

## Optimized growth of *Rhodobacter sphaeroides* O.U.001 using response surface methodology (RSM)

Jacqueline Xiao Wen Hay<sup>1</sup>, Ta Yeong Wu<sup>1\*</sup>, Chee Yang Teh<sup>1</sup> and Jamaliah Md. Jahim<sup>2</sup>

<sup>1</sup>Chemical and Sustainable Process Engineering Research Strength, School of Engineering, Monash University, Jalan Lagoon Selatan, Bandar Sunway, 46150, Selangor Darul Ehsan, Malaysia

<sup>2</sup>Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, Bangi, 43600, Selangor Darul Ehsan, Malaysia

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This study presents optimization of growth parameters (temperature, pH, ammonium conc. and inoculum size) to achieve an economical viable biohydrogen production process by *Rhodobacter sphaeroides* O.U.001 using response surface methodology (RSM) followed by analysis of light and agitation effects on bacterial growth using optimized conditions. Optimized growth conditions on third day of fermentation, when *R. sphaeroides* O.U.001 was cultured aerobically under the presence of light (510 lux), were found as follows: temperature, 30.3°C; pH, 4.71; ammonium conc., 0.60 g/l; and inoculum size, 6.02% (v/v). Light did not give a significant effect on overall growth of *R. sphaeroides* O.U.001. Agitation greatly influenced bacterial growth as a result of higher dissolved oxygen and dispersion of macromolecules in shake culture.

**Keywords:** Biohydrogen, Photofermentation, Response surface methodology (RSM), *Rhodobacter sphaeroides* O.U.001

### Introduction

Among all gaseous fuel, hydrogen (H<sub>2</sub>) has the highest ability of generating large amount of energy per unit weight by combustion and does not contribute to greenhouse gas (GHG) emission as H<sub>2</sub> is carbon-free<sup>1</sup>. When combusted, H<sub>2</sub> only produces water as waste product and can be utilized as fuel for direct combustion in internal combustion (IC) engines or to power up fuel cells<sup>1</sup>. Biological H<sub>2</sub> production is one of the most promising renewable processes that only require low processing energy, ambient temperature and pressure<sup>2</sup>. Among all bacteria, *Rhodobacter sphaeroides* (Family: *Proteobacteria*)<sup>3</sup> has been intensively studied due to its ability to grow under a variety of environmental conditions and its ability to produce high amount of H<sub>2</sub> under light conditions. These bacteria are able to produce H<sub>2</sub> when it grows in photoheterotrophic mode under the presence of light in anaerobic condition<sup>4</sup>. H<sub>2</sub> production rate is proportional to growth rate<sup>5</sup>. Therefore, in order to utilize *Rhodobacter* sp. for biological H<sub>2</sub> process, high bacterial

growth should be obtained to ensure high process yield. This study presents optimizing growth parameters (temperature, pH, ammonium conc. and inoculum size) of *R. sphaeroides* O.U.001 using response surface methodology (RSM), and investigating the effects of light and agitation on optimized growth of *R. sphaeroides* O.U.001.

### Experimental Section

#### Microorganism and Preparation of Seed Culture

For liquid inoculum preparation, *R. sphaeroides* O.U.001, preserved at 4°C, were activated and grown aerobically using modified LMG Medium 80 agar<sup>6</sup> for 3 days at 32°C under light inside an incubator. Modified medium (1 l) consisted of: yeast extract, 1.0 g; succinic acid, 1.0 g; K<sub>2</sub>HPO<sub>4</sub>, 0.5 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 g; NaCl, 0.4 g; (NH<sub>4</sub>)SO<sub>4</sub>, 0.4 g; CaCl<sub>2</sub>, 50.0 mg; ethanol, 0.5 ml; and trace elements solution, 1.0 ml. Trace elements solution (1 l) contained: H<sub>3</sub>BO<sub>3</sub>, 0.3 g; CoN<sub>2</sub>O<sub>6</sub>·6H<sub>2</sub>O, 0.2 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 30.0 mg; MnCl<sub>2</sub>·4H<sub>2</sub>O, 30.0 mg; NiCl<sub>2</sub>·6H<sub>2</sub>O, 20.0 mg; and CuCl<sub>2</sub>·2H<sub>2</sub>O, 10.0 mg. pH of medium was adjusted to 6.8 using 1 mol/l NaOH.

