doi.org/10.36721/PJPS.2024.37.4.REG.863-871.1

Identification of lovastatin production in *Aspergillus terreus* strain (KF971363.1) and its antifungal role

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Abstract: Lovastatin has received interest for its potential therapeutic use in treating numerous diseases, for example, the blood cholesterol level by restraining hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. The research utilized the fungal growth bioassay technique to disengage and evaluate filamentous organism for the lovastatin creation. The clever type of *Aspergillus terreus* (KF971363.1) was embraced for lovastatin creation by solid-state fermentation (SSF). Lovastatin production was optimized using physiological parameters such as pH and temperature at SSF. The addition of nitrogen source enhanced the production of lovastatin by the breakdown of lignocellulose that improved the production of lovastatin. The research verified a yeast growth inhibition bioassay approach, in addition to thin-layer chromatography and liquid chromatography-mass spectrometry (LC-MS). All of these techniques were used to confirm lovastatin production. The purified extract subjected to the TLC analysis showed retention factor (Rf) value of 0.73. Moreover, the inhibition bioassay method reassures the lovastatin production by comparing the zone of inhibition against *C. albicans.*

Keywords: Lovastatin, *Aspergillus terreus,* hypercholesteremia, liquid chromatography-mass spectrometry, thin layer chromatography, solid state fermentation.

INTRODUCTION

Lovastatin is used to treat high cholesterol levels (Faseleh Jahromi *et al*., 2012). In the cholesterol production pathway, lovastatin is a competitive inhibitor of the essential enzyme HMG-CoA reductase. HMG-CoA reductase, a crucial rate-regulating enzyme, is necessary for the cholesterol production pathway to begin (Alberts, 1990). A 27-carbon steroid called cholesterol fills in as a significant piece of the cell membrane, a moderate forerunner of steroid chemicals and the substrate for the development of bile corrosive, which the body needs to retain fats and fat-solvent nutrients from the gut. Because of the high cholesterol production, many conditions like obesity, hypercholesterolemia and fatty liver may occur (Alberts, 1988). Through this process statins can be used medicinally to treat hypercholesterolemia and perhaps lessen cancer-related mortality (Wang *et al*., 2021).

An analogue of lovastatin, simvastatin prevents the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) from producing cholesterol. These chemicals are referred to as “Statins” in the field of pharmacology (Samiee *et al*., 2003). Each one has an HMG-like component that could take the form of an inert lactone. Statin structural resemblance to HMG-CoA which opens the hydroxy acid form to have an inhibitory effect (Istvan & Deisenhofer, 2001). These prodrugs are converted *in vivo* by enzymes to their functional hydroxy-acid forms. Stiff, hydrophobic groups are covalently bonded between the statin and the HMG-like moiety (Schachter & pharmacology, 2005). Statins perform their function by sterically preventing the substrate from binding by binding to the active site of an enzyme. Through the assurance of the catalyst's substrate-restricting pocket (Mulder *et al*., 2015). Lovastatin is made by fungi like *Penicillium* spp., *Monascus* spp., *Trichoderma* spp. and *Aspergillus terreus* (Mouafi *et al*., 2016) say that *A. terreus* is the best species for making lovastatin. Lovastatin is a secondary product that is most often made by fermenting *A. terreus* (Gupta *et al*., 2007). The majority of *A. terreus*, a type of soil fungus, is found in soils all over the world. Despite being primarily thought of as an asexual fungus, new investigations have revealed that it is capable of sexual reproduction (Arabatzis & Velegraki, 2013). Lovastatin is a secondary metabolite and a potent hypocholesterolemic agent, generally produced from *A. terreus* using the process of fermentation (Gupta *et al*., 2007). Warmer climates, such as tropical and subtropical areas, are known to harbor *A. terreus* in soil, compost and decaying plant matter. The early colonies of *A. terreus* are shown brownish in medium and as they grown mature, they turn to dark brownish color. *A. terreus* is known to grow best between 35°C and 40°C and due to the tolerance to heat, it can develop to the greatest potential between 45°C and 48°C (Sreelatha *et al*., 2017). The study was planned to analyze the lovastatin production from the novel strain of *A. terrus* (KF971363.1).

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MATERIALS AND METHODS

Inoculum preparation and growth

The fungal strain, *A. terreus* (KF971363.1), was obtained from the fungal data bank of Department of Biochemistry, The Islamia University of Bahawalpur. *A. terreus* KF971363.1 was allowed to inoculate on potato dextrose agar (PDA) and incubated at 32°C for seven day with Subsequent to addition of 10mL of 0.1% Tween 80 asceptically. The spores were scratched with a sterile loop. The spores were further suspended into sterilized media of 100mL in the Schott bottle that had a similar arrangement. (Faseleh Jahromi *et al*., 2012).

