Bioprospecting lovastatin production from a novel producer *Cunninghamella blakesleeana*

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Abstract

Beside anti-cholesterol activity, lovastatin garners worldwide attention for therapeutical application against various diseases especially cancer. A total of 36 filamentous fungi from soil samples were isolated and screened for lovastatin production by yeast growth bioassay method. C9 strain (later identified as *Cunninghamella blakesleeana*) was screened as potential strain of lovastatin production. Further confirmation of the compound was made using TLC, HPTLC and HPLC in which similar R_f value, densitogram peak and chromatogram peak against the standard lovastatin were observed, respectively. The purified lovastatin subjected for IR analysis showed a lactone ring peak at 1763.63 cm⁻¹ similar to standard lovastatin. Further structural analysis including NMR and LC–MS of the purified lovastatin reassures the molecular formula and molecular weight similar to standard. In quantitative terms, *C. blakesleeana*, *Aspergillus terreus* and *Aspergillus flavus* produced 1.4 mg g⁻¹ DWS, 0.83 mg g⁻¹ DWS and 0.3 mg g⁻¹ DWS of lovastatin, respectively, ($p < 0.0001$) without any optimization. Lovastatin showed significant antioxidant property with IC₅₀: 145.9 µg mL⁻¹ (140 µL), and the percentage of inhibition is maximum at 199.5 µg/mL which is statistically significant ($p < 0.0001$).

Keywords Lovastatin · *Cunninghamella blakesleeana* · Antioxidant · Anticancer

Introduction

Statins, a precise effective competitive inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34) which catalyzes the rate-limiting reduction of HMG-CoA to mevalonate in mevalonate pathway of cholesterol biosynthesis has revolutionized the treatment of hypercholesterolemia (Endo 2004; Istvan 2003; Bizukojc and Ledakowicz 2007; Kamath and Janakiraman 2012). They are the polyketide compounds produced by fungi during their secondary metabolism. It is widely used as a lipid-lowering drugs (lowers LDL cholesterol Levels) to reduce the risk of cardiovascular events in dyslipidemic patients (Endo 2004; Davidson and Robinson 2006; Ludman et al.

2009). Among the statins, lovastatin (C₂₄H₃₆O₅) formerly known as Mevinolin, Monacolin K and Mevacor was the first accepted statin by United States Food and Drug Administration (USFDA) in 1987 as a potent hypercholesterolemia agent (Manzoni and Rollini 2002; Tobert 2003). Lovastatin, a secondary metabolite produced during the secondary phase (idiophase) of fungal growth exists as open β-hydroxyl form (mevinolinic acid—biologically active) and the synthetic lactone form (mevinolin—biologically inactive), while the latter is hydrolyzed to biologically active (open ring) form through liver enzyme when administered invasive (Gupta et al. 2007). Apart from its anti-lipidemic activity, it is also widely used for various functions as anti-inflammatory agent, apoptotic inducer in cancer cells, restorer of renal function, suppressor of tumor necrosis factor in coagulation process and protective agent during host cellular damage (Tobert 2003; Zamvil and Steinman 2002). It is also commonly employed as a potent therapeutic agent in the treatment of major diseases that involves coronary artery, peripheral vascular, cerebrovascular, renal disease, multiple sclerosis, ischemic condition, bone fracture, Alzheimer's and to a negligible extent in cancer (Seraman et al. 2010; Kumar et al. 2000; Tandon et al. 2005).

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The first anti-cholesterol drug (Triparanol-1958) was banned due to its side effect and the second drug ML236B (Compacitin) isolated from *Penicillium citrinum* was also withdrawn owing to its carcinogenic effect (Kirby 1967; Endo et al. 1976). Finally, the lovastatin (Mevinolin) was discovered from *Aspergillus terreus* which gained popularity due its multiple therapeutic potential advantages with no side effects. This attracted the interest of many researchers to identify a better alternate source of fungi that could produce lovastatin which lead to the identification of fungi such as *Monascus (M.) ruber*, *A. flavus*, *M. pilosus*, *M. purpureus*, *A. fischeri*, *A. parasiticus*, *A. umbrosus*, *Acremonium chrysogenum*, *A. flavipes*, *P. funiculosum*, *Trichoderma (T) viridae* and *T. longibrachiatum* (Endo and Monacloin 1979; Sayyad et al. 2006; Wang et al. 2003; Valera et al. 2005; Samiee et al. 2003). In spite of long-term search for about three decades, a commercially successful fungal strain was not yet identified for efficient production of lovastatin apart from the conventional *A. terreus*. Solid-state fermentation was employed for lovastatin production in the present study. There are several reasons and a vital aspect is higher production. For instance, Barrios-González and Miranda (2010) stated that solid-state fermentation (SSF) showed better lovastatin yield than submerged fermentation (Smf). Pandey et al. (2001) stated that SSF is highly advantageous than submerged with the reasons such as usage of wide range of agricultural wastes and thereby reduced overall cost, better yield and stability of the product and ease of optimization due to low-cost constituents in the media. In addition, Suraiya et al. (2018) stated that the products obtained can be directly consumed after sterilization. However, the several prevailing patents in lovastatin production deal with submerged fermentation; Biocon, India Ltd, a leading commercial sector in India, has successfully produced lovastatin using SSF and promoted the same for commercialization (Suryanarayan 2003).

