DEVELOPMENT OF SIMVASTATIN PRODUCTION BY MONASCUS PURPUREUS IN SOLID-STATE FERMENTATION USING AGRICULTURAL PRODUCT

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ABSTRACT

Monascuspurpureus is a non-pathogenic fungus that can produce statin called simvastatin, which can lower blood cholesterol level. The objectives of this research were to explore the potential of agricultural product on simvastatin and identify the optimal condition of simvastatin production in solid-state fermentation by Monascuspurpureus FTC 5356. The local agricultural products used were banana, guava, pumpkin, coconut meat, corn and papaya. Initially, the local agricultural products were ground and the initial moisture content of the agricultural products was fixed at 50% and pH 6. The mixtures were then incubated at 30°C for 11 days. Later, variety conditions of initial moisture content and nitrogen supplementation were introduced and examined on the simvastatin. Further experimental work was carried out using Central Composite Design (CCD) of Response Surface Methodology (RSM), with two factors of initial moisture content and nitrogen source. The results suggested that, among the agricultural products tested; only corn powder was able to produce simvastatin. The optimal condition for simvastatin production on corn was at 50% initial moisture content with supplementation of 0.2% nitrogen source.

KEYWORDS*: simvastatin; Monascus; solid-state fermentation; response surface; central composite design*

1.0 INTRODUCTION

Statins are a group of drugs that mainly used in lowering blood cholesterol. Statins are generally capable of bringing down the cholesterol level by from 20% to 60%. The discovery of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A),which acts as inhibitors was a step forward in the prevention of hypercholesterolemia and related diseases such as atherosclerosis, peripheral arterial disease, cerebro vascular disease, sepsis, ischemic disease and bone fracture (Khaled, 2007; Sultana et al., 2010). Recently, cardiovascular diseases are the major causes of death. Hence, there is a need for in developing processes for production of statins with approval by the Food and Drug Administration (FDA).

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Statins are fungal secondary metabolites and the first enzyme in cholesterol biosynthesis (Manzoni & Rollini, 2002). *Monascus sp.*is a unique fungi which is able to produce various potential useful metabolites consists of statins, pigments and antimicrobial agent (Chang et al.,2002; Manzoni & Rollini, 2002). Natural statins have a common molecular structure, a hexahydro-naphthalene system and α -hydroxy-lactone, but they differ on the side chains and a methyl group around the ring. There are five statins are used as for clinical use, Lovastatin and pravastatin (mevastatin derived) which are natural statins of fungal origin, while simvastatin is a semi-synthetic lovastatin derivative. Atorstatin and fluvastatin which are synthetic statins, which derived from omevalonate and pyridine (Manzoni & Rollini, 2002). Lovastatin, simvastatin and pravastatin are all derived from fungi.

Generally, the statins differ with respect to their ring structure and substituent. These differences in structure affect the pharmacological properties of the statins. 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 gained intense interest because of its importance in the pharmaceutical industry. Pharmaceutical product is known as pricey product because of the high production cost. Therefore, by using solid-state fermentation process on a local agricultural product is an alternative way to reduce the production cost.

Till date, no prior work has been done on this particular strain of *Monascuspurpureus FTC 5356* especially on solid state fermentation of agricultural product on simvastatin production. Hence, this study was focusing on identifying the potentiality of agricultural product and investigating the effect of various conditions on simvastatin by *Monascuspurpureus FTC 5356* in solid-state fermentation.

2.0 METHODOLOGY

2.1 Culture preparation

A culture of *Monascuspurpureus* FTC 5356 was obtained from the Malaysian Agricultural Research and Development Institute (MARDI). It was maintained on potato dextrose agar (PDA) slants and petri dish. Spores and hyphae were scrapped off from the agar slant and suspended in 10 mL sterilized deionized water at room temperature. The suspension was adjusted to approximately 10^7 spores mL^{-1} (Said, 2010). The spore suspension obtained was used as the inoculum suspension.

2.2 Substrate Preparation

Six different local agricultural products, which are pumpkin, papaya, banana, guava, coconut meat and corn, were prepared. The local agricultural products were purchased locally. The products/substrates were cut into 2-4 mm thick and dried at 60°C overnight.

The dried substrates were ground (Carvalho et al., 2006). The ground substrates were sieved to attain the size particle of 315µm.