Solid state fermentation

Solid state fermentation was carried out in flasks of 500 mL capacity containing 20 gm of the rice straw (RS) substrate. Rice straw is the most abundant agricultural byproduct. Rice straw was acquired from the local market as the cost effective economical resource. The material was ground to obtain fine particles size and dried for 48 hrs in the hot oven and used for SSF (Wei *et al*., 2007).

Optimal conditions for fermentation

The production of lovastatin was optimized using physiological factors including temperature, pH, and moisture contents. The pH of solution was adjusted to 5, 6, 7 and 8 by using 1M hydrogen chloride and 1M sodium hydroxide before adding substrate in to the solution (Kumar *et al*., 2000). The moisture level was adjusted till 75%.The moisture content of the substrate was modified using mineral solution (KH2PO4: 2.1g/L, MgSO4: 0.3g/L, CaCl2: 0.3g/L, FeSO4: 0.11g/L, ZnSO4: 0.3g/L) to generate a moisture level at 75% (Ikeda *et al*., 1999). Inoculating media was autoclaved for 15 minutes at a temperature of 121°C, a modest amount of the inoculum was used. After ten days of incubation at 32°C, the biomass in the flask filtered and dried for 48h in an oven at 60°C. The dried biomass of culture was grinded in to a fine powder and proceeded for further extraction process.

Influence of nitrogen on the production of lovastatin

In order to investigate the effect of different nitrogen sources on the production of lovastatin, urea, ammonium sulphate, and sodium glutamate were used (Pansuriya & Singhal, 2010). 10% inoculum was added into the autoclaved media and the mixture was thoroughly mixed. The flasks was incubated at 32°C for 10 days. After that, 0.5g of the dry culture was extracted using 15mL of methanol with 60 minutes of shaking at 220 rpm. Thin layer Chromatography was used to measure the concentration of lovastatin in the filtrate following the membrane filtration (Babu *et al*., 2011).

In solid state fermentation, Candida albicans and A. terreus

At the end of the incubation period for the culture slants containing *C. albicans* and *Aspergillus terreus*, 10mL of sterile 0.1% Tween-80 solution was added, as shown in fig. 3. Subsequently, a sterile loop was used to scratch from the biomass of the culture media. The spores were cultured in the subsequent media with Tween-80 solution and transferred into a Schott container with vigorous shaking (Babu *et al*., 2011).

Fig. 1: *A.terreus* (KF971363.1) growth on potato dextrose agar medium (PDA).

Solid state fermentation was conducted in two 500ml flasks containing 20g of the appropriate substrate (RS). Using mineral solution, the moisture content was adjusted to approximately 75% (CaCl2: 0.3g/L, KH2PO4: 2.1g/L, FeSO4: 0.11g/L, MgSO4: 0.3g/L, and ZnSO4: 0.3g/L). The pH levels of both solutions were adjusted to 6. Ten percent of both strains were added separately to two containers. Both containers were incubated for ten days at 32°C. Then, 0.5 gm of the dried cultures were extracted using 15mL of methanol and 60 minutes of stirring at 220 rpm following membrane filtration, the lovastatin concentration in the filtrate was analyzed using TLC (Jaivel & Marimuthu, 2010).

Thin layer chromatography (TLC)

Thin-layer chromatography was used to establish the presence of lovastatin in the extracts after the solid-state fermentation operations were completed. TLC was used to detect the lovastatin's presence in the extract. 10 mg of Atorvastatin from Pfizer was dissolved in 15mL methanol to make standard solution. A 20µL of the extract was spotted to TLC silica gel plate. TLC plate of sample and standard solution was developed in a solvent system of 70% dichloromethane: 30% ethyl acetate. After the development of chromatogram in the mobile phase, TLC plates were observed under the UV light.

The distance travelled by each spot on the TLC plates were marked. It was possible to identify lovastatin in the samples by comparing the relative Rf values by selected mobile phase to the conventional Rf values (Dikshit *et al*., 2015).

