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Isolation and Screening of Lovastatin Producing Fungi: *Fusarium pseudocircinatum* IBRL B3-4 as a Potential Producer

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In the present study, 17 fungal isolates were isolated from various soils and substrate samples. However, only 11 identified isolates were found to be lovastatin producers. Lovastatin is known as a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), a rate-limiting enzyme in the cholesterol biosynthesis. The lovastatin production was carried out by solid substrate fermentation technique using a combination of rice bran and brown rice as substrate. The production was primarily determined using thin layer chromatography (TLC) and all the positive results were evaluated by confirmatory HPLC. The results revealed that *Fusarium* sp. IBRL B3-4 produced the highest lactone type lovastatin with 281.7±44.4 mg/g dry solid of lovastatin after seven days incubation period. The initial profile of the lovastatin production by *Fusarium* sp. IBRL B3-4 in solid substrate fermentation using a combination of rice bran and brown rice (1:1 ratio) showed the highest lovastatin production of about 425.0±33.3 mg/g dry solid of lovastatin was achieved on day twelfth of cultivation with 2.8±0.1 mg/g fungal growth. The lovastatin and fungal growth production decreased after achieving its maximal production. *Fusarium* sp. IBRL B3-4 was identified as *Fusarium pseudocircinatum* IBRL B3-4 by using macroscopic, *in situ* microscopic and 18S rRNA molecular approach.

Key words: *Fusarium* sp., lovastatin, solid substrate fermentation (SSF), flask system.

Lovastatin ($C_{24}H_{36}O_5$), a potent drug used for lowering blood cholesterol is also called Mevinolin, Monacolin K and Mevacor. It is a fungal secondary metabolite which inhibits hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase, the first enzyme in cholesterol biosynthesis[1]. These compounds, which are members of a drug family generally called statins, are known to exist in open (hydroxy acid) and closed ring (lactone). The major form of lovastatin in fermentation broth is the open hydroxy acid form (mevinolinic acid). However, it is generally in lactone form when administered to the patients as drug. *In vivo*, the lactone form of the compound is converted to the open hydroxy acid, which is the biologically active form of the statin[2]. The inhibitory effect of open hydroxy acid form of statins is due to their structural homology with HMG-CoA[3]. Therefore, the lactone form of lovastatin receives great attention in the treatment of cholesterol as it can be taken orally. Recent evidence shows that statins are not only able to reduce cardiac disease-related mortality, but cancer incidence is also reduced by 28-33 %[4]. Lovastatin has shown great promise as a cholesterol lowering agent and also suppress a variety of leukemic cell lines[5] and a wide array of solid tumors cells *in vivo*, by inhibiting the synthesis of non-sterol isoprenoid compounds[6].

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Lovastatin was first isolated as a secondary metabolite from a red yeast *Monascus ruber*[7] and a filamentous fungus *Aspergillus terreus*[8]. Then, several fungal genera including *Aspergillus* spp.[9], *Penicillium*[10], *Monascus*[11, 12], *Paecilomyces, Scopolariopsis, Doratomyces, Phoma, Phythium, Gymnoascus, Trichoderma*[13], *Hypomyces* and *Pleurotus*[14, 15] are also reported as lovastatin producers[13,16,17] Even though *Aspergillus* sp. especially *A. terreus* has been reported to be the highest lovastatin producer with 180 mg lovastatin/L[18], further investigations have to be conducted on screening of high yielding strains from hitherto under-utilized natural sources. In the present study, local potential fungal lovastatin producers were isolated from various soil samples and screened for the highest activity in a flask system.

MATERIALS AND METHODS

Isolation of lovastatin producing fungi

Various soils (from paddy field and oil palm soils) and substrate samples were used to isolate potential producers of lovastatin. One gram of substrates (rice bran and brown rice) and also soil samples which was taken from Perak and Penang (the northern regions of Malaysia) were scooped out into 9 ml sterile distilled water. After well mixed, a serial dilution was carried out until the final dilution of 10^{-9}. Only dilution of 10^{-5} until 10^{-9} were applied on potato dextrose agar (PDA, Merck) using spread plate technique. Plates were incubated at 30 °C for 7 days. Later on, a pure single colony was transferred onto PDA slant.

