FACTORIAL DESIGN SCREENING FOR THE RED PIGMENT PRODUCTION BY *MONASCUS PURPUREUS* FTC 5356
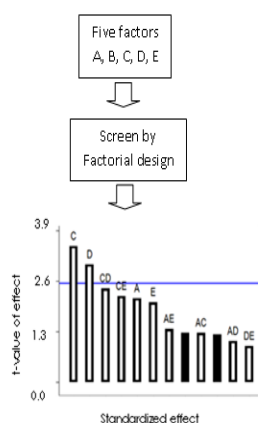
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Abstract

Factorial design experiment was used to obtain maximum information from the least amount of experimental runs. The goal of this study was to screen the significant factors from multivariable parameters which can influence the production of red pigment by *Monascus purpureus* FTC 5356 under solid state fermentation (SSF) using oil palm frond (OPF). Five factors such as initial moisture content (A), nitrogen source concentration (B), percentage of OPF leaflet to petiole (C), initial pH of substrate (D), and inoculum size (E) were screened in sixteen experimental runs. To reduce cost, the feasibility of OPF as an alternative substrate was investigated. Percentage of OPF leaflet and initial pH of OPF substrate contributed significantly (P-values of 0.0121 and 0.0229, respectively). Meanwhile, other factors such as initial moisture content (P- value 0.0766) and inoculum size (P-value 0.0895) had least impact to the red pigment production.

Keywords: Red pigment, factorial design, *Monascus purpureus*, solid state fermentation, oil palm fronds.

1.0 INTRODUCTION

Monascus sp., which is a filamentous fungus has been used to make rice wine, soy bean cheese and anka (red rice) in many Asian countries. Moreover, *Monascus* sp. can synthesize many secondary metabolites such as *Monascus* pigments (MPs), monacolins, ɣ-aminobutyric acid, dimerumic acid and antioxidants [1]. With the carcinogenic and mutagenic risks of synthetic pigments [2], developing *Monascus* pigment as an alternative source in producing natural coloring agents is very important. The *Monascus* sp. is known to produce red color during its growth on substrates. These red color are due to the formation of rubropunctamine and monascorubramine pigments, synthesized in the cytosol from acetyl CoA by a multienzyme complex polyketide synthase [3].

2.0 METHODOLOGY

The strain used in this study was *Monascus purpureus* FTC 5356 obtained from Mardi, Serdang, Malaysia. The stock culture was maintained on Potato Dextrose Agar (PDA) media and incubated at 28-30°C for 7 days, preserved at 4°C and sub-cultured once every 4 weeks [4]. To fully sporulated agar slant culture (6-8 days old), 5ml of sterile distilled water was added into the slant culture bottle. Then the spores were scrapped, transferred and suspended in a sterile universal bottle under aseptic conditions at room temperature. The spore numbers was counted using Neubauer hemacytometer (Cole-Parmer 79001-00). The adjusted spore suspension (10% v/w) was used for further solid-state fermentation [4].

Fresh oil palm frond (OPF) was obtained from a local palm in FELDA Bukit Goh, Kuantan, Pahang, Malaysia. The OPF was cut into small pieces approximately 3-4 cm in length, washed and dried at

60°C for 3 days, before pulverized into powder (1.0 mm) by using a commercial grinder (Retsch ZM-200).

Two-factorial design was used to evaluate the factors. Factorial design of 2^x, where x is the number of factor was prepared by using Software Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA) for screening design. Percentage of OPF leaflet, initial pH of OPF substrate, initial moisture content, inoculum size, and peptone concentration were the five selected factors for this study. The factorial screening design involves runs at every possible combination at the defined upper and lower limit for each parameter (Table 1). The upper and lower limits of the factors were based on the preliminary experiment done.

Table 1 Level of variables in experimental design

Parameter	Term	Upper Limit (+)	Lower Limit (-)
Initial Moisture content (%)	A	55	35
Peptone concentration (%)	B	3	1
Percentage of leaflet (%)	C	100	0
pH value (pH)	D	5	7
Inoculum size (spores/ml)	E	10^9	10^7

The experimental work was done based on the experimental design being set by Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA). All experiments have been carried out in duplicates. Each substrate was inoculated and incubated in the dark at 30°C for 8 days.

Harvested fermented solid was dried at 60°C for 24 hours in an oven (Memmert UFB-500). Dried fermented solid (1g) was extracted with 95% ethanol (10ml) for 1 hour at 200rpm, in an incubator shaker (Infors AG-CH-4103).The extract was then allowed to stand for 15 min, and filtered through a Whatman No1 filter paper. Ethanol extract of unfermented substrates were used as blanks. Analysis of pigment concentration was done using a UV-VIS spectrophotometer (Hitachi U-1800) by monitoring the absorbance at 500nm. Pigment yield was expressed as absorbance units (AU) per gram of dried solids [4,5,6].

