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The influence of physical parameters towards hyper cholesterol reducing agent production, lovastatin, under solid substrate fermentation (SSF) condition

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Abstract. Two potential substrates namely rice bran and unprocessed brown rice indicated positive result of lovastatin existence. *Aspergillus niger* SAR I, our local isolated fungus, took a responsibility to cooperate with those substrates in SSF system. Further experiment including initial profile production, effect of physical parameters (temperature, inoculum size and substrate quantity) and final profile production, were carried out. For initial profile, a basic condition of SSF which consisted 70% (v/w) of moisture content (adjusted to pH 6.0), 5 g substrates mixture (ungrounded size), $1x10^7$ spore/ml of inoculum size and incubation temperature at 30±2 ^{0}C, was conducted in a flask system and fermented for 7 days. Those conditions allowed 160.03±3.79 mg lovastatin/g dry substrate of lovastatin production during initial stage. After a study of effect of physical parameters, it showed that the optimum temperature was still at ambient temperature (30±2 ^{0}C) and substrate quantity of 5 g but different inoculum size ($1x10^5$ spore/ml). Each parameters specifically temperature, inoculum size and substrate quantity produced 253.98±5.92 mg lovastatin/g dry substrate, 297.64±0.56 mg lovastatin/g dry substrate and 298.72±44.12 mg lovastatin/g dry substrate, respectively. Throughout the final profile, the production was 305.08±14.15 mg lovastatin/g dry substrate which made the total increment hit to almost 91%. In this experiment, lovastatin was subjected into high performance liquid chromatography (HPLC) with acetonitrile and phosphoric acid (pH 3.0) as a mobile phase.
Keywords: Lovastatin, temperature, inoculum size, substrate quantity, SSF, *Aspergillus niger*, HPLC

Introduction

A report of human mortality is made by World Health Organization (WHO). 23.6 million of human race will face death by the year 2030 with cardiovascular diseases (CVD). Hypercholesterolemia is the key of this disease and it is controlled by two important cholesterol known as high density lipoprotein (good cholesterol) and also low density lipoprotein (bad cholesterol). When it comes to hypercholesterolemia condition, reducing LDL level by medications is the best option instead of diet and workout. Fungi are a potent microorganism in producing a wide fraction of active compounds mostly pharmacological properties. Those compounds are dynamically underwent polyketide biosynthetic pathway including statin (anti-cholesterol agent). Statins are categorized under few classes viz. natural, synthetic and combination or modification of natural and synthetic statin. Compactin, lovastatin, pravastatin, simvastatin, rosuvastatin, atorvastatin and fluvastatin are the available statins in world's market. According to Praveen and Savitha (2012), the starting production of statin based on acetate units linked to each other in a head to tail manner to structure out a polyketide chain.

Among statins, lovastatin is a common drug that has been used in worldwide as an anti cholesterol agent and most hospital in Malaysia prescribed this drug to CVD patients. A core mission of lovastatin is to inhibit the rate-limiting enzyme of cholesterol biosynthesis namely 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) (Pansuriya and Singhal, 2010). There are two form of lovastatin; lactone (closed lactone form) and acid (open ring salt). Acid form is an active form of lovastatin that functioning to reduce the LDL in blood. The available tablet in our market was in native form (lactone). So, once it is carried to the human liver, it will transform into acid form and handle its duty as anti-cholesterol agent. An informative report by Marovjan *et al*. (1997) provided a new method in facilitating acid form of lovastatin which is by adding the lactone form with water and phosphoric acid. But, the major problem dealing with acid form is its stability.

Lovastatin production has gained large scale importance with more emphasis on use of solid substrate fermentation (SSF) approach. This system promises a lot advantages over submerged fermentation (SmF) such as higher productivity, simpler operation and lower cost (Li and Jia, 2008). However, scientists pay special interest on both systems. *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopolariopsis, Doratomyces, Phoma, Phythium, Gymnoascus, Hypomyces* and *Pleurotus* are some microorganism generas that been reported by

Bizukojc and Stanislaw (2009) and Cabral *et al*. (2010) as lovastatin producers either under SSF or SmF. Based on potentiality of lovastatin production by filamentous fungi in with the cooperation of rice bran and bran rice, this project was carried out under SSF condition. Effect of three compelling physical parameters towards lovastatin production was investigated.

