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ORIGINAL ARTICLE

New method for isolating antibiotic-producing fungi

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A convenient and efficient method was established for isolating antifungal antibiotic-producing fungi from soil samples. In this method, soil samples were diluted and directly plated in agar medium by the standard fungi-isolating method, and the plates were cultured at 27 °C for 2–3 days to permit the growth of fungal colonies. Then, the suspension of pathogenic *Candida albicans* in saline (40 µl, 5–10 × 10^5 CFU ml^{-1}) was overlaid by spraying on the plates under controlled conditions in the safety cabinet. After 1-day incubation, fungal colonies showing an antagonistic effect with the inhibition zone against sprayed *C. albicans* were selected. Among 151 isolates, 26 strains were found to reproduce anti-*C. albicans* activity in liquid medium, yielding a higher selection rate (17.2%) than that (3.1%) by the traditional method. This new method can be applied for isolation of microorganisms (fungi and actinomycetes) that produce antibiotics active against pathogenic microorganisms.
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INTRODUCTION

It is well known that fungi remain one of the most important resources for the discovery of new bioactive compounds.[1] It is thought that fungi rank as the second biggest kingdom of organisms in nature and that as many as 1.5–5.1 million fungal species exist.[2–4] From the history of drug discovery from microorganisms, fungal secondary metabolites have provided a number of important drugs, such as the antibiotic penicillin,[5] the immunosuppressant cyclosporine[6] and the antihypercholesterolemic agents lovastatin[7] and compactin.[8,9] Recent DNA sequencing technology has markedly advanced, revealing whole-genome sequences of a number of organisms. The first whole-genome sequence was published for a free-living organism, *Haemophilus influenza*.[10] Since then, a number of organism genome sequences, including bacteria (more than 1500 species), archaea (more than 100 species) and eukaryotes (more than 100 species, including *Homo sapiens*), have been determined.[11] From genomic information about microorganisms, *Streptomyces* species have 7–9-Mb-long genomes containing 20–30 genes of secondary metabolites biosynthesized via pathways of polyketide synthases or non-ribosomal peptide synthases,[12–14] while fungi such as *Aspergillus* species have 30–40-Mb-long genomes containing 30–80 polyketide synthase and non-ribosomal peptide synthase genes.[15–17] Interestingly, only a few genes can be expressed to produce the corresponding secondary metabolites, but most of them remain silent.[18,19] Thus, these microorganisms were proved to have high potential to produce many secondary metabolites.

Many antibiotics have been discovered from microorganisms. The process of discovering antibiotics usually includes several steps: (1) isolation of microorganisms mainly from soil (usually 2–3 days are needed), (2) pure culture of isolated microorganisms (3–5 days), (3) identification of isolated microorganisms mainly by morphological characteristics to eliminate duplicate strains (14–21 days), (4) testing whether or not these culture broths or extracts of microorganisms show antimicrobial activity to select candidate microorganisms that produce antibiotics (7–8 days) and (5) re-culture of candidate microorganisms to observe the reproducibility of antimicrobial activity (6–8 days). This routine process (named the traditional method in this study) usually takes 5–7 weeks. In this study, we show a more efficient and faster method to select candidate fungi that can produce anti-*Candida albicans* antibiotics.

RESULTS

Media for fungal isolation

As *C. albicans* was usually cultured in GY medium, the growth of *C. albicans* on media 1–4 was investigated. *C. albicans* was found to grow normally in media 1–4; therefore, all the media were used throughout this study.

Preparation of soil plates

By plating method A, in which a soil sample (15 mg) was directly dispensed into each medium (media 1–4), fast-growing fungi such as *Trichoderma*, *Aspergillus* and *Penicillium* species predominantly appeared on the plate and inhibited the growth of other fungi. Colonies of slow-growing fungi such as *Virgaria* and *Humicola* species were isolated on the plates of highly diluted soil samples by plating method B. Thus, both plating methods A and B were used to prepare soil plates. Soil plates were incubated at 27 °C for 2–3 days to form fungal colonies.