Conidial morphology and molecular identification: DNA extraction, PCR amplification and sequencing

PDA was employed for colony characteristics whilst carnation leaf agar (CLA) and Spezieller Nahrstoffarmer agar (SNA) were used for conidial morphologies examination under light microscope[19]. Important appeared structures were investigated under scanning electron microscope (SEM). For molecular part, fungal mycelia mats were freeze-dried under temperature of -50 °C and Â 0.133 mBar vacuum pressures. By using pestle and mortar, dry sample was crushed into powder form in liquid nitrogen. Genomic DNA was extracted using DNeasy kit according to the method provided by manufacturer (Qiagen)[20]. Further identification was done by sequencing the partial translation elongation factor 1-alpha gene (*tef-1α*) and also polymerase chain reaction (PCR). Sequences of *tef-1α* consisted primer EF-1 (forward: 5' -ATGGGTAAGGAGGACAAGAC-3') and EF-2 (reverse: 5' GGAAGTACCAGTGATCATGTT-3')[21]. The amplification was done under conditions of 94 °C for 1 min, followed by 34 cycles of 94 °C for 30 sec, 59 °C for 45 sec, and 72 °C for 1 min, and then 4 °C until analyzed. The product was cleaned up with QIAquick PCR Purification Kit (Germany) and the obtained DNA fragments were sequenced.

Production of lovastatin by solid substrate fermentation (SSF)

A combination of 5 g rice bran and brown rice in the ratio of 1:1 was placed in a 250 mL Erlenmeyer flask. Moisture content was adjusted to 70% (v/w) by adding sterile distilled water (pH 6.5). The flasks were sealed with cotton plug and aluminium foil and autoclaved at 121 °C for 15 min. After cooling, the media was inoculated with 1.0 mL spore solution size of $1x10^7$ spores/mL and mixed thoroughly to have a uniform spore distribution in the substrate. The inoculated media were incubated at room temperature (30±2 ºC) for 7 days aerobically.

Screening of the production of lovastatin

The primary stage of lovastatin existence was determined using thin layer chromatography (TLC) plate (20 x 20 cm Merck silica gel $60F_{254}$). Two hundred microliter of extracted aliquot was dotted on the plate, parallel with the commercial lovastatin as standard (Calbiochem, 99.7% HPLC purity). The plate was air dried and soaked in a suitable glass chamber consisting dichloromethane (Qrec) and Bendosen ethyl acetate (70:30; v/v). It was followed by observation under hand held uv lamp (254 nm) and then exposed in iodine vapor (Samie *et al*., 2003). The retention factor (R_f) of sample and standard lovastatin was compared. All TLC-positive samples, were analyzed using confirmatory High Performance Liquid Chromatography (HPLC).

Reversed phase analytical procedure via HPLC

Analysis of closed ring of lovastatin was carried out using HPLC system (Waters Corporation) at UV range wavelength (238 nm) in reversed phase Symmetry column which comprised of acetonitrile, dimethyloctadecylsilyl bonded

amorphous silica (C18), 4.6 mm x 250 mm and 5.0 µm particle diameter. Under Breeze software system (Waters Breeze System), all elutions were employed at 1.0 ml/min flow rate. Acetonitrile (Merck) and Fluka ortho phosphoric acid (mixed in distilled water and adjusted to pH 3.0 using concentrated ortho phosphoric acid) were labeled as mobile phase eluents. Breeze system was equilibrated isocratically with the mobile phase ratio of 77:23 (v/v), respectively. For each run, 20 µl samples were injected into the column by using a needle sample loop. A standard solution was prepared by dissolving 25 mg of lovastatin in acetonitrile for lactone detection and then concentrated ortho phosphoric was added[22] to assist the acid form of lovastatin. The retention time (R_t) between standard lovastatin and samples were quantified to identify lovastatin via HPLC. Results were expressed as µg lovastatin per g dry solid.

Fungal growth determination

By taking the advantage of chitin presence in fungal's cell wall, the growth can directly be measured[23,24]. Glucosamine was detected spectrophotometrically at 530 nm and the growth was expressed as mg glucosamine per g of substrate. Glucosamine powder manufactured by Sigma was used as a standard with the concentration range of 0.1 -1.0 mg/mL.

