

## A New Latent Lovastatin Producer viz. *Fusarium pseudocircinatum* IBRL B3-4, Produced in Laboratory Tray System

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### ABSTRACT

A proximate analysis study of local rice bran and brown rice disclosed a distinguish level of chemical compositions. Lipid, carbohydrate and ash occupy in rice bran, while fibre is a predominant component in brown rice. Both perceptible anti-cholesterol substrates were grown with locally isolated *Fusarium pseudocircinatum* IBRL B3-4 to obtain the best lovastatin activity via dichloromethane extraction. Evaluation of lovastatin production at different substrate thickness ranges of 0.25 to 1.5 cm in a static tray system (20x20x6 cm<sup>3</sup>) exposed the highest production at 0.5 cm level (1135.0±6.7 µg/g dry solid of lovastatin). Meanwhile, effects of physical parameters investigation interpreted that the original substrate size, 60% (v/w) moisture content and ambient local temperature of 30±2°C as the most suitable conditions to generate lovastatin at the utmost level. Meanwhile, the maximum production was synthesized at day 12<sup>th</sup> (twelfth) under solid substrate fermentation system. A significant activity increment was revealed after 60% (v/v) moisture content had been applied into tray system. In more specific, it boosted up to 2271.7±14.4 µg/g dry solid of lovastatin.

**Keywords:** Solid substrate fermentation, tray system, *Fusarium pseudocircinatum*, rice bran, brown rice, proximate analysis

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### INTRODUCTION

World Health Organization (WHO) has reported a threat caused by cardiovascular diseases (CVDs) against human race. Annually, 7.5 million deaths have been

recorded with 51% fatality cases were caused by stroke and 45% due to coronary heart disease. If this pattern continues, it is estimated that by 2030, almost 25 million human beings are predicted to perish due to these silent killers. A waxy steroid substance known as cholesterol is the main element that causes CVDs. It is synthesized by the liver from a small precursor known as acetoacetyl-CoA, a vital isoprene unit (from acetoacetyl-CoA) and then constructed via a lengthy process which leads to cholesterol production. Cholesterol must be wrapped to be transported in the blood circulation by lipoproteins as carriers. There are two types of cholesterol produced, namely, low density lipoprotein (LDL) and high density lipoprotein (HDL). Dietary cholesterol and genetic factors are the controllers of CVDs. These include tobacco use, low intakes of fibrous food, extreme alcohol drinking, physical inactivity and also psychosocial factors. The extension of hypertension, abnormal lipid level and diabetes mellitus can also lead to cardiovascular problem.

Statins are reversible inhibitors of hydroxymethyl glutaryl-CoA (HMG-CoA) reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, which is an early and rate limiting step in the biosynthesis of cholesterol via the mevalonate pathway. It is categorized under secondary metabolite cluster of fungi and divided into natural produced statin, semi synthetic and also synthetic. Lovastatin was the first administered as anti cholesterol lactone prodrug, announced by Food and Drug Administration. It appears

in two major forms, namely, lactone and acid. Lactone is hydrolyzed *in vivo* to the corresponding  $\beta$ -hydroxyacid which occurs in mammal's liver. The active acid form (also known as mevinolinic acid) acts on the HMG-CoA reductase. Basically, it consists of two polyketide chains viz. nonaketide (go through cyclization to a hexahydronaphthalene ring system) and diketide, 2-methylbutyrate (Hendrickson *et al.*, 1999). Research by Endo *et al.* (1985) on lovastatin biosynthesis in *Aspergillus terreus* has proven that polyketide pathway is involved in statin formation. On the other hand, Casas-Lopez *et al.* (2004) reported that lovastatin retarded its own synthesis in *A. terreus* under submerged fermentation (SmF) which indirectly coronated solid substrate fermentation (SSF) as a better medium for lovastatin production. In submerged culture, lovastatin stays in the mycelium that may suppress further synthesis, while in SSF, the provided space in solid substrate allowed absorption for lovastatin. Throughout the time, the definition of SSF keeps on changing because it is distinguished based on substrate utilization during the experiment that is divided into two; natural and inert substrate support. Recently, it is defined as a growing microorganism process on or within natural or inert substrate's particle, in absence or near absence of free flowing water (Pandey, 1992). The hallmarks of SSF are simple, inexpensive and high yield of secondary metabolites.

