IMPROVEMENT OF LOVASTATIN PRODUCTION by *Fusarium pseudocircinatum* IBRL B3-4 via SOLID SUBSTRATE FERMENTATION

by

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TABLE OF CONTENTS **Page**

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATION

ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
BEA	Beauvericin
CLA	Carnation leaf agar
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
FUM	Fumonisin
GCMS	Gas chromatography mass spectrometry
HDL	High density lipoprotein
HMG CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
IVC	Individually ventilated cage
LC	Liquid chromatography
LC_{50}	Lethal concentration 50
LDKS	Lovastatin Diketide Synthase
LDL	Low density lipoprotein
LNKS	Lovastatin Nonketide Synthase
MEGA	Molecular Evolution and Genetic Analysis
MON	Moniliformin
MS	Multiple sclerosis
NCBI	National Center for Biotechnology Information
OECD	Organization for Economic Cooperation and Development
OPA	Ortho phthaldialdehyde
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
RSM	Response surface methodology
SmF	Submerged fermentation
SNA	Spezieller Nährstoffarmer agar
SSF	Solid substrate fermentation
TBAHS	Tetrabutylammonium hydrogen sulphate
TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography
VLDL	Very low density lipoprotein
WA	Water agar
WHO	World Health Organization
ZEN	Zearalanone
psi	Pounds per square inch
mL	Milliliter
µg/g	Microgram per gram
µl	Microliter
µg/µL	Microgram per microliter
µm	Micrometer
mg	Milligram
mg/g	Milligram per gram

mg/kg	Milligram per kilogram
rpm	Revolutions per minute
kg	kilogram
min	Minute
mm	Millimeter
v/w	Volume per weight
w/w	Weight per weight
w/v	Weight per volume
v/v	Volume per volume
°C	Degree celsius
G	Gravity
M	Molar
cm	Centimeter
nm	Nanometer
R_f	Retention factor
R_t	Retention time

LIST OF PUBLICATIONS AND CONFERENCES

a) Publications

1) Syarifah, A.R., Darah, I. and I Nyoman, P.A. (2013). Effect of cultural conditions on lovastatin production by *Aspergillus niger* SAR I using combination of rice bran and brown rice as substrate. *International Journal of Applied Biology and Pharmaceutical Technology*, 4(2): 150-156

2) Syarifah, A.R., Darah, I. and I Nyoman, P.A. (2014). Isolation and screening of lovastatin producing fungi: *Fusarium pseudocircinatum* IBRL B3-4 as a potential producer. *Journal of Pure and Applied Microbiology*, 8(3): 1763-1772

3) Syarifah, A.R., Darah, I. and I Nyoman, P.A. A new latent lovastatin producer viz. *Fusarium pseudocircinatum* IBRL B3-4, produced in laboratory tray system. *Pertanika Journal of Tropical Agricultural Science*, 37(4): 509-522

b) Conferences:

Oral presentation

1) Syarifah, A.R., Darah, I. and I Nyoman, P.A. (2010). Screening of novel lovastatin producer under solid substrate fermentation by using rice bran as a substrate and brown rice as a support material. 1st Joint Symposium of ITB and USM, Institut Teknologi Bandung Indonesia, 20-21 December 2010

2) Syarifah, A.R., Darah, I. and I Nyoman, P.A. (2011). A potential cooperation of rice bran and brown rice with mesophilic fungi in producing lovastatin under solid substrate fermentation (SSF). International Congress of the Malaysian Society for Microbiology (ICMSM), Bayview Beach Resort, Batu Feringgi, Penang, 8-11 December 2011

3) Syarifah, A.R., Darah, I. and I Nyoman, P.A. (2012). The influence of physical parameters towards hypercholesterol reducing agent production, lovastatin, under solid substrate fermentation (SSF) condition. 8th IMT-GT UNINET Biosceince Conference, Darussalam-Banda Aceh, Indonesia, 22-24 November 2012

b) Poster presentation

1) Syarifah, A.R., Darah, I. and I Nyoman, P.A. (2012). Assessment of double screening programmes via solid substrate fermentation (SSF) in a flask system and identification of lovastatin potential producer. 8th IMT-GT UNINET Biosceince Conference, Darussalam-Banda Aceh, Indonesia, 22-24 November 2012

PENAMBAHBAIKAN PENGHASILAN LOVASTATIN OLEH *Fusarium pseudocircinatum* IBRL B3-4 MELALUI FERMENTASI SUBSTRAT PEPEJAL

ABSTRAK

Keupayaan bran beras dan beras perang dalam menurunkan kolesterol dan menyokong sistem fermentasi substrat pepejal (SSF) tidak dapat dinafikan. Tambahan pula, kemudahan untuk mendapatkan substrat-substrat tersebut di kilang adalah mudah serta harga yang tidak mahal. Sejak beberapa tahun yang lepas, penyelidik-penyelidik telah mengkaji keupayaan padi serta komponennya yang lain seperti bran, beras, jerami dan juga sekam, mempunyai kebolehan dalam menghasilkan metabolit sekunder termasuklah statin. Hospital tempatan di Malaysia mendapat permintaan yang tinggi terhadap statin dan pengambilan lovastatin adalah yang paling ketara di mana sebanyak 51% preskripsi telah dibuat kepada pesakit-pesakit berkolesterol tinggi. Maka, kajian ini menekankan kepada penambahbaikan penghasilan lovastatin oleh *Fusarium pseudocircinatum* IBRL B3-4 melalui SSF. Bran beras dan beras perang memberikan nilai komposisi berbeza terhadap kelembapan, protein, lipid, fiber, karbohidrat dan abu. Komponen tertinggi yang didapati dalam bran beras adalah karbohidrat iaitu sebanyak 41.20 ± 2.10% manakala fiber memonopoli komposisi beras perang (48.53 ± 0.58%). Daripada jumlah 78 kulat berfilamen, 55 pencilan menunjukkan titik gelap yang positif di atas plat kromatografi lapisan nipis (TLC) dengan faktor pengekalan (R_f) antara 0.26 hingga 0.32. Walau bagaimanapun, hanya 28 pencilan kulat telah dikesan mensintesiskan lovastatin melalui menerusi sistem kromatografi cecair berprestasi

