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# Screening of different *Fusarium* species for bioassay of lovastatin

**Vijay Vardhan Pandey, Sucheta Verma, Nidhi Gupta, Vinay Kumar Varshney and Amit Pandey**

**Abstract**
The aim of this research is to Bioassay of lovastatin from *Fusarium* spp. obtained from NTCC, Forest Pathology Discipline, Forest Protection Division, Forest Research Institute, Dehradun, India. Lovstatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A- reducatase (HMG-CoA reducatse) enzyme and a competitive inhibitor of the biosynthesis of cholesterol. In the present study, six different fungal species viz., *Fusarium oxysporum (NTCC 367)*, *F. moniliforme (NTCC 390)*, *F. avenaceum (NTCC 1056)*, *Fusarium equiseti (NTCC 1142)*, *Fusarium solani (NTCC 1145)* and *Fusarium semitectum (NTCC 1150)* were screened for lovastatin production in liquid and solid growth media. The lovastatin extraction from the fungal colonies was done by centrifugation and filtration method. Different techniques viz., paper disc and well preparation were employed to compare their relative sensitivity and efficacy. All the *Fusarium* spp. Shows positive result for lovastatin bioassay. According to results it was concluded that in comparison to the liquid medium (Potato Dextrose Broth), the solid medium (Potato Dextrose Agar) was more able to produce lovastatin.

**Keywords:** bioassay, *Fusarium*, loastatin, *Neurospora*

## Introduction
Statin based compounds have an extremely high therapeutic value and other biological activities. Some of the statins which are naturally produced like lovastatin and compactin are produced by the fermentation, while there are a number of other semisynthetic statins produced by biotransformation. Simvastatin, the second leading statin in the market is also derivative of lovastatin [1]. Lovastatin ($C_{24}H_{36}O_5$, Mevinolin, Monacolin K) is a fungal polyketide based secondary metabolite widely used as a prominent drug in hypercholesterolemia. It stops cholesterol synthesis by competitively inhibiting the rate limiting enzyme 3-hydroxy 3-methylglutaryl Coenzyme-A reductase (HMG Co-A reductase) of the cholesterol synthetic pathway [2, 3].

This compound decrease the level of cholesterol concentration in blood; particularly bad cholesterol (low density lipoprotein, LDL); while slightly increasing the level of good cholesterol (high-density lipoprotein, HDL), thus, preventing plaque build-up inside the arteries and hence, it decreases the risk of heart attack or stroke or cardiovascular diseases[4]. Besides these the recent studies also showed that statins can be used for treatment of alzheimer's disease [5], multiple sclerosis [6], renal disease treatment[7], bone maturation [8] and to some extent in treatment of cancer [9].

Lovastatin lactone form has comparatively higher activity as compared to its acid form. Fungi are important sources for the production of several pharmaceutical compounds. They produce a large variety of compounds mainly through the polyketide biosynthesis pathway. Statins, among these classes of fungal metabolites have become the focus of great attention due to their ability to influence the de novo synthesis of endogenous cholesterol [10]. Of many statin molecules, lovastatin and mevastatin are natural, while other statins like rosuvastatin, simvastatin, pravastatin, fluvastatin, atoravastin, cerivastatin, are produced semi-synthetically from lovastatin [11] Lovastatin is produced as a secondary metabolite by a variety of filamentous fungi. The production starts from the acetate units in head to tail function to form two polyketide chains [12]. Commercially it is produced from Aspergillus terreus [13] but, it can also be synthesized from various fungal strains like *Penicillium* sp. [12], *Monascus ruber* [14], *Monascus purpereus*, *A. paraciticus*, *Acremonium chrysogenum*, *Trichoderma viridae*, *Pleurotus* sp. [10]. Commercial production of lovastatin through batch fermentation using *A. terreus* has been investigated extensively [15]. In submerged fermentation, the yield of lovastatin is proportional to the biomass accumulated. Lovastatin can also be produced by solid state fermentation, which uses substrates that require lesser processing

with better yield than submerged fermentation [16]. Screening of different fungi from natural resources [17]. Besides these, many edible fungi of higher basidiomycetes are capable of producing cholesterol lowering drug; especially *Pleurotus* sp. which is used for production of single cell protein [18]. In the present study, Lovastatin bioassay has been screened from different strains of *Fusarium* sp. in liquid (Potato Dextrose Broth i.e. PDB) and solid growth media (Potato Dextrose Agar i.e. PDA).

## Methodology

### Organisms and fermentation condition

Different *Fusarium* sp. cultures were obtained from NTCC, Forest Pathology Discipline, Forest Protection Division, Forest Research Institute, Dehradun, India and bioassay microbial culture *Neurospora crassa* (MTCC 159) was obtained from MTCC, Chandigarh (Figure 1 and 2). These cultures were used screened for the lovastatin production by submerged fermentation. The stock culture was periodically grown on PDA plates for 6-7 days at $28^0$C. The production of lovastatin was carried out in shake flask under agitation speed of 170-180 rpm for 7-8 days at $28^0$C in the production media at pH 6.5 [19]. The 50 ml of medium was prepared in conical flask and it was sterilized at $121^0$C for 20 minutes. 6 mm size of each freshly grown PDA cultures was aseptically transferred to the production media and production of lovastatin was monitored.

**Bioassay:** The method for screening of lovastatin producing molds by bioassay was adopted from using *Neurospora crassa* as indicator organisms [20]. *Neurospora crassa* was grown for 12 h on PDA dishes at $28^0$C, then harvested and transferred at a concentration of $5\text{-}7X10^2$ cells per ml on fresh PDA slants. Two extraction methods were used for lovastatin detection:

1. **Centrifugation method:** Centrifuge method is used for separation of statins from culture containing media. the cultures was punched out with the help of sterile cork borer(6mm in diameter) and taken in a centrifuge tube with the help of needle, in which 1000µl of ethyl acetate was added. This was macerated with the help of sterile needle and, centrifuged at room temperature at 10000rpm for 10 min for layer separation. The supernatant was collected and bioassay was carried at using *Neurospora crassa* to find the concentration of lovastatin present in the culture extract [21, 22].
2. **Filtration method:** This is another technique for extraction of lovastatin. Whole Fungal culture plates were extracted with ethyl acetate and mixed or macerated properly with the help of sterile needle. Then mixture was filtered through muslin cloth with a pore size of 0.45µm in sterile petri-plates. The filtered crude suspension was used for further analysis and bioassay was carried at using *N. crassa* to find the conc. of lovastatin in the culture extract.