A special feature of filamentous fungi is their capability to generate assorted of

structurally natural products or secondary metabolites (Cole *et al.*, 2003). The first outbreak of lovastatin producer (*Monascus ruber*) was reported by Endo in 1979, followed by Alberts and co-workers (1980) using *Aspergillus terreus*. This 'epidemic' consecutively spread around lovastatin academia and resulted in a variety of other potential producers such as *Aspergillus* sp., *Penicillium* sp., *Monascus* sp., *Paecilomyces* sp., *Trichoderma* sp., *Scopulariopsis* sp., *Doratomyces* sp., *Phoma* sp., *Phythium* sp., *Gymnoascus* sp., *Hypomyces* sp. and *Pleurotus* sp. (Gunde-Cimerman *et al.*, 1973; Shindia, 1997). A recent report by Raghunath *et al.* (2012) stated that *Fusarium* sp. isolate has the capacity to synthesize lovastatin too. A lot of secondary metabolites fungi are enriched with polyketide synthase (PKS) genes and *Fusarium* sp. gained this gene (Xiangcheng *et al.*, 2007; Brown *et al.*, 2012) enabling them to produce lovastatin. Generally, *Fusarium* sp. is known in producing mycotoxin such as beauvericin, fumonisin, zearalenone, moniliformin and trichothecene. Although *Fusarium pseudocircinatum*, it does not produce detectable levels of beauvericin, it can produce moniliformin, low levels of fusaproliferin and trace levels of fumonisins (Fotso *et al.*, 2002).

Up until now, various reports of analytical methods, parameters optimization and purification of lovastatin have been well published. Our recent report concentrated on the potentiality of chemical composition in the used substrates (rice bran and brown rice), effects of solvents during extraction

programme and effects of cultural conditions (physical parameters) on lovastatin production in a tray system.

## MATERIALS AND METHODS

### *Substrates, F. pseudocircinatum and Chemicals Sources*

Substrates viz. rice bran and brown rice were bought at a local rice mill factory in Penang, Malaysia. The *F. pseudocircinatum* culture obtained from Industrial Biotechnology Research Laboratory's stock which had previously been isolated from oil palm farm soil located in the northern region of Malaysia. The culture was revived on potato dextrose agar (PDA) every fortnightly. Only fresh culture was used in performing solid substrate fermentation (SSF) for lovastatin production. All chemicals and solvents used in this experiment were purchased from different companies. Yeast extract (Scharlau Microbiology, Spain), sucrose (Bendosen, Norway) and calcium chloride (Fluka Chemika, Switzerland) were the optimized conditions for the tray system. Organic solvents for the extraction process [namely, acetonitrile (Merck, Germany), dichloromethane (Qrec, New Zealand), buthyl acetate (Merck, Germany), ethanol 99.7 % (Qrec, New Zealand), ethyl acetate (Bendosen, Norway), methanol (Qrec, New Zealand) and toluene (J.T. Baker, USA)] were obtained in AR grade, except for the acetonitrile used during the HPLC analysis. Lovastatin standard was procured from Calbiochem (Merck, Germany) with 99.7 % HPLC purity. Standard powder was dissolved in acetonitrile (HPLC

grade, Merck, Germany) and different concentrations were set up (10 to 100 µg/ml).

#### *Proximate Analysis of Local Brown Rice and Rice Bran*

Substrates used in SSF were evaluated for crude protein, ash, carbohydrate, moisture, fibre and lipid content based on the method of AOAC (1997). Total carbohydrate content of the substrates was measured by deducting the sum of the weight of moisture content, crude protein, lipid, ash and fibre from the total dry solid.

#### *Influence of Assorted Solvents in Extraction*

Various solvents with different polarity were screened to determine the best extraction agent for lovastatin recovery. Then, 10 mL of polar and nonpolar solvents (namely, methanol, acetonitrile, butyl acetate, ethanol, dichloromethane, ethyl acetate and toluene) were added in 1 g of dried fermented substrate which had been exposed to 80°C for 24 hour. The samples were sonicated for 5 min, followed by shaking at 30 °C, 200 rpm for 2 hours. The aliquot was centrifuged at 3000 g for 8 min to separate the solvent and substrate. Then, 1 ml of supernatant was collected and mixed up with 1 % (v/v) of trifluoroacetic acid (TFA) for lactonization purpose. The mixture was concentrated at 80°C without applying vacuum. They were then subjected into high performance liquid chromatography (HPLC) by diluting the concentrated mixture with 5 ml acetonitrile. Samples were filtered with nylon syringe

filter size of 0.45 µm prior to HPLC injection (Pansuriya *et al.*, 2010; Panda *et al.*, 2010).

