoor fungi on a similarly composed medium
148 with crushed chipboard/wallpaper instead of wheat bran/sphagnum peat resulted in no growth, probably
149 because the fungi needed a higher water activity to grow on this medium than WB/SP.

150

151 The study of Pedersen et al. (2009) also showed that there was variation between strains of the same
152 species. This was also seen in this study, for example, with the three *Penicillium chrysogenum* strains that
153 showed similar enzyme profiles in most assays, except for mannose, where *P. chrysogenum* (IBT 30128) had
154 no activity, while the other two strains had the highest activity.

155

156 The cellulolytic and xylanolytic activities of *Chaetomium* and *Stachybotrys* have been reported since the
157 1920s, where the fungi destroyed military equipment and other outdoor cotton fabrics (Greathouse and
158 Ames, 1945; Domsch et al., 2007). The results in this study show, contrary to the expected, that the
159 *Chaetomium* and *Stachybotrys* strains originating from water-damaged building materials showed little or
160 no cellulolytic and xylanolytic activities using AZCL-assays. Even *C. globosum* (IBT 7029 = CBS 148.51 = USDA
161 1042.4), which has been used for material testing (Reese et al., 1950), showed only average activities in the
162 AZCL assays. The fact that these fungi still are able to grow and destroy cellulose-rich building materials,
163 suggests that they have only exo-enzymes and/or membrane bound endo-enzymes, since the AZCL assays
164 screen for extracellular endo-enzymes (Vidal-Melgosa et al., 2015). Also the low β -glucosidase activity
165 suggests that these enzymes are membrane bound or intracellular. Another reason could be that
166 *Chaetomium* and *Stachybotrys* can utilize other carbon sources than cellulose in the building materials.
167 Similar results were seen for *Trichoderma*. The low β -glucosidase activity for *T. reesei* in this study is in
168 accordance with other studies (Okeke, 2014) and it has been suggested that this enzyme is membrane
169 bound or intracellular (Kubicek et al., 2009).

170

171 The other indoor fungi have larger varieties and higher activities of endo-enzymes compared to
172 *Chaetomium* and *Stachybotrys*, which might explain their higher frequency on and lesser specificity for
173 water-damaged building materials (Andersen et al., 2011). *P. chrysogenum* showed the highest activities for
174 most of the screened enzymes, which might explain its occurrence on most damp indoor surfaces. *C.*
175 *sphaerospermum* showed a similar result to that of *P. chrysogenum*, however, this fungus is more
176 specialized and also associated with plaster and grouts in bathrooms, due to its ability also to tolerate high
177 fluctuations in humidity (McGinnis, 2007).

178 The hypothesis of this study, that mouldy buildings constitute a good source for high cellulase and xylanase
179 producers, was partly proven, but not with the fungal species expected. However, both *C. sphaerospermum*
180 and *P. chrysogenum* would be good candidates for over-producers of enzymes needed to supplement or
181 boost e.g. *T. reesei* in bioconversion of e.g. garden/park waste or other lignocellulosic biomass into bio-fuel.

182

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187

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224

225 Figure legends

226

227 **Fig.1.** Comparisons of enzyme activity for all 22 fungal strains for A: AZCL-HE-cellulose, B: AZCL- β -
228 galactomannan and C: AZCL-galactan.

229

230 **Fig.2.** Comparisons of enzyme activity for all 22 fungal strains for β -D-glucopyranoside. A: *Chaetomium* and
231 *Stachybotrys* strains compared with *T. reesei* (gray). B: *T. reesei* (gray) compared with *Trichoderma*,
232 *Ulocladium*, *Cladosporium*, *Aspergillus* and *Penicillium* strains.

233

234 **Fig.3.** Comparisons of enzyme activity for all 22 fungal strains for A: AZCL-arabinoxylan (wheat), B: AZCL-
235 arabinoxylan (birchwood) and C: AZCL-arabinan.

236

237 **Fig. 4.** Principal Component Analysis of all 7 enzyme activities and all 22 fungal strains. Arbitrary scales.

Figure

Figure 1

Figure 2

Figure 3

Figure 4

Table 1. Fungal strains used in this study with identification numbers and origin.

Genus	Species	IBT no.	Other no.	Origin
<i>Aspergillus</i>	<i>versicolor</i>	21890	KD 252-2	Indoor, USA
<i>Aspergillus</i>	<i>versicolor</i>	28028	NRRL 3499, SRRC 108	-, NL
<i>Chaetomium</i>	<i>elatum</i>	41944	BA Home A	Dust on curtain rail, DK
<i>Chaetomium</i>	<i>elatum</i>	42179	BA Sample 3009	Cardboard page from photo album, DK
<i>Chaetomium</i>	<i>globosum</i>	7029	CBS 148.51, USDA 1042.4	Stored cotton, USA
<i>Chaetomium</i>	<i>globosum</i>	42177	Krydsfiner X-a	Plywood, DK
<i>Cladosporium</i>	<i>sphaerospermum</i>	41877	BAV-KD-C1	Indoor air sample, DK
<i>Cladosporium</i>	<i>sphaerospermum</i>	41982	B221/914c	Pipe insulation, DK
<i>Penicillium</i>	<i>chrysogenum</i>	5304	LH 107	Indoor air sample, DK
<i>Penicillium</i>	<i>chrysogenum</i>	30128	DTO 78-E5	Indoor air sample, DK
<i>Penicillium</i>	<i>chrysogenum</i>	31451	GR11BA 10b-1-1b	Dust from vacuum cleaner, GL
<i>Stachybotrys</i>	<i>chartarum</i> (A)	9290	XX	Plaster wall, DK
<i>Stachybotrys</i>	<i>chartarum</i> (A)	14915	ALK 57	Gypsum board, DK
<i>Stachybotrys</i>	<i>chartarum</i> (M)	7711	Dyrup-J	Wood, DK
<i>Stachybotrys</i>	<i>chartarum</i> (M)	40293	201	Indoor, USA
<i>Stachybotrys</i>	<i>chlorohalonata</i>	40285	204	Indoor, USA
<i>Stachybotrys</i>	<i>chlorohalonata</i>	40292	103	Indoor, USA
<i>Trichoderma</i>	<i>harzianum</i>	40876	TMW 4.1880	-, -
<i>Trichoderma</i>	<i>harzianum</i>	41332	16534-a	Indoor air sample, DK
<i>Trichoderma</i>	<i>reesei</i>	-	RUT C30	-, -
<i>Ulocladium</i>	<i>alternariae</i>	9058	ALK 124	Indoor air sample, DK
<i>Ulocladium</i>	<i>alternariae</i>	41862	BA 1886	Wallpaper, DK