ORIGINAL ARTICLE

Combination cellulose plate (non-agar solid support) and agar plate method improves isolation of fungi from soil

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This is the first report describing the improved isolation of common filamentous fungi via a method combining cellulose plate and agar plate system. A cellulose plate is a porous plate made of nanofibrous crystalline cellulose. Isolating fungi from soils using these types of media separately resulted in the number of fungal colonies appearing on cellulose plates being lower than that on agar plates. However, the number of actual fungal species isolated using cellulose plates alone was more or less the same as that found using agar plates. Significantly, the diversity of isolates using a combination of the two media was greater than using each media individually. As a result, numerous new or rare fungal species with potential, including previously proposed new species, were isolated successfully in this way. All fungal colonies, including the *Penicillium* species, that appeared on the cellulose plate penetrated in potato dextrose were either white or yellow. Cultivation on cellulose plates with added copper ion overcomes the change in coloration, the colonies appearing as they do following cultivation on potato dextrose agar.

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INTRODUCTION

Natural products are useful for possible development into drugs and other chemical agents. The rate of known compounds re-isolated from natural sources has been increasing during the past decade.¹ So, there is a pressing need for developing more efficient fermentation and methods to isolate microorganisms, including fungi, to discover new natural compounds. As a fermentation method, a coculture mechanism has the potential to produce novel natural products as well as increase the production of known compounds.^{2,3}

By 2011, around 100 000 species of fungi had been described,⁴ although it is estimated that there are actually 1.5–5.1 million fungal species on the earth.^{4–6} Cultivation of microorganisms using agar media has been the commonly and most widely used method since Koch's invention of the process in the 19th century. Isolation of fungi using new, non-agar solid media offers great potential for the discovery of a variety of fungal species including new species.

Recently, a few new fixation agents composed of other materials have been developed for the cultivation of microbes.⁷ A cellulose plate is one of them. It is a porous plate made of nanofibrous crystalline cellulose and can be used for cultivation following penetration of a nutritional solution.⁸ Mesophilic bacteria and yeast (*Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*) grow on the cellulose plates, just as well as on the conventional agar plates.⁸ *In situ* optical microscopic examination revealed that a cellulose plate can remain unchanged up to 280 °C at a constant pressure of 25 MPa. Due to this structural stability at high temperatures, a thermophile, *Thermus*

thermophilus, was cultured successfully on a cellulose plate at 80 °C, the temperature at which a conventional agar or gellan gum plate does not remain solid (unless the media composition has been carefully adjusted).⁸ Moreover, due to the structural stability at low and high pH value, extremophiles, such as acidophilic *Acidiphilium acidophilum* or alkaliphilic *Bacillus clarkii*, can be cultured successfully on a cellulose plate.⁹ However, the cultivation of common filamentous fungi on cellulose plates has not previously been thoroughly evaluated.

We describe here an innovative isolation method using a cellulose plate (non-agar) as the isolation medium and show how employment of a combination method can significantly enhance the diversity of organisms isolated from a soil sample.

MATERIALS AND METHODS

Isolation

Three soil samples (no. 1–3) from around plant roots were collected from Haha-jima, the Bonin Islands, Tokyo, Japan in March 2011. Sample no. 1, no. 2 and no. 3 were collected from under the *Crepidiastrum ameristophyllum*, *Ficus nishimiyrae* and *Hibiscus glaber* plant species, respectively.

Soil samples (1 g) were suspended in 9 ml of modified Winogradsky's salt solution (K₂HPO₄ 0.38%, KH₂PO₄ 0.12%, MgSO₄ · 7H₂O 0.51%, NaCl 0.25%, Fe₂ (SO₄)₃ · nH₂O 0.005% and MnSO₄ · 5H₂O 0.005%) with 0.01% surfactant Tween-80 (Sigma-Aldrich Co., Saint Louis, MO, USA) and then sonicated for 2 min and subsequently diluted 10²- to 10³-fold with the above Winogradsky's solution. Of the diluted soil suspension, 200 µl was spread on a plate with solidified potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA)

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and potato dextrose 1% (PDC1%: non-agar) and 3% cellulose (PDC3%: non-agar), as well as with all media, with 50 mg l⁻¹ rose bengal and 100 mg l⁻¹ kanamycin. PDC was prepared by adding 2.4% potato dextrose broth (PDB, Difco) into an Exedia Cellulose Plate *Advanced* (cellulose 1%) and *Original* (cellulose 3%) (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. Incubation was at 25 °C for 5 days.

