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FERMENTATION OF BIODIESEL-DERIVED WASTE FOR 1,3-PROPANEDIOL PRODUCTION WITH RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The present study aimed to investigate the fermentation conditions favouring the maximum production of 1,3-propanediol (1,3-PD) from crude glycerol. Response surface methodology (RSM) based on central composite design (CCD) was applied in designing the experiments to evaluate the interactive effects of glycerol concentration (20 to 50 g litre^{-1}), pH (6 to 8), temperature (30°C to 40°C) and incubation time (48 to 72 hr) on 1,3-PD production. A total of 30 experimental runs consisting of 16 factorial points, eight axial points and six centre points were conducted. It was found that the derived optimum conditions were: 39.9 g litre^{-1} glycerol; pH: 7.6, temperature: 33°C and incubation time: 59.1 hr. Under these conditions, the 1,3-PD produced (9.85 g litre^{-1}) was ~2% higher than the predicted value by RSM (9.69 g litre^{-1}), hence, the experimental design employed in validating the results obtained was significant. The analysis of variance (ANOVA) showed high coefficient of the determination values (R^2) of 0.9444. The fermentation using RSM was able to increase the 1,3-PD production by two-fold.

Keywords: 1,3-propanediol, bioprocess, biodiesel by-product, central composite design, optimisation.

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INTRODUCTION

Biodiesel, an environmental-friendly biofuel compared to fossil fuel, is made from plant oils, animal fats and recycled greases. The glycerol to biodiesel production ratio is 1:10 (Yang *et al*., 2012). The rapid development of the biodiesel industry in recent years has generated an inevitable abundance of waste glycerol, the application of which is limited as compared to pure glycerol due to the presence of impurities. Thus, it is economically beneficial to convert this by-product into higher value products, in order to improve the sustainability of the biodiesel business and overcome environmental challenges associated with crude glycerol disposal.

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At present, considerable efforts have been devoted to explore the potential applications of crude glycerol as valuable products such as biofuels, chemicals, polymers and animal feed. The bioconversion via biological routes seems to be more practical and feasible as it will be able to circumvent the disadvantages of conventional processes (Da Silva *et al*., 2015). A wide range of value-added products could be obtained through the microbial fermentation of crude glycerol. These include polyhydroxyalkanoates (PHA), 1,3-propanediol (1,3-PD), citric acid, hydrogen and many others. Among the many promising applications for glycerol, microbial conversion into 1,3-PD has received much attention especially in recent years. Therefore, the main objective of this study is to utilise crude glycerol as low-cost carbon source to produce 1,3-PD via the biological approach.

The 1,3-PD is a starting material used in polymerisation, mainly in polytrimethylene terepht-

halate (PTT) manufacturing. It can be synthesised chemically. However, the chemical route entails expensive catalysts, higher temperature and pressure condition as well as extreme safety measures for handling. In contrast, the biological route is more desirable as it is fermentation-based, uses low-cost raw materials and is safe. Hence, converting glycerol into 1,3-PD via the bioprocess route seems promising due to the better economics, more environmental-friendly and plus the fact that the production yield is much higher compared to chemical synthesis as proven in previous studies (Jun *et al*., 2010; Wilkens *et al*., 2012).

Biologically, glycerol can be used to make valuable chemicals, *e.g*. 1,3-PD (Loh and Stasha, 2016; Jun *et al*., 2010), citric acid (Morgunov *et al*., 2013), succinic acid (Sadhukhan et al., 2016), ethanol (Chanthoom *et al*., 2016) and butanol (Jensen *et al*., 2012). Previously, several bacteria species had been identified for their ability to produce 1,3-PD such as *Klebsiella* (Wojtusik *et al*., 2015), *Clostridium* (Szymanowska-Powalowska, 2014), *Enterobacter* (Waghmare and Naik, 2016) and *Citrobacter* (Ferreira *et al*., 2012). Among these, *K. pneumoniae* is one of the most studied because it has been evidently shown to have potential for glycerol synthesis into 1,3-PD with high productivity (Jalasutram *et al*., 2011; Rossi *et al*., 2012; Da Silva *et al*., 2015).

As the fermentation conditions play an important role in achieving maximum 1,3-PD production, the optimisation of the culture condition is desired. According to Ibrahim *et al.* (2011), the conventional 'one-factor-at-a-time' (OFAT) approach is less practical because the estimation of the factor effects is imprecise and leads to an incomplete understanding of the research findings. Besides, the OFAT system is time-consuming due to the requisite of performing a number of experimental runs. These limitations can be replaced by optimising the factors via designing a statistical experiment using response surface methodology (RSM).

