Mycoscience (2012) 53:45–48
DOI 10.1007/s10267-011-0132-5

NOTE

Primary simple assays of cellulose-degrading fungi

Tsuneo Watanabe · Manabu Kanno · Masahiro Tagawa · Hideyuki Tamaki · Yoichi Kamagata

Received: 24 February 2011 / Accepted: 8 June 2011 / Published online: 25 June 2011

Abstract Some 25 fungi, including at least 14 basidiomycetes, one ascomycete, and five anamorphic fungi were evaluated for their cellulose-degrading abilities in Difco potato dextrose broth or Difco malt extract broth cultures with cellulosic substrates (e.g., filter paper) in plastic Petri dishes. Among them, *Peniophora* sp. 06-13 and *Phlebia* sp. 99-335 reduced the dry weights of the whole cultures with these substrates more than the dry weights of the respective original substrates after 30 days of culture, showing definite cellulose degradation. In the cultures with more than 10 test fungi including *Pycnoporus coccineus* 84-117, such weight losses did not occur. This assay technique for the primary screening for cellulose degrading fungi is simple, inexpensive, reproducible and accurate.

Keywords Absorbent cotton · Cellulose powder · Filter paper · *Peniophora* sp. · *Phlebia* sp.

The use of biofuels in place of fossil fuels has been studied as way to solve global warming (global increase in both atmospheric CO_2 and temperature) and pollution problems practically. To produce biofuels, cellulose must be degraded, so fungi that can degrade cellulose must be found. The work reported in this paper was primarily aimed at assaying fungi with such degrading abilities simply, inexpensively, and accurately.

T. Watanabe (✉) · M. Kanno · M. Tagawa · H. Tamaki · Y. Kamagata
Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
e-mail: tsuneo-watanabe@aist.go.jp

Cellulose degradation has already been assayed on the basis of the weight loss due to the removal of substrates from cultures, loss of enzyme activity, or the amount of breakdown products present (Dashtban et al. 2010; Denison and Koehn 1977; Geng and Li 2002; Sutherland 1984). However, some of the degraded material is often lost during the experimental procedures or treatments employed in such studies. In this work, on the other hand, the degradation was evaluated directly in the whole culture, which was kept in a plastic Petri dishes. In preliminary repeated studies, plastic Petri dishes were found to be stable in terms of shape and weight under continuous drying at 55°C for more than 10 days.

Among various fungi collected during the studies on dioxin- or lignocellulose decomposers (Sato et al. 2002; Watanabe et al. 2003), one of the authors (T.W.) tested several potential cellulose-degrading fungi in the preliminary assays in series, and *Peniophora* sp. 06-13 and *Phlebia* sp. 99-335 were found to be the most powerful cellulose-degrading fungi.

Some 25 fungi were tested in this study as candidates for cellulose-degrading fungi, including at least 14 basidiomycetes, one ascomycete, and five anamorphic fungi, and these were mostly isolated from fruit bodies, rice husks and soil that had recently been collected at various places in Tsukuba in Ibaraki, and Iwaki in Fukushima prefecture, Japan. These fungi were stocked at the National Institute of Advanced Industrial Science and Technology (AIST), Ministry of Economy, Trade and Industry, Tsukuba, Ibaraki, Japan with TW stock numbers. Some of them were also deposited at NIAS Genebank, National Institute of Agrobiological Sciences, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan (with MAFF numbers): *Coprinopsis cinerea* (Schaeff.: Fr) Redhead, Vilgalys & Moncalvo 06-150 (=MAFF240343) (Watanabe et al. 2011), *Cyathus*

stercoreus (Schw.) de Toni 08-10, *Flamullina velutipes* (Fr.) Karst 09-16, *Gliocephalotrichum simplex* (J. Meyer) B. J. Wiley & E. G. Simmons 06-9 (= MAFF240319), *Lentinula edodes* (Berk.) Pegler 09-11, *Morchella conica* Pers. 08-11, *Peniophora* sp. 06-13, *Phlebia* sp. 99-335, *Pycnoporus coccineus* (Fr.) Bond. & Sing. 84-117, *Pyrenochaeta* sp. 10-3, *Sporidesmium* sp. 10-1, and six basidiomycetous or three anamorphic fungi from unknown basidiocarps, including the isolate 06-110 (=MAFF240344). Some of these were illustrated or recorded by Watanabe (2010).