Extraction of lovastatin

The dried samples were extracted using 10 ml dichloromethane. The samples were then sonicated for 5 min, followed by incubation at 30 °C with shaking speed of 200 rpm for 2 hours and centrifuged at 3000 g for 8 min to separate the aliquot and biomass. 1 ml of analyte was taken out and layered with 1 % (v/v) trifluoroacetic acid for lactonization purpose. It was dried out at 80 °C without applying vacuum. Then, it was dissolved in 5 ml acetonitrile before been filtered through 0.45 µm nylon syringe filter and subjected into HPLC[25,26].

Time course of lovastatin production under SSF condition

A sixteen days profile was conducted in a shake flask system and the basic conditions of SSF were applied. For every 48 hours, the samples were harvested, dried out at 80 °C (for 24 hour) and proceed to HPLC analysis. All experiments were carried out in triplicates and the results were presented in mean ± standard deviation (n = 3).

RESULTS AND DISCUSSION

The confirmation of the lovastatin presence was carried out using thin layer chromatography (Fig. 1), and the initial occurrence was done by comparing the R_f values of the extracts against standard lovastatin namely 0.47. The presence of lovastatin in the extract was confirmed when the formation of dark spots on TLC plate observed under the UV light at short wave length, 254 nm. (Fig. 1A). However, the dark spots can be seen clearer under iodine vaporization (Fig. 1B). Commonly, qualitative screening is applied to discover a rough existence of lovastatin in fermented sample. This method is more suitable to disclose the polarity of compound including lovastatin. By referring to Fig. 1, lovastatin was a non polar compound, even though showing a slight affinity towards stationary phase.

HPLC analysis confirmed the presence of lovastatin in the fungal extract (Fig. 2). The sample was subjected into HPLC and retention time of standard lovastatin and samples were compared. The results showed that there was a peak appeared at 7.9 min (Fig. 2A) and 5.5 min (Fig. 2B) which represent lactone and hydroxy acid, respectively. As recorded by Fig. 2C, this fungal covered the same peak area with both authentic standards. Open ring salt of lovastatin eluted earlier than closed lactone form. A report suggested that the R_t for open ring was at 6.41 min and 8.89 min reserved for lactone peak[27].

The highest lovastatin production was produced by *Fusarium* sp. IBRL B3-4 which was isolated from oil palm soil sample. As reported in Table 1, *Fusarium* sp. IBRL B3-4 can produce a significant production of lovastatin (p <0.05) up to 281.7±44.4 ug/g dry solid. It was followed by *Aspergillus terreus* (212.1±48.6 mg/g substrate), *Collectotrichum* sp. (205.4±3.6 ug/g dry solid of lovastatin), *Penicillium citrinum* ITB (205.0±23.3 ug/g dry solid of lovastatin), *Trichophyton mentagophytes* (120.0±4.2 ug/g dry solid of lovastatin) with minor production by other fungi namely *Aspergillus flavus* (95.8±3.0 ug/g dry solid of lovastatin), *Trichoderma viridae* (72.9±3.0 ug/g dry solid of lovastatin), *Penicillium* sp. (59.2±6.1 ug/g dry solid of lovastatin), *Penicillium roquefortii* ITB (47.5±5.8 ug/g dry solid of

Table 1. Lovastatin production by newly isolated fungal isolates using TLC and HPLC

Fungal isolates and sources	Dark spot appearance on TLC plate	Lovastatin activity (lactone form) (µg/g) (Secondary screening on HPLC)	Fungal growth (mg/g)
Rhizopus oligosporus (oil palm soil)	+	0	0.77 ± 0.03^{g}
Aspergillus flavus (rice bran)	+	95 ± 9.7^{cd}	1.03 ± 0.05^{e}
Trichophyton mentagophytes (oil palm soil)	+	120.0 ± 4.2^{c}	0.70 ± 0.04^{g}
Trichoderma reesei (oil palm soil)	+	0	1.52 ± 0.10^{c}
Trichophyton rubrum (oil palm soil)	+	0	1.82 ± 0.04^{b}
Aspergillus fumigatus (rice bran)	+	45.0 ± 3.3^{ef}	1.03 ± 0.05^{e}
Penicillium roquefortii ITB (courtesy from Institut Teknologi Bandung)	+	47.5 ± 5.8^{de}	1.99 ± 0.09^{a}
Trichoderma viridae (Paddy field)	+	72.9 ± 3.0^{cd}	1.07 ± 0.04^{e}
Aspergillus nidulans (Paddy field)	+	0	1.28 ± 0.01^{d}
Collectotrichum sp. (Paddy field)	+	205.4 ± 3.6^{b}	0.43 ± 0.03^{h}
Rhizopus sp. (rice bran)	+	27.9 ± 2.8^{fg}	1.86 ± 0.08^{ab}
Penicillium sp. (Paddy field)	+	59.2 ± 6.1^{ef}	0.50 ± 0.03^{h}
Gliocladium roseum (oil palm soil)	+	0	1.57 ± 0.11^{c}
Penicillium citrinum ITB (courtesy from Institut Teknologi Bandung)	+	205.0 ± 23.3^{b}	0.95 ± 0.04^{ef}
Aspergillus niger USM F4 (oil palm soil)	+	0	0.74 ± 0.04^{g}
Aspergillus terreus USM H36 (oil palm soil)	+	212.1 ± 48.6^{b}	0.81 ± 0.03^{fg}
Fusarium sp. IBRL B3-4 (oil palm soil)	+	281.7 ± 44.4^{a}	0.94 ± 0.11^{ef}

