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Optimization of Hydrogen Production Process by Response Surface Methodology

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Abstract: Enhancement of bio-hydrogen production by green microalgae *Chlamydomonas reinhardtii* has captivated tremendous interest. The yield of bio-hydrogen production by *C. reinhardtii* was enhanced by co-culturing with ragi tapai followed by immobilization and culturing in the right TAP-S medium. In this study, the three factors that affects the bio-hydrogen production by *C. reinhardtii* were evaluated with Response Surface Methodology (RSM). The amount of microalgae and ragi tapai as well the pH of the culture medium were simultaneously altered to determine the maximum bio-hydrogen yield. The maximum bio-hydrogen production yield obtained was 702317 ppm under conditions of 2.46 g of microalgae, 0.45 g of ragi tapai and pH medium of 7.23.

Key words: Bio-hydrogen, Response Surface Methodology (RSM), optimization, microalgae, ragi tapai, amount

INTRODUCTION

Hydrogen is one of the most abundant elements in the world that accounts for about 75% of the universe mass (Johnston *et al*., 2005). Hydrogen holds a promise as a potential clean, renewable and environmental friendly energy source. It is seen as fuel of the future because it has a very high energy density, three times that of the petrol or diesel. Besides, it produces water only without any greenhouse gases or other harmful pollutants. Furthermore, using petrol and diesel in combustion engines wastes at least two thirds of the energy in fuel, whereas hydrogen can be used in fuel cells which are about twice as efficient. So, much more of the fuel's energy is put to good use and at the same time less fuel is needed for the same energy output. However, the viability of a future hydrogen economy depends entirely upon the development of efficient, large-scale and sustainable hydrogen production systems (Esper *et al*., 2006). In order to unravel the possible of hydrogen economy, it requires the availability of low cost hydrogen produced in a carbon-neutral manner. This has led to an investigation of a biological system for hydrogen production from renewable resources. Hydrogen produced through the action of living organism is called as bio-hydrogen. A comprehensive overview of different hydrogen production technologies has been reviewed previously (Holladay *et al*., 2009).

The biological processes of hydrogen production are fundamentally dependent upon the presence of hydrogen producing enzyme. These enzymes catalyze the chemical reaction $2H^{+}+2e^{-} \leftrightarrow H_2$. Three enzymes that carry out this reaction are known as: nitrogenase, Fe-hydrogenase and NiFe-hydrogenase (Eroglu and Melis, 2011). Fe-hydrogenase enzyme is used in the bio-photolysis process whereas photo-fermentation processes utilize nitrogenase. Among many types of microorganism studied for bio-hydrogen production microalgae have received a great deal of attention as a novel biomass resources.

However, the presence of oxygen inhibits the action of hydrogenase enzyme in producing hydrogen. One of the solution was to sulfur deprive the culture medium in order to decline the rate of oxygenic photosynthesis while maintaining the rate of mitochondrial respiration (Melis *et al*., 2000; Torzillo *et al*., 2009). This would then lead to the decrease in photosynthetic oxygen evolution and the culture medium would then become anaerobic a condition where the hydrogenase enzyme is induced and as the result, hydrogen is released. Although, the sulfur deprivation enables the microalgae cells to produce hydrogen efficiently but it is still inefficient to provide high yield of hydrogen. There is other method to further enhance the hydrogen production yield by microalgae, that is through immobilization in chitosan and co-culturing it with ragai tapai. In the previous study, it was reported that the bio-hydrogen production yield increased about 31% when compared to free microalgae cells (Saifuddin and Hussain, 2011). Nevertheless, in that study, only eight different amount of ragi tapai with a constant amount of microalgae cells were tested. Hence,

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in order to determine the optimal conditions for bio-hydrogen production, it was essential to use a statistical experimental design to optimize the key parameters that effects bio-hydrogen production. The classical methods of optimization involve a relatively large number of experiments which makes it laborious and time consuming, especially when the number of factors is large (Wang and Wan, 2008, 2009). For hydrogen production by co-cultures, the ratio of microalgae and ragi tapai immobilized in chitosan and the pH of the culture medium play a vital role during the production of hydrogen. Some statistical methods including the Plackette-Burman design and Response Surface Methodology (RSM) have been successfully employed for optimization in some bioprocesses such as bio-hydrogen production (Pan *et al*., 2008). In this study, the parameters that affect bio-hydrogen production were screened using the RSM approach. Using RSM, the matrix was designed by using low, medium and high level values of each variable. The experimental design was created using Design Expert® Software. Version 7.0. RSM was chosen for this study as multiple factors are involved and using One-Factor-At-a-Time (OFAT) will not be appropriate. This is because OFAT will not be able to disclose the interactions between the factors, hence the results obtained can be biased from the true optimal (Montgomery, 1991). Besides laborious and time consuming, OFAT does not interpret the effect among the multiple variables to obtain optimal conditions (Wang and Wan, 2008). On the other hand, RSM is a time saving method which is able to reveal the interaction among the factors and minimizes the error in determining the effects of each parameters (O'Thong *et al*., 2008; Argun *et al*., 2008).

