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THE USE OF SECONDARY METABOLITE PROFILING FOR EFFICIENT DIFFERENTIATION OF STATIN-PRODUCING SOIL MICROFUNGI ISOLATED FROM LITTLE-EXPLORED HABITATS

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ABSTRACT. *Soil microfungi are considered unique sources of different biotechnologically valuable products, including natural statins. In the present study, five statin-producing fungal cultures were used to evaluate secondary metabolite profiling to aid fungal isolates' differentiation. These microfungi were previously isolated from uncultivated and cultivated soils collected in little-explored tropical ecosystems. Based on their phenotypic characteristics, isolated fungal cultures were initially identified as belonging to the genus Penicillium. For further differentiation of fungal isolates, a profile of secondary metabolites of each fungal strain was studied. The results of the evaluation of secondary metabolite profiling as an aid in efficient differentiation of the Penicillium isolates were further confirmed by molecular identification of these microscopic fungi. In conclusion, the current study has demonstrated that secondary metabolite profiling is a useful approach to reliable differentiation of statin-producing Penicillium species isolated from little-explored mangrove and oil palm plantation soils.*

KEYWORDS: *cholesterol-lowering agents, filamentous fungi, identification, soil, tropical ecosystems*

INTRODUCTION

Elevated plasma cholesterol level has long been recognized as a major risk factor for atherosclerosis leading to coronary heart disease or stroke. The biosynthesis of cholesterol in the human organism is a major contributing factor to hypercholesterolemia. Natural statins are a group of microbial

metabolites with a powerful inhibitory effect on 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the regulatory and rate-limiting enzyme in cholesterol biosynthesis (Manzoni & Rollini 2002, Barrios-González & Miranda 2010). The literature shows that several fungal genera, including *Aspergillus*, *Penicillium*, *Monascus*, *Trichoderma*, *Doratomyces*, *Paecilomyces*, *Scopulariopsis,* and *Pleurotus,* can synthesize natural cholesterol-lowering agents (Endo et al. 1986, Shindia 1997, Seydametova 2013).

Although microfungi are commonly distributed in soils, our knowledge of fungal diversity in tropical ecosystems such as mangrove forests and oil palm plantations is limited. There is a lack of information, particularly on the rhizosphere fungi of tropical plants, which, due to their diversity, include many unstudied fungal species that could provide new pharmaceutical compounds, including natural lipid-lowering agents (Carlile et al. 2001, Kirk et al. 2004).

Previously, several potent statin-producing microfungi were isolated by our research group from the rhizosphere of plants naturally growing in mangrove forests and from peat soil samples collected in an oil palm plantation in the Pahang State of Malaysia. The overall aim of the present study was to characterize these fungal isolates and evaluate secondary metabolite profiling to aid in their differentiation.

Identifying microfungi has always been difficult, and many misunderstandings and misidentifications can be found in the literature (Mantle 1987). A literature survey showed that traditionally, mycologists use primarily morphological criteria for fungal identification. However, morphological features can overlap or be difficult to record precisely, as they may depend on different brands of the media used (Samson & Pitt 2000). Moreover, as new species are described each year, it might be difficult to obtain up-to-date keys for large fungal genera. Therefore, applying more complex approaches, such as secondary metabolite profiling and molecular biological methods, is necessary to provide more reliable differentiation and further identification of fungal isolates at the species level.

Microorganisms, and in particular microfungi, are known to produce a vast number of secondary metabolites. For some industrially important fungal genera, the production of these compounds has been demonstrated to be very consistent at the species level (Larsen et al. 2005). It should be noted that previously, fungal taxonomy based on secondary metabolites

has been used successfully in large genera such as *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium,* and others (Frisvad et al. 2008). Therefore, we hypothesized that secondary metabolite profiling could be used to differentiate fungal strains isolated from different natural samples collected in little-explored tropical habitats.

MATERIALS AND METHODS

Isolation of microfungi

Fungal strains were isolated from mangrove and oil palm plantation soils in the Pahang State (Malaysia) following the soil-plate method of Warcup (1950). The strains ESF2M and ESF19M were isolated from clay soil collected from the rhizospheres of *Rhizophora apiculata* and *Bruguiera gymnorrhiza* in the mangrove forest in Kuantan, respectively. The strains ESF20P, ESF21P, and ESF26P were isolated from peat soil samples collected from a 15-year-old oil palm plantation in Gambang.