*All values are mean ± SD (n = 3). Comparison of means between the lovastatin activity and fungi growth were determined using one way analysis of variance (ANOVA). Different superscript letters indicates significant different ($p < 0.05$).

Table 2. Identification of *Fusarium* sp. IBRL B3-4

Characters of *Fusarium* sp. IBRL	Examination
PDA- Incubation at 25 °C under dark condition	Colony diameter: 54 mm
Conidium color, observe	Rapid mixture of white feathery and violet pigment mycelium
Conidium color, reverse	Violet pigment
PDA- Incubation at 25 °C under light condition	Colony diameter: 60 mm
Conidium color, observe Conidium color, reverse	Moderate, mixture of white feathery and violet pigment mycelium Violet pigment
SNA- Incubation at 25 °C under dark condition	
Conidium color, observe	Rapid, violet pigment mycelium on filter paper
Conidium color, reverse	Dark violet pigment
SNA- Incubation at 25 °C under light condition	
Conidium color, observe	Moderate, violet pigment mycelium on filter paper
Conidium color, reverse	Dark violet pigment
CLA- Incubation at 25 °C under dark condition	
Conidium color, observe	
Conidium color, reverse	Rapid, brownish mycelium
CLA- Incubation at 25 °C under light condition	
Conidium color, observe	
Conidium color, reverse	Slow, light brown

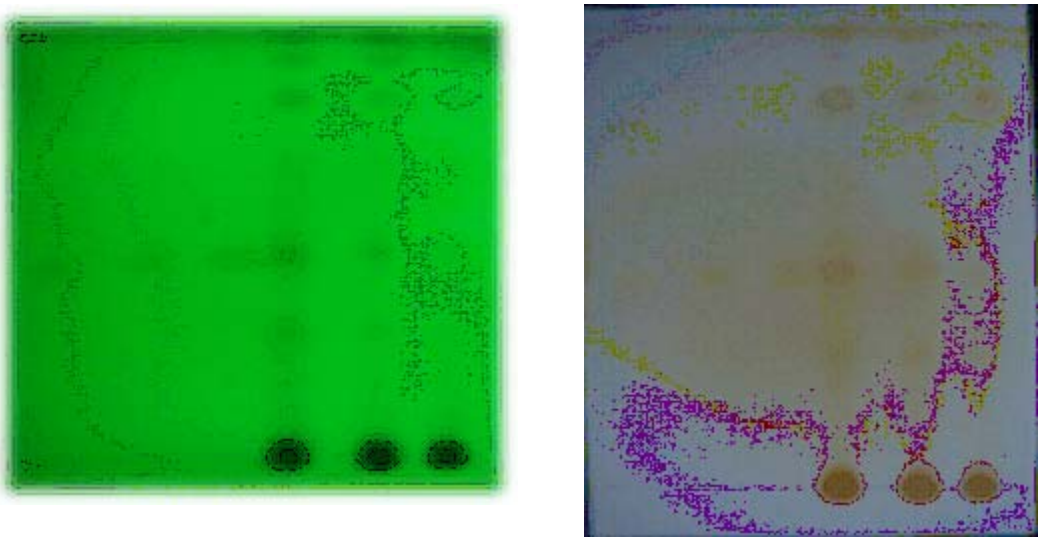


Fig. 1. A dark spot on TLC equivalent to lovastatin existence in fermented sample under different viewing. (A) was observed under hand-held ultra violet (UV) lamp at 254 nm and (B) was appeared after been vaporized with iodine