RSM approach has been used by many researches to improve the efficiency of bio-hydrogen production. Mu *et al*. (2008) used central composite design to study the individual and interactive effects of pH, temperature and substrate concentrations on anaerobic hydrogen production. Shi and Yu (2005) applied Box-Behnken analysis to explain the interactive effect of cell concentration and light intensity on the performance of hydrogen production by *Rhodopseudomonas capsulate*. In this research, the effects of the amount of microalgae cells and ragi tapai as well as the pH of the culture medium on the bio-hydrogen production yield by the microalgae was investigated by using Box-Behnken Response Surface Methodology (RSM).

MATERIALS AND METHODS

Microalgae culture media: The microalgae species *Chlamydomonas reinhardtii* C137(+) was obtained from Culture Collection of Algae and Protozoa (CCAP) of SAMS Limited in United Kingdom its strain number is CCAP 11/32A. *C. reinhardtii* was cultivated in Tris-Acetate-Phosphate (TAP) growth medium.

All the chemicals used in the growth medium were of analytical gradient. Reverse Osmosis (RO) water was used for all media and solution preparations. The TAP medium was prepared following the original TAP medium of Gorman and Levine (1965). The recipe for TAP stock solution are shown in Table 1.

In order to make the final TAP medium, 2.44 g of Tris, 25 mL of TAP salt, 0.375 mL of phosphate solution, 1 mL of Hutner's Trace Element and 1 mL of glacial acetic acid was mixed in ~600 mL RO water which was then made up to 1 L by topping up with more RO water.

For the preparation of sulfur free TAP medium (TAP-S) the sulfate salts were replaced with equimolar of chloride salts. In TAP salt, $MgSO_4.7H_2O$ was replaced with $MgCl_2.H_2O$. Where as in the Hutner's trace element, $ZnSO_4.7H_2O$ was replaced with 1.0 g of $ZnCl_2$, $CuSO4.5H_2O$ was replaced with 0.1 g of $CuCl_2.2H_2O$ and $FeSO_4.7H_2O$ was replaced with 0.36 g of $FeCl_2.4H_2O$. The final volume of TAP-S medium was same as TAP medium.

Culture media and ragi tapai cultivation: The ragi tapai was obtained from a local market and cultivated in YEP broth media. The YEP broth media preparation was adapted from Saifuddin and Refal. The broth media consists of the following solutions:

Table 1: TAP stock solution content

Stock solutions	Compounds	Amount (g)	Water (mL)
TAP salt	NH_4Cl	15.00	Dissolvein ~850 mL RO water, once all is dissolved top up to 1 L
	$MgSO_4.7H_2O$	4.00	
	$CaCl_2.2H_2O$	2.00	
Phosphate solution	K_2HPO_4	28.80	Dissolve in ~70 mL RO water, once all is dissolved top up 100 mL
	KH_2PO_4	14.40	(Top up to 1 L with RO water) (mL)
Hutner's trace element	EDTA	5.00	25
	$ZnSO_4.7H_2O$	2.20	10
	H_3BO_3	1.14	20
	$MnCl_2.4H_2O$	0.50	5
	$CoCl_2.6H_2O$	0.16	5
	$CuSO_4.5H_2O$	0.16	5
	$(NH_4)_6MO_7O_{24}.4H_2O$	0.11	5
	$FeSO_4.7H_2O$	0.50	5

- The 10 g/L yeast extract
- The 10 g/L peptone
- The 5 g/L Sodium Chloride (NaCl)
- The 1 L reverse osmosis water (RO-H_2O)

The 200 mL of YEP broth media was autoclaved at 121°C for 15 min. It was then cooled down before adding in 6 g of dry ragi tapai. The YEP broth media with ragi tapai were then aerobically propagated at 37°C while being shaken at 250 rpm on a mechanical shaker. After about 24 h, the ragi tapai were collected from the YEP broth media by centrifugation.