Hence, the present study aims in identifying and characterizing the alternative source of high-yielding lovastatin fungal producer through solid-state fermentation, characterizing the purified lovastatin in terms of structural and functional aspects and feasible primary application.

Materials and methods

Chemicals

Analytical grades of the chemicals with high purity obtained from Sigma-Aldrich and Merck were used in the experiments.

Microorganism and inoculum preparation

Filamentous fungi were isolated using nylon net litter bag technique from the collected soil samples of Patanam village (coconut area soil), Coimbatore (sugarcane soil) and Nilgiris (tea plantation area) which are present in the southern regions of Tamil Nadu, India (Kondo et al. 1994). Isolation of desired fungal cultures were done initially by plating the collected soil samples and then the individual colonies of micro-fungi were picked and purified by streaking onto the fresh Potato Dextrose Agar (PDA) medium. The pure fungal isolates were kept on PDA medium at 4 °C and re-cultured every 4 weeks. For preparing a spore suspension, 10 mL of spore suspension medium (0.9% NaCl, 0.1% Tween80) was added to a well-sporulated PDA slants of the isolates. The surface was scrapped with a loop and then the suspension was collected. The suspension was agitated completely using a cyclomixer and finally filtered through a glass wool. The concentration of the spore suspension was measured using a hemocytometer and the final concentration was adjusted to 1×10^8 spores/mL which was used as inoculums for the whole study (Alexopoulos et al. 1996).

Cultivation conditions

Each fungal species was cultivated in a solid-state fermentation. The solid substrate (wheat bran) was initially dried in an oven at 60 °C and accurately weighed to 10 g in Petri plates (100 mm × 17 mm) followed by moistening with distilled water and autoclaved for 40 min at 121 °C. After cooling, the medium was inoculated with 1 mL (10%, v/w) of spore suspension and the moisture content of the media was adjusted to 60% by adding sterile distilled water. The medium was thoroughly mixed and incubated at 28 °C in a humidity-controlled incubator for 7 days. After incubation, the Petri dishes were harvested and analyzed for lovastatin content (Valera et al. 2005).

Extraction of lovastatin

After 8 days of incubation, the solid substrate was dried at 40 °C for 24 h and gently crushed. Lovastatin extracted was carried out with 10 mL of ethyl acetate by shaking at 180 rpm for 2 h followed by filtration through Whatman No. 1 paper. After which, 1 mL of 1% trifluoroacetic acid was added to 1 mL of extract and finally the solution was incubated for 10 min (lactonization of hydroxyl acid form of lovastatin). The resultant extract was used for analytical studies (Kamath et al. 2015a, b).

Detection of lovastatin

Yeast growth inhibition bioassay

In our study, the method of screening lovastatin by bioassay was adopted from Babu et al. (2011) in which *Candida albicans* 3102 was used as indicator organisms. In bioassay method, a clear inhibition zone around the indicator organisms found to be observed and the diameter of the inhibition zone is proportional to the concentration of lovastatin in the samples. Yeast growth inhibition bioassay was performed by seeding 15 mL of glucose yeast peptone agar medium with 0.25 mL of yeast culture (*C. albicans*, purchased from NCIM, Pune). Yeast-inoculated medium was poured into a 15 cm diameter of glass Petri dish. After solidifying, 6-mm-diameter wells were made with the aid of sterile cork borer and to which 10–70 μ L of the extracts were added. Ethyl acetate and the standard solution of lovastatin (commercial lovastatin tablet) were used as negative and positive controls, respectively. The standard was prepared based on Friedrich et al. (1995) with a slight modification in which the lovastatin tablet obtained from Merck was crushed using mortar and pestle, and suspended in ethyl acetate followed by sonication and filtration. Antifungal drug (fluconazole) was also added to the wells. *A. flavus* and *A. terreus* [gifted by Indian Institute of Technology (IIT), New Delhi] were also used as reference strains. All the plates were incubated at 26 °C for 16 h. The concentration of standard lovastatin with respect to the diameter of zone of inhibition against indicator organisms were observed and used for calculating the presence of lovastatin in the crude samples.

Thin-layer chromatography (TLC)

Lovastatin in the fungal extracts was analyzed by TLC performed on silica gel-G (200 meshes) with chloroform:methanol (90:10 v/v) as the mobile phase. Lovastatin (10 mg) was dissolved in 100 mL of ethyl acetate and is used as a standard solution. For TLC detection, 10 μ L of standard Lovastatin and tenfold-concentrated fungal extracts were spotted onto TLC plates. Plates were developed three times with mobile phase and spots were made visible in iodine chamber. The distances travelled by each spot in baseline and relative RF values were calculated. By comparing the standard RF values for the chosen mobile phase, the lovastatin present in the samples was identified (Nidhiya et al. 2015; Lingappa et al. 2004).