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Inoculation of *C. albicans* to soil plates

The fundamental aim of this study was to directly observe fungal colonies derived from soil that show antagonistic effects on the growth of *C. albicans*. Five *C. albicans*-inoculating methods were tested. In inoculating method I, *C. albicans* and the soil sample were mixed and incubated at the same time. In inoculating method II, *C. albicans*-containing agar was first prepared, and soil-containing agar was overlaid on the plate, which was then incubated. Thus, in inoculating methods I and II, pathogenic *C. albicans* and soil-derived fungi began to grow almost at the same time. As a result, the plate was covered with *C. albicans* (Figure 1a), because *C. albicans* grew much faster than soil-derived fungi. By these methods, soil-derived fungi could not form colonies and antagonistic effects were not observed on the plates. In inoculating method III, *C. albicans* was spread on soil plates after soil-derived colonies were formed on the plates (2–3-day incubation at 27 °C); however, the spreader shaved soil-derived fungal colonies, which were spread on the plate. Unfortunately, *C. albicans* could not grow evenly on the plate (Figure 1b). In inoculating method IV, *C. albicans*-containing agar was overlaid on the soil plates after the formation of soil-derived fungal colonies (2–3-day incubation at 27 °C); however, soil-derived fungal colonies were also diffused on the plate when *C. albicans*-containing agar was overlaid (Figure 1c).

Thus, *C. albicans*-inoculating methods I–IV were not suitable for our purpose; therefore, we tested another method of overlaying *C. albicans* on the soil plate by spraying (inoculating method V). In this method, it is expected that soil-derived fungal colonies remain intact, and that *C. albicans* can be overlaid evenly on the soil plate. To establish this method, the concentration of *C. albicans* suspension for spraying and the spraying time after formation of soil-derived fungal colonies were investigated.

Establishment of inoculating method V

Using the atomizer in this study, a 40-μl solution can be sprayed. Five concentrations of *C. albicans* suspension (40 μl, 1.0×10^5–5.0×10^6 CFU ml^{-1}) were tested for spraying on the soil plate and the growth of *C. albicans* was compared after 1–3-day incubation at 27 °C. Figure 2 shows *C. albicans* colonies sprayed on the agar plate (without soil) at the five concentrations 1 day after inoculation. *C. albicans* grew rapidly, so 1-day incubation was enough for *C. albicans* to form colonies. As shown in Figure 2, about 1000 and 2000 colonies of *C. albicans* had formed on the plates 1 day after inoculation by spraying at (c) 5.0×10^5 and (d) 1.0×10^6 CFU ml^{-1}, respectively. These two concentrations appeared appropriate to observe antagonistic effects.

The conditions were established accordingly; that is, in inoculating method V, soil plates were prepared by plating method A or B and incubated at 27 °C. After 2- or 3-day incubation to form soil-derived fungal colonies, *C. albicans* suspension (40 μl, 5–10 $\times 10^5$ CFU ml^{-1}) was sprayed on the soil plates in the plastic bag installed in the biological safety cabinet (Figure 3). After 1-day incubation at 27 °C, fungal colonies showing antagonistic effects on the growth of *C. albicans* were selected (arrowed colony (a) in Figure 4).

Screening result

From 18 soil samples, 151 fungal strains that showed antagonistic effects on the growth of *C. albicans* were selected (Table 1); therefore, they were re-cultured in the liquid medium to confirm whether their culture broths exhibited an inhibition zone against *C. albicans*. Among them, the culture broths of 26 strains were reproduced to show an inhibition zone against *C. albicans*.

Taxonomy of selected 26 strains

The results of the taxonomy of 26 selected fungi by morphological observation are summarized in Table 1. They were identified as *Penicillium* sp. (16 strains), *Trichoderma* sp. (3), *Cylindrocarpon* sp. (2), *Aspergillus* sp. (1), *Metarhizium* sp. (1), *Humicola* sp. (1), *Acremonium* sp. (1) and *Beauveria* sp. (1).

