

Mangrove Rhizosphere Soils: A Unique Natural Source of Pravastatin-Producing *Penicillium* Microfungi

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Abstract

The pravastatin-producing potential of rhizosphere soil microorganisms from mangrove forests has not been investigated in detail. In this study, a total of 20 *Penicillium* isolates were tested for pravastatin production. Six strains were able to synthesize pravastatin directly. Among these, the isolate *Penicillium* sp. ESF19M was the most active pravastatin producer with a yield of 28.43 mg/L. Molecular identification of this strain showed the highest homology with *Penicillium citrinum*.

Keywords: Mangrove rhizosphere soil, *Penicillium citrinum*, pravastatin, screening.

Introduction

Clinical and nutritional studies indicate that high cholesterol levels in the blood may be one of the major causes of atherosclerosis and coronary artery problems. In humans more than 50% of the total body cholesterol is derived from *de novo* synthesis. A major rate-limiting step in the cholesterol biosynthetic pathway is at the level of the microsomal enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Manzoni and Rollini, 2002). This enzyme therefore is a prime target for pharmacological intervention.

Statins are known to be potent HMG-CoA reductase inhibitors. This group of compounds can be divided into natural statins and statins of synthetic origin. The chemical structures of the synthetic statins are quite different from the natural forms. Only the HMG-

CoA-like moiety, responsible for HMG-CoA reductase inhibition, is common to both natural and synthetic statins (Manzoni and Rollini, 2002; Barrios-Gonzalez and Miranda, 2010). Due to their hypocholesterolemic activity, statins are currently considered as a class of extremely successful drugs that lower the cholesterol level in blood, decreasing the risk of heart attack or stroke (Maron et al. 2000). Amongst the range of natural statins produced by microorganisms, pravastatin has significant advantages in that it exhibits stronger and highly tissue-selective inhibition of cholesterol synthesis (Koga et al. 1990).

Investigations carried out since the 1970s have indicated the possibility of obtaining a wide range of natural statins as both the intermediate and final products of secondary microbial metabolism, or as products of biotransformation processes. Despite this, until now large-scale processes have been developed only for a few of them. Lovastatin and compactin are produced by fermentation, using microorganisms of different species belonging mostly to the genera *Aspergillus* and *Penicillium* (Endo et al. 1977; Shin-

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dia, 1997; Bazarra et al. 1998; Manzoni et al. 1998; Shaligram et al. 2008). Pravastatin is currently produced by microbial hydroxylation of its precursor, compactin (Hosobuchi et al. 1993; Wang et al. 2001). This bioconversion can be carried out by a number of microorganisms, belonging to the genera *Streptomyces*, *Actinomadura*, *Pseudonocardia* and others (Matsuoka and Miyakoshi, 1993; Demain et al. 1996; Lee et al. 2001; Lin et al. 2007). Although bioconversion of compactin to pravastatin is still considered as the most efficient method, one step de novo production of pravastatin could be an effective and economically viable alternative. Therefore, the search for new strains of microorganisms which are able to accumulate this natural statin directly has gained considerable momentum. The lipid-lowering activity of some secondary metabolites from different *Penicillium* species has focused the search for potent pravastatin producers amongst this taxonomic group of microfungi (Endo et al. 1977; Bazarra et al. 1998; Wang et al. 2001; Shaligram et al. 2008).

Mangrove forests host a unique variety of microorganisms, plants and animals. These natural systems have complex interactions with the surrounding environment wherein the growth of individual species is influenced by both physical and chemical characteristics of soil and seawater. In Malaysia, mangroves occupy 564,606 ha, with nearly 16% (91,779 ha) distributed along the west coast of Peninsular Malaysia (Shamsudin and Nasir, 2005). Mangroves are more prevalent here due to the sheltered environment in comparison to the east coast (where mangrove extent is 5,738 ha), which is exposed to the South China Sea (Mohd-Lokman and Sulong, 2001). Currently, these unique habitats are threatened by various human activities, in particular pollution and land use change through reclamation and development. Watson (1928) was perhaps the first to present an overview of the mangrove forests of the Malay Peninsula. In previous reports on the microbiota of natural mangrove ecosystems, most attention has been given to the examination of bacterial community composition (Bharath Kumar et al. 2008; Sakami et al. 2008). In the few available papers on the mycobiota of mangrove areas, some researchers focused on investigation of fungi on wood, whilst others have addressed the diversity of fungal endophytes isolated from mangrove plant species (Alias et al. 1995; Xiaoke Xing and Shunxing Guo, 2011). However, until now no substantial efforts have been made to as-