### Methods for detection of lovastatin production

#### 1. Paper disc method

The 10 days old culture of *N. crassa* (159) was harvested with 4000µl distilled water and macerated with the help of sterile needle ; 50µl of spore suspension (0.4-0.5 X $10^8$/ml) is poured into a PDA containing solidified Petri-plates and spreaded over the Petri-plates with the help of sterile spreader. After 10-15 min of solidification of *N. crassa* of spore suspension on PDA plates; the dried disks were placed on the surface of *N. crassa* bioassay plate.10µl of crude lovastatin extract were transferred on to 6mm diameter paper disc with the help of pipette. The disks containing 10µl of ethyl acetate were used as negative control standard. Inhibition zones were recorded in mm after 16-18h incubation at 28°C. A large diameter of the inhibition zone indicated a high titre of lovastatin.

#### 2. Well preparation method

After solidification of PDA medium, wells were made using a sterile cork borer of 6mm diameter and 50µl of the extract was loaded into wells with the help of pipette, simultaneously placing ethyl acetate into wells were used as control. Plates were allowed for incubated at 28°C for 24-48h (incubation beyond this specified period resulted in overgrowth of the test organism and thus the boundaries of inhibition zone could not be measured clearly. After incubation period *N. crassa* spore suspension (0.4-0.5 X $10^8$/ml) was made as above and spreaded over petri-plates and again incubated for 24-48h. After incubation, zone of inhibition was measured using Hi-Antibiotic measuring scale (Hi-Media).

## Results

A total of six *Fusarium* sp. were screened for HMG CoA reductase inhibitor activity. All fungal cultures were grown under favourable conditions to assess their potential for lovastatin production. The well and disc in the Petri plates were bored sufficiently apart to prevent coalescence of the zones of inhibition of the extract and lovastatin. Ethyl acetate was used as the control and it did not show any inhibition zone against *Neurospora crassa* lawn culture. The assay was carried out on the solid media (PDA) and liquid media (PDB) separately and inhibition zones were recorded. Zones of inhibition of different fungus are shown in tables.

Observation of different experiments conducted during the course of study are compiled and detailed in this section. Experiment-wise results/ observation are as follow-

**Culture plates of different fungal species on PDA medium:** All fungal cultures were inoculated on solid and liquid medium. After incubation period, the pure culture plates of different fungal species and *Fusarium* species are as follows-

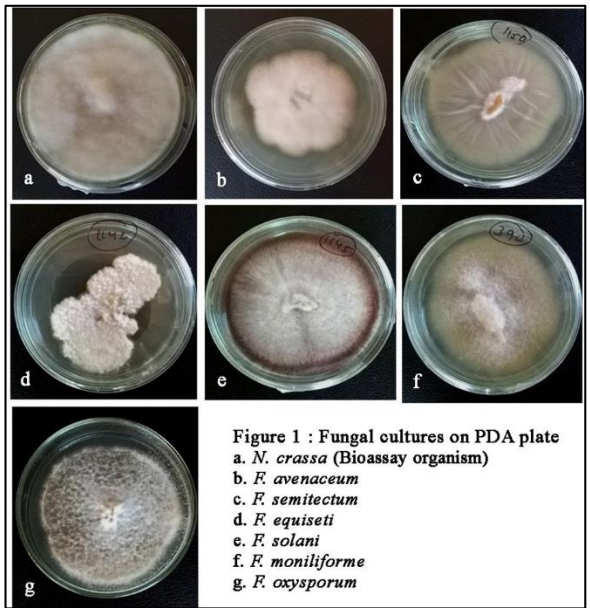
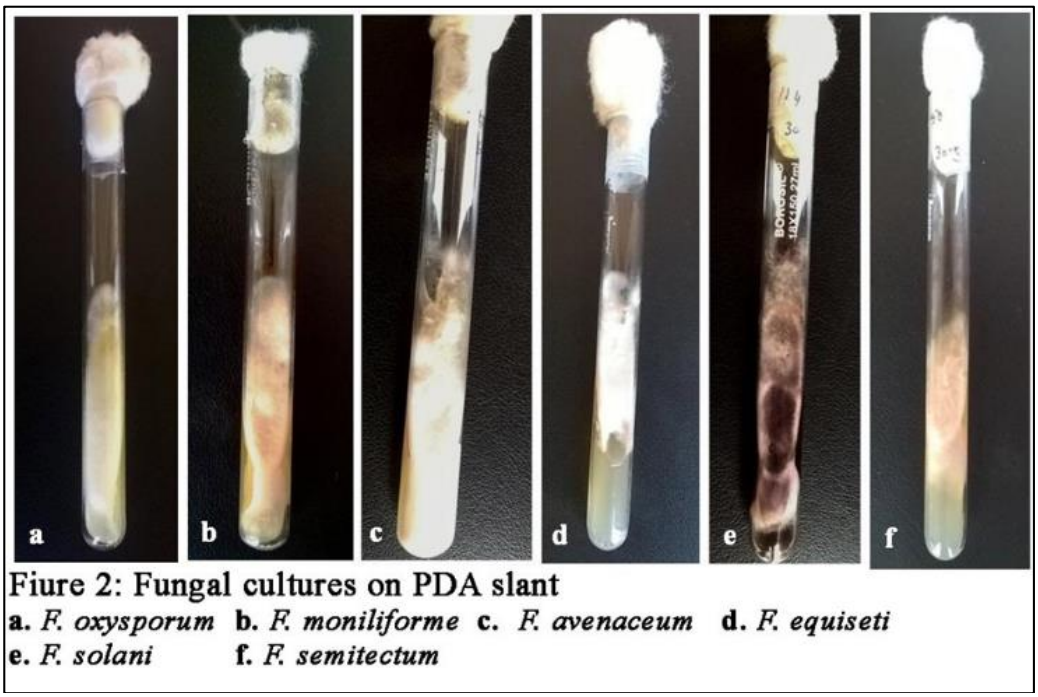