DISCUSSION

In this study, we established an efficient and rapid method for directly isolating fungi from soil samples that can produce anti-*C. albicans* antibiotics. As described in inoculating method V, after soil-derived colonies had formed on the plates (on days 2–3), *C. albicans* (40 μl, 5–10 $\times 10^5$ CFU ml^{-1}) was overlaid by spraying on the plates. After 1-day incubation, soil-derived fungi that showed antagonistic effects on the growth of *C. albicans* were selected. By this method, 151 candidate fungi from 18 soil samples showing antagonistic effects were selected within a week and, among them, 26 fungi exhibited anti-*C. albicans* activity in their re-culture broths. Thus, this method permits us to obtain antifungal antibiotic-producing fungi from soil samples in a shorter time (2 weeks vs 5–7 weeks) and at a higher rate (17.2% vs 3.1%) than the traditional method. The 151 candidate fungi were re-cultured in one liquid medium; therefore, if they were cultured in other media, the hit rate would increase. As summarized in Table 1, *Penicillium* was most highly selected as an antifungal-producing genus (16/26), followed by *Trichoderma* (3/26). Using the traditional method carried out by our group in 2008, 730 fungi were isolated from 18 soil samples. *Penicillium* was the most highly isolated genus (173), followed by *Paecilomyces* (100) and *Trichoderma* (93) (unpublished data); therefore, it is intriguing that two *Cylindrocarpon* strains were selected as anti-*C. albicans* antibiotic-producing fungi by

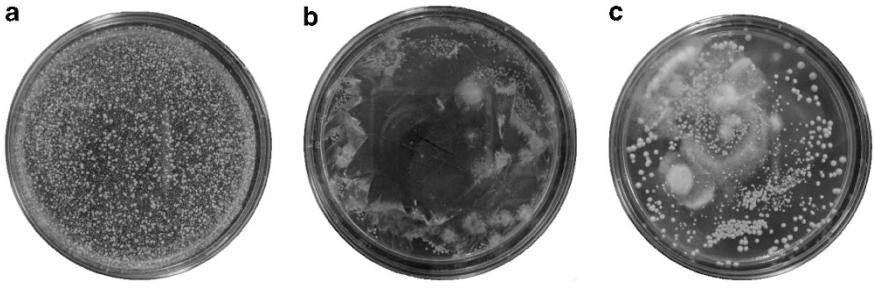

Figure 1 Inoculation of *C. albicans* into soil plates. (**a**) Inoculating method I: the plate was covered with *C. albicans*, and soil-derived fungi could not form colonies. (**b**) Inoculating method III: The spreader-shaved soil-derived fungal colonies and *C. albicans* could not grow evenly on the plate. (**c**) Inoculating method IV: soil-derived fungal colonies were diffused on the plate by *C. albicans*-containing agar. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