Filter paper [Whatman no. 3, one sheet (70 mm diameter, ca. 0.68 g)/plate] (Fig. 1), medical absorbent cotton (Cut Cotton, one piece: 3 × 3 mm, Iwatsuki, Tokyo, two (ca. 0.36 g) or four pieces/plate, and processed cellulose powder (fibrous, Sigma Chemical Co., Milwaukee, WI, USA, ca. 0.2 or 1 g/plate) were used as cellulosic substrates, based on previous works (Deacon 1985; Denison and Koehn 1977; Garrett 1962; Singh and Kunene 1980; Sutherland 1984; Sutherland et al. 1983; Wabnegg et al. 1980). The respective samples were always weighed accurately.

The test fungi were cultured in 10 ml Difco potato dextrose broth (PDB) or Difco malt extract broth (MEB) in tubes for 5 days at 25°C with 4 mm agar culture discs removed from the periphery of 3–7-day-old Difco potato-dextrose agar (PDA) or Difco malt extract agar cultures (MEA). PDB- or MEB-bearing 4 mm agar discs that were separated from noninoculated PDA or MEA served as controls. The pHs of PDB and MEB were 6.2 and 5.5–5.8, respectively, without the adjustment.

Plastic Petri dishes (9 cm in diameter) and cellulosic substrates were prepared aseptically by drying at 55°C for at least 2 days until a constant weight was attained before the start of culture. The substrates were always checked to ensure they were free from contamination before the start of the experiment. Plates bearing the respective substrates were inoculated by transferring the whole test tube cultures of the respective fungi. The inoculated plates were placed in plastic cases (32 × 25 × 10 cm) on the open shelf, and kept moist in the dark under aerobic conditions. The test fungi were incubated at 25°C, the mostly favorable temperature for the growth of any fungus. After incubating for 5, 10, 15, 20 or 30 days, the whole plate cultures were dried for at least for 2 days at 55°C with their lids open until a constant weight was obtained. The dried PDB cultures are shown in Fig. 1, and Table 1 shows one assay as an example after 30 days of culture. The dry weight of each plate included the plastic Petri dish, the broth culture, and the metabolites produced during culture. The cultured weight of each plate was obtained by subtracting the weight of the dish from the total weight.

Considering their dry weights, filter papers in some of the fungal PDB or MEB cultures degraded over time. For example, the dry weights of *Phlebia* sp. 99-335 and *Peniophora* sp. 06-13 in PDB with filter paper cultures approached the dry weight of the filter paper (ca. 0.68 g) after 15 days of culture, as the dry weight dropped by 10.0–12.2% after 5 days, 11.5–14.9% after 10 days, and by up to 17.2% after 15 days of culture as compared with the control. On the other hand, the *P. coccineus* 84-117 culture showed decreases in dry weight of 1.1, 2.3, and 6.9% after 5, 10, and 15 days of culture, respectively (Fig. 2). After 30 days of culture, the dry weight of *Peniophora* sp. 06-13 was found to be the lowest of the four fungi (0.56 g on average in F + PDB; the other three fungi had dry weights of 0.74–0.76 g). This represented a 31.7% reduction in the dry weight as compared with the control (0.82 g) (Table 1), and it was definitely lighter than the filter paper (0.68–0.69 g in F), clearly indicating cellulose degradation. Although other test fungi also showed reduced dry weights as compared with that of the control, these reductions were not large enough to unambiguously indicate cellulose degradation. Similar results were always obtained in PDB

