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METHODS AND PROTOCOLS

Development of a simple cultivation method for isolating hitherto-uncultured cellulase-producing microbes

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Abstract Although enrichment culture is typically employed to isolate cellulolytic microbes, this approach tends to favor fast-growing species and discriminates against all others. Therefore, efforts to prevent the overgrowth of fast-growing species are necessary to isolate novel cellulase-producing strains. In this study, we developed a simple culture method for isolating hitherto-uncultured microbes that possess cellulase activity, particularly exocellulase. In this method, the microbial source (a forest soil) was suspended in sterilized water and inoculated onto a mineral salts agar medium, which was then overlaid with filter paper to sandwich the microbial suspension between the agar surface and paper. The filter paper fibers served to immobilize the microbial cells and were the dominant carbon source. Following cultivation at 30°C for 2 weeks, emerging colonies were isolated based on their morphology and were then subjected to phylogenetic and enzyme analyses. Using this method, 2,150 CFUs/g dry soil were obtained, and the ratio of fungal to bacterial isolates was approximately 4:1. Phylogenetic analyses revealed that most fungal and bacterial isolates belong to ten and two genera, respectively. Notably, all isolates possessed exocellulase activity, and several strains showed strong activity that was comparable to *Trichoderma* cellulase. Many isolates also exhibited cellulase and xylanase activity, and several strains possessed laccase activity. It is expected that the culture method described here will be useful for the isolation of hitherto-uncultured cellulolytic microbes and the identification of novel cellulases.

Keywords Cellulolytic microbe · Hitherto-uncultured strain · Exocellulase · Endocellulase · Xylanase · Laccase

Introduction

A number of cellulolytic microbes have been isolated from various environments and are known to contribute to aerobic and anaerobic cellulose degradation (Bhat and Bhat 1997). However, culture-independent metagemone studies suggested that a majority of microbes in nature has not been cultivated or isolated yet by microbiologists (Amann et al. 1995; Buée et al. 2009); many cellulolytic strains likely remain to be characterized. To fully elucidate their role in cellulose biodegradation on a global scale, it is necessary to isolate these uncultured cellulolytic microbes. In addition, the exploration of novel cellulolytic microbes is also valuable from a biotechnological viewpoint because cellulose is regarded as an abundant, renewable substrate for biofuel production (Mielenz 2001).

Cellulases can be classified into three families based on the type of reaction catalyzed: Endocellulases catalyze cleavage of amorphous regions in cellulose, exocellulases catalyze depolymerization of crystalline regions in cellulose, and β-glucosidases hydrolyzes cellobiose into glucose (Bhat and Bhat 1997). Since cellulosic biomass contains both crystalline and amorphous regions, its complete saccharification requires a suite of appropriate cellulases (Lynd et al. 2002). However, as the crystalline regions of cellulose are considerably more difficult to degrade than amorphous regions (Zhang and Lynd 2004), microbial strains which possess exocellulase as well as endocellulase

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activities are desirable for the efficient saccharification of cellulosic biomass.

To date, a number of methods to culture cellulolytic microbes have been reported. Although liquid enrichment and agar media using cellulose as a sole carbon source are typically employed to isolate cellulolytic microbes, these techniques tend to promote colonization by fast-growing species and discriminate against all others, which become difficult to isolate (Da Rocha et al. 2009; Joint et al. 2010). Therefore, approaches to prevent the overgrowth of fast-growing species are necessary for isolating and characterizing hitherto-uncultured microbes with cellulolytic activity.

In this study, we developed a simple culture method for isolating hitherto-uncultured microbes which possess cellulase activity, particularly excocellulase, and evaluated its usefulness for characterizing cellulolytic microbes in soil.

Materials and method

Chemicals

Cellulose (Avicel) was purchased from Merck (Darmstadt, Germany), and carboxymethyl cellulose and syringaldazine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Reagents for molecular biology, including *Taq* DNA polymerase, were purchased from Takara Bio (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemicals (Kyoto, Japan).

