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Effect of Factors on The Red Pigment Production in The Stirred Drum Bioreactor: Fractional Factorial Design Approach

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Abstract. Public demands on natural colouring over the synthetic colouring are growing due to consumer health concern towards the effect of synthetic colouring. The colorants production by employing fermentation process shows certain advantages. This research has emphasized on the capability of stirred drum bioreactor on performing red pigment in solid-state fermentation (SSF) of Monascus purpureus FTC 5357 by using oil palm frond (OPF). A Fractional factorial experimental design 2^4 (FFD) was applied in order to evaluate the effect of initial moisture content (IMC), inoculum size, percentage of peptone, pH, aeration rate, loading capacity and agitation programme on the red pigment production. In the FFD experimental studies, the highest pigment production was obtained at 75% (v/w) initial moisture content, 10^8 spores/mL of inoculum size, initial pH of 4, aeration rate of 1.21 vvm, loading capacity of 35% (v/v), agitation programme of 6 cycles/day, with the yield of 71.86 AU/g. The initial moisture content had the most pronounced affects to the red pigment production, followed by the aeration rate of stirred drum bioreactor, peptone concentration and inoculum size. The loading capacity of the bioreactor, agitation programme and the initial pH had the lesser effect to the red pigment production. The result indicated that FFD was a useful tool to improve the red pigment production in SSF using stirred drum bioreactor by considering all the factors involved.

INTRODUCTION

Generally, most of the food manufacturer imposed to use colorant to enhance the desirability of the food product. Public demands on natural colouring over the synthetic colouring are aggressively growing due to the consumer health concern towards the effect of synthetic colouring [1]. In 2014, the natural food colorant market has reached around the value of US$1.14 billion [2,3], and it was expected to rapidly increased, and continue growing by 10% to 15% annually [4].

Therefore, natural food colorants are largely being extracted from plant sources [5]. However, different concerns are about to rise due to the large-scale production of natural food colorants, especially on the sustainability of the resources. Therefore, the colorants production by utilizing fermentation process shows certain advantages over the other sources [6]. The production of natural colorants (pigments) using microorganisms gained a significant attention due to certain advantage such as able to be grown rapidly under highly controlled conditions compared to the other available sources, which were results in a high productivity [7,8,9]. One of the potential microorganisms for large scale pigment production is from *Monascus* sp., as its competency to produce an intense red pigment as well as other beneficiary metabolic by-products [10]. Regardless of the high pigment yields in solid-state fermentation compared to the submerged cultivation [11], the mechanical aspects such as the bioreactor design for solid-state fermentation are vastly unexplored. Hence, this study was emphasized on *Monascus purpureus* in the solid state fermentation (SSF) using oil palm frond (OPF) in stirred drum bioreactor for red pigment production.

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A fractional factorial experimental design 2^4 (FFD) was applied in order to evaluate the effect of seven factors such as initial moisture content (IMC), inoculum size, percentage of peptone, initial pH, aeration rate, loading capacity and agitation programme to the red pigment production. The fermentation process was conducted in a 2.3 L stirred drum bioreactor.

MATERIALS AND METHODS

Microorganism, inoculum preparation and substrate preparation

Monascus purpureus FTC 5357 culture was maintained on Potato Dextrose Agar (PDA) and incubated in at 30°C for 8 days [12]. Fully sporulated agar slant culture was prepared prior to inoculum preparation. Sterile distilled water was added to the slant culture, followed by gentle scrapping on the slant surface to harvest the spore. The spore concentration was measured and adjusted accordingly to the desired concentration for fermentation.

The fresh oil palm fronds (OPF) were obtained from a local palm oil plantation in Federal Land Development Authority (FELDA) Bukit Goh, Kuantan, Pahang, Malaysia. The fresh OPF was cut into smaller pieces, cleaned and prepared as mentioned by Hamid and Said (2018) [13].

Experimental design and cultivation condition

A specifically fabricated 2.3 L stirred drum bioreactor from the Faculty of Chemical and Natural Resources Engineering (FKKSA) laboratory, UMP, Malaysia, was used [14]. The experimental work was done based on the experimental design, using fractional factorial design (FFD) 2^4, 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 each run has been harvested on day 8 after inoculation.