Phenotypic characterization of fungal isolates

Microfungi, initially isolated by plating collected soil samples on Potato-Dextrose Agar, were transferred to appropriate diagnostic agar media and identified to the genus level as described earlier (Seydametova 2021).

Micro-scale extraction of fungal secondary metabolites

The micro-scale extraction procedure for standardized determination of fungal metabolite production described by Smedsgaard (1997) was applied. The Yeast Extract Sucrose (YES) Agar medium employed was inoculated in a Petri dish at three points with a conidium suspension made from 7-day-old cultures grown on Czapek Yeast Autolysate (CYA) Agar (Svendsen and Frisvad 1994). Culture extracts were prepared by cutting three plugs of 6 mm in diameter from one colony using a cork drill as follows: one in the center of the colony, one at the rim of a colony as far away from other colonies as possible, and one at the rim of a colony as near to another colony as possible, to represent any variability within a colony. The plugs were transferred to a 1.5-mL disposable autosampler screw-cap vial, and 500 µL of the solvent mixture methanol-dichloromethane-ethyl acetate (1:2:3) containing 1 % (v/v) formic acid was added. The plugs were extracted ultrasonically for 60 min. The extract was transferred to a clean vial, evaporating the organic phase to dryness. Evaporation of the extraction solvent was necessary to make the sample compatible for injection into the high-performance liquid chromatography (HPLC) mobile phase, removing the ethyl acetate. The residues were re-dissolved ultrasonically for 10 min in 400 µL methanol containing 0.6 %

(v/v) formic acid, 0.02 % (v/v) hydrochloric acid and 2.5 % (v/v) water. All samples were filtered through 0.45-µm syringe filters into clean vials before analysis.

Analysis for secondary metabolite profiling

The HPLC analyses were performed on Agilent 1200 HPLC (Agilent Technologies, USA) using 10-µL injections and diode array detection (DAD). Separations were carried out on a 250×4.6 mm ID Zorbax Eclipse Plus C_{18} column (Agilent Technologies, USA) packed with 5 µm particles. The column was maintained at 40°C. A linear gradient starting from 85% water and 15% acetonitrile and increasing to 100% acetonitrile over 40 min, then maintaining 100% acetonitrile for 3 min, was used at a flow rate of 1 mL/min. Both eluents (water and acetonitrile) contained 0.005% (v/v) trifluoroacetic acid. Detection of compounds eluting from the HPLC column was made by UV detection (DAD) by using the absorbance of the column eluent at 280 nm with a bandwidth of 4 nm. The analytical conditions were kept as constant as possible to reduce the alignment needed. Secondary metabolites with identical UV-Vis spectra obtained from different fungal isolates were considered the same compounds. The full chromatogram as a profile was used for fungal identification without identifying the metabolites (Nielsen et al. 1999, Thrane et al. 2001).

The results from secondary metabolite profiling for the five isolates were combined in a single dendrogram with clusters containing the strains with similar profiles. Cluster analysis was performed using the software SPSS version 16.0, based on the retention times of the peaks using the unweighted centroid linkage method. The dendrogram was drawn based on the Euclidian distance.

Molecular identification of fungal isolates

Molecular identification of the fungal strains was carried out using universal fungal primers ITS1 and ITS4, following the procedure described earlier (White et al. 1990, Seydametova 2015). This allowed the fungal isolates to be identified to the species level.

RESULTS

Phenotypic characteristics of fungal isolates

It is well known that the colony appearance on the diagnostic agar media recommended for a particular group of microfungi and the structure of fungal conidiophores are valuable in determining the generic level. All fungal isolates used in the current study were characterized by filamentous growth on diagnostic agar media. Table 1 shows the cultural properties of

the examined isolates, and Table 2 describes their morphological features.

The examined phenotypic characteristics of the fungal isolates indicated that they were characterized by macromorphology (Table 1) and microscopic elements (Table 2) typical of the genus *Penicillium*.

