Supplementary Information

Title: Optimization of Methane Feed and N:C Ratio for Biomass and Polyhydroxybutyrate Production by the Alphaproteobacterial Methanotroph *Methylocystis* sp. Rockwell

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Figure S1 Growth of *Methylocystis* sp. Rockwell in nitrate (NMS; red) and ammonium (AMS; blue) mineral salts medium provided with 6 mmol methane gas injected into the headspace of a 100-ml culture in a 1-L bottle (a). Dotted trendlines represent the exponential growth phase of the bacteria, and the doubling times were calculated using ln(2)/B, where value of B is obtained from exponential growth equation ($\mathbf{Y = ae^{Bx}}$) (b). Doubling times were calculated as 9.8 h in NMS and 8.5 h in AMS. Error bars represent SD (n = 4). PHB production from *Methylocystis* sp. Rockwell during growth phases (c). Culture was grown in AMS medium provided with 6 mmol CH_4 and an N:C ratio of 0.016; samples (n = 3) were harvested every 36 h for biomass and PHB analysis.

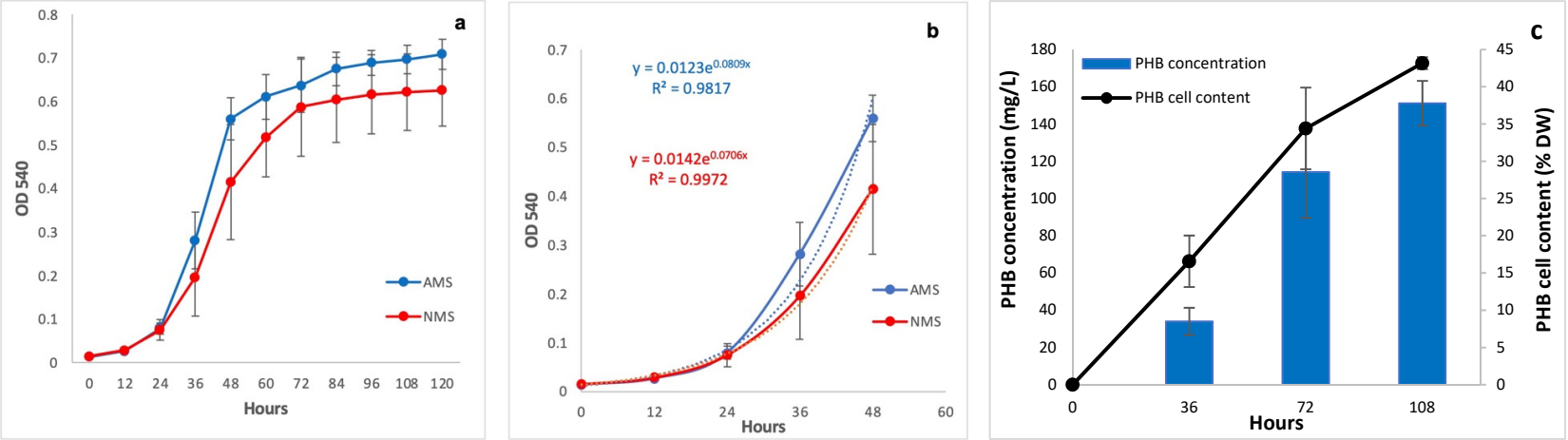


Figure S2 Comparison of the regression model between predicted and actual values for PHB concentration (a) and %PHB cell content (b) when *Methylocystis* sp. Rockwell cultures were provided with various amounts of methane in AMS media. The colour points represent the square root values. The square root transformation was used to normalize the data, reduce heteroscedasticity and for clearer visualization. The R^2 values are indicators of the fit of the regression model.