High-performance thin-layer chromatography (HPTLC)

The spot obtained after TLC analysis of the fungal extract was scrapped and dissolved in 1 mL of ethyl acetate. The mixture was centrifuged at 3000 rpm for 5 min and the

supernatant was used as test solution for HPTLC analysis. Test samples (0.6 μ L) along with lovastatin standard were loaded on to the plate coated with silica gel 60 F254 using Hamilton syringe and run in CAMAG Linomat5 instrument. The sample-loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapor) with respective mobile phase (chloroform:methanol—9.5:0.5). The hot air was used to evaporate the solvent from the developed plate. The plate was kept in photo-documentation chamber (CAMAG TLC SCANNER 3) and the image was captured at UV 254 nm. For each TLC run, lovastatin standard was applied for R_f comparison. The area of the graph was used to calculate the yield of lovastatin (Lingappa et al. 2004).

High-performance liquid chromatography (HPLC)

The prepared sample extracts and a commercial lovastatin standard were quantitatively analyzed for the presence of lovastatin in the fungal extracts. For HPLC, a C18 column (250 mm_4.6 mm_5 mm internal diameter) with diode array detector was used. Acetonitrile and water which was acidified with 1.1% phosphoric acid in the ratio of 70:30 v/v were used as mobile phase. The eluent flow rate was maintained at 1.5 mL/min and the detection was carried out at 238 nm with an injection volume of 20 μ L (Samiee et al. 2003).

Identification and taxonomic positioning of 18S rRNA gene sequence of C9

Based on the screening results, the isolate C9 was selected for further studies. The isolate C9 was identified to the genus level by observing its morphological and conidial features in the culture growth (Alexopoulos et al. 1996). The selected fungi were identified at species level based on the 18S rRNA gene nucleotide sequence obtained by PCR amplification of the ITS region, followed by DNA sequencing, BLAST analysis and sequence similarity comparison using GenBank nucleotide databases. The ITS region of 18 s rRNA fragment (ITS1/ITS4) was successfully amplified using the fungal universal primers (ITS15'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3'). The DNA sequencing was outsourced to MacroGen Ltd. The 18S rRNA sequence was analyzed for its similarity and homology with the existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using the BLAST search tool (<http://www.ncbi.nlm.nih.gov/>). The sequences were aligned using the ClustalW program in BioEdit version 7.0.5.3. A phylogenetic tree was constructed by neighbor-joining method using PHYLIP software version 3.69. Treeview 1.6.6 was used to visualize the phylogenetic tree along with a bootstrap analysis for carrying out 1000 replicates (Saitou and Nei 1987).

Purification of lovastatin

Conventional purification

Unlactonized crude lovastatin extract was concentrated (10 mL) under reduced pressure to get viscous mass (Skoog et al. 1988). The elute was initially purified by silica ranging from 60 to 120 mesh column chromatography using a dichloromethane:ethyl acetate step gradient (100:0–0:100). A volume of 250 mL was collected at each step and analyzed for the presence of compound by TLC. The fractions containing lovastatin compound were pooled and concentrated in vacuum.

Purification by preparative HPLC

The concentrated sample was subjected to preparative HPLC equipped with an octadecylsilane column (Delta-pak C18) equipped with an UV detector. The column was eluted with acetonitrile and water which was acidified with 1.1% phosphoric acid (70:30 v/v) at a flow rate of 20 mL/min. The UV-absorbing peak at 238 nm with a retention time of 13.687 min was collected and concentrated. The purity was determined by analytical HPLC using a Nova-Pak C18 column eluted with acetonitrile and water which was acidified with 1.1% of phosphoric acid (70:30 v/v). The purified sample was concentrated by rotary evaporator and stored at -20°C for further study (Samiee et al. 2003).

Characterization of purified compound

Liquid chromatography–mass spectrometry (LC–MS) analysis

The purified sample was subjected to LC–MS analysis according to the protocol of Beer et al. (2012) at Sophisticated Analytical Instrument Facility in IIT-Bombay, India. LC–MS analyses were conducted on Q-TOF LC–MS Agilent Technologies Q-TOF B.05.01 (B5125.1) system equipped with a binary pump, in-line degasser, auto-sampler and column oven. LC–MS spectra data were compared with the literature to identify the peaks of bioactive compound.

Infrared spectrometry

A pinch of KBr pellet was oven dried and transferred into a mortar. The purified sample at about 0.1–2% was added, mixed and ground to a fine powder. The mid-range of IR spectra ranging from about 4000 to 400 cm^{-1} was recorded

by a Nicolet Avatar Model FT-IR spectrophotometer (Nazal et al. 2002).

Nuclear magnetic resonance spectrometry (NMR)

The final structure of the test compound was identified by ^1H NMR and ^{13}C NMR. The purified lovastatin was dissolved in chloroform for NMR experiments according to Berger and Baun (2004). The NMR spectrum was recorded using a Varian Mercury plus 500 MHz.

