

Bioprospect of Lovastatin in *Aspergillus* spp. From University of Indonesia Culture Collection (UICC)

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Abstract: The aim of this research is to select and analyse lovastatin from isolated molds of *Aspergillus* spp. from University of Indonesia Culture Collection (UICC). Lovastatin is an inhibitor 3 hydroxy-3-methylglutaryl-coenzyme-A reductase (HMG-CoA reductase) enzyme and a competitive inhibitor of the biosynthesis of cholesterol. The results revealed that out of 40 cultures, 18 cultures (45%) produced lovastatin and 22 cultures (55%) were negative. *Aspergillus flavus* UICC 360 showed the highest lovastatin production compared to a number of selected cultures. Thin Layer Chromatography (TLC) analysis showed an amount of *Aspergillus* with same similitaties of Rf value compared to the standard. High Performance Chromatography (HPLC) analysis which confirmed that lovastatin *Aspergillus flavus* UICC 360 has the same retention time with the standard (13.2 minutes).

Key words: *Aspergillus* spp · Extraction · Lovastatin · HPLC · Screening · TLC

INTRODUCTION

Lovastatin has been used as a drug for reduction of hypercholesteromia diseases. Thus, if there is a way to efficiency produce lovastatin, it will be beneficial to patients and scientific community. The pharmaceutical component of lovastatin acts as an inhibitor for hydroxy3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the biosynthesis of cholesterol [1].

HMG Co-A reductase is an important enzyme in the process of converting HMG CoA to mevalonat. HMG CoA reductase will assosiate with lovastatin as a substrate with higher concentration than HMG CoA. As a result, it acts as an competitive inhibitor with the substrate and blocking the production of mevalonat and then inhibits the cholesterol biosynthesis [2].

The screening method for lovastatin uses Thin Layer Chromatography (TLC) followed by a confirmation test that is the High Performance Liquid Fermentation (HPLC). Screening of a total of 110 fungi from 22 genera in Persian Type Culture Collection (PTCC) revealed that 4 genera e.i. *Aspergillus*, *Penicillium*, *Trichoderma* and *Acremonium* were able to produce lovastatin while 18 genera had

negative results. A screening test using TLC 49 sample showed positive result which was confirmed by HPLC. The test showed that only 31 specimens could produce lovastatin e.i. *Asp. terreus* (55 mg/L), *Asp. parasiticus* (4,5 mg/L), *Asp. flavus* (9,0 mg/L). *Asp. terreus* (55 mg/L) produced the highest result among those species potential in producing lovastatin [3].

Reported that the screening was conducted using clear zone (halo) method of *Asp. terreus*, had been induced by chemical mutation *methanesulphonic acid ethyl ester* (EMS) assessed in yeast *C. albicans* [4]. Screened of 134 fungi isolated from soil and found that only 38 specimens produced lovastatin in various concentrations [5]. Also, screened mutant *Asp. terreus* using agar plug method and found that the specimen showed clear zone as positive results of lovastatin against *Neurospora crassa* [6].

The aims of this research is to screen, ferment, extract and analyze *Aspergillus* spp taken from University of Indonesia Culture Collection (UICC) in producing lovastatin. Bioprospecting selected microorganisms from UICC collection will have contribution for the potential in an industrial purposes.

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MATERIALS AND METHODS

Microorganisms: An amount of 40 samples of *Aspergillus* spp. and *C. albicans* UICC Y-29 were obtained from University of Indonesia Culture Collection (UICC), Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia.

Methods

Screening Lovastatin Production with Paper Disc

Method: Spore and mycelium were grown on Potato Dextrose Agar (PDA) medium and were then incubated under room temperature for 7 days. *Candida albicans* Y-27 was streaked in the 5 ml medium Yeast Malt Agar (YMA) slant and incubated under room temperature (26-29°C) for 18 hours. A total of 200 µL yeast inoculum of $5-7 \times 10^7$ cfu/ml was added into 15 ml YMA medium at 50°C. After being rotated for 30 seconds, it was poured into petri dish and allowed to solidify.

