Random Mutagenesis of *Aspergillus sclerotiorum* PSU-RSPG 178 for Improvement a Lovastatin Production

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Abstract:

Aspergillus sclerotiorum PSU-RSPG 178 is a natural fungus producing lovastatin which is commonly used as cholesterol reduction medication. Random mutagenesis method was used to increase the production of lovastatin via UV radiation (255 nm) with a different time of exposure at 30, 45 and 60 min, fifty colonies appeared and screened for lovastatin production by agar plug and *Neurospora crassa* bioassay. The results showed seven colonies had greater diameter of clear zone than that of wild type. After analysis of the quantity of lovastatin by HPLC, the mutant colonies from UV mutation at 30 min provided the maximum amount of lovastatin at 354.69 mg/L compared with that from the control at 113.4 mg/L. However, after sub-culture until 3rd generation, the potential in lovastatin production declined. This evidence indicated that UV mutagenesis could be used for improving the lovastatin production.

Keywords: Lovastatin, *Aspergillus*, *Aspergillus sclerotiorum* PSU-RSPG 178, random mutagenesis

Introduction

A. sclerotiorum PSU- RSPG 178 is a fungus that produced lovastatin. It was isolated from soil sample collected from the Plant Genetic Conservation project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn at Ratchaprapa Dam in Suratthani Province, Thailand [1]

Lovastatin ($C_{24}H_{36}O_5$) is a secondary metabolite produced from fungi such as *Monascus purpureus*, *Monascus ruber*, *Monascus pilosus*, *Monascus pubigerus*, *Monascus vitreus and Aspergillus terreus* [2]. The important function of lovastatin known as a reducer of plasma cholesterol level by competitive inhibition of 3-hydroxy 3-methylglutaryl Coenzyme A (HMG-CoA) reductase which is the rate-limiting enzyme in cholesterol biosynthesis [2] and also found to be effective in treatments of hypercholesterolemia, Parkinson's disease, bone fracture, atherosclerosis, Alzheimer's disease, cerebrovascular disease and cancer [3]. Lovastatin consisted of two forms including lactone and acid form which is soluble in water. Ninety percent of lovastatin fermentation exists as β-hydroxy acid form which can be function as an anti-fungal exhibitor against *N. crassa* [4]. Therefore, agar plug and *N. crassa* bioassay was performed to screen for lovastatin production.

Due to the low amount of lovastatin production from natural *A. sclerotiorum* PSU-RSPG 178, in this study, random mutagenesis was performed by using UV radiations method with a different time intervals (30, 45 and 60 min) to enhance the production of lovastatin.

Materials and methods

Microorganism

A. sclerotiorum PSU-RSPG 178 was cultured on potato dextrose agar (PDA) and incubated at 28º C for 7 days.

Strain improvement by random mutagenesis

The spore was collected in 0.85% NaCl containing 2% Tween 20 and shaken for 10 min, then it was counted using hemocytometer and further diluted with PBS (pH 7.4) to $1x10^6$ spores/mL followed by spread on PDA. After that, plates were exposed to UV radiation at 255 nm with a different time interval (30, 45 and 60 min) at the distance of 5 cm from the UV source and incubated at 28ºC for 5 days.

Screening of lovastatin by using *Neurospora crassa* bioassay

The colonies were grown on PDA to screening the lovastatin producing fungi by using *N. crassa* bioassay [5]. The colonies with the biggest in diameter of clear zone were selected to culture on potato dextrose broth (PDB) at 28ºC for 14 days, and then extraction by ethyl acetate was performed.