2.3 Solid State Fermentation

For solid state fermentation, known amount of each substrate (pumpkin, papaya, banana, guava, coconut meat and corn) was placed in 250 mL of Erlenmeyer flask and distilled water was added to achieve an its initial moisture content of approximately 50% (w/w) in different flask. A 40% (w/w) solution of zinc sulphate ($ZnSO_47H_2O$) (0.128 M) and 0.2% (w/w) of nitrogen source (peptone) were added to each flask. The pH of each flask was adjusted to pH 6. The flasks were then autoclaved. Later, the flask was inoculated with 10% (w/w) spore suspension (10^7 spores mL^{-1}) and incubated at 30°C for 11 days, unless stated otherwise.

In a separate experiment, simvastatin was further examined on the selected substrate for their simvastatin production, on day 5 and 15. Based on the preliminary experiment, corn powder was selected.

For the effect of initial moisture content, 10 g of corn powder was placed in an Erlenmeyer flask and distilled water was added to achieve initial moisture of approximately 40% to 80%(w/w) in different flasks. $ZnSO_47H_2O$ and peptone were fixed at 40% and 0.2% (w/w), respectively. pH was adjusted to pH 6 and the flasks were incubated at 30°C. In a separate experiment, 10 g of corn powder was placed in an Erlenmeyer flask and peptone was added to achieve approximately 0.05% to 0.4% (w/w) in different flasks. All of the flasks were autoclaved. Then, each flask was inoculated with 10% spore suspension and incubated at 30°C for 5 days.

2.4 Analytical Methods

Fermented solids were dried for 24 hours at 60°C in the oven. The powder was stored at -20^{o}C for further analysis. For simvastatin extraction, an amount of dried fermented solids powder was mixed with a mixture of methanol/ water (1:1) and the pH was adjusted to pH 7.7. Then, the mixture was kept at 200 rpm, 2hours at 30^{o}C. Later, the solution was centrifuged and the supernatant was filtered through 0.45µm nylon membrane filters. Analysis of simvastatin was carried out in Quaternary HPLC Agilent Technologies 1200 Series using Luna C_{18} column (250 x 4.6 mm, 5µm). The mobile phase was composed of acetonitrile (0.1 % orthophosphoric acid):water in the ratio of 60:40 (v/v) (Subhagar et al., 2010).The mobile phase was pumped at a flow rate of 1mL min^{-1} at 28^{o}C and 237nm with IR detector. The binary gradient system was used and the samples were injected manually using loop injector of 10 µl. Pharmaceutical grade Simvastatin powder obtained from Sigma Chemical Co was used as a standard solution. Calibration curve was linear, ranging from 2 µg/mL to 10 µg/mL. The standard solution of simvastatin existed in two forms, which were lactone and β-hydroxy acid. About 2 mg of simvastatin powder accurately weighed and transferred into volumetric flask 10

mL, methanol was added and mixed well. The solution was filtered through a 0.45µm membrane filter and further diluted for lactone standard preparation. For β-hydroxy acid, about 2 mg of simvastatin powders accurately weighed and suspended in 0.1 M NaOH, heated at 50°C for one hour. The pH was adjusted to pH 7.7 and the volume was filled up to 10 mL. The solution was then filtered through 0.45µm membrane filter.

2.5 Experimental Design By Response Surface Methodology (RSM)

The investigation on the combined effect of initial moisture content and additional nitrogen source (peptone) on simvastatin production was analyzed by experimental design, which was established by the Design Expert (Stat-Ease, Inc, Version 6.0.8). The values of factors, initial moisture content and additional nitrogen sources were based on the effect of sole factors on simvastatin production, as in the earlier study. Experimental run was carried out using Central Composite Design (CCD) of RSM.

3.0 RESULTS AND DISCUSSION

3.1 Substrate Selection For Simvastatin Production

Six different local agricultural products were evaluated on the efficacy of simvastatin production. The local agricultural products used were pumpkin, papaya, banana, guava, coconut meat and corn. Figure 1 shows the amount of simvastatin produced by each substrate at day 11, which known as simvastatin yield (µg/mL).