3.0 RESULTS AND DISCUSSIONS

Factorial designs are very efficient for studying two or more factors [7]. The effect of factors can be referred to as the change in response produced by a change in the level of the factor, known as the main effect. On the other hand, in some cases, it may be that the difference in the response between levels of one factor was fluctuated at all levels of the other factors, a situation known as an interaction effect between factors. Collectively, main effect and interaction effect can be called factorial effect.

In the present study, after obtaining the range value of factors from one factor at one time (OFAT) experiments, screening of the significant factors was analyzed by two fractional experimental designs to eliminate the insignificant factors in order to obtain more effective red pigment production. The advantage of using factorial design in the experiment was that the effect of factors can be evaluated over a wide range of conditions with a minimum number of experiments; therefore it was economically important in achieving the most efficient use of cost, time and other resources.

The Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA) was used for the statistical design and regression analysis of the experimental data. The general response surface model of the selected design is as follows:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \ldots \ldots (1)$$

Where, Y is the predicted response, β_0 is the interception coefficient, β_1 and β_2 are coefficients of the linear effect, β_{11} and β_{22} are coefficients of the quadratic effect, β_{12} is the coefficient of the interaction effect, x_1 and x_2 are the coded value for variables.

From the experimental designs, four factors (initial moisture content, percentage of OPF leaflet, initial pH value of substrate, and inoculum size) out of total five factors were chosen as the significant factors, whereas only one factor (peptone concentration) was identified as an insignificant factor.

Half normal probability plot displays the absolute value of all positive and negative effects. Figure 1 shows half normal probability plot for red pigment production. It shows that all significant effects were on the right side of the origin. Conversely, the insignificant effects were on the left side of the origin. The advantage of the half normal probability plot is in the arrangement of effect from factors of the "noise" line (red line in the Figure 1). The more distant variable from the "noise" line, the greater the significant effect of the factor to the red pigment. From the figure, it was revealed that the percentage of OPF leaflet(C) and initial pH of substrate (D) pronounced the significant effects for red pigment production. Besides, the interaction between the percentage of OPF leaflet and initial pH value of substrate (C-D) was below the 5% significance level .
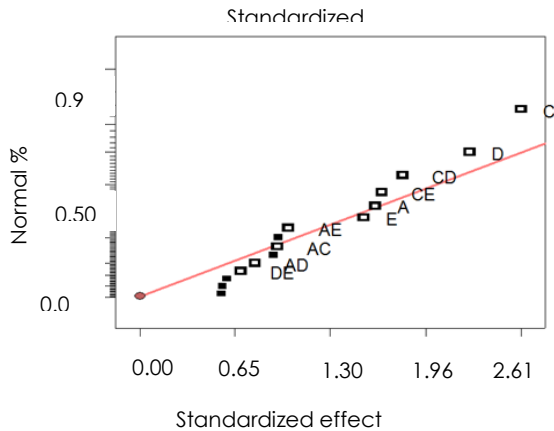
Figure 1 Half-Normal probability plot for red pigment production

The Pareto chart to all factors is shown in Figure 2. This figure represents the estimated effects of the factors and their interactions on the responses variables, in decreasing order of magnitude. The length of each bar is proportional to the t-value of the effect. The horizontal line can be used to determine which effects are statistically significant (Figure 2). Bars extending beyond the line correspond to the statistically significant effects at a confidence level of 95%. The most significant factors were percentages of OPF leaflet and initial pH of substrate (Figure 2). The other factors were not significant at 95% confidence level.
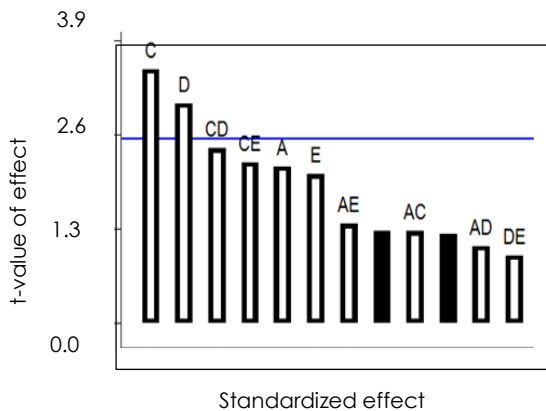

Figure 2 Pareto chart for red pigment production

From the factor that had been chosen, the validity of the model was verified by the analysis of values in the ANOVA table. The predication limit is set to 95.0%. The ANOVA of the quadratic regression model demonstrated that the model is significant with low p-value (0.0497). It demonstrated that the model has a high correlation with the experimental data. There was only 4.97% chance that the "Model F-value" could occur due to noise. The P value lower than 0.0500 indicated the model terms were significant. Factors C (percentage of OPF leaflet) and D (initial pH of substrate) in this model were shown as the most significant for red pigment production. The fit of the

model was evaluated by the determination coefficient (R^2). In this case, R^2 of the quadratic response surface model was 0.9047, which found to be close to 1, indicating that 90.47% of the variability in the response could be explained by the model equation. This model also advocated a high correlation between the observed and predicted values.