Table 1. Cultural characteristics of fungal isolates on different diagnostic agar media (all measurements are presented as extremes; abbreviations: CD - colony diameter; COC - colony obverse color; CRC - colony reverse color; CZ - Czapek-Dox Agar; CYA - Czapek Yeast Autolysate Agar; MEA - Malt Extract Agar; YES - Yeast Extract Sucrose Agar)

Character	Fungal isolates				
	ESF2M	ESF19M	ESF20P	ESF21P	ESF26P
CZ					
CD, mm	11-12	8-10	16-17	11-13	17-18
COC	green	green	glaucous	white to cream	dull grayish-green
CRC	yellow	yellow	yellowish-brown	bright yellow	yellowish-brown
CYA					
CD, mm	19-20	19-20	26-27	24-27	31-32
COC	green	green	grayish-green	white to cream	grayish-green
CRC	yellow	yellow	yellowish-brown	yellow-orange	yellowish-brown
MEA					
CD, mm	14-15	14-16	38-40	50-52	40-42
COC	green	green	greenish-gray	pale bluish-green	greenish-gray
CRC	yellow	yellow	yellowish-brown	yellow-orange	yellowish-brown
YES					
CD, mm	22-25	20-23	33-34	29-35	26-27
COC	green	green	glaucous	grayish blue-green	glaucous
CRC	yellow	yellow	yellowish-orange	yellow-orange	yellowish-orange

Table 2. Morphological characteristics of fungal isolates

Character	Fungal isolates				
	ESF2M	ESF19M	ESF20P	ESF21P	ESF26P
Conidiophore pattern	monoverticillate				
Stipe length (µm)	21.1	67.0	39.4	31.1	31.0
Phialide shape	ampulliform	ampulliform	ampulliform	flask-shaped	ampulliform
Phialide length (µm)	11.2	7.8	10.8	11.7	8.6
Conidia size (µm)	2.0×1.8	2.2×2.0	2.0×1.5	2.3×2.3	2.0×1.2

Differentiation of fungal isolates using secondary metabolite profiling

In addition to the morphological features, the ability of microfungi to produce certain secondary metabolites is also considered a taxonomically significant criterion by fungal taxonomists. Therefore, in the present study, secondary metabolite profiling was evaluated to aid in the differentiation of statin-producing fungal isolates. The chromatograms (plots of absorbance versus time) showed peaks representing the compounds eluting from the HPLC column. Fungal isolates were clustered based on the chromatographic profiles obtained by comparing retention times in UV spectra detected at 280 nm. In the dendrogram, a clear separation of fungal strains and the relationship between them are apparent (Figure 1).

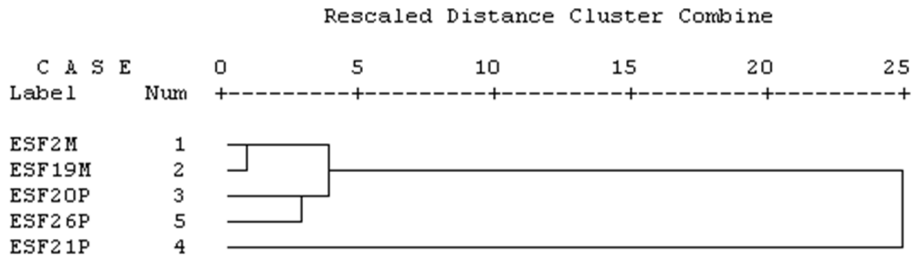

Figure 1. Dendrogram of the *Penicillium* isolates studied using Euclidean distance and centroid clustering method.

Molecular identification of fungal isolates

In recent times, molecular biological methods are also widely used in modern mycology to objectively identify microfungi up to species level. For the molecular approach, our strategy was to identify each statin-producing fungal isolate by means of its nucleotide sequence comparison of the ITS1-5.8S-ITS2 region of the rDNA with reference sequences. It should be noted that each fungal strain used in the current study amplified successfully, producing a single PCR product of the desired length, approximately 550 - 600 base pairs. The obtained sequence data were compared to sequences from known *Penicillium* species deposited in GenBank. Table 3 shows the fungal isolates that are most similar to each other based on comparisons of their ITS1-5.8S-ITS2 regions.