\*Author for correspondence

E-mail: wu.ta.yeong@monash.edu; tayeong@hotmail.com

Table 1—Range and coded levels for temperature, pH, ammonium concentration and inoculum size

Variable	Symbol		Coded levels				
	Actual value	Coded value	-2	-1	0	1	2
Temperature, °C	$X_1$	$x_1$	22.00	27.00	32.00	37.00	42.00
pH	$X_2$	$x_2$	4.50	5.50	6.50	7.50	8.50
Ammonium conc., g/l	$X_3$	$x_3$	0.10	0.60	1.10	1.60	2.10
Inoculum size, % v/v	$X_4$	$x_4$	1.50	6.00	10.50	15.00	19.50

### Experimental Design and Statistical Analysis for Growth of *R. sphaeroides* O.U.001

RSM<sup>7</sup> was used to optimize growth of *R. sphaeroides* O.U.001. Experimental design was a 2<sup>4</sup> full factorial central composite design (CCD). For regression model, variables were transformed to coded variables using  $x_i = \frac{X_i - X_i^*}{\Delta X_i}$  where  $x_i$  = coded value,  $X_i$  = uncoded value,  $X_i^*$  = uncoded value of  $X_i$  at the center value chosen, and  $\Delta X_i$  = step size, for  $i$ th test variable<sup>8</sup>.

An inoculum from exponential phase of the growth curve is the most suitable for a higher yield of H<sub>2</sub> production for purple-non-sulfur (PNS) bacteria<sup>2</sup>. Cell growth of *R. sphaeroides* O.U.001 was proven with the starting of exponential phase in 48 ± 4 h and stationary phase was attained at 80 ± 4 h<sup>9</sup>. A total of 30 experimental runs with different combination of factors in 3 days of fermentation of the growth of *R. sphaeroides* O.U.001 were performed for optimization under aerobic and light conditions inside an incubator without agitation. Bacteria were transferred according to prescribed inoculum size (Table 1) for each run into 100 ml liquid medium inside a clear serum vial. After 3 days of fermentation, growth of *R. sphaeroides* was measured through its optical density at 660 nm using a UV spectrophotometer. By using Design Expert software (Version 6.0.10, Stat-Ease Inc., Minneapolis, USA), all responses from a total of 30 experimental runs were fitted using a predictive polynomial quadratic equation to obtain a correlation of both the response and independent variable. General form of second-order polynomial quadratic model used to describe the effects of different factors on the response<sup>8</sup> is given as

$$y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i < j} b_{ij} x_i x_j \quad \dots(1)$$

where  $y$  = response,  $b_0$  = constant,  $b_i$  = linear coefficient,  $b_{ii}$  = quadratic coefficient,  $b_{ij}$  = interactive

coefficient and  $x_i$  = coded factor level. Optimum conditions of variables were obtained by solving Eq. (1), and also by analyzing contour and response surface plots using the same software.

Predicted optimum conditions were verified by measuring growth of *R. sphaeroides* through its optical density at 660 nm after growing the culture for 3 days. Effect of light (510 lux) without agitation was investigated by incubating *R. sphaeroides* in light and dark conditions. For effect of agitation, *R. sphaeroides* was cultivated in light condition with shaking culture using magnetic stirrer (250 rpm) and without shaking culture.