sess the statin-producing potential of rhizosphere soil mycobiota in mangrove forests. The rhizosphere is a site of complex interactions between plants and microorganisms, where environmental and plant growth factors may have large effects on the physiological properties and biochemical activities of rhizosphere-inhabiting microorganisms, including rhizosphere microfungi. Therefore, there is a need to examine the processes of secondary metabolite production by these microfungi. *Penicillium* species are one of the most commonly occurring soil microfungi associated with rhizosphere of many plants in natural systems (Carlile et al. 2001). We hypothesized that *Penicillium* strains isolated from rhizosphere soils of plants in mangrove forests may have ability to synthesize pravastatin directly. As the initial step in the search for microfungi capable of synthesizing pravastatin *de novo*, the objectives of this study were: (1) to isolate rhizosphere soil microfungi of the genus *Penicillium* from little-explored mangrove habitats, and (2) to assess their potential ability to produce pravastatin.

Materials and Methods

Soil Sampling

The present study was carried out with rhizosphere soils from the following mangrove plant species: *Acrostchum aureum*, *Avicennia alba*, *Avicennia marina*, *Bruguiera gymnorrhiza*, *Excocaria agallocha*, *Hibiscus tiliaceus*, *Rhizophora apiculata*, *Rhizophora mucronata* and *Sonneratia alba*. These plants occur naturally in mangrove forests of Pahang State, Malaysia. Eleven sampling sites were selected in Balok (4 sites), Cherating (4 sites) and Kuantan (3 sites) districts (**Table 1**). At each site, three replicates of each rhizosphere soil were taken from the surface layer at the depth of 10 cm, packed into labeled sterilized polyethylene bags using a sterilized spatula, and returned to the laboratory for further processing. There, the three replicates of each rhizosphere soil were pooled together to form a composite sample and stored in sterilized polyethylene bags at 4 °C. These soil samples were used to determine the soil texture (Brady and Weil, 1999) and soil pH (Muniandy et al. 2009) in the laboratory.

Isolation and Morphological Characterization of Microfungi

Microfungi were initially isolated using the soil plate method of

Table 1. Rhizosphere soil sampling from different plants in mangrove forests

Sampling Site	Geographical Location	Plant Species
MFB1		<i>Avicennia alba</i>
MFB2	Balok	<i>Hibiscus tiliaceus</i>
MFB3	(3.94°-N 103.38°-E)	<i>Rhizophora apiculata</i>
MFB4		<i>Acrostchum aureum</i>
MFC1		<i>Excocaria agallocha</i>
MFC2	Cherating	<i>Sonneratia alba</i>
MFC3	(4.12°-N 103.38°-E)	<i>Rhizophora mucronata</i>
MFC4		<i>Avicennia marina</i>
MFK1	Kuantan	<i>Rhizophora mucronata</i>
MFK2	(3.79°-N 103.31°-E)	<i>Rhizophora apiculata</i>
MFK3		<i>Bruguiera gymnorrhiza</i>

Warcup (1950). A small amount of soil (5-15 mg) was dispersed in liquid (40 °C) potato dextrose agar (PDA) medium in a sterile Petri dish without prior suspension in water. Control and experimental plates were prepared in triplicate. Inoculated Petri dishes were incubated at 25 °C until the colonies were fully formed. No growth was observed on control plates containing PDA medium alone. Individual fungal colonies were picked and purified by streaking onto fresh PDA medium. After several cycles of picking and replating, separate colonies were transferred to PDA slants.