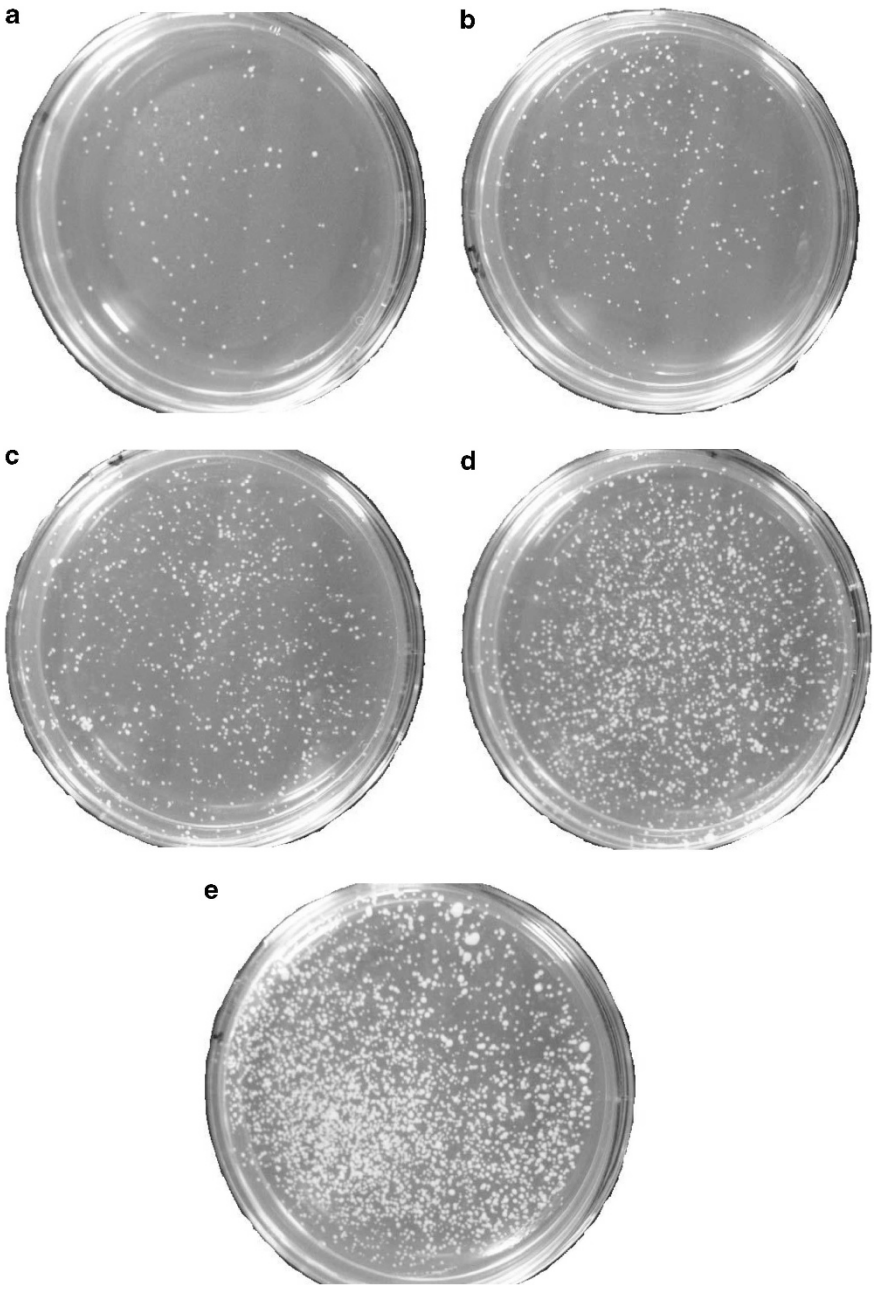

Figure 2 *C. albicans* grown on agar plates by spraying. The concentrations of the *C. albicans* suspension were (**a**) 1.0×10^5, (**b**) 2.5×10^5, (**c**) 5.0×10^5, (**d**) 1.0×10^6 and (**e**) 5.0×10^6 CFU ml^{-1}. The plates were inoculated for 1 day. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

this method. In the accompanying study,[20] we describe that a known antifungal antibiotic, ascochlorin, was isolated from the culture broth of one *Cylindrocarpon* strain. It is expected that this method will be applicable for isolating soil-derived microorganisms (fast-growing/slow-growing fungi and actinomycetes) showing antagonistic effects on other pathogenic microorganisms.

MATERIALS AND METHODS

Soil samples

We used 18 soil samples. Eighteen samples (No. 1–18) were collected in Kanagawa and Tokyo (Japan).

Media for fungal isolation

Four media were used for isolation of fungi from samples (soil). Kanamycin (Meiji Seika Pharma, Tokyo, Japan) was added to all media to protect against bacteria growth in soil samples.

Medium 1 contains sucrose 2.0%, glucose 1.0%, corn steep powder (Marcor Development, Carlstadt, NJ, USA) 0.5%, bonito extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) 0.5%, KH_2PO_4 0.1%, $CaCO_3$ 0.3%, kanamycin 0.1 mg ml^{-1} and agar 1.5%, adjusted to pH 6.0 before sterilization.