Observation of morphological characteristics

For the classification at genus level, micromorphology (conidia, conidiophore and conidial form) of all isolates grown on Miura's medium slant (glycerol 0.1%, KH₂PO₄ 0.08%, K₂HPO₄ 0.02%, MgSO₄ · 7H₂O 0.02%, KCl 0.02%,

NaNO₃ 0.2%, yeast extract 0.02% and agar 1.5% (adjusted to pH 6.0 before sterilization)) was observed under a CKX41 microscope after 14 days (Olympus, Tokyo, Japan).

For the observation of conidia and penicilli of *Penicillium raphiae* FKI-6604, microscope slides were prepared from PDA and PDC cultures. The slides were observed under a Vanox-S AH-2 microscope (Olympus) and digital photographs were obtained with a DP25 digital camera (Olympus).

DNA analysis

DNA extraction and amplification by polymerase chain reaction (PCR) of the ITS region were conducted under the conditions described by Nonaka *et al.*¹⁰

Figure 1 (a–c) 10²-fold diluted soil no. 1 suspension; (d–f) 10²-fold diluted soil no. 2 suspension; (g–i) 10²-fold diluted soil no. 3 suspension. (a, d and g) Photograph of colonies on potato dextrose agar after 5 days. (b, e and h) Photograph of colonies on potato dextrose cellulose 1% after 5 days. (c, f and i) Photograph of colonies on potato dextrose cellulose 3% after 5 days.

Sequencing products were purified using a BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, USA), and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences, with the SeqMan Pro program from the Lasergene 10 package (DNASTAR Inc., Madison, WI, USA). To determine the most closely related species, the DNA sequence of the internal transcribed spacer region of isolates was compared with sequences in the GenBank database by BLASTN 2.2.28 analysis.¹¹ The isolates had more than 99.0% similarity with the known fungal species, proving their identity. Also, they were not classified as a genus level with less than 90.0% similarity.

RESULTS

Isolation of fungi

Three soil samples were used for isolation of fungi. Five PDA, PDC1% and PDC3% plates were used for each sample. A diluted soil suspension (10^2 -fold dilution) was spread on two plates and 10^3 -fold diluted soil suspension was spread on three plates for each soil sample. Filamentous fungi grew successfully on the cellulose plates, similar to their growth on the conventional agar plate (Figure 1). After 5 days, the CFU numbers of fungi, bacteria and total microbe as per gram of soil were calcd and the percentage of fungal and bacterial colonies was shown (Table 1). In soil sample no. 1, the percentage of fungal colonies on PDA, PDC1% and PDC3% was 98.5, 100 and 92.9%, respectively. In soil sample no. 2, the percentage of fungal colonies on PDA, PDC1% and PDC3% was 19.1, 90.9 and 77.3%, respectively. In soil sample no. 3, the percentage of fungal colonies on PDA, PDC1% and PDC3% was 50, 55.6 and 100%, respectively. (Table 1).

Identification of fungal isolates

All fungal isolates were identified on the basis of a combination of the micromorphological characteristics and the internal transcribed spacer sequences. In soil sample no. 1, the 215 strains from PDA were identified as 20 species in 11 genera, 88 from PDC1% were 24 species in 18 genera and 113 from PDC3% were 16 species in 10 genera. The total 416 strains from all three media were 34 species in 22 genera (excluding duplicated species) (Table 2). In soil sample no. 2, 69 strains from PDA were identified as 14 species in 12 genera, 41 from PDC1% were 10 species in 7 genera and 48 from PDC3% were 14 species in 12 genera. The total 158 strains were 25 species in 19 genera (Table 2). In soil sample no. 3, 28 strains from PDA were identified as 9 species in 7 genera, 18 from PDC1% were 14 species in

10 genera and 15 from PDC3% were 10 species in 8 genera. The total 61 strains were 20 species in 14 genera (Table 2). In all soil samples combined, the 312 strains from PDA were identified as 34 species in 20 genera, 147 from PDC1% were 37 species in 23 genera and 176 from PDC3% were 32 species in 18 genera. The combined total of 635 strains was 58 species in 33 genera (Table 2).

