

BIOPROSPECTING OF *PENICILLIUM* GENUS SOIL MICROFUNGI FOR PRAVASTATIN PRODUCTION

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ABSTRACT

Pravastatin is an effective cholesterol-lowering agent currently produced based on a two-step fermentation process, involving the initial production of compactin (mevastatin) and its subsequent microbial biotransformation to pravastatin. The development of a one-step fermentation process using active strains of pravastatin-producing microfungi could be a more attractive approach. Therefore, the isolation of novel fungal strains from different natural sources and their screening for pravastatin production has gained increasing importance. In this study, *Penicillium* genus soil microfungi were isolated from little-explored oil palm plantations. The cultures were cultivated in submerged fermentation followed by screening for pravastatin production using high-performance liquid chromatography. The selected pravastatin-producing fungal isolate was identified to species level based on cultural and morphological characteristics and using Biolog microbial identification system. As a result, 30 soil samples from 10 sampling sites in oil palm plantations of different ages were collected and examined. A total of 34 *Penicillium* genus isolates were recovered from these soils. From the tested fungal cultures, the isolate *Penicillium* sp. ESF21 was able to produce pravastatin directly with a yield of 162.33 mg/l. This previously unknown wild-type pravastatin producer was identified as *Penicillium brefeldianum* ESF21.

Keywords: Microfungi, *Penicillium* spp., pravastatin, screening, soil

INTRODUCTION

Cardiovascular disease remains the leading cause of mortality worldwide, with hyperlipidemia recognized as a significant contributing factor (Pahan, 2006). Control of plasma cholesterol by inhibiting its *de novo* synthesis is a crucial strategy for reducing blood cholesterol levels. Statins are effective cholesterol-lowering agents. They competitively inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Therefore, statins have an extremely high therapeutic value (Barrios-González and Miranda, 2010; Manzoni and Rollini, 2002). Of the known natural statins produced by microorganisms, pravastatin exhibits highly tissue-selective inhibition of cholesterol synthesis in the liver (Koga *et al.*, 1990). Beyond its hypocholesterolemic effect, pravastatin has also been shown to reduce the risk of developing Alzheimer's disease (Wolozin, 2002).

Filamentous fungi are known for their ability to synthesize natural statins, with *Aspergillus* and *Penicillium* genera being the most extensively studied. Based on the published reports, *Aspergillus terreus* was identified as the most effective producer of lovastatin within the *Aspergillus* genus (Manzoni *et al.*, 1999; Shindia, 1997). A few strains have been documented to produce compactin, including *Penicillium citrinum* (Endo *et al.*, 1976), *Penicillium cyclopium* (Doss *et al.*, 1986), and *Penicillium brevicompactum* (Brown *et al.*, 1976; Shaligram *et al.*, 2008). While compactin itself is not directly used as a pharmaceutical, it serves as an important intermediate compound in the industrial production of pravastatin, which is based on a two-step fermentation process (Seydametova, 2013). Microbial biotransformation is regarded as a more practical and cost-effective approach for pravastatin production compared to chemical modification. However, the conversion of compactin to pravastatin is inhibited by its high concentration. Therefore, the development of a one-step fermentation process, utilizing an active microbial producer that accumulates pravastatin directly, could be an attractive alternative to the existing process.

Manzoni *et al.* (1999) reported the ability of several strains of the *Aspergillus* and *Monascus* genera to produce pravastatin directly in culture. Our previous research results have indicated that certain *Penicillium* species were also capable of producing this statin directly (Seydametova *et al.*, 2012; Seydametova, 2015; Seydametova *et al.*, 2015). In addition to these findings, the developments in molecular biology have enabled direct genetic modifications aimed at developing a one-step biosynthesis by transforming *P. citrinum* with a hydroxylase gene from *Streptomyces carbophilus* (Ykema *et al.*, 1999).

Soils from oil palm plantations in Malaysia are generally characterized by high levels of organic matter, including lipids (Muniandy *et al.*, 2009). This investigation aims to increase our knowledge of the potential of *Penicillium* genus soil microfungi isolated from such habitats to produce pravastatin directly.