Preparation of culture medium

Isolation of microbes was performed using a mineral salts agar medium (pH 5.3) supplemented with either glucose or filter paper (overlaid on the agar) as the sole carbon source. Mineral salts agar contained 20 g/l Bacto agar, 5.0 g/l $(NH_4)_2SO_4$, 1.0 g/l KH_2PO_4, 500 mg/l $MgSO_4 \cdot 7H_2O$, 100 mg/l NaCl, 100 mg/l $CaCl_2 \cdot 2H_2O$, 0.5 mg/l H_3BO_3, 0.4 mg/l $MnSO_4 \cdot 5H_2O$, 0.4 mg/l $ZnSO_4 \cdot 7H_2O$, 0.2 mg/l $FeCl_3$, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg/l KI, 0.04 mg/l $CuSO_4 \cdot 5H_2O$, 2.0 mg/l inositol, 0.4 mg/l Ca–pantothenate, 0.4 mg/l niacin, 0.4 mg/l thiamine−HCl, 0.4 mg/l pyridoxine–HCl, 0.2 mg/l riboflavin, 0.2 mg/l *p*-aminobenzoic acid, 2 mg/l folate, and 2 mg/l biotin.

Collection of a soil sample

The soil sample used in this study was taken from a forest located in the Experimental Agriculture Station of Yamaguchi University, Japan. The forest is a thicket composed mainly of beech (*Fagus crenata*) trees. The sample was collected from A-horizon (0–3 cm approximately in depth from a soil surface) and was screened through a 2-mm sieve. Moisture, pH (H_2O), and pH (KCl) of the soil were 22.0 wt.%, 5.3, and 3.2, respectively. The organic carbon and nitrogen in the soil were 11.7 and 0.064 wt.% (in dry soil), respectively, which were determined by the Tyurin and Kjeldahl methods (Marumoto et al. 1978; Bremner and Mulvaney 1982).

Preparation of soil extracts

Six soil extracts, water (W extract), boiling water (B extract), autoclave (A extract), NaOH (N extract), ethanol (E extract), and hexane (H extract) were prepared as follows. W, N, E, and H extracts were prepared by shaking 30 g dried soil and 300 ml deionized water, 50 mM NaOH, ethanol, and hexane, respectively, at 200 rpm for 20 min at 25°C. B and A extracts were prepared by boiling or autoclaving (121°C) respectively a mixture of 30 g dried soil and 300 ml deionized water for 20 min. All of the prepared extracts were filtered through Whatman no. 1 filter paper. After filtration, N extract was neutralized to pH 7.0 by 4 N HCl. The six different types of soil extracts were used to supplement glucose agar at a final concentration of 1.0% (*v*/*v*).

Cultivation and isolation of soil microbes

The sieved soil sample (1.0 g) was suspended in 5 ml autoclaved deionized water, and was then voltexed vigorously for 1 min. The resultant soil suspension was serially diluted with water and used as an inoculum. Colony forming unit (CFU) counts of total soil microbes were performed by inoculating mineral salts agar containing 1% glucose (glucose agar) with the diluted soil suspension. For isolation of cellulolytic microbes, 1-ml diluted soil suspensions were inoculated onto mineral salts agar, which was then overlaid with a Whatman no. 5 filter paper (70-mm diameter) to sandwich the soil suspension between the agar surface and the filter paper. The agar plates (FP agar) were then incubated statically in the dark at 30°C for 2 weeks under aerobic condition. Emerging colonies on the filter paper were counted, and unique isolates, as determined by their morphology, were purified three times on fresh YM agar (10 g/l glucose, 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, and 20 g/l Bacto agar). For microaerophilic and anaerobic cultivation, AnaeroPak-Microaero™ and AnaeroPak-Anaero™ oxygen absorbers (Mitsubishi Gas Chemical, Tokyo, Japan), respectively, were used according to the manufacturer's instruction. Oxygen concentration in closed containers for the microaerophilic cultivation was at 8%, while that in containers for anaerobic cultivation was <0.1%.

For liquid enrichment, the same soil suspension was inoculated to a liquid mineral salts medium containing filter

paper as a carbon source (300 mg filter paper per 30 ml culture) and cultivated for 2 weeks at 30°C with shaking at 150 rpm. An aliquot of the 2-week-old culture was then inoculated on glucose agar and incubated for 1 week at 30°C to grow microbes on agar medium.

Phylogenetic study of isolates

Cell mycelia of fungal isolates were obtained from 20 ml pure cultures in YM broth (10 g/l glucose, 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone), while cell pellets of the bacterial isolates were harvested from 1 ml aliquots of pure cultures. DNA of the isolates was extracted using a Master Pure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) and was used as a PCR template. PCR was performed to amplify inter-transcribed spacer regions (approximately 500 bp, including ITS1, 5.8S, and ITS2 regions) of fungal ribosomal DNA with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as developed by White et al. (1990), while partial 16S ribosomal DNA (approximately 290 bp) of bacterial isolates was amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 380R (5′-ACTGCTGCCTCCCGTAGGAG-3′), as developed by Lane (1991). The PCR reactions consisted of 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final elongation step of 7 min at 72°C. PCR products were stored at −30°C until subjected to sequencing.