Figure 3.1 shows only corn powder was able to produce simvastatin with a yield of 34.09 µg/mL. While the other substrates such as pumpkin, guava, banana, coconut meat and papaya were unable to produce simvastatin. This phenomenon occurred due to the high amount of carbohydrate contained in corn compared to other agricultural products tested (Nutrition Fact, 2014). In this context, carbohydrate is referred to carbon source. Carbon is a main compound of carbohydrate (C_m (H_2O) $_n$) and a crucial component in producing simvastatin ($C_{25}H_{38}0_5$), which contained only carbon, hydrogen and oxygen. In view of this finding, it shows that chemical composition of the substrate plays an important role for fungal growth and product development.

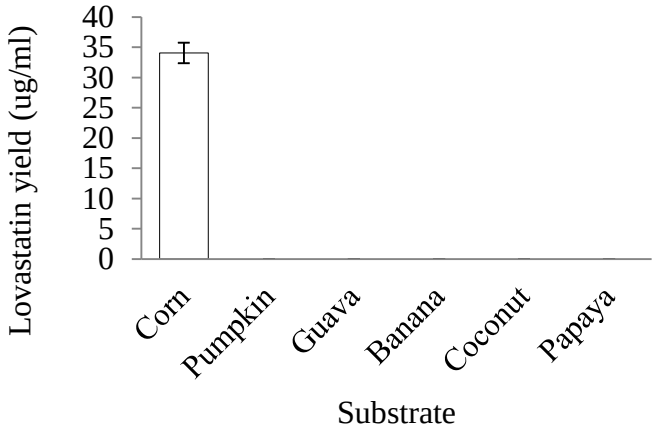

Figure 3.1: Simvastatin yield on different substrate.

The extended incubation period was applied to the selected substrate; corn. Table 3.1 shows that prolong incubation period tend to produce better yield of simvastatin. Simvastatin production increased from 8.61μg/mL (day 5) to 97.69 μg/mL (day 15). During fermentation, the carbohydrate was degraded into simple sugar; hence, more products were synthesized by the fungus.

Table 3.1. Simvastatin yield on rice and corn powder as a substrate

Substrate	Incubation period	Simvastatin yield (ug/mL)
Corn powder	Day 5	8.61
	Day 11	34.09
	Day 15	97.69

3.2 Effect of Initial Moisture Content

Moisture content is a crucial factor in solid state fermentation; significantly influence fungal growth and product development (Pandey, 2003; Singhania et al., 2009; Harsha et al., 2013; Pyo & Seo, 2010). In order to determine the effect of initial moisture content on simvastatin production, a series of experiment was performed. Initial moisture content of approximately 40%, 50%, 60%, 70% and 80% were investigated on the different flask of corn powder. The fermentation was ran for 5 days at 30^{o}C. Figure 2 shows the maximal amount of simvastatin was observed at 50% initial moisture content, with a yield of 34.60 μg/mL. A decrease in simvastatin yield was observed when the moisture level was higher (> 50% initial moisture content), or lower (< 50% initial moisture content) than the optimal level. Optimum moisture content is depending on the water holding capacity of the substrate and the nature of microorganism used (Harsha et al., 2013). Higher moisture content leads to water logging in the substrate

and caused aggregation of substrate particles which reduced the mass transfer process of oxygen and carbon dioxide (Pyo & Seo, 2010), hence, *Monascus*sp. was unable to penetrate completely into the substrate. While, lower initial moisture content (< 50%) resulted incomplete and poor fermentation due to the low water activity of the substrate which caused fewer nutrients to be available (Pyo & Seo, 2010; Said, 2010). Similar result was obtained by Pattanagul et al. (2008) who reported that initial substrate moisture contents less than 40% resulted in less mevinolin production, but moisture contents between 50% and 56% resulted in the highest metabolite production (Figure 3.2).