A 48% of total taxa (28 species) were duplicated on both three isolation media. On the other hand, unique taxa in each of the three isolation media were composed of 30 species (8 species, PDA; 14 species, PDC1%; 8 species, PDC3%), which was 52% of the total taxa (Table 2).

Conidiation of *Penicillium* strain

Several of the same fungal species were isolated from both PDA and PDC plates (Table 2). Fungal colonies on PDA had a diversity of color (Figure 1), whereas colonies on PDC were white or yellow only (Figure 1). *Penicillium* strains were isolated from both media. In general, almost all *Penicillium* species exhibit green colored conidia on rich media such as PDA (Figure 2a) and Czapek yeast extract agar.¹² However, the colony color of *Penicillium* strain on PDC in our experiments was white (Figures 2b and c). To verify that *Penicillium* strains are unable to conidiate or produce colorless conidia on PDC, microscope slides prepared from PDA and PDC cultures were observed under a light microscope (using *Penicillium raphiae* FKI-6604). Its penicilli on PDA were very crowded and long (5–20) conidial chains were produced at the apex of the phialides (Figure 3a), whereas its penicilli on PDC were very sparse and only very short (1–3) conidial chains were produced (Figure 3b).

Sporulation of fungal colonies on cellulose plate

Due to being free of sporulation factor in the PDC plate, the colony of *Penicillium raphiae* FKI-6604 grown on this medium was white in color (Figures 2 and 3). Therefore, 0.0005% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ as a sporulation factor was added to PDC, and it was used as isolation medium. Fungal colonies on PDC with added copper ion had a diversity of color as is the case of PDA (Figure 4). In this examination, fresh soil (from under leaf-litter) was used.

DISCUSSION

Robert Koch developed a solidified medium for cultivating microorganisms for the first time in the 19th century. At first, the gelatin was used as a fixation agent, after which agar became the medium of choice. Meanwhile, other fixation agents were used intermittently and infrequently (for example, gellan gum (gelrite) has been used for the cultivation of bacteria^{13,14}). Recently, a few new fixation agents composed of other materials have been developed for the cultivation of microbes.⁷ A cellulose plate is one of them. However, the cultivation of common filamentous fungi on cellulose plates has not previously been thoroughly evaluated.

Altering the composition of isolation media affects the variation of fungal colonies appearing on them. We tried to alter the fixation agent in the isolation media without otherwise disrupting its normal composition. Filamentous fungi grew successfully on the cellulose plates, similar to their growth on a conventional agar plate. Also, all fungal strains isolated from cellulose plates were able to grow on PDA. But, in the case of isolating fungi from soil samples using cellulose plates, the number of fungal colonies appearing was lower than the number growing on agar plates (Table 1).

In addition, all fungal colonies appearing on cellulose plates were either white or yellow (Figure 1). At first, we suspected that this was caused by the diversity of fungal species being very low. However,

Table 1 Isolation frequency of fungi and bacteria from each isolation medium

Colony forming unit $\times 10^4$ per gram of soil (% of total count)				
Soil no.	Microbe	PDA	PDC1%	PDC3%
Soil 1	Fungi	6.7 (98.5%)	2.9 (100%)	3.9 (92.9%)
	Bacteria	0.1 (1.5%)	0 (0%)	0.3 (7.1%)
	Total	6.8 (100%)	2.9 (100%)	4.2 (100%)
Soil 2	Fungi	2.1 (19.1%)	1.0 (90.9%)	1.7 (77.3%)
	Bacteria	8.9 (80.9%)	0.1 (9.1%)	0.5 (22.7%)
	Total	11 (100%)	1.1 (100%)	2.2 (100%)
Soil 3	Fungi	0.6 (50%)	0.5 (55.6%)	0.4 (100%)
	Bacteria	0.6 (50%)	0.4 (44.4%)	0 (0%)
	Total	1.2 (100%)	0.9 (100%)	0.4 (100%)

Abbreviations: PDA, potato dextrose agar; PDC, potato dextrose cellulose.