Lovastatin extraction was prepared in two agar medium and included the mycelium that was plugged with a 10 mm diameter cork borer for each colony of *Aspergillus* spp. The samples of 500 µL ethylacetate were added to the eppendorf and incubated at 50°C for 15 minutes and every 2 minutes interval, it was shaken and finally centrifuged at 10.000 rpm for 2 menit.

Screening lovastatin activity using paper disc methods. Amount 40 µL lovastatin extract from each sample of *Aspergillus* spp. were dropped onto the paper disc. The negative control used ethylacetate and the positive controle used candistatin of 100 unit/µL. They were incubated for 18 hours at room temperature (26-29°C). The zone inhibition (halo) was measured and the experiment was carried out in triplicate [7].

Fermentation of Lovastatin Produced from Selected Potential *Aspergillus* spp:

Fermentation was done in 250 ml Erlenmeyer flask containing 50 ml Czapek Dox Broth (CDB) medium with pH 6.5. Next, an amount of 1 ml spores suspension ($3,5 - 4 \times 10^7$ cfu/ml) was inoculated, followed by incubating it for 48 hours at room temperature (26-29°C), shake at 110 rpm [7].

Lovastatin Extraction: The result of culture fermentation was filtrated using filter paper (Whatman no.1), acidified with sulfuric acid 2 N up to the pH 3, homogenized the mycelium and then mixed the filtrate. This extraction was

carried out 3 times. After using ethylacetate in an equal volume, the extract was evaporated. As a result, the β-hydroxyacid of lovastatin was obtained.

Analysis of Lovastatin Concentration Using Thin Layer

Chromatography (TLC): TLC was used to analyze the concentration of lovastatin. Activated silica gel was applied on 20X20 cm of TLC plate at 30°C for 30 minutes. The samples were diluted with 1 mL methanol. 5iL samples and standard were spotted on silica plate. The mobile phase was dichloromethan and ethylacetate (70:30, v/v). All plates were observed under UV lamp (254 nm). The Rf value from the standard was compared with the samples. Concentration analysis was used in TLC-Scanner CAMAG 3 [3,7].

Lovastatin Confirmation Using High Performance Liquid

Chromatography (HPLC): One gram of lovastatin sample was prepared and dissolved in 2 ml acetonitrile and 0.1 mL phosphate acid 0.1 %, incubated for 30 minutes and centrifuged at 1500 rpm for 10 minutes. Supernatant was injected in the coloum of 1 ig/mL HPLC. HPLC coloum C-18 (250 cm x 46 cm) was added with eluent acetonitrile and phosphate acid 60:40 (v/v), the eluent rate was 1.5 mL/minute, detector ultraviolet (UV) at λ 235 nm and the temperature of the coloum was 30°C [3].

RESULTS AND DISCUSSION

Lovastatin Screened from *Aspergillus* spp. Through

Paper Disc Method: Only 18 out of 40 samples of (45%) *Aspergillus* spp. showed clear zones around the paper disc. The clear zones (halo) varied with a range between 1.23-5.20 mm.. The smallest halo was 1.23 mm and it was produced by *Asp. awamori* (9), while the highest clear zone was 5.2 mm and it was produced by *Aspergillus* sp. UICC 317 (Fig. 1). The difference clear zones were produced due to the variations of physiology and genetic characteristics of the specimens, that the influencing factors of the paper disc methods were the ability of the lovastatin to diffuse the thickness of agar, total microbial assay and the incubation period [4].

