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Assessment of double screening programmes via solid substrate fermentation (SSF) in a flask system and identification of lovastatin potential producer

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Abstract. Local economical substrates namely rice bran and unprocessed brown rice was applied into fermentation condition to produce a potent secondary metabolite compound, lovastatin. A basis condition of fermentation viz. 70% (v/w) of moisture content (adjusted to pH 6.0), 1×10^7 spore/ml of inoculum size, mixture of 1:1 substrates and 7 days of incubation period, was applied into SSF system. During a preliminary test, all of 72 fungi disclosed positive dark spot onto the thin layer chromatography plate (TLC). In order to verify the existence of lovastatin, the secondary screening which involving high performance liquid chromatography (HPLC) was conducted. Out of 72, only 71 fungi were detected as lovastatin producers and the highest production was stated from SAR I isolate with 68.72±0.84 mg lovastatin/g dry substrate and 0.87±0.03 mg glucosamine/g dry substrate of fungal growth. SAR I isolate was identified via colony and microscopic morphologies. Through the observations, SAR I isolate was identical to *Aspergillus niger*.
Keywords: lovastatin, HPLC, solid substrate fermentation, screening, thin layer chromatography, identification, *Aspergillus niger*

Introduction

An estimation of 30% increment in human mortality from year 2008 to 2030 is caused by cardiovascular diseases (CVD). Coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism are some common problems which clustered under CVD. Heart attack and stroke are the acute phase of those problems. The unhealthy diet, physical inactivity, harmful use of tobacco and alcohol are the major causes of welcoming hypercholesterolemia, an abnormal range of cholesterol in blood. CVD has a close relation with hypercholesterolemia as it is controlled by two important component (combination lipid and protein) namely low density lipoprotein and high density lipoprotein. Maintaining HDL level in a safe range is very crucial in evading CVDs problem.

Prevention is better than cure. Some people start to live in a healthy lifestyle but when it comes to a hypercholesterolemia condition, mostly for those who inherit it from family, medications treatment is the best solution. Statins are one of the options and they are categorized under secondary metabolites compound. Lovastatin which also known as Mevacor or mevinolin was the first statin to be permitted by US Food and Drug Administration (FDA) as an agent for reducing cholesterol (Pansuriya and Singhal, 2010). Basicly, there are two forms of lovastatin; inactive lactone and acid. Lactone form will be hydrolyzed into acid form in human liver and transported via blood. The principal of lovastatin is to inhibit 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) in mevalonate pathway or generally in cholesterol biosynthesis pathway.

The discovery of lovastatin attracted tremendous interest from scientists. At the beginning, only submerged fermentation (SmF) is carried out in producing this compound by varying the physic-nutritional parameters. But, the advantages which are offered by solid substrate fermentation (SSF) have urged researchers for deep surfing this system. Better controlling process, maximum substrate utilization, lower chances of contamination, easy downstream processing and etc. (Pandey *et al*., 2001) are some promises of SSF which allow it gaining popularity for multiple commercialized products including enzymes and secondary metabolites. A GRAS microorganism, *Aspergillus niger* is broadly applied for biotechnological application (Schuster *et al*., 2002). Not many reports are done regarding *A. niger* as lovastatin producer. A good news from www.uniprot.org, actually, *A. niger* consisted lovastatin gene known as lovB (a heterodimer of a nonaketide chain and a diketide) which means it can potentially generates lovastatin.

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This project deals with primary and secondary screening of producers and identification programme for influential fungus towards lovastatin production under SSF conditions. By taking an advantage of rice bran and brown rice as a cholesterol lowering agent, both were used as substrate.

Materials and Methods

Isolation of microorganism and inoculum preparation

All of 72 fungi used in this study were isolated from substrates (rice bran and brown rice), stock cultures of Industrial Biotechnology Research Laboratory (IBRL) and paddy field soils in Penang and Perak, Malaysia. A serial dilution was performed in order to get precise dilution for spread plate method. Fungi were subcultured fortnightly on potato dextrose agar (PDA) slants and stored at 4 ^{0}C. For inoculum preparation, 10 ml of sterile distilled water into the agar slant containing mature fungal culture and shake it vigorously using vortex. After diluting the concentrated stock serially, haemocytometer was used to determine the inoculum size.

Solid substrate fermentation (SSF) system and Identification of the potential lovastatin producer

A 1:1 ratio of rice bran and brown rice were applied into a shake flask system (250 ml). All cultures were grew in basis SSF condition containing 5 g substrate, 70% (v/w) distilled water and inoculum size of 1×10^7 spore/ml. After the inoculation with spore suspension, the flasks were mixed up and incubated up till 7 days at 30±2 ^{0}C. All experiments were carried out in triplicates and the results were presented as mean of the triplicates experiments.