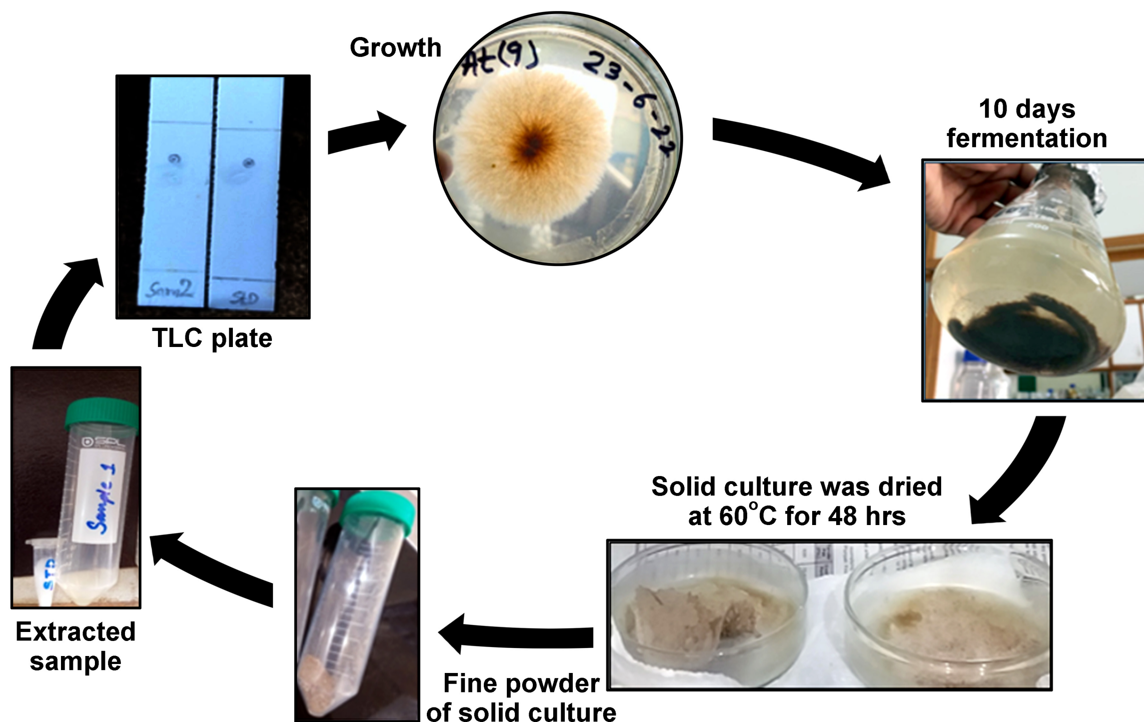

Fig. 2: A schematic illustration of *A. terreus's* lovastatin synthesis via solid-state fermentation. The complete schematic cycle started from the growth of *Aspergillus* strain on the potato dextrose agar medium (PDA), after gaining the best growth of the strain at the day 14th, the fermentation process was conducted for 10 days. Then solid culture was dried and grounded in to a fine powder to extract sample for TLC.

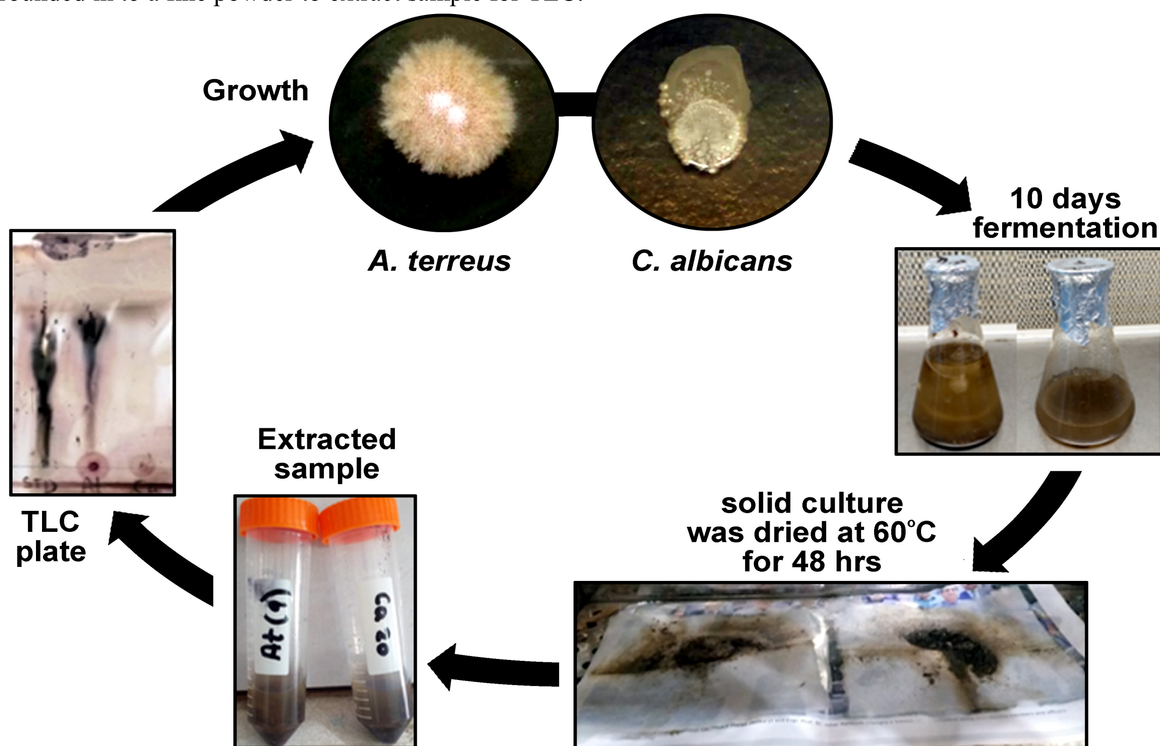

Fig. 3: Solid state fermentation by *A. terreus* and *C. albicans* to produce lovastatin is shown schematically. The complete schematic cycle started from the growth of both strains on the potato dextrose agar medium (PDA), after gaining the best growth of the strains, the fermentation process was conducted for 10 days. Then solid culture was dried and grounded in to a fine powder to extract sample for TLC.