Lovastatin in nature is found in the form of β-hydroxy-acid and lactone. The first one is an active form (Kumar *et al.* 2000). The lovastatin in ethylacetate extract in the form of β-hydroxyacid is an antifungal. The cell membrane of *C.. albicans* is lipidbilayers. The composition o f the cell wall consists of sterol which is a

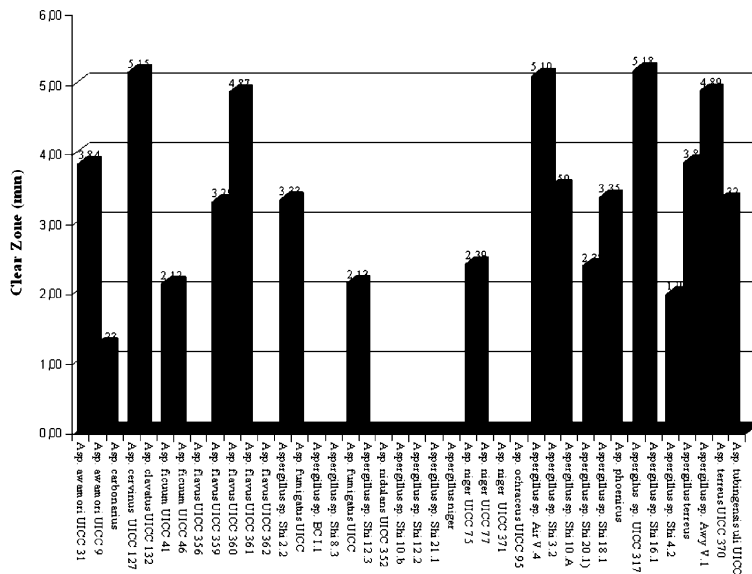
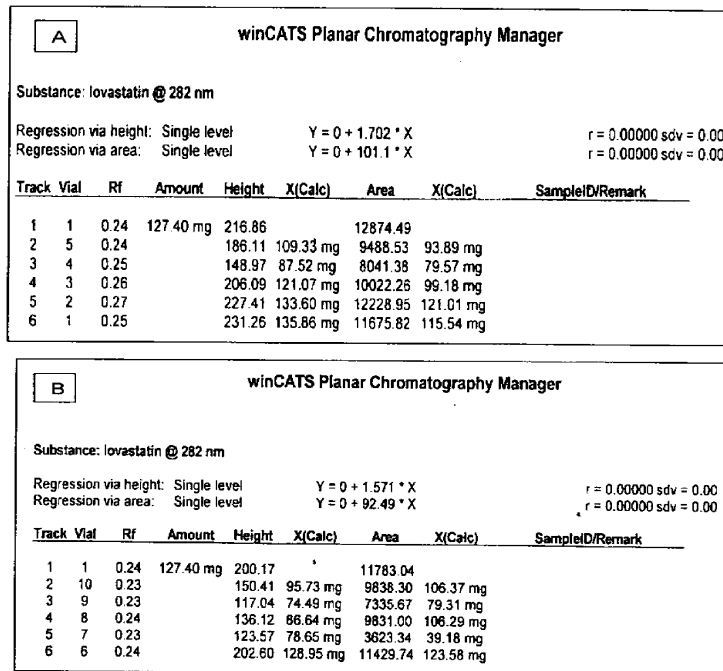
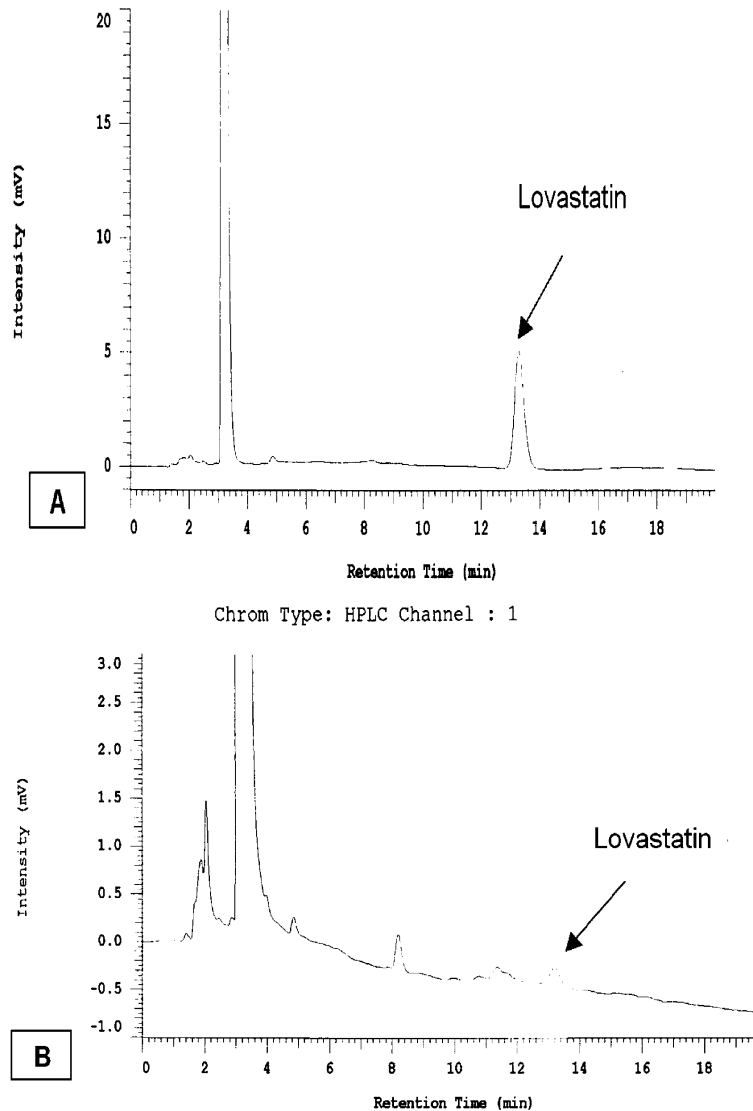