MATERIAL AND METHODS

Collection of soil samples

Thirty soil samples were collected from 10 sampling sites in oil palm plantations of different ages (Yayasan Pahang Plantation Holding Sdn. Bhd., Gambang, Pahang State, Malaysia). Replication is crucial for establishing confidence in research findings. Therefore, at each site, three replicates of each soil were taken, packed into labeled sterilized polyethylene bags using a sterilized spatula, and returned to the laboratory for further processing. Then, the three replicates of each soil were pooled together to form a composite sample. These soil samples were used to determine the soil texture (Brady and Weil, 1999), soil pH (Muniandy *et al.*, 2009), and for the isolation of microfungi.

Isolation and purification of soil microfungi

Microfungi were isolated using the soil plate method of Warcup (1950). Inoculated Petri dishes were incubated at 25 °C until the colonies were fully formed. No growth was observed on control plates containing the PDA medium alone. Individual fungal colonies were picked and purified by streaking onto a fresh PDA medium. After several cycles of picking and replating, separate colonies were transferred to PDA slants.

Characterization of fungal isolates

Isolated fungal cultures were transferred to appropriate diagnostic agar media for identification of *Penicillium* genus microfungi (Frisvad and Samson, 2004; Pitt, 1979): 1) Czapek-Dox (CZ) agar; 2) Czapek yeast autolysate (CYA) agar; 3) malt extract agar (MEA); 4) yeast extract sucrose (YES) agar. 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 colors, and the presence of exudate droplets. The standard medium used for microscopic observations was MEA. Conidiophore branching patterns and microscopic

elements 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

Thirty-four 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 (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) NaNO₃, 0.1% (w/v) MgSO₄•7H₂O. The pH of the screening 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 Multitron® Version 2 (Infors AG, Switzerland).

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.* (1999) were used for 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 1 M trifluoroacetic acid, and then an equal volume of ethyl acetate was added. Extraction was performed in an Ecotron incubator shaker (Infors HT, Switzerland) at 200 rpm and 30 °C for 1 hour. The fermentation samples were subsequently filtered through a Whatman filter paper no. 41, and the organic phase of each sample was collected. The ethyl acetate filtrates were then dried over anhydrous Na₂SO₄ 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, 5µm 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 the addition of 1 M H₃PO₄ (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 (≥ 95% purity) and pravastatin (≥ 98% purity) used in this study as standards were obtained from Sigma-Aldrich (USA).

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.

Identification of selected isolate

The selected fungal isolate was identified on an appropriate diagnostic agar media (Frivstad and Samson, 2004; Pitt, 1979; Stolk and Samson, 1983). The macroscopic features examined included colony diameter, obverse and reverse colony colors. The microscopic features were examined following a standard procedure after incubation of the fungal culture on the MEA medium at 25 °C for 7 days. In addition, a semi-automated Biolog microbial identification system (Biolog Inc., USA) was used for the identification of the fungal isolate based on its ability to assimilate or oxidize different substrates. All necessary nutrients and biochemicals were pre-filled and dried into the wells of the Biolog FF MicroPlate™. All the steps were performed according to the manufacturer's instructions. Utilization reactions were indicated when a purple color formed in the wells, whereas no reactions remained colorless. The Biolog's MicroLog™ 3 computer software automatically cross-referenced the obtained pattern to an extensive library of fungal species. These techniques allowed the fungal isolate to be identified at the species level.

Statistical analysis

Each experiment was conducted in three replicates. Based on the obtained results, mean values with standard deviations were calculated.

RESULTS

Recovery of fungal isolates from soil samples

Soil microbial communities are very diverse, yet many soil microorganisms remain unknown. Soil sampling is a known source of variability. To limit this variability, it is recommended that soils be randomly sampled and composited into a single bulk sample for analysis. The area that is covered when collecting these samples

may vary and will depend upon the objectives of the studies. In this study, 30 soil samples from 10 sampling sites in oil palm plantations of different ages in Gambang (3.72°-N 103.12°-E) were collected and examined. A total of 34 fungal isolates were recovered, with all of the source soils showing acidic conditions (pH ranging from 3.12 to 5.27) favorable for the growth of filamentous fungi (Table 1).