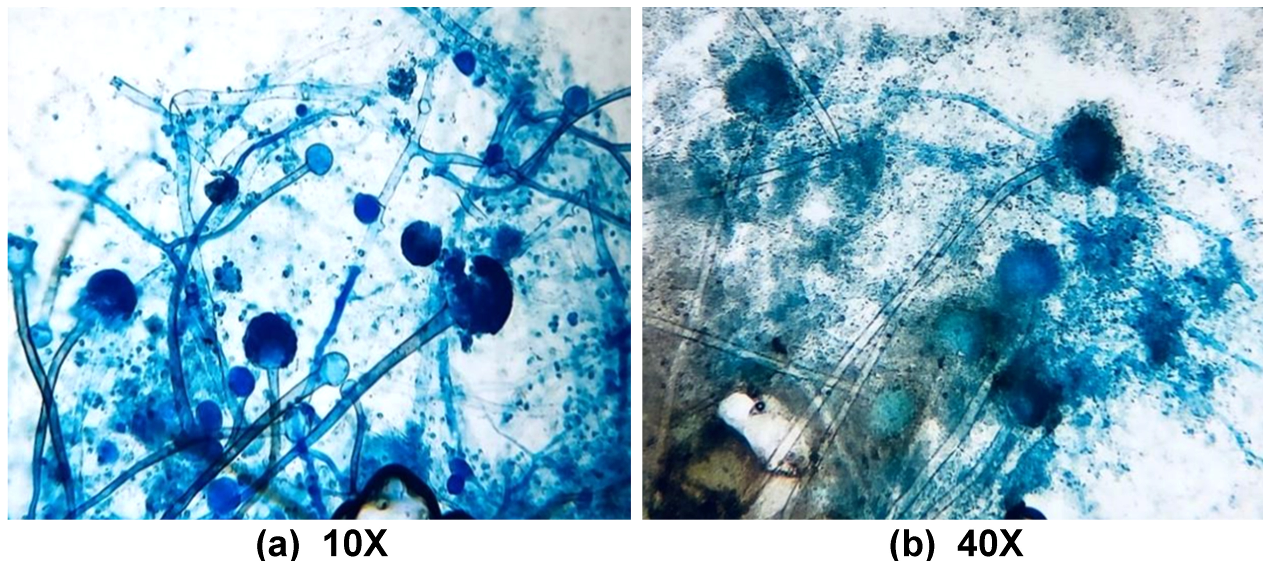

Fig. 4: Microscopic morphological appearance of Aspergillus strain (KF971363.1).

Table 4.1: Rf value of extract and standard lovastatin

Sample Spot	Distance travelled by spot (mm)	Rf value
Spot (At)	31	0.73
Standard spot	32	0.76

Table 4.2: Rf value of extract with and without different nitrogen sources

Sample Spot	Distance travelled by spot (mm)	Rf value
Spot A (without any nitrogen source)	25	0.60
Spot B (urea)	29	0.70
Spot C (sodium glutamate)	16	0.39
Standard spot	31	0.75

Table 4.3: Rf value of extract from *A. terreus* and *C. albicans*

Sample Spot	Distance travelled by spot (mm)	Rf value
Spot A (*At*)	2.9	0.64
Spot B(Ca)	0	0
Standard spot	3.5	0.77

Effect of nitrogen source

Secondly, same TLC was carried out to determine how the nitrogen source affected the production of lovastatin in the extract. TLC plate was created using a 10% Methanol: 90% Ethyl acetate solvent solution. After the development of chromatogram in the mobile phase, the TLC plate was observed under the UV light. Lovastatin was found to be by contrasting the Rf values of the extracts with the typical RF values for the chosen mobile phase (Kan *et al*., 2015).

Comparison between A. terreus and C. albicans

Thirdly, for the comparison between *C. albicans* and *A. terreus* in the production of lovastatin, the same procedure of TLC of the extract of was performed. TLC plate was created in a solvent system of 70% Methanol: 30% Ethyl acetate. After the development of chromatogram in the mobile phase, the TLC plate was observed under the UV light.

The distances travelled by each spot on the TLC plates were marked. Comparing the relative Rf values with the conventional Rf values for the mobile phase allowed researchers to determine if lovastatin was present (Lingappa *et al*., 2004).