### Results and Discussion

Final polynomial equation, representing growth of *R. sphaeroides* O.U.001,  $Y(A)$ , can be expressed in terms of actual factors as

$$Y = -4.57796 + 0.31471x_1 + 0.20141x_2 + 0.197x_3 + 1.65972 \times 10^{-3}x_4 - 5.14875 \times 10^{-3}x_1^2 - 0.02384x_2^2 - 0.06278x_3^2 - 3.68827 \times 10^{-4}x_4^2 + 4.125 \times 10^{-4}x_1x_2 - 7.975 \times 10^3x_1x_3 + 1.02778 \times 10^{-4}x_1x_4 + 0.02212x_2x_3 - 4.30556 \times 10^{-4}x_2x_4 + 2.80556 \times 10^{-3}x_3x_4 \quad \dots(2)$$

where  $x_1$ ,  $x_2$ ,  $x_3$  and  $x_4$  are actual value of temperature (°C), pH, ammonium concentration (g/l) and inoculum size (% (v/v)), respectively. For this equation to be applicable, bacteria should be grown in aerobic condition and with the presence of light.

ANOVA (Analysis of variance) for fitting model (Table 2) showed high model  $F$ -value (8.08) and low  $P$ -value (0.0001), suggesting that the model was significant. Besides, Lack of fit  $F$ -value (2.66) shows that lack of fit was not significant relative to pure error, indicating that model fits experimental data for the growth of *R. sphaeroides* O.U.001. Value of coefficient of determination ( $R^2$ , 0.8829) indicates that 88.29% of variability of response variable was explainable with the model.  $R^2$  is close to 1, thus indicating that the model could be used to effectively describe the effects of four

Table 2—ANOVA for fitting model for growth of *Rhodobacter sphaeroides* O.U.001

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	0.850	14	0.061	8.080	0.0001
$x_1$	0.240	1	0.240	31.480	<0.0001
$x_2$	0.140	1	0.140	18.280	0.0007
$x_3$	3.243E-3	1	3.243E-3	0.430	0.5205
$x_4$	3.060E-3	1	3.060E-3	0.410	0.5324
$x_1^2$	0.450	1	0.450	60.660	<0.0001
$x_2^2$	0.016	1	0.016	2.080	0.1697
$x_3^2$	6.777E-3	1	6.777E-3	0.900	0.3566
$x_4^2$	1.530E-3	1	1.530E-3	0.200	0.6578
$x_1x_2$	6.806E-5	1	6.806E-5	9.085E-3	0.9253
$x_1x_3$	6.360E-3	1	6.360E-3	0.850	0.3714
$x_1x_4$	8.556E-5	1	8.556E-5	0.011	0.9163
$x_2x_3$	1.958E-3	1	1.958E-3	0.260	0.6166
$x_2x_4$	6.006E-5	1	6.006E-5	8.017E-3	0.9298
$x_3x_4$	6.376E-4	1	6.376E-4	0.085	0.7745
Residual	0.095	15	7.492E-3	-	-
Lack of fit	0.095	10	9.460E-3	2.660	0.1458
Pure error	0.018	5	3.555E-3	-	-
Cor Total	0.960	29	-	-	-

\*  $x_1$ ,  $x_2$ ,  $x_3$  and  $x_4$  are actual values of temperature, pH, ammonium conc. and inoculum size, respectively

parameters studied. Eq. (2) can be further simplified by only taking account for the significant terms ( $x_1, x_2, x_1^2$ ) as

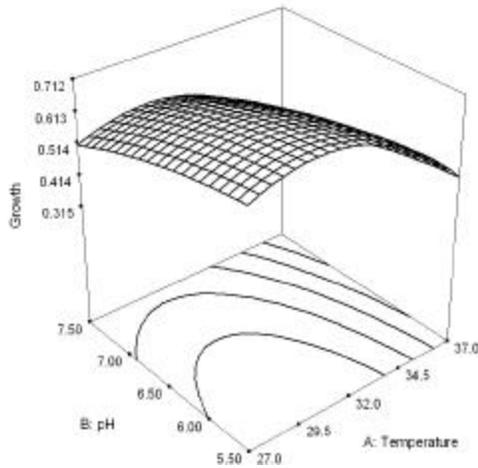
$$Y = -4.57796 + 0.31471x_1 + 0.20141x_2 - 5.14875 \times 10^{-3}x_1^2 \dots(3)$$