tinggi (HPLC). Pencilan IBRL B3-4 menunjukkan penghasilan lovastatin tertinggi berjumlah 281.67 ± 44.44 µg lovastatin/g. Berdasarkan morfologi koloni dan struktur pada agar kentang dektrosa (PDA), koloni pencilan IBRL B3-4 tumbuh sehingga 54 mm dan 60 mm masing-masing di bawah keadaan gelap dan bercahaya. Untuk penelitian struktur di atas agar bunga teluki (CLA) dan agar Spezieller Nahrstoffarmer (SNA), pencilan tersebut mempunyai mikrokonidia berbentuk bujur (4.5-7.0 x 1.7-2.6 µm), makrokonidia yang melengkuk serta berbentuk sabit (32.0-46.4 x 1.2-2.9 µm), kepala palsu, hifa berlingkar serta rantaian pendek. Melalui pendekatan molekular pula, pencilan ini memberikan persamaan urutan sebanyak 99.33% (pangkalan data Fusarium-ID) dan 99.00% (pangkalan data GenBank) dengan *F. pseudocircinatum*. Kondisi terbaik bagi *F. pseudocircinatum* IBRL B3-4 untuk menghasilkan lovastatin dalam sistem kelalang adalah menggunakan saiz asal substrat, kandungan kelembapan sebanyak 70% (i/b), suhu pengeraman pada 30 ± 2°C, saiz inokulum 1 x 10^5 spora/mL, pH 6.5, 5 g kuantiti substrat (nisbah 1:1) dengan keadaan statik tanpa pengadukan. Sistem ini juga memerlukan nutrisi tambahan iaitu 1.5% (b/b) sukrosa, 1% (b/b) ekstrak yis dan 0.5% (b/b) kalsium klorida. Sistem dulang juga memerlukan kondisi yang hampir sama dengan sistem kelalang kecuali kandungan kelembapan (60%; i/b) dan kuantiti substrat (100 g atau ketebalan 0.5 cm). Produktiviti akhir yang dikesan daripada sistem kelalang ialah 1770.00 ± 60.00 µg lovastatin/g pepejal kering manakala dalam sistem dulang pula sebanyak 2436.67 ± 15.56 µg lovastatin/g pepejal kering, mewakili 38% peningkatan. Kedua-dua sistem memerlukan 12 hari untuk mencapai produktiviti yang maksima. Semasa penelitian toksin kulat, *F. pseudocircinatum* IBRL B3-4 telah mensintesiskan moniliformin (MON) dan fumonisin B1 (FUM B1) masing-masing sebanyak 4.20 ± 1.12 µg MON/g substrat and 1.73 ± 0.71 µg FUM/g

substrat. Walau bagaimanapun, nilai kepekatan maut 50% (LC_{50}) untuk fraksi lovastatin menunjukkan aktiviti tidak toksik kerana nilai tersebut melebihi 1 mg/mL. Sepanjang 4 minggu, fraksi lovastatin berkepekatan 110 mg sampel fraksi/kg berat badan (yang mewakili 550 to 750 µg lovastatin/g berat kering lovastatin) memberikan dos terbaik untuk merendahkan lipoprotein ketumpatan rendah (LDL) dan meningkatkan lipoprotein ketumpatan tinggi (HDL) dalam tikus jantan dan betina Sprague dawley. Nilai akhir LDL yang dikesan melalui pembaca mikroplat Termo Saintifik Multiskan® Spektrum ialah 0.006 ± 0.001 µg/µL and 0.004 ± 0.001 µg/µL, masing-masing untuk tikus jantan dan betina. Manakala nilai HDL pula, ia meningkat menjadi 0.023 ± 0.001 µg/µL (tikus jantan) dan 0.022 ± 0.003 µg/µL (tikus betina). Keputusan yang diperolehi daripada kajian ini mencadangkan penggunaan fraksi lovastatin daripada *F. pseudocircinatum* IBRL B3-4 sebagai agen merendahkan kolesterol.

IMPROVEMENT OF LOVASTATIN PRODUCTION by *Fusarium pseudocircinatum* IBRL B3-4 via SOLID SUBSTRATE FERMENTATION

ABSTRACT

The ability of rice bran and brown rice in lowering cholesterol and supporting solid substrate fermentation (SSF) system are undeniable. Plus, the accessibility for these substrates is reachable at the mill factory with inexpensive price. For the last few years, researchers have investigated paddy and its other components like bran, rice, straw and husk, own the potentiality in producing secondary metabolite including statin. Malaysian local hospital has experienced a high demand on statin and lovastatin is the most outstanding drug with 51% prescription done for hypercholesterolemia patients. Thus, this recent investigation emphasized the improvement of lovastatin production by *Fusarium pseudocircinatum* IBRL B3-4 via SSF. Rice bran and brown rice denoted different composition values in moisture, protein, lipid, fibre, carbohydrate and ash. The highest component in rice bran was carbohydrate with 41.20 ± 2.10% while fibre was the superior composition in brown rice (48.53 ± 0.58%). Out of 78 filamentous fungi, only 55 isolates displayed positive dark spot on the thin layer chromatography plate (TLC) with retention factor (R_f) of 0.26 to 0.32. However, only 28 fungal isolates were detected to synthesise lovastatin through high performance liquid chromatography system (HPLC). IBRL B3-4 isolate depicted the highest lovastatin production with 281.67 ± 44.44 µg lovastatin/g dry solid. Based on the colony and structural morphologies on potato dextrose agar (PDA), the colony of IBRL B3-4

isolate was grown to 54 mm and 60 mm under dark and light conditions, respectively. For structural observation on carnation leaf agar (CLA) and Spezieller Nahrstoffarmer agar (SNA), the isolate owned oval shape microconidia (4.5-7.0 x 1.7-2.6 µm), slender and falcate shape macroconidia (32.0-46.4 x 1.2-2.9 µm), false head, coiled hyphae and also short chain structure. As a respond to the molecular approach, this isolate depicted 99.33% (Fusarium-ID database) and 99.00% (GenBank database) sequence similarity with *F. pseudocircinatum*. The best condition for *F. pseudocircinatum* IBRL B3-4 to produce lovastatin in the flask system was under the original substrate size, 70% (v/w) moisture content, incubation temperature of 30 ± 2°C, inoculum size of 1 x 10^5 spore/mL, pH 6.5, 5 g of substrate quantity (1:1 ratio) with static condition. It also needed external nutrients namely 1.5% (w/w) sucrose, 1% (w/w) yeast extract and 0.5% (w/w) calcium chloride. The tray system also required almost the same conditions with the flask system except for moisture content (60%; v/w) and substrate quantity (100 g or 0.5 cm thickness). The final productivity detected from flask system was 1770.00 ± 60.00 µg lovastatin/g dry solid while in tray system was 2436.67 ± 15.56 µg lovastatin/g dry solid, which represented 38% increment. Both systems required 12 days incubation period to synthesis the maximal productivity. During mycotoxin investigation, *F. pseudocircinatum* IBRL B3-4 has synthesized moniliformin (MON) and fumonisin B1 (FUM B1) at 4.20 ± 1.12 µg MON/g substrate and 1.73 ± 0.71 µg FUM/g substrate, respectively. However, the lethality concentration 50% values (LC_{50}) for fractional lovastatin signified non-toxic activities as the value was higher than 1 mg/mL. Within 4 weeks treatment, fractional lovastatin concentration of 110 mg fraction sample/kg body weight (which represented 550 to 750 µg lovastatin/g dry solid of lovastatin) was slightly the best dose to reduce the low density lipoprotein

(LDL) and increase the high density lipoprotein (HDL) in male and female Sprague dawley rats. The final LDL value detected via Thermo Scientific Multiskan® Spectrum microplate readers in male and female was 0.006 ± 0.001 µg/µl and 0.004 ± 0.001 µg/µl, respectively. While for the HDL, it amplified to 0.023 ± 0.001 µg/µl (in male) and 0.022 ± 0.003 µg/µl (in female). The results obtained from this work suggested the application of fractional lovastatin from *F. pseudocircinatum* IBRL B3-4 can greatly appointed this compound as an anti cholesterol lowering agent.