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

Symbol	Factors	-1	1
A	Initial moisture content (% v/w)	55	75
B	Inoculum size (spores/mL)	10^8	10^{12}
C	Peptone concentration (% w/w)	4	6
D	Initial pH (pH)	6	8
E	Aeration rate (vvm)	0.87	1.21
F	Loading capacity (% v/v)	25	35
G	Agitation programme (cycles/day)	6	10
	Responses		
Y_1	Red pigment production (AU/g)		
Y_2	Biomass (mg/g)		

An empty bioreactor drum and the treated OPF having specified initial moisture content, peptone and adjusted pH, as stated in Table 2, were separately autoclaved. After being cooled to room temperature, the substrates were inoculated with specified inoculum volume as in Table 2, evenly mixed and aseptically transferred to the bioreactor. The cultures were cultivated for 8 days at room temperature.

TABLE 2. The fractional factorial design of the independent variables.

Run	A	B	C	D	E	F	G
1	55	10^8	4	6	0.87	25	6
2	75	10^8	4	6	1.21	25	10
3	55	10^{12}	4	6	1.21	35	6
4	75	10^{12}	4	6	0.87	35	10
5	55	10^8	6	6	1.21	35	10
6	75	10^8	6	6	0.87	35	6
7	55	10^{12}	6	6	0.87	25	10
8	75	10^{12}	6	6	1.21	25	6
9	55	10^8	4	8	0.87	35	10
10	75	10^8	4	8	1.21	35	6
11	55	10^{12}	4	8	1.21	25	10
12	75	10^{12}	4	8	0.87	25	6
13	55	10^8	6	8	1.21	25	6
14	75	10^8	6	8	0.87	25	10
15	55	10^{12}	6	8	0.87	35	6
16	75	10^{12}	6	8	1.21	35	10

Analytical methods

Red pigments were determined using a UV-VIS spectrophotometer (Hitachi U-1800) [13]. The yield was expressed as absorbance units (AU) per gram of dried solids [15]. Total fungal biomass was determined using spectroscopy method, by measuring the N-acetylglucosamine released by acid hydrolysis of the chitin in the fungal cell walls [13,16].

Statistical analysis

A Design Expert (Version 7.1.6, 2008, Minneapolis MN, USA) software, was used for the experimental design of fractional factorial design. Statistical parameters were estimated using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

A fractional factorial design (FFD) was conducted to screen out the insignificant factors in order to gain effective red pigment production in stirred drum bioreactor. Factorial design is efficient to evaluate the effect of factors over a wide range of conditions with a minimum number of experiments.

The selections of significant model factors were done using design expert software. Figure 1 and 2 show the pareto charts of red pigment and biomass productions, which represented the estimated effects of the factors and their interactions on the responses variables. The pareto charts show 2 different t-limit; Bonferroni corrected t-limit (or Bonferroni limit) and standard t-limit (or t-value limit) (Figs. 1 and 2). Effects that are above Bonferroni limit are considered almost certainly significant, while the t-value limit indicated the possibly significant effects. While, effects that are below t-value limit are not likely to be significant, at a confidence level of 95%. On the other hand, the factors in Figs. 1 and 2 also characterized into two categories; factors with positive and negative effects. Factors with positive effect are directly correlated to the responses' value (red pigment and biomass), and factors with negative effect are inversely correlated. For instance, initial moisture content (A) (Fig. 1) showed the high positive effect, which indicated that the red pigment was greater in higher initial moisture content. Contrarily, for negative effect, higher peptone (C) would contribute to low red pigment production (Fig. 1). This can be explained due to the excessive nutrient provided in the substrate medium make the medium become toxic and inhibit the growth of the *Monascus* sp. [17].

Fig. 1 clearly exposed that the most statistically significant factors to the red pigment were initial moisture content (A), followed by aeration rate (E), peptone concentration (C), initial moisture-inoculum size (AB) and initial moisture-aeration rate (AE). While, inoculum size (B) was the least significant effect to the red pigment.

