OPTIMIZATION OF STATIN (SIMVASTATIN) BY *MONASCUS PURPUREUS* FTC 5356 IN SOLID-STATE FERMENTATION

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OPTIMIZATION OF STATIN (SIMVASTATIN) BY *MONASCUS PURPUREUS* FTC 5356 IN SOLID-STATE FERMENTATION

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SUPERVISOR DECLARATION

"I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the Bachelor in Chemical Engineering (Biotechnology)

Signature

Name of Supervisor: Dr Farhan binti Mohd Said

Position:

Date: 30 March 2012

STUDENT DECLARATION

I hereby declare that the work in this thesis entitled **"Optimization of Statin (Simvastatin) by *Monascus purpureus* FTC 5356 in Solid-State Fermentation"** is the results of my own research accept as cited in references. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature:

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Date: 30 March 2012

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In order to complete this research, I was in contact with many peoples, researchers, academicians and practitioners. All of them have assisted me in many ways towards completing this research. They also have contributed towards my understanding and thoughts. I would like to express my sincere appreciation to my supervisors, Dr. Farhan binti Mohd Said for her encouragement, guidance, and critics during in finishing my research.

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ABSTRACT

Monascus sp. is a non-pathogenic fungus that can produce statin called simvastatin that can lower blood cholesterol in human body. The objective of this research is to investigate the optimization condition of the simvastatin production in solid-state fermentation by *Monascus purpureus* FTC 5356. The local products that used as substrates were banana, guava, pumpkin, coconut meat, corn, papaya and white rice. The fermentation was conducted using the optimum condition of 50% initial moisture content, pH6 at 30°C for 12 days in order to obtain the best substrate. Among these local products, corn can produce the simvastatin while other five fruits do not produce simvastatin. Further experimental carried out using Central Composite Design (CCD) of Response Surface Methodology (RSM) by setting two parameters which are moisture content and nitrogen source by setting the lower and higher range for each of the parameters. From the analysis from RSM, there are 14 runs conducted to achieve the optimum condition to get the maximum production of simvasatin.

ABSTRAK

Monascus sp. ialah sejenis kulat bukan patogen yang boleh menghasilkan statin dikenali sebagai simvastatin yang boleh menurunkan kolesterol darah di dalam tubuh manusia. Objektif kajian ini adalah untuk mengkaji keadaan optimum penghasilan simvastatin dalam keadaan pepejal penapaian *Monascus purpureus* FTC 5356. Produk tempatan yang digunakan sebagai substrat ialah pisang, jambu, labu, kelapa , jagung, betik dan beras. Penapaian dijalankan menggunakan keadaan optimum yang awal ialah kandungan lembapan 50% l, pH6 pada suhu 30 ° C selama 11 hari untuk mendapatkan substrat yang terbaik. Antara produk tempatan, hasil menunjukkan jagung boleh menghasilkan simvastatin manakala lima jenis lagi buah-buahan tidak menghasilkan simvastatin. Kajian lanjut yang dijalankan menggunakan Design Pusat Komposit (CCD) Kaedah Tindakbalas Permukaan (RSM) dengan menetapkan dua parameter dengan kandungan kelembapan dan sumber nitrogen dengan menetapkan julat yang lebih rendah dan lebih tinggi bagi setiap parameter. Dari analisis dari RSM, terdapat 14 eksperimen dijalankan untuk mencapai keadaan yang optimum untuk mendapatkan pengeluaran maksimum simvasatin.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Statins is a group of drugs that used primarily in lowering blood cholesterol. Statin is generally capable in lowering cholesterol by 20 to 60 percent. The discovery of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A which is act as inhibitors called statin that was a breakthrough in the prevention of hypercholesterolemia and related diseases (Najma et al., 2010). As cardiovascular diseases related to high levels of cholesterol are among the main causes of death in our societies, there is a high incentive for developing processes for the production of statins, an FDA approved drug. All natural statins have a common molecular structure, a hexahydro-naphthalene system and a -hydroxy-lactone, but they differ from each other due to side chains and a methyl group around the ring (Gerardo et al., 2004). Statins also are fungal secondary metabolites and was the first enzyme in cholesterol biosynthesis (Manzoni et al., 2002).

Statins are available either in Tablet or capsule form, statin's are usually taken with dinner or bedtime. The results are typically evident after a period of four to six weeks of use. Medications in this group are usually easy to tolerate and cause few side effects (Najma et al., 2010). The mechanism that involved in controlling the production of plasma cholesterol

levels is the reversible inhibition of HMG-CoA reductase by the statins that is related to the structural similarity of the acid form of the statins to HMGCoA, the natural substrate of the enzymatic reaction (Manzoni et al., 2002).

The statins differ with respect to their ring structure and substituents. These differences in structure affect the pharmacological properties of the statins. Sometimes, statins have been grouped into two groups of statins according to their structure. Statins that belong to type 1 are pravastatin and simvastatin. Statins that are fully synthetic and have larger groups linked to the HMG-like moiety is often referred to as type 2 statins. Statins that belong to this group are atorvastatin and rosuvastatin (Najma et al., 2010). The biosynthetic pathway involved in statin production, starting from acetate units linked to each other in head to-tail fashion to form polyketide chains, has been elucidated by both early biogenetic investigations and recent advances in gene studies. Natural statins can be obtained from different general and species of filamentous fungi (Monzani et al., 2002).

There are five statins currently used as clinical use. Lovastatin and pravastatin (mevastatin derived) are naturally statins of fungal origin while simvastatin is semi-synthetic lovastatin derivative. Atorstatin and fluvastatin are synthetic statins, which derived from mevalonate and pyridine (Monzani et al., 2002).

Simvastatin and lovastatin are well-known hyperlipidemia and hypercholesterolemia drugs that act as cholesterol-lowering agents (Caron et al., 2007). Simvastatin (marketed under the trade names ZOCOR, SIMLUP, SIMCARD, and SIMVACOR) is metabolized to at least four primary metabolites, namely 6'β-OH simvastatin, 6'-exomethylene simvastatin, 6'β-hydroxymethyl metabolite, and 3"-OH simvastatin. After oral ingestion, simvastatin and lovastatin, which are inactive lactones, are hydrolyzed to the corresponding β-hydroxyacid form (Vickers et al., 1990a). This molecule is a principal metabolite and an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, which is an early and ratelimiting step in the biosynthesis of cholesterol (Keon et al., 2010).

The metabolites resulting from microsomal oxidation of simvastatin and lovastatin by P450 enzymes are effective inhibitors of HMG-CoA reductase. Therefore, it has been suggested that the metabolites may contribute to the cholesterol-lowering effect of

simvastatin and lovastatin. However, systematic studies of the safety, efficacy, and toxicity of these metabolites have not been performed (Keon et al., 2010)

Lovastatin or also called Monacolin K is a potent drug for lowering blood cholesterol in human body. Lovastatin also a specific and a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is in the cholesterol biosynthesis, lovastatin act as a reductase that catalyzes the rate limiting step (Chang et al., 2002). Lovastatin also active to lower plasma cholesterol level in human and also animal, therefore it is the effective treatment for the patients that suffering hypercholesterolemia which is a primary risk for the artery disease (Frishman et al., 1989). Other research also indicated that lovastatin also indicated as a potential therapeutic agent for the various kind of tumors disease because lovastatin have ability to suppress the growth of the tumors (Chang et al., 2002).

Lovastatin can be extracted from the *Monascus sp*. especially *Monascus purpureus* by using several of substrates such as banana, papaya, guava, pumpkin, coconut meat, corn and also white rice. *Monascus sp*. is a non-pathogenic and widely used in Chinese foods and also as traditional Chinese medicine. *Monascus sp*. also extensively used in the food industry as a one of the colouring agent for the food such as red and also yellow pigment.

Lovastatin also have been investigated can therapeutically and can give an effective treatment also to prevent the diseases like atherosclerosis, sepsis, peripheral arterial disease, peripheral vascular disease, cerebro vascular disease, ischemic disease and bone fracture (Seraman et al., 2010). Lovastatin is extracted from the variety filamentous fungi for example *Monascus sp*. In particular monascus purpureus, monascus ruber and also monascus pilosus were found to be the most popular and also the most monascus used in production of lovastatin (Negishi et al., 1986).

Simvastatin is a compound derived from the natural lovastatin which is a secondary metabolites produced by filamentous fungus. The synthesis from lovastatin is a multistep process and has been intense interest because of its importance in the pharmaceutical industry.

Simvastatin a lactone analog of lovastatin which is used in the treatment of hypercholesterolemia. Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, is administered in the form of lactone prodrug. Simvastatin lowers plasma cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (Khaled, 2007)

Currently, two semisynthetic processes are widely used to synthesize simvastatin starting from lovastatin. One commonly adapted process starts with the hydrolysis of lovastatin to yield the key intermediate monacolin J, followed by the lactonization of the acid to protect the C11 hydroxyl group and trimethylsilylation protection of the C13 hydroxyl. The protected monacolin J is then subjected to acylation by dimethylbutyryl chloride to yield the protected form of simvastatin, which is subsequently deprotected to yield simvastatin. Both multistep processes are laborious, thus contributing to simvastatin being nearly five times more expensive than lovastatin. Therefore, a new semisynthetic scheme that can decrease the number of chemical transformations and increase the overall efficiency of the conversion can be of significant utility (Xinkai et al 2007).

For over thousands of years, the *Monascus sp.* was used on food which is called as Chinese traditional fermentation fungus. On the other hand, *Monascus sp.* also was very unique because either can extract to the lovastatin, monascus also can produces pigments like rubropunctatin (red colour), monascin (yellow colour), monascorubrin (red colour), anfaklavin(yellow colour), rubropunctamine (purple colour) and monascorubramine (purple colour) which now can be used to replace synthetic dyes by natural colourant and now already widely used (Manzoni et al.,1998; Chang et al.,2002). Statins currently available in different types and can classified also into natural statins which is can obtained directly by fermentation, semisynthetic and synthetic. Natural statins is like lovastatin and also pravastatin, while semisynthetic, atorvastatin and fluvastatin are synthetic statins (Manzoni and Rollini, 2002).

A variety of statins are available to lower plasma lipids to guideline levels, but all differ in their pharmacokinetic properties, drug interaction profiles, and risk of myotoxicity. This has been highlighted by the withdrawal of cerivastatin from the market as a result of serious safety concerns (Alberto, 2003).

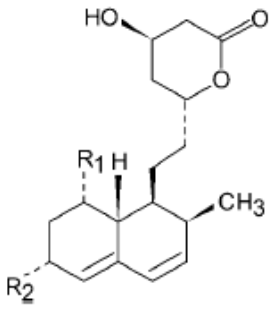

Figure 1.1: Base structure of statins - naphthalene ring and β-hydroxylactone (Manzoni et al., 2002)

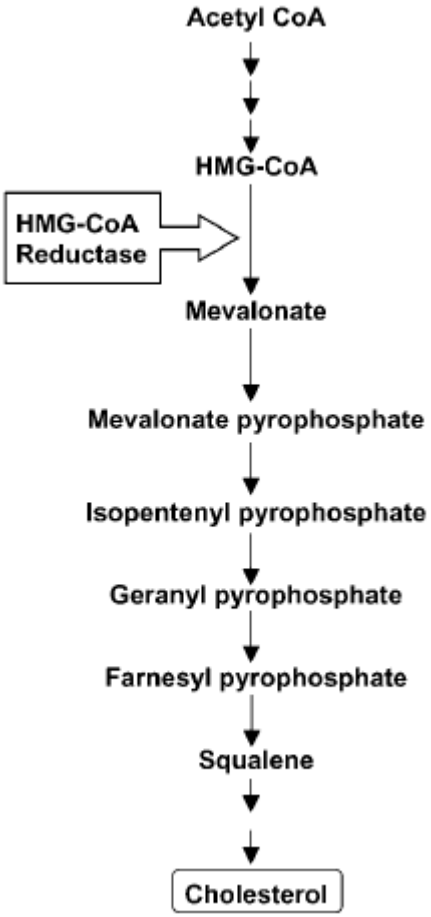

Figure 1.2 : Cholesterol biosynthetic pathway (Manzoni et al., 2002)

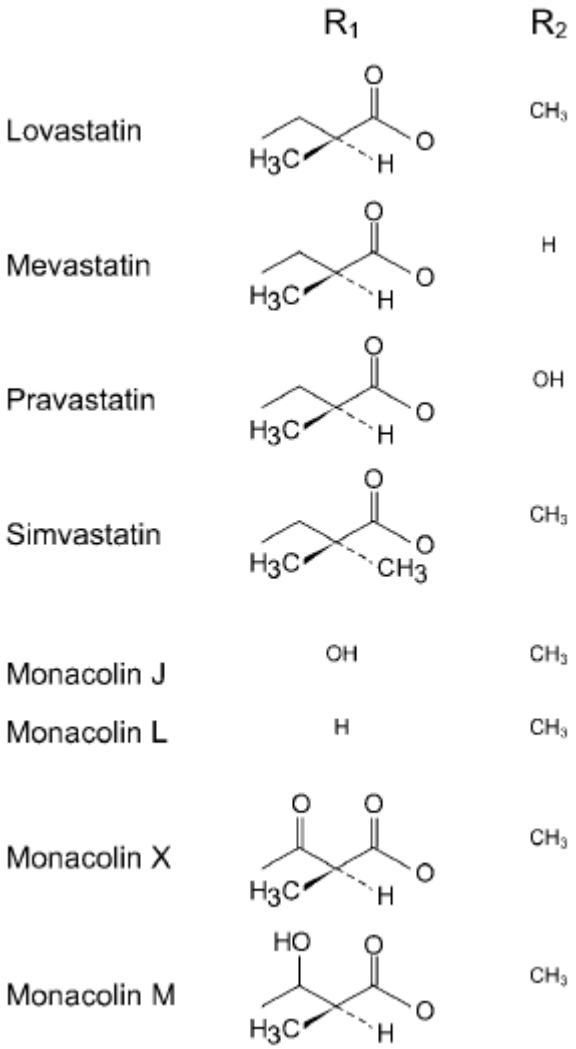

Figure 1.3 : Statin side chains linked at C8 (R1) and C6 (R2) of the base Structure (Manzoni et al., 2002)

1.2 PROBLEM STATEMENT

Nowadays, hypercholesterolemia is one of the world public health problem and also being the major cause of death in the western countries. Hypercholesterolemia is a primary risk factor for the country artery disease, heart attack and also stroke. Therefore, statins or also called blood-reducing cholesterol substance can inhibits the production of cholesterol by blocking of a key enzyme which is HMG-CoA reductase that activates cholesterol synthesis (Erdogrul and Azirak, 2004; Chen and Hu, 2005). In the industries, the production is the main focus, therefore industries need to produce high production but they also have to consume low. Therefore by using solid-state fermentation and also using local product as substrates which are banana, papaya, guava, pumpkin, coconut meat, corn and also white rice, therefore the production cost can be decreased. Statin such as lovastatin and simvastatin can produce by extraction of *Monascus purpureus* using solid-state fermentation. Solid-state fermentation is more advantages compared to submerged fermentation due to the substrate costs is little and also widely available. Solid-state fermentation also using less water and energy than submerged fermentation and the most important is in solid-state fermentation can yield higher production of lovastatin (Lian et al., 2006). Then, the ability of the *Monascus purpureus* to produce lovastatin in different solid substrates and also ability to produce high yield of lovastatin. The substrate chosen are banana, papaya, guava, pumpkin, coconut meat, corn and white rice.