Table 2 List of soil-inhabiting fungi isolated from soil samples using potato dextrose agar (PDA), potato dextrose cellulose 1% (PDC1%) and potato dextrose cellulose 3% (PDC3%) cultivation media

Fungus	Isolation media			Total
	PDA	PDC1%	PDC3%	
<i>Acremonium variegatum</i>	—	+	—	+
<i>Chaunopycnis</i> sp.	—	+	—	+
<i>Chloridium</i> sp.	—	+	+	+
<i>Clonostachys rosea</i>	+	+	+	+
<i>Dokmaia montheadangii</i>	+	+	+	+
<i>Doratomyces stemonitis</i>	+	—	+	+
<i>Fusarium solani</i>	—	+	—	+
<i>Fusarium oxysporum</i>	+	—	—	+
<i>Hypoxyton</i> sp.	—	+	—	+
<i>Lecanicillium primulinum</i>	—	+	—	+
<i>Lecanicillium</i> sp.	—	+	—	+
<i>Mariannaea elegans</i> var. <i>punicea</i>	+	—	—	+
<i>Metarhizium anisopliae</i>	+	—	+	+
<i>Microdiplodia</i> sp.	+	—	+	+
<i>Mortierella alpina</i>	+	+	—	+
<i>Mucor</i> sp.	—	—	+	+
<i>Nemania diffusa</i>	—	+	—	+
<i>Neocosmospora vasinfecta</i>	+	—	—	+
<i>Ochroconis</i> sp.	+	—	—	+
<i>Oidiodendron setiferum</i>	+	—	—	+
<i>Paecilomyces carneus</i>	+	+	+	+
<i>Paecilomyces marquandii</i>	+	+	+	+
<i>Penicillium brevicompactum</i>	—	—	+	+
<i>Penicillium citrinum</i>	+	+	+	+
<i>Penicillium cremeogriseum</i>	—	+	—	+
<i>Penicillium melinii</i>	—	—	+	+
<i>Penicillium sclerotiorum</i>	+	+	+	+
<i>Penicillium smatrense</i>	+	—	+	+
<i>Penicillium paxilli</i>	+	+	—	+
<i>Penicillium raphiae</i>	+	+	+	+
<i>Penicillium virgatum</i>	+	+	+	+
<i>Pestalotiopsis virgatula</i>	—	—	+	+
<i>Pochonia bulbilosa</i>	+	—	—	+
<i>Pochonia chlamydosporia</i> var. <i>catenulata</i>	—	+	—	+
<i>Pochonia chlamydosporia</i> var. <i>chlamydosporia</i>	+	+	+	+
<i>Pochonia chlamydosporia</i> var. <i>spinulosporea</i>	—	+	—	+
<i>Pochonia</i> sp.	+	+	+	+
<i>Pseudobotrytis</i> sp.	—	+	+	+
<i>Purpureocillium lilacinum</i>	+	+	+	+
<i>Purpureocillium</i> sp.	+	—	+	+
<i>Simplicillium chinense</i>	+	+	+	+
<i>Staphylotrichum boninense</i>	+	—	—	+
<i>Talaromyces ruber</i>	—	—	+	+
<i>Talaromyces</i> sp. 1	+	+	+	+
<i>Talaromyces</i> sp. 2	+	—	—	+
<i>Talaromyces</i> sp. 3	—	—	+	+
<i>Tolypocladium</i> sp.	—	—	+	+
<i>Trichoderma atroviride</i>	—	—	+	+
<i>Trichoderma brevicompactum</i>	+	+	+	+
<i>Trichoderma harzianum</i>	+	+	+	+
<i>Trichoderma</i> sp. 1	+	+	—	+
<i>Trichoderma</i> sp. 2	+	+	—	+
<i>Verticillium leptobactrum</i> -like 1	+	+	+	+
<i>Verticillium leptobactrum</i> -like 2	+	+	+	+
<i>Virgaria boninensis</i>	—	+	—	+
<i>Xylaria</i> sp.	—	+	—	+
Unidentified sp. 1	—	+	—	+

Table 2 (Continued)

Fungus	Isolation media			Total
	PDA	PDC1%	PDC3%	
Unidentified sp. 2	–	+ (S1)	–	+ (S1)
The number of species (genera): S1	20 (11)	24 (18)	16 (10)	34 (22)
The number of species (genera): S2	14 (12)	10 (7)	14 (12)	25 (19)
The number of species (genera): S3	9 (7)	14 (10)	10 (8)	20 (14)
Total number of species (genera)	34 (20)	37 (23)	32 (18)	58 (33)

Abbreviations: S1, soil no. 1; S2, soil no. 2; S3, soil no. 3.
+: isolated (soil number), -: nonisolated from all soil samples.

