MYCOBIOLOGY
2020, VOL. 48, NO. 4, 326–329
https://doi.org/10.1080/12298093.2020.1766648

RESEARCH NOTE

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The Antifungal Test: An Efficient Screening Tool for the Discovery of Microbial Metabolites with Respiratory Inhibitory Activity

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ABSTRACT

Valuable natural compounds produced by a variety of microorganisms can be used as lead molecules for development of new agrochemicals. Furthermore, high-throughput *in vitro* screening systems with specific modes of action can increase the probability of discovery of new fungicides. In the current study, a rapid assay tested with various microbes was developed to determine the degree of respiratory inhibition of *Saccharomyces cerevisiae* in two different liquid media, YG (containing a fermentable carbon source) and NFYG (containing a non-fermentable carbon source). Based on this system, we screened 100 fungal isolates that were classified into basidiomycetes, to find microbial secondary metabolites that act as respiratory inhibitors. Consequently, of the 100 fungal species tested, the culture broth of an IUM04881 isolate inhibited growth of *S. cerevisiae* in NFYG medium, but not in YG medium. The result is comparable to that from treatment with kresoxim-methyl used as a control, suggesting that the culture broth of IUM04881 isolate might contain active compounds showing the inhibition activity for respiratory chain. Based on the assay developed in this study and spectroscopic analysis, we isolated and identified an antifungal compound (-)-oudemansin A from culture broth of IUM04881 that is identified as *Oudemansiella venosolamellata*. This is the first report that (-)-oudemansin A is identified from *O. venosolamellata* in Korea. Taken together, the development of this assay will accelerate efforts to find and identify natural respiratory inhibitors from various microbes.

ARTICLE HISTORY
Received 24 February 2020
Revised 10 April 2020
Accepted 30 April 2020

KEYWORDS
High-throughput screening system; oudemansin; phytopathogenic fungi; respiratory inhibitor; *Saccharomyces cerevisiae*

Natural compounds showing antifungal activities have become the basis for synthesis of commercial fungicides. For example, the antifungal compounds strobilurins and structurally close oudemansins, were first discovered in the wood rotting basidiomycete fungus *Strobilurus tenacellus* in the 1960s and in *Oudemansiella mucida* in the late 1970s, respectively [1]. These compounds are respiratory inhibitors belonging to the larger group of Quinone outside Inhibitors (QoI), which inhibit generation of ATP by binding cytochrome bc_1 complex in the electron transfer chain [2,3]. Since their discovery, natural strobilurins have been modified to identify analogous compounds with improved antifungal activity, stability, and dissemination, because those compounds are considered as lower-risk molecules for human health and environment. Thus, valuable natural compounds can be used as lead molecules for development of new agrochemicals.

In this study, we developed effective *in vitro* screening methods for identification of microbial metabolites showing respiratory inhibition, based on the inhibition of growth of *Saccharomyces cerevisiae* using a non-fermentable carbon source such as glycerol and lactate in a medium [4]. When a QoI fungicide is treated to *S. cerevisiae* grown in a medium containing a fermentable carbon source, the fungicide does not have much effect on growth inhibition. This is because *S. cerevisiae* is able to grow by producing ATP through anaerobic glycolysis, even though mitochondrial respiration of *S. cerevisiae* is inhibited by QoI fungicides [4,5]. Therefore, non-fermentable carbon sources can be used for screening of substances that inhibit mitochondrial respiration.

To find microbial secondary metabolites that act as respiratory inhibitors, we screened 100 fungal isolates classified into basidiomycetes, which were deposited in the "Culture Collection of Mushrooms" at Incheon National University, Korea. Each fungal isolate was maintained in potato dextrose agar (PDA) medium, and for preparation of culture

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Supplemental data for this article can be accessed here.