Figure 1 : Fungal cultures on PDA plate
a. *N. crassa* (Bioassay organism)
b. *F. avenaceum*
c. *F. semitectum*
d. *F. equiseti*
e. *F. solani*
f. *F. moniliforme*
g. *F. oxysporum*




Fiure 2: Fungal cultures on PDA slant
**a.** *F. oxysporum* **b.** *F. moniliforme* **c.** *F. avenaceum* **d.** *F. equiseti*
**e.** *F. solani* **f.** *F. semitectum*

**Microscopic examination of fungal cultures**
*Fusarium* species microscopic characters were identified by the description given by Mycobank.

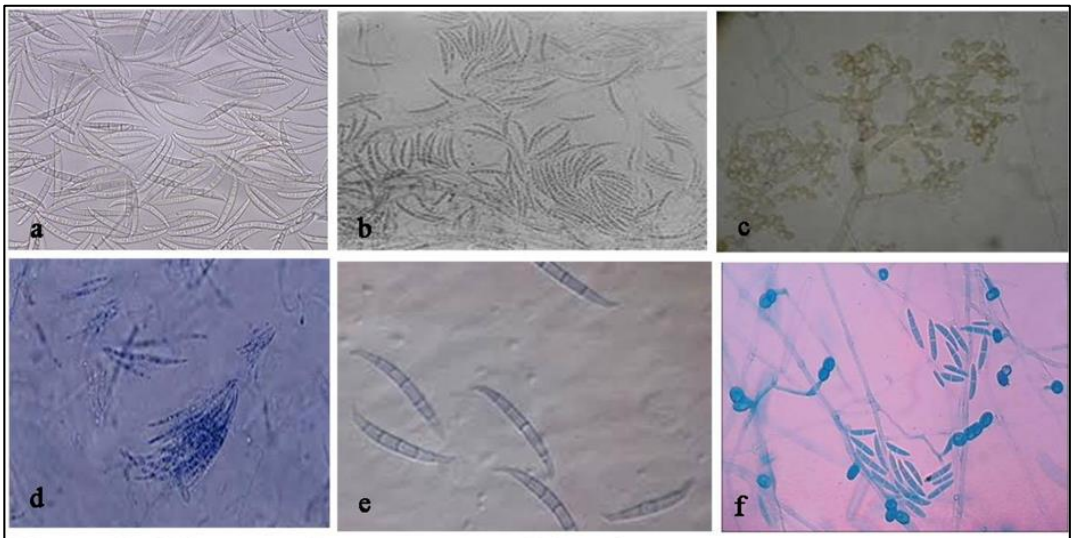



**Figure 3: Microscopic structures of fungi**
**a.** *F. avenaceum* **b.** *F. equiseti* **c.** *F. moniliforme* **d.** *F. oxysporum*
**e.** *F. semitectum* **f.** *F. solani*

**Screening of fungal species for lovastatin production**
Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (centrifugation extraction method) of different fungal species grown on PDA (Table 1).

- In the above experiment assessing *F. oxysporum, F. moniliforme, F. avenaceum, F. equiseti F. solani* and *F. semitectum* on PDA it was found that in well preparation method no clear zone was observed.
- In paper disk method maximum clear zone was formed in *F. moniliforme* followed in *F. oxysporum, F. semitectum* and *F. equiseti* were at path followed by *F. avenaceum*. No clear zone was found in *F. solani.*

Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (filtration extraction method) of different fungal species grown on PDA (Table 2).

- Maximum inhibition was found in *F. semitectum* followed by *F. solani, F. avenaceum, F. moniliforme and F. oxysporum.* No clear zone was found in *F. equiseti.*
- In the above experiment assessing *F. oxysporum, F. moniliforme, F. avenaceum, F. equiseti F. solani* and *F. semitectum* on PDA it was found that in well preparation method no clear zone was observed.

Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (centrifugation extraction method) of different fungal species grown on PDB and Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (filtration extraction method) of different fungal species grown on PDB (Table 3 and 4).

- In paper disk method showed clear zone in only three species *F. moniliforme, F. avenaceum, F. equiseti* and *F. semitectum*. The remaining species did not show any clear zone.
- In contrast the culture grown on PDA five species showed clear zone in paper disk method.
- *F. oxysporum* and *F. solani* did not show clear zone were grown on PDB and assayed using paper disk method.

Clear zone showing inhibition in test fungi for most of the fungal species were hire when grown on PDA as compared to PDB.

*F. moniliforme* and *F. semitectum* were the potential lovastatin producing species showing high inhibition zone in different methods.

From the above experiment it was also concluded that irrespective of culture medium and method of detection *F. moniliforme* and *F. semitectum* showed clear zone.

Lovastatin production in *F. oxysporum* was detected only when grown on PDA.

Lovastatin production in *F. equiseti* was detected on both culture mediums only through centrifugation extraction method.

*F. solani* was detected only through filtration extraction method when grown on PDA.

Detection of lovastatin in *F. avenaceum* was better using filtration extraction method. The best suited method for *F. avenaceum* was PDA grown cultures with filtration extraction method and its detection on paper disk.

The major findings of the study were:

1. The liquid and solid media extracts of *Fusarium* spp. were assayed separately. According to results it was concluded that in comparison to liquid medium, solid medium was more able to produce lovastatin.
2. According to lovastatin extraction techniques (centrifugation and filtration) both the extraction methods gave comparable results.
3. Maximum results for lovastatin production were found through paper disk method.