RSM is a useful model for designing and analysing the experiments statistically in determining the optimal conditions desirable for the responses with minimum run of experiments by assessing the relative significance of numerous independence variables (Sivasubramanian and Namasivayam, 2014). RSM constitutes various techniques, for instance, central composite design (CCD), one factor design, Box-Behnken design and the historical data design. The CCD is more desirable that is ideal for sequential experimentation and doesn't involve a huge number of design points. Several studies have successfully utilised CCD for the optimisation process of 1,3-PD (Hong *et al*., 2013; Li *et al*., 2014). The present study attempted applying RSM to evaluate the combined effect of pH, temperature, incubation time and concentration of glycerol on the production of 1,3-PD.

MATERIALS AND METHODS

Materials

Crude glycerol was obtained from Sime Darby Biodiesel Sdn Bhd (Selangor, Malaysia). The properties of crude glycerol are 79.3% glycerine, 6.69% water, 16.2% soap, 7.3% methanol and 0.97% oil. All chemicals were purchased from Sigma-Aldrich (USA) and Merck.

Microorganism, Inoculum Preparation and Culture Conditions

The commercial bacterium, *Klebsiella pneumoniae*, was used throughout this study. This bacterium was purchased from the Unit of Bacteriology, Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The strain was cultured on growth medium containing (g litre^{-1}): yeast extract, 5; peptone, 10; NaCl, 9; glycerol, 20 and agar, 20. Mineral salt medium (MSM) consisted of (g litre^{-1}): K_2HPO_4, 0.69; KH_2PO_4, 0.25; $(NH_4)_2SO_4$, 6; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract, 1.5 and 1 ml of trace element solution was used as the basal medium for the experiments. The solution consisted of (mg litre^{-1}): $MnSO_4.4H_2O$, 100; $ZnCl_2$, 70; $Na_2MoO_4.2H_2O$, 35; H_3BO_3, 60; $CoCl_2.6H_2O$, 200; $CuSO_4.5H_2O$, 29.3; $NiCl_2.6H_2O$, 25 and 0.9 ml of 37% HCl solution.

Analytical Methods

For the seed culture, the strain was grown on the growth medium overnight. The 1,3-PD fermentation was conducted in a 250-ml Erlenmeyer flask containing 100 ml of the basal MSM and 10% (v/v) seed culture at varying conditions according to the experimental design as described in *Table 1*. During the cultivation, the culture samples were taken at specific time interval and centrifuged at 4000 rpm for 10 min to remove cells. The supernatant was then filtered through a nylon-based membrane filter (0.45 μm). The concentration of 1,3-PD was measured by a high performance liquid chromatography (HPLC) system with a refractive index detector (RID). A mobile phase *i.e.* aqueous sulfuric acid (0.5 mM) was pumped at the flow rate of 1.0 ml min^{-1}. Samples injection and metabolites analysis was conducted at 60°C. All experimental trials were conducted in triplicate.

Experimental Design and Optimisation by RSM

CCD was employed to firstly determine the optimum culture conditions and secondly

to show the statistical significance of the four independent variables (pH, incubation time, glycerol concentration and temperature) in 1,3-PD production. The experiments were designed using Design-Expert® software (version 9.0, Stat-Ease, Inc. Minneapolis, USA). The CCD primarily consists of a 2^4 factorial experimental design which has 16 factorial points, eight axial points and six replicated centre points leading to 30 runs covering the full design of four variables investigated at five different levels ($-\alpha$, -1, 0, 1, $+\alpha$) as illustrated in *Table 1*. The presence of curvature of response and estimation of pure error were examined by applying the replicated center points (Mabilia *et al*., 2010). In developing the regression equation, the variables were coded according to Equation (1):

$$x_i = \frac{(X_i - \overline{X}_i)}{\Delta X_i} \quad i = 1,2,3 \ldots . k \qquad \text{Equation (1)}$$

where x_i is the independent variable coded value, X_i the independent variable real value, X_i is the independent variable real value at centre point and ΔX_i the value of step change. The response variable was fitted into a second order model polynomial [Equation (2)] in order to correlate the response variable to the independent variables.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad \text{Equation (2)}$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interaction effect and x_i represents the coded value of variables. The variable $x_i x_j$ represents the first-order interaction between x_i and x_j $(i < j)$.

