OPTIMIZATION OF RED PIGMENT PRODUCTION BY *MONASCUS PURPUREUS* FTC 5356 USING RESPONSE SURFACE METHODOLOGY

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(Received: 20th Feb 2017; Accepted: 23rd Feb 2018; Published on-line: 1st June 2018)

https://doi.org/10.31436/iiumej.v19i1.814

ABSTRACT: Factors such as environmental conditions and nutrients are significant for successful growth and reproduction of microorganisms. Manipulations of the factors are the most effective way to stimulate the growth of the microorganism, which can be used to optimize the yield of a product. In this study, Central Composite Design (CCD) of Response Surface Methodology (RSM) was used to optimize the production of red pigment by *Monascus purpureus* FTC 5356 using the petioles of oil palm fronds (OPF) as a substrate in solid state fermentation (SSF). The data was analyzed using Design Expert Software. The optimum combination predicted via RSM was confirmed through experimental work. The interactions between three variables such as initial moisture content (%), initial pH value (pH), and peptone concentration (%) were studied and modelled. The statistical analysis of the results showed that the optimal conditions for red pigment production 47 AU/g with the biomass of 425.1 mg/g was at 55% initial moisture content, 3% of peptone, and at pH 3. The RSM results showed that the initial pH value had a significant effect on red pigment production (P-value <0.05). The validation of these results was also conducted by fermentation with predicted conditions and it was found that there was a discrepancy of 0.39% between the values of the experimental result and those of the predicted values.

ABSTRAK: Keadaan persekitaran dan nutrien merupakan faktor-faktor penting dalam pertumbuhan mikroorganisma. Manipulasi faktor-faktor tersebut adalah kaedah terbaik bagi meningkatkan pertumbuhan mikroorganisma dan mengoptimumkan penghasilan produk. Kajian ini mengguna pakai Rekaan Gabungan Pusat (CCD) melalui Kaedah Tindak balas Permukaan (RSM) bagi penghasilan pigmen merah optimum oleh *Monascus purpureus* FTC 5356 menggunakan batang pelepah kelapa sawit (OPF) sebagai perumah dalam proses penapaian pepejal (SSF). Data telah dianalisis menggunakan perisian Design Expert. Gabungan parameter optimum seperti cadangan RSM telah disahkan secara eksperimen. Interaksi antara tiga pemboleh ubah seperti kandungan lembapan awal (%), nilai pH awal (pH), dan kepekatan pepton (%) telah dikaji dan dimodelkan. Analisis statistik menunjukkan penghasilan optimal pigmen merah adalah pada 47 AU/g dengan biomas sebanyak 425.1 mg/g, pada 55% lembapan awal, 3% pepton dan pada pH 3. Hasil keputusan RSM menunjukkan pH awal memberikan kesan signifikan kepada penghasilan pigmen merah (nilai P <0.05). Pengesahan analisis juga telah dijalankan melalui proses penapaian dan hasil ujikaji mendapati 0.39% lebih tinggi daripada nilai jangkaan.

KEYWORDS: *response surface methodology; red pigment; oil palm frond; Monascus pigment*

1. INTRODUCTION

In recent years, colorants have been extensively used in the food industry. However, to overcome the unlimited usage of synthetic pigment, which is found to be hazardous and toxic to human health, the development of alternate sources for the production of natural pigment has been focused on. Nowadays, productions of pigment from microbial origin have attracted more attention from the food industry. Particular focus has been given to *Monascus* sp., which is a nontoxic fungi that has been widely used as a natural colorant and food additive in East Asia. *Monascus* pigment can produce three groups of pigment: orange, red, and yellow. Among these pigments, the red pigment is gaining high market demand for its use [1].

It is important to study the effect on the red pigments produced by *Monascus* sp. under different culture conditions, for the safe and successful application in food and pharmaceutical industries [2]. Previous study was done on the usage of petioles and leaflets of oil palm frond (OPF) as a substrate [3]. The finding revealed that 100% petiole rendered the best results. Thus, the goal of this study was to optimize the most significant of the multivariable factors for substrates made solely of petiole, in order to influence red pigment production. Factors observed include initial moisture content, peptone concentration, and initial pH value.

The traditional 'one factor at a time' (OFAT) approach used for optimizing a multifactor system is not only effort and time consuming, but also often misses in representing the interaction effect between different factors [4]. However, OFAT could be used as a preliminary experiment to set the range of the factor efficiently, making the results more reasonable and credible [5]. Therefore, the traditional approach of OFAT still can be applied. The range of factors obtained in OFAT can be used by adopting a statistical approach, such as response surface methodology (RSM), to solve the complexity involved in red pigment production. Recently, many types of statistical experimental design methods have been discovered for optimization [6-8]. Among them, RSM is the most suitable technique to reduce the number of experimental trials needed. It is also used to evaluate the most significant single factors and to effectively seek the optimum conditions for the multivariable system [8]. Several studies have applied RSM for optimization of red pigment [9-11]. However, to the best of our knowledge, no research has been reported on the application of RSM for optimization the red pigment production using *Monascus purpureus* FTC 5356 on petioles of oil palm fronds (OPF). Therefore, in order to determine the significant optimization factors in red pigment production, response surface methodology was applied in the present study.