**Table 1:** Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (centrifugation extraction method) of different fungal species grown on PDA

| S. No. | Fungal species | Zone of Inhibition (mm) | |
|---|---|---|---|
| | | Paper disc method | Well preparation method |
| 1. | *F. oxysporum* | 10.6 | ND |
| 2. | *F. moniliforme* | 17.4 | ND |
| 3. | *F. avenaceum* | 2.4 | ND |
| 4. | *F. equiseti* | 3.6 | ND |
| 5. | *F. solani* | ND | ND |
| 6. | *F. semitectum* | 3.4 | ND |
| 7. | Control | ND | ND |

**Table 2:** Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (filtration extraction method) of different fungal species grown on PDA

| S. No. | Fungal species | Zone of Inhibition (mm) | |
|---|---|---|---|
| | | Paper disc method | Well preparation method |
| 1. | *F. oxysporum* | 3.4 | ND |
| 2. | *F. moniliforme* | 6.5 | ND |
| 3. | *F. avenaceum* | 10.4 | ND |
| 4. | *F. equiseti* | ND | ND |
| 5. | *F. solani* | 11.4 | ND |
| 6. | *F. semitectum* | 13.6 | ND |
| 7. | Control | ND | ND |

**Table 3:** Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extra ct (centrifugation extraction method) of different fungal species grown on PDB

| S. No. | Fungal species | Zone of Inhibition (mm) | |
|---|---|---|---|
| | | Paper disc method | Well preparation method |
| 1. | *F. oxysporum* | ND | ND |
| 2. | *F. moniliforme* | 6.4 | ND |
| 3. | *F. avenaceum* | ND | ND |
| 4. | *F. equiseti* | 3.2 | ND |
| 5. | *F. solani* | ND | ND |
| 6. | *F. semitectum* | 4.6 | ND |
| 7. | Control | ND | ND |

**Table 4:** Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (filtration extraction method) of different fungal species grown on PDB

| S. No. | Fungal species | Zone of Inhibition (mm) | |
|---|---|---|---|
| | | Paper disc method | Well preparation method |
| 1. | *F. oxysporum* | ND | ND |
| 2. | *F. moniliforme* | 3.4 | ND |
| 3. | *F. avenaceum* | 3.6 | ND |
| 4. | *F. equiseti* | ND | ND |
| 5. | *F. solani* | ND | ND |

| | | | |
|---|---|---|---|
| 6. | *F. semitectum* | 13.4 | ND |
| 7. | Control | ND | ND |

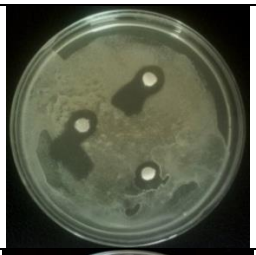
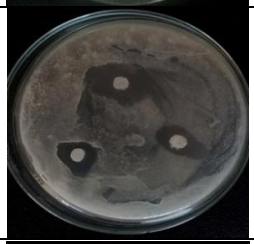
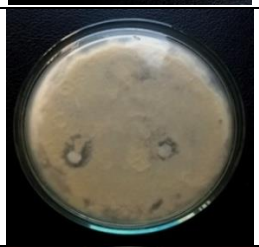
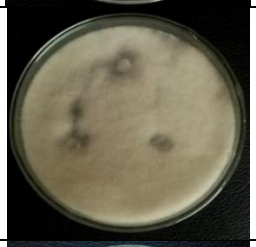
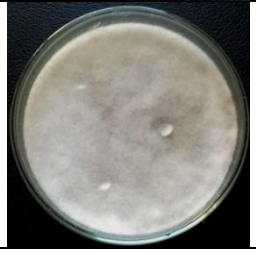
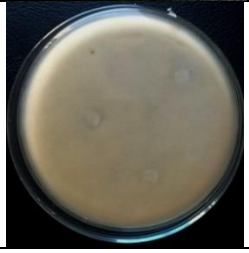
| Species | Front view | Reverse view |
|---|---|---|
| *F. oxysporum* |  |  |
| *F. moniliforme* |  |  |
| *F. avenaceum* |  |  |
| *F. equiseti* |  |  |
| *F. solani* |  |  |
| *F. semitectum* |  |  |
| Control |  |  |

**Fig 1:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing paper disk method by mycelial extract (centrifugation extraction method) of different fungal species grown on PDA

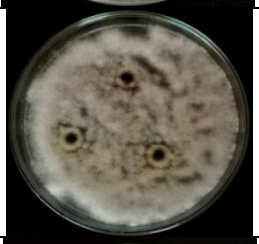
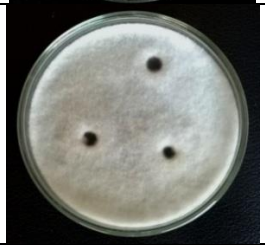
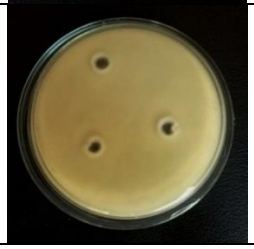
| Species | Front view | Reverse view |
|---|---|---|
| *F. oxysporum* |  |  |
| *F. moniliforme* |  |  |
| *F. avenaceum* |  |  |
| *F. equiseti* |  |  |
| *F. solani* |  |  |
| *F. semitectum* |  |  |
| Control |  |  |

**Fig 2:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing well preparation method by mycelial extract (centrifugation extraction method) of different fungal species grown on PDA