Cultivation of microalgae: Fifteen millimeters (mL) of *C. reinhardtii* were cultivated into 150 mL of TAP growth media as prepared previously. The microalgae cells were then left to be cultivated for 4 days at room temperature and were continuously illuminated from the top using cool white fluorescents light with an intensity of 200 μmol m^2/sec (14800 Lux). The distance between the microalgae suspension and the fluorescent light was 25 m which was adequate in order to avoid photo inhibition. To avoid adherence of the microalgae to the sides of the culture flask, they were hand shaken once or twice daily. Cell density of the microalgae cell suspension were measured daily for 6 days using a spectrophotometer at 750 nm.

Immobilization of microalgae and ragi tapai on chitosan beads: Immobilization of the co-cultures of microalgae cells and ragi tapai on chitosan beads were adapted from Kaya and Picard (1996) with some slight modification. One gram of chitosan powder was dissolved in 50 mL of distilled water to get 2% (m/v) of chitosan solution. It was then stirred and heated at 50°C on a hot plate. The pH of chitosan solution was adjusted to pH 5 5.5 by dropwise addition of acetic acid. Once all the chitosan flakes have been dissolved, the appropriate ratios of microalgae to ragi tapai were added into the chitosan solution and left to be stirred for 30 min to get a homogenous suspension. Once a uniform suspension was achieved, the microalgae suspension was added dropwise into 200 mL of gently stirred 1.5% (m/v) sodium pyrophosphate (pH 7.5) using a model 100 push-pull syringe pump. It was then cross left to be stirred for 50 min for chelation. Then, the linked chitosan gel beads were washed 3 times with 0.1 M phosphate buffer (pH 7.5) till the washing solution becomes neutral and were then stored in RO water.

Optimization of bio-hydrogen yield under sulfur deprived conditions: This experiment was conducted to determine the best ratio of microalgae to ragi tapai and pH of the TAP-S medium in order to obtain the maximum bio-hydrogen yield. Three variables which are the amount of microalgae (1, 2, 4 g) the amount of ragi tapai (0.2, 0.5, 2 g) and the pH value of culture medium (6, 7, 8) are served as the critical variables X_1-X_3, respectively as shown in Table 2. A total of 15 experimental runs was carried out and the central points was replicated three times to evaluate the experimental errors. The number of experimental trails and their corresponding setting conditions are summarized in Table 3.

Table 2: Level of variables selected for bio-hydrogen production yield by *C. reinhardtii*

Parameters	Amount of algae x_1 (g)	Amount of ragi tapai x_2 (g)	pH value (X_3)
Low level (L)	2	0.40	7.0
Medium level (M)	3	0.50	7.5
High level (H)	4	0.60	8.0

Table 3: Overall experimental design and setting conditions for the bio-hydrogen production yield

Trial	Amount of algae (g)	Amount of ragi tapai (g)	pH
1	L	M	H
2	M	H	H
3[a]	M	M	M
4	H	H	M
5	L	M	L
6	M	L	H
7	L	L	M
8	M	L	L
9	H	M	L
10[a]	M	M	M
11	L	H	M
12	H	M	H
13	H	L	M
14[a]	M	M	M
15	M	H	H

[a]The center point of this experiment was replicated 3 times

Measurement of bio-hydrogen production yield: Bio-hydrogen production yield was determined by collecting the gas produced at the headspace of the bottle by using a gas tight syringe. About 10 mL of the gas sample was withdrawn and injected into the sensor of the hydrogen meter to obtain the amount of hydrogen gas.

RESULTS AND DISCUSSION

Optimization of bio-hydrogen production using RSM: RSM was used to evaluate the relationship between a set of controllable experimental factor and its observed responses. In order to identify the optimum conditions for maximum hydrogen production yield, fifteen trials were run. The amount of microalgae and ragi tapai as well as the pH of the culture medium are closely related to enhance bio-hydrogen production. Thus, those three factors were selected as the key parameters in order to maximize the bio-hydrogen production. The center point for microalgae, ragi tapai and pH were 3, 0.45 and 7.5 g,

Table 4: Optimization study of bio-hydrogen production yield using RSM box-behnken design