2. MATERIALS AND METHODS

2.1 Microorganism

The strain used in this study was *Monascus purpureus* FTC 5356 obtained from Malaysian Agricultural Research and Development Institute, Serdang, Malaysia. The stock culture was maintained on potato dextrose agar (PDA) media and incubated at 28-30 ^{o}C for 7 days, preserved at 4 ^{o}C and sub-cultured once every 4 weeks [12].

2.2 Inoculum Preparation

Monascus purpureus FTC 5356 was grown on PDA slants at 30 ^{o}C for 7 days. The spores were then scrapped off and suspended in 5 ml sterile distilled under aseptic conditions at room temperature. The suspension was adjusted to 10^8 spores/ml with sterile distilled

water. The spore numbers were counted using a Neubauer hemacytometer (Cole-Parmer 79001-00). The adjusted spore suspension (10% v/w) was used for further solid state fermentation [12].

2.3 Substrate Preparation

Fresh oil palm fronds (OPF) were obtained from the Federal Land Development Authority (FELDA) Bukit Goh, Kuantan, Pahang. The leaflets and petioles were separated from the OPF. The petioles were then cut into small pieces approximately 3-4 cm in length, washed, and dried at 60 ^{o}C for 3 days. The dried petiole was shredded and ground using a commercial grinder (Retsch ZM-200, Germany) to a particle size smaller than 1 mm by passing through 1 mm sieve screens using a vibrator sieve shaker (Retsch, Germany).

2.4 Solid State Fermentation

The experimental work was done based on the experimental design being set by Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA), (Table 1 and Table 2). The best range of each factor was selected by applying the One Factor at A Time (OFAT) method as in the preliminary experiment (data not shown). All experiments have been carried out in replicates and the whole flasks were discarded. Each substrate was inoculated and incubated in the dark at 30 ^{o}C for 8 days.

Table 1: Independent variables, responses and the levels in the experimental design.

No	Designation	Factors	-1	0	+1
1	X_1	Initial moisture content (%)	40	55	70
2	X_2	Peptone concentration (%)	2	35	5
3	X_3	Initial pH value	6	8	10
		Response			
4	Y_1	Red pigment production (AU/g)			
5	Y_2	Biomass (mg/g)			
6	Y_3	Glucose concentration (µg/g)			

Table 2: The central composite design matrix developed for three independent variables

Run	X_1	X_2	X_3
1	0	0	-1
2	-1	-1	-1
3	+1	-1	-1
4	-1	+1	-1
5	+1	+1	-1
6	0	0	0
7	-1	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	+1	0	0
12	0	+1	0
13	0	-1	0
14	+1	-1	+1
15	+1	+1	+1
16	0	0	+1
17	-1	+1	+1
18	-1	-1	+1

2.5 Pigment Extraction and Determination

The harvested fermented solid was dried at 60 ^{o}C for 24 hours in an oven (Memmert UFB-500). The dried fermented solid was extracted with 95% ethanol in a ratio of 1:10 w/v for 1 hour at 200 rpm, in an incubator shaker (Infors AG-CH-4103 Bottmingen). The extract was then allowed to stand for 15 min, and filtered through Whatman No.1 filter paper. Ethanol extracts of unfermented substrates were used as blanks. Analysis of pigment concentration was done using a UV-Vis spectrophotometer (Hitachi U-1800). The wavelength used was 500 nm. Pigment yield was expressed as absorbance units (AU) per gram of dried solids [12-14].

2.6 Reducing Sugar Determination

Reducing sugar was measured using a dinitrosalicylic acid (DNS) method [12, 15]. The reducing sugar was measured at 575 nm by UV-Vis spectrophotometer (Hitachi U-1800).