While in Fig. 2, factor initial moisture content (A) also showed the most significant factor to the biomass production. Followed by peptone concentration(C), aeration rate (E) and initial moisture content-inoculum size (AB), at 95 % of confidence level. Initial moisture content (A) and aeration rate (E) showed positive effects to the biomass production, while and peptone concentration (C) and initial moisture content-inoculum size (AB) show negative effects (Fig. 2). The effectiveness of initial moisture content (A) was in agreement with several reports in literature stating that the moisture content of the substrate significantly affects the biomass growth of the *Monascus* sp. [15,18].

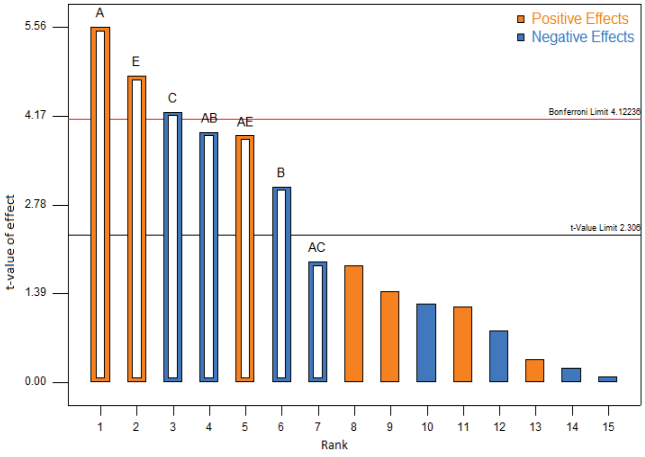

FIGURE 1. Pareto chart for red pigment production

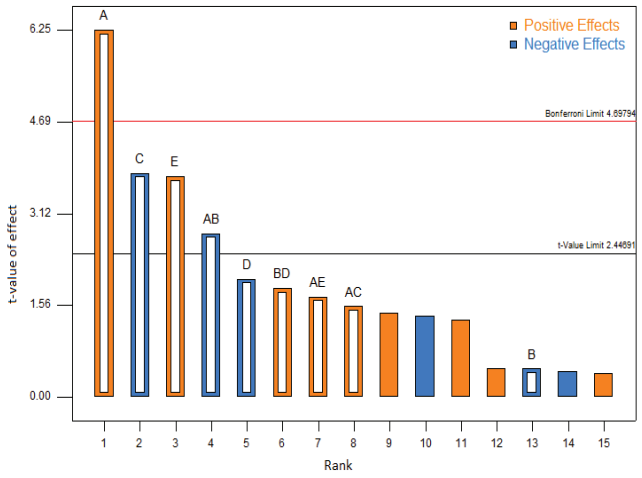

FIGURE 2 Pareto Chart of biomass production

Analysis of variance (ANOVA) for red pigment and biomass productions

The significance of the factors shown in Figs. 1 and 2 were verified by the analysis of variance (ANOVA) in Table 3 and 4, respectively. The quadratic regression model of ANOVA showed that the model was significant to the red pigment with low p-value (0.0004) (Table 3). It also demonstrated that the model has a high correlation with the experimental data. In addition, Table 3 also reveals that the most significant factors to the red pigment was initial moisture content (A), followed by aeration rate (E), peptone concentration (C), initial moisture-inoculum (AB), initial moisture-aeration (AE), and inoculum (B); where all the factors showed the p-value of <0.05. The results were consistent with the pareto chart discussed earlier (Figure 1). The R-squared of the quadratic response surface model of the red pigment obtained was 0.933, which found to be close to 1, indicated the good relation of the predicted and the experimental data of the red pigment. This model also showed a reasonable agreement between the

adjusted (0.875) and predicted (0.734) R-squared values. The adequate precision for the red pigment was 12.3, indicated that the quadratic models obtained were significant for the process [13].