CHAPTER 1

INTRODUCTION

1.1 Hypercholesterolemia versus its reversal agent

Hypercholesterolemia is an anomalous cholesterol level in blood which is out of normal range and regularly welcomes the cardiovascular-relating events. Any substances or compounds which can obstruct the extra production of cholesterol in liver are recognized as hypercholesterolemia agent. Cardiovascular diseases are a cluster of heart and blood vessel abnormalities which can be controlled by cholesterol antidotes like statins. Besides anticholesterol agent, statin is also well established as a stroke preventer and able to block the peripheral vascular disease (Maron *et al*., 2000). At the same time, it possesses biological and pleiotropic effects including antithrombotic and anti-inflammatory which may lead to atherosclerotic plaque reduction (Rosenson and Tangney 1998; Fenton and Shen 1999; Vaughan *et al*., 2000) and also as a treatment against Alzheimer disease, multiple sclerosis, hypertension, ostreoporosis, ventricular arrhythmia and immune response (Glorioso *et al*., 1999; De Sutter *et al*., 2000; Meier *et al*., 2000; Chong *et al*., 2001; Zamvil and Steinmann, 2002; Eckert *et al*., 2005). The controllers for the hypercholesterolemia are low density lipoprotein (LDL) and high density lipoprotein (HDL). Both of these carriers are responsible to translocate the cholesterol in blood within cell which results changes in cholesterol.

1.2 Fermentation and idiolites perspectives

Fermentation is an ample process to produce complex molecules and active compounds such as antibiotics, enzymes, vitamins, amino and organic acid that are not viable to be chemically constructed (Reddy *et al*., 2012). It is a technique of biological modification of complex substrates to become simpler compounds by microorganism including bacteria, fungi and yeast. Apart from carbon dioxide and alcohol, the metabolic breakdown also releases the additional compounds which are also known as secondary metabolites (Subramaniyam and Vimala, 2012) or idiolites. Chemical structures of these metabolites are secreted by certain microbes and some plants. They are spawned throughout trophophase period (rapid growth phase or log phase) and are further synthesized at the later phase namely iodophase (stationary phase). The accomplishment of any biosynthesis extension in iodophase relies on the tropophase (Barrios-González *et al*., 2003). Secondary metabolism in the iodophase employs primary metabolites to generate species-specific and chemically vary end products that are not vital for microbial growth (Waites *et al*., 2001). The booster of secondary metabolite production is when the growth is restricted due to the lack of nutrients either carbon, nitrogen or phosphate (Barrios-Gonzales *et al*., 2003).

1.3 Lovastatin in action

Statins are the recommended drugs for hypercholesterolemia which are derived via fermentation as a secondary metabolite compound. Together with recognized diets and physical exercises, statins can be a navigator for the cholesterol level in blood. A

few statins have been manufactured as a single-ingredient products with their own commercial names by US Food and Drug Administration namely atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin (United State Food and Drug Administration, 2012). The standard recommended dose by medical practitioners was 20 to 40 mg. These drugs' concentration can deduct LDL by about 25% to 40% and increase HDL nearly 5% to 10% (Scirica and Cannon, 2005).

Lovastatin is categorized under natural statin and formerly known as mevinolin, monacolin K and Mevacor. This persuasive drug is responsible to demolish 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG CoA) which is attracted to trap at the early step of cholesterol biosynthesis. Due to the heat and mass transfer problems which occurs in solid substrate fermentation system (SSF), submerged fermentation (SmF) has become a favorite mediator for lovastatin production (Pandey, 2003). Nevertheless, it can also be generated by the emerging technology of SSF which displays more advantages. This system promises more biotechnological benefits over than SmF even in a basic laboratory scale. It offers higher fermentation yield and product concentration packaged with better stability, lower in catabolic suppression, sterility demand (Hölker *et al*., 2004), energy requisite, producing less dissipate water and biological friendly (Pandey, 2003). SSF has proven in exhibiting much distinctiveness which is indeed very useful and has become a major industrial microbial producer.

Production of lovastatin by SmF and SSF has been widely investigated and commonly, filamentous fungi exhibit tremendous potentiality. Reports from 2003 onwards recognized that other than *Aspergillus terreus* (Casas López *et al*., 2004; Pansuriya and Singhal, 2010), there are numbers of different genus or species of fungi

that hold abilities to generate lovastatin specifically *Aspergillus niger*, *Fusarium* sp. (Raghunath *et al*., 2012), *Penicillium funiculosum* (Reddy *et al*., 2011), *Aspergillus fisheri* (Latha *et al*., 2012), *Monascus purpureus*, *Monascus ruber* (Seraman *et al*., 2009; Panda *et al*., 2010), *Monascus pilosus* (Miyake *et al*., 2006), *Aspergillus flavipes* (Valera *et al*., 2005), *Trichoderma viridae* and *Trichoderma longibrachiatum* (Samiee *et al*., 2003).

1.4 Rationales of Study and Objectives

Increment of hypercholesterolemia patients, high demand in statin drugs prescription, large capacity of agriculture wastes in Malaysia and advancement in SSF which can turn the waste into myriad of value added products, are the concrete rationales for this research.

An outbreak of world's bestselling drugs in 2013 which is recently highlighted by Forbes magazine exposes that hypercholesterolemia generic remedy namely statin, still holding the throne of the greatest selling drugs totaling of USD13,696 million in 2006. Until now, the number has not yet been broken by sales of any other drugs. According to the World Health Organization's report, in 2008, 30% of global deaths (equal to 17.3 million of people) are caused by cardiovascular diseases and hypercholesterolemia was the main trigger. It has been predicted that by 2030, a total mortality can potentially hit to 23.3 million figures (World Health Organization, 2013). Cardiovascular disease is one of the forefront diseases that cause death at Malaysian government hospitals in 2009. The report indicates a total of 55.9% of patients suffered

from acute coronary syndrome (ACS) due to dyslipidemia condition (an elevation of lipoprotein level which leads to hypercholesterolemia) (Ministry of Health Malaysia, 2010). A local data collection of cholesterol level among Penangites at Sungai Pinang Township indicated 3 out of 12 persons hit the cholesterol reading of 5.2 mmol/L or more (Kiew and Chong, 2013). The worrisome increases after National Cardiovascular Disease Database Malaysia (2008) reveals that the development of ACS among Malaysians is at a younger age (58) compared to other countries i.e. China, Thailand and the West (above age of 60).