Figure 2 *Penicillium raphiae* FK1-6604: (a) Photograph of colonies grown on potato dextrose agar after 7 days. (b) Photograph of colonies grown on potato dextrose cellulose 1% after 7 days. (c) Photograph of colonies grown on potato dextrose cellulose 3% after 7 days.

Figure 3 *Penicillium raphiae* FK1-6604: (a) Micrograph of penicilli grown on potato dextrose agar after 7 days. (b) Micrograph of penicilli grown on potato dextrose cellulose 1% after 7 days. Scale bar, 10 μ m.

when the isolates were identified, the number of fungal species isolated using cellulose plates was approximately equal to (or exceeded) the number found on agar plates, although the fungal diversity differed slightly. In this study, we isolated a total of 58 species in 33 genera using the dual system. On the other hand, unique taxa in each of the three isolation media were composed of 30 species (8 species, PDA; 14 species, PDC1%; 8 species, PDC3%), which was 52%

of the total taxa (Table 2). Significantly, the diversity of isolates using a combination of the agar plate and the cellulose plate was greater than that using each medium individually. The cellulose plate was slightly sagging downwards at the periphery of many fungal colonies (data not shown). The phenomenon was thought to be due to breakdown by cellulase-producing fungi.¹⁵ The carbon sources available differed between the agar plate and the cellulose plate.

Figure 4 A 10^2 -fold diluted suspension of soil collected from under leaf-litter: (a) Photograph of colonies on potato dextrose agar after 5 days. (b) Photograph of colonies on potato dextrose cellulose 1% after 5 days. (c) Photograph of colonies on potato dextrose cellulose 1% with 0.0005% CuSO_4 after 5 days.

Therefore, it is speculated that this influences the difference in the fungal diversity.

Using the dual system, 25 new (or potential) fungal species (43% of total species) have been isolated successfully (Table 2). Two of the new species, *Lecanicillium primulinum* and *Virgaria boninensis*, were described elsewhere.^{16,17} In a previous study, we had discovered two new species, *Staphylotrichum boninense* and *Pochonia chlamydosporia* var. *spinulosporea*, which were re-isolated using this new system.^{18,19} We discovered more than 30 novel compounds (including unpublished) from screening using 1500 fungal strains from 2005 to 2010.^{20–22} The producers of the novel compounds were classified as species level, and 63% of them were new (or potential) species.^{10,17,23} In this study, *Trichoderma* sp. 1 FKI-6626 isolated by the cellulose plate method produced a new compound, cytosporone S.²⁴ In addition, other novel compounds were discovered from other fungal strains isolated by using the dual system (in preparation for report). The results of our studies demonstrate the use of the dual system in isolating new fungal species and the novel compound productivity of these new fungal species.

Significantly, the number of bacterial colonies appearing on cellulose plates was much lower than that on agar plates (Table 1), which means that the cellulose plate technique could be very useful in isolating fungi and minimizing bacterial contamination of the process. Influence of various factors (for example, medium composition, pH and bacterial population) on antibacterial activity of antibiotics was reported.²⁵ In this case, influence of cellulose on antibacterial activity of kanamycin and rose bengal has potential to be greater than that of agar. On the other hand, rapid-growth fungi, genus *Trichoderma*, were repressed on PDC (Figure 1). It is speculated that each minimizing bacterial appearance and growth suppression of *Trichoderma* was also one of the difference factors in the fungal diversity.

For isolation of various fungal strains efficiently from one plate, macromorphology on the isolation medium was used as a reference. If all colonies on any isolation medium are of the same color, they look so much alike that it is difficult to tell them apart. *Penicillium implicatum* production of the yellow pigment citrinin was induced when copper ions were added to the medium.²⁶ From this phenomenon, identification media of *Aspergillus* and *Penicillium* (for example, Czapek yeast extract agar, Czapek Dox agar and 25% glycerol nitrate agar) were generally added with 0.0005%

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to distinguish the species level on the basis of colonial color.^{12,27} To overcome the problem of using a cellulose plate as an isolation medium, adding copper ions to the cellulose plate penetrated with potato dextrose allowed various color colonies to appear, as with PDA (Figure 4). In this study, we used only potato dextrose as the medium in cellulose plates and agar plates, and soil as the sole isolation source. Further studies using varying compositional cellulose plates, varying culture conditions and various natural isolation sources (for example, soil, plants, insects and sea water) offer potential for isolating a far greater variety of fungi.

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