Antioxidant assay

Free radical scavenging activity measurement

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was determined using 1 mL of the sample with 5 mL of 0.1 mM methanol solution of DPPH followed by a vortex and incubation at 27°C for 20 min. 0.1 mM methanol solution of DPPH alone served as the control. For negative control, 4.0 mL methanol and 1.0 mL of DPPH solution were used. The absorbance of sample was measured at 517 nm using methanol to set the blank (Iwashima et al. 2005). The ability of the sample to scavenge DPPH radical was calculated by the following formula:

DPPH radical scavenging activity (%)

$$= \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{(\text{Abs}_{\text{control}})} \times 100, \text{ Abs} = \text{absorbance.}$$

Reducing power

Total reducing power was determined as described by Oyaizu (1986) by adding 0.1 mL of sample solution at different concentrations with 2.5 mL of phosphate buffer (0.2 mol L^{-1} , pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. 2.5 mL of trichloroacetic acid (TCA 10%) was added to the mixture and centrifuged at 3000 g for 10 min. The supernatant (5 mL) was mixed with 1 mL of ferric chloride (0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates its activity.

Iron chelating activity

The iron chelating activity was measured by the decrease in absorbance at 562 nm of the iron (II)—ferrozine complex (Yamaguchi et al. 2000). The reaction mixture contained 0.5 mL of ferric chloride (0.6 mM) and 900 μL of methanol. The mixture was shaken and left at room temperature for 10 min. To this, 0.1 mL of ferrozine (5 mM) in methanol was added, then mixed and left for 5 min to complex the residual

Fe^{2+} . The control sample contained iron and ferrozine only. The absorbance of the resulting solution was measured at 562 nm. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

$$\text{Chelating effect (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100,$$

A = absorbance.

Statistical analysis

All the experimental samples were done in triplicates to validate the test results. Statistical analyses were performed using the latest SPSS 10 version. The mean and standard deviation values of reading were obtained from the test replicate. The efficiency of test sample was compared between the commercially available standard and lovastatin sample by employing independent sample t test and paired sample t test. A ' p ' value of less than 0.0001 was considered as statistically significant.

Results

36 fungal strains were isolated from the collected soil sample and the information related to its source is represented in Table 1. All the 36 fungal strains that were grown under solid-state fermentation conditions for the production of lovastatin were screened by bioassay method against *C. albicans* indicator.

Screening of lovastatin producers by bioassay method

The screening of potential lovastatin-producing fungal strain was carried out by measuring the zone of inhibition against indicators. 25 μL , 50 μL , and 100 μL of

standard lovastatin and crude extract of lovastatin of 36 fungal strains along with fluconazole and reference strains extract were loaded into the well to assess the zone of inhibition against *C. albicans*. The graph in Fig. 1a showed that the C3 and C9 strain crude extracts were much effective against *C. albicans* than all other strains when compared to controls and reference strains. The standard plot of zone of inhibitions against different concentrations of standard lovastatin and C9 strain crude extract is also presented in Fig. 1b, c, respectively. This graph showed that C9 strain crude extract activity is similar to that of standard lovastatin. The statistical insignificance was observed when the effect of C9 lovastatin crude extract was compared with standard lovastatin, fluconazole, *A. flavus* and *A. terreus* ($p > 0.0001$). This showed that C9 strain crude extract activity is similar to that of lovastatin, fluconazole, extracts of *A. flavus* and *A. terreus*. This also demonstrated that extract was compared with all the other strains ($p < 0.0001$) as represented in Table 2. Moreover, a statistical significant difference was documented between standard lovastatin and C3 strain which was lower at 25 μL and 50 μL except 100 μL . Hence from the above observations, C9 strain and its crude extract were selected for further evaluation.

Rapid confirmation of C9 statin by TLC, HPTLC and HPLC method

The C9 crude extract showed a R_f value of 0.53 by TLC (Fig. 2) and their R_f value was compared against the authentic lovastatin R_f value of 0.52 which confirms that the C9 crude extract as a lovastatin compound. HPTLC profile of the commercial standard lovastatin and C9-extracted crude lovastatin extract was carried out by measuring the absorbance at 254 nm. The peak height observed in densitogram of the standard lovastatin showed a correlation with C9 crude extract as shown in Fig. 3a, b which confirms once again

Table 1 Details of fungal strain isolated from different agricultural sites of southern region in Tamil Nadu

S. no.	Name of sampling area	Source isolation	No. of strains obtained from soil
1	Pattanam village, Coimbatore	Coconut soil (C)	14
2	Pattanam village, Coimbatore	Sugarcane soil (S)	10
3	Nilgiris tea plantation area	Tea soil (T)	12
Total no. of strains			36

For our conventions, organisms or strains were designated with consecutive number based on the source of sample collection

Fig. 1 Inhibition zones formed at varying concentrations of fungal extracts in significant strains compared against standards and references against *C. albicans*. **a** Yeast growth bioassay of all compared strains at different concentrations. L1 (stan), Standard lovastatin; AF (Flucan), flucanazole; R1 (*A. flav*), *A. flavus*; R2; *A. terreus*. **b** The

effect of standard lovastatin at its different concentration in producing the zone of inhibition against *C. albicans*. **c** The zone of inhibition produced by lovastatin crude extract isolated from C9 strain at different concentrations which is very similar to the effect of standard lovastatin

that C9 crude extract as lovastatin compound. The objective of HPLC analysis of any drug is to confirm the identity of a drug and provide quantitative results. The HPLC chromatogram standard peak was detected at a retention time (RT) of 13.486 and 13.687 min for standard lovastatin and C9 strain crude sample, respectively, which resembled each other as represented in Fig. 4a, b. Hence, the presence of lovastatin is confirmed in a confounding way.