Direct sequencing of the amplified DNA fragments was carried out as described by Satomi et al. (1997), and similarities of the obtained sequences with known species were evaluated by a comparison with sequence data in the GenBank, EMBL, and DDBJ databases using the BLAST algorithm (Altschul et al. 1990). We also analyzed the data by the Ribosomal Database Project (http://rdp.cme.msu.edu/) and confirmed their accuracy. Phylogenetic trees were constructed using the neighbor-joining method contained within the Clustal W program (Saitou and Nei 1987; Thompson et al. 1994).

ITS region DNA or 16S rDNA partial sequences for the strains isolated in this study have been deposited in the GenBank database under accession numbers shown in Electronic supplementary material (ESM) 1.

Enzyme assay

The selected isolates were cultivated in 20 ml Mandels and Weber medium for 1 week at 30°C with shaking at 150 rpm (Mandels and Weber 1969). The culture supernatants (20 ml) of each isolate were separated from microbial biomass and insoluble materials by centrifugation at 3,000×*g* for 10 min and were then filtered through a Vivaspin-20 concentrator (GE Healthcare, Little Chalfont, UK). The residue on the filter membrane was suspended with 1 ml of 50 mM citrate buffer (pH 4.8) and used as an enzyme solution. Protein concentration in the enzyme solution was determined using the method of Bradford (1976).

Exocellulase, endocellulase, and xylanase activities were determined using Avicel, carboxymethyl cellulose (CMC), and birchwood xylan as substrates, respectively, by DNS method (Ghose 1987). Laccase activity was determined using syringaldazine as a substrate by the method of Leonowicz and Grywnowicz (1981).

Results

CFU counts of culturable soil microbes in forest soil

The number of culturable microbes in the forest soil sample when plated on glucose agar was determined to be 3.3×10^6 CFUs/g dry soil. To examine whether soil constituents affected the culturability of soil microbes, soil suspensions were inoculated onto glucose agar which was supplemented with six different types of soil extracts at final concentrations of 1.0% (*v*/*v*) and were cultivated under identical conditions. Despite the addition of soil extract to the culture medium, no significant differences in fungal and bacterial colony numbers were observed when compared with the unsupplemented medium by Student's *t* test, $p<0.05$ (data not shown).

Isolation of cellulolytic microbes from the soil sample

To culture and isolate cellulolytic microbes present in the forest soil sample, diluted soil suspensions were first sandwiched between the mineral salts agar surface and an autoclaved filter paper, which served as the dominant carbon source and immediately absorbed the soil suspensions. After a 2-week cultivation, microbial colonies with various morphologies emerged on the filter paper (Fig. 1), and the concentration of microbes was determined to be 2,150 CFUs/g dry soil. Based on microscopic observations, approximately 80% of total colonies were fungal and 20% were bacterial.

A total of 67 fungal and bacterial isolates (strains 1–67) were obtained based on colony morphology, among which 62 isolates could grow through repeated subculture, while five isolates (strains 7, 40, 54, 57, and 65) failed to grow upon subculturing for unknown reasons. Sixty representative cellulolytic isolates included 53 fungal and nine bacterial strains.

To compare the microbial profiles obtained using FP agar with those in liquid culture, we next cultivated the same soil suspension in liquid mineral salts medium

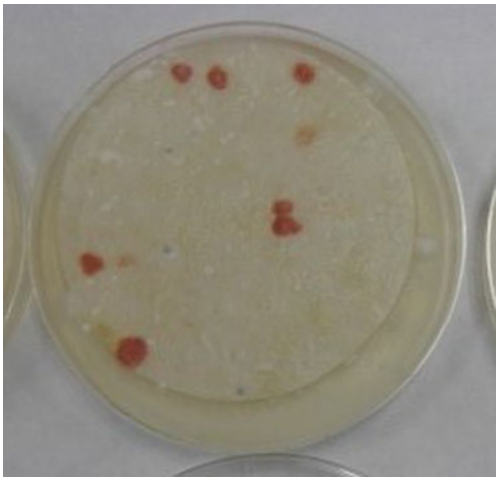


Fig. 1 Image of the microbes formed on the FP agar after a 2-week cultivation at 30°C

containing filter paper, and an aliquot of 2-week-old culture was then incubated on glucose agar. However, only fungal colonies with the identical morphology emerged on the agar medium. Three colonies were randomly isolated and designated as strains A, B, and C.

