Optimization of lovastatin in solid-state fermentation using oil palm frond

IOP Conf. Series: Materials Science and Engineering **736** (2020) 022056 doi:10.1088/1757-899X/736/2/022056

Optimization of lovastatin in solid-state fermentation using oil palm frond

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Abstract. Lovastatin plays a role in lowering the cholesterol level in the human blood, especially the bad cholesterol or low density lipoproteins (LDL). Concurrently, lovastatin increase the good cholesterol or high density lipoproteins (HDL), to prevent the formation of plaque inside the blood vessels. The objective of this research was to experimentally optimize the lovastatin compound produced by *Monascus purpureus* FTC5357 under solid state fermentation (SSF) using oil palm frond (OPF). In order to identify the optimal condition to produce lovastatin, four parameters which were pH, initial moisture content, peptone and potassium, were optimized using Box–Behnken design. Based on the ANOVA analysis performed, initial moisture content, potassium and peptone contributed significantly to the lovastatin production. Meanwhile, pH had the least impact to the lovastatin production.. Peptone pronounced to be the most contributed factor, as the lovastatin production increased with the increasing of peptone in the substrate. Under optimized condition (pH 5.50, moisture content at 60%, 3.40 g of potassium, and 3.30 g of peptone) maximum lovastatin yield was 45.84 µg/g. The lovastatin produced through SSF using OPF as a substrate by *Monascus purpureus* FTC 5357 has a great potential to be utilized as a source of lovastatin in future.

1 Introduction

Hypercholesterolemia is the accumulation of cholesterol in blood plasma that causes atherosclerosis (blockage of the artery), leading to the coronary heart disease and heart attack [1]. According to the report of the World Health Organization (WHO), cholesterol problem is estimated to cause 2.6 million deaths annually, which simultaneously increases the risks of heart disease and stroke [2]. Further, hypercholesterolemia stimulates the chances of diabetes development, obesity and certain types of cancers [3].

3–hydro–3–methyl glutaryl coenzyme–A (HMG–CoA) is the key enzyme which inhibits the rate limiting step in cholesterol biosynthesis. Structurally similar to the substrate HMGA–CoA, such as lovastatin can compete the key enzyme and used as a drug for hypercholesterolemia treatment because of their proven efficiency and safety profile [1-4]. Lovastatin belongs to a group of fungal secondary metabolites known as statins. Lovastatin and pravastatin are natural statins; simvastatin is semi-synthetic while atorvastatin and fluvastatin are synthetic statins. Natural statins can be produced through microbial fermentation [5].

Lovastatin can be naturally produced by several fungal species, such as *Aspergillus* species; *Aspergillus niger, Aspergillus terreus, Aspergillus flavus, Aspergillus flavipes, Aspergillus parasiticus* and some *Monascus* species including *Monascus ruber, Monascus paxi, Monascus anka,* and *Monascus purpureus* [2, 6]. Compared to the other *Aspergillus* species, *Monascus* species possess several

advantages, such as non–pathogenic and traditionally acted as fermentation food in Asian countries [2, 7].

Both solid-state fermentation (SSF) and submerged fermentation (SMF) can be applied to produce lovastatin [2]. Compared to SMF, SSF is considered to be the best approach for lovastatin production as it offers numerous advantages such as high concentration of the end-product, less waste water, low sterility and low product repression effect [1, 2, 6, 7]. In addition, production of lovastatin by fermentation decreases the production cost compared to the costs of chemical synthesis [4].

The microbial process especially through SSF process may utilize the agro industrial product such as long-grain rice, sorghum grain, barley, wheat bran, sago, corn, bagasse, barley, soybean meal, gram bran and fruits waste [1, 8]. However, these substrate materials are normally expensive and are need to compete with the human being and the livestock [9]. Thus, there is a need to find alternative substrate for the microbial process. Recently, large quantity of agro-industrial biomass such as from palm oil industry is produced globally especially in the tropical countries, including Malaysia. Oil palm frond (OPF) has made up approximately 70% of total biomass of palm oil industry [10]. Global production of OPF is found to be around 250 million metric tonnes (MMT) in weight which can accommodate a sustainable production of 34.6 MMT of structural carbohydrates [11].