Microfungi, initially isolated by plating collected soil samples on PDA, were transferred to appropriate diagnostic agar media for identification (Pitt, 1979; Frisvad and Samson, 2004): Czapek-Dox agar (CZ), Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and yeast extract sucrose agar (YES). Cultures were incubated in the dark at 25 °C for 7 days. Fungal isolates were identified morphologically following Pitt (1979) and Frisvad and Samson (2004). The macroscopic features examined included colony diameter, obverse and reverse colony colours and presence of exudate droplets. The standard medium used for microscopic observations was MEA. Conidiophore branching patterns were examined using an optical microscope (Primo Star Carl Zeiss, Germany). These techniques allowed the fungal isolates to be identified at the genus level.

Fermentation Procedure

Twenty fungal isolates obtained from the soil samples were tested for their ability to produce mevastatin (precursor of pravastatin) and pravastatin. The fungal strains were maintained on PDA slants and incubated at 25 °C for 7 days. A spore suspension was prepared by suspending spores from the appropriate slant in 10 ml of sterilized distilled water containing 0.01% (v/v) Tween-80. The number of spores was counted using a haemocytometer after vortexing and appropriate dilution. Ten percent (2.0 mL, v/v) of the medium volume of the spore suspension (10^8 spores/mL) prepared from each slant was used to inoculate sterile screening medium containing: 3% (w/v) glucose, 3% (w/v) glycerol, 0.4% (w/v) peptone, 0.2% (w/v) NaNO_3 , 0.1% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH of the medium was adjusted to 6.5 with either 10% HCl or 10% NaOH before sterilization (Konya et al. 1998). Shake flask fermentation was carried out for each fungal culture in a 100-mL flask at 25 °C, 180 rpm, for 7 days in an incubator shaker.

Analytical Determination of Mevastatin and Pravastatin

Ethyl acetate extracts from the whole cell broths obtained according to the slightly modified extraction procedure of Manzoni et al. (1998 and 1999) were used for high-performance liquid chromatography (HPLC) analysis (Agilent 1200; Agilent Technologies, USA) of mevastatin and pravastatin. The pH value of the whole cell broth was adjusted to 3 ± 0.2 with 1M trifluoroacetic acid, and then an equal volume of ethyl acetate was added. Extraction was performed at 200 rpm and 30 °C for 1 h. The fermentation samples were subsequently filtered through a Whatman filter paper no. 41 and the organic phase of each sample was then collected. The ethyl acetate filtrates were then dried over anhydrous Na_2SO_4 followed again by filtration from the drying agent, and concentrated using a rotary evaporator to a final volume of 4 mL. Five microliters from the organic phase were then injected for HPLC analysis on a 250×4.6 mm ID Zorbax Eclipse Plus C18 column, 5 µm particle size (Agilent Technologies, USA). The mobile phase consisted of acetonitrile and water (60:40, v/v) with a pH value adjusted to pH

3 ± 0.2 by addition of 1M H_3PO_4 (Konya et al. 1998; Manzoni et al., 1999). The flow rate was maintained at 0.8 mL/min and detection was measured at 238 nm. HPLC grade mevastatin ($\geq 95\%$ purity) and pravastatin ($\geq 98\%$ purity) from Sigma-Aldrich (USA) were used as standards.

Dry Cell Weight Estimation

Dry cell weight (DCW) was measured by filtering the contents of each flask through a pre-weighed Whatman filter paper no. 41. The collected biomass was washed with distilled water and dried at 105 °C in a laboratory oven until constant weight.

Molecular Identification of the Selected Strain

The *Penicillium* sp. ESF19M strain selected in this study was maintained on a PDA slant. The pure isolate was cultured in a 100-mL conical flask containing 20 mL of CYA liquid medium. The culture was inoculated with a spore suspension and incubated at 25°C with shaking (150 rpm) in a microbiological incubator. Mycelial biomass from 2-day-old culture was harvested by filtration through Whatman filter paper no. 1 and used for DNA isolation. Genomic DNA was obtained using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, Canada), according to the manufacturer's instructions. The concentration of DNA, after dilution 25 times by mixing 1 µL of sample with 24 µL of molecular grade water, was determined at 260 nm using an Eppendorf Biophotometer Plus (Eppendorf AG, Germany). Then, the final concentration of DNA was adjusted to 2.5 µg/mL by preparation of an appropriate dilution of a stock DNA solution. The DNA purity was checked using the Eppendorf BioPhotometer Plus (OD 260/280 nm), and the DNA integrity was checked by electrophoresis at 70V for 30 min on 1% agarose gel in a Sub-Cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, USA). The purified genomic DNA was stored at -20°C in a laboratory freezer until required.