Morphological characterization of selected C9 strain

The selected C9 strain was characterized using standard microbiological methods such as morphological properties (shape, margins evaluation and growth rate) and microscopic properties (conidial head, conidiophores, vesicles and conidia). The organism was observed to have conidial features as shown in the Fig. 5a–c.

Table 2 Details of fungal isolates studied for lovastatin production through yeast growth inhibition bioassay

S. no.	Name of fungal extract	Diameter of zone of inhibition against <i>C. albicans</i> (cm)			C9 crude lovastatin statistical significance ($p < 0.0001$) 'p' value compared with all		
		25 μ L	50 μ L	100 μ L	25 μ L	50 μ L	100 μ L
1	Lovastatin (standard)	1.3	1.5	1.7	0.000128	0.000913	0.004025
2	Fluconazole (Antifungal drug)	0.6	0.9	1.3	0.004025	0.000913	0.004025
3	<i>A. flavus</i> (Reference compound 1)	–	0.5	1.0	–	0.000034	0.008538
4	<i>A. terreus</i> (Reference compound 2)	0.1	0.9	1.3	0.111034	0.000913	0.004025
5	C1	–	–	0.5	–	–	0.000913
6	C2	–	–	1.2	–	–	0.000913
7	C3	0.5	0.7	1.2	0.000913	0.000128	0.000913
8	C4	–	–	0.68	–	–	0.000062
9	C5	–	–	1.2	–	–	0.000913
10	C6	–	–	1.4	–	–	0.004025
11	C7	–	0.3	0.7	–	0.000013	0.00002
12	C8	–	–	1.1	–	–	0.000128
13	C9	0.8	1.2	1.5	–	–	–
14	C10	–	–	1.2	–	–	0.000913
15	C11	–	–	0.9	–	–	0.000034
16	C12	–	–	1.1	–	–	0.000128
17	C13	–	–	1.1	–	–	0.000128
18	C14	–	–	1	–	–	0.000128
19	S1	0.2	0.6	1.2	0.000062	0.000062	0.000913
20	S2	–	–	1.1	–	–	0.000128
21	S3	–	0.3	0.8	–	0.000013	0.000034
22	S10	0.3	0.44	1.3	0.000128	0.000017	0.004025
23	T1	–	–	–	–	–	–
24	T2	–	–	0.3	–	–	0.000013
25	T3	–	0.2	0.4	–	0.000013	0.000913
26	T4	–	–	0.15	–	–	0.000011
27	T5	0.22	0.5	1.0	0.000071	0.000034	0.000128
28	T7	–	0.1	0.5	–	0.000034	0.000128
29	T10	–	0.6	1.0	–	0.000062	0.000128
30	T11	–	0.3	0.5	–	0.000013	0.000128

Values in bold indicate significance

Phylogenetic tree analysis—C9 strain

Alignment and cladistic analysis of homologous nucleotide sequences had shown that the sequence belonged to *Cunninghamella blakesleeana* UBOCC-A-101341 (KF225029.1) strain with a bootstrap value of 100%. Of the species included in the phylogenetic analysis, *C. bertholletiae* strain E15 was found to be closely related to *C. blakesleeana* UBOCC-A-101341 (KF225029.1). From the Fig. 6, it was observed that *C. polymorpha* CBS 779.68 and *C. echinulata* strain ISa species formed a well-supported clade with 88% bootstrap support.

Purification and quantification of lovastatin

The purification of lovastatin was done using conventional method followed by preparative HPLC method (Fig. 7a, b). The peak observed between standard and sample lovastatin was similar. Subsequently, lovastatin was quantified using spectrophotometer at 238 nm. The standard plot of lovastatin was used to evaluate the concentration of the purified lovastatin as shown in the Fig. 8a, b, respectively. *C. blakesleeana* (C9) strain had shown higher concentration than all other strains ($p < 0.0001$). The concentration of lovastatin by *C. blakesleeana* was found to be 1.4 mg g⁻¹

Fig. 2 TLC chromatogram of standard lovastatin (left) and C9-produced lovastatin crude extract (right)

DWS followed by *A. terreus* with 0.834 mg g^{-1} DWS. Both the data reflect that definitely *C. blakesleeana* is a better strain than *A. terreus*.