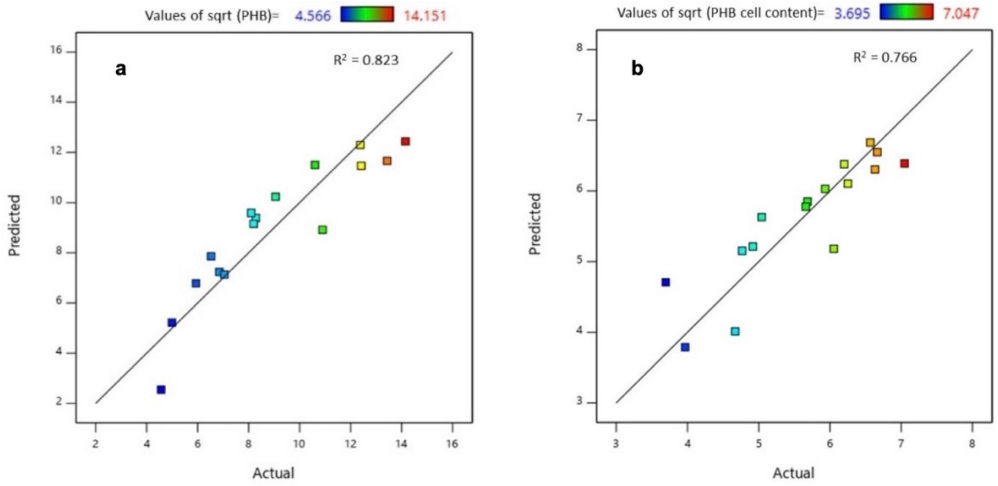


Figure S3 Comparison of regression models between predicted and actual values for PHB concentration (a) and %PHB cell content (b) when *Methylocystis* sp. Rockwell cultures were provided with various amounts of methane in NMS medium. The colour points represent the square root values. The square root transformation was used to normalize the data, reduce heteroscedasticity and for clearer visualization. The R^2 value represents the fit of the regression model.

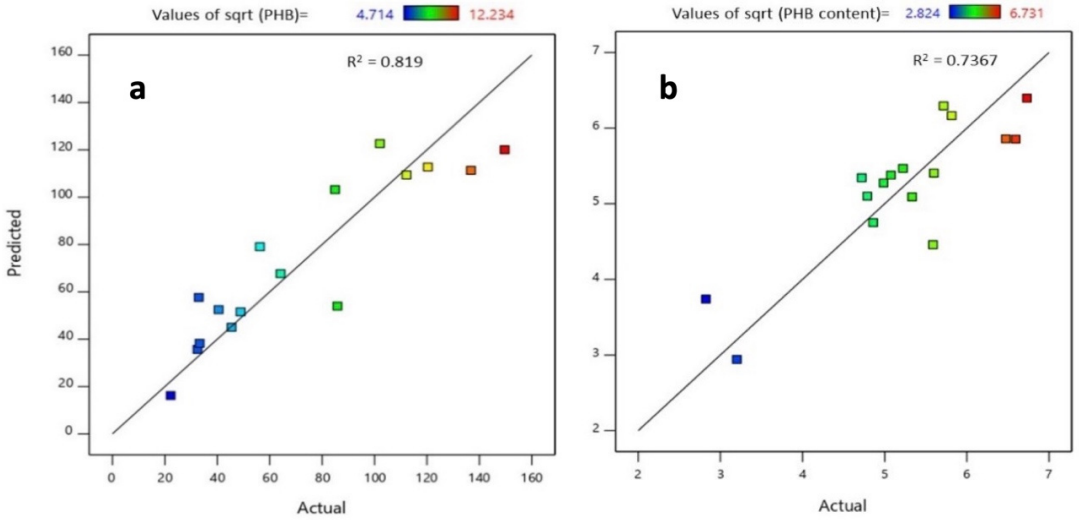


Figure S4 One-way ANOVA followed by post hoc Tukey's test of PHB production. Cultures were grown with the indicated amounts of methane and N:C ratios with either nitrate (a) or ammonium (b) as N-source. Error bars indicate standard deviation (n = 3). Conditions such as- 6 mmol methane with N:C of 0.012 for nitrate (a), 6 mmol of methane with N:C of 0.022 and 8 mmol methane with N:C of 0.012 for ammonium (b) showed significantly high levels of PHB were indicated by asterisk sign (*).