Medium 2 contains soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast (Fermipan, GB Ingredients, Dordrecht, Zuid-Holland, Netherlands) 3.0%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, kanamycin 0.1 mg ml^{-1} and agar 1.5%, adjusted to pH 6.5 before sterilization.

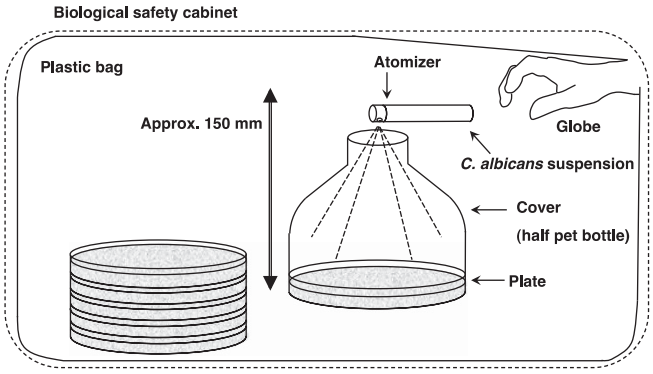

Figure 3 Facilities for inoculation of *C. albicans* to soil plates. In inoculating method V, *C. albicans* suspension (40 µl) was sprayed on the soil plates kept in the plastic bag installed in the biological safety cabinet.

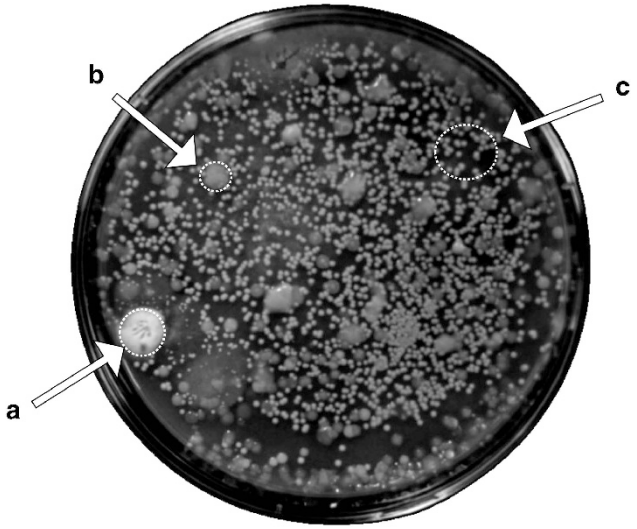

Figure 4 Selection of soil-derived fungi showing antagonistic effects on *C. albicans*. Soil plates were prepared by plating method A. After 2-day incubation to form colonies, *C. albicans* suspension (40 µl, 1.0×10^6 CFU ml^{-1}) was sprayed on the soil plates by Inoculating method V. (**a**) A fungal colony showing antagonistic effect on *C. albicans* was selected. (**b**) A fungal colony showing no antagonistic effect on *C. albicans*. (**c**) Colonies of *C. albicans*.

Medium 3 contains non-glutinous brown rice (Cigario Co., Ltd., Tokyo, Japan) 3.0%, glucose 0.5%, glycerol 0.5%, yeast extract (Oriental Yeast, Tokyo, Japan) 0.2%, potato dextrose broth (Becton Dickenson, Sparks, MD, USA) 0.2%, ammonium acetate 0.2%, $NaNO_3$ 0.03%, KCl 0.005%, $MgSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $ZnSO_4 \cdot 7H_2O$ 0.0001%, $CuSO_4 \cdot 5H_2O$ 0.00005%, kanamycin 0.1 mg ml^{-1} and agar 1.5%, adjusted to pH 6.0 before sterilization.

Medium 4 contains sucrose 3.0%, soluble starch 3.0%, malt extract (BD) 1.0%, brewer's yeast (Asahi Food and Healthcare, Tokyo, Japan) 0.3%, KH_2PO_4 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, kanamycin 0.1 mg ml^{-1} and agar 1.5%, adjusted to pH 6.0 before sterilization.