1.3 RESEARCH OBJECTIVE

The purpose of doing this study is to achieve the objective which is to investigate the optimization condition of the statin production which is simvastatin in solid-state fermentation by *Monascus purpureus* by using local products which is banana, papaya, guava, pumpkin, coconut meat, corn and also white rice.

1.4 SCOPE OF RESEARCH

To achieve the objective for this experiment, there are few types of parameters has been identified which is first of all, the substrate selection. First of all, the first step is to obtain the best substrate from the seven local fruits. From the seven local products that been investigated, the best substrate chooses due to the two parameters that will be set which is percentage of nitrogen source added which is peptone and moisture content which being set under optimum moisture content which is 50%. Optimum amount of zinc sulphate also added which is 10%. All of the substrate was set under optimum condition which by using pH6 and setting under 30°C temperature inside incubator and left to undergo solid state fermentation for 11 days and the optimum days for the *Monascus purpureus* to obtain optimum yield is for 11 days. After get the best substrate, the investigated will be continued to study the effect of the moisture content and the concentration of the nutrient media added which is from nitrogen sources. Then, the experiment will continue to optimize the initial moisture of the substrate and the nutrient media which nitrogen sources is the best nutrient for simvastatin production. This study also using solid-state fermentation that is more advantages compared to the submerged fermentation.

1.4 RATIONALE AND SIGNIFICANCE OF RESEARCH

Statin is a very valuable product that can lowering the cholesterol in human body and already investigated and proven can be effective treatment of hypercholesterolemia and other major kind of diseases such as atherosclerosis, sepsis, peripheral arterial disease, cerebro vascular disease, ischemic disease and bone fracture (Seraman et al., 2010). In addition, statin now already been indicated for the therapeutic agent for the treatment of the variety kind of tumors because this statin have the ability to suppress tumors growth (Chang et al., 2002).

This research using local products which are banana, papaya, guava, pumpkin, coconut meat, corn and white rice as a substrate. Banana, guava, pumpkin and coconut meat never been use as a substrate from previous research. Therefore, this is an advantage if this research is success because it will become a new discovery in the science field.

From here we know that, simvastatin have a very valuable significant in the pharmaceutical industries. Therefore, hopefully this research can be commercialize and perhaps can increasing the pharmaceutical industries for our country.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

For over thousands of years, the *Monascus sp.* which is also called as Chinese traditional fermentation fungus was widely used on food and also been used as the essential part of wine production and for the other fermented foods (Seraman et al., 2010). From the cultivation of *Monascus sp.* on the rice grain, red mold rice which is also contains a large amount of γ-aminobutyric acid and also contain anti-hypertensive effects for human (S.Seraman et al., 2010). *Monascus sp.* also was very unique because either can extract to the lovastatin, *Monascus sp.* also can produces pigments like rubropunctatin (red colour), monascin (yellow colour), monascorubrin (red colour), anfaklavin(yellow colour), rubropunctamine (purple colour) and monascorubramine (purple colour) which now can be used to replace synthetic dyes by natural colourant and now already widely used (Manzoni et al.,1998; Chang et al.,2002).

The primary causes for the coronary artery disease is called hypercholesterolemia which also causes the major death in the western countries (Chang et al., 2002). Lovastatin is the best treatment for this disease. Statins currently available in different types and can classified also into natural statins which is can obtained directly by fermentation, semisynthetic and synthetic. Natural statins is like lovastatin and also pravastatin, while

semisynthetic, atorvastatin and fluvastatin are synthetic statins (Manzoni and Rollini, 2002). In the food processing, to add the aroma, nutrition and colour of the fermentation products, the *Monascus sp.* was commonly used because this species is such a non-pathogenic (Chang at el., 2002). Lovastatin also was first determined by Endo from the *Monascus ruber* and independently from *Aspergillus terreus* (Alberts et al.,1980). The first natural statin was from fungal secondary metabolite that being approved by the US Food and Drug Administration in August 1987 (Tobert 2003; Demain 1999; and Rollini 2002).

In the pharmaceutical study, after being experimented by three animal models, the study showed that this lovastatin can lower the blood cholesterol of hypercholesterolemia (Li et al., 1998). In addition, for the human clinical trial, the lovastatin showed a significant value in lowering cholesterol levels after tested in 83 tested individual (Heber et al., 1999).

2.2 STATIN

Statins are group of drugs that used primarily in lowering blood cholesterol. Statin are generally capable in lowering cholesterol by 20 to 60 percent. The discovery of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A which is act as inhibitors called statin that was a breakthrough in the prevention of hypercholesterolemia and related diseases (Najma et al., 2010). As cardiovascular diseases related to high levels of cholesterol are among the main causes of death in our societies, there is a high incentive for developing processes for the production of statins, an FDA approved drug. All natural statins have a common molecular structure, a hexahydro-naphthalene system and a -hydroxy-lactone, but they differ from each other due to side chains and a methyl group around the ring (Gerardo et al., 2004). Statins also are fungal secondary metabolites and was the first enzyme in cholesterol biosynthesis (Manzoni et al., 2002).

The most commonly prescribed drugs in medicine are statins. Statins significantly reduce the risk of heart attack and death in patient through clinical studies and proven that coronary artery disease (CAD) and also can reduce cardiac events in patients with high cholesterol levels who are at increased rosk for heart disease. Statin also was best known as drugs that can lower cholesterol also have several other beneficial effects that also may improve cardiac risk and that may turn out be even more important than their cholesterol reducing properties (Richard, 2011).

2.2.1 TYPE OF STATIN

Statins include well-known medications such as atorvastatin (Lipitor), simvastatin (Zocor), lovastatin (Mevacor), pravastatin (Pravachol), rosuvastatin (Crestor) and others. Lower cost generic versions of many statin medications are available (Mayo clinic, 2011).

The statins differ with respect to their ring structure and substituents. These differences in structure affect the pharmacological properties of the statins. Sometimes, statins have been grouped into two groups of statins according to their structure. Statins that belong to type 1 are pravastatin and simvastatin. Statins that are fully synthetic and have larger groups linked to the HMG-like moiety are often referred to as type 2 statins. Statins that belong to this group are atorvastatin and rosuvastatin (Najma et al., 2010).

There are five statins currently used as clinical use. Lovastatin and pravastatin (mevastatin derived) are naturally statins of fungal origin while simvastatin is semi-synthetic lovastatin derivative. Atorstatin and fluvastatin are synthetic statins, which derived from omevalonate and pyridine (Manzni et al., 2002). Lovastatin, simvastatin and pravastatin are derived from fungi. Simvastatin is chemically modified 2,2-dimethyll butyrate analogue of lovasatin. Provastatin then is a purified active metabolite of mevastatin with an open hydroxyl acid instead of lactone ring (Khaled, 2007).

The first representative of the new class of statin compounds was mevastatin which is derived from a strain of Penicilln citrinum. Lovastatin is a natural products while simvastatin is derived from the lovastatin. Pravastatin also derived from the natural products and fluvastatin is totally synthetic recemic mixture (Illingworth et al., 2001).

The fungal products lovastatin, pravastatin and simvastatin are structurally related since they have a hydronaphthalene in common and differ only at a few sites inhe molecule K shown in Figure 4 (Khaled, 2007).

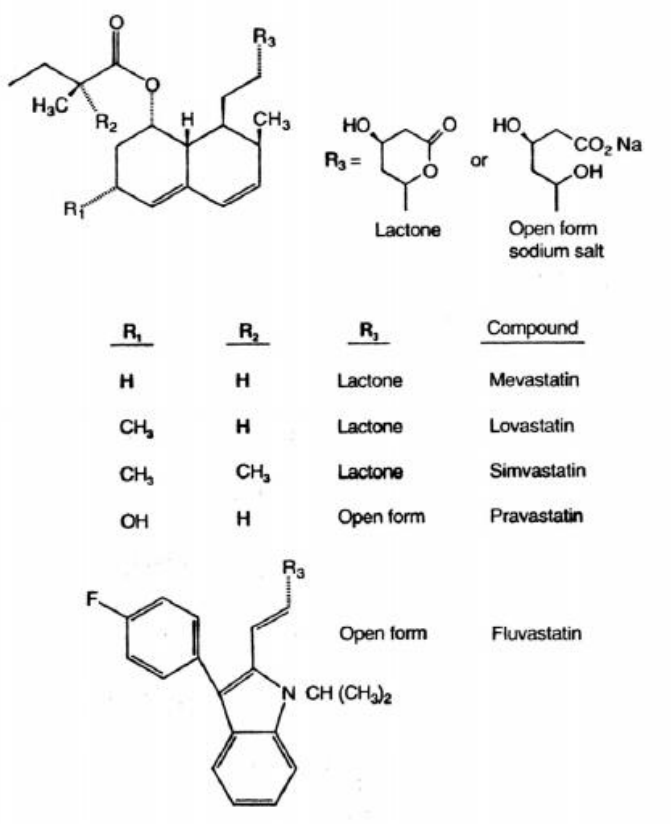

Figure 2.1 : Chemical structures of the main 3-hydroxy-3- methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (Khaled 2007; Desager and Horsmans, 1996)

Lovastatin, simvastatin, pravastatin and fluvastatin have similar pharmacodynamic properties. All can reduce LDL-cholesterol by 20 to 35%, a reduction which has been shown to achieve decreases of 30 to 35% in major cardiovascular outcomes. Simvastatin has this effect at doses of about half those of other 3 statins (Khaled, 2007).

2.2.2 BENEFIT OF STATIN

Statins is a group of drugs that used primarily in lowering blood cholesterol. Statin are generally capable in lowering cholesterol by 20 to 60 percent. The discovery of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A which is act as inhibitors called statin that was a breakthrough in the prevention of hypercholesterolemia and related diseases (Najma et al., 2010). As cardiovascular diseases related to high levels of cholesterol are among the main causes of death in our societies, there is a high incentive for developing processes for the production of statins, an FDA approved drug.

Lovastatin or also called Monacolin K is a potent drug for lowering blood cholesterol in human body. Lovastatin also a specific and a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is in the cholesterol biosynthesis, lovastatin act as a reductase that catalyzes the rate limiting step (Chang et al., 2002). Lovastatin also active to lower plasma cholesterol level in human and also animal, therefore it is the effective treatment for the patients that suffering hypercholesterolemia which is a primary risk for the artery disease (Frishman et al., 1989). Other research also indicated that lovastatin also indicated as a potential therapeutic agent for the various kind of tumors disease because lovastatin have ability to suppress the growth of the tumors (Chang et al., 2002).

Statin is a very valuable product that can lowering the cholesterol in human body and already investigated and proven can be effective treatment of hypercholesterolemia and other major kind of diseases such as atherosclerosis, sepsis, peripheral arterial disease, cerebro vascular disease, ischemic disease and bone fracture (Seraman et al., 2010). In addition, statin now already been indicated for the therapeutic agent for the treatment of the variety kind of tumors because this lovastatin have the ability to suppress tumors growth (Chang et al., 2002).

The four 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors used today are lovastatin, simvastatin, pravastatin and fluvastatin. The HMG-CoA reductase is the key enzyme of cholesterol synthesis. HMG-CoA reductase inhibitors are potent reversible inhibitors of this enzyme, which act by competing for the substrate HMG-CoA (Desager and Horsmans, 1996).

HMG-CoA reductase inhibitors are now widely used and account for the majority of prescriptions for lipid lowering drugs in many countries. HMG-CoA reductase inhibitors are the most effective agents developed to date for the treatment of patients with primary and secondary hypercholesterolaemia associated with increased levels of LDL cholesterol (Khaled, 2007).

The mechanism by which statins may influence prostate cancer is unknown but may involve their cholesterol-lowering properties or their influence on other pathways (Allison et al., 2010). Nowadays, recent evidence suggests that statins may also act as chemoprotective agents against various types of cancers (Vikram et al., 2007). Then, the statins have been reported to protect against stroke events in stroke-prone spontaneously-hypertensive rats and

ameliorate stroke severity by inhibition of superoxide production and modulation of inflammation in the brain (Sung et al., 2004).

2.2.3 EFFECT OF STATIN

The hypocholesterolemic effects of statins are evident after only a few days of therapy. Lovastatin, simvastatin, and pravastatin are well tolerated drugs; at 40 mg lovastatin a mean reduction of 30% in total plasma cholesterol, 40% in LDL (low-density lipoprotein), 35% in VLDL (very low-density lipoprotein) cholesterol, and 25% in triglycerides, and an increase of 10% high density lipoprotein (HDL)-cholesterol was observed (Monzani et al., 2002; Tobert 1987).