Characterization of purified compound

Infrared spectrometry (IR)

Infrared spectrum of the standard lovastatin and purified lovastatin is shown in Fig. 9a, b, respectively. The characteristic peaks of lovastatin were represented at 3541 cm^{-1} (alcohol O–H stretching vibration), 3003.27 cm^{-1} (olefinic C–H stretching vibration), 2335.87 cm^{-1} (methyl and methylene C–H asymmetric stretching), 1629.90 cm^{-1} (lactone and ester carbonyl stretch), 1375 cm^{-1} (methyl and methylene-bending vibration), 1033.88 cm^{-1} (ester C–O–C symmetric bend), 819.77 cm^{-1} (trisubstituted olefinic C–H)

Fig. 3 HPTLC profile of standard lovastatin compared over the C9-produced lovastatin. **a** HPTLC densitogram of standard lovastatin. **b** HPTLC densitogram of lovastatin extracted from C9 strain

Fig. 4 Comparison of HPLC chromatogram of standard lovastatin over for rapid confirmation of the compound. **a** HPLC chromatogram of standard lovastatin. **b** HPLC chromatogram of C9-derived crude extract sample

and 750.33 cm^{-1} (meta-disturbed benzene-strong) which confirmed that purified extract of *C. blakesleeana* is lovastatin.

The observed characteristic peak represented each chemical group present in a compound between the standard and purified *C. blakesleeana* extract which almost remained same with a small difference in numerical value. Moreover, the presence of lactone ring peak at 1629.90 cm^{-1} confirmed that the sample compound was lovastatin (Patel and Patel 2007). In the present study, it is observed that the lactone ring peaks at 1763.63 cm^{-1} and 1762.35 cm^{-1} for standard and *C. blakesleeana* extract sample correspondingly. This confirmed that the purified test sample was lovastatin.

Nuclear magnetic resonance spectrometry (NMR) and liquid chromatography–mass spectrometry (LC–MS) analysis

The *C. blakesleeana* lovastatin data obtained from ^1H and ^{13}C NMR were in accordance with the standard data reported in *A.*

terreus. The characteristic peaks of ^1H -NMR shift difference in the neighborhood of the stereo center C-1; H-1 experienced a downfield shift of δ 4.63, whereas H-4 experienced the higher field compared to the H-4 which is at δ 5.80. Further, ^1H -NMR spectrum of the isolated compound suggests that the presence of characteristic three olefinic protons at 5.80 (1H, *q*, $J=6.00$, 3.6 Hz), 6.00 (1H, *d*, $J=9.6$ Hz) and 5.52 (1H, *brs*) corresponding to H-4, H-5 and H-6, respectively. Four methyl group signals at δ 0.90 (6H, *d*, $J=7.6$ Hz), 1.08 (3H, *d*, $J=7.2$ Hz), 1.12 (3H, *d*, $J=6.8$ Hz) and 0.88 (3H, *t*, $J=7.6$ Hz) were due to H-3/ CH_3 , H-7/ CH_3 , H-2''/ CH_3 and H-3''/ CH_3 , respectively, three 'O' attached methine groups at δ 4.63 (1H, *m*), 5.39 (1H, *m*) and 4.37 (1H, *brs*) were attributed to the position of H-1, H-5' and H-3', respectively. The ^{13}C -NMR spectrum indicated the presence of four methyls as δ 22.81, 13.85, 16.21 and 11.70, six methylenes at δ 32.69, 38.59, 36.17, 32.94, 24.25 and 26.79, three OCH_2 were at δ 67.86, 62.83, 76.35 due to C-1, C-3' and C-5' and three methine carbons which were assigned to the characteristic of

Fig. 5 Morphological characterization of C9 strain. **a** C9 strain on PDA plate. **b** Scanning electron microscopic micrograph of C9 strain. **c** Microscopic image of C9 strain showing conidial features

Fig. 6 Phylogenetic tree using the neighbor-joining method of C9 strain inferred from analysis of the ITS region

Fig. 7 Preparative HPLC profile. **a** HPLC chromatogram of standard lovastatin. **b** HPLC chromatogram of purified lovastatin from *Cunninghamella blakesleeana*

the C-4, C-5 and C-6, respectively. Two ester carbonyl carbons at δ 170.30 and 176.80 are due to C-1' and C-1'', respectively. Finally all the spectral data match with lovastatin which is to be 8-(2-(4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl)ethyl)-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2-methylbutanoate, a known compound. The ^1H and ^{13}C NMR spectra of fungal sample yielded a molecular formula $\text{C}_{24}\text{H}_{36}\text{O}_5$ as shown in Fig. 10a, b. The LC–MS spectral peak of lovastatin produced by *C. blakesleeana* is represented in Fig. 11 which ultimately confirmed the compound.

Antioxidant assay

Free radical—scavenging activity measurement

The hydroxyl radical scavenging activity of the purified extracts of *C. blakesleeana* was dose dependent. At a concentration of $199.5 \mu\text{g mL}^{-1}$ (200 μL), the scavenging activity of purified lovastatin was optimum and it is almost nearly equal to that of the standard lovastatin as shown in the Fig. 12a. The IC_{50} of the purified lovastatin was

Fig. 8 Quantification of purified lovastatin using the standard lovastatin plot. **a** Standard lovastatin calibration curve at different concentrations ($\mu\text{g mL}^{-1}$). **b** Estimation of lovastatin from yielding strains

at $145.9 \mu\text{g mL}^{-1}$ ($140 \mu\text{L}$) demonstrating the inhibitory activity and it was not higher than the standard.