Whereas, in Table 4, the quadratic regression model of the biomass production was significant at 95% confidence level, with relatively low p-value (0.0065). The most significant factors to the biomass was comparable to the red pigment, which was initial moisture content (A), followed by peptone concentration (C), aeration rate (E) and initial moisture-inoculum (AB). Further, the R-squared of the quadratic response surface model of biomass production was 0.934, close to 1. The R-squared obtained showed relatively good relation of both predicted and the experimental data of the biomass production.

TABLE 3. Analysis of variance (ANOVA) for red pigment production

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	4576.75	7	653.82	16.02	0.0004*
A	1261.13	1	1261.13	30.91	0.0005*
B	382.59	1	382.59	9.38	0.0155*
C	731.16	1	731.16	17.92	0.0029*
E	937.89	1	937.89	22.98	0.0014*
AB	625.00	1	625.00	15.32	0.0045*
AC	144.72	1	144.72	3.55	0.0964
AE	607.87	1	607.87	14.90	0.0048*
Residual	326.45	8	40.81		
Cor Total	4903.20	15			
R^2	0.933				
Adj R^2	0.875				
Pred R^2	0.734				
Adeq Precision	12.298				

*significant ($p<0.05$),

Notes: A-initial moisture content, B-inoculum size, C-peptone, E-aeration rate

TABLE 4 Analysis of variance (ANOVA) for biomass production

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	208810.55	9	23201.17	9.41	0.0065*
A	96209.63	1	96209.63	39.02	0.0008*
B	564.54	1	564.54	0.23	0.6492
C	35758.81	1	35758.81	14.50	0.0089*
D	9887.02	1	9887.02	4.01	0.0921
E	34638.79	1	34638.79	14.05	0.0095*
AB	19063.32	1	19063.32	7.73	0.032*
AC	5849.19	1	5849.19	2.37	0.1744
AE	7120.83	1	7120.83	2.89	0.1401
BD	8392.39	1	8392.39	3.40	0.1146
Residual	14793.32	6	2465.55		
Cor Total	223603.87	15			
R^2	0.934				
Adj R^2	0.835				
Pred R^2	0.530				
Adeq Precision	11.129				

*significant ($p<0.05$),

Notes: A-initial moisture content, B-inoculum size, C-peptone, D-pH, E-aeration rate

Interaction factors on the red pigment and biomass production

Figure 3 and 4 denote the interaction factors of initial moisture content and aeration rate (AE) and initial moisture content and inoculum size (AB), to the red pigment, respectively. In Fig. 3, the pigment production was increased as the AE increased. For instance, the higher aeration rates, the higher red pigment production. The red pigment production was in favor at higher initial moisture content and higher aeration rate. The increased of initial moisture content proportionally increased the solubility of nutrients in the substrate. Hence, promoted to the fungal growth, consequently increased the production of the red pigment. In addition, an adequate aeration in the bioreactor facilitates to the oxygen transport process [18], besides stimulated to the transformation of accumulated heat in the substrate bed in the bioreactor [14,19]. These interactions (AE) suggested the synergic effects between initial moisture content and the humidified air supplied throughout the fermentation in the bioreactor, to the red pigment production.

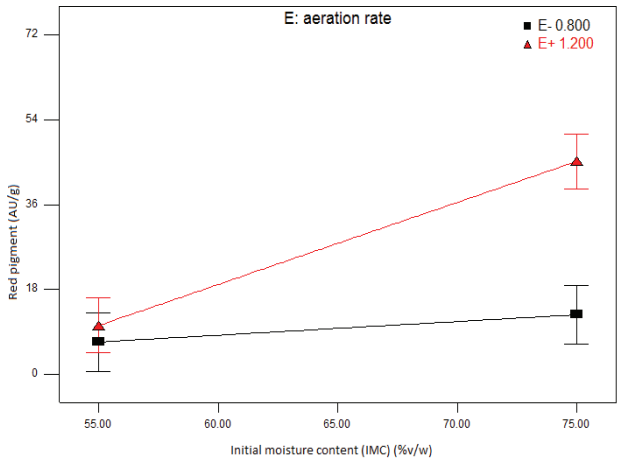

FIGURE 3. Interaction of initial moisture content and aeration rate (AE) on red pigment production. Other factors were fixed at 10^9 spores/ml, 5% (w/w) peptone, pH 7, loading capacity 30% (v/v), agitation programme 8 (cycles/day)