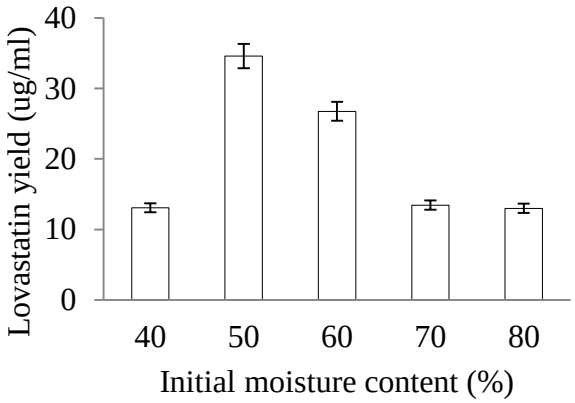

Figure 3.2: Effect of initial moisture content on simvastatin yield

3.3 Effect of Additional Nitrogen Source

Nitrogen source is a substantial component in fungal development (Xu et al, 2005). In the experimental work, peptone was chosen as the supplementation nitrogen source for corn powder. The selection of nitrogen source was based on the ability of peptone to stimulate *Monascus* growth as well as secondary metabolite production (Cho et al., 2002). Peptones of approximately 0.05% to 0.4% were added to each flask, separately. All flasks contained 10g of corn powder. Initial moisture content and pH were fixed at 50% and pH 6, respectively. Figure 3.3 shows the effect of additional nitrogen source on simvastatin yield. The figure shows that additional nitrogen source into corn powder significantly affected simvastatin yield. An upward trend of simvastatin yield was shown with additional of nitrogen source (peptone) from 0.05% to 0.3%. However, excessive nitrogen source (>0.3% peptone) inhibited simvastatin production. Too high nitrogen source may lower the ratio of carbon to nitrogen in the substrate. The molecule of simvastatin ($C_{25}H_{38}0_5$) is known to contain only carbon, no nitrogen. However, the nitrogen compound is essential for fungal growth (Said, 2010), rather than simvastatin. Therefore, the ratio of carbon to nitrogen has an important effect on simvastatin production. The high ratio of carbon to nitrogen may increase the ability of *Aspergillus*sp to produce statin (Casas et al., 2003).The nutrient compositions of

substrate play an important role because they directly linked to the formation of simvastatin and other metabolites.

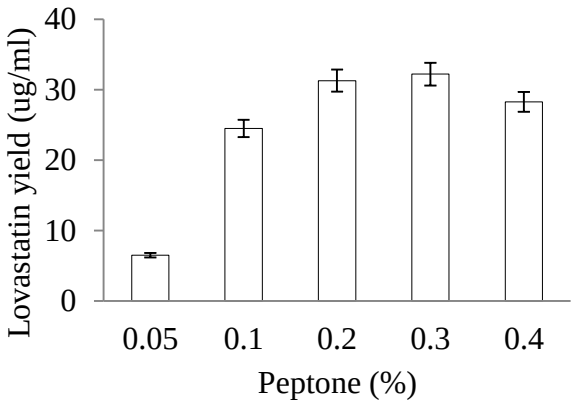

Figure 3.3. Effect of peptone on simvastatin yield

3.4 Optimization of Simvastatin Yield by Response Surface Methodology (RSM)

A 2^2 factorial central composite design (CCD) with two factors was used for optimization of simvastatin yield on *Monascuspurpureus* FTC 5356. The design included two blocks and three centre points of each block, which corresponding to 14 sets of experimental works. All experimental works were done in replicates. The two factors were initial moisture content (ranges of 40% to 60%) and peptone (ranges from 0.1% to 0.3%).

Analysis of variance (ANOVA) was used to determine the significance effect of independent variables on the response activity (Montgomery, 2001). Table 2 shows the ANOVA result of simvastatin production by *Monascuspurpureus* FTC 5356 on corn powder as a substrate. The p-value for the lack of fit of the model was 0.368. An insignificant value for lack of fit at the 95% confidence level (P-value > 0.05) shows that the model is fit, which explains that the response is acceptable and close to the actual values (Said, 2010).

ANOVA result of simvastatin yield shows that the model developed was statistically significant as the F value obtained was 4.83, with low p-value 0.031 (Table 2). The relationship between the simvastatin yield and the two factors was estimated by the second order polynomial function as in Equation 1:

$$\text{Simvastati n yield}\left(\mu g/ml\right) = -26.65 + 1.89x_1 + 20.42x_2 - 0.01x_1^2 - 2.19x_2^2 - 0.23x_1x_2 \quad (1)$$

where, x_1 is initial moisture content and x_2 is peptone.