Trial	Amount of algae (g)	Amount of ragi tapai (g)	pH	Experimental H_2 production (ppm)	Predicted H_2 production (ppm)
1	2	0.50	8.0	650000	642500
2	3	0.60	8.0	300000	300000
3[a]	3	0.50	7.5	670000	670000
4	4	0.60	7.5	350000	342500
5	2	0.50	7.0	655000	647500
6	3	0.40	8.0	650000	650000
7	2	0.40	7.5	640000	647500
8	3	0.40	7.0	650000	650000
9	4	0.50	7.0	680000	687500
10[a]	3	0.50	7.5	665000	670000
11	2	0.60	7.5	250000	257500
12	4	0.50	8.0	685000	692500
13	4	0.40	7.5	660000	652500
14[a]	3	0.50	7.5	675000	670000
15	3	0.60	7.0	300000	300000

[a]The center point of this experiment was replicated 3 times

Table 5: Lack of fit test summary for maximum bio-hydrogen production

Sources	Sum of squares	df	Mean square	F-values	p-value (Prob.>F)	Remarks
Linear	1.420×10^{11}	9	1.557×10^{11}	630.99	0.0016	-
2FI	1.403×10^{11}	6	2.339×10^{10}	935.66	0.0011	-
Quadratic	4.500×10^{8}	3	1.500×10^{8}	6	0.1462	Suggested
Cubic	0.000	0	-	-	-	Aliased
Pure Error	5.000×10^{7}	2	2.500×10^{7}	-	-	

Table 6: Model summary statistics for lack of fit test

Sources	SD	R^2	Adjusted R^2	Predicted R^2	Press	Remarks
Linear	1.13600	0.6368	0.5378	0.3059	2.714×10^{11}	
2FI	1.32500	0.6410	0.3717	-0.5603	6.102×10^{11}	
Quadratic	10000.0	0.9987	0.9964	0.9813	7.312×10^{9}	Suggested
Cubic	5000.00	0.9999	0.9991			Aliased

Table 7: ANOVA table for bio-hydrogen production

Sources	Sum of squares	df	Mean square	F-values	P-value (Prob.>F)	Remarks
Model	3.906×10^{11}	9	4.340×10^{10}	433.970	<0.0001	Significant
A-Microalgae	4.050×10^{9}	1	4.050×10^{9}	40.500	0.0014	
B-Ragi tapai	2.450×10^{11}	1	2.450×10^{11}	2450.000	<0.0001	
C- pH	0.000	1	0.000	0.000	1.0000	
AB	1.60000×10^{9}	1	1.600×10^{9}	16.000	0.0103	
AC	2.500×10^{7}	1	2.500×10^{7}	0.250	0.6383	
BC	0.000	1	0.000	0.000	1.0000	
A^2	5.769×10^{6}	1	5.769×10^{6}	0.058	0.8197	
B^2	1.386×10^{11}	1	1.386×10^{11}	1386.060	<0.0001	
C^2	5.769×10^{6}	1	5.769×10^{6}	0.058	0.8197	
Residual	5.000×10^{8}	5	1.000×10^{8}			
Lack of Fit	4.500×10^{8}	3	1.500×10^{8}	6.000	0.1462	Not significant
Pure error	5.000×10^{7}	2	2.500×10^{7}			
Cor Total	3.911×10^{11}	14				

respectively and this point was replicated three times. Results from the experiment were then modeled in the design expert software using RSM-Box-Behnken Design as in Table 4.

The lack of fit test was done to compare the residual error with "pure error" from the replicated design points. From Table 5, it can be concluded that the quadratic model is the most likely model as its lack of fit (Prob.>5) is not significant. Values of prob>F<0.05 indicates that its "lack of fit" is significant and must be ruled out as it is advisable to select a model that has insignificant "lack of fit". This is because significant "lack of fit" indicates that the variation in the model points is knowingly different from the variation in the replicated points. Therefore, it is suggested that the Prob.>F value for lack of fit should be >0.10 and the obtained result from this test is 0.1462 (>0.10). The cubic model is aliases hence it should not be chosen as adding it would not significantly improve the fitness of model design.

To further confirm the chosen model which was quadratic model Table 6 was viewed. It can be confirmed that quadratic model is the best model as it exhibits a low standard deviation, high R^2 and low PRESS. Moreover, the quadratic model has the most maximized value for both adjusted R^2 and predicted R^2. Predicted R^2 obtained was 0.9813 and it is in reasonable agreement with the adjusted R^2 of 0.9964. The R^2 obtained (0.9987) are very good as well as it is very close to 1.