Hence, this study was conducted to study the optimal condition to produce lovastatin from *Monascus purpureus* FTC5357 using OPF in SSF. In order to identify the optimal condition to produce lovastatin, four parameters which were pH, initial moisture content, peptone and potassium, were studied. The Box–Behnken design approach was applied to achieve the objective of the study.

2 Methodology

2.1 Substrate preparation

The fresh OPF was collected from a local palm oil plantation-Lepar, Pahang, Malaysia. The OPF was cut into smaller pieces, washed thoroughly with tap water and dried in oven at 60 °C for 24 h. Then, the dried OPF were grinded to powder form using grinder (Retsch ZM-200, Germany). The OPF powder were sieved using 1 mm mash hole using a sieve shaker (Retsch AS 200 Basic, Germany). Later, the OPF powder was autoclaved at 121 °C for 20 min with distilled water in a ratio of 1:18 (w/v). The treated OPF were filtered and washed with distilled water, before being oven dried at 60 °C for 24 h before used [12].

2.2 Culture and Inoculum preparation

Monascus purpureus FTC5357 used was collected from Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. *Monascus purpureus* FTC5357 was periodically prepared by maintained on Potato Dextrose Agar (PDA), incubated at 30 °C for 7 days and sub-cultured when needed [13] and then stored at 4 ° [2]. The spore were scraped off from agar slant in distilled water under aseptic condition at room temperature. The spore suspension were calculated using Neubauer hemacytometer (Cole-Parmer 79001-00) and adjusted to 10^4 spores per ml for use as inoculum throughout the study [1, 2, 12, 14].

2.3 Solid State Fermentation (SSF) by Box- Behnken's approach

The experimental work was conducted based on the design generated by Box–Behnken of response surface methodology (RSM) using Design Expert 7.0 software. The ranges of the parameters were determined based on the earlier study by Jahromi et al. (2012). The level of parameters for Box-Behnken design is shown in Table 1.

A 5 g of OPF powder was placed into a 250 ml conical flask. The moisture content, pH, potassium and peptone were adjusted and added to the flask, according to the generated table (Table 2). Next, the medium was autoclaved at 121 °C for 20 min. On cooling, 1 ml of 10^8 spores *Monascus purpereus* FTC 5357 spores were inoculated to the autoclaved medium and incubated at 30 °C for 8 days [12, 15].

Table 1. Level of parameters for Box–Behnken's response surface design

Parameter	Term	Lower limit (-)	Upper limit (+)
pH	A	4	7
Initial Moisture Content (%)	B	40	60
Potassium (g)	C	0.5	3.5
Peptone (g)	D	0.5	3.5

2.4 Lovastatin extraction and determination

The fermented substrates were dried in an oven at 60 °C for 1 day. A gram of dried fermented OPF was taken for lovastatin extraction and adjusted to pH 3.0 by using hydrochloric acid (HCl). Next, an equal volume of ethyl acetate was added and agitated at 200 rpm at ambient temperature for 15 min using incubator shaker (Infors AG CH-4103 Bottmingen) [6]. The extraction liquid was then filtered through Whatman filter paper [3]. The lovastatin concentration was measured using spectrometer (Hitachi, U-1800) at wavelength of 245 nm [3]. The data collected was subjected to Analysis of Variance (ANOVA). The Response Surface Methodology plots (RSM plots) and contour graph were drawn by using Design Expert software. The means and the standard errors (Mean ± S.E) for each treatment were performed.

3 Result and Discussion

3.1 Design of experiment (DOE) for lovastatin production

RSM plays a very critical role in efficiently exploring the optimal values of explanatory variables. The three dimensional response surface and the contour plot obtained from the RSM, are useful in understanding the main and the interaction effects of the parameters. There can be used to describe and examine the regression equations in a visualized way to reflect the effects of experimental variables on the required response [16].

