JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2012. Vol. **6**(2), p. 609-613

Improvement Lovastatin Production of *Aspergillus terreus* by pTRLI plasmid Transformation

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(Received: 20 January 2012; accepted: 27 February 2012)

Protoplasts of *A. terreus* were transformed by pTRLI plasmid containing *lovE* gene as regulator gene in lovastatin biosynthesis and *ptrA* gene as pyritiamine resistance gene. Transformats were selected for ability to grow on Czapex-dox agar containing pyrithiamine. Transformats were stable and grew on Czapex-dox agar containing pyrithiamine for 5 generations. *A. terreus* transformant is tested for lovastatin productivity using medium fermentation. This research showed that copy number of *lovE* gene in *A. terreus* increased significantly lovastatin productivity. The lovastatin productivity of *A. terreus* recombinant was 693 ppm, which is 93% hingher than the parental strain (398 ppm) at 28°C for 7 days and 200 rpm.

Key Words: Lovastatin, *Aspergillus terreus* recombinant, Transformation, pTRLI plasmid.

Hypercholesterolemia is the high level of total cholesterol and low density lipoprotein derived cholesterol in the plasma. Cholesterol in the plasma can be absorpted from diet or biosynthesis in liver. There are some steps for cholesterol bioasynthesis and 3-hydroxy-3methylglutaryl coenzyme A (HMG CoA) reductase is an enzyme in cholesterol biosynthesis to produce mevalonic acid from HMG CoA (one step in cholesterol biosyntesis)[1].

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Lovastatin ($C_{24}H_{36}O_5$, mevinolin, monacolin K, mevacor™) is a potent antihypercholeterolemia drug that inhibit cholesterol biosyntesis by competitively inhibiting HMG CoA reductase[2,3,4,5]. It is active not only in vitro to inhibit cholesterol biosynthesis but also in vivo to lower plasma cholesterol level in humans and animals[6,7,8,9].

Lovastatin is secondary metabolete that were produced by filamentous fungi, such as *A. flavus* (48.4 ppm), *A. niger* (29.9 ppm), *A. oryzae* (37.6), *A. terreus* (59.2 ppm), *Cylindrocarpon radicicola* (7.1 ppm), *Penicillium spinolosum* (15.8 ppm), *Trichoderma viridae* (36 ppm), *Mycellia sterilia* (15.3 ppm)[10], *A. terreus* (116.8 ppm), *Monascus sp.* (21.5 ppm), *A. niger* (4.3), *A. flavus* (5.9 ppm), *Penicillium purpurogenum* (16.9 ppm), *Pleurotus sp.* (18.6 ppm), *Trichoderma viridae* (8.6 ppm)[11]; *A. terreus* (55.0 ppm), *A. parasiticus* (4.5 ppm), *A. fischeri* (2.0 ppm), *A. flavus* (9.0 ppm), *A. umbrosus* (14 ppm), *Penicillium funiculosum* (19.3 ppm), *Trichoderma viridae* (9.0 ppm), *Trichoderma*

longibrachiatum (1.0 ppm), *Acremonium chrysogenum* (2.5 ppm)[12]. From the research above showed that *A. terreus* is the highest fungus in lovastatin production therefore *A. terreus* is used in this research.

Straint Improvement is used to increase secondary metabolte through mutation with phisical (UV rays and X rays) and chemical (N-nitrosoguanidine and ethyl methane sulfonate) agents or genetic engeenering. Genetic engeenering is targeted mutation on the genes unlike mutation with phisical and chemical agents that are random mutation. Random mutation with ethyl methane sulfonate[13,15] and UV rays[13,14] have been reported to increasing of lovastatin production in *A. terreus*. The cluster gene of lovastatin biosynthesis was investigated and analyzed and there were 18 open reading frames (ORF)[16]. *LovE* gene, a regulator of lovastatin biosynthesis was inserted in pTRLI plasmid and pTRLI plasmid was transformed into *A. terreus* protoplast. The objectives of this research were to increase lovasatin production by *A. terreus* recombinant (transformant) and characterization of lovastatin.

MATERIAL AND METHODS

The isolate of *A. terreus* obtained from the Biotech Center Culture Collection was maintained and grown on Potato Dextrose Agar (PDA) at 28°C for 7 days. *Escherichia coli* DH5á containing pTRLI plasmid was grown on Luria–Bertani medium. pTRLI plasmid is derived from pTRI plasmid (Takara) containing l*ovE* gene from *A. terreus* and *pTRA* gene (pyrithiamine resistance gene).