2.7 Biomass (Cell Dry Weight)

Total fungal biomass was determined by measuring the N-acetylglucosamine released by acid hydrolysis of the chitin present in the fungal cell walls. The acid hydrolysis of the sample was carried out by mixing 0.5 g of dry fermented OPF powder with 2 ml of 60% (vol/vol) sulfuric acid, H_2SO_4 and the mixture was incubated at 25 ^{o}C in a fume hood for 24 h [16]. Then the mixture was diluted with distilled water to make a 1 N solution of sulfuric acid that was then autoclaved at 121 ^{o}C for 1 h. The mixture was allowed to cool at room temperature and neutralized with 5 N NaOH to pH 7 and the final volume was brought up to 60 ml with deionized water. Later, the filtered acid hydrolysis sample (1 ml) was mixed with 1 ml of acetyl acetone reagent (2% (vol/vol) of acetyl acetone in 1 N sodium bicarbonate (Na_2CO_3) before being placed in a boiling water bath for 20 min [12]. After cooling, 6 ml of ethanol (95%) was added, followed by 1 ml of Ehrlich reagent (2.67% (w/v) of *p*-dimethylaminobenzaldehyde (Merck) in 1:1 mixture of ethanol and concentrated hydrochloric acid) [17]. The mixture was incubated in a water bath at 65 ^{o}C for 10 min. After cooling, the optical density was read at 530 nm against the reagent blank, N-acetylglucosamine (Sigma-Aldrich) as the external using a UV-visible spectrophotometer [12, 18].

2.8 Experimental Design

The red pigment production was developed and optimized using response surface methodology (RSM) provided by Design Expert Software (Version 7.1.6, 2008, Minneapolis MN, USA). A standard RSM design tool known as Central Composite Design (CCD) was applied to study the significant production factor of red pigment. The three identified independent variables were the initial moisture content (40-70%), peptone concentration (2-5%), and initial pH (pH 6-8). The critical ranges of selected factors were determined by preliminary experiment using OFAT and screening by factorial design (data not shown). During the screening process of petiole used as a substrate, the initial moisture content (IMC), initial pH, interaction of peptone, and pH were found to be significant. Thus, three factors were chosen for optimization. Screening was done to eliminate the insignificant factor. Table 1 lists the ranges and levels of the three independent variables with actual and coded levels of each factor. The lower and upper levels were coded as -1 and +1; the middle level was coded as 0. A total of 18 runs with 4 central points were generated. The center points are usually repeated 4-6 times to determine the experimental error (pure error) and the reproducibility of the result. Three responses, red pigment (AU/g), biomass (mg/g) and glucose concentration (µg/g), were measured. The experiments were run in triplicate. The

complete design matrix corresponding to the CCD design in terms of real and coded independent variables is displayed in Table 2.

2.9 Validation Experiment

The validation experiment was performed by conducting the experiment with the suggested optimal conditions of higher pigment.

3. RESULTS AND DISCUSSION

The statistical significance of the model equation was evaluated by the F-test analysis of variance (ANOVA). The ANOVA statistics for responses Y_1, Y_2, and Y_3 were summarized in Table 3, 4, and 5, respectively. Multiple regression analyses of the response surface design were developed as in Equations 1, 2, and 3. In order to determine the optimal level of each variable for maximum production of red pigment and biomass, a 3D surface plot was designed as a function of two factors at a time, holding all other factors at a fixed level. This design was helpful for understanding both the main and the interaction of the two factors. The response values for the variables can be predicted from these plots.

Table 3: ANOVA analysis for red pigment production (Y_1)

Source	**Sum of squares**	**DF**	**Mean square**	**F-value**	**Prob>F**	
Model	4915.48	9	546.16	52.33	<0.0001	Signi-ficant
X_1- Initial moisture content	9×10^3	1	9×10^3	8.6×10^4	0.9773	
X_2- Peptone concentration	26.9	1	26.9	2.58	0.1471	
X_3- Initial pH value	126.74	1	126.74	12.14	0.0083	
X_1X_2	16.24	1	16.24	1.56	0.2475	
X_1X_3	0.18	1	0.18	0.017	0.8988	
X_2X_3	0.13	1	0.13	0.012	0.9156	
X_1^2	44.47	1	44.47	4.26	0.0729	
X_2^2	444.04	1	444.04	42.54	0.0002	
X_3^2	1195.10	1	1195.10	114.51	<0.0001	
Residual	83.50	8	10.44			
Lack of fit	76.15	5	15.23	6.22	0.0817	Not signi-ficant
Pure error	7.35	3	2.45			
R^2	0.9833					
Adeq precision	18.345					

Table 4: ANOVA analysis for biomass response (Y_2)

Source	**Sum of squares**	**DF**	**Mean square**	**F-value**	**Prob>F**	
Model	1.28×10^5	9	14169.99	7.63	0.0044	Signi-ficant
X_1- Initial moisture content	124.61	1	124.61	0.067	0.8022	
X_2- Peptone concentration	108.24	1	108.24	0.058	0.8153	
X_3- Initial pH value	2982.53	1	2982.53	1.61	0.2408	
X_1X_2	296.46	1	296.46	0.16	0.7	
X_1X_3	5.95	1	5.95	3.2×10^3	0.9563	
X_2X_3	0.66	1	0.66	3.6×10^4	0.9854	
X_1^2	4302	1	4302	2.32	0.1666	
X_2^2	9802.12	1	9802.12	5.28	0.0507	
X_3^2	25466.58	1	25466.58	13.71	0.0060	
Residual	14864.29	8	1858.04			
Lack of fit	13890.42	5	2778.08	8.56	0.0536	Not signi-ficant
Pure error	973.87	3	324.62			
R^2	0.8956					
Adeq precision	7.105					