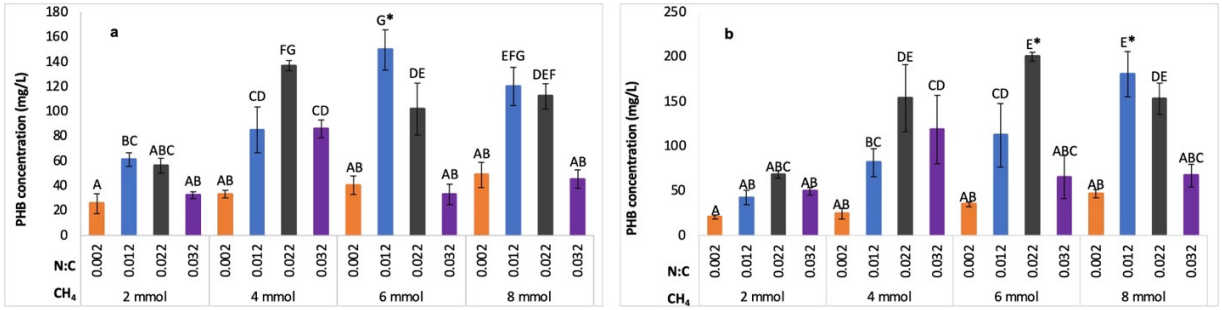
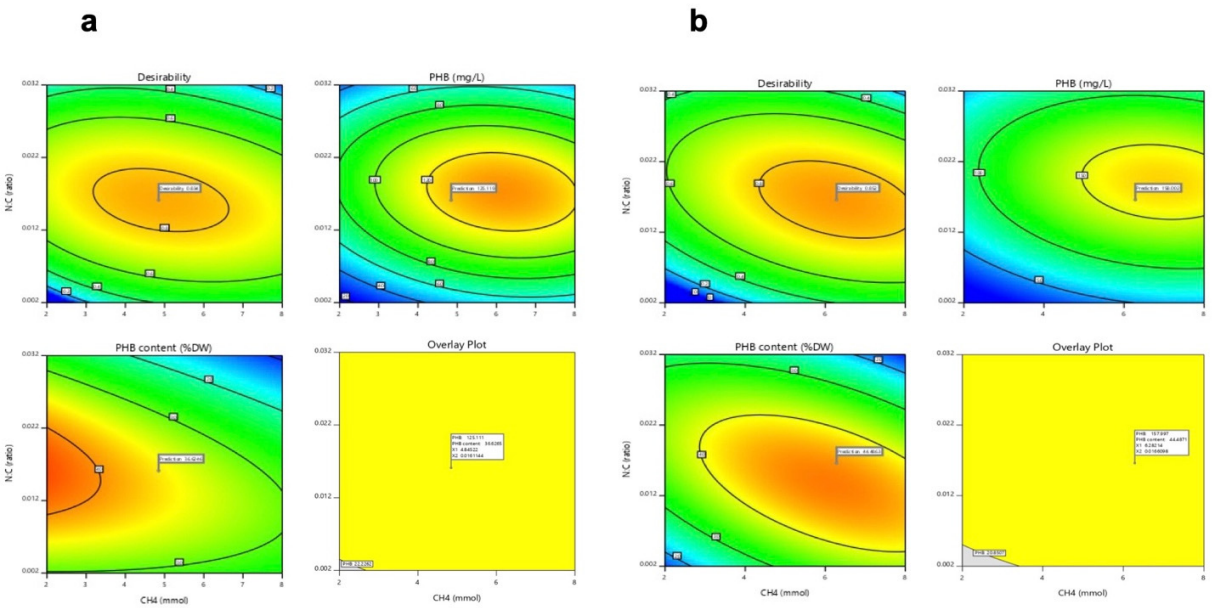


Figure S5 Model prediction for multi-objectives optimal condition (MOOC) to balance higher PHB yield and %PHB cell content. The model predicted N:C ratio of 0.016 for both nitrate (a) and ammonium (b) as N-sources was subjected to experimental validation.