| Species | Front view | Reverse view |
|---|---|---|
| *F. oxysporum* |  |  |

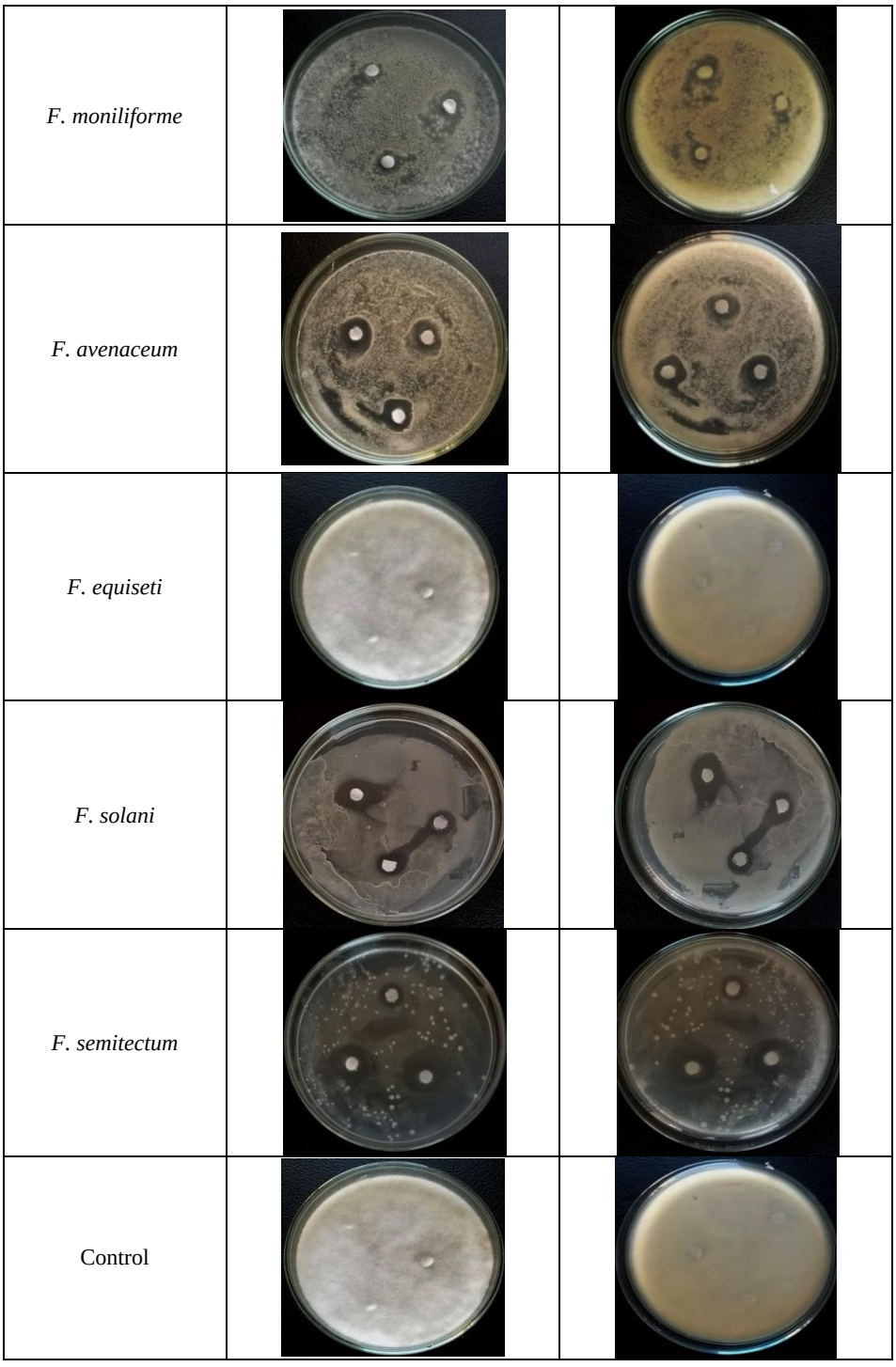
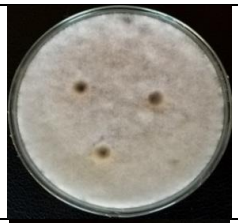
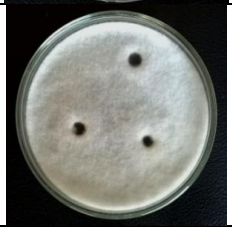
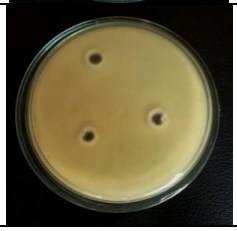



**Fig 3:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing paper disk method by mycelial extract (filtration extraction method) of different fungal species grown on PDA

| Species | Front view | Reverse view |
|---|---|---|
| *F. oxysporum* |  |  |
| *F. moniliforme* |  |  |