Table 1 Characteristics of soil samples and the number of recovered fungal isolates

Sampling site number	Soil type	pH	Number of recovered isolates
1	sandy soil	4.40±0.02	1
2	sandy soil	4.44±0.03	8
3	sandy soil	5.27±0.01	8
4	sandy soil	4.99±0.04	3
5	sandy soil	4.74±0.03	2
6	peat soil	3.61±0.03	-
7	peat soil	3.90±0.01	4
8	peat soil	4.04±0.04	6
9	deep peat soil	3.12±0.05	1
10	deep peat soil	3.18±0.04	1

Legend: pH measurements are presented as mean ± standard deviation from three replicates

Cultural and morphological characteristics of recovered isolates

The fungal isolates were characterized by filamentous growth on the diagnostic agar media CZ, CYA, MEA, and YES. In general, most isolates had white to cream, glaucous or light brown obverse and yellow, orange or dark brown reverse colony color on CZ. Colonies on CYA had white to cream, glaucous, yellow, light brown or dark brown obverse color and yellow, orange, light brown or dark brown on the reverse. Most isolates on MEA had white to cream, glaucous, light green, green or dark green obverse and yellow, orange, light brown or dark brown reverse coloration. On YES agar medium both obverse and reverse colony colors varied from white and cream to dark green and from white to brown, respectively. The colony diameters of the isolates also varied considerably on the different diagnostic agar media: 5-21 mm (on CZ), 11-36 mm (on CYA), 15-61 mm (on MEA), and 23-42 mm (on YES). Most of the isolated cultures produced colorless, brown or dark brown exudate droplets on CZ, CYA, and MEA agar media after 14 days of cultivation. The presence of colorless and brown exudates was also observed on colonies of several isolates grown on YES agar medium. Micromorphological examination identified examples of both simple and branched conidiophores with metulae, phialides, and conidia. Conidia colors for most of the isolates varied on the different agar media from glaucous to dark green and from light brown to brown.

Screening of *Penicillium* isolates for statin production

Of the 34 cultures obtained from oil palm plantation soils, *Penicillium* sp. ESF21 was able to produce pravastatin, achieving a concentration of 162.33 mg/l, equating to 12.48 mg/g DCW. The standard pravastatin used showed a peak with *R_t* of 3.202 min (figure 1A) in the current study, while that extracted from the *Penicillium* sp. ESF21 isolate showed a peak of 3.200 min (figure 1B), both also very similar to the *R_t* (3.19 min) reported by Manzoni *et al.* (1999). From figure 1B, it can be seen that another fungal metabolite with *R_t* of 3.968 min was also accumulated by *Penicillium* sp. ESF21 strain and the amount of it was higher in comparison with our target product. However, this compound has not been identifiable with reference to published spectra.

Identification of pravastatin-producing isolate to species level

To identify pravastatin-producing *Penicillium* sp. ESF21 fungal strain to species level, the macro- and micromorphological characteristics of it were compared with those of the *Penicillium* species reported in the literature (Stolk and Samson, 1983). Table 2 shows the cultural and morphological properties of the pravastatin-producing *Penicillium* sp. ESF21 strain, which was selected in this study.

The Biolog microbial identification system provides a suitable and convenient protocol for the identification of filamentous fungi using Biolog FF MicroPlate™ and a semi-automated Biolog MicroStation. Therefore, the selected pravastatin-producing fungal culture was examined using this system too. The isolate *Penicillium* sp. ESF21 utilized substrates with a similar profile to *Penicillium brefieldianum* (Dodge) Stolk & Scott (Table 3). Moreover, a unique library of macroscopic and microscopic photographs of the fungi in the Biolog FF Database confirmed this result.

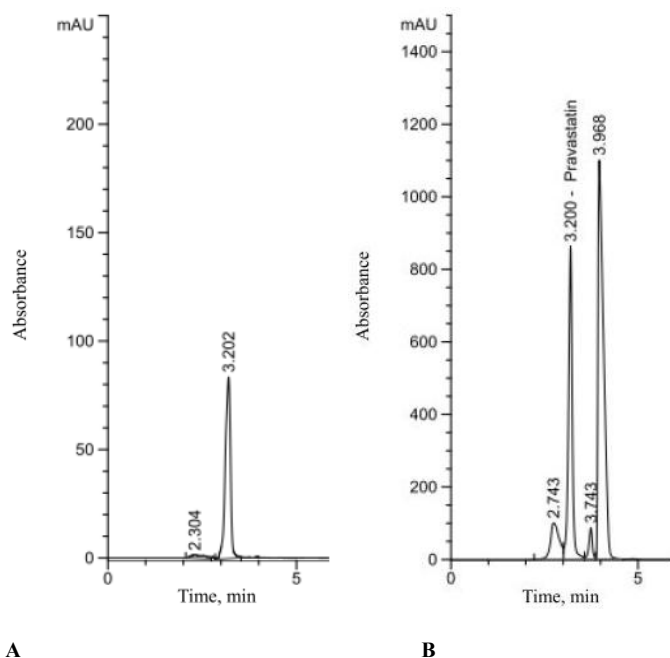


Figure 1 High-performance liquid chromatograms of pravastatin standard (A) and ethyl acetate extract of isolate *Penicillium* sp. ESF21 (B).