Statins are a potent hypercholesterolemia modifying drug in Malaysia as 91% of discharged patients from our local hospitals have experienced the statins treatment (Wan Ahmad *et al*., 2011; Ahmad *et al*., 2011). The figure increases almost 19% from year 2007 to 2008. Among statins, lovastatin is the most outstanding drug in Malaysian hospitals with 51% prescription and this recent status has appointed lovastatin to be under top 40 most wanted drugs in this country. Since year 2007 to 2008, the demand towards lovastatin increased and its rank in National Cardiovascular Disease Database Malaysia changed from 13^{th} to 9^{th} (National Cardiovascular Disease Database Malaysia, 2008). In 2005, the expenditure of general cholesterol lowering drug (including statin) was about RM108.5 million out of RM2.2 billion of total drugs expenses (Sameerah and Sarojini, 2007). After considering all of the provided facts, Malaysians needs to have a new mode of 'drug' to lessen the government burden and obtain the healthiness. One of the best solutions is by considering the traditional or staple food which owns the medicinal value in lowering cholesterol such as 'Angkak' or red yeast rice production, an edible traditional medicinal-food for hypercholesterolemia. The trial on rice bran and

brown rice in producing anticholesterol agents are rarely being investigated especially through solid substrate fermentation system, albeit both of these products are valuable of respect in their own right. Kang *et al.* (2012) recorded a tremendous action of rice bran in reducing lipid level in mice by inspecting the lipid excretion in fecal. Even in humans, rice bran still maintains its anticholesterol greatness by improving 78% of lipid ratios in hypercholesterolemia patients (Gerhardt and Gallo, 1998). Brown rice, on the other hand, is also bringing a parallel impact as same as rice bran. Through research, a brown rice diet in *Sprague dawley* managed to put the LDL condition at ameliorate level (Roohinejad *et al*., 2010). There was also a significant decrease in lipid activity after 10 days of lipid profile in rabbit as reported by Mohd Esa *et al.* (2011). Due to these consistent evidences, the rice bran and brown rice should release no doubt to be selected as a ruling element in SSF for the production of anticholesterol agent purpose.

In addition, the originality of lovastatin is another selection factor for this experiment. It is generated naturally from the fermentation process and holds a low adverse side effect towards animal and human (Tobert, 2003). Up until now, only about 4.6% of patients stop prescribing their lovastatin medicine because of the side effect (Goswami *et al*., 2012). Ever since from its first discovery until the patent approval by Food and Drug Administration (FDA), lovastatin is widely produced under fermentation process and SmF is the foremost system for its production. SmF by batch or fed-batch culture is the well recognized method to generate lovastatin in the system by diversifying the conditions of physico-nutritional parameters. The advancement in SmF gives another idea to develop the SSF system. Isolation, screening, optimization, scale up system and purification of lovastatin via SmF which including Plackett-

Burman study, factorial designs and response surface methodology (RSM), are inclusively reported by researchers (Lai *et al*., 2003; Seraman *et al*., 2010). Thus, it is important to initiate another research of SSF lovastatin-based.

Agro-industry is still an important sector that contributes to Malaysia economical growth. In year 2013, Malaysia produced RM789.9 billion of Gross Domestic Product and out of the figure, RM56.9 billion representing agriculture, forestry and fishing sector (The Malaysian Economy in Figures, 2013). From the statistic, it can be concluded that agricultural (or agro-industry) waste becomes a troublesome burden to the government. Farmers in Malaysia habitually use an easy initiative method to overcome the biomass abundant (mostly paddy and oil palm's left-over) which is by allowing a direct open burning. Under low cost and time saving factors, the burning activity is done to clear the land and fertilize the soil for the next planting cycle. The biomass burning inspires the greenhouse gas emissions such as carbon dioxide, methane, carbon monoxide and nitrous oxide (N_2O), into the atmosphere. However, carbon dioxide is the most widely produced gas from the combustion of biomass (Mastura, 2008). The existence of those gases contribute to the production of chemicals in the ozone layer (troposphere) that directly involve in controlling the concentration of hydroxide radicals during the regulation cycle of other atmospheric gases. Some of the biomass wastes or residues can naturally be used as fertilizer, however, it involves a long decomposition process until at a certain stage it can transfer into pests' territory (Ahmad *et al*., 2002). Some of the damped biomasses such as rice bran and palm kernel cake are also being consumed by ruminant and non-ruminant animal as a feedstuff.

The benefits offered by SSF specifically its high yield and concentrated final product with less energy requirement and effective process (Perez-Guerra *et al*., 2003), provide some promises to disentangle all of the aforementioned problems. Hence, it directly leads into sensible reasons to proceed this experiment. The objectives of the present research are;

1. To isolate, screen and identify potential local fungi which can significantly produce lovastatin under SSF
2. To improve cultural conditions and medium compositions for lovastatin production
3. To compare lovastatin production in flask and laboratory tray systems
4. To purify lovastatin via chromatographic purification
5. To test the effectiveness of fractional lovastatin as cholesterol lowering agent on laboratory animal, Sprague dawley rats.

CHAPTER 2

LITERATURE REVIEW

2.1 Agricultural crops in Malaysia: Rice processing and its byproduct

In a year, a total of 998 million tones of agricultural wastes are produced worldwide and out of the number, 1.2 million tones is represented by Malaysia alone (Twana and Fauziah, 2012). According to the Department of Statistic Malaysia, in 2010, the oil palm sector conquers the landfill agricultural production with 64,282,738 tones of fresh fruit bunches followed by paddy (2,464,831 tones), cocoa (402 tones) and rubber (56 tones).

The world needs to support 600 million tones production of *Oryza sativa* (rice) yearly (Chen *et al*., 2012) and 150 million hectares of land area are preserved for paddy planting (Food and Agriculture Organization, 2004). Rice has a few layers namely hull, bran layer, endosperm and embryo. Once the outer layer (the hull or husk) is eradicated, it will expose the brown rice which is the carrier of the essential nutrients. Rice is a staple food for Asian but the grain polishing process has eliminated a lot of nutrients richness. Cleaning, hulling and post-hulling processing which cover whitening, polishing and grading procedures are the basic steps in rice refining (Mohd Esa *et al*., 2013). Those programs destroy B1, B3 and B6 vitamins at 80%, 67% and 90%, respectively. Others ousted minerals are half of manganese and phosphorus with 60% iron and all of the dietary fiber and vital fatty acid (Babu *et al*., 2009).

The main outcome of rice process is endosperm or rice with 70% production which excludes its minor portion (20% rice husk, 8% rice bran and 2% rice germ)

(Wells, 1993; de Deckere and Korver, 1996; Van Hoed *et al*., 2006). A hundred kilograms of paddy equal to 56 to 58 kg white rice, 10 to 12 kg broken rice, 18 to 20 kg hull or husk and 10 to 12 kg rice bran (Kahlon, 2009). Rice husk and bran are the only leftovers that are applied into feed formulation (Department of Veterinary Services, 2013).

Comparing to the white rice, brown rice is claimed to be the best rice to consume. Within the brown rice, there is a bran layer and it is composed of pericarp, seed coat, nucellus and aluerone layer (Tahira *et al*., 2007). Brown rice becomes the second choice because of low eating quality and poor in palatability. The hard and dark cooked grain which is originated from the bran contributes to difficulty during munching process. But yet, those two elements (bran and brown rice) are the best options for healthy diets.