Inhibitory bioassay for yeast growth

The bioassay approach for screening lovastatin, using *C. albicans* as the indicator organism was followed from (Babu *et al*., 2011). The indicator was surrounded by a distinct inhibition zone in the bioassay approach. The amount of high-quality lovastatin in the sample is closely connected with the width of the inhibitory zone. By seeding 0.25mL of yeast culture into 15mL of glucose

yeast peptone agar medium, a bioassay for yeast growth inhibition was carried out. *C. albicans* was inoculated into a 15-mm-wide Petri dish. Extracts was pumped into wells with a diameter of 6 mm with a sterilized cork borer. As a negative control, ethyl acetate was used, and as a positive control the lovastatin was used (Friedrich *et al*., 1995). All of the plates were kept at 26°C for 16hrs. The diameter of the inhibition zone of the indicator organism and the standard lovastatin concentrations were compared (Balraj *et al*., 2018).

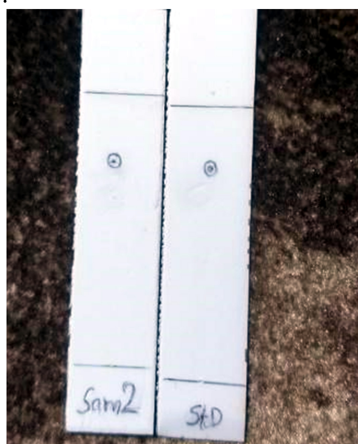

Fig. 5: TLC chromatogram of conventional lovastatin, as well as the crude extract of lovastatin that was generated by *A. terreus.*

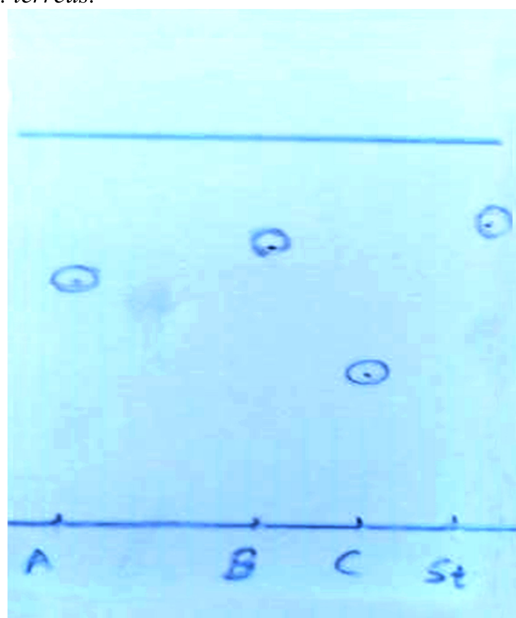

Fig. 6: TLC chromatogram of standard lovastatin, (A) without any nitrogen source, (B) urea and ammonium sulphate as a nitrogen source, (C) sodium glutamate as a nitrogen source.

Analysis with liquid chromatography-mass spectrometry (LC–MS)

The Lovastatin extract was further subjected to LC-MS screening in accordance with the instructions of a previous study (Side, 2018). During this multi-step process, the analyte has to be partitioned into the organic solvent, the organic and aqueous layers need to be separated, the organic solvent needs to be evaporated, and the analyte to be reconstituted in a solvent combination that is miscible with the LC mobile phase.

Identification and taxonomic positioning of A. terreus (KF971363.1)

Using fungal universal primers for the intra transcribed spacer (ITS1/ITS4) obtained by PCR amplification of ITS regions, the selected fungi were identified down to the species level. Using BLAST search engine (http://www.ncbi.nlm. nih.gov/), the sequence homology and Nucleotide NCBI blast was utilized to look at the sequences and Clustal W and MEGA6 were used to line up the sequences (Ismail *et al*., 2021).

Fig. 7: The crude extract of lovastatin was generated by TLC chromatography of standard lovastatin, *A. terreus*, and *C. albicans*.

STATISTICAL ANALYSIS

The data analysis statistically was performed by mean values using Microsoft Excel 2018 2018.

RESULTS

The Fungal strain, *A. terreus* (KF971363.1), was obtained from the fungal data bank of biochemistry department of The Islamia University of Bahawalpur. In order to cultivate the strain, the conditions for solid-state fermentation, which are required for the manufacture of lovastatin, were adhered to.

Microscopic morphology

Standard microbiological techniques were used during the morphological characterization of the *A. terreus* strain. These methods incorporated the assessment of the *A.*