#### *Time Course of Lovastatin Production in Different Substrate Thickness*

Rice bran and brown rice are broadly known as cholesterol reducing agent. Both substrates consisted lovastatin but not in a very high value. In order to induce lovastatin production, 1.5% (w/w) of sucrose, 1% (w/w) of yeast extract and 0.5 % (w/w) of calcium chloride were supplied into four substrate thicknesses (tray size of 20x20x6 cm<sup>3</sup>) under physical environmental of the original particle size, 70% (v/w) of moisture content, ambient incubation temperature (30±2 °C), inoculum size of 1 x 10<sup>5</sup> spore/ml and without mixing effect condition. Substrate thickness ranges of 0.25 cm up to 1.5 cm were autoclaved separately from any solution at 121 °C for 15 min and pH was set as 6.5, prior to autoclave. Afterwards, all the solutions were sterilely pipetted onto the substrate mixture (1:1) and thoroughly stirred for nutrients distribution in a tray. The fermented samples were harvested for every two days interval until day sixteenth.

#### *Modification of Substrate Size, Moisture Content and Temperature*

The combination of rice bran (which has an undefined particle size) and brown rice allowed an inter particle space to occur in SSF. Different particle sizes of the brown rice, ranging from 0.1 mm to original size, were investigated. The effect of moisture content was evaluated by varying the moisture percentages from 50% to

90% (v/w). The temperature influence towards lovastatin production was also determined by incubating the trays within the temperature range of 25 to 40°C.

#### *Analytical Methods*

##### **Lovastatin Analysis**

Retention times of lovastatin's peak produced by the samples were compared with the standard peak via HPLC (Waters, USA) in isocratic motion. The instrument was supplied with aqueous-organic mixture (namely phosphoric acid which was adjusted to pH 3.0) and acetonitrile as a mobile phase (23:77, v/v). A stainless steel column, Symmetry C<sub>18</sub> (250 x 4.6 mm) was responsible to flow out solution at 1.0 mL/min under 238 nm uv absorbance (Huang *et al.*, 2010).

##### **Determination of Fungal Growth**

The complexity in valuing the mycelia biomass or the extent to which the mycelia have penetrated into the solid substrate was one of the main problems came across in the SSF study. The combination methods of Tsuji *et al.* (1969) and Swift (1973) have overcome this problem by hydrolyzing the chemical compound presented in the cell wall of the fungi known as poly-N-acetylglucosamine or chitin into glucosamine. Tsuji *et al.* (1969) developed the conversion part of chitin. Later on, Swift (1973) found the glucosamine's assay method using Ehrlich reagent. Glucosamine was detected spectrophotometrically at the absorbance of 530 nm.

## **RESULTS AND DISCUSSION**

#### *Proximate Analysis*

The choice of substrate in SSF must meet the criteria of non-soluble in water, readily available and cheaper than synthetic substrate, possesses own physical and nutrient support and the most vital characteristic is that whether the substrate can obtain the specific product or not (Pandey, 2003). A study of crude nutrient contents in rice bran and brown rice was executed via proximate analysis. Basically, six categories of chemical properties were investigated and these are moisture content, crude ash, crude protein, fats or lipid, crude fibre and carbohydrate. Table 1 denotes the mean percentage of different compositions of rice bran and brown rice. Our local rice bran consist higher amount of lipid, carbohydrate and ash, while brown rice is enriched with fibre. A previous research by Wanyo *et al.* (2009) indicated that the existence of compositions in rice bran was almost in the same values as those in this study. These essential nutrients play a prominent role in human growth but lipid is always clustered under wrong concept. Both these substrates, rice bran and brown rice, are great carriers of unsaturated fatty acids (with no trans fatty acids) which is a good sign for cholesterol deduction. In addition, a report by the United States Department of Agriculture (USDA) declares that there are also Omega 3-fatty acids (alpha linoleic acid) and omega-6 fatty acid (linoleic acid) in rice bran and brown rice. These elements give an extra boosting factor for lovastatin production by *F. pseudocircinatum* in SSF.