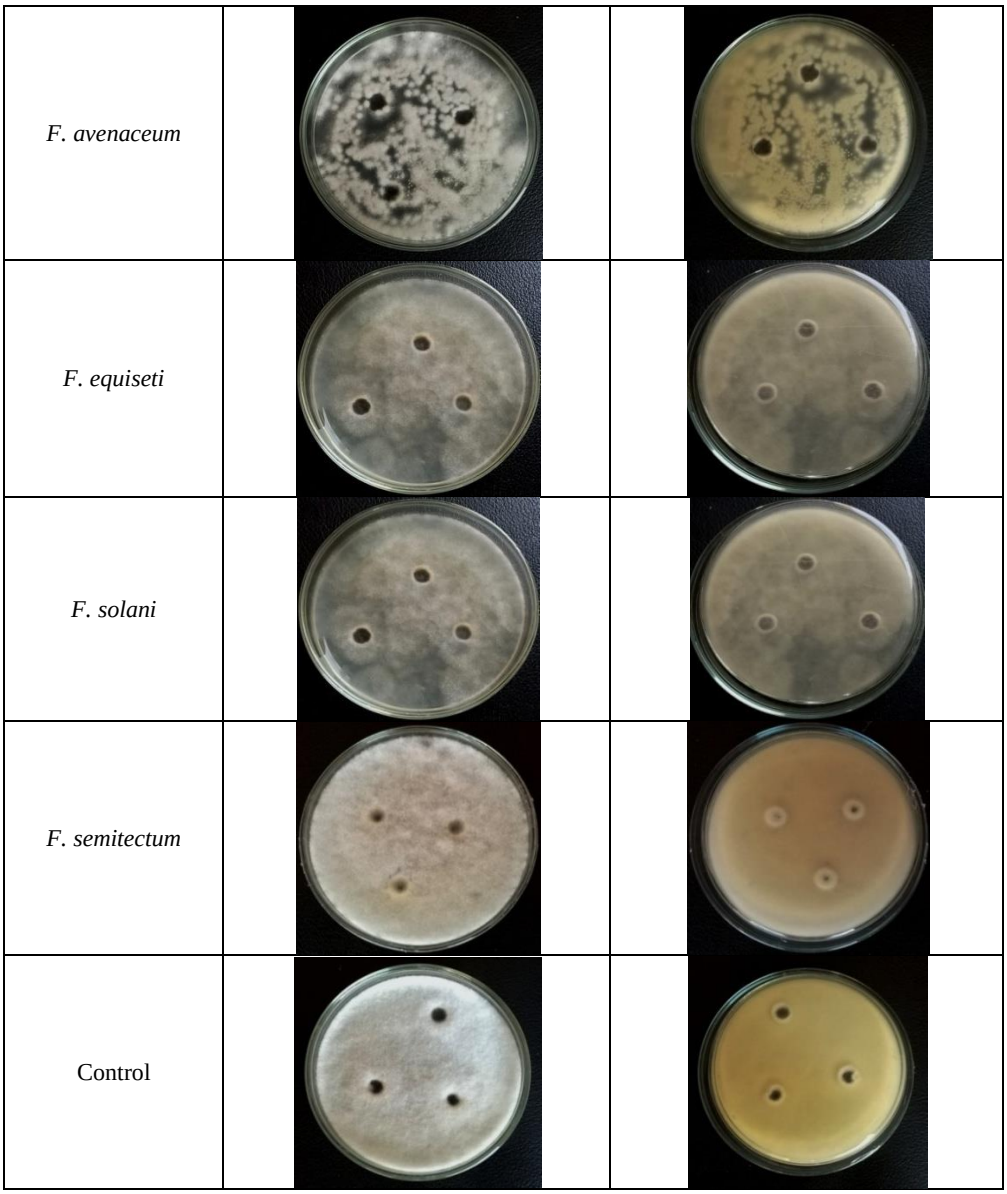



**Fig 4:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing well preparation method by mycelial extract (filtration extraction method) of different fungal species grown on PDA

| Species | Front view | Reverse view |
|---|---|---|
| *F. oxysporum* | | |
| *F. moniliforme* | | |
| *F. avenaceum* | | |

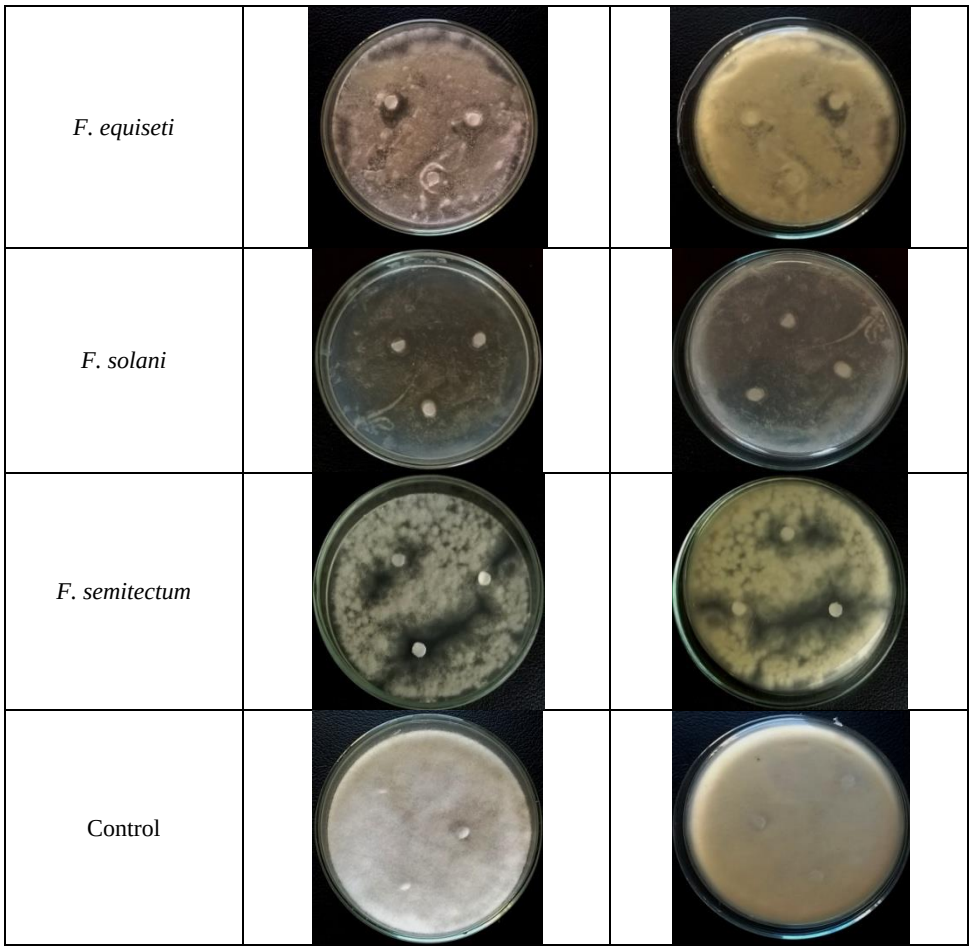
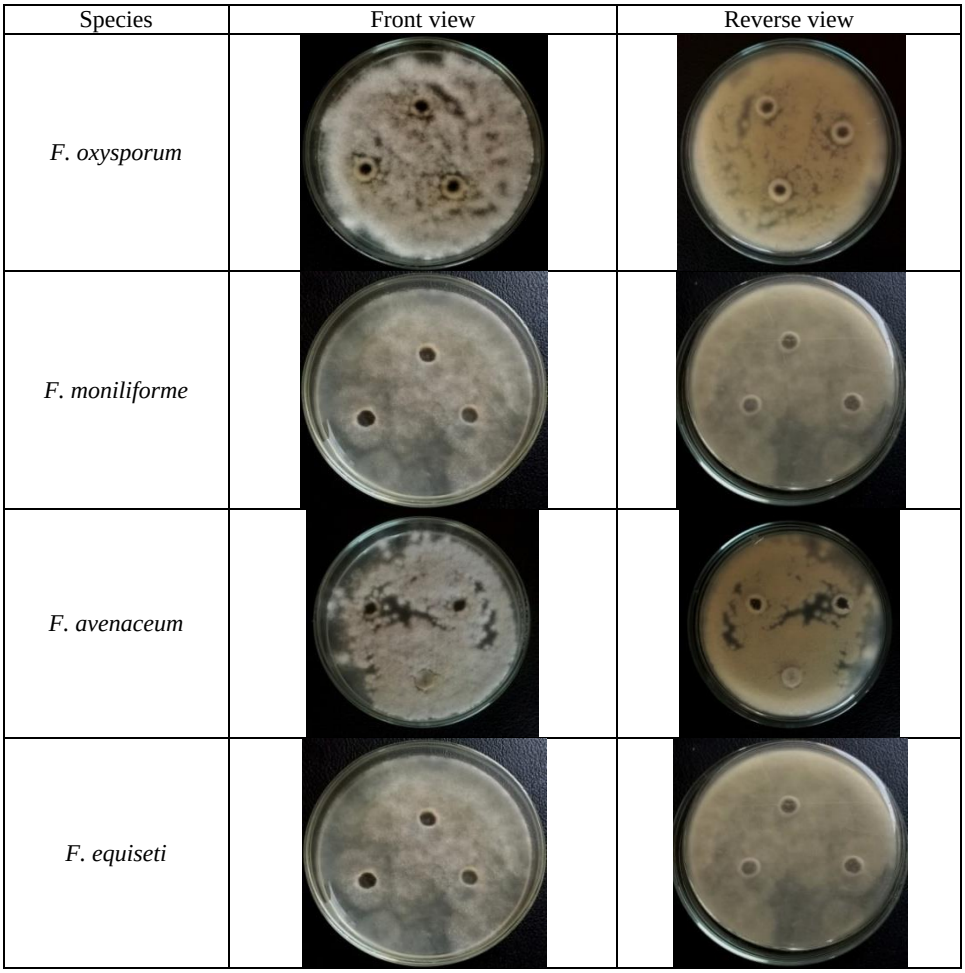