The results reported since 1987, the year of approval of lovastatin as a therapeutic drug by the FDA, indicate that statins can be employed successfully in the treatment of hypercholesterolemia. However, the benefit-risk relationship must always be taken into account. The marked lipid-lowering effects of statins have led to a substantial reduction in coronary events, as revealed by clinical, epidemiological, and pathological studies (Monzani et al.,2002; Chong et al. 2001; Farnier and Davignon 1998; Furberg 1999; Maron et al. 2000).

In addition to reducing the risk of cardiovascular morbidity and mortality, statins can prevent stroke and reduce the development of peripheral vascular disease (Monzani et al., 20020; Maron et al. 2000).

Many years, have proven beyond reasonable doubt that virtually all patients at cardiovascular risk benefit from effective lipid-lowering therapy with statins, even those with normal LDL cholesterol levels (Alberto, 2003). Based on these recent trials, patients with coronary heart disease (CHD), diabetes, the elderly, menopausal women, recipients of donor organs or those with HIV are at highest absolute cardiovascular risk, even though LDL cholesterol levels are not always elevated, and have the most to gain from statin therapy. In addition, many patients with mixed dyslipidaemia are at increased cardiovascular risk as a result of low High Density Lipoprotein (HDL) cholesterol and high triglycerides, and/or abnormal small, dense atherogenic Low Density Lipoprotein (LDL) cholesterol levels (Alberto, 2003).

Statin therapy reduces the incidence of coronary events in part by slowing the progression of atherosclerosis, with coronary angiography studies consistently demonstrating the ability of statin treatment to slow the CHD progression (Alberto, 2003).

Therefore statin treatment suggested to provide clear outcome benefits in patients with average cholesterol levels. These findings suggest that the reduction in coronary risk caused by statin therapy may reflect actions of this drug class independent of their lipid lowering effects (Alberto, 2003).

2.3 APPLICATION OF STATIN

2.3.1 LOVASTATIN

Potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is called as lovastatin which is also known as mevinolin, monacolin K and also mevacor. HMG-CoA is reductase which is rate-limiting enzyme in cholesterol biosynthesis (Chang et al., 2002). Other than that, lovastatin now already been indicated for the therapeutic agent for the treatment of the variety kind of tumors because this lovastatin have the ability to suppress tumors growth (Chang et al., 2002). Lovastatin also have been investigated can therapeutically and can give an effective treatment also to prevent the diseases like atherosclerosis, sepsis, peripheral arterial disease, peripheral vascular disease, cerebro vascular disease, ischemic disease and bone fracture (Seraman et al., 2010).

Lovastatin is extracted from the variety filamentous fungi for example *Monascus sp.* In particular *Monascus purpureus, Monascus ruber* and also *Monascus pilosus* were found to be the most popular and also the most *Monascus sp.* used in production of lovastatin (Negishi et al., 1986). Other than that, also indicated that lovastatin also indicated as a potential therapeutic agent for the various kind of tumors disease because lovastatin have ability to suppress the growth of the tumors (Chang et al., 2002). Lovastatin also active to lower plasma cholesterol level in human and also animal, therefore it is the effective treatment for the patients that suffering hypercholesterolemia which is a primary risk for the arthery disease (Frishman et al., 1989).

Recently, since the death because of the heart disease increasing due to the one of the famous factor which is hypercholesterolemia and because of that lovastatin and it

semisynthetic derivatives became the important natural drugs which is from the natural sources that is *Monascus sp*. (Wei et al., 2007). According to the history, lovastatin was firstly being isolated by Endo from *Monascus ruber* and then independently by the Alberts et al., 1980 from *Aspergillus terreus* (Chang et al., 2002).

2.3.2 SIMVASTATIN

Simvastatin is a water insoluble drug used as a hypocholestrolemic agent (Jaleh et al., 2011). Simvastatin is a compound derived from the natural lovastatin which is a secondary metabolites produced by filamentous fungus. The synthesis from lovastatin is a multistep process and has been intense interest because of its importance in the pharmaceutical industry.

Simvastatin a lactone analog of lovastatin which is used in the treatment of hypercholesterolemia. Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, is administered in the form of lactone prodrug. Simvastatin lowers plasma cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (Khaled, 2007).

Currently, two semisynthetic processes are widely used to synthesize simvastatin starting from lovastatin. One commonly adapted process starts with the hydrolysis of lovastatin to yield the key intermediate monacolin J, followed by the lactonization of the acid to protect the C11 hydroxyl group and trimethylsilylation protection of the C13 hydroxyl. The protected monacolin J is then subjected to acylation by dimethylbutyryl chloride to yield the protected form of simvastatin, which is subsequently deprotected to yield simvastatin. Both multistep processes are laborious, thus contributing to simvastatin being nearly five times more expensive than lovastatin. Therefore, a new semisynthetic scheme that can decrease the number of chemical transformations and increase the overall efficiency of the conversion can be of significant utility (Xinkai et al 2007).

Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, is administered in the form of lactone prodrug. The lactone ring is hydrolyzed *in vivo* to produce the hydroxyl acid derivatives which are the pharmacologically active forms of this drug, and this is believed to take place predominantly in the liver (Khaled, 2007).

2.4 PRODUCTION OF STATIN BY *MONASCUS PURPUREUS*

2.4.1 PRODUCTION OF SIMVASTATIN AND LOVASTATIN

For centuries, *Monascus sp.* has been used widely in Asia as a coloring of fish, Chinese cheese, red wine and sausages (Pattanagul et al., 2008). *Monascus sp.* widely used long time ago as a folk medicine for the food digestion and also blood circulation and also as a treatment of other sickness (Panda at el., 2010). *Monascus sp*. belong to the *Ascomycetes* group and family of *Monascaceae. Monascus purpureus* easily can be distinguished by it ascospores which is in spherical shape (Pattanagul et al., 2007). *Monascus sp*. are non-pathogenic and use in the food processing to obtain the aroma, nutrition and also colour of the fermentation products (Chang et al., 2002). Using *Monascus sp.* for the production of the lovastatin indicate that give advantageous with an increased saving in cost and if using directly as a functional food as long it is proves to be nontoxic (Xu et al., 2005). Various of active ingredients including lovastatin owned by the *Monascus purpureus* and several trials been done for its ability toward lowering the lipid have been conducted (Liu et al., 2006).

Simvastatin is a methyl analogue of lovastatin and is synthesized from a fermentation product of *Aspergillus terreus* (Khaled 2007; Hoffman *et al*., 1986). Simvastatin is a nonhygroscopic white crystalline powder, insoluble in water but quite soluble in chloroform, methanol and alcohol (Mauro, 1993) with pKa of 4.68 (Corsini *et al.,* 1999). The molecular weight of this compound C25H38O5 is 418.57. Simvastatin is the pharmacologically inactive lactone form of simvastatin acid, butanoic acid, 2,2-dimethyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl) ethyl]-1-naphthalenyl ester. Simvastatin is a lactone which needs the opening of the ring for it to become active.

Simvastatin is a crystalline powder, that practically water insoluble that is obtained as a fermentation product of *Aspergills terreus* and is poorly absorbed from the gastro-intestinal (GI) . Therefore, it is very important to enhance its dissolution rate substantially leading to its improved bioavailability (Jaleh et al., 2011).

2.4.2 CONNECTION BETWEEN LOVASTATIN AND SIMVASTATIN

Simvastatin is a semisynthetic derivative of the fungal polyketide lovastatin and is an important drug for lowering cholesterol levels in adults (Xinkai et al., 2007). The synthesis of simvastatin from lovastatin is a multistep process and has been of intense interest because of its importance in the pharmaceutical industry (Xinkai et al., 2007).

Currently, two semisynthetic processes are widely used to synthesize simvastatin starting from lovastatin. One commonly adapted process starts with the hydrolysis of lovastatin to yield the key intermediate monacolin J, followed by the lactonization of the acid to protect the C11 hydroxyl group and trimethylsilylation protection of the C13 hydroxyl. The protected monacolin J is then subjected to acylation by dimethylbutyryl chloride to yield the protected form of simvastatin, which is subsequently deprotected to yield simvastatin. Both multistep processes are laborious, thus contributing to simvastatin being nearly five times more expensive than lovastatin. Therefore, a new semisynthetic scheme that can decrease the number of chemical transformations and increase the overall efficiency of the conversion can be of significant utility (Xinkai et al 2007).

Parameter	Lovastatin	Simvastatin	Pravastatin	Fluvastatin	Atorvastatin	Cerivastatin
Prodrug	Yes	Yes	No	No	No	No
Crosses blood brain barrier	Lactone	Lactone	No	No	N.A	N.A.
Lipophilicity	Lipophilic	Lipophilic	Hydrophilic	Hydrophilic	Lipophilic	N.A.
Oral pharmacokinetics						
• Dose (mg/day)	20-8	10-40	20-4	20-80	2.5-80	0.1-0.3
• Absorption (%)	30	60-85	35	98	N.A.	N.A.
• Bioavailability (%)	< 5	< 5	10	10-35	12	60
• Effect of food	↑ 50%	No	↓ 30%	↓ 15-25%	↓ 13%	↓23%
T_{max}	2-6	1.3-2.4	0.9-1.6	0.5-1.5	2-4	0.5-4
Terminal half-life (hr)	2.5-15	1.9-15.6	1.3-2.6	0.5-3.1	14	1.7-2.7
Hepatic extraction (%)	62-69	>78	46	68	N.A.	N.A.
Renal elimination (%)	30	13	20-60	6	< 2	30
Protein binding (%)	> 90	> 90	43-48	95-98	> 95	N.A.
p-Glycoprotein substrate	Yes	N.A.	Yes	Yes	N.A.	N.A.
CYP substrate	CYP3A	CYP3A	No	CYP2C9	CYP3A	CYP3A
Metabolites effect	Yes	Yes	No	No	Yes	Yes
Mostly eliminated as	Metabolites	Metabolites	Unchanged	Metabolites	N.A.	Metabolites

N.A., not available

Table 2.1: Comparison of HMG-CoA Reductase inhibitors (Khaled, 2007;Christians *et al.*, 1998). The Table show only lovastatin and simvastatin are prodrug and crosses blood drain brain barrier.

2.5 SOLID STATE FERMENTATION

Solid substrate fermentation was done by mixed cultures of different fungal. For better biomass and also secondary metabolite productions, the co culture of fungi during the fermentation process. To enhance enzyme, organic acid production and also microbial bioconversion reaction, there were several reports showed (Banerjee et al., 2005; Pandey et at., 1999; Temudo et al. 2007).

By using different process parameter that can contributing the lovastatin production, the optimum levels was identified, which is carried out the solid state fermentation in conical flasks that contain optimized nutrients (Panda et al., 2008). There are four process parameters been used which is temperature, fermentation time, inoculums volume and pH of the solid medium were chosen for investigating and also the procedure of this process parameters was mostly contribute to the growth of the different fungal strain during solid state fermentation (Panda et al., 2008).

In solid-state fermentation (SSF), the cultivation of *Monascus sp*. in steamed rice is very exuberant. Carbon and nitrogen give an effect to the production of lovastatin and for the fungus growth because there are many natural substrates that showed similar or even higher quantities of carbohydrate and protein because these two nutrients contribute to the production of lovastatin (Soccol et al., 2003).

Nowadays, the solid-state fermentation become the most effective ways to ferment *Monascus sp*. to gain the lovastatin because of its advantages compared to the submerged fermentation. This advantages are widely available, water and also energy is less used and the most important fact is it is can produce high yield of the lovastatin (Wei et al., 2007). For addition, to minimize the production cost, a few efforts have been done using solid-state fermentation for the production of the lovastatin (Szakacs et al., 1998). The capability of fungus like *Monascus sp*. to produce lovastatin in the variety of solid substrates is investigated (Jaivel and Marimuthu, 2010).

2.5.1 EFFECTS OF SUBSTRATES

In the solid state fermentation focused on *Monascus sp*. fermentation, such substrate might showed a potential substrate and gives the best result in the production of other metabolites in solid-state fermentation (Soccol et al., 2003).

There also a report that describe some other raw materials used as a substrate for the *Monascus sp*. growth which is cassava starch, pear juice and also dairy milk. There are also supplement this substrate with the others nutrients such as vitamins and also organic nitrogen supplements (Carvalho et al., 2006). Substrates such as wheat bran, rice bran, maize flour and sorghum grain being used in the solid-state fermentation process to find the suiTable substrate for maximum lovastatin production then incubated to get the yield by the HPLC analysis (Morovjan et al., 1997).

Important factor that affect fungi growth and productivity is the composition of a solid substrate. Rice is a common substrate and soy-bean flour is a common additive substrate for the SSF of M.ruber (Xu et al., 2005). The culture medium has significant influence on the production of a metabolite as with any solid state fermentation product. The important in the industrial scale fermentation in about the screening and also optimization of the substrate constituents. The important thing for the lovastatin production is the selection

and also the composition of the nutrients of a suiTable substrates from the SSF (Xu et al., 2005).

2.5.2 EFFECT OF CARBON AND NITROGEN ADDITIVES

Carbon and nitrogen sources is very important in the fermentation activity because this nutrients is contribute and gives major effect to the formation of biomass and the metabolite (Xu et al., 2005). Peptone is one of the organic nitrogen source for the lovastatin production, the lower concentration of the peptone will be increase the production of lovastatin but the higher the concentration of the peptone will decreasing the production of the lovastatin. The effect of the carbon source such as glucose, maltose and also glycerol also the combination either both of the carbon source will required for the higher of the production of the lovastatin (Miyake et al., 2006).

2.6 ANALYSIS USING HIGH PERFORMANCE LIQUID CHROMATOGRAOHY (HPLC)

Analysis is carried out using high performance liquid chromatographic (HPLC) in an reverse phased C_{18} column (Morovjov et al.,1997). Using HPLC, lovastatin was quantified as β-hydroxy acid form which is unstabble and freshly prepared from lactone form (Friedrich et al., 1995). Using Rheoyne injector of 20µl manually, the binary gradient system was used and the samples injected. The mobile used were acetonitrile and 0.1% orthophosphoric acid in water in the ratio of 60:40 by the flow rate of 1.5 ml min^{-1} (Seraman et al., 2010). Nowadays, for HPLC analysis already processed in the same manner done in the previous study for the preparation of the sample and the standard solution of the HPLC (Xu et al., 2005).