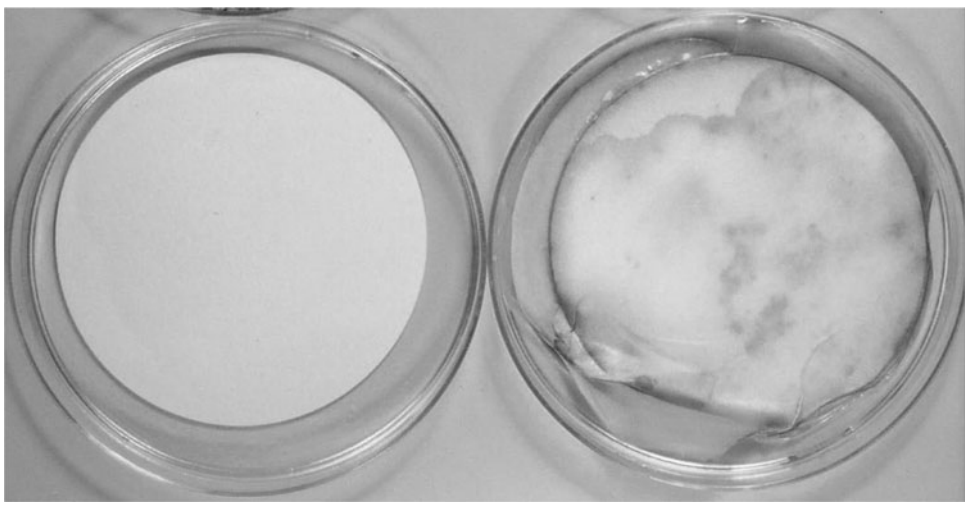

Fig. 1 Dried *Peniophora* sp. 06-13 (*right plate*) and uninoculated control (*left*) in Difco potato dextrose broth cultures with filter paper (70 mm diameter, one sheet/plate) after 30 days of culture. Photographed 3 days after drying at 55°C

Table 1 Dry weights [mean (g) ($n = 10$) ± SD (standard deviation)] of Difco potato dextrose broth (PDB) cultures with filter paper (F) in plastic Petri dishes (D) of four fungi (*Coprinopsis cinerea* 06-150, *Gliocephalatrichum simplex* 06-9, *Peniophora* sp. 06-13, and an unknown basidiomycete 06-110) and an uninoculated control after 30 days of culture at 25°C

Treatment	Before culture			After 30 days of culture	
	D	D + F	F	D + F + PDB	F + PDB
Control	16.81 ± 0.14	17.50 ± 0.13	0.68 ± 0.01	17.63 ± 0.12	0.82 ± 0.06 C
Cop 06-150	16.81 ± 0.13	17.49 ± 0.13	0.68 ± 0.01	17.56 ± 0.13	0.75 ± 0.02 B
Gli 06-9	16.82 ± 0.13	17.51 ± 0.13	0.69 ± 0.01	17.58 ± 0.13	0.76 ± 0.01 B
Pen 06-13	16.81 ± 0.13	17.50 ± 0.13	0.68 ± 0.01	17.38 ± 0.13	0.56 ± 0.03 A
Unb 06-110	16.81 ± 0.14	17.49 ± 0.14	0.68 ± 0.01	17.55 ± 0.13	0.74 ± 0.02 B

Values with the same letter in the "F + PDB" column do not differ significantly ($P = 0.01$) according to the least significant difference test

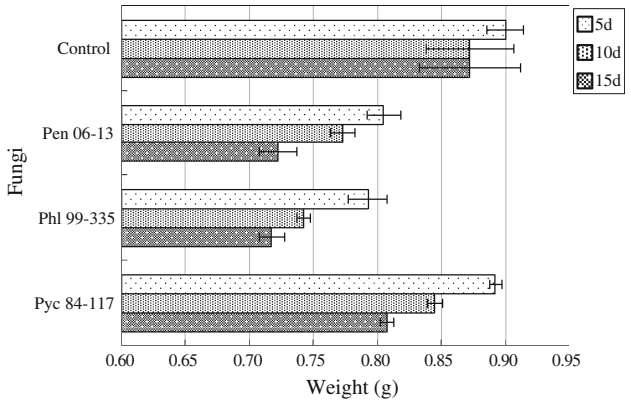

Fig. 2 Dry weights [mean (g) ($n = 4$) ± SD (standard deviation)] of three fungi (*Peniophora* sp. 06-13, *Phlebia* sp. 99-335, and *Pycnoporus coccineus* 84-117) and an uninoculated control in Difco potato dextrose broth (PDB) cultures with filter papers (one sheet, 70 mm diameter, ca. 0.68 g/plate) after 5, 10, and 15 days of culture at 25°C

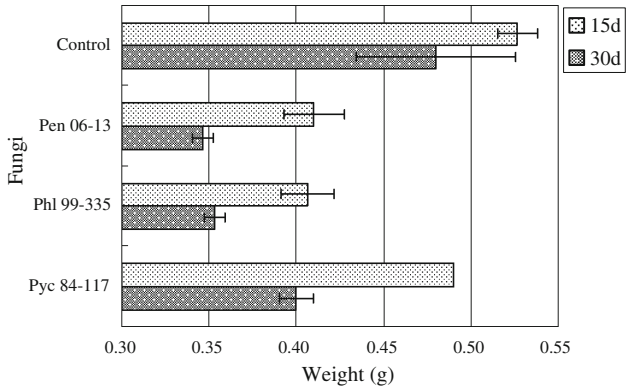

Fig. 3 Dry weights [mean (g) ($n = 3$) ± SD (standard deviation)] of three fungi (*Peniophora* sp.06-13, *Phlebia* sp. 99-335, and *Pycnoporus coccineus* 84-117) and an uninoculated control in Difco potato dextrose broth (PDB) cultures with absorbent cut cotton (two pieces, ca. 0.36 g/plate) after 15 and 30 days of culture at 25°C