**Fig 5:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing paper disk method by mycelial extract (centrifugation extraction method) of different fungal species grown on PDB



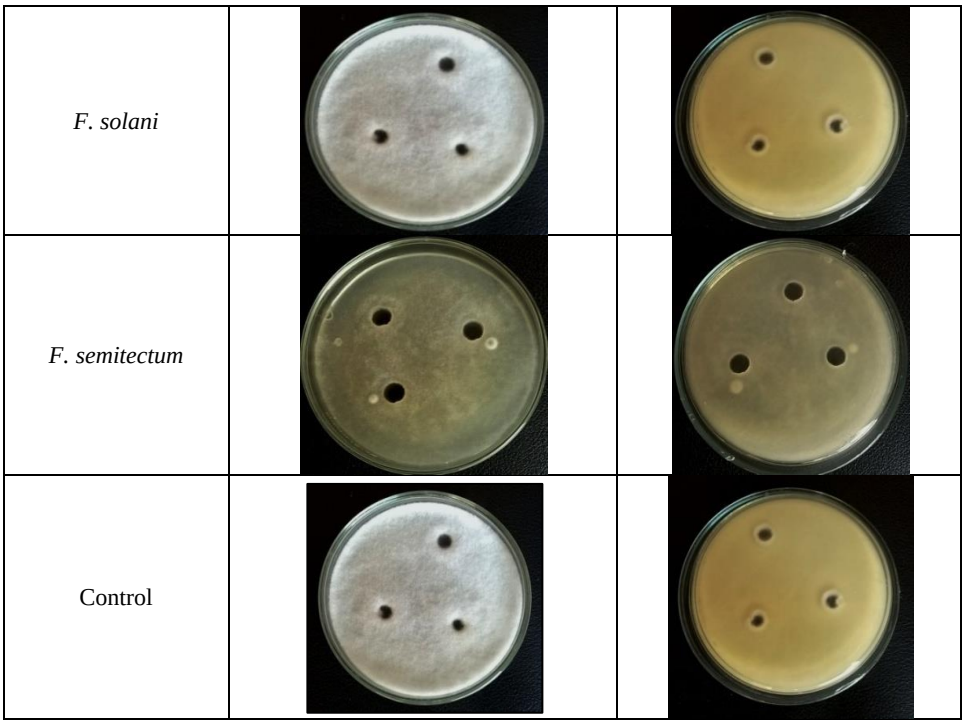
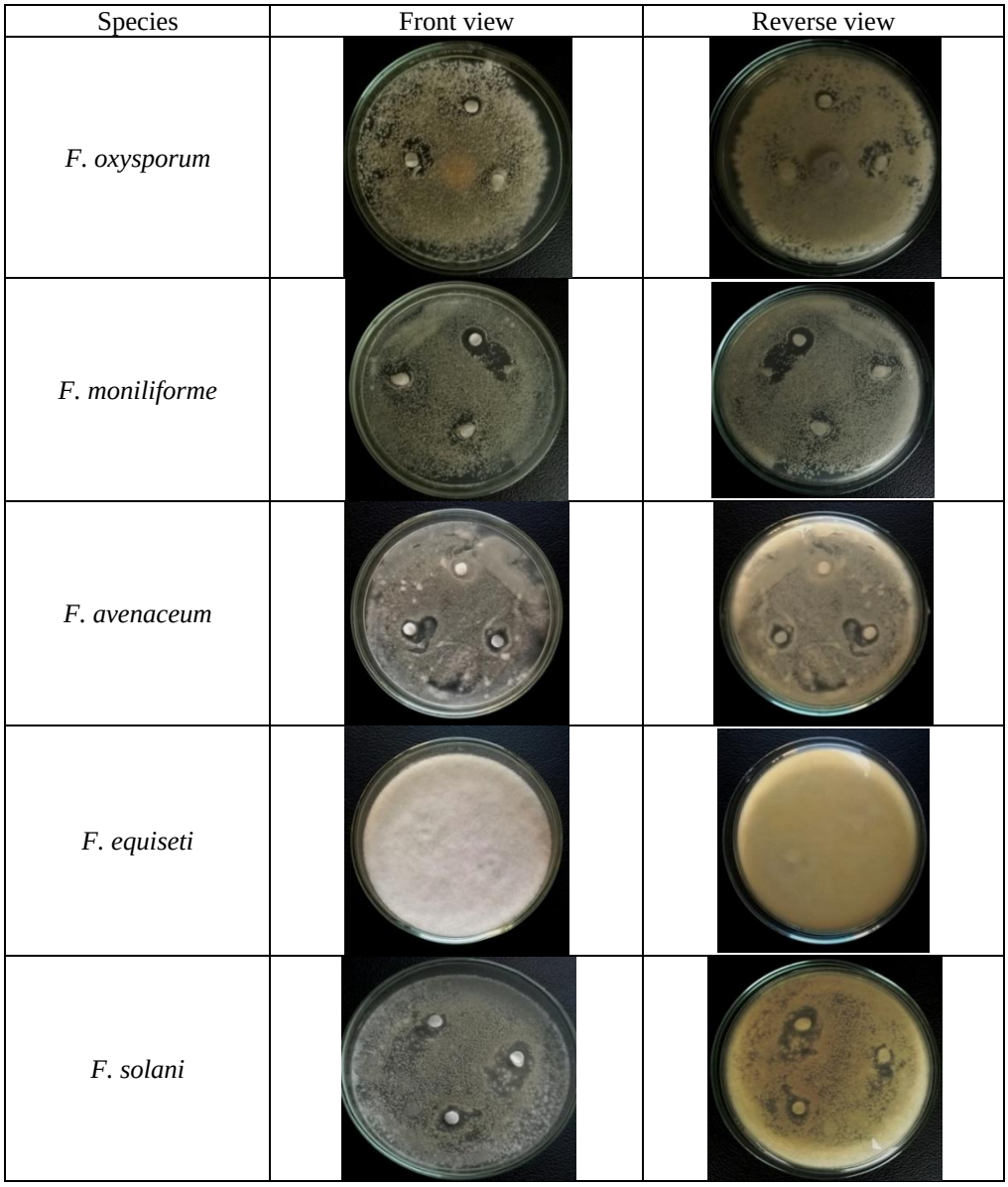