2.2 Cholesterol: a review

Cholesterol is vital during essential bile acids secretion and also very low density lipoproteins (VLDL) production for hormone biosynthesis. Generally, it is a fat substance or steroid molecule which is accumulated in our blood and cells. One-third of body cholesterol (75%) originated via diet and the other two-thirds (25%) are naturally generated from intracellular precursors by organs (Albert *et al*., 1980; Demain 1999; Furberg 1999). Cholesterol comes in two main packages; the bad cholesterol LDL and the good cholesterol HDL. The emergence of extra LDL in blood commonly relates to the increasing risk of heart attack and stroke. By contrast, HDL becomes a life guard

against those diseases. Besides genetic heritage, lifestyle and diet, the accumulation of cholesterol around the arteries is also depending on age factor (Scirica and Cannon, 2005). The animal-based food such as meats, poultry and dairy products and also some seafood are sources for exogenous cholesterol ingestion with 30% to 75% naturally absorbed by human body. For endogenous synthesis, it takes place in liver and spares 600 to 1000 mg/day cholesterol for our body usage. About 750 to 1250 mg cholesterol are secreted each day in bile. Generally, cholesterol and other fats cannot directly suspend in blood. Thus, it has to be carried by its own transporter, HDL and LDL. *In vivo*, cholesterol synthesis is triggered from acetyl CoA, reacts with acetoacetyl CoA to form HMG CoA and then it is narrowed down to mevalonate with HMG CoA reductase intervention (Campbell and Farrel, 2008). During this rate limiting step, the invading of cholesterol inhibitors such as statins are very crucial in controlling cholesterol over production.

2.2.1 Biomedical applications of lovastatin

Cholesterol obstruction by lovastatin not only works on hypercholesterolemia but also in other clinical terms. The trafficking condition during or after the elimination of HMG CoA reductase in cholesterol biosynthesis pathway (specifically in mevalonate pathway) allows bunch of mystifying pathways with unknown mechanisms system that might spare a lot of advantages in healing other chronic diseases.

2.2.1.1 The triumph of lovastatin in heart diseases

Human heart is a pump that comprises four chambers with remarkable ‘lup-dup’ rhythm, symbolic of the opening and closing of its valves. A complete blood pumping travels from right ventricle to left ventricle via pulmonary artery and pulmonary vein and finally, the oxygenated blood will be transported throughout the body by arteries. This tremendous process repeats around 72 times in a minute, more than 100,000 times daily, over 37 million times yearly and almost 3 billion times in a lifetime. Approximately, the human heart pumps 4,000 gallons of blood per day. The hard work of heart may welcome variety of diseases and usually the blood vessels become the main factor of the problematic compare with the heart itself. The failure of coronary arteries to transport blood and oxygen required by the heart muscle is defined as coronary heart disease and the most widespread cause of this disease is atherosclerosis. The event happens due to the plaque attachment to the coronary arteries wall and obstructs the blood flow. The plaque is built of cholesterol deposit, calcium and anomalous cells (Pampel and Pauley, 2004). The lovastatin intakes can increase the good cholesterol level (HDL) in blood system which leads into lesion retardation around the artery. Furthermore, it is also believed to cure all kinds artery related problems including improvement of endothelial function, controlling the inflammatory responses, sustaining plaque constancy and averting thrombus development (Sreenivasan *et al*., 2008).

2.3 Statins

2.3.1 Back to the past: the historical statins

Akira Endo is the best person to enlighten the discovery of statins. In 1971, a research team was set up to isolate fungi and mushrooms. Within two years, they managed to identify *Penicillium citrinum* ML-236B as a mevastatin or compactin producer, an inhibitor for lipid secretion. Pioneering in statin group, mevastatin scientifically denoted a reduction of plasma cholesterol in hens, dogs and monkeys. However, Japan stopped the trial because of the tumor detection in dogs. The next finding is a 'controversy' lovastatin. In Tokyo (February 1979), Endo has isolated mevastatin analogues from *Monascus ruber* namely monacolin K, monacolin J and also monacolin L. Within those compounds, monacolin K generated the major product and vaguely indicated better result in demolishing HMG CoA reductase compared with the original mevastatin. Those three derivatives (monacolin K, monacolin J and monacolin L) are patented by the owner in February, April and October 1979, respectively (Endo, 2004). Meanwhile, in 1978, Alfred Alberts and Julie Chen from Merck Research Laboratory found a new soil fungus, *Aspergillus terreus* which can also produced HMG CoA inhibitor. They called it mevinolin but later it is officially launched as lovastatin (Tobert, 2003). Merck formally patented mevinolin in June 1979 and four months later, it is verified that monacolin K and mevinolin consisted the same structure. Mevinolin is found earlier than monacolin K (November 1978 versus February 1979), but the copyright was four months behind monacolin K (June 1979 versus February 1979). There is no toxic revealed by this compound during toxicity test on animal and via this result, Merck boldly continued the clinical test on hypercholesterolemia patients. The

trials exposed a significant drop of plasma cholesterol with slight side effects. On November 1986, Merck moved one step forward by submitting an application to the Food and Drug Administration (FDA) for lovastatin commercialization. The FDA committees signed an approval on 1 September 1987 with a condition. The drug can be prescribed to the patients only if the diet or non-pharmacological techniques can not lessen the overproduction of cholesterol in blood (Merck and Co., 1987).

Basically, statins are built up of polyketide portion (hydroxyl-hexahydro naphthalene ring system) initiated from acetate units bonded together in a head-to-tail formation (Manzoni and Rollini, 2002). Native lactone (a close ring form) and β-hydroxyl acid (open ring form) are two major forms of statins. After mevastatin failure (compactin) and successful medicinal effect of lovastatin, statins welcoming a new comer into the cluster namely simvastatin (or Synvinolin and Zocor). It contains the same molecular structure as lovastatin but with the additional side chain methyl group. Then, the world witnessed the existence of pravastatin followed by fluvastatin, atorvastatin, cerivastatin and recent finding in 2003, rosuvastatin. As mentioned earlier, statins are triggered from fermentation. Thus, all of the statins are huddled into natural, semisynthetic and synthetic. Mevastatin and lovastatin are certainly natural statins while simvastatin is categorized under semisynthetic as it is synthesized from lovastatin by replacing the 2-methylbutryl side chain with 2,2-dimethylbutyryl. Pravastatin undergoes a biological transformation from mevastatin which involves *Strepromyces carbophilus* during the process. Other new statins members such as fluvastatin, atorvastatin, pitavastatin and rosuvastatin are in synthetic cluster. Even statins share a general action mechanism and structural composition which are very identical to HMG portion, they

diverge in their chemical structures (Figure 2.1). These structures are very potent in showing affinity towards HMG CoA reductase. The differences of each statins lie in the hydrophobic components which covalently tie a linkage to HMG-like moiety. In synthetic class, they have fluorophenyl groups to link to the structure. Decalin ring substituent appears in naturally derived statins and a special case for pravastatin as it consists a hydroxyl substituent at the hexahydronaphthalene nucleus which is considered as hydrophilic (Nigović *et al*., 2012).