In this study, the effect of SSF parameters including initial moisture content, pH, amount of peptone and potassium sulphate, on lovastatin production from *Monascus purpureus* FTC3557 were studied. Box-behnken design suggested a set of experiments based on combination of the four parameters (Table 2). The results of lovastatin production was given as an input to the software for further analysis and predict.

From Table 2, run 25 has the lowest amount of lovastatin, whereas run 15 has the highest amount, which were 7.288 µg/g and 46.487 µg/g, respectively. The differences of both conditions were the former was with 40% moisture content, 2 g of potassium and 2 g of peptone, while the latter was with 50% moisture content 3.5 g of potassium and 3.5 g of peptone. From the results obtained, it showed that the higher the amount of peptone used, the higher the amount of lovastatin obtained. This is consistent with Miyake et al. (2006).

Table 2. Optimal design for optimization of lovastatin production

Run	pH (pH)	Initial Moisture Content (%)	Potassium (g)	Peptone (g)	Response (µg/g)
1	5.50	50.00	2.00	2.00	35.578
2	4.00	50.00	3.50	2.00	21.487
3	5.50	50.00	2.00	2.00	33.630
4	4.00	40.00	2.00	2.00	8.652
5	5.50	50.00	2.00	2.00	33.435
6	5.50	60.00	3.50	2.00	45.794
7	5.50	50.00	0.50	3.50	39.431

8	7.00	50.00	2.00	0.50	15.253
9	4.00	60.00	2.00	2.00	19.496
10	5.50	50.00	0.50	0.50	25.838
11	4.00	50.00	2.00	3.50	23.500
12	5.50	40.00	2.00	0.50	26.119
13	5.50	60.00	2.00	3.50	41.725
14	5.50	40.00	3.50	2.00	29.149
15	5.50	50.00	3.50	3.50	46.487
16	5.50	60.00	0.50	2.00	34.885
17	7.00	50.00	3.50	2.00	9.669
18	4.00	50.00	0.50	2.00	11.704
19	7.00	60.00	2.00	2.00	28.846
20	7.00	40.00	2.00	2.00	10.448
21	5.50	50.00	3.50	0.50	29.668
22	7.00	50.00	2.00	3.50	23.457
23	5.50	40.00	2.00	3.50	33.067
24	5.50	40.00	0.50	2.00	28.500
25	4.00	50.00	2.00	0.50	7.288
26	7.00	50.00	0.50	2.00	10.751
27	5.50	60.00	2.00	0.50	34.171

3.2 Statistical Analysis of lovastatin production

Table 3 shows the Analysis of variance (ANOVA) table of lovastatin production obtained from Box–Behnken experimental design. The ANOVA analysis shows (Table 3) that the developed regression model is highly significant (F-value >16.37), with a p-value of <0.0001. In this study, parameters B, C, D and A^2 are significant model parameters as the p-values were less than 0.05, which implies the regression model is significant. The "lack of fit" F-value of the model was 12.07 with the p-value of 0.0789, implied that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is good as this shows that the suggested model equation fits well with the experimental results. The goodness of fit was evaluated by the R^2 value. The R^2 value found near to 1 indicates that the experimental data are closed to the fitted regression line, where R^2 was 0.9503. The value suggests that the model could predict 95.03% of the variability in the response.

The developed mathematical model for the prediction of lovastatin production can be expressed by equation (1) where the response is the lovastatin production in µg/g. The model suggested as pH (A), initial moisture content (B), amount of potassium (C) and amount of peptone (D). The positive (+) sign in model equation indicates the synergic effects and the negative (-) sign represents antagonistic effects on lovastatin production.

$$\text{Lovastatin Production } (\mu g/g) = -218.0789 + (88.756)\,A - (0.755)\,B + (0.280)\,C + (5.890)\,D + (0.126)\,AB - (1.207)\,AC - (0.890)\,AD + (0.171)\,BC + (0.010)\,BD + (0.358)\,CD - (8.228)\,A^2 + (2.751 \times 10^{-5})\,B^2 - (0.294)\,C^2 + (0.409)\,D^2 \quad (1)$$

Figure 1 shows a graphical representation of the predicted (mathematically calculated) and actual (experimental) plot of model for lovastatin production. The predicted values of lovastatin are quite similar to the experimental values (Figure 1). It demonstrated that the regression model has strong correlation between the model prediction and its experimental results. Thus, the developed regression model is reliable and can be used to predict the lovastatin production [18].