or MEB cultures with filter paper after 30 days of culture for several fungi (data not shown). In another primary assay with eight freshly isolated candidate fungi from unknown basidiocarps, three efficient fungi (*Peniophora* sp. 06-13,*Phlebia* sp. 99-335,*P. coccineus* 84-117) showed significantly greater filter paper degrading ability in PDB culture than eight other test fungi. These three fungi were definitely cellulose-degrading fungi, as their dry weights were significantly less than the original filter paper weight (ca 0.68 g) after 30 days of culture (data not shown).

The test fungi in PDB or MEB cultures with absorbent cotton (two (ca. 0.36 g) or four cut pieces/plate) showed reductions in their dry weights over time, just like the cultures with filter paper. Both *Peniophora* sp. 06-13 and *Phlebia* sp. 99-335 in PDB cultures with cotton (e.g., two pieces, 0.36 g/plate) showed almost the same degrading activities, but their reductions were significantly greater than those of *P. coccineus* 84-117 and the control (Fig. 3) after 15 and 30 days of culture, and indicated clear degradation of the cellulosic substrate. However, for *P. coccineus* 84-117, it was unclear whether the cotton, PDB or both had been degraded.

Peniophora sp. 06-13 and *Phlebia* sp. 99-335 in PDB cultures with cellulose powder showed reductions in their dry weights to below the dry weight of the original cellulose powder (ca. 0.2 g)/plate) after 15 and 30 days of culture, indicating clear degradation of the cellulosic substrate. Although the dry weight of the culture with cellulose powder and *P. coccineus* 84-117 was close to 0.3 g after 15 days of culture, it was unclear whether the cellulose powder had degraded at this stage, but this was definitely clear when the dry weight reached a value of 0.2 g after 30 days of culture (Fig. 4). Similar results were always obtained in either PDB or MEB culture with cellulose powder (1 g/plate) after 30 days of culture for several fungi (data not shown).

The dry weights of the fungi in the broth cultures in plastic Petri dishes with cellulosic substrates were obtained after drying the whole plate culture at 55°C for at least 2 days. During incubations, the cultures dropped in weight

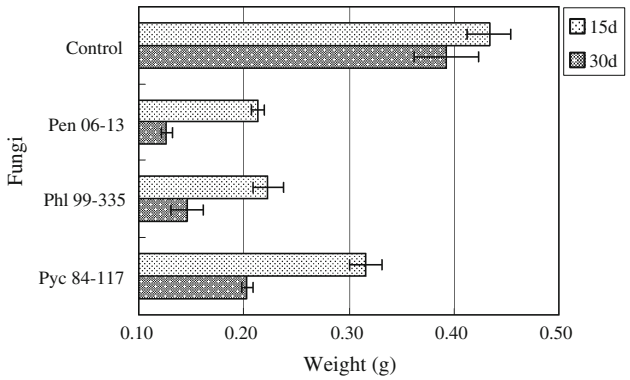

Fig. 4 Dry weights [mean (g) ($n = 3$) ± SD (standard deviation)] of three fungi (*Peniophora* sp.06-13, *Phlebia* sp. 99-335, and *Pycnoporus coccineus* 84-117) and an uninoculated control in Difco potato dextrose broth (PDB) cultures with cellulose powder (ca. 0.2 g/plate) after 15 and 30 days of culture at 25°C

because CO_2 and water were released from the cultures, as demonstrated using ^{14}C-labeled lignocellulosic substrates (Crawford et al. 1977; Sutherland et al. 1983). Using this technique, it is possible to assay the fungal degradation of the substrates, because the degradation of the products and substrates of the culture can be evaluated directly without removing them (and therefore potentially losing some of them) from the culture in plastic Petri dishes.