Table 5: ANOVA analysis for glucose concentration response (Y_3)

Source	**Sum of squares**	**DF**	**Mean square**	**F-value**	**Prob>F**	
Model	90779.97	9	10086.66	91.77	<0.0001	Signi-ficant
X_1- Initial moisture content	1.51	1	1.51	0.014	0.9095	
X_2- Peptone concentration	442.89	1	442.89	4.03	0.0796	
X_3- Initial pH value	3997.20	1	3997.20	3637	0.0003	
X_1X_2	1074.62	1	1074.62	9.78	0.0141	
X_1X_3	7.57	1	7.57	0.069	0.7997	
X_2X_3	25.56	1	25.56	0.23	0.6425	
X_1^2	1516.91	1	1516.91	13.80	0.0059	
X_2^2	9162.68	1	9162.68	83.37	<0.0001	
X_3^2	17555.18	1	17555.18	159.73	<0.0001	
Residual	879.26	8	109.91			
Lack of fit	235.72	5	47.14	0.22	0.9319	Not signi-ficant
Pure error	643.54	3	214.51			
R^2	0.9904					
Adeq precision	25.360					

3.1 Optimization of Red Pigment Production

The second order polynomial equation model for prediction of the optimal point between the response variable (red pigment production) and the independent variables was expressed in Eqn. 1:

$$Y_1(\text{red pigment}) = 45.66 - 0.03X_1 + 1.64X_2 + 3.56X_3 - 1.42X_1X_2 + 0.15X_1X_3 + 0.13X_2X_3 - 405X_1^2 - 12.80X_2^2 - 21X_3^2 \quad (1)$$

where Y_1 is the response for red pigment production, X_1 is the code for initial moisture content, X_2 is for peptone concentration, X_3 is for initial pH value.

Based on the ANOVA Table, as presented in Table 3, the quadratic model indicated that this model could be accepted to navigate the design space. The Model F- value of the response Y_1 with the value 52.33 implies that the model was significant at 95% confidence level. The P-value was used as a tool to check the significance of each coefficient, which in turn designates the pattern of interaction between the factors. The smaller the P-value, the larger the significance of the coefficient was. As in Table 3, the P-values for the Y_1 was <0.0001, which was less than 0.05. Therefore, it can be concluded that the model terms were statistically significant. In addition, the main model terms indicated that the significant factor was the initial pH (X_3), while the significant quadratic terms were peptone concentration (X_2^2) and initial pH (X_3^2). The lack of fit test with P-value (0.0817), which was not significant (p-value> 0.05 is not significant), supported the hypothesis that the model was satisfactorily fitted to the experiment data. The 'not significant' term of lack of fit is most-desired as a significant of lack of fit indicates the presence of the contribution in the regressor-response relationship that is not accounted for by the model [19]. The correlation coefficient (R^2) is a tool to identify the 'goodness of fit' between the experimental and the predicted values. Based on Table 3, the R^2 for Y_1 (0.9833) was found to be close to 1, which indicated the presence of a good relation between experimental and predicted values for red pigment (Y_1). The adequate precision for Y_1 is 18.345. These large values of adequate precision demonstrated that these quadratic models were significant for the process. The evaluation of residuals was analyzed to validate the adequacy of the model. A normal probability plot of the residuals for Y_1 is displayed in Fig. 1. Based on the figure, it clearly shows that the residuals distribution was nearly a straight line. Thus, it can be concluded that the errors were distributed evenly.

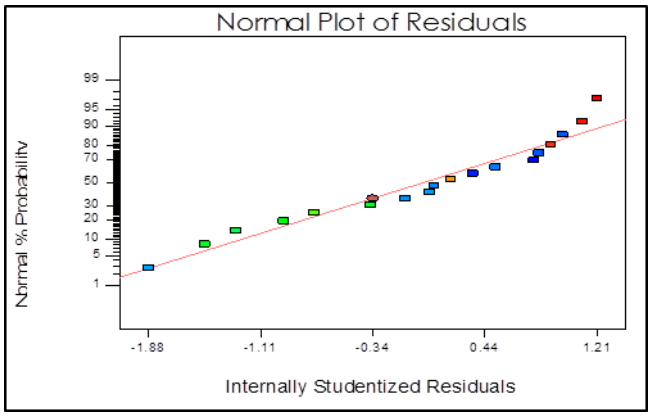

Fig. 1: Normal plot of residuals for red pigment production (Y_1).