Reducing power

Reducing power reflects the antioxidant activity significantly. The reducing capacity of the purified extract was observed maximum at $117.4 \mu\text{g mL}^{-1}$ ($120 \mu\text{L}$) and also optimum at $145.95 \mu\text{g mL}^{-1}$ ($140 \mu\text{L}$) and $166.95 \mu\text{g mL}^{-1}$ ($160 \mu\text{L}$). The reducing power of the purified extracts is also represented in Fig. 12b.

Iron chelating activity

The iron chelating assay was used to evaluate the ability of purified lovastatin to disrupt the complex formation. In our study, it was observed that the percentage of inhibition is maximum at $176.8 \mu\text{g mL}^{-1}$ ($180 \mu\text{L}$) and $199.5 \mu\text{g mL}^{-1}$ ($200 \mu\text{L}$) which is statistically significant ($p < 0.0001$). At other doses also, it is statistically significant ($p < 0.05$) in case of both test sample (*C. blakesleeana* extract) and standard (Fig. 13).

Fig. 9 IR analysis. **a** IR spectrum of standard lovastatin. **b** IR spectrum of purified lovastatin of *Cunninghamella blakesleeana*

Discussion

To date, there are plenty of reports that dealt with the production of pharmaceutical drugs such as antibiotics, anticancer, anti-cholesterol, anti-diabetic, immunosuppressant, anti-anxiety and antiviral from fungal origin and it also acts as obvious potent producers of those useful compounds. At present, organisms that are familiarly reported to produce lovastatin as a secondary metabolite include *A. terreus*, *Monascus* sp., *A. niger*, *A. flavus*, *P. purpurogenum*, *Pleurotus* sp., *T. viride* and *Penicillium* sp., whereas the strains such as *Penicillium* sp., *M. ruber*, and *A. terreus* are commonly used for the

commercial production of lovastatin (Lai et al. 2007; Pecyna and Bizukojc 2011; Subazini and Kumar 2011). However, various compounds have been produced from various fungal species, trying a compound production from a species that are never reported earlier always been crucial and chances of getting an efficient activity could be high. In such perspective, *C. blakesleeana* showed magnificent innate ability of producing lovastatin which is on par with its conventional fungal producers. The purified Lovastatin showed significant zone of clearance and the reasons could be attributed to competitive inhibition of HMG-CoA receptor which in turn prevents ergosterol biosynthesis followed by cell cycle arrest and accompanied by various

Fig. 10 NMR elucidation. **a** ^1H NMR spectrum. **b** ^{13}C NMR spectrum of *Cunninghamella blakesleeana* lovastatin

eventual intracellular activities holistically contributing to cell death. The confirmatory screening techniques including TLC, HPTLC and HPLC all showed similar agreement with standard lovastatin. The proximity of the retention time (standard: 13.486; lovastatin from *C. blakesleeana*: 13.687) between the standard lovastatin and lovastatin obtained from *C. blakesleeana* showed that the lovastatin is no longer distinguishable than the commercially available lovastatin. So far, a large number of microbes were screened for the production of lovastatin in which *A. terreus* was identified to be the best lovastatin-producing fungus (production level up to 55 mg L^{-1}) among other producers (Samiee et al. 2003). Shindia (1997) had reported the synthesis of lovastatin by *A. oryzae*, *A. terreus*, *Doratomyces stemonitis*, *Paecilomyces variotii*, *P. citrinum*, *P. chrysogenum*, *Scopulariopsis brevicaulis* and *T. viridae*, etc. and

concluded *A. terreus* as best producer. Hence, on finding the best lovastatin producers among the studied organisms such as *A. terreus*, *A. flavus* and *C. blakesleeana*, it was evident that *C. blakesleeana* found to be the predominant producer of lovastatin with a yield of 1.4 mg g^{-1} DWS. *C. blakesleeana* produced a good amount of lovastatin without optimization unlike other earlier reported strains which were optimized for attaining considerable production (Table 3). In addition, it was observed that *C. blakesleeana* produced 12% and 23% of lovastatin more than that of *A. terreus* and *A. flavus*, respectively, as shown in the Fig. 8b. Hence, the present study had lucidly established the fact that the unreported lovastatin producer *C. blakesleeana* can be further explored for commercial production after conducting extensive studies such as optimization of medium constituents and other process parameters that

Fig. 11 LC–MS spectra of the purified lovastatin

may influence the production of lovastatin than the present innate capacity of the lovastatin production. Even though *C. blakesleeana* is known to be reported for bromhexine metabolite production (Dube and Kumar 2017), lovastatin productions have not been reported earlier. Thus, the present study makes a sincere note and light on *C. blakesleeana* ability as lovastatin producer and prospecting area to explore further production augmentation and modification of lovastatin.