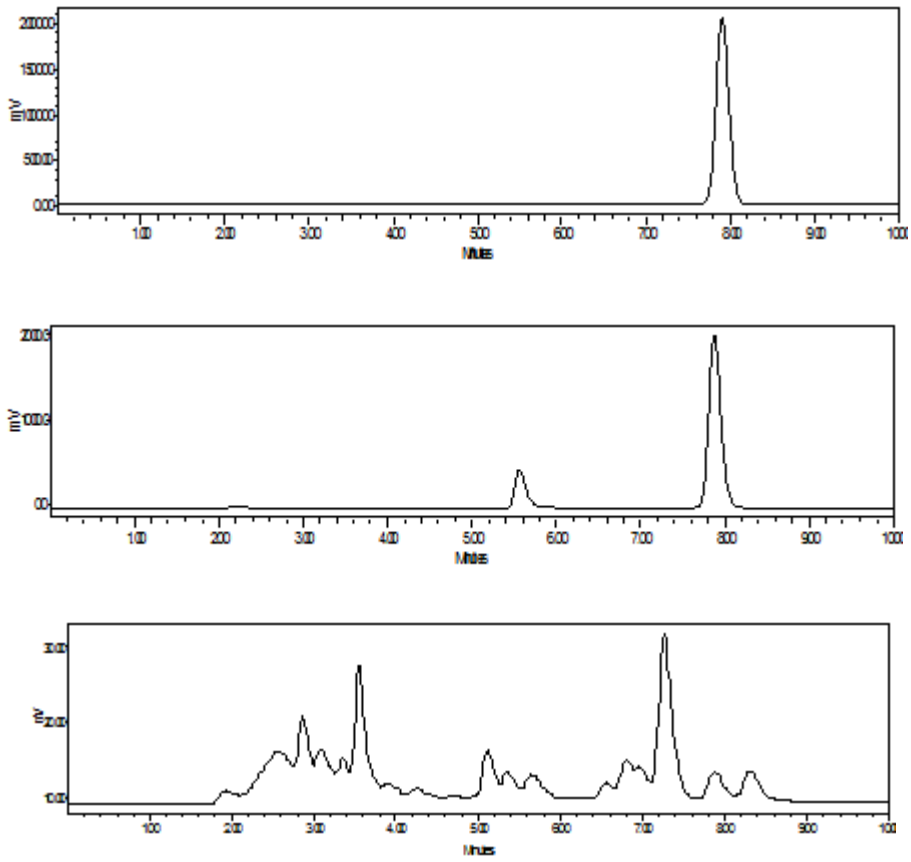


Fig. 2. High performance liquid chromatographic estimation of lovastatin open hydroxyl and lactone forms. (A) Standard lovastatin in lactone form, (B) Standard lovastatin in open hydroxy acid form, (C) Fungal extract containing both open hydroxy acid and lactone forms of lovastatin