The ITS1-5.8S-ITS2 region of the nuclear rDNA from the isolate was amplified using forward primer ITS1 and reverse primer ITS4 (White et al. 1990), obtained from Vivantis Technologies, USA. These primers are considered as universal fungal primers. The amplification reaction was carried out in volume of 25 µL containing 4 µL (10 ng) of template DNA, 1.5 µL of each primer (20 µM), 2.5 µL of 10×PCR buffer, 1 µL of MgCl_2 (50 mM), 0.25 µL of dNTPs (100 mM) and 0.25 µL of Taq DNA polymerase (5 U/µL) supplied by Vivantis Technologies, USA (Patino et al. 2007). A negative control, containing all reagents except for genomic DNA, was also prepared. The PCR reaction was performed in an Eppendorf Mastercycler Vapoprotect (Eppendorf AG, Germany) using 10 ng of genomic DNA. The amplification program used was described by Henry et al. (2000) and included one cycle of 4 min 30 s at 95 °C (pre-denaturing), 40 cycles of 30 s at 95 °C (denaturation), 30 s at 50 °C (annealing), 60 s at 72 °C (extension) and finally one cycle of 3 min at 72 °C. For PCR, 5 µL of PCR product was examined by electrophoresis at 90V for 1 h 50 min in a 1% (w/v) agarose gel stained with GoodView nucleic acid stain in 1×TAE buffer in a Sub-Cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, USA). The molecular mass of the amplified DNA was estimated by comparison with a ready-to-use VC 100-bp Plus DNA ladder (Vivantis Technologies, USA) as a molecular size marker. The agarose gel was visualized under UV light using an Alpha Ease FC Imaging system (Alpha Innotech, Germany).

The obtained PCR product was sequenced by First Base Labo-

ratories Sdn Bhd, Malaysia. The PCR fragment was extracted from the agarose gel and purified with GF-1 AmbiClean Kit (Vivantis Technologies, USA) following the manufacturer's instructions. The purified PCR fragment was sequenced on both strands using forward (ITS1) or reverse (ITS4) primers. Genetic sequencing of the amplified ribosomal sequences was carried out utilizing the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Following removal of dye terminators using the BigDye XTerminator Purification Kit (Applied Biosystems, USA), samples were analysed on an ABI PRISM 3730xL Genetic Analyzer (Applied Biosystems, USA), utilizing Sequence Scanner software version 1.0.

The sequences obtained were pairwise aligned using the SDSC Biology Workbench 3.2 software (Subramaniam, 1998) with default settings. For the identification of the fungal isolate, percentage of sequence identity and coverage were compared with available sequences in GenBank using the Basic Local Alignment Tool, BLASTN program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Phylogenetic Analysis

Phylogenetic analysis was performed from aligned sequences of the rDNA ITS region containing ITS1 and ITS2 and the intervening 5.8S rRNA gene. The sequences were aligned using ClustalW with all multiple alignment parameters used at default settings. The sequence alignment included the strain selected in this study and representatives of other *Penicillium* species available from GenBank. *Talaromyces bacillisporus* CBS 296.48 (JN899329) was the outgroup species. Cladistic analysis using the neighbour-joining method was performed with the MEGA 4.0 computer program (Tamura et al. 2007). Phylogenetic distances were calculated using Jukes-Cantor model. Confidence values for individual branches were determined by 1000 bootstrap replications.

Statistical Analysis

Each experiment was conducted in three replicates. Mean values with standard deviation are presented.