The constituent and structural details of the purified lovastatin were obtained through IR, NMR and LCMS which all showed similar agreement with the standard lovastatin. For instance, in IR analysis, the lactone ring peak observed at 1763.63 cm^{-1} and 1762.35 cm^{-1} for standard and *C. blakesleeana*, respectively. Similarly, the spectral data obtained through NMR had fine accordance

and matches with the reported lovastatin. Researchers have also quantified lovastatin with their hydroxy acid metabolites in rat and mouse plasma and even in humans using LC/MS/MS (Vlčková et al. 2011; Wu et al. 1997). The information derived from the above spectroscopic analysis is also in accordance with our report. This shows that the *C. blakesleeana* is producing lovastatin in a form without any detectable structural differences than that of the commercial producers of lovastatin.

Oxidative stress has been associated with the development of many diseases such as cardiovascular disease and cancer which cause higher mortality worldwide. Certain earlier reports have shown that the lovastatin possesses anticancer and antioxidant activity (reduces oxidative stress) (Singh et al. 1997; Kumar et al. 2011). Bhargavi et al. (2016) have shown that the lovastatin isolated from *A. terreus*

Fig. 12 Antioxidant and reducing power assessment of purified lovastatin. **a** Assessment of radical scavenging activity using DPPH. **b** Reducing power evaluation of the lovastatin extract

(KM017963) was effective to accelerate hydroxyl radical scavenging activity (54.06%) at an IC₅₀ of 3601 µg mL⁻¹ compared to its positive control (catechin) which showed 86.86% at an IC₅₀ value of 350.5 µg mL⁻¹. The same paper had reported that lovastatin showed a moderate reduction in total reduced glutathione (41.58%) compared to its positive control (ascorbic acid) which showed 68.85% at an IC₅₀ value of 19.46 µg mL⁻¹. Our study had observed IC₅₀ value of purified lovastatin at 145.9 µg mL⁻¹ (140 µL) demonstrating its potent inhibitory activity. This difference could be because of the controls (standard lovastatin) used for the study. Hydroxyl radicals formed through ascorbic acid–iron EDTA complex are effectively disturbed by the lovastatin of *C. blakesleeana* which reflected the increased scavenging activity in terms of decreased absorbance as observed in the present study. It is a well-known fact that

cancer cells generate uncontrolled formation of hydroxyl free radicals which result in death of healthy cells, tissues, DNA modifications, membrane peroxidation, enzyme inactivation and protein oxidations (Mohan Kumari et al. 2011). Hence, lovastatin was found to be effective than the standard in accelerating scavenging activity of hydroxyl radical. Hence, the current study had the significant reflection of an antioxidant activity and reducing power ability of the purified lovastatin compound synthesized by *C. blakesleeana*. Thus the reported species *C. blakesleeana* produces lovastatin with good quantity and similar structural and functional features on par with earlier reported lovastatin.

In a commercial perspective, the economy behind the production can only be analyzed. For instance, wheat bran acts as an excellent cheaper medium possessing protein, fat and crude fiber, celluloses, pentoses, vitamins, minerals, and

Fig. 13 Assessment of iron chelating ability of standard lovastatin and purified lovastatin

Table 3 Lovastatin production from various fungal species using solid-state fermentation

S. no.	Reports	Strain	Lovastatin production (mg g ⁻¹ DWS)	Yield after optimization
1	Mouafi et al. 2016	<i>Aspergillus fumigatus</i>	3.353	Yes
2	Kamath et al. (2015a)	<i>Aspergillus terreus</i> (KM017963)	3.6	Yes
3	Kamath et al. (2015b)	<i>Aspergillus terreus</i> (KM017963)	1.3	Yes
4	Dikshit and Tallapragada (2015)	<i>Monascus sanguineus</i>	0.402	Yes
		<i>Monascus purpureus</i>	0.27	Yes
		Both of the above co-culture	0.26	Yes
5	Our current report	<i>Cunninghamella blakesleeana</i> KP780148.1	1.4	No
		<i>Aspergillus terreus</i>	0.83	No
		<i>Aspergillus flavus</i>	0.3	No

phytochemicals and such richness in constituents made it as a suitable substrate for commercialization (Kamath et al. 2015a, b; Jahromi et al. 2012; Chanakya et al. 2011; Arranz and Calixto 2010; Jaivel and Marimuthu 2010; Sramkova et al. 2009; Sun et al. 2008). Wei et al. (2007) stated that either wheat or rice bran in SSF gives maximum lovastatin production in a cost-effective way. Regarding patents, there are plenty of existing patents on lovastatin production

and purification which were comprehensively reviewed by Mulder et al. (2015). Since the present study aims in investigating the possible lovastatin production ability of *C. blakesleeana* KP780148.1 strain, our future work involves a detailed insight on exploring optimized higher production in commercial aspect. Hence, compared with the existing patent is quite early for this *C. blakesleeana* KP780148.1 strain which is newly reported to have lovastatin ability.

Conclusion

This is the first report that documented *C. blakesleeana* having gene bank accession number KP780148.1 as a lovastatin producer. This study suggested that the *C. blakesleeana* strain may be a better alternative source to the conventional sources such as *A. terreus* and *A. flavus* in the production of lovastatin. However, the commercialization of lovastatin production can be feasible after subjecting to further studies on production optimization. Since the characterized lovastatin demonstrated significant antioxidant property, future perspectives are towards improving the yield of lovastatin followed by its further necessary applications for establishing its strong anticancer potentials.

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