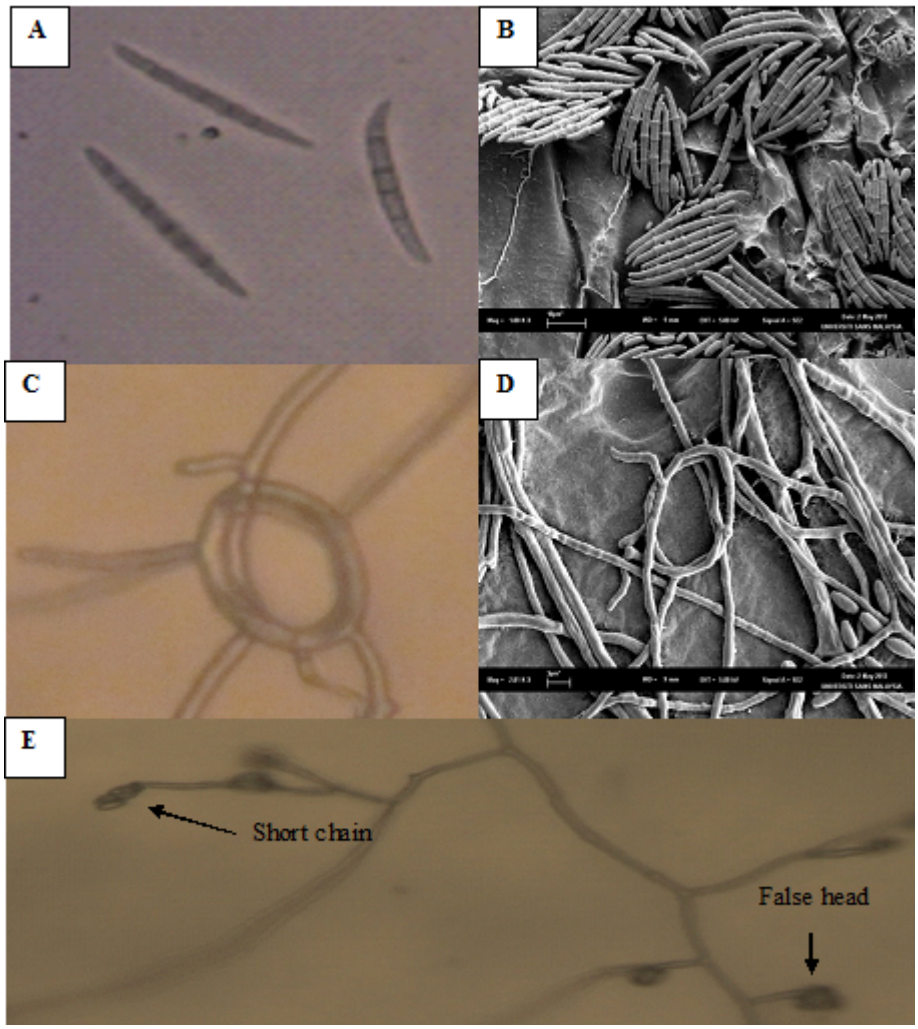


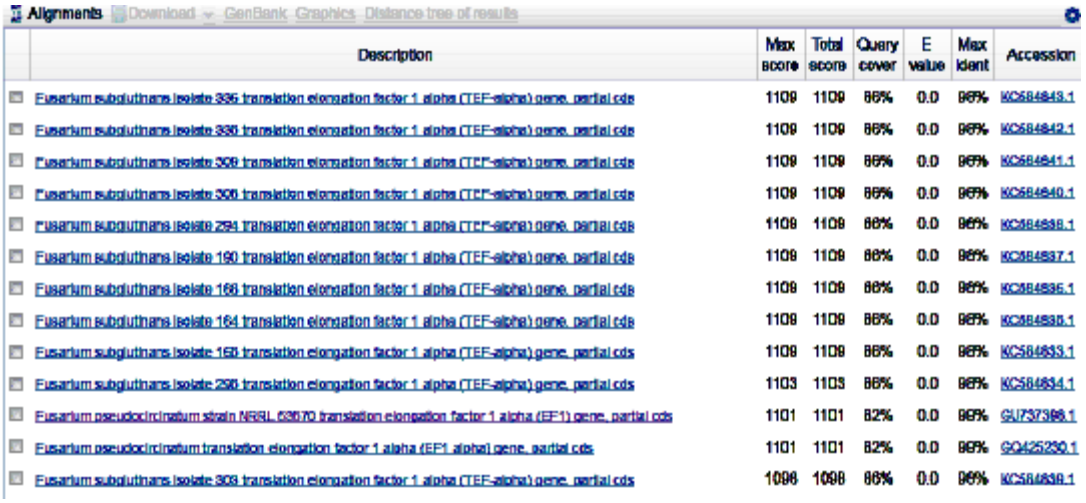
Fig. 3. Microscopic view of *Fusarium pseudocircinatum* IBRL B3-4 under a light microscope and scanning electron microscope. (A) Structure of sporodochia or macroconidia, (B) bunches of microconidia and macroconidia. (C) and (D) Sterile coiled hyphae, a special feature for this species and (E) the existence of false heads and short chain structure