Soil plating methods

Two soil (or sample) plating methods, A and B, were tested on each medium.

Plating method A: A soil sample (15 mg) was directly placed in a plastic plate (90 $\phi \times 15$ mm; Kanto Chemical, Tokyo, Japan). Each melting (at 45–50 °C) medium (15 ml, media 1–4) was poured into the plate and mixed well to disperse the sample. The soil plate was incubated at 27 °C for 2–3 days to form fungal colonies.

Table 1 Summary of isolation of anti-*C. albicans* antibiotic-producing fungi from soil samples

Soil sample	Plating method	Medium	Number of selected strains	Number of reproduced fungi	Genus
1	A	3	7	2	*Cylindrocarpon* (2)
2	A	3	6	0	—
3	A	1	8	3	*Aspergillus* (1)
					Penicillium (2)
4	B (1:10)	1	9	2	*Trichoderma* (1)
					Penicillium (1)
5	A	1	7	4	*Trichoderma* (1)
					Penicillium (3)
6	A	4	16	5	*Metarhizium* (1)
					Humicola (1)
					Trichoderma (1)
					Penicillium (2)
		2	2	1	Acremonium (1)
7	A	4	12	3	*Penicillium* (3)
8	A	4	3	0	—
9	B (1:10)	4	12	0	—
10	A	4	3	1	*Penicillium* (1)
11	B (1:10)	3	10	1	*Beauveria* (1)
12	A	3	6	0	—
13	A	4	13	0	—
14	B (1:10)	4	8	0	—
15	A	2	10	0	—
16	A	3	3	1	*Penicillium* (1)
17	A	4	7	0	—
18	A	3	9	3	*Penicillium* (3)
Total			151	26	

Plating method B: A soil sample (1.0 g) was suspended in Winogradsky buffer (10 ml) (K_2HPO_4 0.38%, KH_2PO_4 0.12%, $MgSO_4 \cdot 7H_2O$ 0.51%, NaCl 0.25%, $Fe_2(SO_4)_3 \cdot nH_2O$ 0.005% and $MnSO_4 \cdot 5H_2O$ 0.005%) by shaking vigorously to make a series of dilutions (1:10–1:1000). Each diluted suspension (200 µl) was placed on the surface of each medium (15 ml, media 1–4 in a plastic plate) (90 $\phi \times 15$ mm) and spread evenly over the surface with a sterilized bacteria spreader. The soil plates were incubated at 27 °C for 2–3 days to form fungal colonies.

C. albicans-inoculating methods

C. albicans ATCC 64548 was used as a pathogenic fungus. A loopful of *C. albicans* from potato dextrose agar (BD) slant (7.5 ml, in a tube with screw cap, $9 \times 16 \times 150$ mm^3; Asahi Glass, Tokyo, Japan) was inoculated in Waksman medium (10 ml, peptone 0.5%, meat extract 0.5%, NaCl 0.3% and glucose 2.0%, adjusted to pH 7.0 before sterilization) and incubated at 27 °C for 2 days, reaching 1.0×10^9 CFU ml^{-1} concentration. This original culture was directly used as a *C. albicans* suspension. Five *C. albicans*-inoculating methods, I–V, were tested.

Inoculating method I: The original *C. albicans* culture (15 µl, 1.0×10^9 CFU ml^{-1}) was directly added to each soil plate at the same time when they were prepared according to plating method A.

Inoculating method II: The original *C. albicans* culture (15 µl, 1.0×10^9 CFU ml^{-1}) was directly placed in plastic plates (90 $\phi \times 15$ mm). Each melting (at 45–50 °C) medium (15 ml, media 1–4) was poured into the plate and mixed well to disperse the *C. albicans*. Soil samples were overlaid according to plating method B.