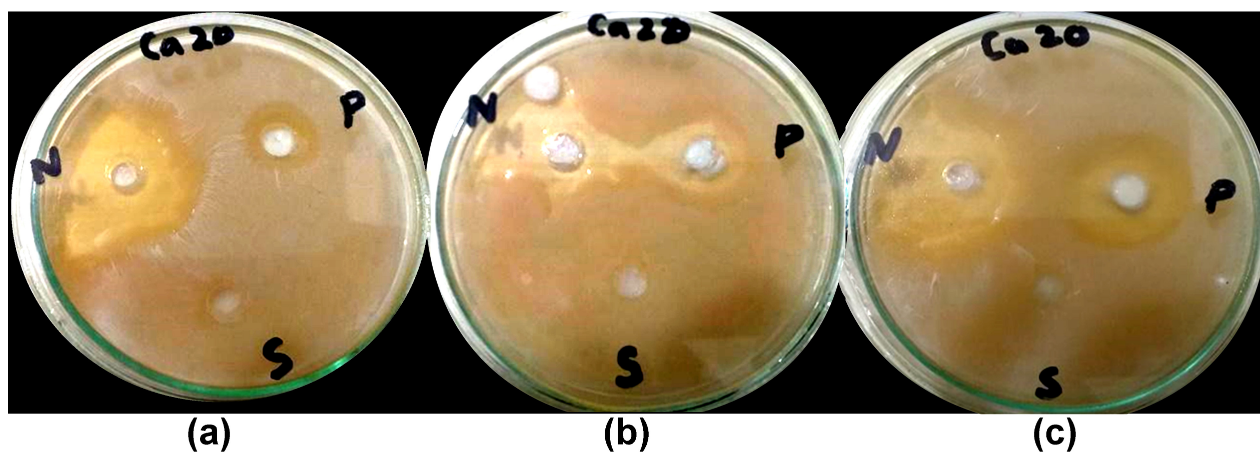

Fig. 8: Inhibition zones formed at varying concentrations of fungal compared with standard and against *C. albicans*. (a) Positive control = 0.8mm, negative control = 1.9mm, sample = 0.6mm. (b) Positive control = 0.9mm, negative control=1.8mm, sample =0.8mm (c) Positive control =1.4mm, Negative control=1.9mm, Sample = 1mm.

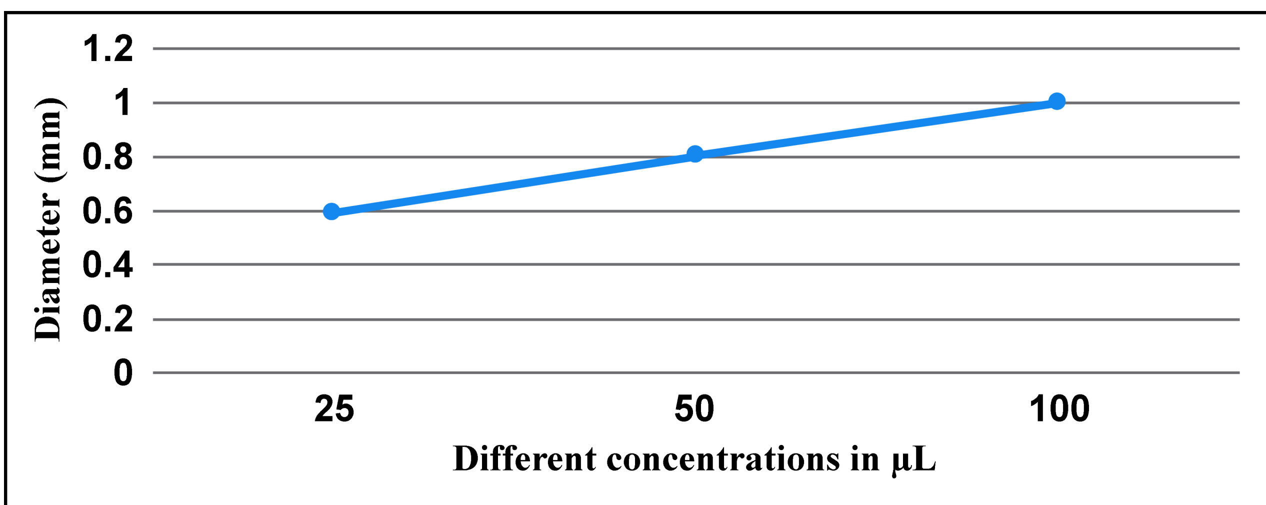

Fig. 9: Inhibition zones created at varying concentrations of fungal extract. The vertical axis shows the zones of inhibition diameter in mm. The horizontal axis shows the varying concentrations of 25, 50 and 100 µL. The whole graph shows that with the increase in the concentration of fungal extract, there was an increase in the zone of inhibition's diameter against *C. albicans*.

terreus morphological characteristics (structure) as well as its minuscule properties (conidial head, conidiophores, vesicles and conidia). The observations revealed the presence of conidial features in the organism as shown in fig. 4. The conidias of *A. terreus* were small, glob-shape, smooth-walled. This fungus was promptly separated itself from different types of *Aspergillus* by its cinnamon-earthy colored state shading.

Optimization of lovastatin production

The pH of solution was adjusted at 6pH by using 1M hydrogen chloride and 1M sodium hydroxide before adding substrate in to the solution. Mineral solution (KH_2PO_4) was used to adjust the initial moisture concentration. 2.1g/L, $MgSO_4$: 0.3g/L, $CaCl_2$: 0.3g/L, $FeSO_4$: 0.11g/L, $ZnSO_4$: 0.3g/L) to deliver a dampness content of roughly 75%.