Table 3. Molecular identification of fungal isolates based on sequencing of the ITS1-5.8S-ITS2 region of rDNA

Fungal isolate	Closely related sequence	Query coverage	E value	Maximum similarity
ESF2M	*Penicillium citrinum* KACC43900 (EU821333.1)	96%	0.0	99%
ESF19M	*Penicillium citrinum* KACC43900 (EU821333.1)	95%	0.0	99%
ESF20P	*Penicillium janthinellum* CBS:340.48 (GU981585.1)	95%	0.0	98%
ESF21P	*Penicillium brefeldianum* 3352 (FJ527878.1)	96%	0.0	99%
ESF26P	*Penicillium janthinellum* P1 (JQ727998.1)	95%	0.0	98%

DISCUSSION

The identification of microfungi has always been considered not easy. Knowledge of the associated mycobiota of different habitats can help identify the most common fungal species. However, our knowledge of fungal diversity in little-explored tropical ecosystems such as mangrove forests and oil palm plantations is very limited (Carlile et al. 2001, Kirk et al. 2004). The overall aim of the present study was to characterize and differentiate statin-producing microfungi, which were isolated from mangrove and oil palm plantation soils collected in the Pahang State of Peninsular Malaysia.

According to the traditional mycological strategy, to characterize filamentous fungi isolated from little-explored habitats, it is necessary to identify them to the genus level before employing other complex approaches (Carlile et al. 2001). Microfungi have historically been determined to genus level based primarily on macroscopic and microscopic morphological characteristics. The examined phenotypic features of the fungal isolates used in the current study indicated that they were characterized by macromorphology (Table 1) typical of the genus *Penicillium*. Classical monographs and taxonomic keys describe the colonies of *Penicillium* species as typically being rapid growing, flat and filamentous (Pitt 1979, Samson & Pitt 2000, Turland et al. 2018). The colony's obverse color is initially white and becomes green, blue-green, gray-green, or olive-gray over time. The colony reverse is usually pale to

yellowish or brownish. It should be noted that, in addition to their cultural properties, all statin-producing fungal isolates were characterized by a monoverticillate conidiophore branching pattern and microscopic elements typical for the genus *Penicillium* (Table 2).

The literature shows that four groups of organisms are particularly good producers of secondary metabolites: plants, fungi, lichen, and actinomycetes. However, other organisms such as animals, yeasts, and protozoa are less efficient producers of these compounds. In addition to the morphological criteria, secondary metabolites are widely used in plant and fungal taxonomy, whereas chemotaxonomy is neglected in bacteriology (Frisvad et al. 2008).

In modern mycology, a secondary metabolite is a chemical compound produced by a limited number of fungal species in a genus. A profile of secondary metabolites consists of all the different compounds a fungus can produce on a given substratum (Frisvad et al. 2008). In its simplest form, metabolite profiling for fungal differentiation and further identification consists of three elements: 1) obtaining the metabolites (extraction), 2) determining the compounds or profiles (analysis), and 3) data processing (chemometrics). This includes all relevant metabolites needed for reliable differentiation and identification of a fungus, and profiles across multiple metabolites rather than single metabolites should be used for identification (Nielsen et al. 2004).

As mentioned above, the first step in metabolite profiling for fungal differentiation and further identification is to obtain the metabolites produced by the fungi growing on a defined substrate. Solid media are generally better than liquid media in terms of the quantity and number of metabolites produced. Moreover, on agar media, any contamination is usually visible, and an agar plug technique can be used to sample different parts of the fungal colony and its surroundings. Generally, agar media for optimal secondary metabolite production have been based on media containing yeast extract. Initially, YES broth was introduced as a semisynthetic broth medium. It was later shown to be a very effective general secondary metabolite production medium when used with a crude yeast extract and formulated as an agar medium (YES Agar) (Frisvad & Filtenborg 1983). This agar medium has been used for *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria,* and many other fungal genera. Other media, including CYA Agar, can be used depending on the considered genus (Thrane 2001, Nielsen et al. 2004).