Phylogenetic analysis of cellulolytic isolates

Phylogenetic positions of the fungal and bacterial isolates were determined based on the ITS region sequence and 16S rDNA partial sequence, respectively. Figure 2a, b shows the phylogenetic trees for fungal and bacterial isolates, respectively, as constructed using the neighbor-joining method. Although nearly all fungal isolates were classified to ten genera, consisting of typical soil fungi, the sequence similarity of the ITS region between ten fungal isolates (strains 9, 11, 18, 19, 33, 34, 44, 50, 55, and 63) and their closest known neighbors was <97%.

On the other hand, all fungal isolates obtained from liquid enrichment culture (strains A, B, and C) were *Penicillium* spp. (Fig. 2a). For the bacterial isolates, they were classified into two known genera (*Burkholderia* and *Kitasatospora*), but the sequence similarity of 16S rDNA for two isolates (strains 25 and 47) and their closest neighbors was <97%.

Enzyme activity of the isolates

Twenty-eight isolates, including 21 fungal and 7 bacterial isolates, were selected as representative strains (indicated in bold letters in Fig. 2) based on their phylogenetic positions, to be examined for cellulase, xylanase, and laccase activities. Table 1 summarizes the enzyme activities in the culture supernatants of each examined isolate. Notably, exocellulase activity was detected in the culture supernatants of all tested strains, with several strains (strains 2, 5, 8, 9, 11, 28, and 42) exhibiting a relatively strong activity in comparison to the other isolates. A number of isolates also showed endocellulase and xylanase activities, and several strains possessed laccase activity. Potent endocellulase and xylanase producers were found mainly in *Penicillium* and *Trichoderma*, while the laccase activity of *Cunninghamella* and *Burkholderia* spp. was relatively strong among the tested strains.

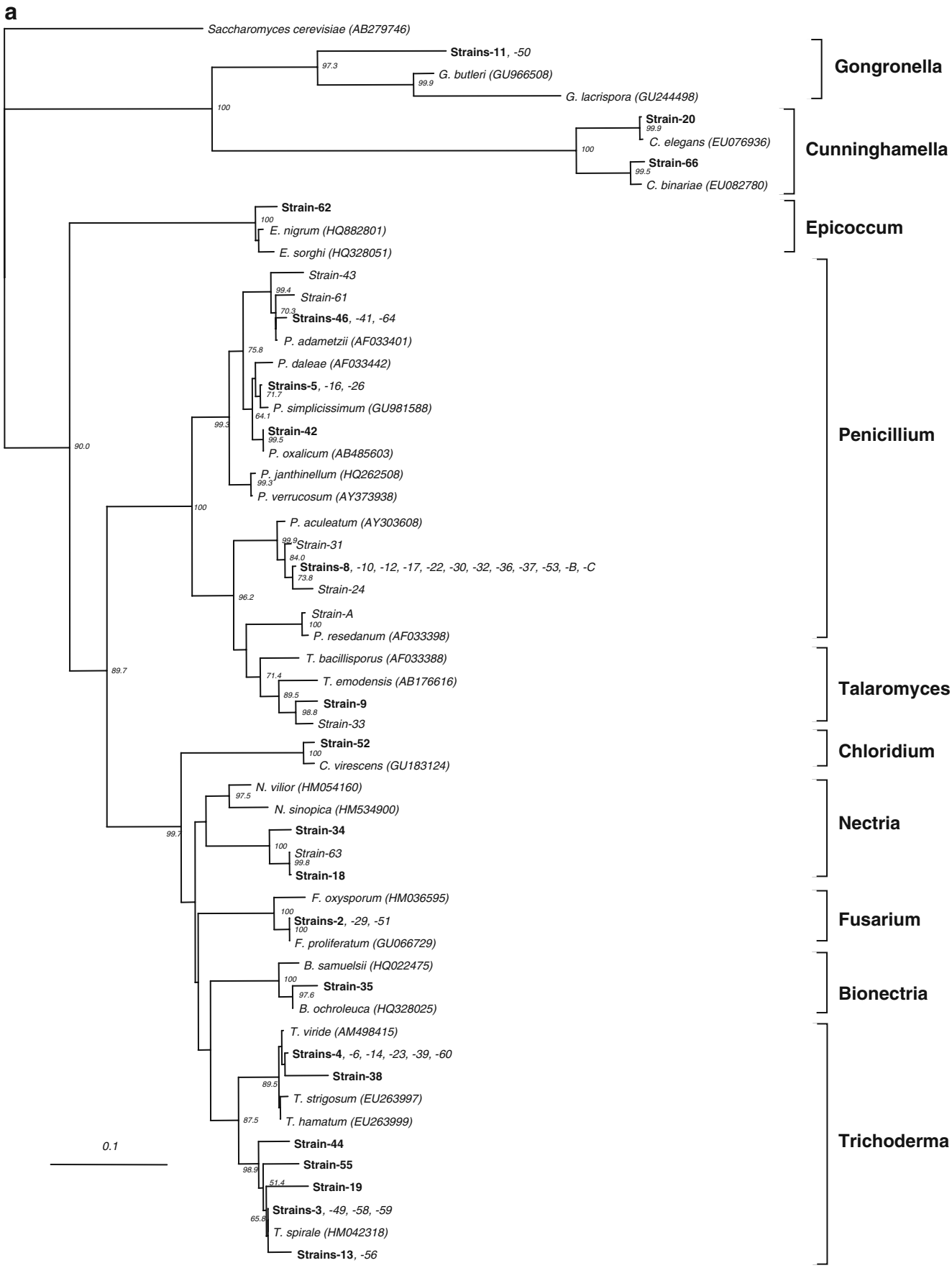
Fig. 2 Phylogenetic trees of cellulolytic isolates and related species constructed using the neighbor-joining method. Trees for fungal isolates based on ITS region sequences (**a**) and bacterial isolates based on partial 16S rDNA sequences (**b**) are shown. Accession numbers for the isolates are shown in ESM 1. *Scale bar* represents 0.1 base substitutions per nucleotide. Bootstrap values (Felsenstein 1985) above 50% (of 1,000 samplings) are shown at the internodes. The strains indicated in *bold letters* were assayed for cellulase, xylanase, and laccase activities