The normality assumptions can be ensured by the construction of normal probability plot of the experimental residual. Figure 2 shows the normal probability plot for the lovastatin. The normal probability shows a straight line, indicated there is no noticeable issue with the normality [18].

Table 3. Analysis of variance (ANOVA) for lovastatin production

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3291.690	14	235.121	16.374	< 0.0001*
A	3.306	1	3.306	0.230	0.6400
B	396.551	1	396.551	27.616	0.0002*
C	80.846	1	80.846	5.630	0.0352*
D	400.543	1	400.543	27.894	0.0002*
AB	14.266	1	14.266	0.994	0.3386
AC	29.516	1	29.516	2.056	0.1772
AD	16.035	1	16.035	1.117	0.3114
BC	26.316	1	26.316	1.833	0.2008
BD	0.092	1	0.092	0.006	0.9376
CD	2.600	1	2.600	0.181	0.6780
A^2	1827.861	1	1827.861	127.295	< 0.0001*
B^2	0.404	1	0.404	0.028	0.8697
C^2	2.331	1	2.331	0.162	0.6941
D^2	4.504	1	4.504	0.314	0.5857

*significant

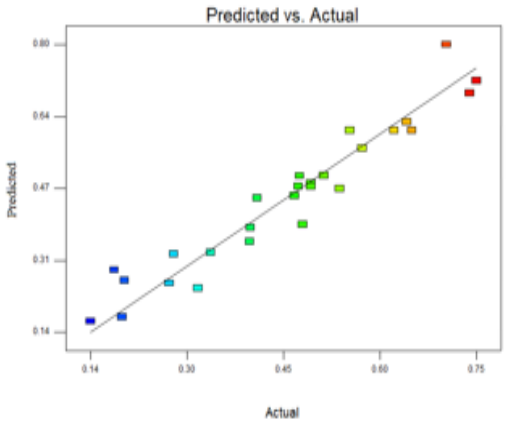


Figure 1. The actual versus predicted plot for lovastatin production

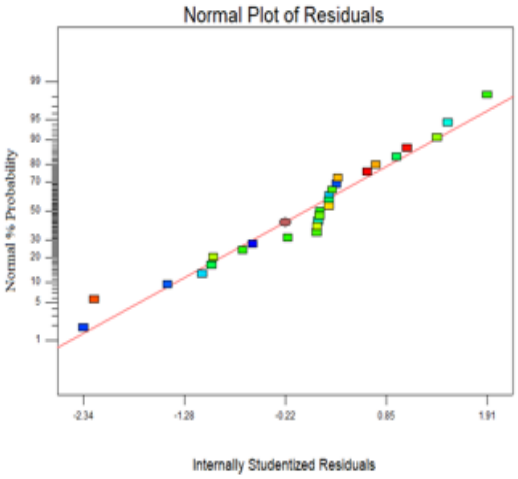
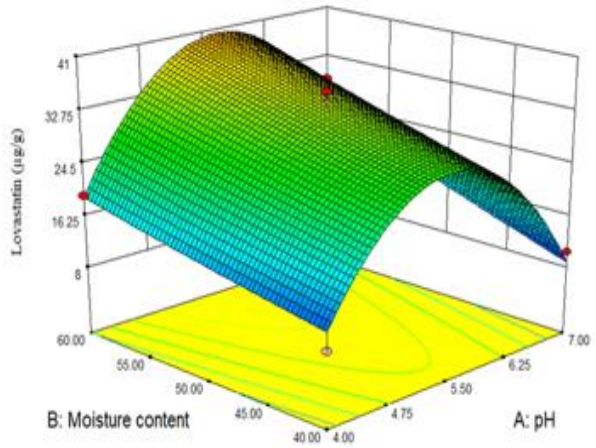