2.7 EXPERMENTAL DESIGN AND OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY (RSM)

Optimization using response surface methodology (RSM) has been widely used for various phases in the fermentation process using various parameters (Panda et al., 2009). Using RSM the researcher can reduce the experimental because RSM is a very systematic technique for testing multiple process variables compared to the study of a variable in one time and such technique also can be determined and quantified for the interaction between variables (Chang et al., 2002). Researcher also can get their optimum result because RSM

will lead with a initial objective rapidly and efficiently. When the optimum can locate therefore it is easy for the researcher to running the experiment because how many time experiment need to be done already been found (Chang et al., 2002). For this lovastatin production, the RSM is base on the Central Composite Design (CCD) to locate the optimum parameters (Chang et al., 2002).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 INTRODUCTION

This chapter includes the methodology applied in order to carry out the experiment for the optimization of simvastatin by *Monascus sp*. in solid state fermentation. The experiments will be carried out at Chemical Engineering Laboratory, Faculty of Chemical of Natural and Resources Engineering, Universiti Malaysia Pahang. Before the experiment is carried out, all the chemical, raw material and also the apparatus need to be set up and ready to run.

3.2 MATERIAL

The fungal culture of *Monascus purpureus* FTC5356, acetonitrile, orthophosphoric acid, sodium hydroxide, peptone, methanol, hydrochloric acid, zinc sulphate, banana, papaya, guava, white rice, coconut, pumpkin and corn.

3.3 PROCEDURE

3.3.1 CULTURE

A culture of *Monascus purpureus* FTC 5356 was obtained from the Malaysian Agricultural Research and Development Institute (MARDI). It was maintained on potato dextrose agar (PDA) slants and also in petri dish. By using inoculums loops, *Monascus Purpureus* FTC 5356 then had been transfer to the slants and petri dish. All the experimental works been done inside the laminar air flow to prevent contaminated. After that, the slants and petri dish were left inside the Microbiological Incubator (Memmert) for the 30℃ for a week and been subculture to avoid out of culture's stocks because we need to use different slants when we doing inoculums sporulated.

3.3.2 SUBSTRATE PREPARATION

Seven of the chosen local fruits which are banana, papaya, pumpkin, guava, coconut, corn and also white rice were purchased from the local market. All of the fruits were dried using 60℃ using oven (memmert) for until the fruits were totally dried. After that, all of the fruits been blend using wiring blender and lastly sieve it by using shieve shaker to obtain the powder condition.

3.3.3 INOCULUM PREPARATION

The fully sporulated (6–8 days old) agar slant was added with sterile distilled water and then poured the sporulated with distilled water inside sterilized universal bottle.The spore suspension obtained was used as the inoculum suspension.

3.3.4 SOLID STATE FERMENTATION

From the seven local products that will be investigated, the best substrate chooses due to the two parameters that will be set which is percentage of nitrogen source added which is peptone and moisture content which being set under optimum moisture content which is 50%. All of the substrates been set under optimum condition which is using pH6 and setting under 30℃ temperature inside microbiological incubator and will be left to undergo solid state fermentation for 11

days because optimum days for the *Monascus purpureus* to obtain optimum yield is for 11 days. After get the best substrate, the investigated continued to study of the effect of the moisture content and the concentration of the nitrogen source added. Then, the experiment will continue to optimize the initial moisture of the substrate and the nitrogen source.

First of all, 10g powder of 7 substrates weighed using the analytical balance and transferred inside conical flasks. The conical flask was closed using cotton and been wrapped by two layers aluminium foil to prevent the substrate to contaminated with the air and other microorganisms. The moisture content of each of the substrates needs to be adjusted because different substrates have different ability to absorb water

Then different percentage of peptone added as the nitrogen source to the each of the substrate because the fruit already contain the nitrogen source as their natural nutrient and the carbon source became constant. The medium pH was adjusted using 1M NaOH to adjust the pH became 6. Then, all of the conical flasks had been autoclave (HIRAYAMA – HICLAVE HVE-50) using 121℃ for 20 minutes.

After autoclaved at 121℃ for 20 minutes, each of the conical flasks was inoculated with inoculums suspension 10% of the volume of *Monascus purpureus* FTC 5356 in distilled water. Fermentation was incubate at 30℃ for 11 days.

3.3.5 EXTRACTION OF SIMVASTATIN

After fermentation, the fermented material was dried at 60℃ for 24 hours and then crushed to powdered material and then by taking 2g of the powder fermented material and the been extracted by mixture of methanol and water for the ratio 1:1 for pH 7.7. After that, the mixture was keep in the incubator shaker (INFORS HT ECOTRON) at 200rpm for 2hours. Then after two hours, the mixture was centrifuged using centrifuge 5810R (eppendorf) at 10,000rpm for 10 minutes and the supernatant was filtered through 0.45µm nylon membrane filters (Valera at el., 2005).

3.3.6 ANALYSIS OF SIMVASTATIN

Analysis of simvastatin was carried out in Quaternary HPLC (Agilent Technologies 1200 Series at 237 nm in Luna C_{18} column. Binary gradient system will used and the samples will be injected manually using loop injector of 10 µl. The mobile phase used is acetonitrile and 0.1 % orthophosphoric acid in water in the ratio of 60:40 respectively. The flow rate will be pumped at 1ml min^{-1}. By using Pharmaceutical grade simvastatin powder containing 5µg obtained from Sigma Chemical Co as standard. Various concentration of standard dissolved in methanol to prepare the standard curve.

3.3.6.1 MOBILE PHASE PREPARATION

Mobile phase used was acetonitrile with 0.1%orthophosphoric acid in water with the ratio of 60:40. For these analysis, 1000ml mobile phase been prepared and being filtered with HPLC standard filter and continue put inside the ultrasonic (ELMASONIC S60 H) for 30 minutes (Subhagar at el., 2010).

3.3.6.2 STANDARD PREPARATION

The standard was prepared by diluted in the methanol. The standard was existing in two forms which is in lactone and in β-hydroxyacid. The β-hydroxy acid need to be suspended in 0.1 M NaOH and heated at 50°C for one hour in a shaking water bath BS-21. The pH adjusted to 7.7 with 1M HCl then filtered through 0.45µm membrane filter. Both of the standard form diluted to 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml. After filtered, all of the standard need to undergo ultrasonic cleaner at least 3 minutes (Jaivel et al., 2010)

3.4 RESPONSE SURFACE METHODOLOGY (RSM)

To optimize the significant parameters using RSM which to obtain the maximize simvastatin production by the *Monascus purpureus* efficiently. Statistical methods provide an alternative methodology to optimize a particular process by considering the mutual interactions among the variables and to give an estimate of the combined effects of these variables (Aravindan et al., 2008; Lim et al., 2005). The response surface optimization involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model (Montgomery, 2001; Box et al., 1978; Murthy et al., 2000; Xin et al., 2005).

In the solid-state fermentation, the process parameters such as moisture of the substrate and also the addition of the nutrient source either carbon or nitrogen sources. Experimental run will be carried out using Central Composite Design (CCD) of RSM (Chang et al., 2002).

CHAPTER 4

RESULT AND DISCUSSION

From the result obtained, the confirmation of simvastatin was carried out by using High Performance Liquid Chromatography (HPLC) and the estimation of simvastatin was calculated. Retention time of standard simvastatin and sample was 3.345 and 3.475. The existed of the other peak on the sample in high performance liquid chromatography might be due the presence of impurities or other unidentified compounds in the samples.

Analysis of simvastatin in high performance liquid chromatography is carried out in the form of β-hydroxyacid form, because the β-hydroxyacid form form elutes earlier in the chromatography column (Jaivel et al.,2010 ; Fredrich et al., 1995). The lactone also exist but at the retention time of 5.401. The lactone phase only exists in the 15 day fermentation time and it take a long time to form. Due to the limited time, the lactone phase could not be analyze.

4.1 STANDARD CURVE ANALYSIS

Table 4.1: Standard for β-hydroxyacid form

Concentration, µg/mL	Retention time, min	Area, mAU*s
0	0	0
2	3.261	26.83171
4	3.259	51.57607
6	3.262	57.44005
8	3.261	65.90139
10	3.277	68.24733

Table 4.2: Standard for lactone form

Concentration, µg/mL	Retention time, min	Area, mAU*s
0	0	0
2	5.410	17.09400
4	5.415	49.14852
6	5.401	97.11134
8	5.422	268.17572
10	5.396	536.64062

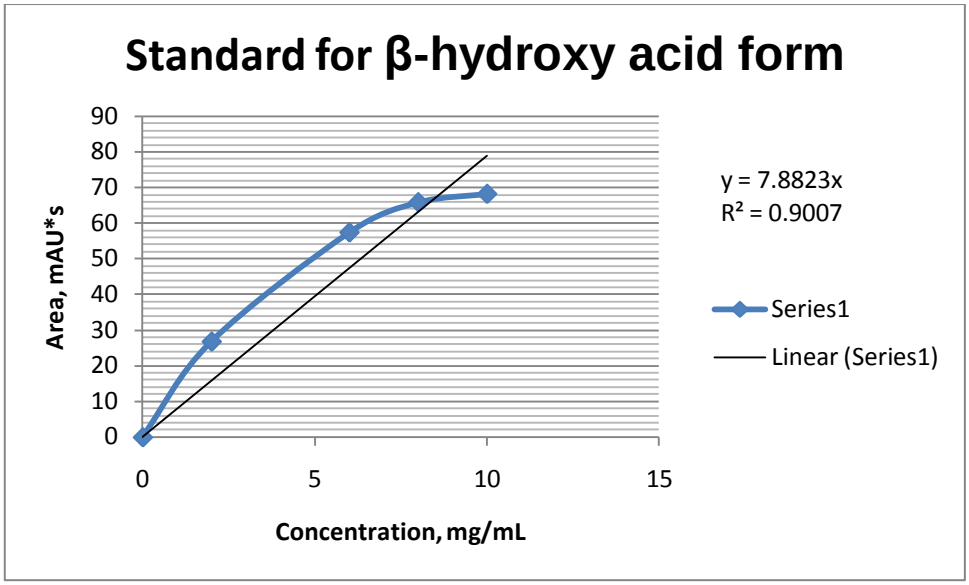

Figure 4.1: Standard curve for β-hydroxyacid form

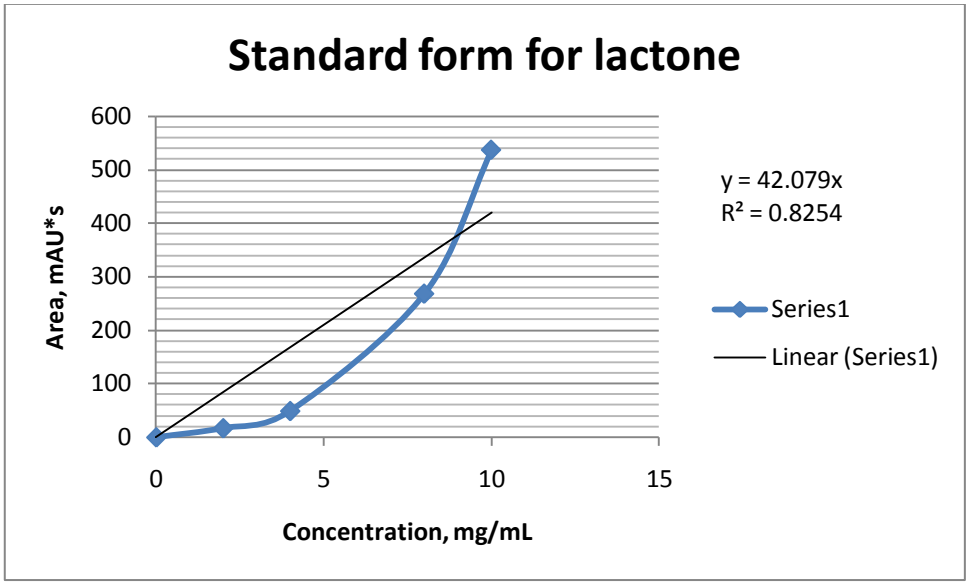

Figure 4.2: Standard curve for lactone form

4.2 ANALYSIS ON SUBSTRATE SELECTION

Table 4.3: Substrate selection analysis

Substrate	Zinc Sulphate, (g)	Peptone, (g)	Water, (mL)	Area, mAU*s	Simvastatin yield, (µg/ml)
Rice	3.97	0.18	4.60	231.31120	29.34565
Corn	3.85	0.15	4.50	180.24139	22.86660
Pumpkin	3.98	0.19	4.50	0	0
Guava	3.98	0.19	4.50	0	0
Banana	3.99	0.16	4.60	0	0
Coconut	3.93	0.13	4.60	0	0
Papaya	4.00	0.20	4.55	0	0

From the Table 4.3 analysis, rice and corn proven can produce simvastatin after calculed using calibration curve in Figure 4.1. Unfortunately, pumpkin, guava, banana, coconut and papaya not produce simvastatin. Zinc sulphate addition for all substrate is 0.128M, therefore after calculation; the amounts added were different for each substrate. Peptone was added as nitrogen source which is one of the parameter for this experiment. All of the substrate already has their natural nitrogen sources from its nutrients. Therefore, 1 % of nitrogen source set for all substrates which need to add the amounts of peptone according to the balance from the actual nitrogen source from each substrate until 1%. For the another parameter which is moisture content, 50% of the moisture content been set and the amount of water added for each substrate are different according to its absorption after undergo try and error amount after autoclave process to get 50% of the moisture contents. The optimum condition was set which is moisture content 50-60%, pH 6 and optimum fermentation days were 11 days.

The composition of a solid state substrate is an important factor that can affects fungi growth and also the productivity. For the solid state fermentation, from the Table 4.2 above, we can see that only corn and rice that produce simvastatin. This might be due to the simvastatin is very complicated components that need a highly potential factors to produce. From the Table 4.2 also, we can see that rice can produce higher simvastatin then corn. But because the rice is our control substrate, therefore corn is being chosen to be proceeding to the RSM.