From the regression equation of simvastatin yield (Equation 1) and Anova result (Table 3.2), both factors (initial moisture content and peptone) of linear coefficient were not significant. However, all quadratic terms were significant at 95% confidence level. Model Equation 1 was used to create a surface plot (Figure 3.4) to clearly visualize the influence of the factors on the simvastatin yield. Maximum simvastatin yield can be attained at initial moisture content approximately 50% with 0.2% peptone. No further incrementat higher or lower initial moisture content and nitrogen source.

Table 3.2: Analysis of variance (ANOVA) for simvastatin production in corn powder

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	55.183	5	11.037	4.825	0.0314*
A	0.526	1	0.526	0.230	0.6462
B	0.959	1	0.959	0.419	0.5379
A^2	14.601	1	14.601	6.383	0.0394*
B^2	14.802	1	14.802	6.471	0.0384*
AB	20.348	1	20.348	8.896	0.0204*
Residual	16.011	7	2.287		
Lack of Fit	8.162	3	2.721	1.387	0.3684
Pure Error	7.849	4	1.962		
Cor Total	71.832	13			

Where, A=initial moisture content, B=peptone, * significant at 95% confidence level

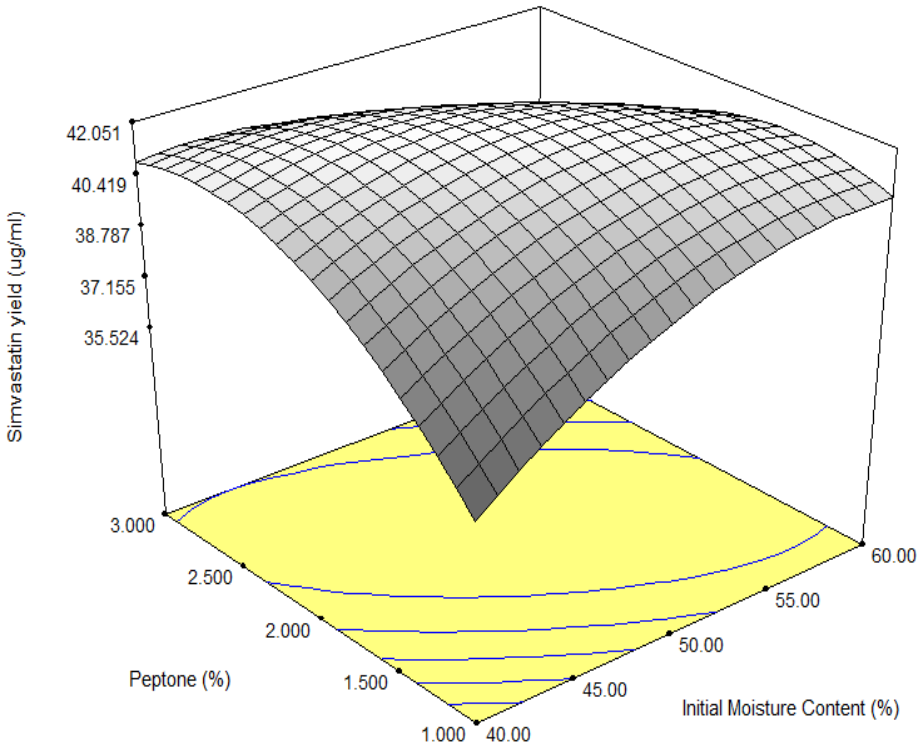

Figure 4. Surface response plot of simvastatin yield

The optimal conditions suggested by the Design Expert software were at 50.3% (w/w) initial moisture content and 0.21% (w/w) peptone. Model obtained was then verified in order to validate the prediction's accuracy. The actual simvastatin yield and the predicted yield were compared. The results indicated that the measured simvastatin yield was 1.05% lower than the predicted value of 42.052 μg/mL. The percentage error was 4.86%. Thus, it was indicated that the experimental results agreed well with the predicted model.

4.0 CONCLUSION

Among the agricultural product tested, corn powder has shown the capability as a substrate for *Monascuspurpureus* FTC 5356 to produce simvastatin. Simvastatin yield was significantly affected by initial moisture content and additional of nitrogen source (i.e. peptone). Optimization by response surface methodology (RSM) indicated that initial moisture content of 50.3% with 0.21% peptone were the best condition for optimal simvastatin production in solid state fermentation by *Monascuspurpureus* FTC 5356 usingcorn powder as a substrate.

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