Isolation of cellulolytic microbes under microaerobic or anaerobic conditions

Isolation of cellulolytic microbes was also attempted under microaerobic (5% oxygen) or anaerobic condition using an identical methodology and FP agar described for the aerobic cultivation. The morphologies of the fungal and bacterial colonies formed under microaerobic conditions were identical to those observed under aerobic conditions. Thirty-four colonies were isolated for phylogenetic analyses, but all the isolates were classified into the genera previously found in the phylogenetic study of the aerobically cultivated strains (data not shown). In contrast to aerobic and microaerobic cultivation, no microbial colonies were observed on filter paper under anaerobic conditions.

Discussion

In this study, a simple method to selectively culture microbes capable of degrading crystalline cellulose was evaluated for its potential to isolate cellulolytic microbes in a forest soil. The developed method was designed to immobilize microbes in the cellulose fibers within the filter paper to prevent the overgrowth of fast-growing species that typically mask or inhibit slower-growing species in standard enrichment cultures. Cellulose fibers are thought to restrict hyphal extension and spore dissemination of fast-growing strains, which easily occur on standard agar media which contain cellulose powders (e.g., Avicel) or a soluble cellulose derivative (e.g., CMC) as a carbon source. Additionally, cellulose fibers also seem to reduce intercolony interactions, permitting longer incubation time for slow-growing strains. We think that these devices are


a
Saccharomyces cerevisiae (AB279746)
Strains-11, -50
G. butleri (GU966508)
G. lacrispora (GU244498)
Gongronella
Strain-20
C. elegans (EU076936)
Strain-66
C. binariae (EU082780)
Cunninghamella
Strain-62
E. nigrum (HQ882801)
E. sorghi (HQ328051)
Epicoccum
Strain-43
Strain-61
Strains-46, -41, -64
P. adametzii (AF033401)
P. daleae (AF033442)
Strains-5, -16, -26
P. simplicissimum (GU981588)
Strain-42
P. oxalicum (AB485603)
P. janthinellum (HQ262508)
P. verrucosum (AY373938)
P. aculeatum (AY303608)
Strain-31
Strains-8, -10, -12, -17, -22, -30, -32, -36, -37, -53, -B, -C
Strain-24
Strain-A
P. resedanum (AF033398)
Penicillium
T. bacillisporus (AF033388)
T. emodensis (AB176616)
Strain-9
Strain-33
Talaromyces
Strain-52
C. virescens (GU183124)
Chloridium
N. vilior (HM054160)
N. sinopica (HM534900)
Strain-34
Strain-63
Strain-18
Nectria
F. oxysporum (HM036595)
Strains-2, -29, -51
F. proliferatum (GU066729)
Fusarium
B. samuelsii (HQ022475)
Strain-35
B. ochroleuca (HQ328025)
Bionectria
T. viride (AM498415)
Strains-4, -6, -14, -23, -39, -60
Strain-38
T. strigosum (EU263997)
T. hamatum (EU263999)
Strain-44
Strain-55
Strain-19
Strains-3, -49, -58, -59
T. spirale (HM042318)
Strains-13, -56
Trichoderma
0.1