Tables S1–S4. Effect of methane amount and N:C ratio on PHB production from *Methylocystis* sp. Rockwell when ammonium is used as the N-source.

Table S1. ANOVA for quadratic model in response to PHB concentration. The model F-value of 9.30, R^2 of 0.823 (from Figure S2a) and $p < 0.05$ implies the model is appropriate. In this case A, B, and B^2, highlighted in bold, are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	112.85	5	22.57	9.30	**0.0016**
A-CH4	25.02	1	25.02	10.31	**0.0093**
B-N:C	23.47	1	23.47	9.67	**0.0111**
AB	2.20	1	2.20	0.9080	0.3631
A^2	4.91	1	4.91	2.02	0.1853
B^2	57.25	1	57.25	23.59	**0.0007**
Residual	24.27	10	2.43		
Total	137.12	15			

Table S2. Final equation for sqrt. and quadratic model in response to PHB concentration.

Coded equation	Parameter	Actual equation	Parameter
+12.02		-2.81	Intercept
+1.68	A	+2.20	CH4
+1.62	B	+825.67	N:C
-0.6680	AB	-14.84	CH4 * N:C
-1.25	A^2	-0.14	$CH4^2$
-4.26	B^2	-18915	$N:C^2$

The correlation among the controlled factors (methane amount and N:C ratio) and response measures (PHB concentration and %PHB cell content) for cultures grown with ammonium as N-source are obtained using the regression analysis technique. The equations obtained for the quadratic model using square root transformation are shown in the following equations.

For PHB concentration, the complete regression was:

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_2 \bullet B) + (a_{12} \bullet A \bullet B) + (a_{11} \bullet A^2) + (a_{22} \bullet B^2) \quad (1)$$

$$= -2.81 + (2.20 \bullet A) + (825.67 \bullet B) - (14.84 \bullet A \bullet B) - (0.14 \bullet A^2) - (18915 \bullet B^2)$$

The final regression, taking into consideration the statistical significance of parameters (Table S2).

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_2 \bullet B) + (a_{22} \bullet B^2) \quad (2)$$

$$= -2.81 + (2.20 \bullet A) + (825.67 \bullet B) - (18915 \bullet B^2)$$

Table S3 ANOVA for quadratic model in response to %PHB cell content. The model F-value of 6.55, R^2 of 0.766 (Figure S2b) and $p < 0.05$ implies the model is appropriate. In this case AB, B^2 highlighted in bold are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	11.46	5	2.30	6.55	**0.0060**
A-CH4	0.25	1	0.25	0.7068	0.4202
B-N:C	0.70	1	0.70	1.97	0.1912
AB	3.82	1	3.82	10.90	**0.0080**
A^2	0.80	1	0.80	2.24	0.1653
B^2	5.92	1	5.92	16.91	**0.0021**
Residual	3.50	10	0.35		
Total	14.96	15			

Table S4 Final equation for sqrt. and quadratic model in response to %PHB cell content

Coded equation	Parameter	Actual equation	Parameter
+6.65		+1.90	Intercept
+0.16	A	+0.94	CH4
-0.28	B	+286	N:C
-0.88	AB	-19.54	CH4 * N:C
-0.50	A^2	-0.05	$CH4^2$
-1.37	B^2	-6084.20	$N:C^2$

The linear regression model for %PHB cell content

$$(\hat{y}') = b_0 + (b_1 \bullet A) + (b_2 \bullet B) + (b_{12} \bullet A \bullet B) + (b_{11} \bullet A^2) + (b_{22} \bullet B^2) \quad (3)$$

$$= 1.90 + (0.94 \bullet A) + (286 \bullet B) - (19.54 \bullet A \bullet B) - (0.05 \bullet A^2) - (6084.20 \bullet B^2)$$

And the final version of the regression model, taking into account the statistical significance of each term is:

$$(\hat{y}') = b_0 + (b_{12} \bullet A \bullet B) + (b_{22} \bullet B^2) \quad (4)$$

$$= 1.90 - (19.54 \bullet A \bullet B) - (6084.20 \bullet B^2)$$

Tables S5–S8. Effect of methane amount and N:C ratio on PHB production from *Methylocystis* sp. Rockwell when nitrate is used as the N-source.