Moreover, the ANOVA test was used to confirm the adequacy of the chosen quadratic model. From Table 7,

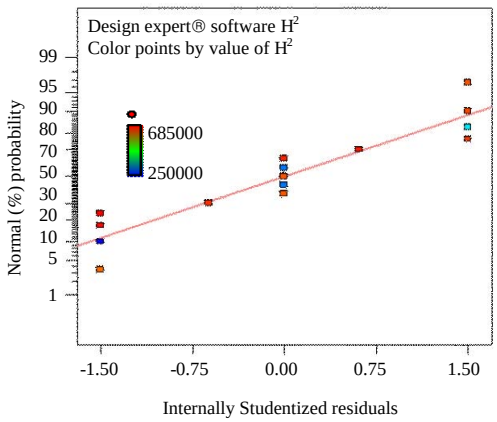

Fig. 1: Normal plot residuals for the whole model of bio-hydrogen production yield; design expert Software H_2; color points by value of H_2

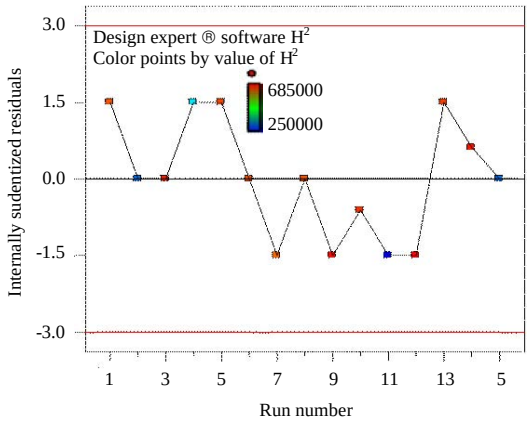

Fig. 2: Residual plot of modified quadratic model for bio-hydrogen production yield; design expert Software H_2; color points by value of H_2

the probability values for each individual term in model can be seen. The model F-value of 433.97 implies that the model is significant and there is only a 0.01% chance that this F-value is occurred due to noise or random error. The p-value (Prob.>F) of <0.0500 indicated that the model terms are significant and so A, B, AB and B^2 are the significant model terms which means they have significant effects on the response. Whereas C, AC, BC, A^2 and C^2 are excluded from the model as their p-value is >0.1 hence they are insignificant. The lack of fit F-value of 6.00 signifies that the lack of fit it not significant relative to the pure error and this is a good sign as it means the model is fit. There is a 14.62% chance that the lack of fit F-value this large could have occurred due to noise. The adequate precision obtained was 53.276. Adequate precision is used to measure the signal to noise ratio and a ratio of >4 is desirable. Ratio of 53.276 obtained implies an adequate signal and that this model can be used to navigate the design space.

Figure 1 is a normal probability plot which specifies whether the residuals follow a normal distribution where the points will follow a straight line. The normal probability plot of studentized residuals is the most important diagnostic (A studentized residual is the quotient resulting from the division of a residual by an estimate of its standard deviation). As can be seen in Fig. 1, the points follow a straight line with some moderate scatter even with the normal data. There is nothing to worry about the moderate scatter as only when a definite pattern (non-linear pattern) such as "S-shaped" curve is observed it signifies that a transformation of there sponse is required in order to obtain a better analsis. A non-linear pattern would indicate non-normality in the error term. There are no signs of any issues in the data obtained in this case.

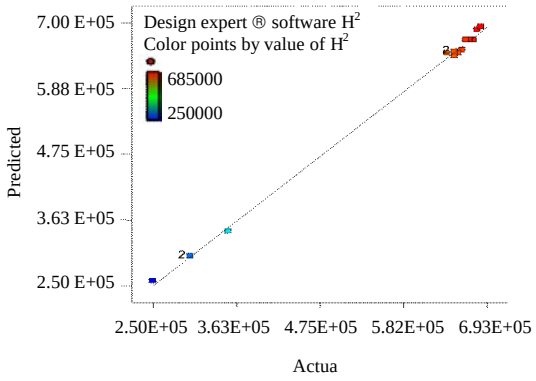

Fig. 3: Relationship between the predicted and experimental response for bio-hydrogen production yield; design expert software H_2; color points by value of H_2

Figure 2 shows the relationship between the residual and experimental run order. In enables to check for lurking variables that might have influence the response during the experiment. Residual illustrates the difference between the actual response (experimental bio-hydrogen production) and the predicted response (predicted bio-hydrogen production). From Fig. 2, a random scatter without any trend pattern was observed. This is a good sign as if a trend pattern was observed it would signify that there is a time related variable lurking in the background. Hence, this shows that the analysis obtained is logical. Figure 3 illustrates the relationship between the actual response (experimental bio-hydrogen production) and the predicted response (predicted bio-hydrogen production).