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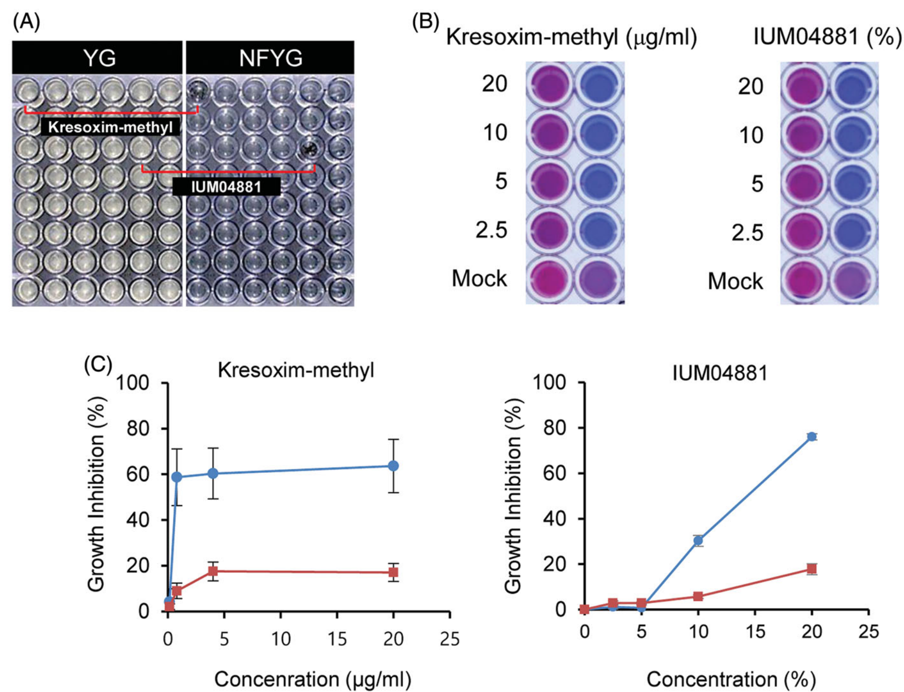

Figure 1. Effects of IUM04881 culture broth on growth of *Saccharomyces cerevisiae* in YG and NFYG medium. (A) Growth inhibition by representatives of fungal culture filtrates (20%, v/v). Photographs were taken one day after treatment. A QoI fungicide kresoxim-methyl was used as a positive control. (B) Visualization of growth inhibition. At each panel, left columns indicate *S. cerevisiae* cultures grown in YG medium, and right columns indicate *S. cerevisiae* cultures grown in NFYG medium. Prestoblue reagent (10%; Invitrogen) was added directly to the *S. cerevisiae* cultures, and then the cultures were incubated for 2 h at 37 °C. Mock indicates a non-treatment control. (C) Growth inhibition based on the optical density (OD_{600}). Blue line, growth inhibition of *S. cerevisiae* grown in NFYG medium; Red line, growth inhibition of *S. cerevisiae* grown in YG medium. The experiment was conducted twice with three replicates.

broth, five agar plugs punched with 8 mm diameter cork border were inoculated into 250 ml of potato dextrose broth (PDB) and incubated at 25 °C for 3 weeks. Each culture was filtrated through four layers of cheese cloth and then added to a 96-well plate in a final concentration of 5–20% (v/v) with 1% of *S. cerevisiae* ($OD_{600} = 0.3$). The growth inhibition of *S. cerevisiae* was compared according to the type of media: YG or NFYG. The YG medium consists of 1% yeast extract and 2% glucose, and is available for ATP production by glycolysis and mitochondrial respiration. In contrast, NFYG medium consists of 1% yeast extract and 1% glycerol, and is capable of supporting respiration only. Distilled water and the QoI fungicide kresoxim-methyl were used as negative and positive controls, respectively. One day after treatment of the culture broth, the optical density (OD_{600}) of each well was recorded using a microplate reader. Growth inhibition (%) of *S. cerevisiae* was calculated as $[1 - (OD_{600} \text{ of treatment}/OD_{600} \text{ of control})] \times 100$.