Table 2 Cultural and morphological characteristics of pravastatin producer in comparison with those reported in the literature for *Penicillium brefieldianum* species

Character	<i>Penicillium</i> sp. ESF21 ^a	<i>Penicillium brefieldianum</i> ^b
Cultural properties		
CZ		
Colony diameter on CZ, mm	10-13	35-45 (rarely 10-20)
Conidial mass color	inconspicuous	inconspicuous
Colony reverse color	bright yellow	bright yellow
MEA		
Colony diameter on MEA, mm	50-54	50-70
Conidial mass color	pale bluish green	grayish blue-green
Colony reverse color	yellow-orange	yellow, yellow-orange
Morphological properties		
Conidiophore branching pattern	monoverticillate	monoverticillate
Stipe length, µm	23.1-39.2	10.0-800.0
Phialide length, µm	11.3-12.1	6.0-15.0
Phialide shape	flask-shaped	flask-shaped
Conidium length, µm	2.3	2.2-6.5
Conidium width, µm	2.3	1.8-4.0

Legend: ^aObservations were recorded after incubation at 25 °C for 7 days. ^bPrimarily adapted from Stolk and Samson (1983).

Table 3 Species identification for *Penicillium* sp. ESF21 isolate using Biolog system

Rank	PROB	SIM	DIST	Organism type	Species
1	0.866	0.645	3.858	FF	<i>Penicillium brefieldianum</i> (Dodge) Stolk & Scott
2	0.088	0.061	4.636	FF	<i>Penicillium restrictum</i> Gilman & Abbott
3	0.033	0.022	4.969	FF	<i>Penicillium javanicum</i> var <i>javanicum</i> (vanBeyma) Stolk & Scott
4	0.009	0.006	5.410	FF	<i>Penicillium simplicissimum</i> (Oudemans) Thom BGB

Legend: PROB – probability, SIM – similarity index, DIST – distance.

DISCUSSION

Fungi play a crucial role in the functioning of soil ecosystems, particularly in agricultural soils. They are essential for various processes, including the decomposition of organic matter and the release of elements through mineralization. The community of microorganisms present in any given soil is influenced by several environmental factors, such as soil pH, nutrient type and availability, moisture content, aeration, and temperature. Among these, soil pH and organic matter content are widely recognized as the primary factors that affect fungal populations and diversity. The acidic soil pH of the plantation soils sampled in this study is consistent with the findings described by Muniandy *et al.* (2009). It is typical of tropical peat soils.

The majority of the fungal isolates (22 out of 34) were obtained from the sandy soils of a 10-year-old oil palm plantation. These soils are characterized by pH ranging from 4.40 to 5.27 (Table 1), which is suitable for the growth of microfungi. In contrast, a smaller number of isolates were recovered from peat soils. In total 10 isolates were obtained from the peat soils of a 15-year-old plantation, while only two isolates were recovered from the deep peat soils of a 20-year-old plantation. Peat soils in oil palm plantations are comprised mainly of disaggregated tree trunks, branches, leaves, roots and fruits, in addition to some mineral fractions such as clay, sand, and silt (Muniandy *et al.* 2009). It is normal practice for old leaves and palm trunks to be shredded and allowed to decompose *in situ*. However, both shallow peat and deep peat soils primarily consist of partially decomposed plant material. This is because the high water content in these soils prevents the aerobic decomposition of plant debris (Omar *et al.* 2012). The highly acidic conditions, particularly in deep peat soil, further inhibit the complete decay of plant material. Therefore, the fungal community in these soils is comprised mainly of endophytic fungi, which are distributed not in the soil itself but in the partially decomposed plant residues (Muniandy *et al.* 2009; Omar *et al.* 2012).