TABLE 1  
A study of chemical composition in local rice bran and brown rice (%)

Components	Rice bran	Brown rice
Moisture	10.5±0.3	9.3±0.4
Protein	13.4±0.2	12.1±0.3
Lipid	17.7±0.1	5.1±0.1
Fibre	7.1±1.9	48.5±0.6
Ash	10.04±0.3	2.5±0.3
Carbohydrate	41.3±2.1	22.5±0.5

Values are expressed as means ± standard deviation

### *Solvents Effect on Lovastatin*

In fermentation, fungal mycelia prefers secreting lovastatin more in acid form (beta hydroxy acid) compared to the lactone form. However, acid, which is an active form of lovastatin, is less stable and by considering this matter, it needs to be converted into native form (lactone) via lactonization process. Lovastatin is a dimly non polar compound (Pansuriya & Singhal, 2009) and different polarity of solvents will hand in very much help to drag out lovastatin compound. Commonly, there are three group of solvents encountered namely polar protic, polar aprotic and non polar. As illustrated in Fig.1, dichloromethane, a 'borderline' polar aprotic solvent, showed a tremendous activity of lovastatin (281.7±44.4 µg/g dry solid) compared to acetonitrile (216.7±5.6 µg/g dry solid), butyl acetate (61.7±2.2 µg/g dry solid), toluene (60.0±3.3 µg/g dry solid), ethyl acetate (58.3±4.4 µg/g dry solid), methanol (38.3±2.2 µg/g dry solid) and ethanol which gained the lowest activity (33.8±2.6 µg/g dry solid). A previous report by Pansuriya and Singhal (2009) affirmed

that the production of the acid form of lovastatin shed with a decrease in the solvent polarity. For this experiment, the addition of trifluoroacetic acid (TFA), a lactonization agent, drew a polar aprotic solvent as the best solvent to synthesize lactone lovastatin.

Meanwhile, the chromatogram pattern for lovastatin appearance using dichloromethane as a chosen extraction solvent was shown in Fig.2. Fermented sample pointed out a peak at retention time ( $R_t$ ) of 7.9 min (Fig.2A) which is paralleled with the standard lovastatin (Fig.2B). Verification was made by overlaying those chromatograms and consequently, the peaks were equivalent (Fig.2C). Reports by Alvarez-Lueje *et al.* (2005), Szakacs *et al.* (1998) and Huang *et al.* (2010) recommended a flexible  $R_t$  for lactone lovastatin; 8.27 min, 9.20 min and 8.89 min, respectively. The cases of varying  $R_t$  depend on a few factors which include size column and types of solution used for mobile phase.

### *Initial Profiles of Substrate Thickness in Tray System*

*F. pseudocircinatum* is clustered under African clan group which gains a rapid growth on potato dextrose agar. According to Nirenberg and O'Donnell (1998), it exposes short chains of microconidia and sterile coiled hyphae as distinguishable structures with other *Fusarium* sp. (Fig.3). A laboratory scale of simple tray system was done by growing *F. pseudocircinatum* in square tray in five thicknesses ranging from 0.25 cm (50 g) to 1.5 cm (200 g), as shown in Fig.4. Fig.4A indicates that

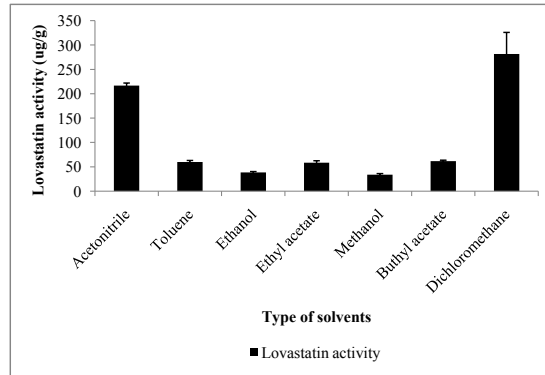


Fig.1: Solvents with different polarity did effect the lovastatin production. The polar aprotic dichloromethane illustrated the best extraction solvent for lovastatin