Isolation of *A. terreus* protoplasts was used one colony of *A. terreus* aged 7 days on PDA plate. *A. terreus* is added with sterile water, homogenized using potter, and filtered. Filtrate was grown in Erlenmeyer 250 ml containing YMP medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.6 %, glucose 2.0%) 50 ml and incubated for 20 hours, 200 rpm at 28°C. Mycelium was collected by centrifugation for 5 minutes at 6,000 g, washed twice with sterile water, and resuspended in enzyme solution (chitinase 0,1%, cellulase 1%, dan macerozyme 1%). This suspension was incubated at 28°C for 4 hours with shaking at 100 rpm. Protoplasts in the suspension were filtered in order to remove mycelium debris. The filtrate was centrifuged at 3,000 g for 20 minutes. Pellet of protoplasts were washed twice with $CaCl_2$ 50 mM – KCl 0.6 M solution, resuspended in KCl 0.6 M – Tris-Cl buffer 10 mM pH 7.5, and counted the number of protoplasts using hemocytometer.

Transfomation pTRLI plasmid into *A. terreus* protoplasts was used PEG-mediated transformation. 100 µl (10^7) of *A. terreus* protoplasts in Eppendorf tube 1.5 ml were added pTRLI plasmid 10 µg and polyethylenglycol (PEG 6000 60%; buffer tris-HCl 10 mM pH 7.5; $CaCl_2$ 50 mM) solution 50 µl. This Eppendorf tube 1.5 ml was incubated on ice for 20 minutes, added polyethylene glycol solution 1 ml, and incubated at 28°C for 15 minutes. The results of transformation were grown in Czapex-Dox medium plates containing pyrithiamine 1 mg l^{-1} at 28°C. The numbers of transformants were counted after 5 days.

Isolation of *A. terreus* genome is used one colony of *A. terreus* aged 7 days on PDA plates. *A. terreus* is added with sterile water, homogenized using potter, and filtered. Filtrate was grown in Erlenmeyer 250 ml containing YMP medium 50 ml and incubated for 20 hours, 200 rpm at 28°C. Mycelium was harvested by paper filter and used immediately for isolation of DNA genome and tranfered to a sterilized Eppendorf tube 1,5 ml containing 300 µl of TES extraction buffer (0.2 M Tris-HCl pH 8.0; 10 mM EDTA pH 8.0; 0.5 M NaCl; 1% SDS) and acid-washed strerilized glass beads. Mycelium was homogenized for 2 minutes, vortexed for 30 second and added 200 ul of TES extraction buffer containing proteinase K (final concentration 50 µg ml^{-1}). Then, the suspension was vortexed, incubated in a water bath at 65°C for 30 minutes. The sample was added 250 µl of 7.5 M amonium acetate, mixed, incubated on ice for 10 minutes, and sentrifused for 10 minutes at 13,000 g. The supernatant was transfered to new Eppendorf tube 1,5 ml, added an equal volume of (500 µl) cold isopropanol, and incubated at -20°C for 2 hours. The sample was centrifused for 10 minutes at 13,000 g. The supernatant was decanted and DNA pellet was washed by 800 ul of cold 70% ethanol. Eppendorf tube 1,5 ml of sample was dried in desicator vaccum for 15 minutes to dry DNA. DNA was added by 50 µl of water free DNAse and RNAse and added 5 µl of RNAse A (20mg/ml) and

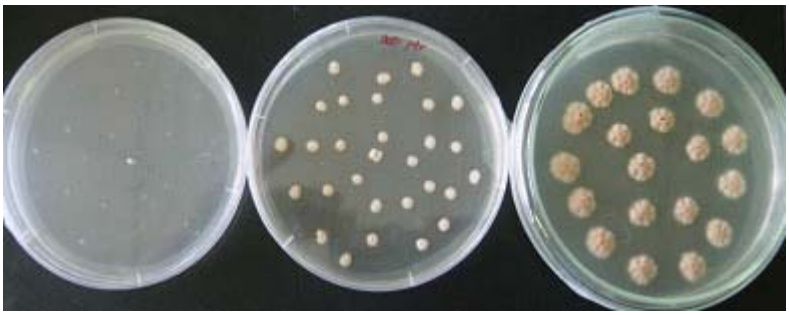
incubated at 37°C for 60 minutes. The DNA concentration and purity were measure by nanodrop apparatus.

Southern blot analysis used Dig High Prime DNA Labeling and Detection Starter Kit I (Roche). Genome DNA was restricted by *Hind*III, separated by electrophoresis on 1% agarose gel. The DNA fragments were transferred to nylon membrane using 20 X SSC. Labeling of the DNA probe, hybridization, and signal detection were detected by DIG-probe synthesis and detection kit (Roche). The detail procedure can be read in manual instruction.