The initial search levels of optimum fermentation time, pH and temperature of the solid medium were selected from the based maximum production of simvastatin (Bibhu et al., 2009).

4.3 ADDITION ANALYSIS

4.3.1 CORN

Table 4.4: Corn analysis for extended day of fermentation

Simvastatin form	β-hydroxyacid		Lactone		Total yield, µg/mL
	Area, mAU*s	Yield, µg/mL	Area, mAU*s	Yield, µg/mL	
Day 11	371.61911	47.14602	0	0	47.14602
Day 15	338.00546	42.88158	33.80373	0.80334	43.68492

4.3.1 RICE

Table 4.5: Rice analysis for extended day of fermentation

Simvastatin form	β-hydroxyacid		Lactone		Total yield, µg/mL
	Area, mAU*s	Yield, µg/mL	Area, mAU*s	Yield, µg/Ml	
Day 11	180.24139	22.86660	0	0	22.86660
Day 15	1226.03027	155.54220	36.69925	0.87215	156.41435

From the Table 4.4, can proven that for the day 15, both of the acid and lactone form are existed, but because of time limited to wait until 15 days for the lactone form, therefore the analysis result for the next fermentation is only taking for acid form because the β-hydroxyacid form form elutes earlier in the chromatography column (Jaivel et al.,2010; Fredrich et al., 1995).

4.4 GROWTH STUDY FOR CORN

Table 4.6: Analysis for corn growth phase

Fermentation times, days	Area, mAU*s	Simvastatin yield, µg/mL
1	19.16789	2.43176
2	19.67785	2.49645
3	22.73696	2.88456
4	32.37396	4.10717
5	67.89967	8.61420
6	35.81486	4.54371

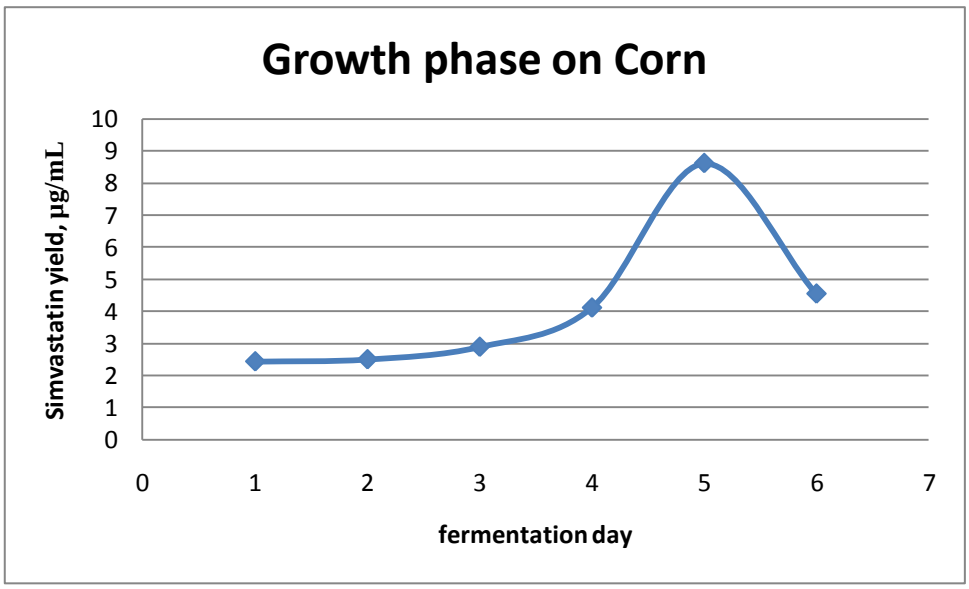

Figure 4.3: Growth study for the corn

From the Figure 4.3, the simvastatin yield decrease on day 6. The highest yield is on day 5. Therefore for the next experimental, the fermentation for response surface methodology only take 5 days to get the optimum yield for the simvastatin production. The simvastatin yield increased from day 1 to day 5, which explained that although simvastatin is a kind of secondary metabolite, its accumulation in mycelia seems growth related, which is different with the phenomena in submerged fermentation (Wet et al., 2007).

4.5 PARAMATER ANALYSIS FOR RESPONSE SURFACE METHODOLOGY (RSM)

4.5.1 NITROGEN SOURCES ADDITION (PEPTONE)

Table 4.7: Analysis for nitrogen addition parameter

Peptone Addition, gram	Area, mAU*s	Simvastatin yield, µg/mL
0.005	71.20158	9.03310
0.01	161.60641	20.50244
0.02	585.89111	74.32997
0.03	148.12372	18.79194
0.04	111.70705	14.17885

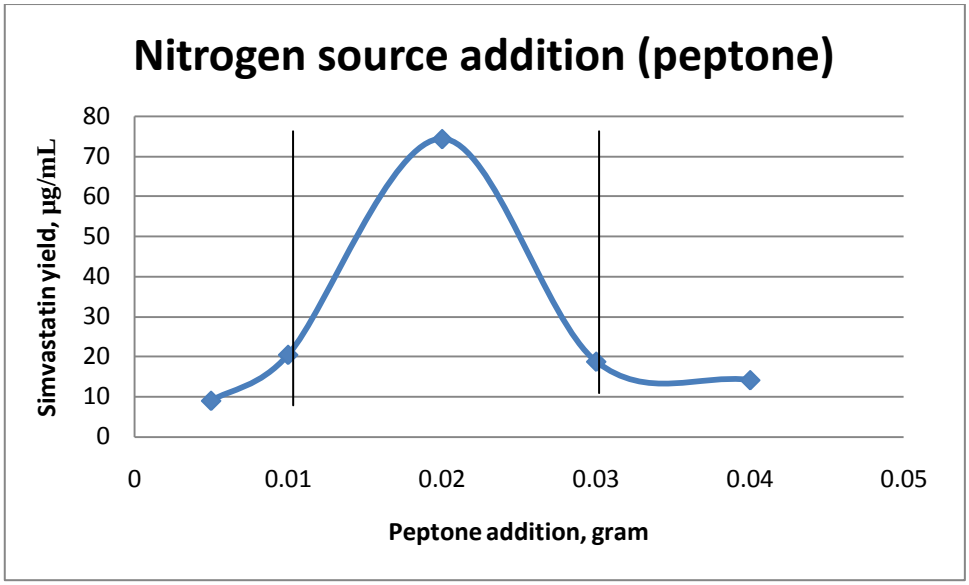

Figure 4.4: Nitrogen source addition (peptone)

From the Table 4.7 and Figure 4.4 above, simvastatin yield is highest when the peptone addition is 0.02g. The lowest value of simvastatin yield is when the peptone is 0.005g. At 0.01g, 0.03g and 0.04g the simvastatin yield is slightly same. Therefore, the range for the RSM parameters is 0.01g and 0.03g because when the addition of peptone is 0.02g, the simvastatin yield highest. Therefore, two points that before and after the optimum point is selected to proceed in RSM.

The nutrient was very important because they directly linked with the formation of the simvastatin and the metabolite. From the Figure 4.4 , we can see that when the addition of peptone increase, the production of simvastatin decrease, this might be due to the decrease in fungal cell permeability and also increased the chances of bacterial contamination.

4.5.2 MOISTURE CONTENT PERCENTAGE

Table 4.8: Analysis for moisture contents parameter

Moiture contents, %	Area, mAU*s	Simvastatin yield, µg/mL
40	268.16299	34.02091
50	710.29248	90.11234
60	549.16742	69.67096
70	276.03778	35.01995
80	266.81842	33.85033

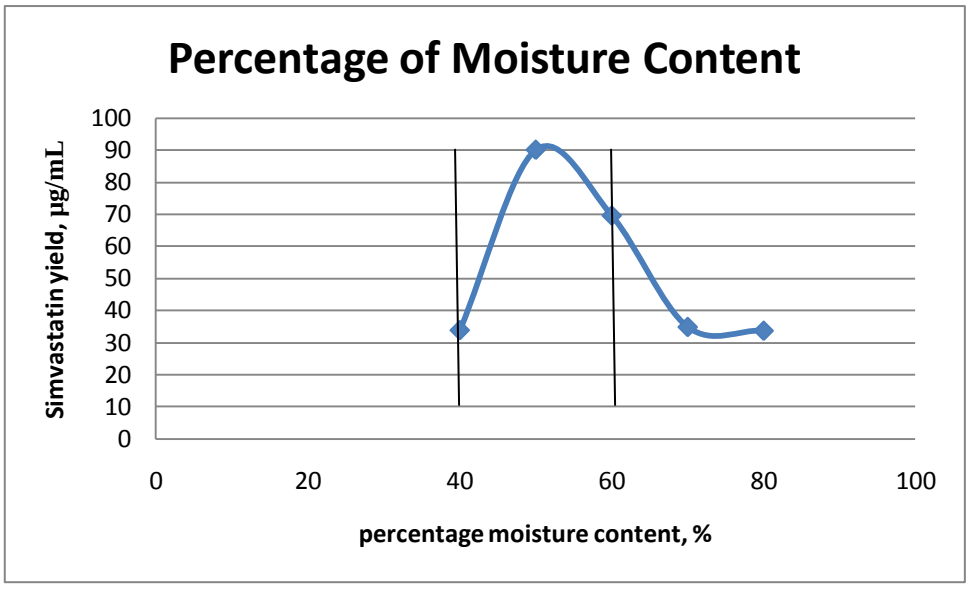

Figure 4.5: Moisture contents percentage

From the Table 4.8 and Figure 4.5, we can see that for the moisture contents that can produce highest simvastatin production is 50%. Therefore, the response surface methodology for moisture contents parameters range between 40%-60%. The value of the moisture percentage used by the smallest and the largest point between the optimum point.

The production of the simvastatin decrease when the moisture contents increase because the starch of the substrate will hydrolysis into oligosaccharide and water by the hydrolase enzyme (Pattanagal et al., 2008 ; Lin et al., 2008; Lotong and Suwarit, 1990).

4.6 RESULT AND DISCUSSION FOR RESPONSE SURFACE METHODOLOGY

4.6.1 ANALYSIS PARAMETER USING CENTRAL COMPOSITE DESIGN (CCD)

Table 4.9: The analysis of variance of the calculated parameters for simvastatin production

STD	RUN	BLOCK	FACTOR 1 A: MOISTURE CONTENT, %	FACTOR 2B: NITROGEN (PEPTONE ADDITION, gram	RESPONSE 1 SIMVASTATIN YIELD, µg/Ml
5	**1**	**BLOCK 1**	**50.0**	**0.02**	**9.23416**
6	2	BLOCK 1	50.0	0.02	8.63733
1	3	BLOCK 1	40.0	0.01	7.19412
4	4	BLOCK 1	60.0	0.03	7.86197
7	5	BLOCK 1	50.0	0.02	8.93578
2	6	BLOCK 1	60.0	0.01	8.48112
3	7	BLOCK 1	40.0	0.03	8.46551
9	8	BLOCK 2	64.14	0.02	8.18052
10	9	BLOCK 2	50.0	0.01	8.55299
11	10	BLOCK 2	50.0	0.03	8.40351
13	11	BLOCK 2	50.0	0.02	8.80877
12	12	BLOCK 2	50.0	0.02	8.81515
8	13	BLOCK 2	35.86	0.02	8.35993
14	14	BLOCK 2	50.0	0.02	8.29251

To identify the optimum levels of different process parameters influencing simvastatin production, solid-state fermentation was carried out in conical flasks containing different optimized parameters. Two parameters were chosen which is percentage of moisture content and nitrogen source (peptone) addition.

Table 4.9 taken from the analysis using Central Composite Design (CCD) at Response Surface Methodology (RSM). After entering the low and high value for each of the parameters which is moisture contents and addition of nitrogen sources, the experimental process need to be run are 14 runs. The simvastatin yield from the run showed that high simvastatin production on the run 1,2,5,11,12 and 14. These 5 runs used percentage moisture contents 50% and nitrogen source (peptone) addition was 0.02 gram. The highest production was for the first run which gives the yield 9.23416 µg/mL. The lowest production was for the third run which simvastatin yield only produces 7.19412µg/mL.

4.7 ANALYSIS RESULT FOR RESPONSE SURFACE METHODOLOGY (RSM)

Table 4.10: Analysis result using RSM

STD	RUN	BLOCK	FACTOR 1 A: MOISTURE CONTENT, %	FACTOR 2B: NITROGEN (PEPTONE ADDITION, gram	RESPONSE 1 SIMVASTATIN YIELD, µg/Ml
5	1	BLOCK 1	50.0	0.02	9.23416
6	2	BLOCK 1	50.0	0.02	8.63733
1	3	BLOCK 1	40.0	0.01	7.19412
4	4	BLOCK 1	60.0	0.03	7.86197
7	5	BLOCK 1	50.0	0.02	8.93578
2	6	BLOCK 1	60.0	0.01	8.48112
3	7	BLOCK 1	40.0	0.03	8.46551
9	8	BLOCK 2	64.14	0.02	8.18052
10	9	BLOCK 2	50.0	0.01	8.55299
11	10	BLOCK 2	50.0	0.03	8.40351
13	11	BLOCK 2	50.0	0.02	8.80877
12	12	BLOCK 2	50.0	0.02	8.81515
8	13	BLOCK 2	35.86	0.02	8.35993
14	14	BLOCK 2	50.0	0.02	8.29251

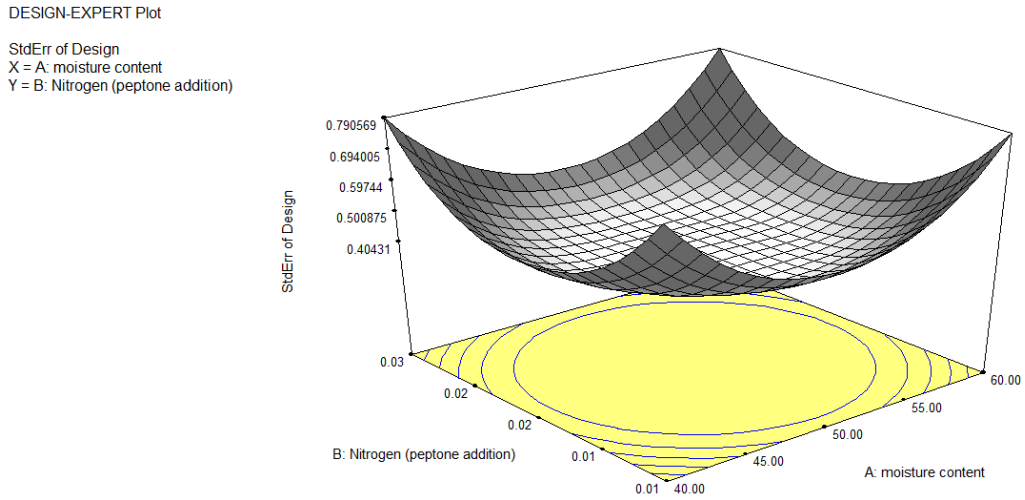

Figure 4.6: Graph for design-expert plot

Figure 4.6 above showed the Surface plot for simvastatin yield of *Monascus purpureus* FTC 5356. Variation in moisture content and addition of peptone revealed that the maximum yield for the simvastatin yield can be obtained when the moisture content was 60% and the nitrogen added From here, can be determined that, when that the Monascus purpureus need the moisture condition more that 50% but less than 70% to produce simvastatin. For addition of nitrogen, the optimization result showed that the amount of peptone added needs more that 0.01 gram but less than 0.02 gram.