Materials and Methods

Microorganism maintenance and inoculum preparation

A. niger SAR I isolated from Kepala Batas Pulau Pinang (Northern region of Malaysia) was used in the present study. It was maintained on potato dextrose agar (PDA) slants, stored at 4 ^{0}C and sub-cultured every fortnightly. Spore inoculum was prepared by adding 10 ml of sterile distilled water containing 0.1% (v/v) Tween 80 to a fully sporulated agar slant. The spores were dislodged using sterile loop under aseptic condition and serial dilution was done. The spore suspension concentration was determined using special square chamber of haemocytometer.

Solid substrate fermentation in a flask system and production profile of lovastatin

A basis condition of SSF was applied into a flask system. 70% (v/w) of moisture content, 5 g substrate mixture (1:1, rice bran: brown rice), 1x10^7 spore/ml inoculum size and distilled water which was adjusted to pH 6.0 using hydrochloric acid and sodium hydroxide. The flasks were incubated at ambient temperature, 30±2 ^{0}C, for 7 days. *A. niger* SAR I was applied into production profile before and after physical parameters optimization. Profile before physical parameters optimization was performed under basis condition while the optimized conditions were applied into production profile after physical optimization. Both profiles underwent sixteen days of incubation period.

Temperature, inoculum size and substrate quantity

Those parameters were the targeted conditions to be optimized. Experiments were presented under different temperature ranging from 25 to 40^{0}C. For inoculum size, size of 1x10^4 to 1x10^8 spore/ml was tested. In order to testify the effect of substrate size towards lovastatin, various quantity was investigated which was 2.5 to 20 g of substrate.

Extraction and analysis

A modified method of Szakacs *et al*. (1998) was selected for extraction purpose. After 48 hours drying procedure at temperature of 60 ^{0}C, substrates were crushed into powder form. Then, 1 g of powdery dry substrates was dissolved in 30 ml acetonitrile and shake for 1 hour at 220 rpm. After that, samples were centrifuged at 3000 gravity for 10 min. The aliquot was filtered through nylon syringe filter (pore size of 0.45 µm) and subjected to high performance liquid chromatography. The Waters HPLC was supplied with C_{18} column with the size of 250x4.6 mm (pore of 5 µm). The eluent system was acetonitrile-phosphoric acid, pH 3.0 (77:23, v/v), solution flowing at 1.0 ml/min. The detection wavelength was 238 nm.

Fungal growth was measured using method of Tsuji *et al*. (1969) and Swift *et al*. (1973) by taking advantage of chitin existence in cell wall.

Results and Discussion

The application of basis conditions in a flask system during production profile before physical optimization indicated minimum production which was 160.03±3.79 mg lovastatin/g dry substrate with 1.31±0.03 mg/g of fungal growth (Figure 1A). After a few physical modifications, 91% increment was depicted at day tenth which made the total production boost to 305.08±14.15 mg lovastatin/g dry substrate (Figure 1B).There was no correlation observed between lovastatin production and fungal growth.

Temperature plays an important role in SSF. As shown in Figure 2.0, lovastatin achieved its optimum level at ambient temperature (30±2 ^{0}C). The influence of *A. niger* SAR I original temperature might affected the temperature selection. However, it has been reported that most of microorganism used in SSF was mesophilic, thus the optimal temperature for growth is between 20 to 40 ^{0}C or below than 50^{0}C (Manpreet *et al*., 2005). In this study, the highest production was 253.98±5.92 mg lovastatin/g dry substrate. Figure 3.0 denoted the effect of inoculum size which ranged of 1x10^4 to 1x10^8 spore/ml towards lovastatin. The optimum size was 1x10^5 spore/ml (297.64±0.56 mg lovastatin/g dry substrate). Size of inoculum initially will influence the lag phase of fungal growth. 1x10^5 spore/ml was suitable for lovastatin production as the incubation period was ten day. It allowed fungal to produce maximum level of lovastatin.