Results and Discussion

Fungal Isolates Recovered from Mangrove Rhizosphere Soils

In this study, 11 composite samples collected from the rhizosphere of plants naturally growing in mangrove forests of Pahang State (Malaysia) were examined for *Penicillium* strains. A total of 20 fungal isolates proposed to belong to the genus *Penicillium* were recovered from five composite samples collected from the rhizosphere of *Avicennia alba*, *Acrostichum aureum*, *Excocaria agallocha*, *Rhizophora apiculata* and *Bruguiera gymnorrhiza*. The soil types, pH, and the number of recovered fungal isolates are shown in **Table 2**.

The isolates were characterized by filamentous growth on the standard agar media for *Penicillium* (Pitt, 1979; Frisvad and Samson, 2004). The data obtained indicated that colony diameters of fungal cultures varied considerably on the different diagnostic agar media: 6-17 mm (on CZ), 9-44 mm (on CYA), 14-47 mm (on MEA), and 25-50 mm (on YES). Almost all isolates had green or dark green obverse and yellow reverse colony colour on CZ. Colonies on CYA were glaucous, light green, green or dark green on the obverse and mostly yellow or orange on the reverse. Most isolates on MEA were green or dark green on the obverse and yellow on the reverse. On YES medium both obverse and reverse colony colours of almost all isolates were dark green and yellow, respectively. Most of the isolated cultures produced uncoloured, yellow, brown or dark brown exudate droplets on CYA and MEA agar media. The presence of uncoloured and yellow exudates was also observed on colonies of several isolates grown on CZ and YES agar.

In addition to colony macromorphology, micromorphological characteristics of the isolated cultures were also examined. Microscopic analysis identified examples of both simple and branched conidiophores with metulae, phialides and conidia. Conidia colours varied on different agar media from yellow-green to green and dark green.

Penicillium microfungi are considered to be amongst the most common fungal species distributed both in uncultivated and cultivated soils. These microfungi have been isolated from numerous

Table 2. Characteristics of composite soil samples and the number of recovered isolates

Sampling Site	Soil Type	pH ^a	Number of fungal isolates
MFB1	sandy clay soil	2.84±0.01	1
MFB2	sandy clay soil	5.86±0.02	-
MFB3	sandy soil	5.82±0.02	-
MFB4	sandy clay soil	3.22±0.01	3
MFC1	sandy clay soil	5.59±0.09	1
MFC2	sandy soil	5.93±0.07	-
MFC3	sandy soil	5.76±0.02	-
MFC4	sandy clay soil	5.75±0.03	-
MFK1	sandy clay soil	5.93±0.020	-
MFK2	clay soil	3.62±0.010	3
MFK3	clay soil	2.88±0.015	12

^a pH measurements are expressed as mean ± standard deviation from three replicates.

soil samples in sub-tropical, tropical, and semi-arid soils (Carlike et al. 2001). Soil characteristics are one of the most important environmental factors directly affecting soil microbial community composition and the productivity of soil microorganisms. One of the typical characteristics of mangrove soils is high salinity. There appear to be marked differences in the ability of mangrove plant species to tolerate very high salinities (Asish Kumar Parida and Bhavanath Jha, 2010). Soil texture and pH are also major features of mangrove soil properties. The rhizosphere soil analyses summarized in **Table 2** show that the soil texture in samples examined here varied from sandy to clay and that pH was acidic for all samples from which fungal isolates were recovered. Most mangrove soils have pH in the range of 6 to 7, but some may have a lower pH value. Low soil pH may be linked with a high level of iron pyrites, which are the common constituent of mangrove soils and can be easily oxidized to sulphuric acid (English et al. 1997). The acidity of the soil influences the chemical transformation of many nutrients and hence their availability to soil organisms. As shown in **Table 2**, the number of recorded fungal isolates proposed to belong to *Penicillium* genus differed between the sampling sites. The maximum number of isolates (15) was recovered from acidic clay soil samples collected from the rhizospheres of *Rhizophora apiculata* and *Bruguiera gymnorrhiza* in mangrove forest in Kuantan district. Considerably fewer isolates were recovered from the acidic sandy clay soils collected from the rhizospheres of *Avicen-*