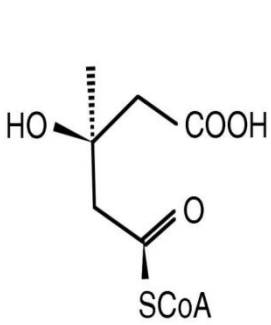
2.3.2 Biosynthesis of lovastatin

The IUPAC name of lovastatin is [(1S,3R,7R,8aS)-8-[2-[(2R,4R)-4-hydroxy-6-oxo-oxan-2-yl]ethyl]-3,7- dimethyl-1,2,3,7,8,8a- hexahydronapthalen-1-yl](2S) 2-methylbutanoate with empirical formula of $C_{24}H_{36}O_5$ and 404.55 molecular weight. It is a white, nonhygroscopic crystalline powder which is unsolvable in water except in ethanol, methanol and acetonitrile (Goswami *et al*., 2012). Lovastatin is consumed as lactone prodrug which is transformed into active HMG CoA inhibitor form (hydroxyl acid) in the hepatocyte organ, liver. As noted above, all statins derived via acetate units to structure out polyketide skeleton. Lovastatin Nonketide Synthase (LNKS) and Lovastatin Diketide Synthase (LDKS) are two polyketides preserved for lovastatin in Polyketide Synthase system (Barrios-González and Miranda, 2010). By referring to previous study by Endo (1979), two main chains are involved to spawn monacolin K or lovastatin which are monacolin J and monacolin L. Figure 2.2 designates the first synthesised chain of monacolin L which is constituted of nine acetate molecules and

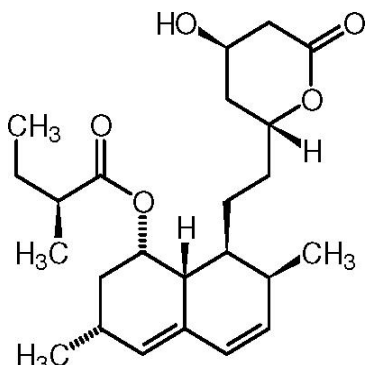
then it alters into monacolin J (assemble from two units of acetate and nine methionine units) under hydroxylation process. Monacolin J subsequently linked to methyl butryl CoA lovastatin which is assembled from two acetate units and one methione unit. Polyketide Synthase system codes the LNKS and LDKS in *Aspergillus terreus* with a few essential genes engaged; *lov* B, *lov* C, *lov* F and *lov* D. LNKS is a gene outcome of *lov* B that interacts with *lov* C (enoyl reductase) to perform dihydro monacolin L which is next synthesised into monacolin J. The other pathway to generate monacolin J is via LDKS system and it is a prominent key to guarantee an efficient production of lovastatin. *lov* F cooperated with *lov* D, a transesterases enzyme, which allow the attachment of 2-methylbutyric acid to monacolin J and proceed to the formation of monacolin K or lovastatin (Manzoni and Rollini, 2002; Sreevanivasan *et al*., 2008; Barrios-Gonzales and Miranda, 2010).

2.3.3 Lovastatin in SSF

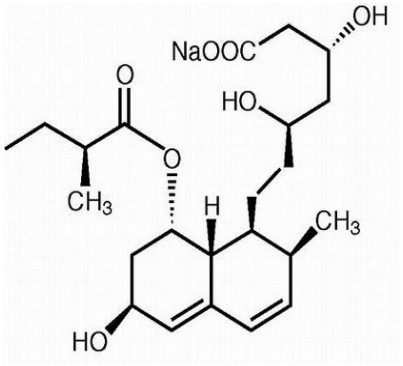
Low cost factor due to dual-duty of solid substrate (as a matrix and nutrients support) is the utmost reason for SSF selection. One of the ancient uses of SSF, which dates approximately at the first century A.D., is angkak (also known as anka or Chinese red rice) production by *Monascus* sp. At that time, in China, Angkak production was secretly produced. However, during Ming Dynasty (A.D 1368-1644), this medicinal-value rice was widely published to treat a lot of diseases including colic dyspepsia, diarrhea, hangovers and bruised muscles. Besides, it is also consumed by Chinese to improve the blood circulation and upgrading the spleen and stomach function



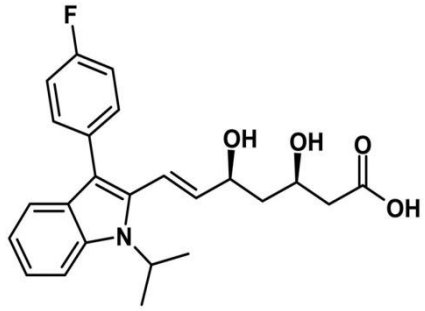
HMG CoA



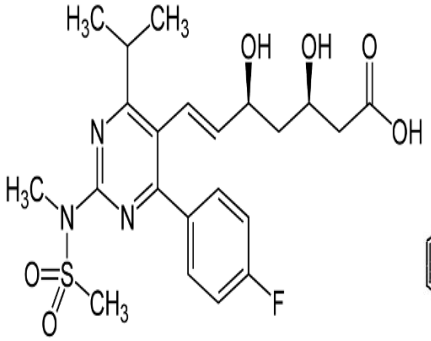
Lovastatin



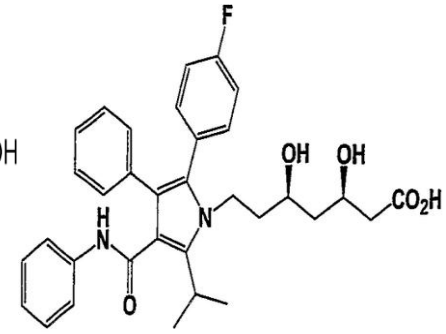
Pravastatin



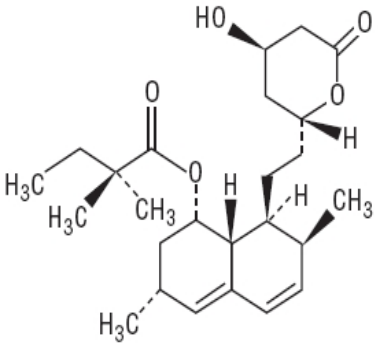
Fluvastatin



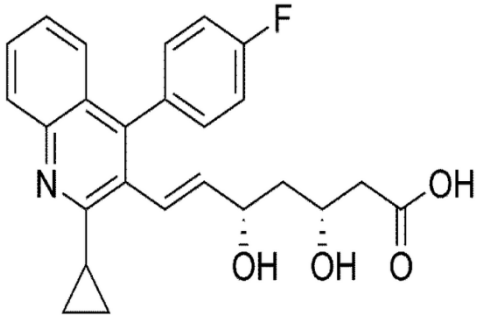
Rosuvastatin



Atorvastatin



Simvastatin



Pitavastatin

Figure 2.1: Different structures of natural, semisynthetic and synthetic statins and its similarity with HMG portion of HMG CoA reductase (Manzoni and Rollini, 2002)