The Design-Expert® software was used to fit the second-order polynomial model and was statistically validated by performing analysis of variance (ANOVA). ANOVA is essential to test the significance and adequacy of the model. It subdivides the total variation of the results into two sources of variation, the model and the experimental error to show whether the variation from the model is significant when compared to the variation due to residual error. The fitness and accuracy of the model was determined by R^2 coefficient and *F*-test was used to check the statistical significance of the model. The main and interactive effects of the independent variables on the dependent ones were illustrated by the three-dimensional surface.

RESULTS AND DISCUSSION

Model Fitting and ANOVA

The maximum production of 1,3-PD by *K. pneumoniae* was achieved using the CCD based method of RSM. The experimental design matrix and their employed responses are presented in *Table 2*. The predicted values were obtained with a model fitting technique using Design-Expert® software and were seen to be sufficiently correlated with the experimental values. In our study, the ANOVA showed that the model was most suitably described with a quadratic polynomial model. The results of the second-order response surface model for the 1, 3-PD production in the form of ANOVA are listed in *Tables 3* and *4*, respectively.

The model was highly significant as demonstrated by the quadratic regression of ANOVA, as was evident from the low *P*-value of the F-test (18.20) [$P_{model} > F$) = <0.0001. The model also showed a statistically insignificant lack of fit, as was evident from the lower calculated *F*-value (0.17) *i.e.* the desired probability value (0.1) for lack of fit; hence indicated that the model was valid for the present work and can be used to predict the response (Suhaila *et al*., 2013). Among the linear terms, the main ones, *i.e.* pH and temperature for 1,3-PD production were highly significant as were shown by their respective *P*-values ($Px_1 = < 0.0001$ and $Px_4 = 0.0001$).

Glycerol concentration also showed a significant effect although in lesser degrees compared to pH and temperature in producing 1,3-PD ($Px_3 = 0.0041$). In contrast, the incubation time was insignificant in affecting 1,3-PD production with *P*-values > 0.05 ($Px_2 = 0.3982$). Based on the ANOVA, significant interactions were found between glycerol and

TABLE 1. EXPERIMENTAL RANGE AND LEVEL OF THE INDEPENDENT VARIABLES

Factor	Variable	Range and level				
		$-\alpha$	-1	0	1	$+\alpha$
X_1	pH	5	6	7	8	9
X_2	Temperature (°C)	25	30	35	40	45
X_3	Incubation time (hr)	36	48	60	72	84
X_4	Glycerol concentration (g litre^{-1})	5	20	35	50	65

Note: x_1 - coded value of the variable X_1
x_1 - (pH-7)/1; x_2 - (temperature-35)/5; x_3 - (incubation time-60)/12; x_4 - (glycerol concentration-35)/15.

TABLE 2. COMPOSITION OF THE VARIOUS RUNS OF THE CENTRAL COMPOSITE DESIGN, ACTUAL AND PREDICTED RESPONSES

Run No.	Factor 1	Factor 2	Factor 3	Factor 4	Response (1,3-PD) g litre^{-1}	
	pH (X_1)	Temperature (X_2)°C	Time (X_3) hr	Glycerol (X_4) concentration g litre^{-1}	Actual value	Predicted value
1	7 (0)	35 (0)	60 (0)	65 (+α)	5.10	4.58
2	7 (0)	35 (0)	36 (-α)	35 (0)	4.28	4.61
3	7 (0)	25 (-α)	60 (0)	35 (0)	4.24	4.53
4	6 (-1)	30 (-1)	72 (1)	50 (1)	5.66	5.30
5	7 (0)	35 (0)	60 (0)	35 (0)	10.65	8.97
6	7 (0)	35 (0)	60 (0)	5 (-α)	1.28	1.97
7	8 (1)	30 (-1)	72 (1)	20 (-1)	4.78	4.35
8	8 (1)	30 (-1)	48 (-1)	20 (-1)	5.37	5.49
9	8 (1)	40 (1)	48 (-1)	50 (1)	4.19	4.05
10	6 (1)	40 (1)	72 (1)	20 (-1)	1.39	1.11
11	8 (1)	40 (1)	48 (-1)	20 (-1)	5.02	5.00
12	8 (1)	40 (1)	72 (1)	50 (1)	2.17	3.53
13	8 (1)	30 (-1)	72 (1)	50 (1)	7.29	7.59
14	8 (1)	30 (-1)	48 (-1)	50 (1)	7.49	7.39
15	6 (-1)	40 (-1)	48 (-1)	20 (-1)	2.08	1.98
16	7 (0)	35 (0)	60 (0)	35 (0)	9.69	8.97
17	7 (0)	35 (0)	60 (0)	35 (0)	10.13	8.97
18	6 (-1)	40 (1)	48 (-1)	50 (1)	1.29	1.34
19	7 (0)	35 (0)	84 (+α)	35 (0)	4.09	3.94
20	7 (0)	35 (0)	60 (0)	35 (0)	8.46	8.97
21	6 (-1)	40 (1)	72 (1)	50 (1)	1.71	1.81
22	6 (-1)	30 (-1)	72 (1)	20 (-1)	1.39	1.74
23	6 (-1)	30 (-1)	48 (-1)	50 (1)	3.72	4.10
24	7 (0)	35 (0)	60 (0)	35 (0)	7.81	8.97
25	8 (1)	40 (1)	72 (1)	20 (-1)	3.32	3.14
26	7 (0)	45 (+α)	60 (0)	35 (0)	0.68	0.56
27	6 (-1)	30 (-1)	48 (-1)	20 (-1)	2.90	1.89
28	9 (+α)	35 (0)	60 (0)	35 (0)	7.70	7.52
29	7 (0)	35 (0)	60 (0)	35 (0)	7.07	8.97
30	5 (-α)	35 (0)	60 (0)	35 (0)	1.84	2.20