4.8 SIMVASTATIN YIELD ANALYSIS

4.8.1 ANOVA FOR RESPONSE SURFACE QUADRATIC MODEL

Table 4.11: Analysis of variance Table [Partial sum of squares**]**

Source	Sum of Square	DF	Mean Square	F Value	Prob > F	
Block	0.026	1	0.026			
Model	2.22	5	0.44	3.28	0.0765	not significant
A	*0.023*	*1*	*0.023*	*0.17*	*0.6918*	
B	*0.024*	*1*	*0.024*	*0.18*	*0.6844*	
A^2	*0.91*	*1*	*0.91*	*6.76*	*0.0354*	
B^2	*0.45*	*1*	*0.45*	*3.36*	*0.1096*	
AB	*0.89*	*1*	*0.89*	*6.61*	*0.0370*	
Residual	0.95	7	0.14			
Lack of Fit	*0.59*	*3*	*0.20*	*2.19*	*0.2316*	*not significant*
Pure Error	*0.36*	*4*	*0.090*			
Cor Total	3.19	13				

The Model F-value of 3.28 implies there is a 7.65% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case A^2, AB are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms, model reduction may improve the model. The "Lack of Fit F-value" of 2.19 implies the Lack of Fit is not significant relative to the pure error. There is a 23.16% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit.

Table 4.12: Table of Lack of fit F value

Std. Dev.	0.37	R-Squared	0.7009
Mean	8.44	Adj R-Squared	0.4873
C.V.	4.35	Pred R-Squared	-0.5774
PRESS	4.99	Adeq Precision	4.875

A negative "Pred R-Squared" implies that the overall mean is a better predictor of the response than the current model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 4.875 indicates an adequate signal. This model can be used to navigate the design space.

Table 4.13: Table for Adeq Precison analysis

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	8.79	1	0.15	8.43	9.14	
Block 1	-0.043	1				
Block 2	0.043					
A- moisture content	0.054	1	0.13	-0.25	0.36	1.00
B-Nitrogen (peptone addition)	0.055	1	0.13	-0.25	0.36	1.00
A^2	-0.35	1	0.14	-0.67	-0.032	1.01
B^2	-0.25	1	0.14	-0.57	0.072	1.01
AB	-0.47	1	0.18	-0.91	-0.038	1.00

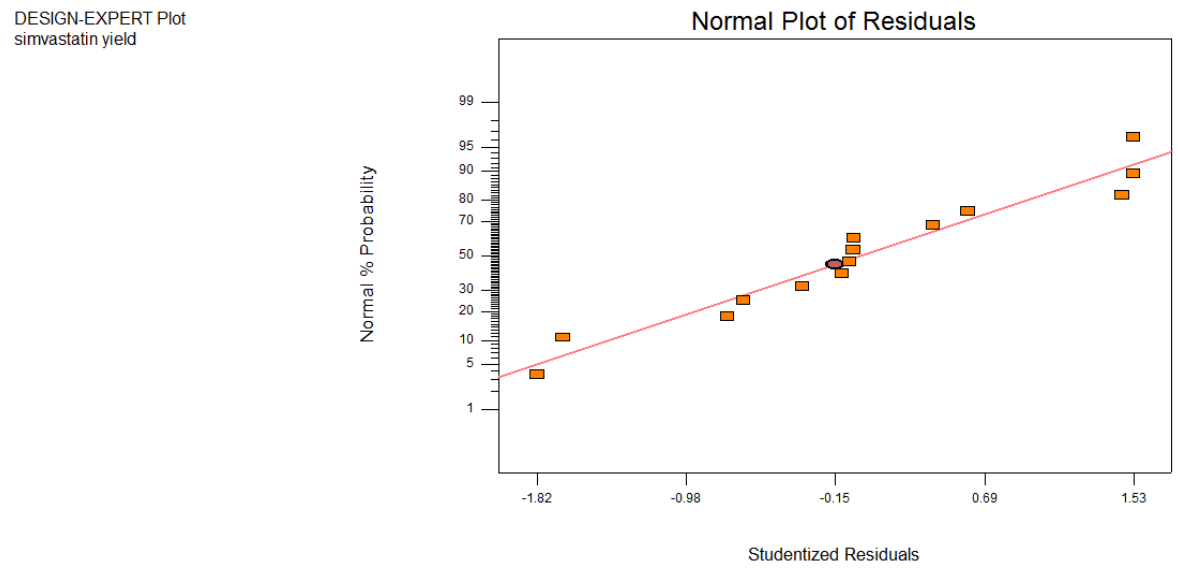

Figure 4.7: Design expert plot for simvastatin yield

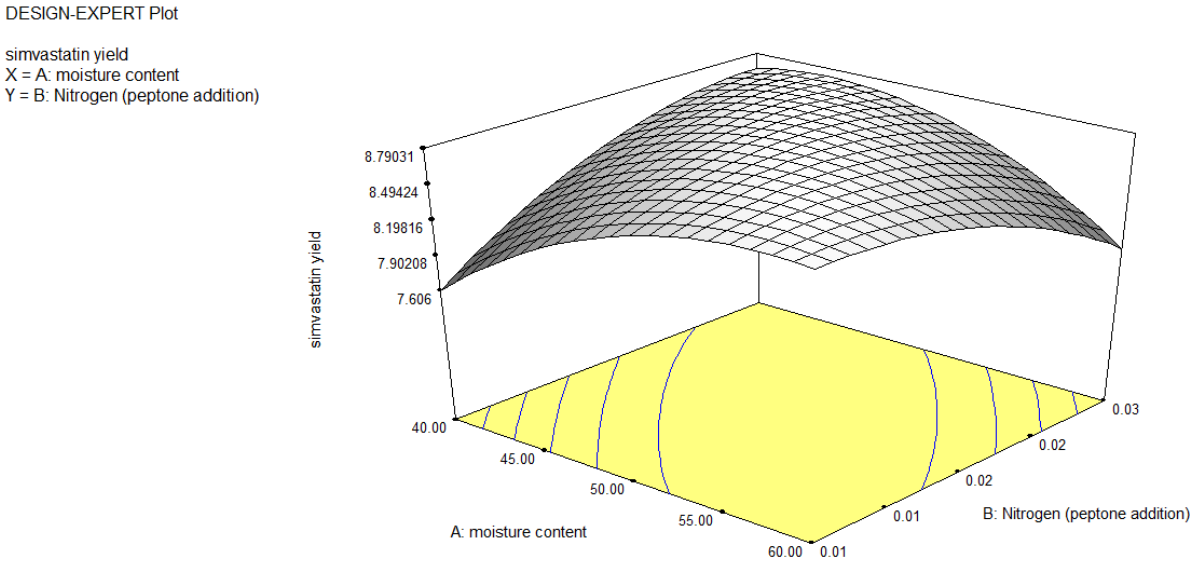

Figure 4.8: 3D graph simvastatin yield

4.9 CONCLUSION ON ANALYSIS OF RESPONSE SURFACE METHODOLOGY

The simplest and most effective way to increase the productivity is to do the variation on the nutritional requirement (Aravindan et al., 2007). Response surface methodology is a statistical method that provides an alternative ways to optimize a particular process by considering the mutual interactions among the variables and to give an estimate of the combined effects of these variables (Aravindan et al., 2005).When doing the analysis of the response surface that used to optimize the production of the required product, this process involved three major steps. First, to perform the statistically designed experiments, second, to estimate the coefficients in the mathematical model and lastly, to predict the response and checking the adequency of the model (Montgomery, 2001).

In order to produce high concentration of protein concentration, two factors were carried out in this study. The factors are percentage of moisture contents and also the concentration on nitrogen added. Apparently, the multiple regression equation for the moisture content (x_1) and nitrogen added (x_2) as the main variables was as follows:

Final equation in terms of coded factors:

$$\text{Simvastatin yield} = 8.79+0.054A+0.055B-0.35A^2-0.25B^2 - 0.47AB \qquad (1)$$

Final equation in term of actual factors:

$$\text{Simvastatin yield} = -6.107 + 0.452X_1+340.99X_2-3.51E0.03\ (X_1)^2 -2479.06(X_2)^2 - 4.73\ X_1X_2 \qquad (2)$$

According to the equation (2), the largest value estimated regression coefficient for simvastatin yield was the nitrogen added (X_2=2479.06). Thus, from here can say that it is noting that production of simvastatin affected by the addition of the nitrogen added when the fermentation process occurs. Therefore, they are helpful in understanding both the main and the interaction effects of the factors on the response value.

Hence, addition of peptone as organic nitrogen sources for the simvastatin production in this study. The more amounts of the peptone added will lower the production of the simvastatin production. From the previous study, there are concluding that a higher concentration of peptone strongly repressed the production of the simvastatin (Tsuyoshi et al.,

2006). While, a lower con concentration of peptone led to production, but unsuiTable biomass levels (Tsuyoshi et al., 2006). Another significant limiting factor influencing the regulation of lovastatin biosynthesis via growth is the nitrogen source, if higher peptone concentration or added nitrogen source without limit and consumption of carbon sources might repress lovastatin biosynthesis and reduce the statin's production respectively (Tsuyoshi et al., 2006). From here, the lovastatin used as the references because the production of simvastatin closely related with the lovastatin, because the simvastatin was the conversion of the lovastatin. So, much greater the production of lovastatin so there is a way to convert it to the simvastatin too.

Results from CCD clearly demonstrated that when the percentage moisture contents 50% and nitrogen source (peptone) addition was 0.02 gram, this give the highest production which gives the yield 9.23416 µg/mL.

From the Figure 4.7 and Figure 4.8 which is 3D graph of simvastatin yield, that is important diagnostic tool to detect and explain the departures from the assumptions that errors are normally distributed, independent of each other and the errors variances are homogenous. Therefore, from the Table 6.9 in the appendix which is the Table of Point Prediction, point prediction of the design expert software was used to determine the optimum values of the factors for maximum simvastatin production. Then, from the analyses on normal probability plot of residuals (Figure 4.7) depicted nearly a straight line residuals distribution, which denoting errors are evenly distributed and therefore support adequacy of the least-square fit.

Lastly, solid state fermentation runs were designed according to Central Composite Design of Response Surface Methodology at randomly selected different level. The process parameters of percentages of moisture contents and also the amounts of addition of nitrogen sources were positively significant factors.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

For the conclusion, corn has been selected among the other fruits which is banana, papaya, guava, pumpkin, coconut and rice because only corn that produces the simvastatin while rice also produce simvastatin but rice was being set as control substrate. From the screening of substrate selection which needs to select the best substrate, only corn and rice give result when analyze through high performance liquid chromatography (HPLC) which corn with the yield of 22.86660µg/mL while rice give the highest yield with 29.34565µg/mL. Even rice give the highest production, but rice already being set as the control substrate because rice already known can produce simvastatin in previous study, therefore corn had been selected to preceed to the next response surface methodology (RSM).

From the growth phase of the corn, the maximum day for the simvastatin production was on day 5 with the yield of 8.61420µg/mL. From the growth phase graph, the yield seem increase rapidly from day 4 to 5 but starting to decrease rapidly on day 6. Then, for the parameters analysis, the maximum percentage of moisture content that can produce highest production of simvastatin was 50% that can produce yield of 90.11234µg/mL while for the addition of peptone, the yield was the highest on the addition of 0.02g with the production of 74.32997µg/mL. Therefore, the range that set for the lower and higher range for the

percentage moisture content that enter the RSM was set for 40%-60% while for addition of nitrogen source, the lower and higher range that enter RSM was from 0.01-0.03g.

After undergo solid state fermentation according to the parameters that been set, there are 14 runs need to be experimented to get the optimum condition after entering the RSM. The highest production was for the first run which gives the yield 9.23416 µg/mL. The lowest production was for the third run which simvastatin yield only produces 7.19412µg/mL. Due to the several factors such as the condition in the laboratory while doing RSM was not in good condition because the temperature inside the laboratory was high cause of unfunction air conditional for a few days. Due to the non air condition at the laboratory, the HPLC also cannot run on the time because the high temperature will cause the HPLC not functioning well. Even the fungal *Monascus purpureus* can live longer, perhaps the condition inside the laboratory was not good enough to growth it. Without the bad condition, the result for the optimization can obtain well enough for the experimental.

5.2 RECOMMENDATION

To improve this research in the future hopefully we can try to used another method, so we can compared the result of the production of simvastatin with the previous study. Also, we can find another local fruits that can produce simvastatin higher than rice and corn, so we can increase the production of the simvastatin in the future. There also recommend that while using High Performance Liquid Chromatography (HPLC), using the new HPLC column that has long separation.