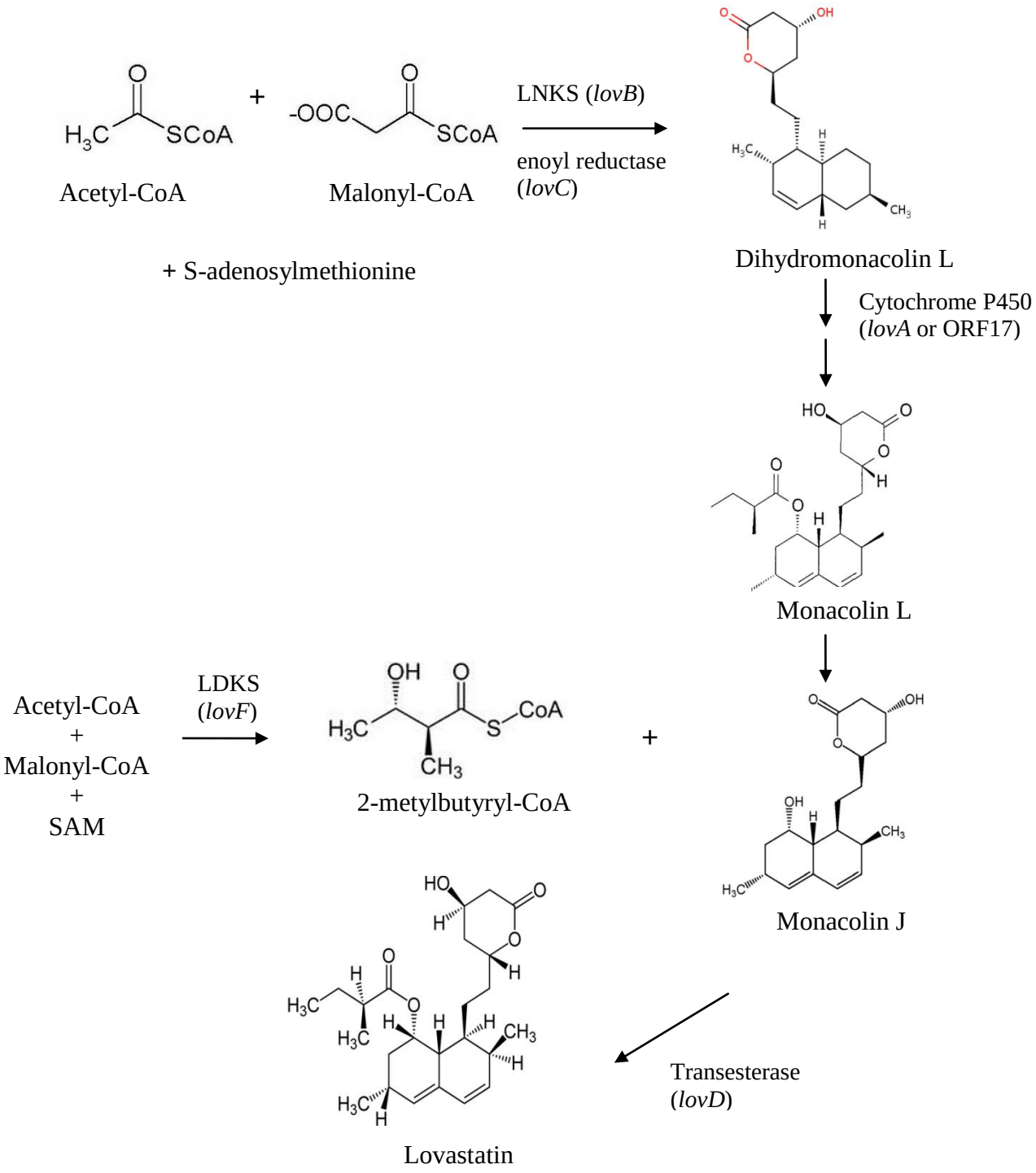


Figure 2.2: Biosynthesis pathway for lovastatin generation in simplified scheme. The different enzymes that impregnated their own specific genes are actively involved in every part of bioconversion (Manzoni and Rollini, 2002).

(Arunachalam and Narmadhapriya, 2011). According to recent studies, single and co-culture inoculation (Panda *et al.*, 2009; Panda *et al.*, 2010) of *Monascus* sp. initiates the activity of lovastatin which indirectly proves that *Monascus* sp. fronting the lovastatin production in SSF compared with other filamentous fungi. Szakacs *et al.* (1998), Valera *et al.* (2005), Wei *et al.* (2007), Pansuriya and Singhal (2010), Reddy *et al.* (2011) and Latha *et al.* (2012) are researchers who gave their efforts in investigating the best lovastatin producers among filamentous fungi under SSF condition. It includes the mutant or wild-type species of suspected fungi. Furthermore, studies on maximizing the production of secondary metabolite lovastatin is started to be emphasized via response surface methodology (RSM), Plackett-Burman, Box-Behnken factorial and as well as fabrication and modeling of bioreactor designs.

2.4 Solid substrate fermentation (SSF) and its consideration factors

2.4.1 Definition of SSF

Solid substrate fermentation is a general term in describing any fermentation process which involves solids including suspensions of solid particles in an unremitting aqueous phase and even trickle filters. Solid state fermentation is also categorized under solid substrate fermentation (Mitchell *et al*., 2006). It is characterized as growing microorganism on adequate moist of solid supports which are comprised of insoluble natural substrates and inert carriers (Hölker *et al*., 2004). A natural territory is created by allowing the absence or near absence of free water in the fermentation process (Pandey *et al*., 2000). Throughout this experiment, SSF term is used to represent solid

substrate fermentation. As reported by Pandey (2003), the selection of microorganism and substrate, physico-chemical parameters, isolation and purification of the product are some critical aspects that should be considered prior to SSF employment.

2.4.2 Rationale of filamentous fungi selection

Common microbes such as bacteria, yeast and fungi are capable to grow and conquering the solid substrate. However, the most highlighted microbe in SSF, especially the ones with lovastatin-producing ability, goes to fungi kingdom. By considering the physiological, enzymological and biochemical properties in a low water activity (A_w) event in SSF, filamentous fungi are the best selection compared with the other unicellular microorganisms (Raimbault, 1998; Pérez-Guerra *et al*., 2003).

Two gigantic groups in macro fungi are Ascomycetes and Basidiomycetes. However, only a few of the members can synchronize their growth with SSF surroundings (Hölker *et al*., 2004). The hallmark of filamentous fungi is its special hyphae with enzymatic tip. The hyphae ability in elongating, branching and attacking the substrate has made this microorganism more superior in host colonisation and nutrients utilisation (Figure 2.3). After inoculation process, spores take 10 hours to germinate. At this time, the substrate bed has to supply some heat to make sure a precise temperature for smooth germination. This progress results in daughter hyphae production which induces new branches and finally expanding the micro colony. Elongation of hyphae stimulates the micro colonies meeting which welcome a negative interaction between hyphae tips. Such this incident forces the tips to change their route