Thus, since term C and term D influenced the significance of the model, they had been chosen as the greater significant factor for red pigment production. Collectively, this model can be used to describe the experimental data well.

A two way design enables to examine the interaction effect of the independent variables on the dependent variable. Figure 3 illustrates the interaction effect of factors on promoting the red pigment production by *Monascus purpureus* FTC 5356 using OPF as a substrate. The findings showed that the most significant factor was the percentage of OPF leaflet, where low percentage of OPF led to produce higher red pigment production. For instance, Figure 3a (0% OPF leaflet, pH 7), 3b (0% OPF leaflet, 10^9 spores/ml of inoculum size), and 3d (0% OPF leaflet, 55% moisture content) show higher red pigment production with yield of 5.09 AU/g, 4.66 AU/g, and 4.34 AU/g, respectively. Conversely, result shows lower red pigment production at Figure 3a (100% OPF leaflet, pH 7), 3b (100% OPF leaflet, 10^9 spores/ml of inoculum size), and 3d (100% OPF leaflet, 55% moisture content). These results explained that by decreasing the percentage of OPF leaflet, the higher the red pigment can be produced. By considering the nutritional value of OPF leaflet,it was expected that a higher percentage of OPF leaflet would favour the production of red pigment [8]. However, the finding in this study contradicted with the expected result. In this case, a lower percentage of OPF leaflet has influenced a more rapid production of red pigment.

Figure 3a, 3e, and 3f show the significant factor of initial pH of the substrate. Initial pH of the substrate was the second most influential factor in terms of red pigment production. The finding showed that by increasing the initial pH, the higher red pigment production can be obtained. For instance, Figure 3a (pH 7, 0% OPF leaflet), 3e (pH 7, 55% moisture content), and 3f (pH 7, 10^9 spores/ml of inoculum size) showed a higher concentration of red pigment production. On the other hand, result showed a lower concentration of red pigment production at Figure 3a (pH 5, 0% OPF leaflet,), 3e (pH 5, 55% moisture content), and 3f (pH 5, 10^9 spores/ml of inoculum size). The same trend was observed by Yongsmith *et al.* (2000) [8] where a lower pH value (pH 5) promoted more yellow pigment synthesis, whereas higher pH pronounced more to red pigment production. Hence, the higher the initial pH of substrate, the higher the red pigment could be produced.

Figure 3b, 3c, and 3f show the significant factor of inoculum size of the strain *Monascus purpureus* FTC 5356. The findings displayed that by increasing the

inoculum size, a higher red pigment production can be obtained. For instance, Figure 3b (10^9 spores/ml of inoculum size, 0% of OPF leaflet), 3c (10^9 spores/ml of inoculum size, 55% moisture content), and 3f (10^9 spores/ml of inoculum size, pH 7) show higher red pigment production. Conversely, result shows lower red pigment production at Figure 3b (10^7 spores/ml of inoculum size, 0% of OPF leaflet), 3c (10^7 spores/ml of inoculum size, 55% moisture content), and 3f (10^7 spores/ml of inoculum size, pH 7). It seems that a higher inoculum size was recommended to produce higher red pigment production. This fact can be explained by considering that insufficient biomass produced to form mycelia, if the inoculum size is too small [9]. Furthermore, there were high possibilities for the unfavorable organism to growth on the substrates if the inoculum size was too low [10].