Based on colony and microscopic morphologies, the fungal identification was done. Colony morphology was carried out on different agar namely potato dextrose agar (PDA), malt extract agar (MEA), creatine agar (CREA) and czapeks yeast agar (CYA). The agars were incubated at temperature of 25, 30 and 37 ^{0}C for seven days. The fungal colony and agar structure (reverse plate) were observed. For microscopic morphology, light and scanning electron microscope (LM and SEM) were used in order to observe fungal's structural characteristics. The fungal characteristics were described and identification was performed based on manual described by Pitt and Hocking (1999) and Cappuccino and Sherman (2005).

Extraction of lovastatin in SSF system and Thin layer chromatography as primary screening of lovastatin

The fermented substrates were crushed into powder after 48 hours of drying procedure at 60 ^{0}C. Later, 1 g of dried matter was dissolved in 30 ml acetonitrile and shake for an hour at 220 rpm. Then, the samples were centrifuged at 3000 gravity for 10 min. 2 ml portions of aliquot were mixed up with 2 ml water and 50µl of concentrated phosphoric acid before filtered through nylon syringe filter (pore size of 0.45 µm). The filtered samples were subjected onto thin layer chromatography (TLC) plate and high performance liquid chromatography (HPLC) for analysis purpose (Szakacs *et al*., 1998).

20 x 20 cm Merck silica gel 60F$_{254}$ TLC plate was used to detect lovastatin presence (Samiee *et al*., 2003). The extracted sample was spotted on the plate and soaked into a mixture of dichloromethane and ethyl acetate (70:30; v/v). All the plates were observed under a hand-held UV lamp (254 nm) and then stained with iodine vapor. The retention factor (R$_f$) was measured and compared with the standard (purity of 99.7%).

Quantification of lovastatin by HPLC and Fungal growth detection

An active form of lovastatin was measured using Waters HPLC which was supplied with Symmetry C$_{18}$ column (4.6 mm x 250 mm, 5 µm pore). Acetonitrile and phosphoric acid (pH 3.0) were used as the mobile phase. The ratio used was 77:23 (v/v), respectively. The retention time between standard and samples were compared to identify lovastatin.

Method of Tsuji *et al*., (1969) and Swift (1973) was applied in order to determine fungal growth. Chitin in cell wall was hydrolyzed and converted into glucosamine before been detected at 530 nm.

Results and Discussion

All of 72 fungi namely C2-1, tempeh, New, BS1, *Aspergillus flavus*, C4-2, B2-2, *Trichophyton mentagophytes*, *Trichoderma reesei*, ED25, *Trichophyton rubrum*, KB1, F4, FI3,

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KB2, BI, P1, II2, BS2, EI3, *Aspergillus fumigatus*, B3-4, RBS1, *Penicillium roquefortii* ITB, B1-2, BS3, C4-1, *Trichoderma viridae*, *Aspergillus nidulans*, PBK2-2, PBK1-3, H36, B2-1, BS4, B2-6, *Collectotrichum* sp, KB3, KB4, *Rhizopus* sp, 7(1), BS5, PBK1-1, 7(14), K5-5, *Penicillium* sp, ED24, NR, BS6, KB5, SAR I, ED19, *Gliocladium roseum*, ED16, P2, K3-4, P3, *Penicillium citrinum* ITB, AI1, BS7, B3-2, D3-5, KC, 7(2), P4, KB6, KB7, KB8, 7(13), P5, P6, P7 and C3 depicted a dark spot which was appeared at almost the same level of standard spot. The R_f for all samples were ranged of 0.19 to 0.39. Primary screening by TLC only reported on compound polarity. As for lovastatin, it was slightly non-polar compound because it has less affinity towards stationery phase.

In this experiment, the retention time (R_t) for acid was 5.5±0.2 min. From the R_t, the activity of lovastatin can be determined. Only 71 fungi depicted positive and active lovastatin compound. Among the fungi, isolate SAR I displayed the highest activity which was 68.72±0.84 mg lovastatin/g dry substrate and 0.87±0.03 mg glucosamine/g dry substrate of fungal growth. While isolate K5-5 gave no lovastatin activity at all.

As isolate SAR I gave the highest production, it was solely selected for identification. The colony was black and spread rapidly on PDA while from the reverse plate view, the agar changed from smooth into wavy structure (only at the inoculation area). Those characteristics were observed after seven days incubation at 25, 30 and 37 ^{0}C. Generally for MEA, the same condition was reported but the agar became wavier compared to PDA. There was also rapid black spore spreading on CYA during 25 and 30 ^{0}C, but, a slow pattern was showed during 37 ^{0}C. The mixture of spore (at the central of inoculation point) with white mycelium was clearly made a different outcome compared to PDA and MEA. CREA was a special agar to detect the ability of the fungal isolate whether it can produce acid or not. The positive result for acid production will expose if the agar color change from purple to yellow. In this experiment, SAR I isolate did produce acid (Figures were not shown).