Inoculating method III: The original *C. albicans* culture was diluted with sterilized saline to adjust to 1.0×10^5 CFU ml^{-1}. The suspension (100 µl) was placed on the surface of the soil plates previously prepared according to plating

methods A and B, and spread evenly over the surface with a sterilized bacteria spreader.

Inoculating method IV: The original *C. albicans* culture was diluted with 0.8% agar at 45–50 °C to adjust to 1.0×10^4 CFU ml^{-1}. Two milliliters of the diluted *C. albicans* suspension was overlaid on soil plates previously prepared according to plating methods A and B.

Inoculating method V: The original *C. albicans* culture was diluted with sterilized saline to adjust to 5–10×10^5 CFU ml^{-1}. The suspension (40 µl) was overlaid by spraying from heights of about 150 mm onto soil plates prepared by plating methods A and B (Figure 4). The atomizer was purchased from Ryohin Keikaku (Tokyo, Japan). With one push of the atomizer, a 40-µl solution was sprayed. Inoculated plates were incubated at 27 °C for 1 day. Fungi showing antagonistic effects (inhibition zone) on the growth of *C. albicans* were selected.

Taxonomical identification of fungi

Taxonomical identification[21,22] of fungi was carried out by observing the precise arrangement of the conidiophores and the way in which spores were produced (conidial ontogeny) by fungi grown on potato dextrose agar and potato carrot agar[23] (potato 2.0%, carrot 2.0% and agar 2.0%). For this, the slide culture method was adopted.[24] Potato dextrose agar and potato carrot agar blocks (5×5 mm^2) of the cultures were cut out aseptically, and placed on sterile microscope slides (26×76 mm^2; Toshinriko, Tokyo, Japan). Four edges of the agar block were inoculated with fungal strains. Inoculated agar blocks were covered with a sterile micro cover glass (18×18 mm^2; Matsunami Glass, Osaka, Japan) and incubated in moist chambers at 25 °C for 5 days. Cover slips were carefully removed from the agar blocks and mounted in a drop of Shear's Mounting Medium (potassium acetate 1.0%, glycerol 20% and EtOH 30%) on a clean glass slide. The slide was sealed with transparent nail polish. The structure and branching pattern of conidiophores were observed under a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan).

Fermentation

Fungi from soil samples were maintained on Miura's medium (LCA) (glycerol 0.10%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02% and agar 1.5%, adjusted to pH 6.0 before sterilization). To confirm the antifungal antibiotics produced by selected strains, a loopful of strains from LCA slant was inoculated into a test tube containing one of the media (10 ml, media 1, 2, 3 or 4 without kanamycin and agar). The tube was incubated on a rotary shaker (300 r.p.m.) at 27 °C for 6 days. After treatment of the culture broth (10 ml) with EtOH (10 ml), the mixture was centrifuged to obtain the EtOH extracts. Anti-*C. albicans* activity was investigated by the agar diffusion method using paper disks.

Assay of anti-*C. albicans* activity

Anti-*C. albicans* activity was measured by the established agar diffusion method[25–27] using paper disks (8 mm, Advantec, Tokyo, Japan) containing EtOH extracts of isolated fungi (60 µl). The original *C. albicans* culture (15 µl, 1.0×10^9 CFU ml^{-1}) was directly placed on plastic plates (90 $\phi \times 15$ mm). Melting (at 45–50 °C) GY agar (15 ml, glucose 1.0%, yeast extract 0.5% and agar 0.8%, adjusted to pH 6.0 before sterilization) was poured into the plate and mixed well to disperse *C. albicans*. The *C. albicans* plate was incubated at 27 °C for 24 h.

Traditional isolation method

The traditional isolation method was carried out by using the established method.[28] Soil plates were prepared by the same method (plating methods A and B) using other media. Soil-derived fungi, isolated from the soil plates after 2- or 3-day incubation at 27 °C, were re-cultured on LCA and YpSS (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 2.0%, adjusted to pH 6.0 before sterilization) plated for 2 weeks. After duplication of the fungal strains, fungi were re-cultured in four liquid media (media 1–4) by using the same method as described above.

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