Figure 2. The studentized residuals and normal percentage for lovastatin production

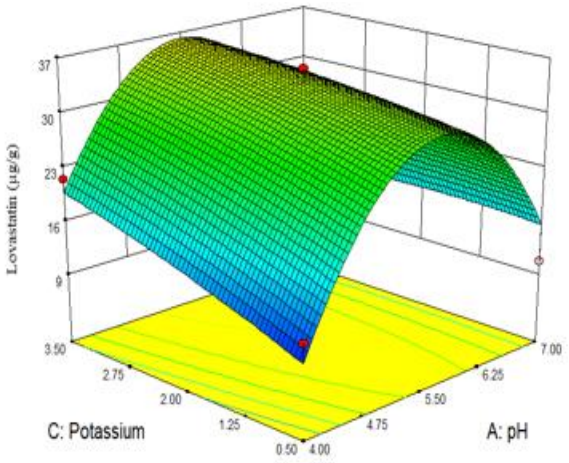
3.3. Response surface analysis of parameters on lovastatin production

3.3.1. Interaction of pH with other parameters. The three dimensional response surface plots for lovastatin production on pH and the interaction with other parameters are shown in Figure 3. The interactive relationship between AB (pH and moisture content), AC (pH and potassium) and AD (pH and peptone) on the amount of lovastatin produced, are shown in Figure 3(a), 3(b) and 3(c), respectively. The lovastatin productions were maximum at 41 µg/g, 40 µg/g, and 36 µg/g, with respect to interactive parameters of AD, AB, and AC, respectively. At the same condition of pH 5.5, peptone had the most significant effect to the lovastatin, which affected to the better lovastatin production, compared to the other two parameters (initial moisture content and potassium). No further increment of lovastatin at pH beyond the optimum value (pH 5.5). This is because, pH is strongly influences the transport of various components across the cell membrane which in turn supports the cell growth and product formation [4]. Lower or higher pH than the optimum pH during SSF, it will cause the denaturation and inactivation of *Monascus purpereus* FTC 5357 leading to lower lovastatin production [4]. Similar finding had also been reported by Kumar et al., (2000), where the lovastatin production by *Aspergillus terreus* DRCC 122 in the batch process, was optimum at pH range 5–6.5. On the other hand, the optimum pH for lovastatin production by *Monascus purpureus* MTCC 369 was pH 6 [14] and pH 5 by *Aspergillus flavipes* [1]. The above information, suggested that the optimum pH for lovastatin production in SSF is in the ranges of pH 5–6.5, depending on the types of substrate and microorganism used.

(a)



(b)



(c)

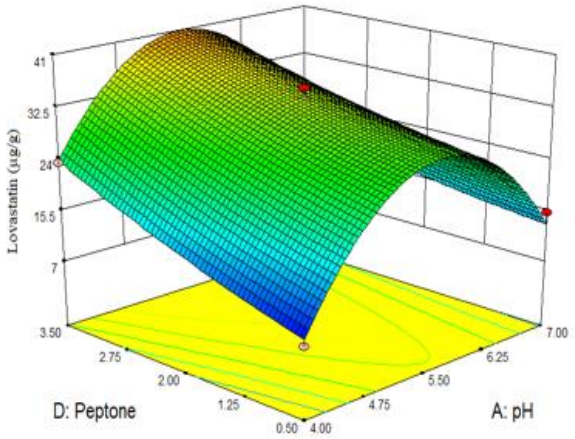


Figure 3. The 3D Response Surface of (a) pH and Moisture Content, (b) pH and Potassium, (c) pH and Peptone on Lovastatin Activity

3.3.2. Interaction between parameters at pH 5.5. The three dimensional response surface plots for lovastatin production at pH 5.5 and the interactive relationship between BC (moisture content and potassium), BD (moisture content and peptone) and CD (potassium and peptone), are shown in Figure 4(a), (b) and (c), respectively. The lovastatin productions were maximum at 44 µg/g, 47 µg/g, and 43 µg/g, with respect to interactive parameters of BC, BD, and CD, respectively. Interaction parameter of BD shows the most significant effect leading to the highest lovastatin production, compared to the interaction parameters of BC and CD.