ANOVA of fitting model (Table 2) showed that linear and quadratic effect of temperature and linear effect of pH on growth were highly significant ( $p < 0.01$ ), indicating that small variation of temperature and pH could greatly influence the growth of *R. sphaeroides* O.U.001. However, interaction between four parameters were not significant ( $p > 0.05$ ). In response surface plots (Fig. 1), two independent variables were kept constant at the suggested center point while varying other two variables to observe the growth. There were no clear peaks for most of the surface plots. Contour plots (Fig. 1) also shows that a wide range of ammonium concentration and inoculum size did not give significant effects on the growth and were applicable for this experiment. Higher ammonium chloride ( $\text{NH}_4\text{Cl}$ ) concentration provides higher nitrogen source for bacteria. Although biomass of *R. sphaeroides* O.U.001 would increase in parallel with  $\text{NH}_4\text{Cl}$  concentration under anaerobic condition, no

significant difference for growth was observed under aerobic condition in medium with different  $\text{NH}_4\text{Cl}$  concentration<sup>10</sup>. Experimental observation from this study also agrees well with reported<sup>10</sup> results, may be due to toxic effect of the combination of oxygen and ammonium ion. Besides, variation of inoculum size also did not give any significant impact on bacterial growth, may be due to the ability of certain bacteria (in specific medium) to reach an apparent steady-state density, which is independent of initial inoculum density<sup>11</sup>.

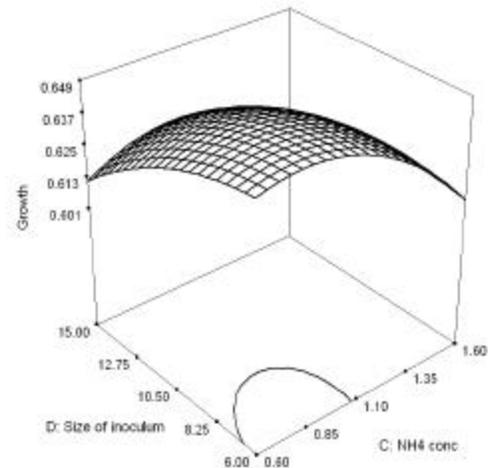
Based on Eq. (2) and Fig. 1, maximum growth of *R. sphaeroides* O.U.001 would be 0.73 A at: temperature, 30.3°C, pH, 4.71; inoculum size, 6.02% (v/v); and  $\text{NH}_4$  conc., 0.60 g/l. Typical growth temperature for most of the photosynthetic bacteria is reported<sup>12-15</sup> as 30-35°C. Optimum pH (4.71) in this study differed greatly from reported<sup>16,17</sup> optimum pH 7, probably due to different modified medium used for culturing. *R. sphaeroides* O.U.001, cultivated using optimized conditions from the model, gave a maximum growth of 0.624 A, which differed from predicted growth by 17.9%, could be due to time interval of sampling. With the assumption that experimental errors for normal biological selection experiment fall between 30-40%<sup>18</sup>, this model can still

DESIGN-EXPERT Plot  
 Growth  
 X = A: Temperature  
 Y = B: pH  
 Actual Factors  
 C: NH<sub>4</sub> conc = 1.10  
 D: Size of inoculum = 10.50



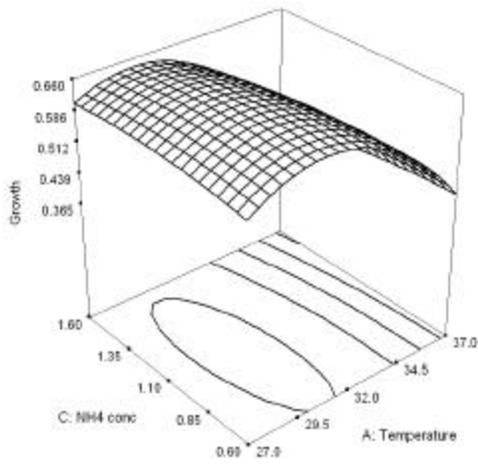
a)

DESIGN-EXPERT Plot  
 Growth  
 X = C: NH<sub>4</sub> conc  
 Y = D: Size of inoculum  
 Actual Factors  
 A: Temperature = 32.0  
 B: pH = 6.50