REFERENCES

Alberto C., (2003). The Safety of HMG-CoA Reductase Inhibitor in Special Populations at High Cardiovascular Risk. *Journal of Cardiovascular Drugs and Therapy 17 285-285 2003.*

Allison M. M., Elizabeth S., Angelo M.D.M., Stephan J. F., Elizabeth A. P., (2010). Statin Drugs, Serum Cholesterol, and Prostate Specific Antigen in the National Health and Nutrition Examination Survey 2001-2004. *Journal of Cancer Causes Control (2010) 21: 671-678 DOI 10.1007/s10552-009-9494-9*

Amal H. A., Abdessamad D., Peter P., (2011). Fifty years of Drug Discovery from Fungi. *Journal of Fungal Diversity 2011) 50:3-19 DOI 10.1007/s13225-011-0116-y*

Bao J. X., Qi J. W., Xiao Q. J., Chang K. S., (2005). Enhanced Lovastatin Production by Solid State Fermentation of Monascus Ruber. *Retrived from Biotechnology and Bioprocess Engineering 2005, 10:78-84.*

Bibhu P. P., Saleem J., Mohamed A., (2008). Optimization of Fermentation Parameters for Higher Lovastatin Production in Red Mold Rice through Co-culture of Monascus purpureus and Monascus ruber. *Food Bioprocess Technol DOI 10.1007/s11947-008-0072-z*

Bibhu P. P., Saleem J., Mohamed A., (2009). Engineering Rice Based Medium for Production of Lovastatin with Monascus Species. *Czech J. Food Sci., 27:352-360*

Bibhu P. P., Saleem J., Mohd A., (2009). Statistical Analysis and Validation of Process Parameters Influencing Lovastatin Production by Monascus purpureus MTCC 369 under Solid-state Fermentation. *Biotechnology and Bioprocess Engineering 2009, 14:123-127*

Bin Z., Hui Y. F., Hai Y., Zhi M. D., Wei S., Jian S., Jian Z., (2008). Bioconversion of Lovastatin to a Novel Statin by Amycolatopsis sp. *Journal of Appl. Microbial Biotecnol (2008) 79:289-216, DOI 10, 1007/s00253-008-1430-5*.

Chang C. N., Fuu S., Chun L. W., Yuan T. S. Fermentation of Monascus Purpureus on Agri-By-Products to Make Colourful and Functional Bacterial Cellulose (NATA). *Department of Horticulture National Taiwan University*.

Jaleh V., Naser T., Fatemah A. S., (2011). Effect of Temperature and Stirring Rate of Flow and Compact- Ability Properties of Simvastatin Spherical Crystals. *International Journal of Pharmacy and Pharmaceutical Sciences ISSN- 0975-1491, Vol 3, Issue 3, 2011*.

Javier B. G., Roxana U.M., (2010). Biotechnological Production and Application of Statins. *Journal of Appli. Microbial Biotechnol (2010) 85:869-803, DOI 10, 1007/s00253-009-2239-6*.

Jianping L., Jiang Z., Yi S., Sameline G., Terje A.,Vinjar F., (2005). Chinese Red Yeast Rice (Monascus Purpureus) for Primary Hyperlipidemia: A Meta-Analysis of Randomized Controlled Trials. *Retrived fromhttp://www.cmjournal.org/content/1/1/4*

Julio A., Sergio A., Patricia A.A., Oscar F., Enrique Z.P., Margarita H., (2003). Production and Purification of Statins from Pleurotus ostreatus (Basidiomycetes) Strains. *Retrived from: www.znaturforsch.com*

Keon H. K., Ji Y. K., Dong H. K., Sun H. P., Seon H. P., Dooil K., Ki D. P., Young J. K., Heung C. J., Jae G. P., Taeha A., Chul H. Y., (2010). Generation of Human Chiral Metabolites of Simvastatin and Lovastatin by Bacterial CYP102A1 Mutants. *School of Biological Sciences and Technology, Chomam National University, Gwangju 500-757*

Khaled M. A., 2007. *The Application of LC-MS-MS To Study The Effects of Pharmacokinetics of Simvastatin in Healthy Malaysian Subjects.* PhD Thesis University Sains Malaysia, Malaysia.

Lennart N., Per E., Pierre C., Lena J., (2011). Effects of Simvastatin on Proinflammatory Cytokines and Matrix Metalloproteinases in Hypercholesterolemic Individuals. *Journal of Inflammation, Vol. 34, No. 4, August 2011 (2010) DOI:10.1007/s10753-010-9227-y*

M.Manzoni and M.Rollini, (2002). Biosynthesis and Biotechnological Production of Statins by Filamentous Fungi and Application of These Cholesterol Lowering Drugs. *Journal of Appl Microbiol Biotechnol (2002) 58:555-564 DOI 10.1007/s0053-002-0932-9.*

Najma S., M.Saeed Arayne, Waseem S., (2010). Simultaneous Determination of Cetraxone Sodium and Statin Drugs in Pharmaceutical Formulations and Human Serum by RP-HPLC. *Journal of J. Chil. Chem. Soc, 55, Nº2 (2010).*

N.Jaivel and P.Marimuthu, (2010). Optimization of Lovastatin Production is Solid-state Fermentation by Aspergillus terreus. *International Journal of Engineering Science and Technology Vol.2 (7), 2010, 2730-2733*

Patcharee P., Renu P., Aphirak P., Somsak T., (2008). Mevinolin, Citrinin and Pigments of Adlay Angkak Fermented by Monascus species. *Retrived from www.elsevier.com/locate/ijfoodmicro*

Ruchir C. P., Rekha S. S., (2010). Response Surface Methodoly for Optimization of Production of Lovastatin by Solid State Fermentation. *Brazillian Journal of Microbiology (2010) 41:164-172, ISSA 1517-8382.*

Sadik A. S., Bibhu P. P., Saleem J., Mohd A., (2007). Screening of Nutrients Parameters for Lovastatin Production MTCC369 Under Submerged Fermentation Using Plackett-Burman Design. *Research Journal of Microbiology 2(7): 601-605, 2007*

Sarah J. B., Susyu L.P., Karl L.O., Thomas J. K., Jane K., (2005). Clinical and Economics Outcomes of Conversion of Simvastatin to Lovastatin in a Group Model Health Maintanance Organization. *Journal of Managed Care Pharmacy.*

Shaheen E. L, Sanjit B., Magdalena H., (2010). Statins and Clinical Outcome of Acute Ischemic Stroke: A Systematic Review. *Journal of Lakhan et al International Archieves of Medicine 2010, 322.*

Stephen J. N., Nicholls, E. Murat T., Ilke S., Adam W. G., Paul S., Tingfei H., Kathy W., Tim C., Milind Y. D., Stanley L. H., Samir R. K., Steven E. N., (2007). Statins, High-Density Lipoprotein Cholesterol and Regression of Coronary Atherosclerosis. *Journal of American Medical Association.*

Subhagar S., Aravindan R., Viruthagiri T., (2010). Statictical Optimization of Anticholesterolemic Drug Lovastatin Production by the Red Mold Monascus Purpureus. *Retrived from www.elsevier.com/locate/fbp*

Sumathy B., Carlos R. S., Ashok P., (2005). Jackfruit Seed- A Novel Sustrate for Production of Monascus Pigment through Solid State Fermentation. *Food Technol. Biotechnol. 44 (4) 465–471 (2006)*

Sumathy B., Carlos R.S., Ashok P., (2006). Solid-state fermentation for the production of Monascus pigments from jackfruit seed. *Retrived from www.sciencedirect.com*

Sung S. Y., James D., Julio C., Mustapha E., Steven W., Joseph H., Lisa D., Alison E. B. Rising Statin Use and Effect on Ischemic Stroke Outcome. *Research Article retrived from http://www. biomedcentral.com/1741-7015/2/4*

T.Miyake, K.Uchitomi, M.Y.Zhang, I.Kono, N.Nozaki, H.Sammoto and Kenji Inagaki, (2006). Effects of the Priciple Nutrients on Lovastatin Production by Monascus Pilosus. Biosel.*Biotechnol.,70(5).1154-1159.2006*

Tom B. V., Erik D., Ivan U., Stefan H. K., Miroslav R., IJsbrand M., (2003).

Differences Between Lovastatin and Simvastatin. Hydrolysis of Lovastatin is Twice that of Simvastatin. *The Scientific World JOURNAL.*

Venkateswaran V, Vijayalaksmi G, (2010). Finger Millet (Eleusine Cococana) an Economically Viable Source for Antihypercholesterolemic Metabolites Production by Monascus Purpureus. *Journal from Jfood Sci Technol (July-August 2010).*

Vikram B. W. and Paul W. S., (2007). Synergistic Antiproliferative Effects of ɣ- Tocotrienol and Statin Treatment on Mammary Tumor Cells. *Journal of Lipids (2007) 42: 1113-1123 DOI 10.1007/s11745-007-3102-0.*

Vikram B. W., Sunitha V. Bachawal, Paul W. S., (2009). Suppression in Mevalonate Synthesis Mediates Antitumor Effects of Combine Statin and ɣ- Tocotrienol Treatment. *Journal of Lipids (2009) 44:925-934 DOI 10.1007/s11745-009-3344-0.*

Vineet C., Palak U.C., Erdal C., (2007). Beyond Lipid Lowering: The Anti-Hypertensive Role of Statins. *Journal of Cardiovasc Drugs Ther (2007) 21: 161-169 DOI 10.1007/s10557-007-6025-3.*

Wei K. and Yi Z., (2011). The Pleiotropic Effects of Statins in the prevention of Artherosclerosis. *Journal of Cardiovasc Drugs Ther DOI 10.1007/s10557-011-6353-1*

Wei P. L., Xu Z. N., Cen P. L., (2006). Lovastatin Production by Aspergillus Terreus in Solid-state Fermentation. *Journal of Zheijang University SCIENCE A, 2007 8(9):1521-1526*

Xinkai X. and Yi T., (2007). Efficient Synthesis of Simvastatin by Use of Whole-Cell Biocatalysis. *Journal of Appl. Environ. Microbial. 2007, 73(7):2054. DOI:10.1128/ AEM. 02820-06.*

Yaw N. C., Jen C.H., Chih C.L., Ing L. S., Yew M.T.,(2002). Use of Response Surface

Methodology to Optimize Culture Medium for Production of Lovastatin by Monascus ruber. *Retrieved from www.elsevier.com/locatc/enzmictcc*

Young H. P. and Shin Y. S., (2010). Simultaneous Production of Natural Statins and Coenzyme Q_{10} by Monascus Pilosus Fermentation Using Different Solid Substrates. *Journal of Food Sci Biotechnol, 19(6): 1635-1641(2010), DOI 10: 1007/s 10068-010-0231-7.*

APPENDIX

6.1 DESIGN SUMMARY

STUDY TYPE = RESPONSE SURFACE
INITIAL DESIGN = CENTRAL COMPOSITE
DESIGN MODEL = QUADRATIC
EXPERIMENT = 14
BLOCKS = 2

Table 6.1: Design Summary

RESPONSE	NAME	UNITS	OBS	MINIMUM	MAXIMUM	TRANS	MODEL
Y1	SIMVASTATIN YIELD	MICROGRAM/ML	14	7.19	9.23	NONE	QUADRATIC
FACTOR	NAME	UNITS	TYPE	LOW ACTUAL	HIGH ACTUAL	LOW CODED	HIGH CODED
A	MOISTURE CONTENT	PERCENTAGE, %	NUMERIC	40.0	60.0	-1.000	1.000
B	NITROGEN (PEPTONE ADDITION)	GRAM, g	NUMERIC	1.000E-002	0.030	-1.000	1.000

6.2 EVALUATION RESULTS

2 FACTORS: A, B
DESIGN MATRIX EVALUATION FOR RESPONSE SURFACE QUADRATIC MODEL
NO ALIASES FOR QUADRATIC MODEL
DEGREES OF FREEDOM FOR EVALUATION

BLOCK	1
MODEL	5
RESIDUALS	7
LACK OF FIT	3
PURE ERROR	4
CORR TOTAL	13

Table 6.2: Evaluation of result from RSM

				Power at 5% alpha level for effect of		
TERM	STDERR**	VIF	RI-SQUARED	½ STD.DEV	1 STD DEV	2 STD DEV
BLOCK 1	0.27					
A	0.35	1.00	0.0000	9.4%	23.2%	68.1%
B	0.35	1.00	0.0000	9.4%	23.2%	68.1%
A^2	0.37	1.01	0.0059	21.8%	64.7%	99.6%
B^2	0.37	1.01	0.0059	21.8%	64.7%	99.6
AB	0.50	1.00	0.0000	7.2%	14.0%	40.8%

****basis std. dev. = 1.0**

6.3 MEASURED DERIVED FROM THE $(X'X)^{-1}$ MATRIX

Table 6.3: Table of measured derivation from matrix

STD	LEVERAGE	POINT TYPE
1	0.6964	Fact
2	0.6964	Fact
3	0.6964	Fact
4	0.6964	Fact
5	0.2381	Center
6	0.2381	Center
7	0.2381	Center
8	0.6964	Axial
9	0.6964	Axial
10	0.6964	Axial
11	0.6964	Axial
12	0.2381	Center
13	0.2381	Center
14	0.2381	Center

Average = 0.5

Maximum Prediction Variance (at a design point) = 0.696

Average Prediction Variance = 0.5

Condition Number of Coefficient Matrix = 1.167

G Efficiency (calculated from the design points) = 71.8 %

Scaled D-optimality Criterion = 1.683

Determinant of $(X'X)^{-1}$ = 3.633E-7

Trace of $(X'X)^{-1}$ = 1.009

Table 6.4: Correlation Matrix of Regression Coefficients

	Intercept	Block 1	A	B	A^2	B^2	AB
Intercept	1.000	-	-	-	-	-	-
Block 1	-0.000	1.000	-	-	-	-	-
A	-0.000	-0.000	1.000	-	-	-	-
B	-0.000	-0.000	-0.000	1.000	-	-	-
A^2	-0.555	-0.000	-0.000	-0.000	1.000	-	-
B^2	-0.555	-0.000	-0.000	-0.000	0.077	1.000	-
AB	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000	1.000

Table 6.5: Correlation matrix of factors [Pearson's r]