Depending on the group of metabolites of interest, two main schemes can be applied to collect metabolites for a profile: 1) an extraction approach is used for the non-volatile metabolites in combination with HPLC or mass spectrometry (MS); 2) a headspace approach is used for the volatile metabolites in combination with gas chromatography (GC) or GC-MS (Nielsen et al. 1999, Thrane et al. 2001).

It should be noted here that Frisvad and Filtenborg (1983) first demonstrated the advantage of secondary metabolite profiling in fungal taxonomy within the genus *Penicillium*, using a simple agar-plug-thin layer chromatography (TLC) technique. Later, Frisvad and Thrane (1987) introduced a standardized HPLC method with DAD. Members of the same biosynthetic family usually have the same UV-Vis spectra. HPLC is the most common analytical technique used for determining non-volatile metabolites due to its versatility, relative ease of operation, and the broad spectrum of metabolites that can be determined directly. Metabolite profiling using HPLC is almost always carried out using gradient elution on reversed phase material (C_{18} phase or similar) with a polar mobile phase (water-acetonitrile or water-methanol) containing some form of modifier (e.g., trifluoroacetic acid). Detection of compounds eluting from the HPLC column is made by UV detection (DAD) by using the absorbance of the column eluent at a specific wavelength (280 nm). The full data matrix is then used for fungal differentiation and identification without identifying the metabolites (Nielsen et al. 1999, Thrane et al. 2001). Dendrogram constructed based on the Euclidean distance and centroid clustering method shows the relationship between clusters of statin-producing *Penicillium* isolates used in this study (Figure 1). Of the *Penicillium* strains included in this dendrogram, ESF2M and ESF19M fell in the same cluster, indicating that these two fungal cultures most probably belong to the same species. Moreover, the isolates ESF20P and ESF26P were grouped, justifying that they might be different strains of the same *Penicillium* species.

The isolate ESF21P did not fall into the clusters mentioned above, proving that it most probably belongs to a different *Penicillium* species.

In addition to other approaches, molecular biology has brought many powerful new tools to fungal taxonomists. These provide rapid identification of fungal isolates and do not rely on the presence of reproductive structures. DNA sequences provide large amounts of data that can be compared among microfungi and analyzed to determine sequence

relatedness, which is assumed to reflect phylogenetic relatedness among species. Nowadays, molecular biological methods can also distinguish between closely related species with few morphological differences and strains within a species (Carlile et al. 2001).

Analysis of the ITS1-5.8S-ITS2 region of the rDNA has been widely employed to characterize fungal species since it is highly conserved intraspecifically but variable between different species (Carlile et al. 2001). Therefore, in the present study, molecular identification of the statin-producing fungal isolates to species level was conducted based on nucleotide sequence analysis of this region.

From Table 3, it can be seen that the ITS1-5.8S-ITS2 sequences of amplicons from the isolates ESF2M and ESF19M showed the highest homology (99%) to that of *Penicillium citrinum* KACC43900 (EU821333.1). The nucleotide sequences of the isolates ESF20P and ESF26P showed 98% identity with the sequences of *Penicillium janthinellum* CBS:340.48 (GU981585.1) and *Penicillium janthinellum* P1 (JQ727998.1), respectively. The sequence of the ESF21P amplicon showed 99% identity to *Penicillium brefeldianum* 3352 (FJ527878.1). The results of molecular identification of the statin-producing *Penicillium* species examined in this study (Table 3) concur with the tree constructed based on secondary metabolite profiling data (Figure 1). This provides strong evidence that the proposed dendrogram is accurate.

CONCLUSIONS

In conclusion, examination of morphological features may not be sufficient for precise fungal characterization and classification. Therefore, additional techniques are necessary to provide reliable differentiation of different fungal species. In this study, we successfully evaluated different methods employed in modern mycology for fungal characterization and identification. It was highlighted that secondary metabolite profiling can be used for reliable differentiation of statin-producing *Penicillium* strains isolated from mangrove and oil palm plantation soils. While this report only presents one example, the secondary metabolite profiling approach used in this study could be employed to differentiate other newly isolated fungal strains efficiently.

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