A. terreus recombinant (transformant) is tested for lovastatin productivity using medium fermentation. Isolate of *A. terreus* recombinant on Potato Dextrose Agar (PDA) plate at 28°C for aged 7 days was inoculated in seed medium contained 0.5% corn steep liquor, 4% tomato paste, 1% oat flour, 1% glucose, and 1% trace element (0.16% $MnSO_4$, 0.34% $ZnSO_4.7H_2O$, 0.2% $CoCl_2.6H_2O$, 0.5% $FeSO_4.7H_2O$) at 28°C for 3 days with 200 rpm. 10% *A. terreus* recombinant from seed medium was inoculated in fermentation medium (4.5% glucose, 2.4% pepton, 0.25% Bacto yeast extract, and 0.25% polyethylene glycol 2,000) at 28°C for 7 days with 200 rpm. Culture of *A. terreus* recombinant was harvested after incubation for 7 days at 28°C with 200 rpm. Broth pH was adjusted by adding HCl until pH 3.0, centrifugated at 15,000 g, and supernatant was filtrated by filterer 0.2 µl. Concentration of filtrate was determined by HPLC, using C18 column, 2 solvents (solvent A and solvent B are acetonitrile and 0.1% phosphoric acid) respectively), detector UV 238 nm, and flow rate 1.5 ml $minute^{-1}$. As control is used the parental strain of *A. terreus*.

RESULTS AND DISCUSSION

The transformation for filamentous fungi is necessary for the manipulation of genes. In this research, pTRLI plasmid containing *lovE* gene was transformed into protoplasts of *A. terreus*. The aim of this research is obtained *A. terreus* recombinant that has pTRLI plasmid in chromosome of *A. terreus* and increases lovastatin productivity.



a b c

Fig.1. a. Parental strain of *A. terreus* did not grow in Czapex-Dox containing pyrithiamine agar, b. Transformant of *A. terreus* from PEG-mediated transformation grew in Czapex-Dox containing pyrithiamine agar, c. Parental strain of *A. terreus* grew in Czapex-Dox agar

pTRLI plasmids are transformed into *A. terreus* protoplast mediated PEG and calcium chloride. The efficiency of transformation by polyethylenglycol-mediated of *A. terreus* were 19 transformants μg^{-1} of PTRLI plasmid. Transformants grew in selection medium (Czapex-dox containing pyrithiamine medium agar) because this transformant contain pTRLI plasmid that have pyrithiamine resistance as resistance marker. PEG serves to assist the entry of plasmid into protoplast by lowering the surface tension and calcium ions accelerate the entry of plasmid into protoplast by forming a bond with negative charge on surface membrane. Southern blot analysis was used to analyze number integrative plasmid in *A. terreus* chromosome and know type of integration. Transformant stable for 5 generation was chosen in this study.

A. terreus is transformed by pTRL plasmid as integrative plasmid. Southern hybridization analysis of parental strain and transformant DNA digested with *Hind*III. Southern blot showed that copies of pTRLI plasmid integrated in chromosome *A. terreus* recombinant but the parental strain did not have copy of pRTLI plasmid.

The isolate of *A. terreus* is used in this research because this fungal can produce lovastatin higher than other filamentous fungi[10,11,12]. *A. terreus* recombinant was 93% higher than the parental strain because *A. terreus* recombinant had copy of *lovE* gene from pTRLI plasmid. This is supported by previous research that copy of gene coded FPS (farnesyl diphosphate synthase) in *Artemisia annua* recombinant was higher 34% than parental (concentration of artemesin in *A. annua* parental 0.9%)[17] and copy of *pcbC – penDE* gene cluster in *Penicillium chrysogenum* increase in penicillin production up to 40%[18].

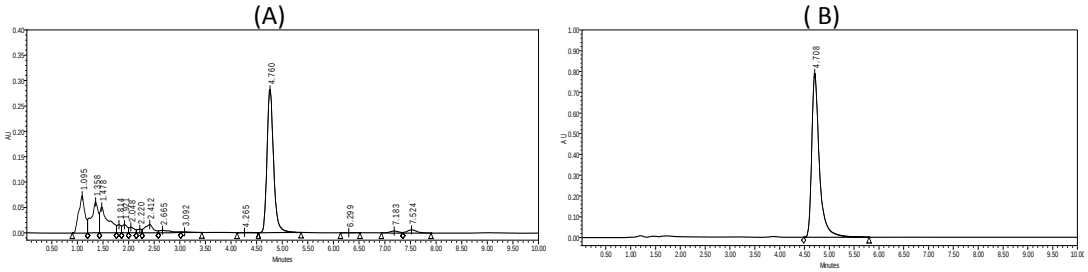


Fig.2. (A) HPLC chromatogram of lovastatin in open hydroxyl acid form from broth fermentation. (B) HPLC chromatogram of lovastatin standard in open hydroxyl acid form

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