TABLE 3. ANALYSIS OF VARIANCE (ANOVA) FOR 1,3-PD PRODUCTION RESPONSE SURFACE REDUCED QUADRATIC MODEL

Source	Sum of squares	DF	Mean Square	F value	Prob > F
Model	226.51	14	16.18	18.20	< 0.0001
Residual	13.34	15	0.89	-	-
Lack of fit	3.43	10	0.34	0.17	0.9909
Pure error	9.91	5	1.98	-	-
Cor total	239.85	29	-	-	-

Note: SD - 0.94; CV - 19.71; R^2 - 0.9444; Adj R^2 - 0.8925; Pred R^2 - 0.8582; Adeq precision - 12.607

temperature ($P < 0.05 = 0.0084$). The quadric effects of all factors were also found highly significant. Using the findings from the designed experiment (*Table 2*), the polynomial model for the 1,3-PD production (Y) was regressed by only considering the significant terms as in Equation (3):

$$Y = 8.97 + 1.33X_1 - 0.17X_2 + 0.65X_3 - 0.99X_4 - 0.71X_3X_4 - 1.03X_1^2 - 1.17X_2^2 - 1.42X_3^2 - 1.60X_4^2 \quad \text{Equation (3)}$$

where Y is the predicted response (1,3-PD production) and X_1, X_2, X_3 and X_4 are the coded values of the independent variables; pH, temperature, incubation time and glycerol concentration, respectively. The quadratic model in Equation (3) with 10 terms contained four linear terms, four quadratic terms and one two factorial interactions.

The goodness of fit of the model as examined by the model determination coefficient, R^2 was very close to 1.0, thus the better the model fits the experimental data and predicts the response. The R^2 was found to be 0.9444, which implied that the model had 94.44% of the observed response attributed to the variables and their interactions.

TABLE 4. MODEL COEFFICIENT ESTIMATED BY MULTIPLIES LINEAR REGRESSION

Factor	Coefficient estimate	Standard error	P value
Intercept	8.97	0.38	< 0.0001*
X_1-pH	1.33	0.19	< 0.0001*
X_2-Time	-0.17	0.19	0.3982
X_3-Glycerol	0.65	0.19	0.0041*
X_4-Temperature	-0.99	0.19	< 0.0001*
X_1X_2	-0.25	0.24	0.3089
X_1X_3	-0.078	0.24	0.7451
X_1X_4	-0.14	0.24	0.5506
X_2X_3	0.33	0.24	0.1767
X_2X_4	-0.18	0.24	0.4547
X_2X_4	-0.71	0.24	0.0084*
X_1^2	-1.03	0.18	< 0.0001*
X_2^2	-1.17	0.18	< 0.0001*
X_3^2	-1.42	0.18	< 0.0001*
X_4^2	-1.60	0.18	< 0.0001*

Note: *Values of 'probability > F value' less than 0.05 indicate model terms are significant.

Yet, only 5.56% of the total variance could not be explained by the model. These measures indicated that the model fitted well in optimising 1,3-PD production (response) as the polynomial model used was capable of generating accurate data and the response trends analysed was reasonable. Meanwhile, the model's significance level was confirmed by the value of the adjusted R^2 (0.8925) which was very close to the predicted R^2 (0.8582).