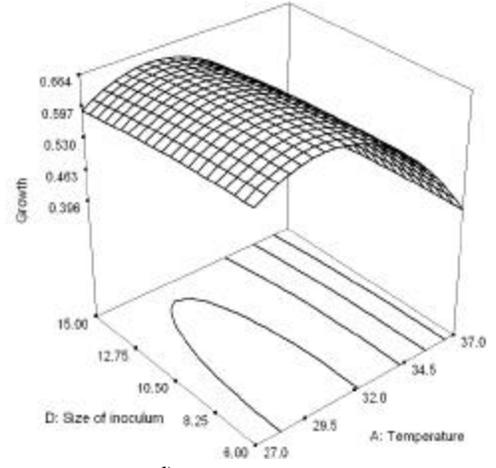
b)

DESIGN-EXPERT Plot  
 Growth  
 X = A: Temperature  
 Y = C: NH<sub>4</sub> conc  
 Actual Factors  
 B: pH = 6.50  
 D: Size of inoculum = 10.50



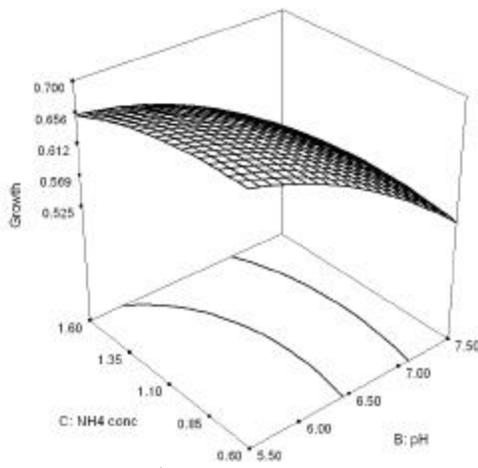
c)

DESIGN-EXPERT Plot  
 Growth  
 X = A: Temperature  
 Y = D: Size of inoculum  
 Actual Factors  
 B: pH = 6.50  
 C: NH<sub>4</sub> conc = 1.10



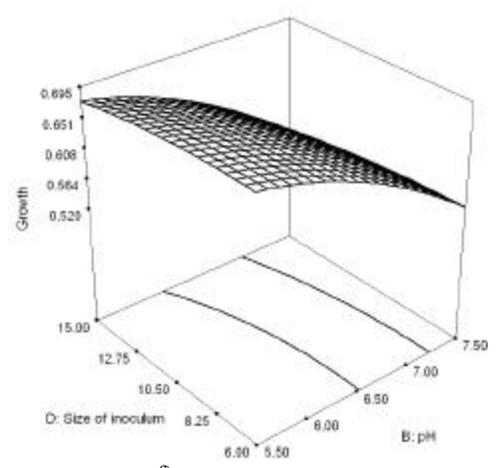
d)

DESIGN-EXPERT Plot  
 Growth  
 X = B: pH  
 Y = C: NH<sub>4</sub> conc  
 Actual Factors  
 A: Temperature = 32.0  
 D: Size of inoculum = 10.50



e)

DESIGN-EXPERT Plot  
 Growth  
 X = B: pH  
 Y = D: Size of inoculum  
 Actual Factors  
 A: Temperature = 32.0  
 C: NH<sub>4</sub> conc = 1.10



f)

Fig. 1—Response surface model between two combination factors: a) temperature & pH; b) NH<sub>4</sub> conc. & size of inoculum; c) temperature & NH<sub>4</sub> conc.; d) temperature & size of inoculum; e) pH & NH<sub>4</sub> conc.; and f) pH & size of inoculum