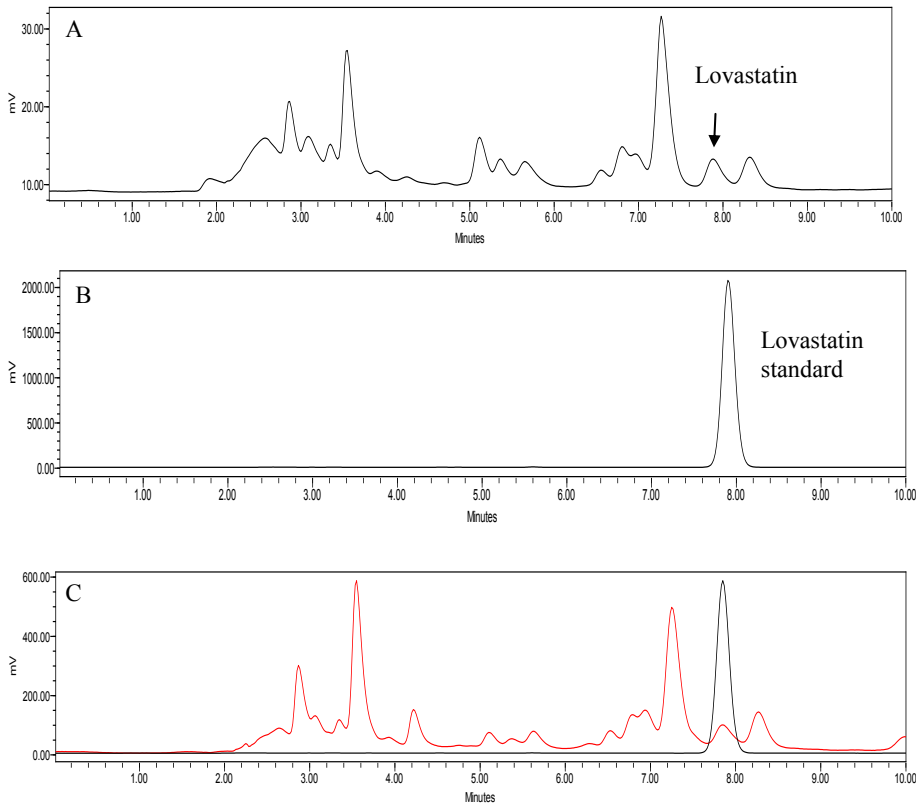


Fig.2: Fermented sample's peak (A) appeared at the same  $R_t$  with the standard lovastatin (B). The overlapping peak between sample and standard verified the lovastatin production by *F. pseudocircinatum* (C).

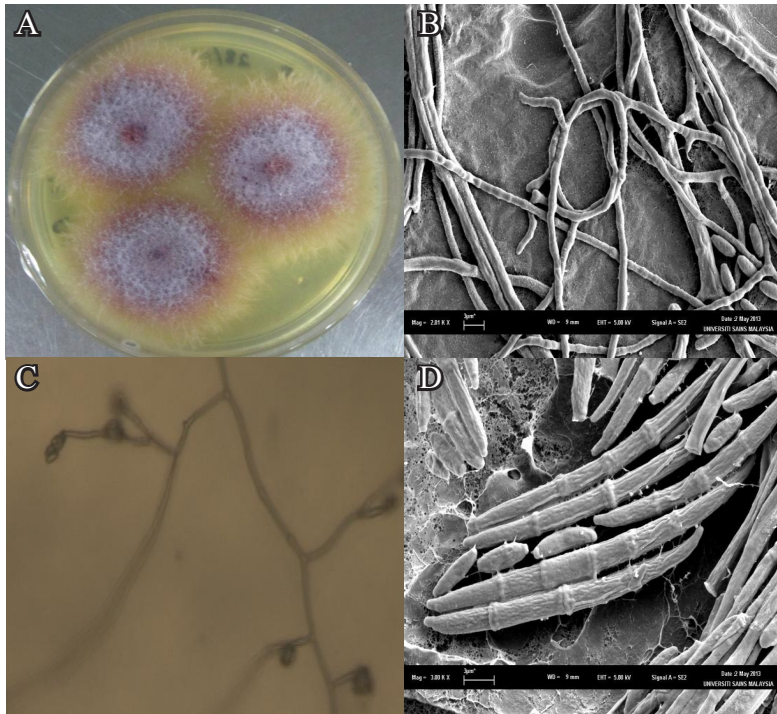


Fig.3: *F. pseudocircinatum* IBRL B3-4 image grew on potato dextrose agar (PDA) for seven day (A) and the special features of it namely coiled hyphae (B). The short chain structure was pointed out in (C) and (D) represented a close up of micro and macroconidia of this fungus