	Block 1	A	B	A^2	B^2	AB
Block 1	1.000	-	-	-	-	-
A	0.000	1.000	-	-	-	-
B	0.000	0.000	1.000	-	-	-
A^2	-0.000	0.000	0.000	1.000	-	-
B^2	-0.000	0.000	0.000	-0.077	1.000	-
AB	0.000	0.000	0.000	0.000	0.000	1.000

6.4 F/T SUMMARY

Table 6.6: Response: Simvastatin Yield (Sequential models sum of squares)

Source	Sum of squares	DF	Mean square	F value	Prob> F	
Mean	**998.34**	**1**	**998.34**	**-**	**-**	**Suggested**
Block	0.026	1	0.026	-	-	-
Linear	0.047	2	0.024	0.076	0.9273	-
2FI	0.89	1	0.89	3.62	0.0896	-
Quadratic	**1.28**	**2**	**0.64**	**4.72**	**0.0503**	**Suggested**
Cubic	0.20	2	0.10	0.68	0.5468	Aliased
Residual	0.74	5	0.15	-	-	-
Total	1001.53	14	71.54			

Table 6.7: Lack of Fit Tests

Source	Sum of squares	DF	Mean square	F value	Prob> F	
Linear	2.76	6	0.46	5.14	0.0676	-
2FI	1.87	5	0.37	4.17	0.0957	-
Quadratic	**0.59**	**3**	**0.20**	**2.19**	**0.2316**	**Suggested**
Cubic	0.39	1	0.39	4.31	0.1606	Aliased
Pure error	0.36	4	0.09	-	-	-

Table 6.8: Model Summary Statistics

Source	Std. Dev	R-squared	Adjusted R-squared	Predicted R-squared	PRESS	
Linear	0.56	0.0150	-0.1820	-1.1241	6.72	-
2FI	0.50	0.2974	0.0632	-1.5634	8.11	-
Quadratic	**0.37**	**0.7009**	**0.4873**	**-0.5774**	**4.99**	**Suggested**
Cubic	0.39	0.7651	0.4362	-17.5017	58.54	Aliased

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Table 6.9: Point Prediction

Factor	**Name**	**Level**	**Low level**	**High level**	**Std Dev**		
A	Moisture content, %	50.0	40.0	60.0	0.000		
B	Nitrogen (Peptone addition)	0.02	0.01	0.03	0.000		
	Prediction	SE Mean	95%Cl low	95% Cl high	SE pred	95%PI low	95% PI high
Simvastatin yield	8.78728	0.15	8.43	9.14	0.40	7.85	9.73

Table 6.10: Solutions for optimization of parameter

Number	moisture content*	Nitrogen (peptone addition)*	Desirability
1	*43.95*	*0.02*	1.000
2	*52.24*	*0.02*	1.000
3	*50.99*	*0.03*	1.000
4	*57.74*	*0.02*	1.000
5	*44.33*	*0.03*	1.000
6	*48.07*	*0.01*	1.000
7	*45.97*	*0.01*	1.000
8	*48.44*	*0.02*	1.000
9	*48.33*	*0.02*	1.000
10	*40.63*	*0.02*	1.000

Table 6.11: Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	7.19	7.56	-0.37	0.696	-1.820	1.086	-2.322	3
2	8.48	8.62	-0.13	0.696	-0.664	0.145	-0.635	6
3	8.47	8.62	-0.15	0.696	-0.754	0.186	-0.728	7
4	7.86	7.78	0.081	0.696	0.402	0.053	0.376	4
5	9.23	8.74	0.49	0.238	1.528	0.104	1.732	1
6	8.64	8.74	-0.11	0.238	-0.332	0.005	-0.310	2
7	8.94	8.74	0.19	0.238	0.595	0.016	0.566	5
8	8.36	8.05	0.31	0.696	1.527	0.764	1.730	13
9	8.18	8.20	-0.022	0.696	-0.108	0.004	-0.100	8
10	8.55	8.26	0.30	0.696	1.463	0.701	1.625	9
11	8.40	8.41	-0.089	0.696	-0.045	0.001	-0.041	10
12	8.82	8.83	-0.015	0.238	-0.047	0.000	-0.044	12
13	8.81	8.83	-0.022	0.238	-0.067	0.000	-0.062	11
14	8.29	8.83	-0.54	0.238	-1.676	0.125	-2.005	14

Note: Predicted values include block corrections.

Proceed to Diagnostic Plots (the next icon in progression). Be sure to look at the:

1) Normal probability plot of the studentized residuals to check for normality of residuals.
2) Studentized residuals versus predicted values to check for constant error.
3) Outlier t versus run order to look for outliers, i.e., influential values.
4) Box-Cox plot for power transformations.

Figure 6.1*: Monascus purpureus* sp.in slant agar

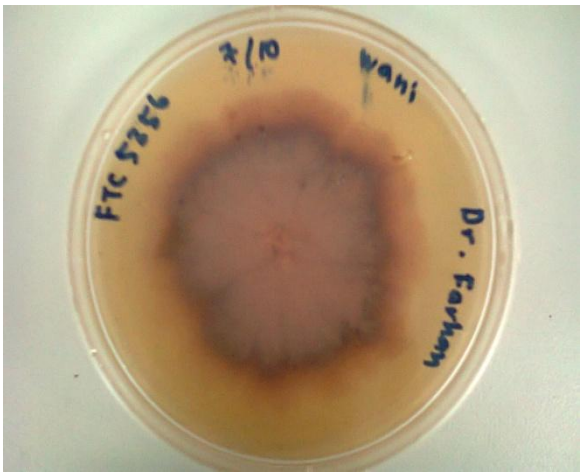

Figure 6.2: *Monascus purpureus* sp. in petri plate

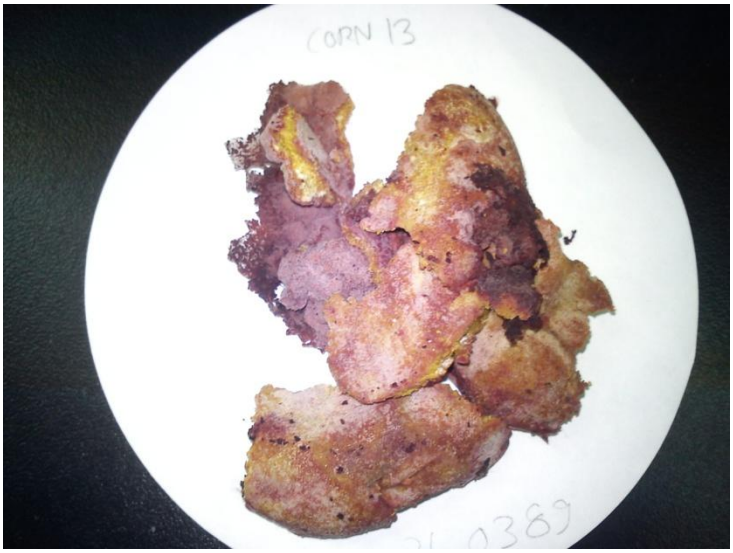

Figure 6.3: Picture of *Monascus purpureus* sp. while in solid state fermentation before drying

Figure 6.4: Picture of *Monascus purpureus* sp. while in solid state fermentation after drying

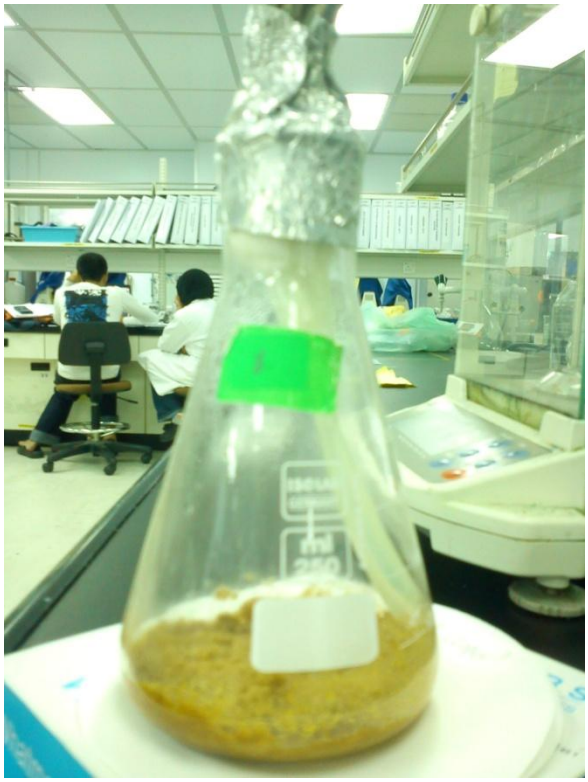

Figure 6.5: Solid state fermentation done in conical flask

Figure 6.6: *Monascus purpureus* sp. with the rice as a substrate

Figure 6.7: *Monascus purpureus* sp. with the corn as a substrate

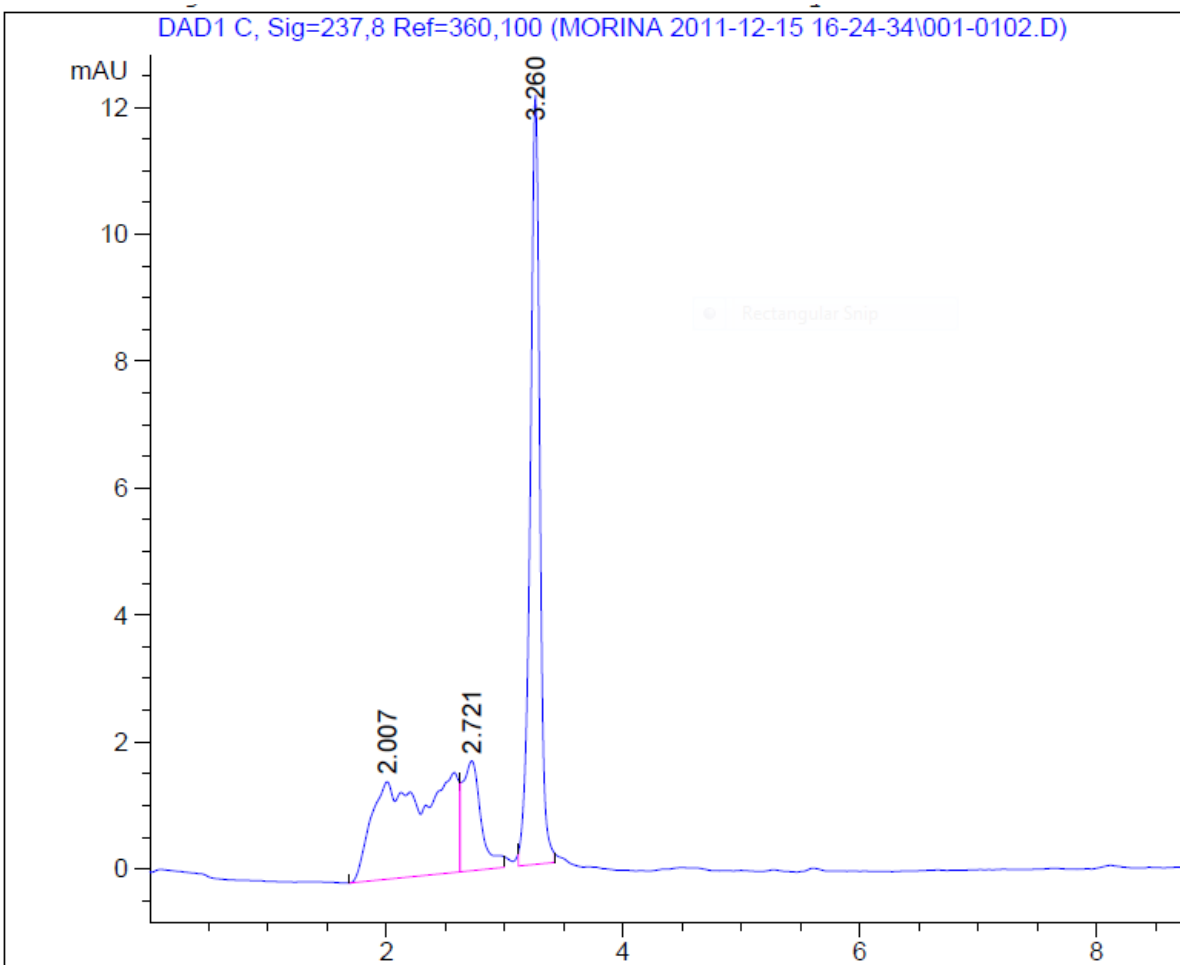

Figure 6.8: Analysis graph using HPLC

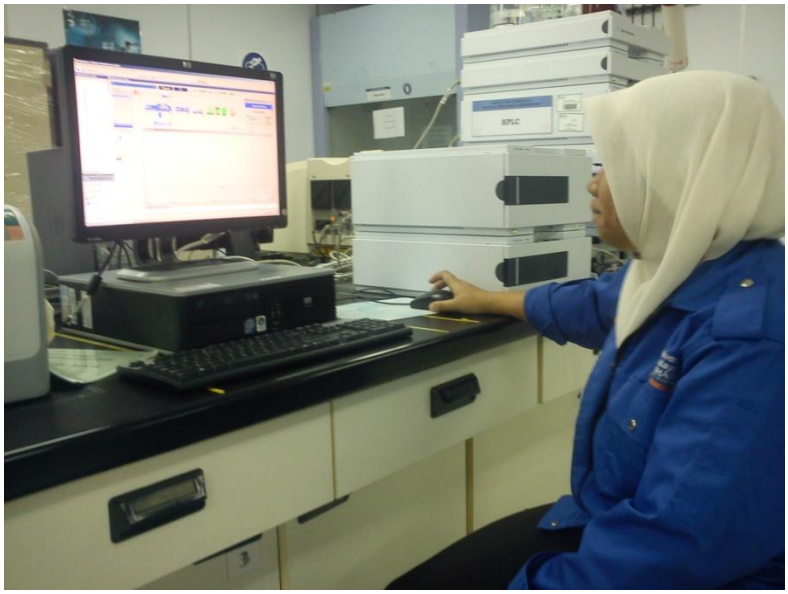

Figure 6.9 HPLC