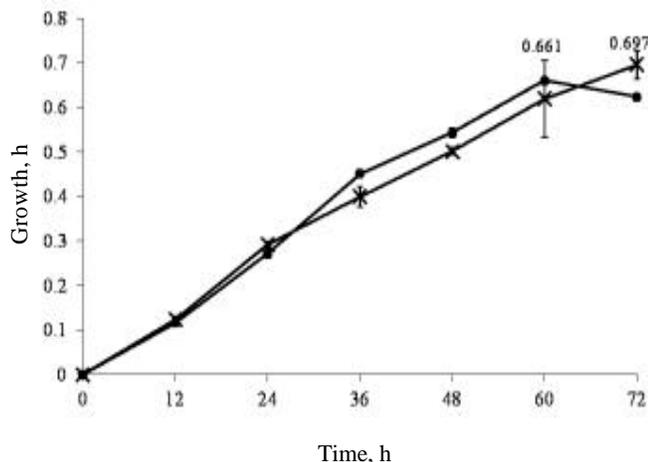


Fig. 2—Growth of *R. sphaeroides* O.U.001 under optimized conditions (●) with and (×) without light

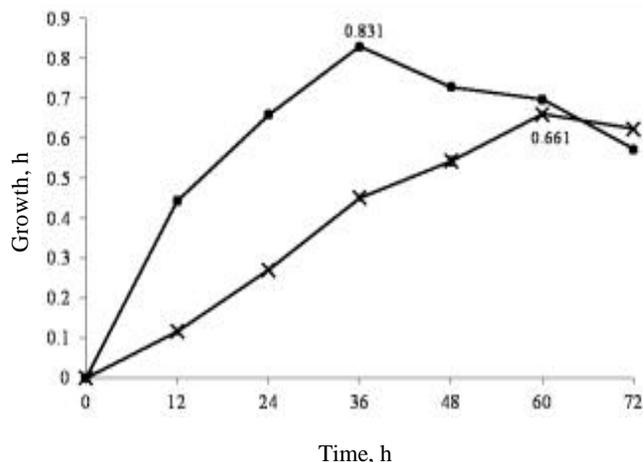


Fig. 3—Growth of *R. sphaeroides* O.U.001 under optimized conditions (●) with and (×) without agitation

be used to predict the growth of *R. sphaeroides* O.U.001 under aerobic condition with the presence of light.

Comparing aerobic growth of *R. sphaeroides* O.U.001 under optimized conditions with light (510 lux) and without light, slightly higher growth (Fig. 2) was observed between 24–65 h under light conditions. Shape of curves (Fig. 2) is similar to reported<sup>19</sup> graph, where higher growth of *Rhodobacter* was observed at exponential growth phase in the presence of light. This result is consistent with reported<sup>20</sup> result. A decrease of growth under light condition after 63 h of fermentation may be due to photo-oxidative stress, which kills cells in biological system of *Rhodobacter* sp. This condition usually occurs in the presence of high light intensity and oxygen saturated condition<sup>20</sup>.

Effect of agitation on the growth of *R. sphaeroides* O.U.001 was studied in two types of culture, incubated with shaking using magnetic stirrer, and without shaking. Within 72 h of fermentation, higher growth (26%) was observed (Fig. 3) in shaking culture (max. growth, 0.831 A) as compared to growth in static culture (max. growth, 0.661 A). Besides, time taken for static culture to reach its maximum value was 1.67 times longer than shake culture. This result is consistent with reported results<sup>21</sup>. Higher agitation speed is reported<sup>22</sup> to increase the amount of dissolved oxygen and dispersion of macromolecules in the medium<sup>14</sup>. Nutrient uptake by bacteria also increased. Agitation is directly related to the efficiency in operation of oxygen mass transfer rate and oxygen solubilization including oxygen consumption of cell. Therefore, agitation contributed to higher growth of *R. sphaeroides* O.U.001 in this study.

## Conclusions

Only temperature and pH influenced significantly the growth of *R. sphaeroides* O.U.001 individually. RSM showed that the highest bacterial growth under aerobic and light conditions could be obtained when bacteria was cultivated at: temperature, 30.3°C; pH, 4.71; ammonium conc., 0.60 g/l; and inoculum size, 6.02% (v/v). However, actual maximum growth differed from predicted growth by 17.9%, reflecting the limitation of a statistical design in biological systems, which overlooks the complex physiological regulatory mechanisms operating<sup>23</sup>. Presence of light did not give a significant effect but agitation during cultivation promoted faster growth of *R. sphaeroides* up to 26%.

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