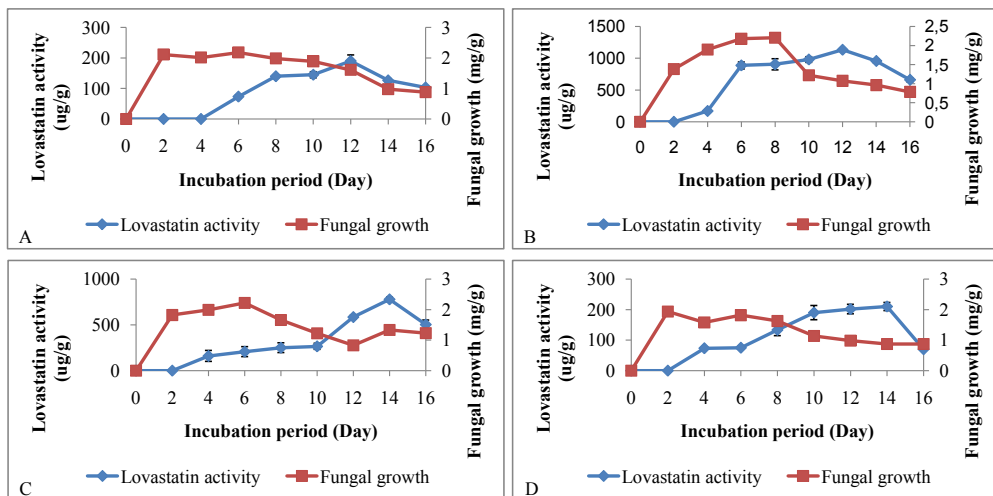


Fig.4: Initial profiles for lovastatin production at different substrate thickness. (A) was 0.25 cm, (B) was 0.5 cm, (C) was 1.0 and (D) was 1.5 cm. The highest activity obtained in (B) which equal to 100 g of substrate



the best production for 0.25 cm thickness is at the same day with 0.5 cm substrate thickness i.e. at day 12<sup>th</sup> (190.0±20.0 µg/g dry solid and fungal growth of 1.6±0.01 mg glucosamine/g substrate). By comparing all the thicknesses, 0.5 cm thickness (Fig.4B), which is equivalent to 100 g substrate mixture, showed the highest lovastatin production with 1135.0±6.7 µg/g dry solid and fungal growth of 1.1±0.03 mg glucosamine/g substrate. As the thickness got deeper, the production slowly became smaller and the optimum day started to extend. This phenomenon was found for the thickness of 1 cm and above. The activity hit the best production at day 14. The best production for 1.0 cm thickness was 778.3±24.4 µg/g dry solid and fungal growth of 1.3±0.02 mg glucosamine/g substrate (Fig.4C). Meanwhile, the activity for 1.5 cm thickness was 210.0±13.3 µg/g dry solid with fungal growth of 0.87±0.09 mg glucosamine/g substrate. Nonetheless, no relationship tangled between lovastatin production and fungal growth.

Previous reports by Pei-Lian *et al.* (2006), Panda *et al.* (2010) and Pansuriya *et al.* (2010) stated that the optimum day for lovastatin produced by fungi was eleventh, fourteenth and tenth, respectively. *F. pseudocircinatum* IBRL B3-4 possesses an efficient mycelia system which permits it to board deep into the bottom part of the tray system and also breaks down the substrate. However, the problem occurred as the oxygen supply was limited beneath the substrate bed. Filamentous fungi preferred spreading between the solid substrate

fragments and on the surface of substrate in order to form a mat, a conquering symbol for fungi in SSF. In the static fermentation conditions, the phenomenon related to mass and energy occurs along within the substrate bed. Metabolic heat production, conduction, diffusion gases, convective heat transfer, evaporation and convective mass transfer, are directly influenced the final product in SSF (Mitchell *et al.*, 2006).