The small inoculums size will lead to slow growth of microorganism needed, but not other organism. Figure 3c, 3d, and 3e show the significant factor of initial moisture content of the OPF substrate. The finding displays that by increasing the initial moisture content, it lead to produce higher red pigment production
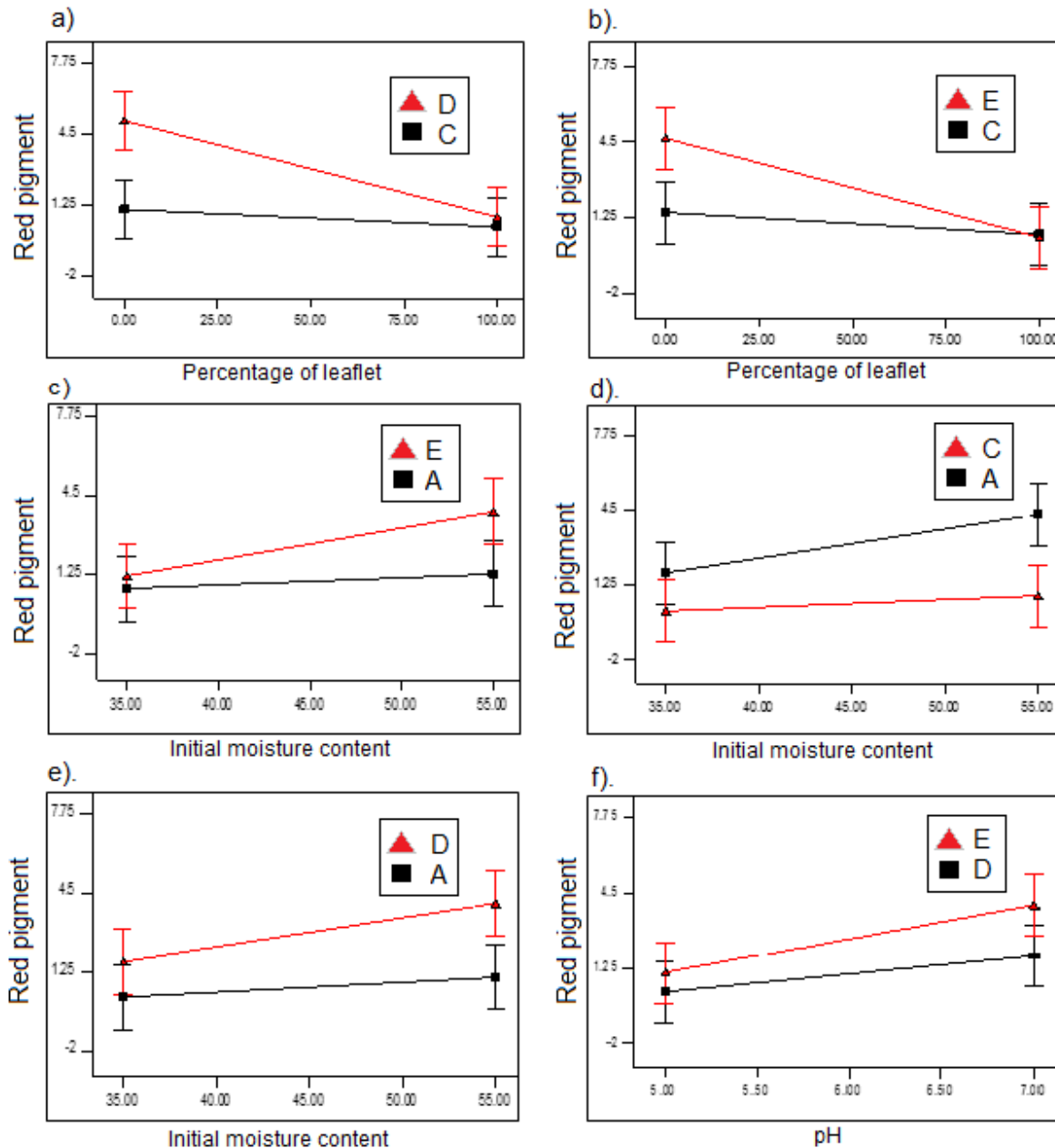

Figure 3 Interaction effect between factors. a) Interaction effect of % OPF leaflet and pH; b) Interaction effect of % OPF leaflet and inoculum size; c) Interaction effect of moisture content and inoculum size; d) Interaction effect of moisture content and % OPF leaflet; e) Interaction effect moisture content and pH; f) Interaction effect of pH and inoculum size

For instance, Figure 3c (55% initial moisture content, 10^9 spores/ml of inoculum size), 3d (55% initial moisture content, 0% of OPF leaflet), and 3e (55% initial moisture content, pH 7) show higher red pigment production. However, result shows lower red pigment production at Figure 3c (35% initial moisture content, 10^9 spores/ml of inoculum size), 3d (35% initial moisture content, 0% of OPF leaflet), and 3e (35% initial moisture content, pH 7) with the yield of 1.21 AU/g, 1.79 AU/g, and 1.69 AU/g, respectively. From the figure, it was expected that higher initial moisture content would favor the red pigment production. The low moisture content can result in the decreasing of red pigment. This phenomenon was due to the low nutrient availability such as nutrient salt dissolution, as well as inefficient heat exchange and oxygen transfer [11,5]

4.0 CONCLUSION

Screening for significant factors for the red pigment production by *Monascus purpureus* FTC 5356 was carried out by applying a fractional factorial design at two levels. The influence of percentage of OPF leaflet, initial pH of substrate, inoculum size and initial moisture content were evaluated. Percentage of OPF leaflet had the most marked effect on the red pigment production. A higher initial pH, inoculum size, and initial moisture content were recommended to optimize the production of red pigment in SSF using OPF as the substrate.

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