Figures 2a, 2b, and 2c show the 3D surface plots of the relationship between the main factors X_1X_2 (initial moisture content and peptone concentration), X_1X_3 (initial moisture content and initial pH), and X_2X_3 (peptone concentration and initial pH), respectively. In Fig. 2a, the increment of initial moisture content from low level 40% to 55% leads to the increase in red pigment to a maximum level. However, a further increase in the initial moisture content (>55%) did not further increase the pigment. This result clearly shows that the red pigment decreased above and below the 55% initial moisture content. The poor yield of red pigment at high moisture content (>55%) was possibly due to the agglomeration of substrate, consequently reducing oxygen supply for the growth of *Monascus*. While, the decrease in red pigment at low moisture content was because of the insufficient nutrient supply due to the low nutrient salt dissolution [19].

A similar trend of effect on the response was observed for the initial moisture content and the initial pH. An increase of the initial moisture content and initial pH, up to the optimum point, maximized the red pigment production and a further increase of the factors decreased the red pigment, as shown in Fig. 2b. This reaction process was in agreement with Orozco and Kilikian [20] in which changing the pH value of the medium from pH 5.5 to pH 8, caused the drastic excretion of the red pigment. In addition, they also claimed that the best condition for red pigment production was at alkaline medium (pH 8.0 or pH 8.5). Between these two pH values, pH 8 had been chosen to be the best condition due to the maximum yield of red pigment production.

The interaction effect of the peptone concentration with initial pH as shown in Fig. 2c clearly suggested the best combination for production of red pigment. An increase in the peptone concentration with initial pH, optimized the red pigment gradually. However, at higher peptone concentration (> 3.5%) and higher initial pH (> pH 8), the pattern is reversed. The decrease in yield may due to excessive nutrients provided in the medium that became toxic and inhibited the red pigment production. Therefore, the optimum conditions for maximum red pigment production were obtained at the initial moisture content of 55%, peptone concentration of 3.5%, and initial pH value of 8. The maximum red pigment achieved was 47.9 AU/g.

3.2 Optimization of Biomass Production

Based on the experimental results and regression analysis, a quadratic polynomial equation was developed to determine the relationship between the biomass of *Monascus purpureus* and the factors. The model of coded units can be stated as in Eqn. 2:

$$\boldsymbol{Y_2(\text{biomass}) = 382.75 + 3.53X_1 + 3.29X_2 + 17.27X_3 - 6.09X_1X_2 - 0.86X_1X_3 - 0.29X_2X_3 - 39.85X_1^2 - 60.15X_2^2 - 96.95X_3^2} \quad (2)$$

where Y_2 is the response for biomass production, X_1 is the code for initial moisture content, X_2 is for peptone concentration, and X_3 is for initial pH value.

From the analysis of variance (ANOVA) as presented in Table 4, the model for biomass was highly significant (P-value, 0.0044) and the R^2 (0.8956) was relatively good, as evidenced by the significance of the model. There was no significance of a single factor or interaction between factors that influenced the biomass production, however, the quadratic terms of initial pH value was found to be significant. Furthermore, the lack of fit was not significant with P-value of 0.0536 (>0.05), indicating that the experimental data obtained fitted well with the model.