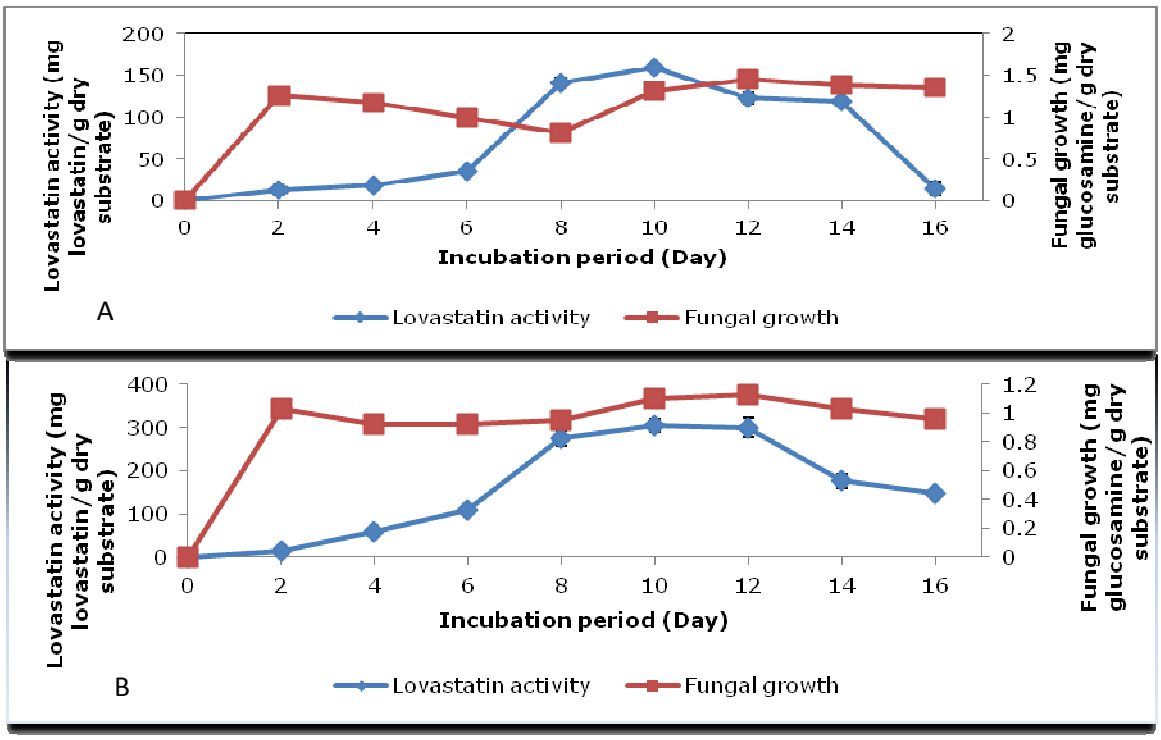

Figure 1. Production of lovastatin before physical parameters optimization (A) and after optimization (B). Total increment was about 91%.

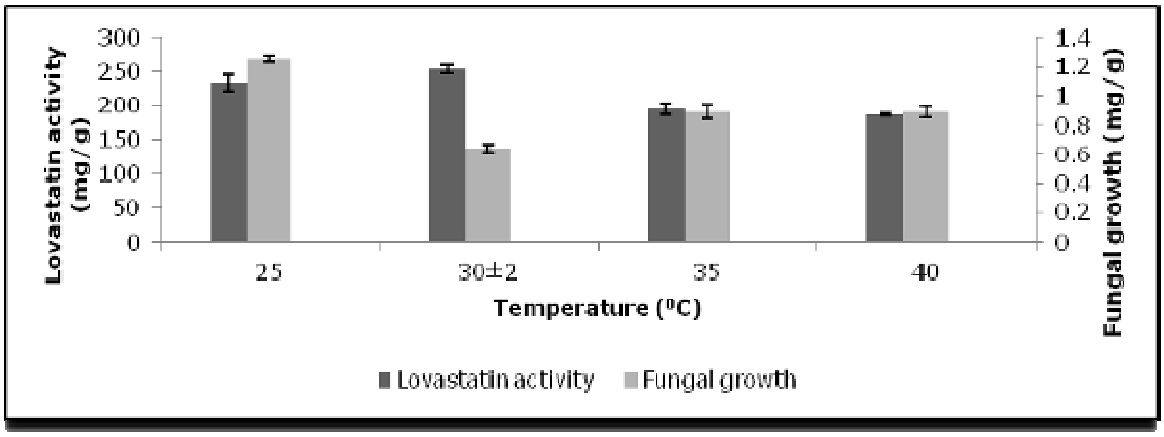

Figure 2. Effect of temperature on lovastatin production by *A. niger* SAR I after ten days incubation period.