The model showed the ratio of signal to noise > 4 *i.e*. 12.607, hence was indicative of adequate model discrimination (Ramanan *et al*., 2010). This ratio indicated the reliability of the experiment data and the higher model adequacy to predict the response. The coefficient of variation (CV = 19.71) and standard deviation (SD = 0.94) of the model further indicated the higher degree of precision. The low values of CV reflected that reliable and precise experiments had been conducted.

Response Surface Plots Analysis

The 3D response surface generated from the regression model illustrated the interactive effects of each independent variable on the response. *Figure 1* shows an example of the effects of two variables in producing 1,3-PD, while the other two variables were constant at their middle levels. It further shows that only glycerol concentration and temperature had significant interactions as displayed in *Figure 1*. Previously, the convex response surfaces suggested that the model used was well-defined in providing optimal solutions. This otherwise indicated that the optimised values may differ very little from single variable conditions, if the response surfaces were rather flat and symmetric near the optimum point.

Model Validation with the Optimised Culture Conditions

The optimal culture conditions as predicted by RSM: pH = 7.6; incubation time = 59.1 hr; glycerol concentration = 39.9 g litre^{-1} and temperature = 33°C were verified. The experiments performed showed pretty close predicted and experimental values (9.69 g litre^{-1} *vs*. 9.85 g litre^{-1}) confirming that the model used was valid with the existence of an optimal point. The optimisation via RSM may improve 1,3-PD production by two-fold as compared with that under unoptimised condition (*Table 5*).

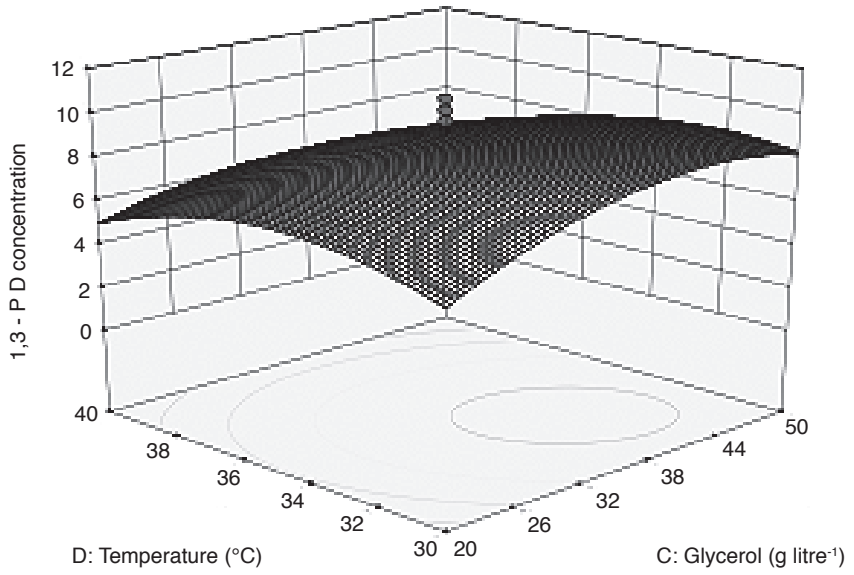

Figure 1. The 3-D graphic plots showing the effect of glycerol concentration and temperature on 1,3-PD production. Value of pH and incubation time was fixed at central point.

TABLE 5. EXPERIMENTAL VERIFICATION OF COMBINED EFFECT OF OPTIMISED CONDITION ON THE RESPONSE OF 1,3-PROPANEDIOL (1,3-PD) PRODUCTION

Variables	Unoptimised condition	Optimised condition	1,3-PD production (g litre^{-1})		
			Before optimisation	After optimisation	
				Predicted	Experimental
pH	7	7.6	-	-	-
Temperature (°C)	30	33	4.89	9.69	9.85
Incubation time (hr)	48	59.1	-	-	-
Glycerol concentration (g litre^{-1})	20	39.9	-	-	-

CONCLUSION

Temperature and pH were shown to be the most significant in influencing the process performance of 1,3-PD followed by glycerol concentration, whereas incubation time showed the least influence. The RSM-based CCD study revealed that the interaction between incubation temperature and glycerol concentration had profound impact, which was vital in achieving exponential increase in 1,3-PD production. The findings were beneficial for future research looking into cost-effective production of 1,3-PD from biodiesel-derived waste.

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