Analysis of lovastatin by Thin- layer chromatography (TLC)

To detect the lovastatin in the extract TLC was carried out. The distance travelled by each spot on the TLC plates was marked. It was determined that lovastatin was present in the samples after comparing the relative Rf values that were derived with the standard Rf values that were determined for the mobile phase. By conducting TLC, the extract exhibited an Rf value of 0.73 (as shown in fig. 5). Through comparison with the standard Rf value for the lovastatin, which is 0.76, it was determined that the extract indeed contains lovastatin compound.

Thin layer chromatography (TLC) to analyze the influence of nitrogen

To detect the lovastatin in the extract TLC was again carried out and distances travelled by each spot on the TLC plates was marked. The presence of lovastatin was

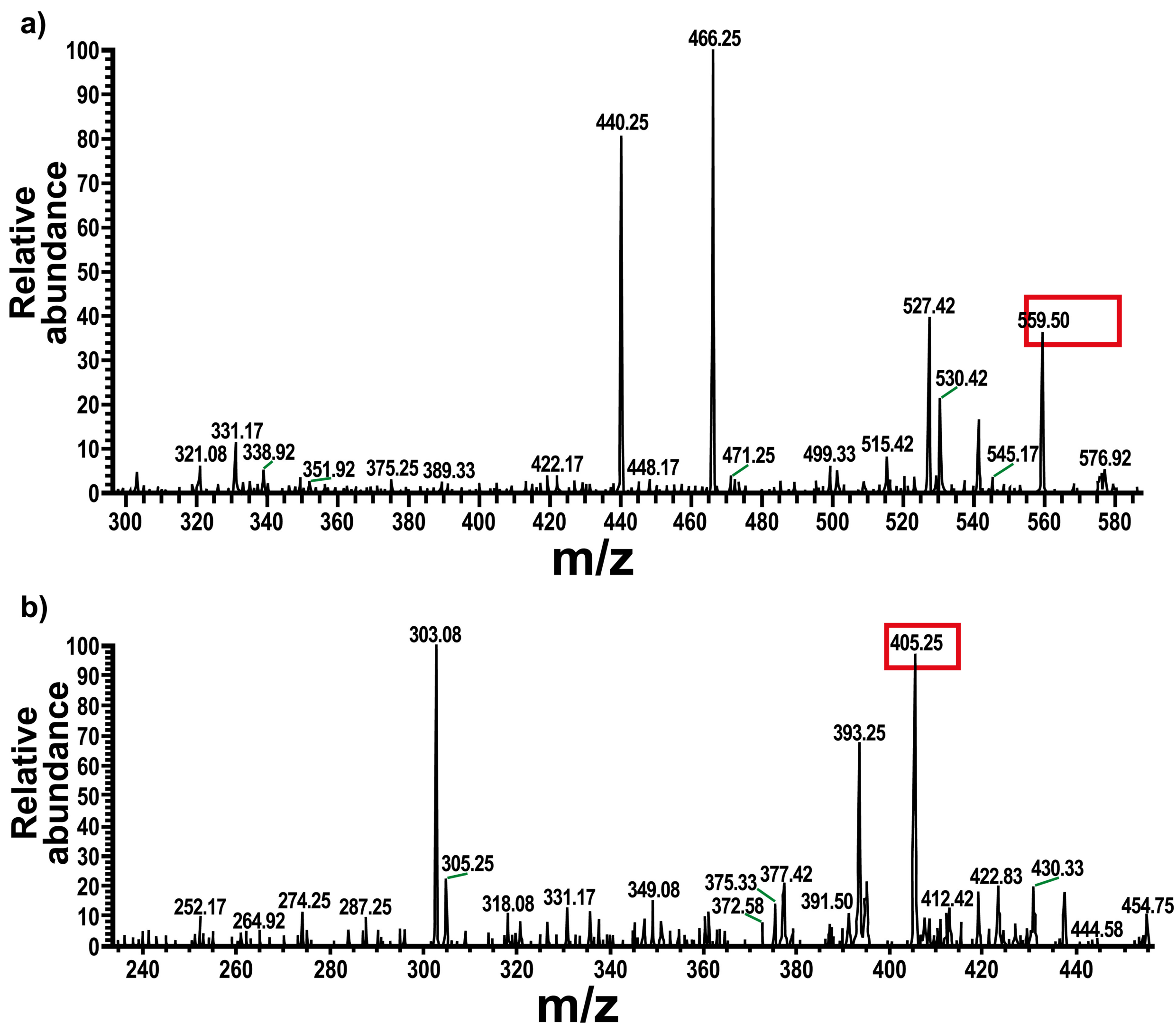

Fig. 10: The standard atorvastatin represented by LC-MS spectral peak of 559.50m/z as (a)The graphical representation of LC-MS spectral peak of lovastatin produced by at 405 mentioned as (b) *A. terreus*.

detected in the samples by first calculating their relative Rf values and then comparing those values to the standard Rf values that were determined for the mobile phase. The extract (A) without any nitrogen source, (B) Urea and ammonium sulphate as a nitrogen source, (C) Sodium glutamate as a nitrogen source displayed an Rf value of 0.60, 0.70 and 0.39 respectively by TLC (fig. 6), and their Rf value was matched against the genuine lovastatin Rf value of 0.75, which demonstrated that the extract B was a lovastatin compound more than that of A and C.