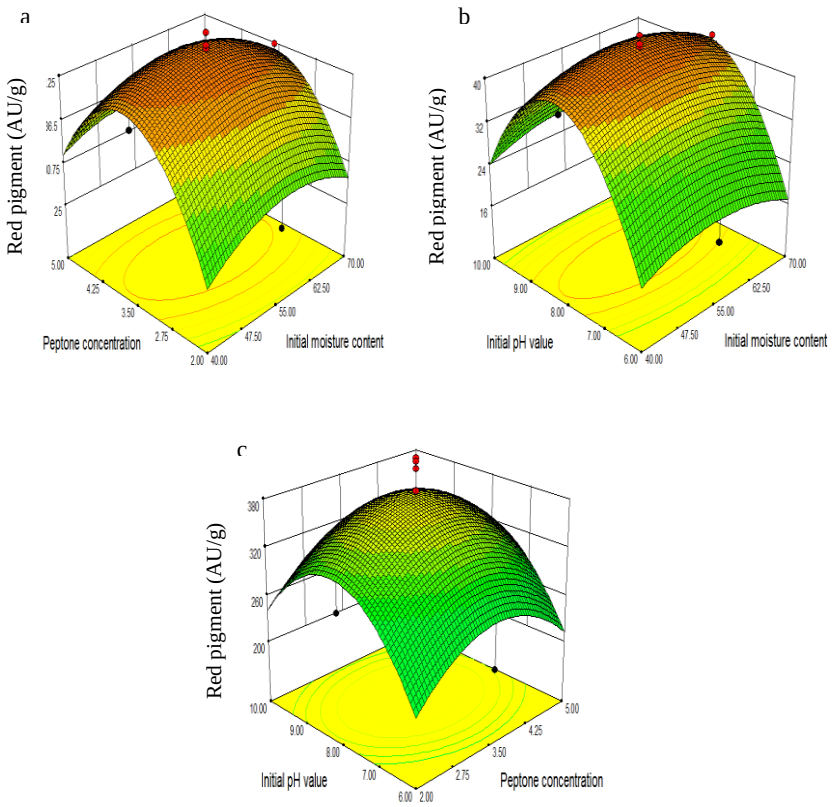

Fig. 2: Response surface curve showing combined effect between the main factors: (a) initial moisture content (X_1) and peptone concentration (X_2), (b) initial moisture content (X_1) and initial pH value (X_3), (c) peptone concentration (X_2)and initial pH value (X_3).

The residual analysis was carried out for the confirmation of the adequacy of the model. This was done by observing the normal probability plot of the residual in Fig. 3 where the residuals were on a straight line, suggesting that the errors were distributed evenly.

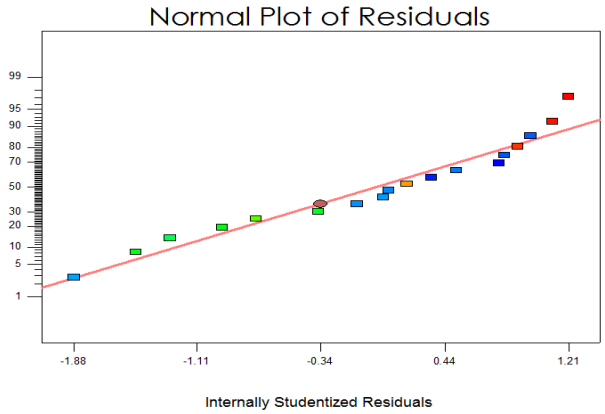

Fig. 3: Normal plot of residuals for biomass response (Y_2).

Figures 4a, 4b, and 4c show the 3D surface plots of biomass responses after combining the effect of the main factors. The effect of the initial moisture content and peptone concentration on the biomass is shown in Fig. 4a. An increase of initial moisture content with peptone concentration up to the optimum point increased the fungal biomass to a maximum level and a further increase in the initial moisture content and peptone

concentration did not further increase the trend. This finding was supported by Krishna [21], who stated that the low initial moisture content could reduce nutrient diffusion consequently affecting the growth of the *Monascus.* However, if the initial moisture content is too high, water will occupy the voids where airflow is required for fungal growth.

Increased factors of initial moisture content and initial pH up to the optimum point, maximized the biomass production (Fig. 4b). From the 3D plot, it was obviously shown that *Monascus* was grown successfully at pH 8 indicating that the biomass achieved the maximum yield. However, the fungal biomass production started to decrease with a further increase of initial pH (> pH 8) of substrate.

The interaction effect of the peptone concentration with initial pH in Fig. 4c clearly suggested the best combination for the production of fungal biomass. An increase in the peptone concentration and initial pH optimized the biomass gradually but at higher peptone concentration and initial pH, the pattern is reversed. It was studied that nitrogen is the major element of cell membranes and nucleic acid, therefore supplying nitrogen sources to the medium may facilitate the growth of the fungus. However, if the nitrogen concentration is too high (> 3.5%), it might inhibit the fungal growth. Therefore, the optimum biomass was observed at the initial moisture content of 55%, peptone concentration of 3.5%, and initial pH of substrate of pH 8. The maximum biomass achieved was 430.8 mg cell dry weight/g dry matter.