TGCAAGTTGGTAGATCAAGACACCGCCTTGGGTAGAGAACCCTACGAGTACTACCCT
CGACGATGAGCTTATCTGCCATCATAATCCCGACCAAAACCTGGCGGGGTATTTCTC
AAAAGCCAACATGCTGACATTACTTCACAGACCGGTCACTTGATCTACCAGTGCGGT
GGTATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCACTTTCCCTTCGAT
CGCGCGTCCTTTATCCATCGATTTCCCCTACGACTCGAAACGTGCCCGCTACCCCGCT
CGAGTCCAAAATTTTTGCGATATGACCGTAATTTTTTTGGTGGGGCCTTTACCCCGCC
ACTCGAGCGGCGCGTTTTTGCCCTCTCTCATTCCACAACCTCACTGAGCGCATCGTC
ACGTGTCAAGTAATCACTAACCGTTCGACAATAAGAAGCCGCTGAGCTCGGTAAGG
GTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTA
TCACCATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCAT
TGGTATGTTGCCGCTCATGCTTCATTCTACATCTCTTCTTACTAACATATCGCTCAGA
CGCTCCCGGTCACCGTGATTTCATCAAGAAGATGATCAACTGGTTCCCTCCAAACAC
CGTGATTTCATCAAGAAGATGATCAACTGGTTCCCTCCAAA

Fig. 4. Aligned sequence for *Fusarium* sp. IBRL B3-4 isolate

lovastatin), *Aspergillus fumigatus* (45.0±3.3 ug/g dry solid of lovastatin) and *Rhizopus* sp. (27.9±2.8 ug/g dry solid of lovastatin). No production recorded by *Rhizopus oligosporus*, *Trichoderma reesei*, *Trichophyton rubrum*, *Aspergillus nidulans*, *Aspergillus niger* USM F4 and *Gliocladium roseum*. As illustrated in the same table, fungal growth did not affect the compound activity. Thus, lovastatin production was not depending on the fungal growth. *Aspergillus terreus*, *Monascus sp*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium purpurogenum*, *Pleurotus sp*, *Trichoderma viride* etc. were among of wide fungi variation that kept a great capability in producing lovastatin. Raghunath and co-workers (2012) reported that out of 57 fungi, 5 of the isolates were capable in producing lovastatin and *Fusarium* sp was one of the outstanding producers. To our best knowledge, that was the only report regarding *Fusarium* sp. as a lovastatin producer even though no species identification was done.

Since *Fusarium* sp. IBRL B3-4 exhibited the potential to produce maximal lactone type lovastatin, it was selected for further investigation. The spore-producing fungus was observed and identified to the genus and species levels based on the morphological (Table 2), microscopic characteristics (Fig. 3) and also molecular identification by sequencing the partial translation elongation factor 1-alpha gene, *tef-1α* (Figure 4).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Fusarium subglutinans isolate 335 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584643.1
Fusarium subglutinans isolate 336 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584642.1
Fusarium subglutinans isolate 308 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584641.1
Fusarium subglutinans isolate 306 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584640.1
Fusarium subglutinans isolate 294 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584638.1
Fusarium subglutinans isolate 190 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584637.1
Fusarium subglutinans isolate 166 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584636.1
Fusarium subglutinans isolate 164 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584635.1
Fusarium subglutinans isolate 155 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1109	1109	86%	0.0	96%	KC584633.1
Fusarium subglutinans isolate 296 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1103	1103	86%	0.0	96%	KC584634.1
Fusarium pseudocircinatum strain NRRL 53670 translation elongation factor 1 alpha (EF1) gene, partial cds	1101	1101	82%	0.0	96%	GU737398.1
Fusarium pseudocircinatum translation elongation factor 1 alpha (EF1 alpha) gene, partial cds	1101	1101	82%	0.0	96%	GQ425230.1
Fusarium subglutinans isolate 309 translation elongation factor 1 alpha (TEF-alpha) gene, partial cds	1098	1098	86%	0.0	96%	KC584639.1

Fig. 5. Blast result of the *Fusarium* sp. IBRL B3-4 (BLAST database)