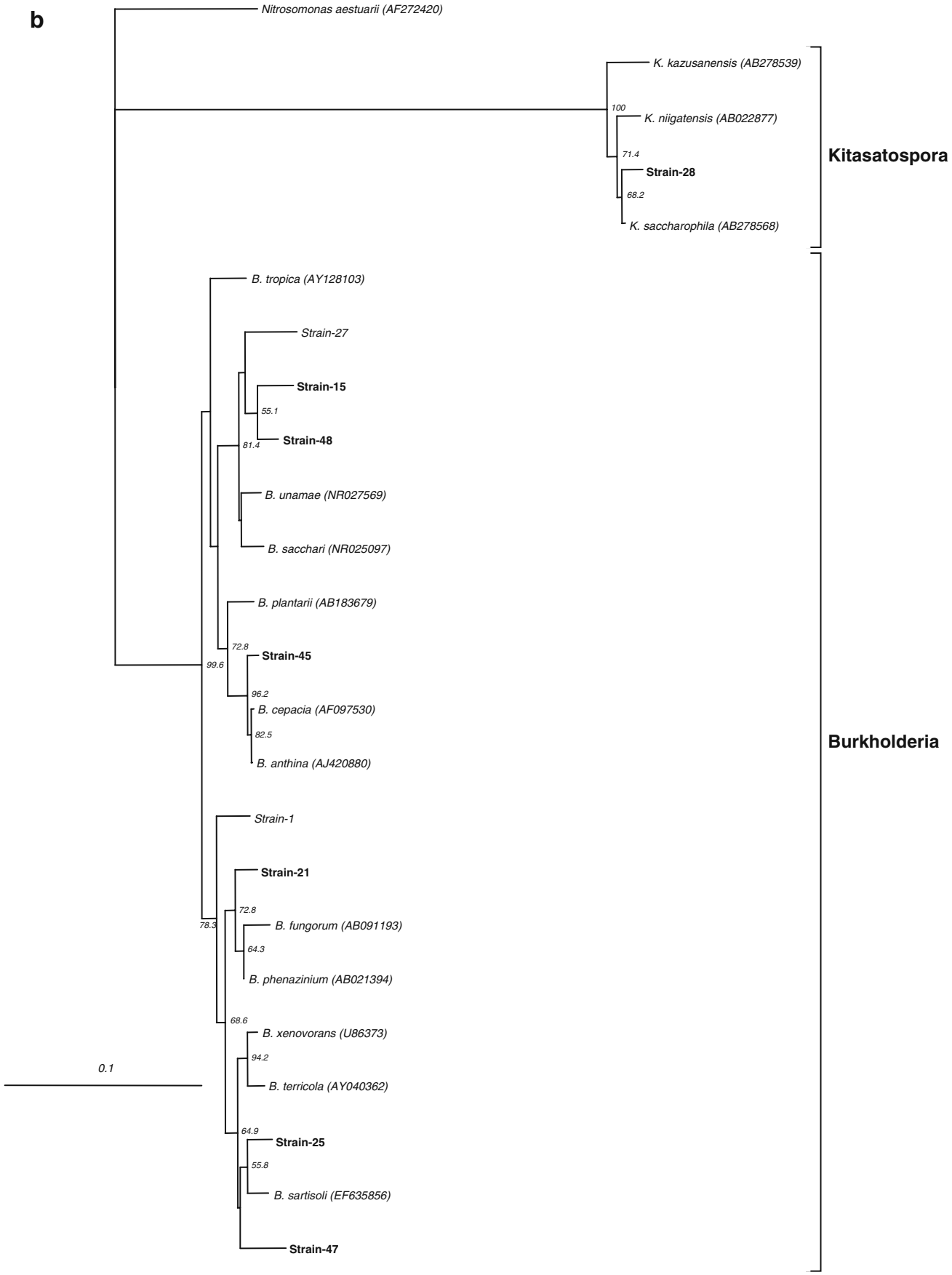


Fig. 2 (continued)