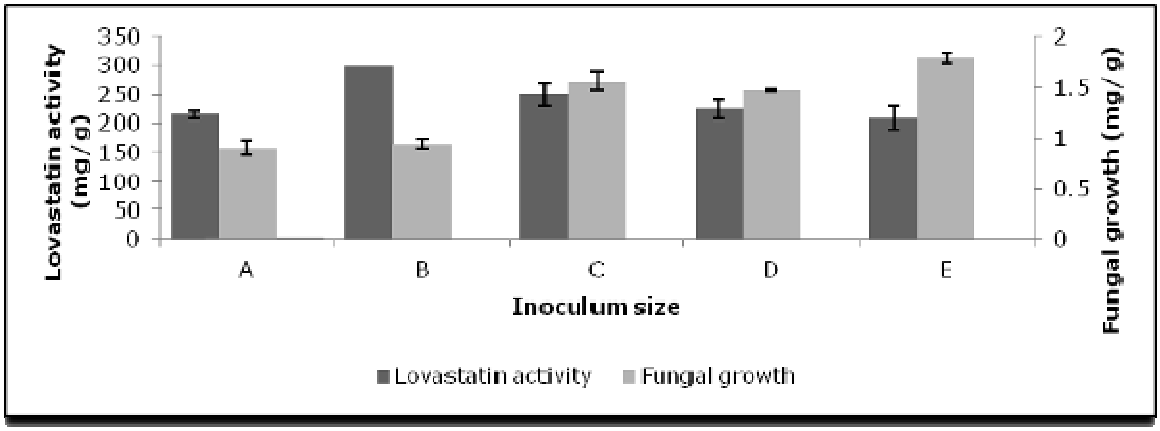

Figure 3. Inoculum size 1x10^5 spore/ml eliminated other sizes as it depicted the highest production of lovastatin (Indicator: A= 1x10^4, B= 1x10^5, C= 1x10^6, D= 1x10^7, E= 1x10^8 spore/ml)

The thicker substrate applied into flask system, the harder *A. niger* SAR I needed to penetrate into the bed as the fungus commonly grow at the surface of substrate. Out of five substrate quantities, 5 g substrate gave the best lovastatin production. It provided 298.72±44.12 mg lovastatin/g dry substrate of lovastatin activity (Figure 4.).

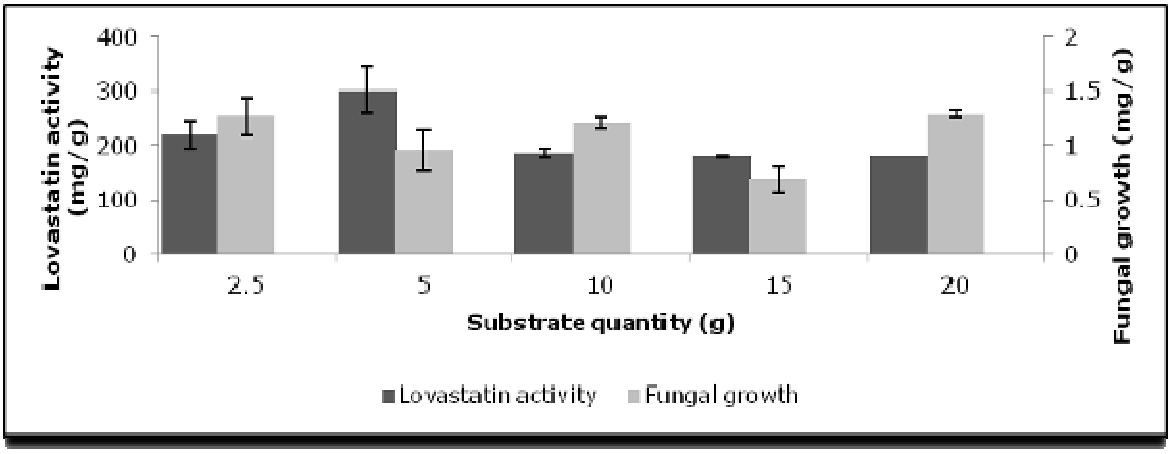

Figure 4. Effect of substrate quantities towards lovastatin production by *A. niger* SAR I

Conclusions

Physical parameters attribution was very crucial in producing final product of SSF. Under temperature of 30±2 ^{0}C, inoculum size of 1x10^5 spore/ml and 5 g of substrate quantity, lovastatin did increase to maximum level.

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