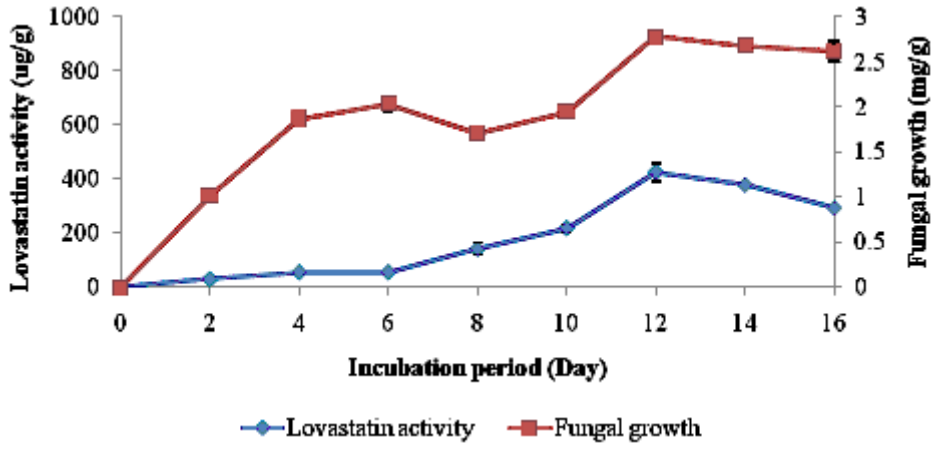


Fig. 6. Initial profile of lovastatin production by *F. pseudocircinatum* IBRL B3-4 in a flask system

Based on the result, the fungal isolate showed 99% similarity with *Fusarium pseudocircinatum* strain NRRL 53570 translation elongation factor 1 alpha (EF1) gene, partial cds (Fig. 5). *F. pseudocircinatum* has morphologically similar to *F. circinatum* and *F. sterilihyphosum* as their special capability in forming coiled hyphae. However, the hallmark of short chain microconidia in *F. pseudocircinatum* makes it distinguishable with other *Fusarium* sp (Fig. 3E). It also has falcate macroconidia (3-5 septate), chlamydospore, false head, beaked apical cell and foot shaped basal cell[28].

This present study is believed to be the first report on the isolation, characterization and identification of a new variant of the *F. pseudocircinatum* IBRL B3-4 that is able to produce high amount of lovastatin in solid substrate fermentation using rice bran and brown rice as substrate. The time course profile of the lovastatin production by *F. pseudocircinatum* IBRL B3-4 in solid substrate fermentation using a combination of rice bran and brown rice (1:1 ratio) showed that the highest lovastatin production with 425.0±33.3 mg/g dry solid and 2.78±0.1 mg/g fungal growth. The achievement obtained at day twelfth of fermentation day (Fig. 6). The lovastatin and fungal growth production decreased after achieving its maximal production. The present study showed that solid substrate fermentation is able to produce higher yields of lovastatin and a combination of rice bran and brown rice is suitable for *F. pseudocircinatum* IBRL B3-4. The main composition of rice is starch and it is easily hydrolyzed into glucose and acetate, which is one of the compounds involved in biosynthesis of lovastatin. Furthermore, rice bran also served as good encourage medium for the fungus and this condition was indicated by the formation of enhanced aerial mycelium on the substrate. It could also provide good substrate porosity for the growth of fungus. Brown rice, one of anti cholesterol stars is useful in avoiding accumulation of rice bran during fermentation process. This condition has allowed an inter particle space which is very vital in macro scale mass transfer. There were many reports showed that the quantity of lovastatin production in solid substrate fermentation is significantly higher than submerged culture[29]. In fact, different substrates were used for lovastatin production in solid substrate fermentation, including sorghum grain, wheat bran and corn[30]. In this respect, Pansuriya and Singhal (2010) has used a strain of *Aspergillus terreus* UV 1718 to be grown on different solid substrates like agricultural wastes to check their suitability for lovastatin production. Of all substrates used, the research found that the use of wheat bran yielded maximum lovastatin. The use of date waste extract as a potential substrate for lovastatin production by *M. purpureus* and various industrial residues including green gram husk, black gram husk and orange peel for lovastatin production by *Penicillium fumiculosum* NCIM 1174 were also reported[31, 30] .Thus, SSF is an alternative step to produce lovastatin as it promises a good environment for fungi to grow at high mycelia density plus high level compound production[32].

CONCLUSION

In the present study, an attempt was made to isolate potential lovastatin-producing fungus and the isolate *F. pseudocircinatum* IBRL B3-4 was found to be the highest lovastatin producer with 425.0±33.3 mg/g dry solid of lovastatin in solid substrate fermentation system. It could therefore be considered as a promising fungal species in the production of lovastatin. However, further experiments will be carried out to optimize the culture conditions and medium compositions in order to enhance the production ability.

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