Fig. 1: Histogram of lovastatin screening activity of 40 *Aspergillus* spp. from University of Indonesia Culture Collection against *C. albicans* UICC Y-29



- Notes :
- | | |
|-----------------------------------|-------------------------------------|
| 1. <i>Aspergillus</i> sp.UICC 317 | 6. <i>Asp. flavus</i> UICC 360 |
| 2. <i>Asp. terreus</i> UICC 370 | 7. <i>Asp. awamori</i> UICC 3 |
| 3. <i>Aspergillus</i> sp. AWy V1 | 8. <i>Asp. flavus</i> UICC 359 |
| 4. <i>Asp. terreus</i> | 9. <i>Aspergillus</i> sp. Shi. 18.1 |
| 5. <i>Aspergillus</i> sp. Shi 2.2 | 10. <i>Aspergillus</i> sp. Shi. 3.2 |

Fig. 2: A. Rf value, height and area standard (track1), samples 1-5 *Aspergillus* spp. B. Rf value, height and area standard (track1), sampel 6-10 *Aspergillus* spp.



Notes: A. Lovastatin standard
 B. Lovastatin *Asp. flavus* UICC 360

Fig. 3: Chromatogram HPLC lovastatin of *Asp. flavus* UICC 360.

target of antifungal activity despite of the enzyme that is involved in the cell wall synthesis. The mechanism of antiyeast inhibition was: (a) mycosin contact directly with the sterol in the membrane cell causing the leak of membrane cell and the loss of the intracellular component, (b) mycosin attached with RNA and inhibited the protein synthesis (c) mycosin inhibited ergosterol synthesis causing the increase of membrane permeability and the damage of the membrane [9].

Lovastatin Qualitative Analysis on Selected *Aspergillus* spp: The Rf value of the ethylacetate extract samples indicated the same Rf value (0.24) as the standard

lovastatin (Rf 0.24), e.i. *Aspergillus* sp.shi 2.2, *Aspergillus flavus* UICC 360 and *Aspergillus flavus* UICC 3. The other samples gave different value of Rf such as, 0.23; 0.25; 0.26 and 0.27. These differences might be due to the impurity of the samples (Fig. 2).