In the classical work by Garrett (1962) using filter papers (Whatman no. 3), *Rhizoctonia solani* Kühn was shown to reduce the mean dry weight of filter papers by 9.2–10.24% after 6 weeks of culture. Similarly, the reductions caused by four isolates of *Pyricularia oryzae* Cav. were 3–12% (Singh and Kunene 1980) after 7 weeks of incubation. In these works, samples removed from the flasks were dried and weighed. In the degradation of three types of cotton-gin trash (green bolls, burr trash and gin motes) by *Pycnoporus cinnabarinus* (Jacq.: Fr.) Karst, inoculated lignocellulose from green bolls lost 58.6% of its dry weight versus 15.6% for noninoculated control samples; inoculated lignocellulose from burr trash lost 38% of its dry weight versus 13.8% for controls, and inoculated lignocellulose from gin motes lost 23.8% of its dry weight versus 8.6% for controls (Sutherland 1984).

Among the many (at least 25) species assayed in this study, *Peniophora* sp. 06-13 and *Phlebia* sp. 99-335 were found to be the most powerful degraders, reducing the dry weights of PDB cultures with cellulosic substrates by up to 31.7%. These results are almost comparable with the wood-degrading abilities of *Coriolus hirsutus* (Wulf.: Fr.) Quél., *Panus rudis* Fr. and *Pycnoporus coccineus*, which reduced the weight of wooden blocks by >30% after 40 days of incubation (Tanesaka et al. 1993). *P. coccineus* 84-117 was also shown to reduce the weight of a pair of chopsticks by 25.7% after 50 days of culture (Watanabe et al. 2003). These assays required at least 40 days of incubation, but the cellulose degraders in our assays were confidently identified after less than 30 days of culture, because the dry weight of the inoculated medium and substrates were significantly less than that of the original substrate. The results of these assays indicate that it is possible to select the most efficient cellulose-degrading fungi from various fungal collections simply, inexpensively and readily.

References

Crawford DL, Crawford RL, Pometto Al III (1977) Preparation of specifically labelled ^{14}C-(lignin)- and ^{14}C-(cellulose)-lignocelluloses and their decomposition by the microflora of soil. Appl Environ Microbiol 33:1247–1251

Dashtban M, Maki M, Leung KT, Mao C, Qin W (2010) Cellulase activities in biomass conversion: measurement methods and comparison. Critical Rev Biotech 30:302–309

Deacon JW (1985) Decomposition of filter paper cellulose by thermophilic fungi acting singly, in combination, and in sequence. Trans Br Mycol Soc 85:663–669

Denison DA, Koehn RD (1977) Cellulase activity of *Poronia oedipus*. Mycologia 69:592–603

Garrett SD (1962) Decomposition of cellulose in soil by *Rhizoctonia solani* Kühn. Trans Br Mycol Soc 45:115–120

Geng X, Li K (2002) Degradation of non-phenolic lignin by the white-rot fungus *Pycnoporus cinnabarinus*. Appl Microbiol Biotech 60:342–346

Sato A, Watanabe T, Watanabe Y, Harazono K, Fukatsu T (2002) Screening for basidiomycetous fungi capable of degrading 2,7-dichlorodibenzo-*p*-dioxin. FEMS Microbiol Lett 213:213–217

Singh N, Kunene IS (1980) Cellulose decomposition by four isolates of *Pyricularia oryzae*. Mycologia 72:182–190

Sutherland JB (1984) Biodegradation of lignocelluloses from cotton-gin trash by *Pycnoporus cinnabarinus*. Mycologia 76:369–372

Sutherland JB, Pometto AL III, Crawford DL (1983) Lignocellulose degradation by *Fusarium* species. Can J Bot 61:1194–1198

Tanesaka E, Masuda H, Kinugawa K (1993) Wood degrading ability of basidiomycetes that are wood decomposers, litter decomposers, or mycorrhizal symbionts. Mycologia 85:347–354

Wabnegg F, Messner K, Röhr M (1980) A screening method for the estimation of filter paper activity. J Gen Microbiol 117:267–269

Watanabe T (2010) Pictorial atlas of soil and seed fungi, 3rd edn. CRC, Boca Raton

Watanabe T, Watanabe Y, Nakamura K (2003) Biodegradation of wood in dual cultures of selected two fungi determined by chopstick method. J Biosci Biotech 95:623–626

Watanabe T, Tagawa M, Tamaki H, Hanada S (2011) *Coprinopsis cinerea* from rice husks forming sclerotia in agar culture. Mycoscience 52:152–156