A matured SAR I isolate was further identified via microscopes (LM and SEM). Figure 1.0 described the observation under LM. This fungal consisted a long conidiophore, globose vesicle (to hold the conidium chain) and also conidia (Cappuccino and Sherman, 2005). While for SEM view (Figure 2.0), it was more interpreted as the colorless conidiophore elongated up to 3.0 mm, globose vesicle with 50-75 µm diameter plus rough and wavy surface of conidia (4 -5 µm diameter) with radiate head. It was all reported by Pitt and Hocking (1999). With these elaborations, those morphologies were very identical to *Aspergillus niger*.
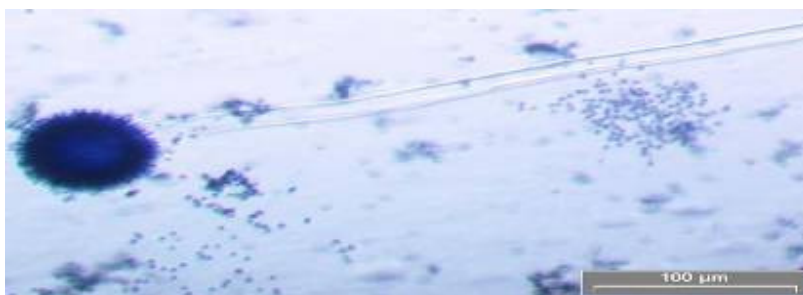

Figure 1. Microscopic view of conidia, conidiophore and vesicle under LM after stain with lactophenol cotton blue
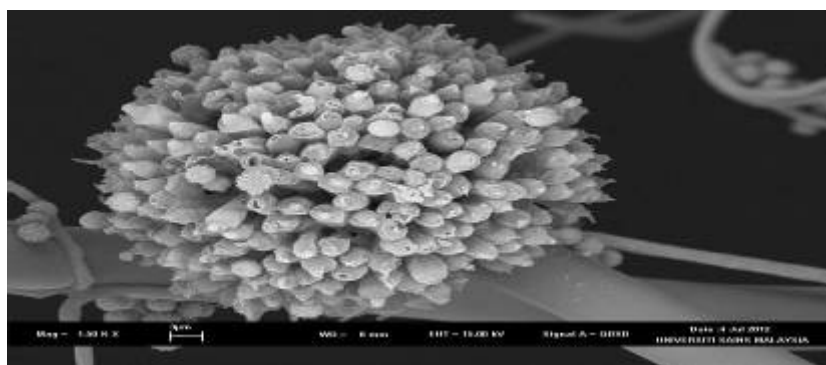

Figure 2. The scanning electron microscope result of SAR I isolate indicated more detail structural characteristics compared to LM

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Conclusions

A slight non polar of lovastatin by various fungi was discover via TLC and then was verified by HPLC. SAR I isolate which was later identified as *A. niger*, gave the highest production of anti cholesterol agent (lovastatin) under SSF system.

Acknowledgements

References

Cappuccino J.G., Sherman N. 2005. Microbiology- A laboratory manual, 7th Edition. Pearson Education, Inc.

Pandey A., Soccol C.R., Rodriguez-Leon J.A., Nigam P. 2001. Solid-state fermentation in biotechnology- fundamentals and applications. Asiatech Publishers, Inc. New Delhi.

Pansuriya R.C, Singhal R.S. 2010. Response surface methodology for optimization of production of lovastatin by solid state fermentation. Brazilian Journal of Microbiology, 41: 164-172

Pitt J.I., Hocking A.D. 1999. Fungi and Food Spoilage, Gaithersburg, Maryland: Aspen Publishers, inc.

Samiee S.M., Moazami N., Haghighi S., Mohseni F.A., Mirdamadi S., Bakhtiari, M.R. 2003. Screening of lovastatin production by filamentous fungi. Iranian Biomedical Journal, 7(1): 29-33

Schuster E., Dunn-Coleman N., Frisvad J.C., van Dijck P.W.M. 2002. On the safety of *Aspergillus niger* – a review. Applied microbiology and Biotechnology, 59: 426-435.

Swift M.J. 1973. The estimation of mycelial biomass by determination of the hexosamine content of wood tissue decayed by fungi. Soil Biology and Biochemistry, 5: 321-332.

Szakacs G., Morovjan G., Tengerdy R. 1998. Production of lovastatin by a wild strain of *Aspergillus terreus*. Biotechnology Letters, 20 (4): 441-415

Tsuji A., Kinoshta T., Hosino, M. 1969. Analytical chemical studies of amino sugars- Determination of hexosamines using 3-methyl-2-benzothiazolone-hydrazone hydrochloride. Chemical and Pharmaceutical Bulletin, 17: 1505-1510