As an initial screening, the culture filtrates (20%, v/v) of 100 fungal species were investigated for their ability of growth inhibition against *S. cerevisiae grown* in YG and NFYG medium. Our results showed that the culture broth of an IUM04881 isolate exclusively inhibited growth of *S. cerevisiae* in NFYG medium, but not in YG medium, which is comparable to that from treatment with kresoxim-methyl (Figure 1(A)). For the visualization of cell viability, Prestoblue reagent (Invitrogen, Carlsbad, CA, USA) was added directly to the *S. cerevisiae* cultures, because metabolically active cells are able to change dye color from blue to red in which resazurin is converted to fluorescent resorufin [6]. Our results showed that the reagent did not change its color in the *S. cerevisiae* cultures of NFYG medium treated with the IUM04881 culture filtrate, which is comparable to that from treatment with kresoxim-methyl. However, all culture filtrate treatment to the *S. cerevisiae* cultures grown YG medium exhibited red color with Prestoblue reagent (Figure 1(B)). Therefore, the colorimetric assay supports that the IUM04881 culture filtrate has much effect on growth inhibition of *S. cerevisiae* cultures of NFYG medium. Although the reagent did not change visually its color in the *S. cerevisiae* cultures of NFYG medium by the treatment of the IUM04881 culture filtrate (2.5% and 5%, v/v), we observed the growth of *S. cerevisiae* in the 2.5% and 5% culture filtrate treatment compared to the 10% and 20% culture filtrate treatment. Based on the optical density (OD_{600}), the growth inhibition values for the 10% and 20% treatment of IUM04881 culture filtrate against *S. cerevisiae* grown in NFYG medium were 30% and 76%, respectively, whereas the growth

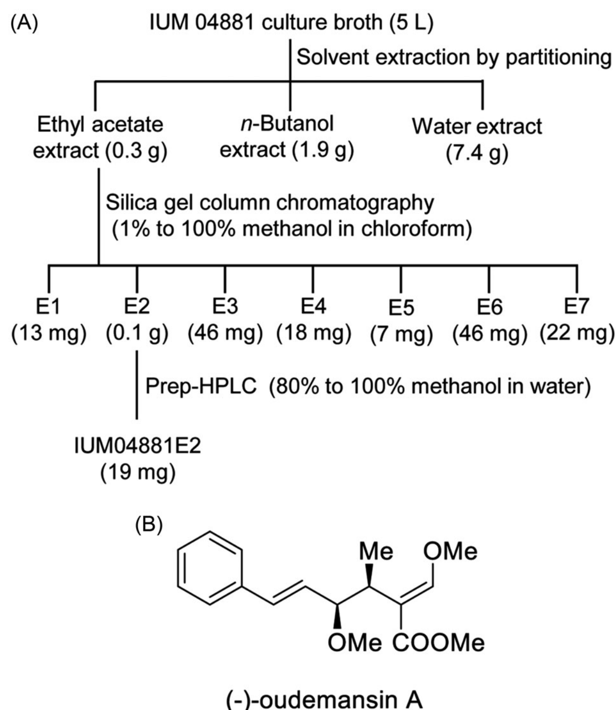

Figure 2. Isolation and chemical structure of an active compound derived from fungal IUM04881 isolate. (A) Procedure for isolation of active compounds showing activity of respiratory inhibition from culture broth of IUM04881 isolate. (B) Chemical structure of an IUM04881E2 compound.

inhibition values for all the other treatments against *S. cerevisiae* grown in YG medium were less than 18% (Figure 1(C)). Together, these results suggest that the culture broth of IUM04881 isolate might contain active compounds showing the inhibitory activity for respiratory chain.