Table 1 Enzyme activities of representative fungal and bacterial soil isolates

Strain no.	Genus	Enzyme activity (U/mg protein)[a]			
		Exocellulase	Endoellulase	Xylanase	Laccase
2	*Fusarium*	1.80±0.52	0.32±0.25	nd[b]	0.49±0.10
4	*Trichoderma*	0.14±0.03	1.12±0.71	1.01±0.45	nd[c]
5	*Penicillium*	2.22±1.56	2.42±1.11	3.29±1.56	0.28±0.15
8	*Penicillium*	2.03±1.34	1.31±0.68	2.57±1.52	0.51±0.40
9	*Talaromyces*	1.94±0.70	0.96±0.50	0.20±0.11	nd[c]
11	*Gongronella*	0.72±0.22	nd[b]	0.54±0.34	0.61±0.41
13	*Trichoderma*	0.24±0.17	2.43±1.06	1.43±0.76	nd[c]
15	*Burkholderia*	0.32±0.24	nd[b]	nd[b]	0.71±0.52
18	*Nectria*	0.13±0.08	0.50±0.26	0.80±0.48	nd[c]
19	*Trichoderma*	0.25±0.04	nd[b]	nd[b]	nd[c]
20	*Cunninghamella*	0.28±0.04	nd[b]	nd[a]	1.59±0.79
21	*Burkholderia*	0.55±0.44	nd[b]	nd[b]	0.78±0.10
25	*Burkholderia*	0.30±0.09	0.43±0.29	nd[b]	0.35±0.25
28	*Kitasatospora*	0.89±0.20	nd[b]	0.86±0.38	0.68±0.55
31	*Penicillium*	0.43±0.32	0.94±0.46	3.07±1.41	nd[c]
34	*Nectria*	0.59±0.32	0.94±0.34	1.92±1.23	0.40±0.39
35	*Bionectria*	0.29±0.25	nd[b]	nd[b]	nd[c]
38	*Trichoderma*	0.35±0.21	0.18±0.14	nd[b]	nd[c]
42	*Penicillium*	1.35±0.91	0.83±0.45	1.95±0.82	nd[c]
44	*Trichoderma*	0.27±0.21	1.70±1.14	nd[b]	nd[c]
45	*Burkholderia*	0.13±0.11	0.60±0.44	nd[b]	nd[c]
46	*Penicillium*	0.24±0.09	1.32±0.74	0.61±0.41	nd[c]
47	*Burkholderia*	0.22±0.03	nd[b]	nd[b]	nd[c]
48	*Burkholderia*	0.25±0.12	0.29±0.14	nd[b]	nd[c]
52	*Chloridium*	0.24±0.15	0.86±0.57	nd[b]	nd[c]
55	*Trichoderma*	0.21±0.13	1.47±1.00	0.19±0.17	nd[c]
62	*Epicoccum*	0.17±0.12	nd[b]	nd[b]	nd[c]
66	*Cunninghamella*	0.38±0.26	nd[b]	nd[b]	0.86±0.45

[a] Mean ± standard deviation of independent triplicates

[b] *nd* not detected (<0.1 U/mg protein)

[c] *nd* not detected (<0.2 U/mg protein per second)

unique features of the method developed in this study. Following 2 weeks of cultivation, numerous fungal and bacterial colonies with distinct morphological characteristics emerged on the filter paper. As cellulose fibers served as the sole carbon source in the medium, all isolates expectedly exhibited exocellulase activity, which suggests that our method is suitable for the isolation of exocellulase-producing strains. In addition, as only *Penicillium* spp. were isolated from the liquid enrichment culture, our method appears to overcome the shortcomings of enrichment cultures because it permits the growth of a significantly wider range of microbial genera.

Phylogenetic analyses revealed that the 54 examined fungal isolates belong to ten known genera, although one isolate could not be classified at the genus level. Since *Trichoderma*, *Penicillium*, *Talaromyces*, *Nectria*, and *Fusarium* are common soil fungi with reported cellulolytic activity (Folan and Coughlan 1978; Reese and Mandels 1989; Singh and Kumar 1991; Samuels 1996; Pardo and Forchiassin 1998; Krogh et al. 2004; Jørgensen et al. 2005; Fujii and Takeshi 2007; Picart et al. 2007) and *Cunninghamella* was also recently shown to have cellulolytic activity (Gopinath et al. 2005), our data appear to reflect the likely cellulolytic microbiota present in the forest soil. Interestingly, the ITS region sequence similarity between ten fungal isolates and their closest known neighbors was <97%. In most fungal genera, ≥3% sequence variability in the ITS region between strains suggests that they are heterospecific (Nilsson et al. 2008); hence, the ten fungal strains may be novel species in each respective genus. While there are no reports of cellulolytic activity among the other identified genera, which included *Chloridium*, *Bionectria*, *Epicoccum*, and *Gongronella*, the sequence similarity of the isolated strains and their closest neighbors was more than 98%. Our analyses therefore suggest that the isolates are conspecific to their closest known neighbors (strain 52, *Chloridium virescens*; strain 35, *Bionectria ochroleuca*; strain 62, *Epicoccum nigrum*; strains 11 and 50, *Gongronella*

butleri) and represent the first findings of cellulolytic activity among members of those genera.