The screening of lovastatin activity using TLC on the genus *Pleurotus* especially *Pleurotus ostreatus*, *Pleurotus saca* and *Pleurotus sapidus* [10]. The determination of lovastatin *Pleurotus ostracesus* on fruiting body and mycelium. The Rf value was the same as the standard which was Rf 0.46. The peak of the standard curve was lovastatin (216.86); the lowest peak was *Aspergillus* sp. Shi 18.1 (117.04) and the highest was

Table 1: Quantitative analysis of selected *Aspergillus* spp..

Molds	Lovastatin extract (mg/L)
<i>Aspergillus</i> sp. UICC 317	77.6
<i>Asp. terreus</i> UICC 370	52.8
<i>Aspergillus</i> sp. Awy V.1	31.0
<i>Asp. terreus</i>	36.7
<i>Aspergillus</i> sp. Shi 2.2	38.5
<i>Asp. flavus</i> UICC 360	85.8
<i>Asp. awamori</i> UICC 31	9.9
<i>Asp. flavus</i> UICC 359	48.5
<i>Aspergillus</i> sp. Shi 18.1	39.5
<i>Aspergillus</i> sp. Shi 3.2	69.7

Aspergillus sp. UICC 317 (231.26). The value area from TLC scanner was indicated by the area spotted on the silica gel [11].

Quantitative Analysis on Lovastatin from Selected *Aspergillus* Spp: Quantitative analysis TLC spectrodensitometry results were analysed using TLC Scanner CAMAG 3, based on calibration curve and lovastatin standard. The calibration curve was based on the area of serial concentrations of standard lovastatin from 5-25 ppm. (Tabel 1). The peak standard was then analysed using winCATS Planar Chromatography Manager program.

Quantitative analysis of lovastatin production varied between 85.8 mg/L - 9.9 mg/L. The highest was *Asp. flavus* UICC 360 (85.8 mg/L) and the lowest was *Aspergillus* awamori UICC 31 (9.8 mg/L). The research reported that *Asp. terreus* (55 mg/L), *Asp. parasiticus* (4.5 mg/L), *Asp. flavus* (9.0 mg/L) and *Asp. terreus* (55 mg/L) produced lovastatin during fermentation process [3]

HPLC Analysis on Lovastatin from *Aspergillus flavus* UICC 360: Identification of the highest lovastatin production of *Asp. flavus* UICC 360 was conducted through HPLC methods. Chromatogram analysis indicated that there was only one peak of lovastatin standard appeared with a retention time of 13.2 minutes (Fig. 3 A), whereas the chromatogram for lovastatin extract of *Asp. flavus* UICC 360 amounted to the same peak with a retention time of 13.2 minutes (Fig. 3B). The results confirmed that the ethylacetate extract from *Asp. flavus* UICC 360 was lovastatin. Mevinolin analysis from product fermentation using the coloum 250 x 4 mm in a temperature of 40°C and a detection at λ 237 nm showed one peak in the form of lovastatin β -hydroxyacid.. Lovastatin in the form of β -hydroxy-acid was detected with HPLC in the coloum C-18 with acetonitril-methanol-phosphate buffer pH 4 as a mobile phase, a temperature of 45°C, a flow rate of 1.5 ml and a detection at λ 238 nm [12, 13].

CONCLUSION

Screening of 40 *Aspergillus* spp. samples from University of Indonesia Culture Collection (UICC) with paper disc method showed only 18 samples (45%) were positive with lovastatin while the remaining 22 samples (55%) were negative. *Asp. flavus* UICC 360 produced the highest lovastatin compared to other selected molds. Thin Layer Chromatography (TLC) analysis indicated the Rf of selected *Aspergillus* spp. (0.23 - 0.27) and the lovastatin standard (0.24). High Performance Chromatography (HPLC) analysis confirmed that *Asp. flavus* UICC 360 had 13.2 minutes retention time which was the same as the lovastatin standard (13.2 minutes).

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