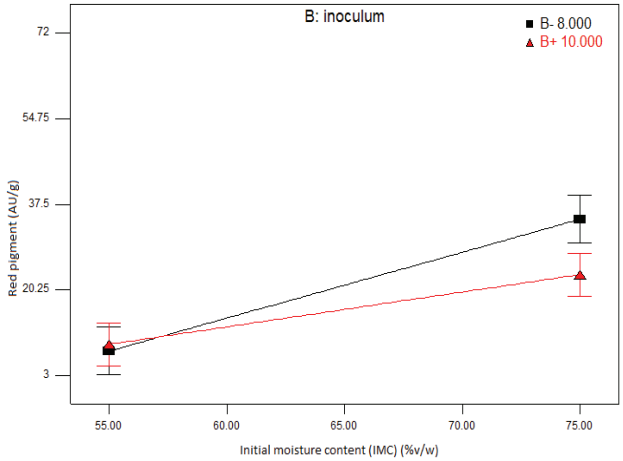

FIGURE 4. Interaction of initial moisture content and inoculum size (AB) on red pigment production. Other factors were fixed at 5% (w/w) peptone, pH 7, loading capacity 30% (v/v), agitation programme 8 (cycles/day), aeration rate 1 (vvm)

In Fig. 4, at low initial moisture content, the response showed relatively in low pigment production to both high and low inoculum size. However, at higher moisture content, the response favored to the lower inoculum size, although generally there was slight increment of response at higher inoculum size. Various possible explanations can be accounted for this occurrence. From the mechanical aspect of fermentation, the high water content would likely to facilitate the heat regulation process in the drum bioreactor. While, on the other aspects, at higher inoculum size, the overpopulation of the fungal might be occurred in the bioreactor. The overpopulation of the *Monascus* sp. in the bioreactor may result to the nutrient exhaustion and oxygen depletion [18], thus caused the unsustainable biological system in the bioreactor. This phenomenon would lead to the disruption of the pigment producing performance in the bioreactor.

Figure 5 shows the interaction factors of initial moisture content and inoculum size (AB), to the biomass production. At low initial moisture content, the biomass production slightly favored to the high inoculum size. However, at higher moisture content, the biomass was favored to the lower inoculum size (Fig. 5). This result is highly corresponded to the earlier discussion in Fig. 4.

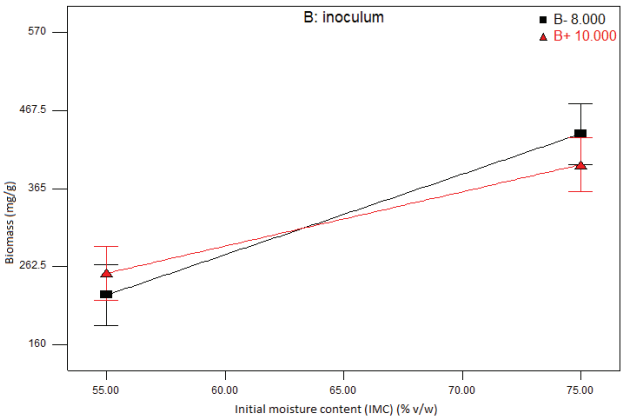

FIGURE 5. Interaction of initial moisture content (A) and inoculum size (B) on biomass production. Other factors were fixed at 5% (w/w) peptone, pH 7, loading capacity 30% (v/v), agitation programme 8 (cycles/day), aeration rate 1 (vvm)

CONCLUSION

The study showed that in the stirred drum bioreactor, the initial moisture content had the most pronounced affects to the red pigment and biomass productions. Followed by the aeration rate and the peptone concentration. The inoculum size, loading capacity of the bioreactor, agitation programme and the initial pH had the lesser effect to the red pigment and biomass production. The result indicated that FFD was a useful tool to screen the significant factors that contributed to the red pigment production to further optimize for larger scale production in stirred drum bioreactor.

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