Table S5 ANOVA for quadratic model in response to PHB concentration. The model F-value of 9.05, R^2 of 0.819 (from Figure S3a) and $p < 0.05$ implies the model is appropriate. In this case A and B^2 highlighted in bold are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	70.07	5	14.01	9.05	**0.0018**
A-CH4	8.85	1	8.85	5.72	**0.0379**
B-N:C	1.31	1	1.31	0.8492	0.3785
AB	1.92	1	1.92	1.24	0.2915
A^2	5.25	1	5.25	3.39	0.0954
B^2	52.74	1	52.74	34.06	**0.0002**
Residual	15.48	10	1.55		
Total	85.55	15			

Table S6 Final equation for sqrt. and quadratic model in response to PHB concentration.

Coded equation	Parameter	Actual equation	Parameter
+11.21		-0.90	Intercept
+0.9978	A	+2	CH4
+0.3846	B	+712.20	N:C
-0.6235	AB	-13.85	CH4 * N:C
-1.29	A^2	-0.14	$CH4^2$
-4.08	B^2	-18155	$N:C^2$

The correlation among the control factors (methane amount and N:C ratio) and response measures (PHB concentration and %PHB cell content) for cultures grown with nitrate as N-source are obtained using the regression analysis technique. The equations obtained for the quadratic model using square root transformation are shown in the following equations.

For PHB concentration, the complete regression was:

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_2 \bullet B) + (a_{12} \bullet A \bullet B) + (a_{11} \bullet A^2) + (a_{22} \bullet B^2) \quad (5)$$

$$= -0.90 + (2 \bullet A) + (712.20 \bullet B) - (13.85 \bullet A \bullet B) - (0.14 \bullet A^2) - (18155 \bullet B^2)$$

The final regression, taking into consideration the statistical significance of parameters (Table S6).

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_{22} \bullet B^2) \quad (6)$$

$$= -0.90 + (2 \bullet A) - (18155 \bullet B^2)$$

Table S7 ANOVA for quadratic model in response to PHB cell content. ANOVA for quadratic model in response to %PHB cell content. The model F-value of 5.6, R^2 of 0.737 (Figure S2b) and $p < 0.05$ implies the model is appropriate. In this case A, B, and B^2 highlighted in bold are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	12.60	5	2.52	5.60	**0.0102**
A-CH4	3.40	1	3.40	7.55	**0.0206**
B-N:C	3.35	1	3.35	7.44	**0.0213**
AB	1.05	1	1.05	2.33	0.1576
A^2	0.02	1	0.02	0.0548	0.8196
B^2	4.77	1	4.77	10.61	**0.0086**
Residual	4.50	10	0.45		
Total	17.10	15			

Table S8 Final equation for sqrt. and quadratic model in response to %PHB cell content

Coded equation	Parameter	Actual equation	Parameter
+5.95		+5	Intercept
-0.62	A	+0.07	CH4
-0.61	B	+196.04	N:C
-0.50	AB	-10.25	CH4 * N:C
-0.10	A^2	-0.01	$CH4^2$
-1.23	B^2	-5461.76	$N:C^2$

The linear regression model for %PHB cell content

$$(\hat{y}') = b_0 + (b_1 \bullet A) + (b_2 \bullet B) + (b_{12} \bullet A \bullet B) + (b_{11} \bullet A^2) + (b_{22} \bullet B^2) \quad (7)$$

$$= 5 + (0.07 \bullet A) + (196.04 \bullet B) - (10.25 \bullet A \bullet B) - (0.01 \bullet A^2) - (5461.76 \bullet B^2)$$

And the final version of the regression model, taking into account the statistical significance of each term is:

$$(\hat{y}') = b_0 + (b_1 \bullet A) + (b_2 \bullet B) + (b_{22} \bullet B^2) \quad (8)$$

$$= 5 + (0.07 \bullet A) + (196.04 \bullet B) - (5461.76 \bullet B^2)$$