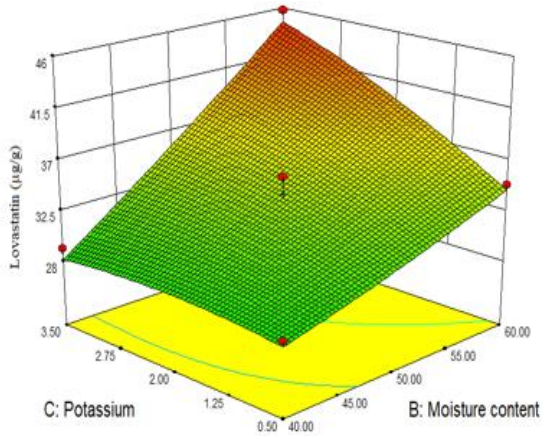
Figure 4(a) and 4(b) show that increase of moisture content from 40% to 60% facilitate to the production of lovastatin. Initial moisture content of the medium is a key parameter affecting SSF, which control the diffusion of nutrients in the reaction system, maintain the stability and the functional of biological molecules such as proteins, carbohydrates, and nucleotides [19]. *Monascus purpureus* FTC5357 were utilized the substrate at 60% initial moisture content (optimum) and favourable for high production of lovastatin. Furthermore, moisture content basically influence the physical properties of the substrate [7], where the porosity of the substrate is directly decrease as the moisture content increase beyond the optimum value. Resulted in low diffusion of gas exchange. As a result, the lovastatin production started to decline. This is due to the aggregation of substrate particles, reduction of aeration and leading to the anaerobic conditions [3, 9]. Conversely, lower moisture content reduce the metabolic activity and may account for lower lovastatin production [2, 3, 6]. Similar result was obtained by Latha et al. (2012) whom conducted optimization of lovastatin using coconut oil cake by *Aspergillus fischeri*.

They reported that the highest lovastatin yield at 60% moisture content. The other researchers claimed that the optimal initial moisture content of *Monascus ruber, Monascus purpureus* 9901 and *A. terreus* were set in the range of 50–55 %, in SSF [2, 7, 9].

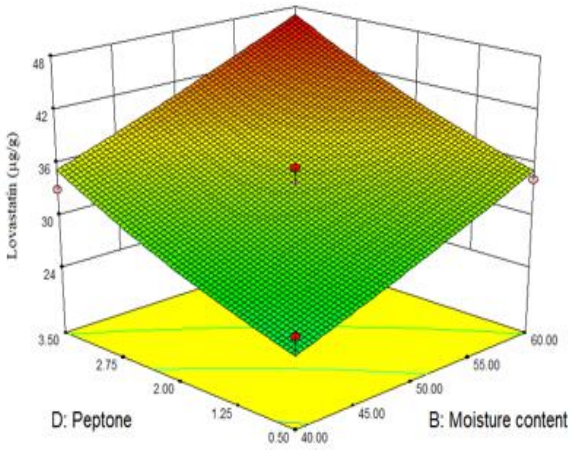
Figure 4(b) and 4(c) clearly show that lovastatin production increased with the increase of peptone. Slow-acting organic nitrogen source, such as peptone, was more favour, due to the long-term biosynthesis of lovastatin [2]. It was because organic nitrogen source possess complex nutrients compared to the inorganic nitrogen source [2]. Selection of carbon and nitrogen in the fermentation medium are key parameters to the lovastatin production, as they acted as precursors and cofactors for the formation of biomass and lovastatin product [2, 4, 7]. Higher concentration of peptone contributed to the better growth of *Monascus purpureus* FTC5357 by shortening the growth period and rapid entry to the stable phase, and consequently synthesize the product [7]. However, extreme level of peptone will repressed the production. There have been several reports of the production of lovastatin using SSF from organic nitrogen source [2, 7, 20]. However, the optimum value of organic nitrogen to give the maximum lovastatin yield, are different. These results might be directly due to the proportion variance of the carbon nature and the nitrogen sources [4].