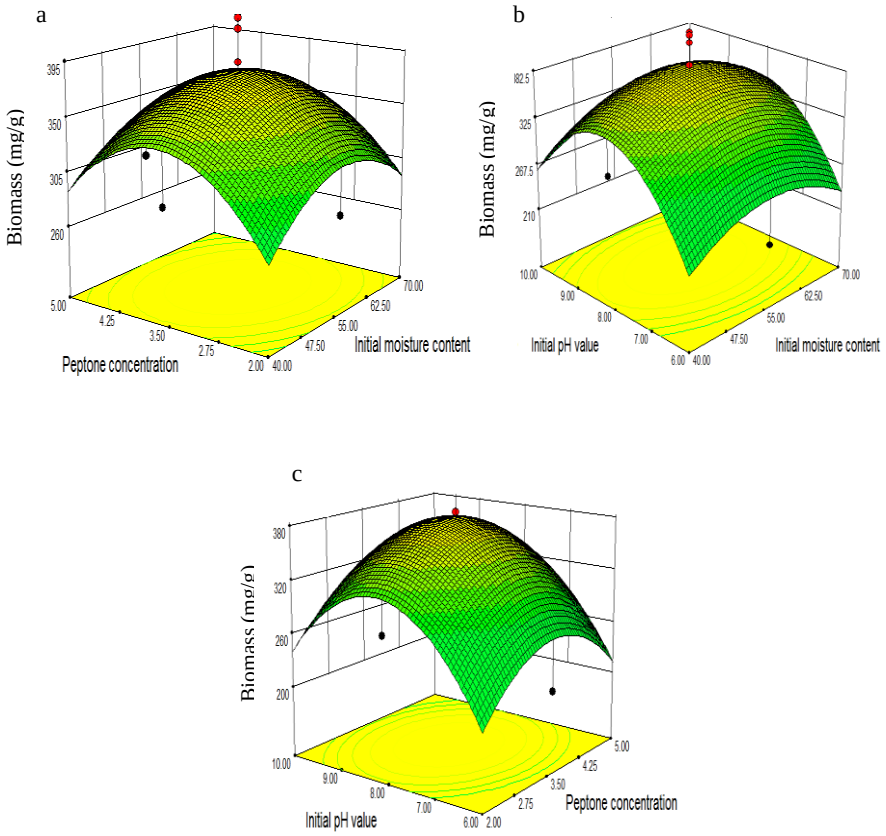

Fig. 4: Response surface curve showing combined effect between the main factors:
(a) initial moisture content (X_1) and peptone concentration (X_2),
(b) initial moisture content (X_1) and initial pH value (X_3),
(c) peptone concentration (X_2) and initial pH value (X_3).

3.3 Glucose Concentration

On the glucose consumption, a second order polynomial can be obtained by the Design Expert. Multiple regression equations (in term of coded factors) were represented in Eqn. 3:

$$Y_1(\text{glucose concentration}) = 130.65 - 0.39X_1 - 6.66X_2 - 19.99X_3 + 11.59X_1X_2 - 0.97X_1X_3 - 1.79X_2X_3 + 23.66X_1^2 + 58.15X_2^2 + 80.49X_3^2 \quad (3)$$

where Y_3 is the response for glucose concentration, X_1 is the code for initial moisture content, X_2 is for peptone concentration, X_3 is for initial pH value.

The ANOVA Table implies that the model was significant with the F-value of 91.77 (Table 5). The P-value (<0.0001) was less than 0.05, which indicated the model terms were highly significant. In addition, the main model terms indicated that the significant factor was initial pH value (X_3) and the interaction terms were found to exist between initial moisture content (X_1) with peptone concentration (X_2). While, the significant quadratic terms were initial moisture content (X_1^2), peptone concentration (X_2^2), and initial pH value (X_3^2). The lack of fit value of 0.22 confirmed that the lack of fit was not significant, relative to the pure error when p-value was 0.9319 and > 0.05. The insignificant lack of fit demonstrates the good predictability of the model. In addition, the value of R^2 was 0.9914, indicating that the model was fitted and explains 99.14% of the variability in glucose concentration. The high values of adequate precision with the value of 25.360 demonstrated that these quadratic models were significant for the process.

Figure 5 displays the normal plot of residuals of response Y_3 glucose concentration. It was obviously shown that the points cluster around the diagonal line which indicated the good fit of the model.

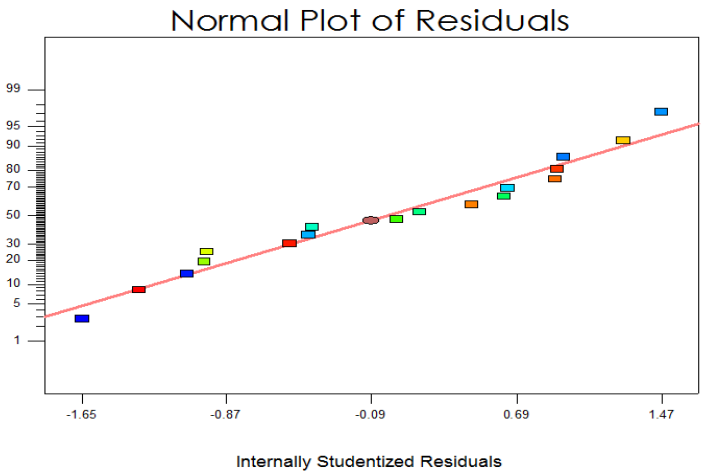

Fig. 5: Normal plot of residuals for glucose concentration response (Y_3).