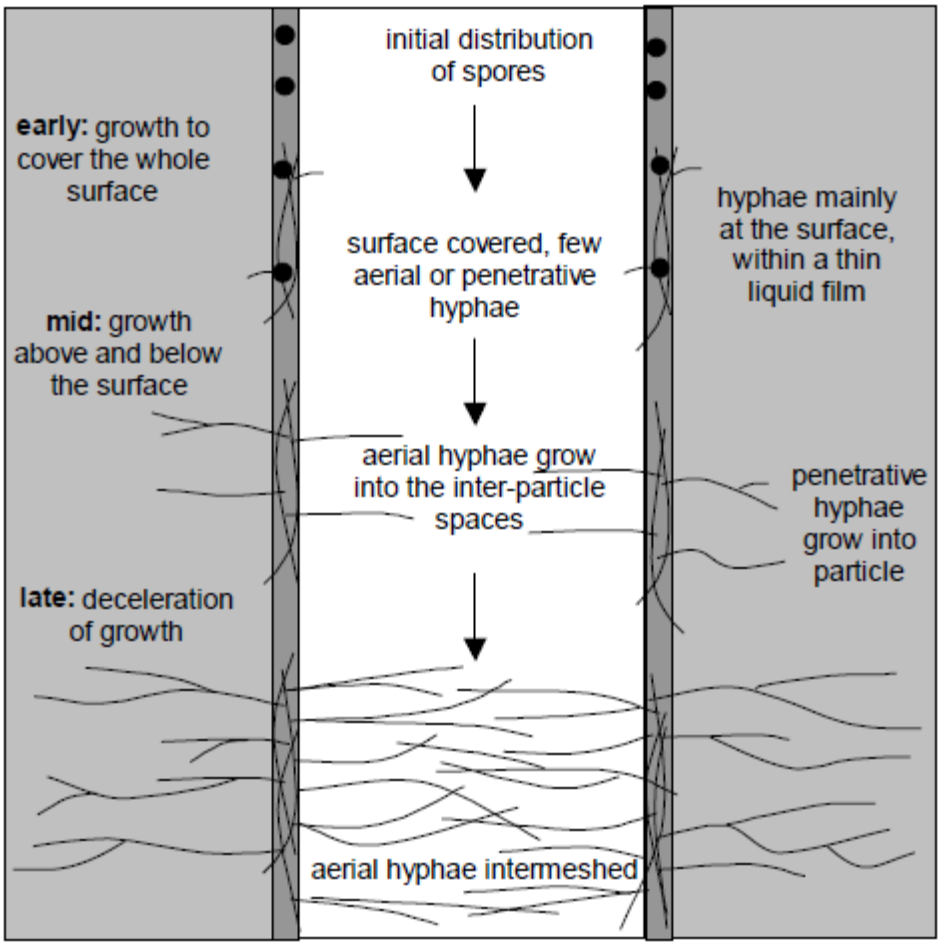


Figure 2.3: The illustrations of filamentous fungi growth invading the substrate matrix during fermentation process (Rahardjo *et al*., 2006).

or retarding the growth. For those that keep on extending, they can choose to grow at the surface of the liquid film or injecting into the matrices (Mitchell *et al*., 2006). Hyphae are grown in linear and constant pattern to permit a contact to the cell wall of solid substrate. Once it grips the substrate, the hyphal apex emits an amount of hydrolytic enzyme to ensure a smooth penetration into the substrate. Unlike SmF, the enzyme excretion in SSF is more concentrate and contribute a lot in penetration efficiency. Other than invading the host, a close connection between hyphae and substrate also encourage biological synthesis and fungal metabolic activities (Raimbault, 1998) like secondary metabolites production.

2.4.2.1 *Fusarium* sp. and its major mycotoxins

This genus is defined as plant destructor with pan-tropical distribution via air, seeds, soils or plant debris. Commonly, the members of this genus are actively contributed in economic losses involving cereal grains (Goswami and Kistler, 2004), vascular wilts on banana (Liew *et al*., 1998), bakanae on rice (Nur Ain Izzati, 2007), pokkah boeng on sugarcane (Siti Nordahliawate, 2007), root rots on vegetable such as asparagus (Al-Amodi and Salleh, 2005) and some researchers reported cankers production on soft and hardwood trees (Wingfield *et al.*, 2008).

This species is literally divided into three clades; African, American and Asian. According to the hypothesis of O'Donnell *et al.* (1998), the originality of fungi host including its evolvement should be taking into account during clade clustering. However, numbers of species composition does not suit with the hypothesis. Among

these three groups, African is announced as the biggest clade which has 23 phylogenetic lineages and it huddles as productive chlamydospore-formers. The so-called 'American clade' has 18 phylogenetic lineages while the Asian is the smallest clade with 10 phylogenetic lineages (Kvas *et al*., 2009).

Mycotoxins are classified as unavoidable natural contamination in most of foods and feeds. Fumonisins (FUM), zearalenone (ZEN), moniliformin (MON) and beauvericin (BEA) are the most regular toxins produced by *Fusarium* sp. (Logrieco *et al*., 2002; Leslie *et al*., 2004; Sopterean and Puia 2012). The sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (Figure 2.4) is the precise criterion to elaborate moniliformin (MON). Its first outbreak was in 1973 after the extraction process of *F. proliferatum* which was inoculated in a corn culture. However, the identified species shown a similar characteristic with *F. moniliforme*, thus the mycologists came out with moniliformin name (Cole *et al*., 1973). At least 30 *Fusarium* species produce this compound in the cereal grains (commonly corn) and the most influential species are *F. proliferatum* and *F. subglutinans*.

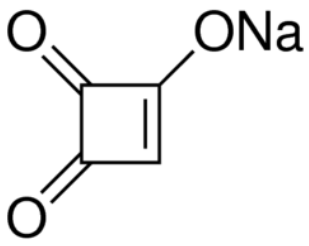


Figure 2.4: The skeleton structure of moniliformin (sodium salt) (Munimbazi and Bullerman, 2001)

This metabolite is very toxic either to plants or animals. The symptoms of afflicted animal may be varied and commonly muscular failing, respiratory suffering, cyanosis, coma and also death, are the ordinary signs. Analyte that contains moniliformin has to go through cleanup process before it is further analysed via liquid chromatography, capillary electrophoresis or immunochemical reactions (Munimbazi and Bullerman, 2001).

Fumonisin is named after *F. verticilloides* which previously known as *F. moniliforme*. It comprises B_1, B_2 and B_3 and commonly B_1 is found at the highest level compared with others (Marasas, 1996). (Rheeder *et al*., 2002). Approximately, 70 to 80% of the total fumonisins are represented by $FUMB_1$ and $FUMB_2$ reaches up 15 to 25%. The $FUMB_3$ produces the lowest level which is around 3 to 8%. These percentages occur in solid or liquid medium condition (Branham and Plattner, 1993; Marín *et al*., 1995). Fumonisin B_2 ($FUMB_2$) and fumonisin B_3 ($FUMB_3$) show up after the finding of $FUMB_1$ in 1988. The backbone of $FUMB_2$ and $FUMB_3$ are lacked in free hydroxyl groups compared with $FUMB_1$ (Figure 2.5) (Plattner *et al*., 1992).

The detection of $FUMB_1$ in a sample usually is done via liquid chromatography (LC) completed with fluorescence detector. This toxin contains a least UV-light absorbing chromophore and has no fluorescence production because of the simple long chain alcohols, thus a derivatization of the free amine is required for detection purpose (Shephard *et al*., 1990; Kedera *et al*., 1999).

There are a few of diseases related to fumonisins such as leukoencephalomalacia (ELEM), pulmonary oedema syndrome (PES) and hepatocarcinoma. These diseases