This graph was plotted to analysis if all the data points are predicted by the model chosen. As can be seen

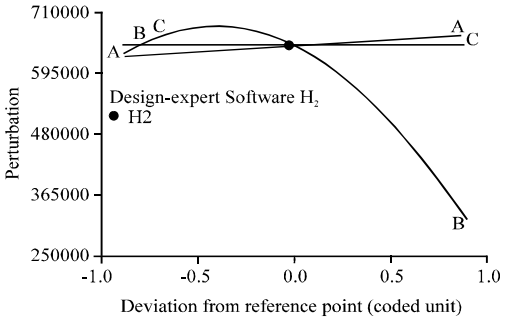

Fig. 4: Perturbation plot for bio-hydrogen production yield; actual factor (A: Algae = 3; B: Ragi = 0.50; C: pH = 1.50)

on Fig. 3 the data points are distributed evenly on a 45° line. This shows that the modified quadratic model chosen is competent of predicting all of the data points. By examining Table 5-7, Fig. 2 and 3, the modified quadratic model was proven to be sufficient to analyze this experiment. The modified quadratic model in this experiment can be described using the following equation:

Pridicted biohyrogen production yield = 6.7+22500×amount of algae-1.75×amount of ragi tapai+200000×amount of algae×amount of ragi tapai+2500×amount of algae×pH-1250×amount of algae^2-1.937×amount of ragi tapai^2-1250×1250×pH^2

Using the above equation, the predicted bio-hydrogen production yield was calculated and recorded in the last column of Table 4.

Figure 4 shows the perturbation plot which helps to set priorities on what to graph first as there are more than two factors in this study. The perturbation plot is able to show the consequences on the response by changing each factor from the reference point while holding the other factors at constant. The reference point by default is set at the middle of the design space (the coded zero level of each factor). A, B and C illustrate the individual effect of amount of microalgae, amount of ragi tapai and pH on the amount of bio-hydrogen production yield consequently. As shown in Fig. 4 among all three factors selected, the most significant factor is the amount of ragi tapai. This is because slight changes in the amount of ragi tapai would cause major difference on the response outcome. The amount of ragi tapai has a slight bell-shaped effect on the predicted bio-hydrogen production yield. Whereas the amount of microalgae and the pH value has a linear effect on the predicted

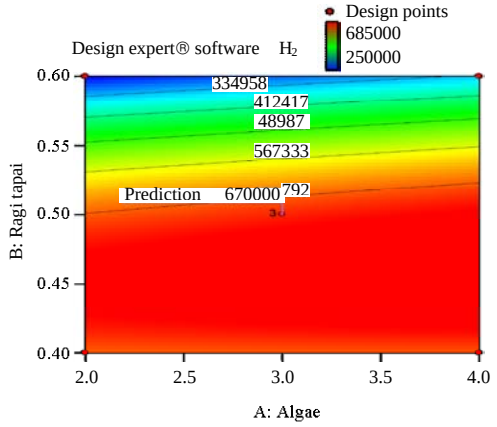

Fig. 5: Two-dimensional contour plot for the bio hydrogen production yield; X1 = A: Algae; X2 = B: Ragi; actaul factor C: pH = 1.50

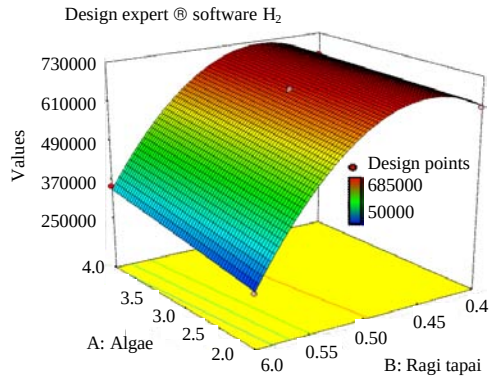

Fig. 6: Three-dimensional response for the bio-hydrogen production yield; X1 = A: Algae; X2 = B: Ragi; actaul factor C: pH = 1.50