nia alba (1 isolate) and *Acrostchum aureum* (3 isolates) in Balok district, and *Excocaria agallocha* (1 isolate) in Cherating district. Fungal isolates were not recovered from sandy mangrove soils, which have lower water holding capacity. It is well established that the proportions of clay, silt and sand, together with the grain size, dictate the permeability of the soil to water, which then further influences soil salinity and water content. Nutrient status is also affected by the physical composition of the soil, with clay soils generally being higher in nutrients than sandy soils (English et al., 1997). Sandy soils and sandy clay soils with a high level of sand may not retain a high enough water and nutrient content to support fungal growth (Latiffah et al. 2009).

The properties of the isolates in current study were consistent with those of *Penicillium* microfungi described by other researchers (Pitt, 1979; Frisvad and Samson, 2004). The colonies of *Penicillium* species are characterized as rapid growing, flat, and filamentous. The colony obverse colour is initially white and becomes dark green, blue green, gray green, olive gray, yellow or pinkish in time. The colony reverse is usually pale to yellowish or brownish. The presence of exudates on colonies again is consistent with literature descriptions of *Penicillium* species able to produce distinct exudate droplets, for instance, yellow in *Penicillium chrysogenum* and dark brown in *Penicillium venetum* (Frisvad and Samson, 2004). Microscopic features of the isolated cultures are also broadly in agreement with the literature (Pitt, 1979; Frisvad and Samson, 2004).

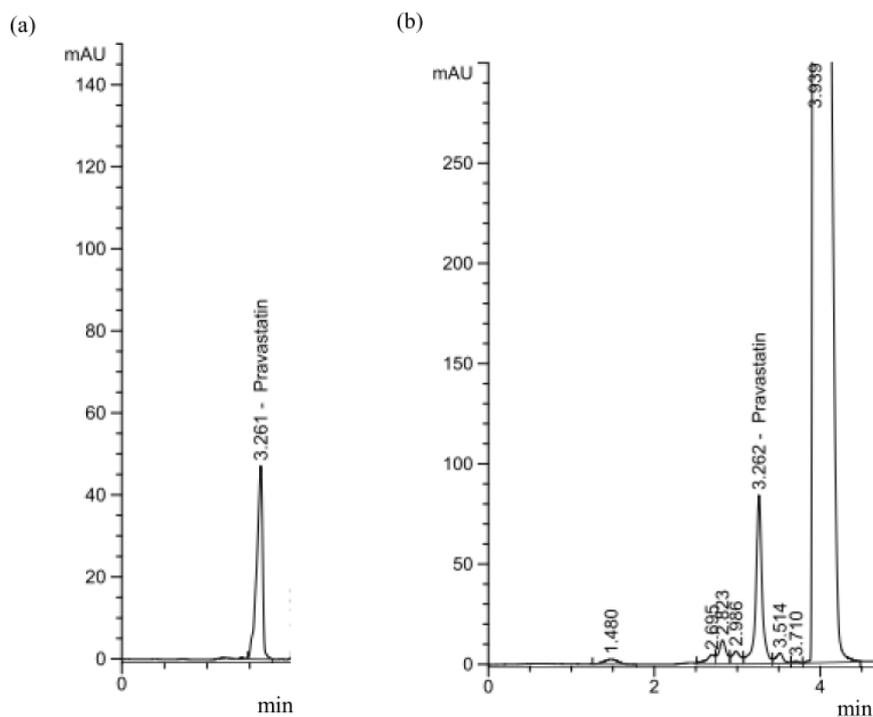


Figure 1. High-performance liquid chromatograms: (a) standard pravastatin; (b) ethyl acetate extract of isolate *Penicillium* sp. ESF19M

Table 3. Pravastatin production by *Penicillium* microfungi^a

Fungal Culture	Origin	Pravastatin, mg/L	Pravastatin, µg/g DCW
<i>Penicillium</i> sp. ESF1M	MFK2	3.00±0.188	231.77±29.310
<i>Penicillium</i> sp. ESF2M	MFK2	8.17±0.622	711.69±24.318
<i>Penicillium</i> sp. ESF5M	MFK3	1.15±0.056	69.92±3.782
<i>Penicillium</i> sp. ESF6M	MFK3	2.55±0.070	205.60±10.316
<i>Penicillium</i> sp. ESF12M	MFB4	1.17±0.061	92.65±6.353
<i>Penicillium</i> sp. ESF19M	MFK3	28.43±2.522	2301.11±138.665

^a Results are presented as mean ± standard deviation from three replicates.