Figure 4(a) and 4(c) show that potassium ions contribute to the increase of lovastatin. However, if the potassium amount is higher than the optimum value (3.40 g), it reduced the production. It was due to the interactions with lower moisture content (Figure 4 a) and lower peptone (Figure 4 c), which were affected much to the fungal growth, consequently lowering the lovastatin production. Although potassium is an essential growth nutrient and affected to the lovastatin production [21], though peptone and moisture content are more important to the lovastatin production. This behaviour was also observed by Jahromi et al. (2012), where the lovastatin yield increased using OPF in *A. terreus* ATCC 74135 by added the minerals.

(a)



(b)



(c)

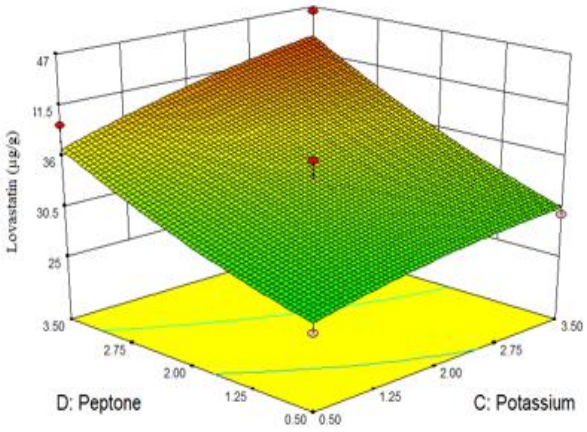


Figure 4. The 3D Response Surface of (a) Moisture Content and Potassium, (b) Moisture Content and Peptone, (c) Potassium and Peptone on Lovastatin Activity

To validate the optimal condition for lovastatin production by *Monascus purpereus* FTC5357, an experiment was conducted under the predicted optimal conditions. Table 4 shows the differences between predicted and actual results on lovastatin production. Under the suggested conditions, the predicted optimal values of parameters were at 60% of moisture content, pH 5.5, 3.30 g of peptone and 3.40 g of potassium. The predicted amount of lovastatin obtained was 49.63 µg/g. While, the actual value obtained through triplicate analysis was 45.84 µg/g. The percentage error obtained was 8.63, which was less than 10%. Thus, it was considered as acceptable conditions to optimize the lovastatin by SSF using *Monascus purpereus* FTC5357.

4 . Conclusion

Response surface methodology was proven suitable to be applied for the optimization of SSF using OPF as substrate. Box-Behnken experimental design which developed the ANOVA analysis showed that the second-order polynomial model was valid and adequate to study the effects of parameters on lovastatin production. Based on the ANOVA analysis, peptone was the most significant parameter, followed by moisture content, potassium and pH values. In short, it can be observed that the higher the pH, the higher the production of lovastatin, until optimal pH of 5.5. The production efficiency will drop beyond the optimal point. The optimal conditions for lovastatin production via SSF were at pH 5.5, 3.30 g peptone, 3.40 g potassium and 60% moisture content. The maximum lovastatin produced was 49.626 µg/g. Validation experiment was done to confirm the adequacy of the model by producing lovastatin under the optimal condition. The experimental value was 45.8376 µg/g of lovastatin, confirming the validity of the model with percentage error of 8.63%. Based on the results of the experimental study, the usage of *Monascus purpureus* FTC5357 in SSF which acts as a natural source to produce desired amount of lovastatin. The lovastatin produced through SSF by *Monascus purpureus* FTC5357 using OPF had indicated that it has a great potential to be utilized as the source of lovastatin in future. The results in this study may expand our understanding on the application of SSF using agricultural waste as substrate.

Acknowledgements

The authors highly appreciate and acknowledge the supported of a Fundamental Research Grant Scheme (FRGS) FRGS/1/2015/SG05/UMP/03/2 (RDU150105) granted by the Ministry of Higher Education (Malaysia).

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