The identification of *Penicillium* genus microfungi commonly involves examining both the macroscopic features of colonies and the microscopic characteristics of conidiophores. In this study, the macro- and micromorphological properties of the 34 fungal isolates were consistent with those of *Penicillium* microfungi described by other researchers (Frisvad and Samson 2004; Pitt 1979). Notably, the pravastatin-producing *Penicillium* sp. ESF21 strain exhibited characteristics broadly consistent with the literature description of *Penicillium brefieldianum* (McNeill *et al.* 2012; Pitt 1979; Stolk and Samson, 1983).

In addition to cultural and morphological characteristics, the biochemical uniqueness of microfungi is often used for their identification. The Biolog FF MicroPlate™ was designed for the rapid identification of common filamentous fungi based on their ability to utilize 95 distinct substrates (Rice and Currah, 2005; Singh, 2009). This microbial identification system was effectively employed in the present study to rapidly identify a pravastatin-producing microfungus based on its unique substrate utilization pattern. An important added feature of the Biolog FF Database is its unique library of macroscopic and microscopic photographs of fungi, which assists in species identification. This tool was also successfully used in our study to confirm the identification of the tested pravastatin producer by providing visual and morphological verification of the identified species.

Table 4 Pravastatin production by *Penicillium brefieldianum* ESF21 in comparison with known producers reported in the literature

Microbial producer	Pravastatin, mg/l	Reference
<i>Monascus paxii</i> AM12M	45	Manzoni <i>et al.</i> , 1999
<i>Aspergillus terreus</i> A1R	204	
<i>Aspergillus terreus</i> A2R	34	
<i>Aspergillus terreus</i> BST	121	
<i>Penicillium citrinum</i> ESF19M	28.43	Seydametova <i>et al.</i> , 2015
<i>Penicillium janthinellum</i> ESF20P	15.8	Seydametova, 2015
<i>Penicillium brefieldianum</i> ESF21	162.33	This study

Microfungi of the *Penicillium* genus have a well-established history of producing various natural products of biotechnological importance. In the present study, the pravastatin yield from the newly isolated wild-type *Penicillium brefieldianum* ESF21 strain was comparable to that of known producers of this natural statin, as detailed in Table 4. Manzoni *et al.* (1999) reported that *Monascus paxii* AM12M strain, a spontaneous mutant macromorphologically different from the original *M. paxii* AM12 strain, produced 45 mg/l pravastatin after 14 days of growth on defatted soybean flour medium. Under the same conditions *A. terreus* yielded 121 mg/l pravastatin for the BST strain, and 204 and 34 mg/l for A1R and A2R strains, respectively. Importantly, *Penicillium brefieldianum* ESF21 synthesized pravastatin in quantities that were more than 5-fold and 10-fold higher than those previously reported for *Penicillium citrinum* ESF19M and *Penicillium*

janthinellum ESF20P, respectively. From figure 1B, it is evident that this strain also accumulated another fungal metabolite with a retention time (*Rt*) of 3.968 min, which was present in a higher concentration than our target product. Despite our efforts, we were unable to identify this compound through comparisons with published spectra. Therefore, future studies will focus on isolating and characterizing this unknown metabolite using advanced analytical techniques.

CONCLUSION

In the present study, a previously unknown *Penicillium* sp. ESF21 microfungus showing relatively high pravastatin-producing ability (162.33 mg/l) was isolated from oil palm plantation soil. Notably, it was recovered from peat soil, which is characterized by high organic matter content, including lipid compounds. Based on its cultural and morphological features, the pravastatin producer was identified as *Penicillium brefeldianum* ESF21. The unique substrate utilization pattern obtained using the Biolog microbial identification system confirmed the highest similarity of this fungal culture with *Penicillium brefeldianum* species. Compared to previous studies, this strain can already be considered as a novel fungal pravastatin producer of scientific interest. To further explore the potential of *Penicillium brefeldianum* ESF21, several future research directions can be proposed. Implementing a strain improvement program through mutagenesis or genetic engineering could lead to the development of strains with considerably enhanced pravastatin production capabilities. Furthermore, conducting a detailed analysis of the metabolic pathway involved in pravastatin biosynthesis in this strain may help to identify potential bottlenecks and targets for optimization. Lastly, optimizing key fermentation parameters could significantly improve pravastatin yield.

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