Microscopic analysis supports the isolated microfungi as being characterized by conidiophores and microscopic elements typical for the genus *Penicillium*.

Screening of Statin-Producing Isolates

The identified *Penicillium* strains were screened for pravastatin production. Of the 20 fungal isolates examined, only six were able to produce pravastatin (Table 3). Of these, the isolate *Penicillium* sp. ESF19M, isolated from the rhizosphere soil of *Bruguiera gymnorrhiza*, was the best producer, achieving a concentration of 28.43 mg/L pravastatin, equating to 2301.11 µg/g dry cell weight. The standard pravastatin (≥ 98% purity) used in current study showed a peak with R_t of 3.261 min (Fig. 1a), while that extracted from the isolate *Penicillium* sp. ESF19M showed a peak of 3.262 min (Fig. 1b). From Fig. 1b, it can be seen that another fungal metabolite with R_t of 3.939 min was also accumulated by this strain in a higher concentration than our target product. However, this compound has not been identifiable with reference to published spectra. The next active pravastatin producer was *Penicillium* sp. ESF2M, giving a lower concentration of 8.17 mg/L using this method of assessment (Table 3). The most active pravastatin producers were recovered from clay soils, which are typically charac-

terized by a high level of organic matter in comparison with sandy soils (English et al. 1997).

All 20 isolates were also screened for mevastatin production (R_t of 14.602 min), with no traces being found. This is consistent with any mevastatin produced being immediately transformed into pravastatin.

Identification of the Selected Strain to Species Level

The identification of the best pravastatin producer to species level was based on its morphology as well as nucleotide sequence analysis of the enzymatically amplified ITS1-5.8S-ITS2 region of the rDNA. Traditionally, colony characters and diameters on specific agar media are considered important for *Penicillium* species identification. Characterization of conidiophore branching patterns and microscopic elements is also of great taxonomic value for *penicillia* (Pitt, 1979; Frisvad and Samson, 2004). Table 4 shows the cultural and morphological features of the best pravastatin-producing isolate selected in this study in comparison with literature data. Both macro- and micro-morphological characteristics of the examined strain were in agreement with the literature description of *Penicillium citrinum* (Bridge et al. 1989).

For molecular identification, genomic DNA from the best

Table 4. Cultural and morphological properties of *Penicillium* sp. ESF19M in comparison with those reported in the literature for *Penicillium citrinum*

Character	<i>Penicillium</i> sp. ESF19M	<i>Penicillium citrinum</i> ^a
Cultural properties		
CD on CZ agar, mm	12-16	16-19
Velvet colonies	yes	yes
Colony profile	flat and spreading	flat and spreading
Conidial mass color	green	green
CRC on CZ agar	yellow	yellow
Morphological properties		
Conidiophore branching pattern	monoverticillate	monoverticillate
Phialide length, µm	10-12	9-14
Phialide shape	ampulliform	ampulliform
Conidium length, µm	2	2
Conidium width, µm	1.8	1.5-1.8

^a Literature data were primarily adapted from Bridge et al. (1989)