#### *The Effect of Substrate Size, Moisture Content and Temperature*

Critical parameters in SSF (namely substrate size, moisture content and temperature) were studied. During substrate size investigation, various sizes of brown rice were inspected as the rice bran has an undefined size. Substrate particle size and shape affect the ability to access nutrients by microorganism. *F. pseudocircinatum* IBRL B3-4 demanded the original size of brown rice to achieve the highest production of lovastatin (see Fig.5). In order to beat other sizes (0.1 mm, 1 mm, 3 mm and 6 mm), the original size of brown rice has to set some suitable physical conditions including surface area, porosity, penetration space (Nandakumar *et al.*, 1996) and a permission to allow a better ventilation. A study by Panda *et al.* (2010) using the original size of rice mold also showed a positive result of lovastatin production after growing with *Monascus* sp. In this experiment, a combination of brown rice with undefined size of rice bran permitted a large attacking area without causing substrate agglomeration, a common problem that occurred during small particle

size application (Couto & Sanroman, 2006). The indicated result showed that the highest production obtained by *F. pseudocircinatum* IBRL B3-4 was  $1171.7 \pm 55.5 \mu\text{g/g}$  dry matter and fungal growth of  $1.57 \pm 0.05 \text{ mg glucosamine/g}$  substrate. Meanwhile, the lowest activity was omitted by the size of 1.0 mm and it produced  $53.33 \pm 4.44 \mu\text{g/g}$  dry matter with  $1.27 \pm 0.05 \text{ mg glucosamine/g}$  substrate fungal growth. Couto and Sanroman (2006) informed that smaller sizes only approved the accumulation of substrate that unswervingly resulted in poor growth of fungi. In large scale fermentation, Mitchell *et al.* (2006) stated the particle size could affect the packing within the substrate bed and hence the airing of the bed. By comparing two different bed particle sizes with similar porosity, more trouble of forcing air through a bed of smaller particles (the phenomenon of pressure drop) was found. This condition causes air to choose an extra-large route (channeling phenomenon).

Moisture content is closely related to the definition of the SSF system; a process that involves solids in the absence or near absence of free water and the substrate itself must contained enough humidity to accommodate the growth and metabolism of microorganism. The key to the biological processes in SSF for elongation of hyphae, spores and metabolites production were detained by moisture content and water activity (Lenz *et al.*, 2004). It is important to note that the optimal content of water is very crucial to identify the productivity of the SSF process as it comes in restricted amounts. As demonstrated in Fig.6, a moisture content of 60% (v/w) boosted up the lovastatin production up to  $2271.7 \pm 14.4 \mu\text{g/g}$  dry matter with fungal growth of  $2.3 \pm 0.03 \text{ mg glucosamine/g}$  substrate. The lowest production was obtained by 50% (v/w) moisture content, with  $468.3 \pm 64.4 \mu\text{g/g}$  dry matter and fungal growth of  $1.3 \pm 0.03 \text{ mg/g}$ . Pei-Lian *et al.*

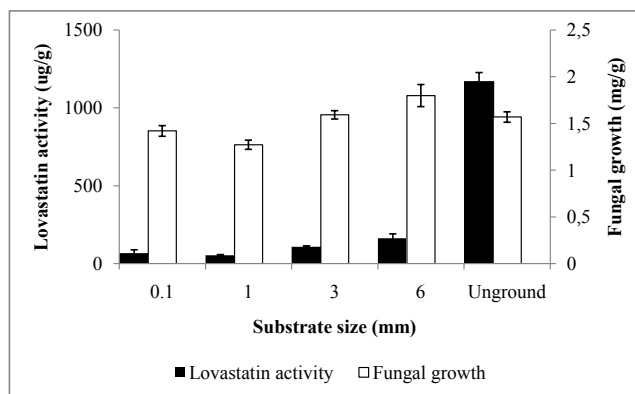


Fig.5: Effects of substrate size towards lovastatin production in a static tray system. After twelve-day incubation time, the natural size of brown rice maintained as the suitable size for lovastatin production by *F. pseudocircinatum* IBRL B3-4

(2006) in a paper work entitles ‘Lovastatin production by *Aspergillus terreus* in solid state fermentation’, generated 2.9 mg/g lovastatin under moisture level of 50-60%. Water presents in the SSF system in either a complex condition within the solid matrix (substrate) or as a thin layer which can absorb into substrate surface or slightly bonded to the substrate capillary (Raimbault, 1998). Lower moisture content encourages microorganism sporulation and at the same time deducts nutrient absorption, problem during mixing or agitation and results in high pressure of water. A high water percentage persuades decrement of substrate porosity, and this contributes to the viscosity of the medium and enhances chances of contamination. This matter was further amplified by Perez-Guerra *et al.* (2003) who reported that porosity reduction could limit the transfer of oxygen and attract bacteria contamination risk.