It was revealed that bacterial isolates belonged to two genera, *Burkholderia* and *Kitasatospora*, which are ubiquitous soil bacteria (Omura et al. 1982). Although xylanolytic, pectinolytic, and chitinolytic strains of *Burkholderia* have been identified (Kong et al. 2001; Massa et al. 2007; Mohana et al. 2008), there are no reports of cellulolytic activity among the identified strains of *Burkholderia* and *Kitasatospora*. The sequence similarity of 16S rDNA between the bacterial isolates and closest known neighbors was >98%. As Stackebrandt and Goebel (1994) proposed that a 16S rDNA similarity exceeding 97% between tested strains indicates conspecificity, we concluded that all isolates are conspecific to the closest known neighbors. However, our study is the first report of cellulase activity in the genera *Burkholderia* and *Kitasatospora*.

It is also interesting to compare exocellulase activities determined for the isolates with characterized cellulase producers. The specific activity of exocellulases produced by fungal strains 2, 5, 8, 9, 11, 28, and 42 were comparable to that of *Trichoderma* exocellulase (Esterbauer et al. 1991; Martins et al. 2008), which is considered the most potent microbial exocellulase with an activity that ranges from 0.15 to 1.83 IU/mg (international units per milligram protein; 1 IU corresponds to 1 μmol reducing sugar equivalent formed during cellulose hydrolysis), suggesting the potential usefulness of these strains for biotechnological applications. In addition to the exocellulase activity, many isolates were found to produce endocellulase, required for the degradation of the amorphous part of cellulose, and xylanase, while several isolates also exhibit laccase activity, which is necessary for lignin degradation. Taken together, these results strongly suggest that the isolates identified here contribute to the decomposition of cellulosic biomass, such as leaf litter or dead grass, in forest soil.

Despite the apparent potential of our developed method, a few of the present results suggest the necessity for an improvement of our approach. For instance, *Cellulomonas* spp., which are aerobic cellulolytic bacteria, were not isolated in this study. Although we speculate that *Cellulomonas* spp. were minor components of the microbiota with slow growth rates in a forest soil, as reported previously (Sarathchandra et al. 1997; Wang et al. 1999), it is possible the FP agar was not suitable for the growth of these species. As it is known that some cellulolytic microbes found in the rumen are introduced in neonates from an outer environment (Williams and Dinusson 1972), we attempted to isolate them in the soil sample under anaerobic conditions, but were unsuccessful. Since it was reported that many rumen microbes have strict nutritional requirements (Slyter and Weaver 1971), the composition of FP agar may be too nutritionally poor to support the growth of such species. Therefore, to target certain cellulolytic microbes using this method, it may be necessary to supplement FP agar culture medium with specific micronutrients.

In conclusion, the method established in this study was applicable for isolating and characterizing hitherto-uncultured exocellulase-producing species. Although culture-independent approaches were employed recently to isolate novel cellulase genes from environmental samples, including soil metagenomes, most studies reported the isolation of only endocellulase genes (Healy et al. 1995; Voget et al. 2006; Feng et al. 2007; Kim et al. 2008; Wang et al. 2009). To date, the only successful work has been accomplished by Ferrer et al. (2005) who isolated exocellulase genes from rumen microflora. However, as the discovery rate of cellulase genes in these studies only ranged from 0.001% to 0.5%, isolating exocellulases from soil metagenomes appears challenging. In contrast, many researchers have shown that simple improvements to culture conditions can significantly increase the number of obtainable isolates, including as yet unculturable strains (Janssen et al. 2002; Kaeberlein et al. 2002; Zengler et al. 2002, 2005; Joseph et al. 2003; Svenning et al. 2003; Stevenson et al. 2004; Davis et al. 2005; Ferrari et al. 2005, 2008; Rasmussen et al. 2008; Ferrari and Gillings 2009; Tamaki et al. 2009; Matsuzawa et al. 2010). Therefore, we consider that culture-dependent approaches remain an attractive way to identify novel cellulolytic microbes with potentially useful enzymes for biotechnological applications. Although slight modifications to the culture medium may be needed depending on the physiological characteristics of target microbes, it is anticipated that the simple method described in this work will contribute to the isolation of hitherto-uncultured cellulolytic microbes from environmental samples.

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