Cultivation and extraction

A. sclerotiorum PSU-RSPG 178 and mutant colonyies were culture on PDA at 28 ºC for 7 days. Five pieces (0.5×0.5 cm^2) of mycelial agar plugs were incubated in PDB (potato dextrose broth) 150 ml in 250 ml Erlenmeyer flask at 28º C for 14 days. After incubation, the mycelia and medium were separated by filtration. The medium was extracted with an equal volume of ethyl acetate and the cells were cut into small pieces and incubated with an equal volume of methanol at room temperature for 3 days. The medium was extracted twice with ethyl acetate (2x300 ml). The organic layer was evaporated by an evaporator to dryness under a reduced pressure (BE). The mycelia were separated from methanol by filtration. The methanol was removed by evaporator and hexane: H_2O (1:1) 200 ml, was added. Then, upper layer was evaporated by an evaporator to dryness under a reduced pressure (CH) and the lower layer was extracted twice with ethyl acetate (2x300 ml) and also evaporated to dryness (CE). All of crude extract were determined by Nuclear Magnetic Resonance (^{1}HNMR).

Analysis qualitative of lovastatin by High-performance liquid chromatography (HPLC)

Estimation of lovastatin by HPLC system (Agilent 1200 series DAD using an ACE ® Generix 5 C-18 column) was performed by using acetonitrile and 0.1 % phosphoric acid in a ratio of 60:40 (V/V) as a mobile phase. Sample was injected by flow rate adjustment at 1.0 ml/min and further detected at 238 nm. The identity of sample was confirmed by lovastatin standard.

Results and discussion

Genetic improvement

Genetic improvement is one of the rising approaches for increasing the production of secondary metabolites by UV radiation method. Culture plates with the mutant colonies survival rate at less than 90% compared with control plate were selected (Fig.1).

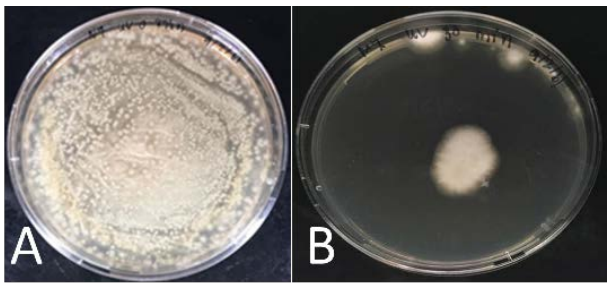

Figure 1 Comparison of mutant strains of *A. sclerotiorum* PSU-RSPG 178 grown on PDA between normal strain with irradiated (A) and radiated to UV radiation (B).

Screening and isolation of lovastatin production through bioassay method

The results of bioassay revealed seven colonies (Fig. 2) out of fifty showed the bigger size in diameter of zone of inhibition (cm) than control. Then, those seven colonies were cultured on PDB for 14 days and further extraction for lovastatin by ethyl acetate.

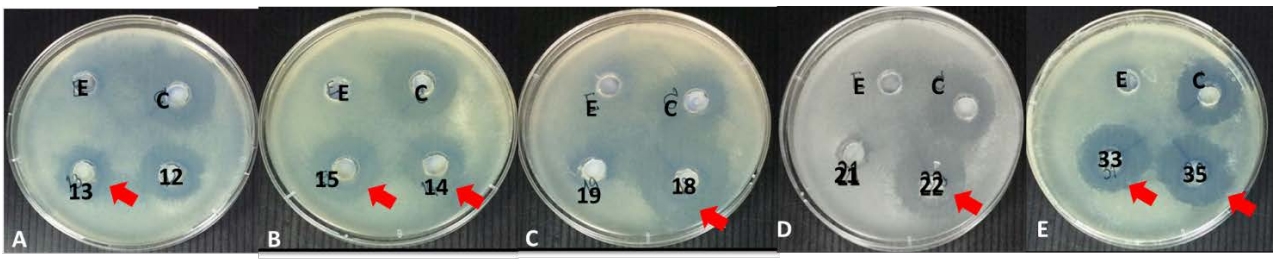

Figure 2 Bioassay of samples from different fungal mutants. Clear zone of selected seven colonies were showed by red arrow (E = negative control, C = *A. sclerotiorum* PSU-RSPG 178) mutant colony UV07.30_13 (A), UV07.30_14 and UV07.30_15 (B), UV07.30_18 (C), UV07.30_22 (D), UV07.45_33 and UV07.60_35 (E)