To identify active compounds from an IUM04881 isolate, culture broth (5 L) of IUM04881 was filtered through cheese cloth. Then, the filtered culture broth was successively extracted with equal volumes of ethyl acetate and *n*-butanol. Ethyl acetate extract (0.3 g) was subjected to silica gel (70–230 mesh; Merck, Darmstadt, Germany) column chromatography with elution of a gradient of chloroform/methanol (99:1–0:100, v/v), to afford seven fractions (E1–E7) (Figure 2(A)). Based on *in vitro* yeast assay in the NFYG and YG media, fraction E2 (0.1 g) was determined to contain active compounds. Consequently, a pure compound IUM04881E2 (19 mg) was purified using a Shimadzu LC-6AD HPLC system (Kyoto, Japan) equipped with a Polaris C18-A column (250×21.2 mm, 10 μm; Agilent, Santa Clara, CA, USA) and a SPD-M10Avp photodiode array detector. The column was eluted at a flow rate of 5 ml/min with 80–100% aqueous methanol, over a linear gradient for an uninterrupted interval of 50 min.

The chemical structure of IUM04881E2 was determined using electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) spectroscopy. The ESI-MS data of IUM04881E2 was recorded on a single-quadruple mass spectrometer equipped (Acquity QDa; Waters, Manchester, UK). The ^{1}H-NMR spectrum was measured using a Bruker Avance 300 spectrometer (Burker BioSpin, Rheinstetten, Germany) in a chloroform-*d* (Cambridge Isotope Laboratories, Tewksbury, MA, USA). Chemical shifts were referenced to trimethylsilane. IUM04881E2 showed an ESI-MS spectrum having a quasi-molecular ion at m/z 313 $[M+Na]^+$. After comparison of the spectroscopic data with the literature [7], the compound IUM04881E2 was identified as (-)-oudemansin A (Figure 2(B) and Supplementary Table 1).

For identification of IUM04881 isolate, the ITS region was amplified from gDNA of IUM04881 isolate, and resulting sequences (616 bp of amplicon) were searched using the BLASTn program of NCBI (http://www.ncbi.nlm.nih.gov). Pairwise sequence similarities of the ITS region were determined with the most closely related strains using the BLASTn analysis. The sequences were aligned using ClustalW implemented in MEGA version 7, and distances were estimated based on the model of Tamura and Nei [8]. A phylogenetic tree was generated using the neighbor-joining method [9] with 1,000 bootstrap analyses (Supplementary Figure 1). Phylogenetic analysis showed that the IUM04881 isolate belongs to genus *Oudemansiella* and mostly relates to *Oudemansiella venosolamellata* SU200510011 (AB688120) and *O. venosolamellata* SU20100425 (AB688121) with 99.8% similarity, which resulted in that the IUM04881 was identified as *O. venosolamellata.*

It has been reported that oudemansins were first discovered in *O. mucida* in the late 1970s [1]. Given that the culture filtrate of *O. venosolamellata* IUM04881 showed the activity of respiratory inhibition and contained (-)-oudemansin A, we found another *O. venosolamellata* isolate (IUM02548) from 100 fungal isolates used in this study (data not shown). Based on *in vitro* yeast assay in the NFYG and YG media, we observed that the culture filtrate of IUM02548 isolate does not have a significant effect on respiratory inhibition (data not shown), suggesting that the activity of respiratory inhibition seems not commonly found in all of *Oudemansiella* species. Indeed, only few *Oudemansiella* species such as *O. mucida* and *O. canarii* have been reported to be a producer of oudemansins [10,11]. To our knowledge, this study is the first report that (-)-oudemansin A is identified from *O. venosolamellata* in Korea.

In the current study, a rapid assay tested with various microbes was developed to determine the

degree of respiratory inhibition of *S. cerevisiae* in two different liquid media, YG and NFYG. Further, the identification of oudemansin A from *O. venosolamellata* will make this approach accessible for a wide variety of fungicide research. The development of this assay will accelerate efforts to find and identify natural respiratory inhibitors from various microbes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This study was financially supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) [2019R1A2C1008103].

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