Figures 6a, 6b, and 6c show the 3D surface plots of glucose concentration response after combining the effect between the main factors. From the figure, it was observed that the glucose was decreased when the initial moisture content, peptone concentration and initial pH value were 55%, 3.5% and pH 8, respectively. The 3D surface plots of glucose concentration were totally different with the previous figures (Figures 2a, 2b, 2c, 4a, 4b, and 4c). The glucose concentration decreased when the fungal biomass and red pigment production achieved the maximum yield. This phenomenon suggested that the rapid consumption of glucose by *Monascus* caused the depletion of glucose, consequently

resulting in an insufficient glucose supply that reached its supply limitation [22]. The lowest final glucose concentration of 114.73 µg/ g was obtained.

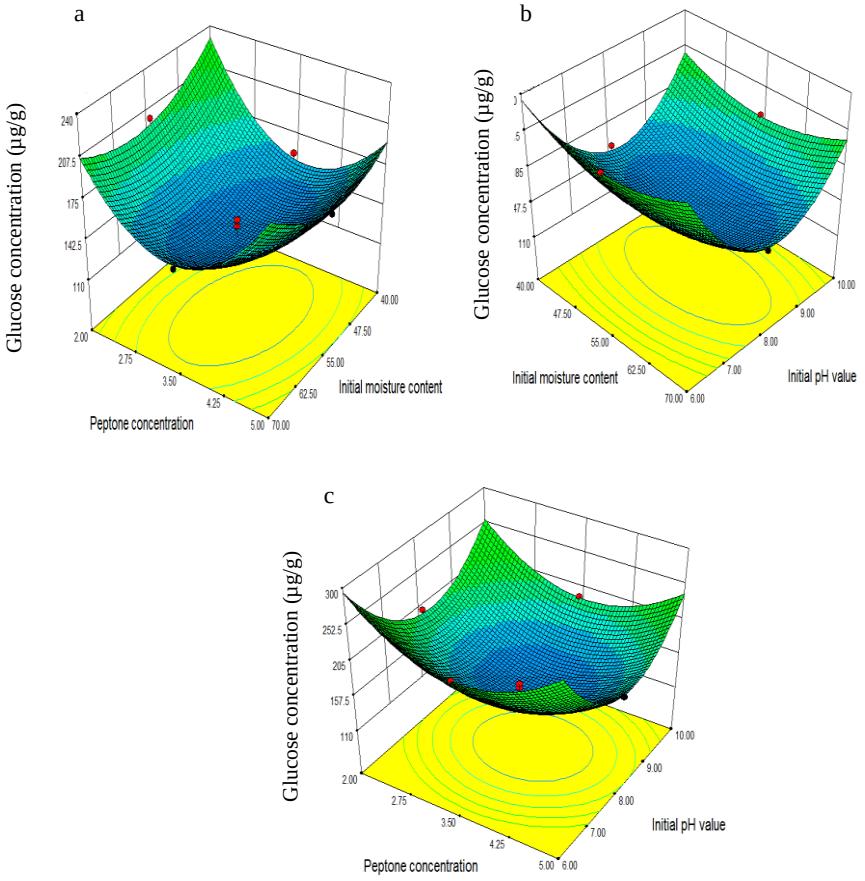

Fig. 6: Response surface curve showing combined effect between the main factors: (a) initial moisture content (X_1) and peptone concentration (X_2), (d) initial moisture content (X_1) and initial pH value (X_3), (e) peptone concentration (X_2)and initial pH value (X_3).

3.4 Validation

In order to confirm the optimization of red pigment production by *Monascus purpureus* FTC 5356, an experiment was performed under the predicted optimal conditions. This experiment was conducted in triplicate. Under these suggested conditions, the predicted optimal values of the variables were 56% initial moisture content, 3.5% peptone, and pH 8.2. The prediction of the total red pigment was 45.85 AU/g and the actual value obtained through the triplicate experiments was 46.03 AU/g, as shown in Table 6. The percentage error calculated based on the Eqn. 4 was 0.39%. Therefore, the experimental results agreed well with the model predicted values.

$$Percentage\ error = \frac{(\text{Experimental value} - \text{predicted value})}{\text{Experimental value}} x\ 100\% \quad (4)$$

Table 6: Optimum factors of RSM on red pigment

Factor	Value	Predicted (AU/g)	Actual (AU/g)	Percentage error (%)
Initial moisture content (%)	56	45.85	46.03	0.39
Peptone (%)	3.5			
pH	8.2			

4. CONCLUSION

This study shows that response surface methodology is a fast and error-free approach for optimization of media composition to obtain the best performance of red pigment production and biomass. Besides, an interaction study among all the components was an additional advantage of employing RSM. Results obtained from response surface methodology critically point out the importance of initial pH value of the substrate for red pigment production as well for the fungal biomass.

ACKNOWLEDGEMENT

This work was funded by research grant granted by Ministry of Higher Education of Malaysia under FRGS grant no. RDU 150105.

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