Analysis qualitative of lovastatin by HPLC

To ensure whether the maximum zone of inhibition by mutant colony UV07.30_22 on bioassay plate was appeared due to lovastatin activity, crude extract was injected to HPLC for quantitatively confirming the amount of lovastatin in the fungal extraction. The resemblance in retention time (RT) of peak shown in chromatogram of control (*A. sclerotiorum* PSU-RSPG 178) and mutant colony UV07.30_22 was similar at 16.6 min (Fig. 2A and B). The results found the maximum amount of lovastatin by *N. crassa* bioassay was 354.69 mg/L (UV07.30_22) followed by 297.68 mg/L (UV07.30_18), 296.02 mg/L (UV07.45_33), 221.84 mg/L (UV07.60_35), 186.33 mg/L (UV07.30_13), 175.6 mg/L (UV07.30_15), 144.93 (UV07.30_14) and 113.4 mg/L (control) (Fig. 4). This result indicated that UV mutagenesis could improve the lovastatin yield.

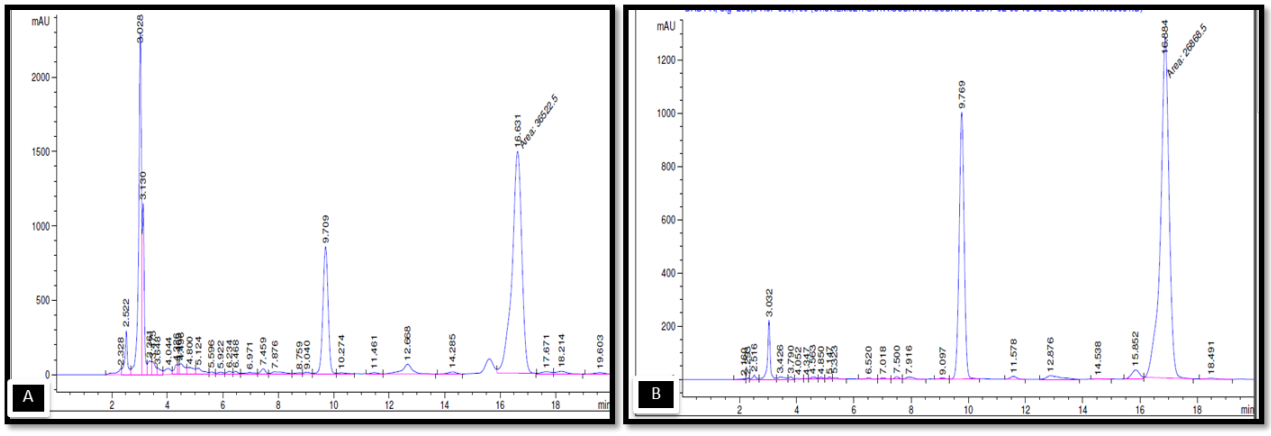

Figure 3 High-performance liquid chromatography chromatogram of *A. sclerotiorum* PSU-RSPG 178 (A) and mutant colony UV07.30_22 (B)

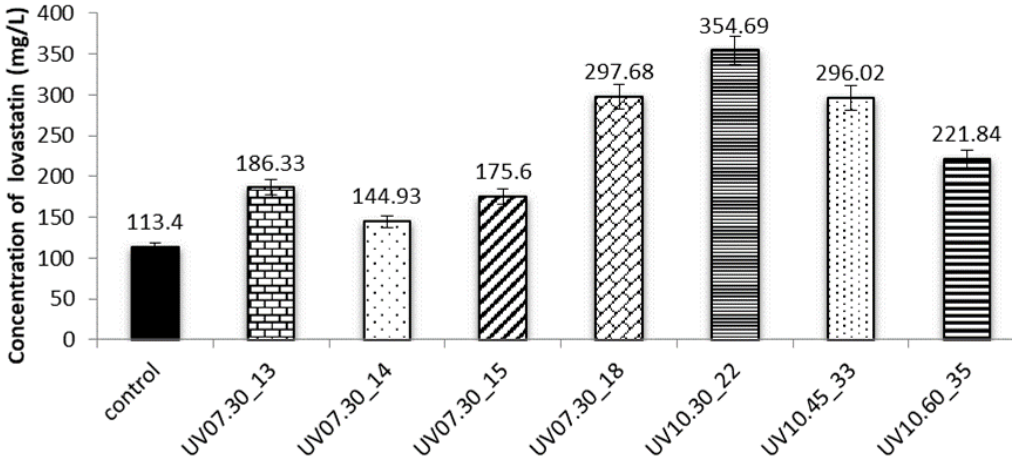

Figure 4 Concentration of lovastatin produced by the fungal *A. sclerotiorum* PSU-RSPG 178 and mutants