A mesophilic temperature is commonly chosen by filamentous fungi as it is similar to the original terrestrial. Many researchers have agreed that the filamentous fungi growth rapidly in 20 to 40°C (Manpreet *et al.*, 2005). Fig.7 designates an ambient temperature, 30±2°C, as the best surrounding in the tray system for lovastatin production. The activity increased to 2298.3±8.9 µg/g dry matter with 2.7±0.09 mg glucosamine/g substrate fungal growth. Meanwhile, a temperature of 40°C has been indicated as an unfavourable condition for the production of this secondary metabolite compound. The obtained activity was 75.0±13.3 µg/g dry matter with 1.64±0.04 mg glucosamine/g substrate fungal growth. According to Jahromi *et al.* (2012) and Pei-Lian *et al.* (2007), the temperatures of 25°C and 28°C are the optimum surroundings for lovastatin production by filamentous fungi, *Aspergillus terreus*. The application of high temperature

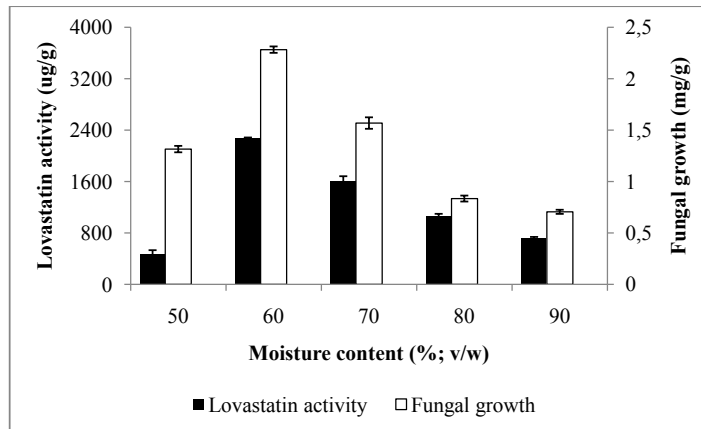


Fig.6: The presence of 60% (v/w) moisture content successfully increased lovastatin activity at the highest rate compared with other percentages

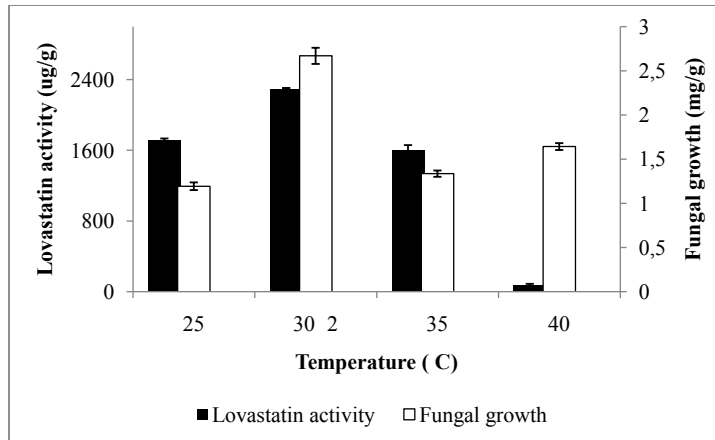


Fig.7: Ambient temperature ( $30\pm 2^{\circ}\text{C}$ ) sustained as the most suitable parameter for production and fungal growth. This was closely followed by  $25^{\circ}\text{C}$  and  $35^{\circ}\text{C}$ .

will have significant impacts towards fungal growth and final product production. In SSF, a large amount of heat is generated to be used in microorganism metabolic activity which means the heat condition inside the tray is higher than the outside. In addition, there is a heat capacity spawned within substrate bed but this depends on the existing elements such as dry solid, liquid water, dry air and water vapour. As a result, plenty of upscaling bioreactors are fabricated from metal with aeration package to control the overheat problem. Frequently, convection or heat transfer is done in a few alternatives including via bioreactor wall, removal from solids to air and also removal due to air flow through the bed (Mitchell *et al.*, 2006).

## CONCLUSION

This is the first report of *F. pseudocircinatum* as an anticholesterol agent, lovastatin. Extraction by dichloromethane gained the

best solvent mediator in SSF. Under 0.5 cm of substrate thickness, lovastatin activity was found to achieve the highest production with some physical modifications such as unaltered substrate size, 60% (v/w) of moisture content and ambient temperature of  $30\pm 2^{\circ}\text{C}$ .

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