bio-hydrogen production yield. However, the steepnes of line C which is the pH is smaller when compared to line A which is the amount of microalgae. This shows that pH has very little effect on the predicted bio-hydrogen production yield. Hence, the predicted bio-hydrogen production yield changes more due to the amount of ragi tapai and the amount of microalgae than the pH value. However, the perturbation plot cannot be dependent too much as it only shows one-dimensional paths through a multifactor surface. Nevertheless, the perturbation plot points out the relatively influential factors which is the amount of ragi tapi and the amount of microalgae which are then used as the axes to obtained a more significant contour and 3D response surface plots. Whereas the factor that must be held constant on any given plot would be pH.

Figure 5 displays the two-dimensional contour plot whereas Fig. 6 illustrates the three-dimensional

Table 8: Constraints set for the optimization analysis of bio-hydrogen production yield

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Microalgae	Is in range	2.0	4.0	1	1	3
Ragi tapai	Is in range	0.4	0.6	1	1	3
pH	Is in range	7.0	8.0	1	1	3
H_2	Maximize	250000	685000	1	1	3

response for the predicted bio-hydrogen production yield as a function of A (amount of microalgae) and B (amount of ragi tapai). From Fig. 5, it can be observed that the optimum point is located inside the design boundary. Based on the shape of the contour plot and three dimensional response, it shows that the bio-hydrogen production yield is sensitive to the interactions between the factors (amount of microalgae and amount of ragi tapai). The peak on the graph in Fig. 6 delineate the optimal point for the predicted bio-hydrogen production yield by the software.

Therefore, the optimal conditions to maximize the bio-hydrogen production yield by the co-culturing of *C. reinhardtii* and ragi tapai immobilized in chitosan beads is 3 g of microalgae 0.5 g of ragi tapai and pH medium of 7.5. The optimized bio-hydrogen production yield is 670000 ppm. Similar results were also recorded in other studies for the effect of pH and temperature (Zhu and Yang, 2004). However, these results were all based on the "one-variable-at-a-time" approach and the mutual interactions between the independent variables could not be observed (Long *et al*., 2010).

Predicted bio-hydrogen production yield was then used as the response for the software optimization analysis. This was done as predicted bio-hydrogen production yield was mainly based on the equation generated. Software optimization analysis will be a higher degree of accuracy compared to the experimental data. To run the optimization analysis, constraints were set as shown in Table 8.

The constraints set as shown on Table 8 enables the software to maximize the predicted bio-hydrogen production yield. The optimization study combines the individual desirability into one single number and searches for the best overall desirability. A desirability value of one would be the perfect case whereas a desirability value of zero signifies that one or more of the response falls out of the desirable limits. From the 100 solutions proposed by the software the combination of factors that attained the maximum predicted bio-hydrogen production yield was selected. All of the 100 solutions proposed by the software gave a desirable value of one thus the lowest amount of algae that is required to obtain the highest predicted bio-hydrogen yield was selected. This was done to accomplish an optimal condition for bio-hydrogen production yield by minimizing the amount of microalgae which would that save up on the experimental cost. Therefore, the best combination of factors is 2.46 g of algae 0.45 g of ragi tapai and pH of 7.23 giving a maximum bio-hydrogen production yield of 702317 ppm.

CONCLUSION

Previous studies have indicated substantial improvement and development in both the yield and volumetric production rates of hydrogen fermentations. Hydrogen yields and production rates must at least surpass considerably the present achievements for realistic applications. The main of this study was to determine the optimal conditions for bio-hydrogen yield based on the immobilized co-culture by using RSM Box-Behnken design. An optimized condition for bio-hydrogen production by *C. reinhardtii* was successfully accomplished using Box-Behnken design experiment. To determine the optimize conditions, amount of microalgae amount of ragi tapai and pH of culture medium was selected as the independent variables. Fifteen experiments were done to obtain the optimal conditions and their responses were depicted with mathematical equation. The design expert predicted that optimized conditions to obtained maximum amount of bio-hydrogen yield of 702317 ppm is by co-culturing 2.46 g of microalgae with 0.45 g of ragi tapai and pH 7.23 of TAP-S medium. The statistical methodology effectively established the optimal conditions that maximized the bio-hydrogen production yield with respect to the amount of microalgae, the amount of ragi tapai and the pH of the culture medium.

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