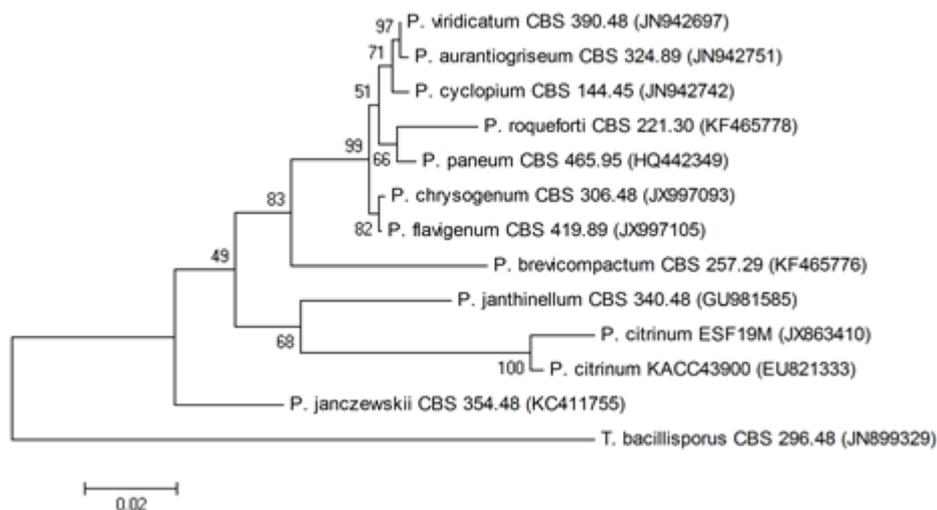


Figure 2. Phylogenetic tree, using the neighbor-joining method of representatives of *Penicillium* sp. inferred from analysis of the ITS region. Bootstrap values calculated from 1000 replicates are given at the branches

pravastatin-producing strain was extracted. The ITS1-5.8S-ITS2 region of the rDNA was amplified successfully producing a single PCR product of the desired length (Dean et al. 2006). The section of rDNA sequenced from *Penicillium* sp. ESF19M included 497 base pairs. The partial ITS1 region occupied nucleotides 1 to 149, the complete 5.8S rDNA gene from nucleotides 150 to 306 and ITS2 from nucleotides 307 to 476. This ITS1-5.8S-ITS2 nucleotide sequence showed 99% homology with that of *Penicillium citrinum* KACC43900 (EU821333). The sequence data obtained in this study have been submitted to GenBank under the accession number JX863410. Thus, based on the morphological characteristics described, and the ITS1-5.8S-ITS2 nucleotide sequence obtained, the selected strain was identified as *Penicillium citrinum* ESF19M. This novel fungal producer of pravastatin has been deposited at the Microbial Culture Collection of the Universiti Malaysia Pahang (strain number: UMPCC/F/019) and at the Microbial Culture Collection Unit (UNiCC) of the Universiti Putra Malaysia (strain number: UNiCC/UPMC 940).

Phylogeny

A phylogenetic tree (Fig. 2) shows the evolutionary relationships between the strain selected in this study and a range of fungal species whose sequence information was obtained from GenBank. Through the alignment and cladistic analysis of homologous nucleotide sequences, it was shown that the sequence obtained from *P. citrinum* ESF19M fell in the same sub-clade as of the known strain *P. citrinum* KACC43900 with a bootstrap value of 100%. Of the species included in the phylogenetic analysis, *P. janthinellum* CBS 340.48 was the next most closely related strain.

Conclusions

The work outlined herein has investigated pravastatin-producing ability of 20 *Penicillium* strains isolated from the rhizosphere soils of various plants in mangrove forests. Six of them were able to synthesize this statin directly, confirming that rhizosphere soils of plants in mangrove forests can be considered as a unique natural source of pravastatin-producing *Penicillium* microfungi. Our study has demonstrated that clay rhizosphere soils in mangrove forests host wild-type *Penicillium* strains which are able to produce pravastatin in relatively high concentrations. The strain *Penicillium citrinum* ESF19M, selected in this study, can already be considered as a novel promising pravastatin producer of scientific value. A strain improvement program and fermentation optimization studies may significantly enhance the production of this valuable natural statin.

Abbreviations

- CD – colony diameter
- CRC – colony reverse colour
- CYA – Czapek yeast autolysate agar
- CZ – Czapek-Dox agar
- DCW – dry cell weight
- HMG-CoA – 3-hydroxy-3-methylglutaryl-coenzyme A
- HPLC – high-performance liquid chromatography
- MEA – malt extract agar
- PDA – potato dextrose agar
- YES – yeast extract sucrose agar

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Conflict of interests

The authors declare that they have no competing interests.

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