**Fig 6:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing well preparation method by mycelial extract (centrifugation extraction method) of different fungal species grown on PDB



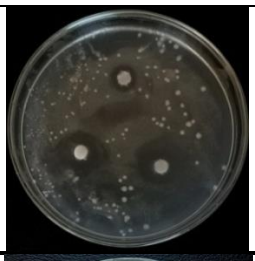
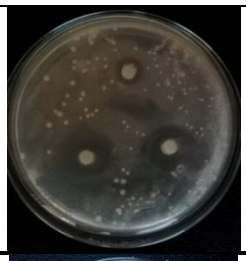
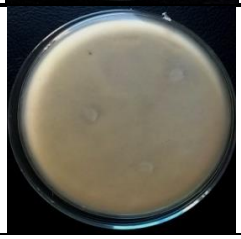
| | | |
|---|---|---|
| *F. semitectum* |  |  |
| Control |  |  |

**Fig 7:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing paper disk method by mycelial extract (filtration extraction method) of different fungal species grown on PDB

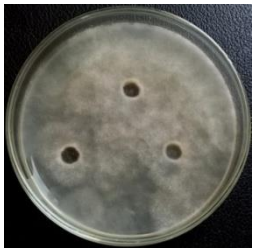
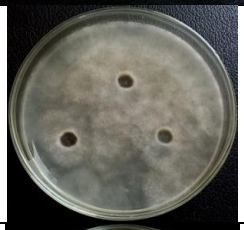
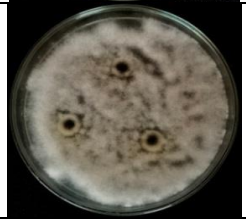
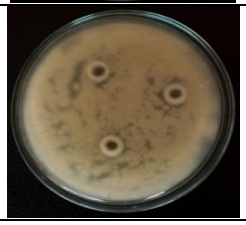
| Species | Front view | Reverse view |
|---|---|---|
| *F. oxysporum* |  |  |
| *F. moniliforme* |  |  |
| *F. avenaceum* |  |  |
| *F. equiseti* |  |  |
| *F. solani* |  |  |
| *F. semitectum* |  |  |


**Fig 8:** Lawn culture inhibition of *Neurospora crassa* (test fungus) employing well preparation method by mycelial extract (filtration extraction method) of different fungal species grown on PDB

## Discussion

In the present study, attempts were made to isolate potential, high yielding 3- hydroxy-3-methyl glutaryl CoA (HMG CoA) reductase inhibitor producing fungal species from natural samples.

All species of *Fusarium* e.g. *F. avenaceum, F. solani, F. semitectum, F. moniliforme, F. equiseti and F. oxysporum* were able to produce inhibition zone against bioassay organism so these *Fusarium* species were lovastatin producers.

The results of *Neurospora crassa* (NTCC-159) bioassay revealed that all isolated fungal cultures were shown zone of inhibition of growth after incubation period.

*Fusarium moniliforme* exhibited maximum zone of inhibition against *N. crassa* which related to the maximum capacity to produce the drug lovastatin. In the bioassay plate method using *N. crassa* as the test organism, lovastatin causes inhibition of growth. From the results it is clear that all species of *Fusarium* fungal cultures were able to produce lovastatin.

The liquid and solid media extracts of *Fusarium* spp. were assayed separately. According to results it was concluded that in comparison to liquid medium, solid medium was more able to produce lovastatin.

According to lovastatin extraction techniques (centrifugation and filtration) filtration technique was recorded for better result to produced inhibition zones against bioassay organism. Maximum results for lovastatin production were found through paper disk method.

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