Analysis qualitative of lovastatin by Nuclear Magnetic Resonance (^{1}HNMR)

Qualitative of lovastatin was observed by ^{1}HNMR spectrum. Both the ^{1}HNMR spectrum of *A. sclerotiorum* PSU-RSPG 178 and mutant colony UV07.30_22 showed the similar signal of ^{1}HNMR peak. This evidence indicated that the mutant colony UV07.30_22 could produced lovastatin (Fig.5).

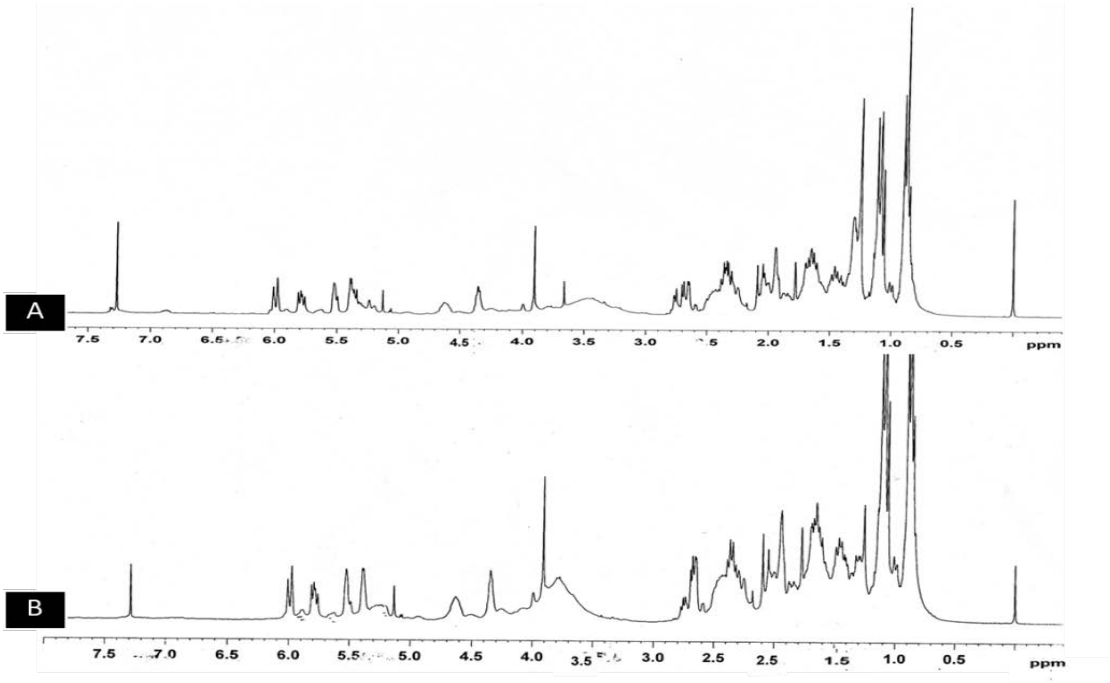

Figure 5 ^{1}H NMR spctrum of *A. sclerotiorum* PSU-RSPG 178 (A) and mutant colony UV07.30_22 (B).

Conclusions

Considering these references as discussed above, it could be concluded that *A. sclerotiorum* PSU-RSPG 178 UV07.30_22 exposed to UV radiation could produce an increasing of lovastatin yield at 354.69 mg/L suggesting the physical mutagenesis (UV random mutation) can probably be used for lovastatin yield improvement.

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