Confirmation of lovastatin by TLC in A. terreus and C. albicans

To detect the lovastatin in the extract TLC was once again carried out. The distance travelled by each spot on the TLC plates were marked. Following the computation of the relative Rf values, a comparison with the conventional Rf values for the mobile phase made it possible to identify lovastatin in the samples.

After comparing the Rf values of the extracts from *A. terreus* and *C. albicans* to the standard lovastatin Rf value of 0.77, it was determined that the extract from *A. terreus* is a lovastatin compound. The Rf value of the extract from *C. albicans* was 0.64, while the Rf value of the extract from *A. terreus* was 0.64.

Bioassay-based lovastatin producer screening

By comparing the zone of inhibition as shown in fig. 8, lovastatin-producing fungus strain was screened. To determine the zone of inhibition compared with *C. albicans*, 25µL, 50µL and 100µL of the fungal strain extract were injected into the wells as previously published study (Babu *et al*., 2011).There was increase in the diameter of zone of inhibition in comparison with *C. albicans* with the increase in the concentration of fungal extract as shown in fig. 8 and fig. 9.

Screening by Liquid chromatography-mass spectrometry (LC-MS)

Standard (Atorvastatin)

In standard graph atorvastatin presence was shown by LC-MS spectral peak of 559.50m/z. In test sample, LC-MS spectral peak of 405.25 m/z represented the lactone form of lovastatin (fig. 10).

DISCUSSION

Numerous studies have addressed the pharmaceutical products from fungal origin, which served as an obvious potent producer of bioactive compounds, such as anti-cholesterol, anticancer, anti-diabetic, antibiotics, immunosuppressant and anti-anxiety. Fungi such as *A. terreus*, *A. niger*, *A. flavus, Moascus spp, Pleurotus spp, penicillium spp* and *T. viride* are currently known to produce lovastatin as a secondary metabolite, whereas *Penicillium spp, M. ruber* and *A. terreus* are frequently utilized for the creation of lovastatin (Pecyna & Bizukojc, 2011).

In the present research, study checked the affirmation of lovastatin from the *A. terreus* (KF971363.1). The organism was seen to have conidial characteristics under a microscope (Valera *et al*., 2005).

The impact of nitrogen source on the development of lovastatin was additionally seen by utilizing Dainty layer chromatography (López *et al*., 2003). Additional nitrogen source gave slightly greater production of lovastatin because it helps in the breakdown of lignocellulose-based substrate which enhances the production of lovastatin.

For the purpose of lovastatin screening, quick screening method was demonstrated. The screening process was based on lovastatin inhibitory effect against the *C. albicans* (yeast). Inhibitory zones observed on *C. albicans* plates. Further lovastatin production was compared between the *A. terreus* and *C. albicans* using TLC.

Lovastatin was isolated as its lactone form as depicted in (fig. 10). The hydroxyl type of lovastatin is the more dynamic type and unstable form of this drug however, it is temperamental. The lactone form of lovastatin is the main lovastatin found in fermented products and it is also confirmed by liquid chromatography-mass spectrometry (LC-MS) (Jaivel & Marimuthu, 2010).Thus, study proved that *A. terreus* (KF971363.1) has been demonstrated to be a powerful strain for the synthesis of lovastatin (lactone form) as per previously reported study (Hassan *et al*., 2019).

CONCLUSION

The present study demonstrated that in solid state fermentation, the novel strain of *A. terreus* (KF971363.1) produces lovastatin. Expanded nitrogen source gives marginally more noteworthy creation of lovastatin. Lovastatin creation of *A. terreus* (KF971363.1) was affirmed by tender loving care with maintenance factor (Rf) worth of 0.73 contrasted and standard atorvastatin's (Rf) value of 0.76. Furthermore, LC-MS determined the stable lactone form of lovastatin from *A. terreus* (KF971363.1) with spectral peak of 405.25 m/z. Moreover, study proved that lovastatin production from *A. terreus* (KF971363.1) has antifungal inhibitory effect for *C. albicans*. Thus, study suggested that the novel strain of *A. terreus* (KF971363.1) had potent antifungal capability that should be further investigated with pharmacodynamic perspectives.

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