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ENHANCEMENT OF POLYHYDROXYALKANOATE PRODUCTION BY *Priestia aryabhattai* ML113 ISOLATED FROM ME LINH, HANOI

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that microorganisms naturally accumulate as intracellular carbon and energy reserves. They are considered environmentally friendly alternatives to petroleum-based plastics. This study evaluated *Priestia aryabhattai* ML113 for its ability to synthesize PHA and employed a two-step optimization strategy to enhance polymer yield. In the preliminary one-factor-at-a-time (OFAT) experiments, temperature, fermentation time, and the carbon-to-nitrogen (C/N) ratio were identified as the most influential factors, yielding a maximum PHA concentration of 1.16 g L^{-1} under conditions of 35°C, 72 h, and a C/N ratio of 28:1. Subsequently, response surface methodology (RSM) using a Box–Behnken design (BBD) was applied to analyze factor interactions and quadratic effects. The developed regression model predicted an optimal PHA concentration of 1.3014 g L^{-1}, achieved under 34°C, 72 h, and a C/N ratio of 30:1. Experimental validation confirmed a comparable yield of 1.4473 ± 0.03 g L^{-1}, corresponding to a 1.25-fold improvement compared with the OFAT result. The strong correlation between predicted and observed values confirms the model's reliability. It highlights the effectiveness of integrating OFAT screening with RSM optimization as a scalable strategy for improving PHA biosynthesis. This study represents the first systematic optimization of PHA production by *P. aryabhattai* ML113, providing a methodological framework for exploiting this strain and low-cost carbon sources toward sustainable bioplastic production.

Keywords: Biopolymer optimization, Box–Behnken design (BBD), carbon-to-nitrogen ratio, polyhydroxyalkanoate (PHA), *Priestia aryabhattai* ML113, response surface methodology (RSM), sustainable bioprocess.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a group of biodegradable polyesters synthesized by a wide variety of microorganisms under specific environmental conditions, typically in response to nutrient limitations. These biopolymers have received significant attention due to their environmental benefits, particularly as sustainable alternatives to conventional petroleum-based plastics. PHAs are biocompatible, biodegradable, and exhibit thermoplastic properties, making them suitable for a wide range of applications, including packaging, medical devices, and agricultural films (Chen, 2010; Koller *et al*., 2017). The rising global concern over plastic waste has driven researchers and industries to seek environmentally friendly materials, with PHAs offering a promising solution to plastic pollution (Kourmentza *et al*., 2017).

The biosynthesis of PHAs is primarily influenced by environmental factors, such as temperature, initial pH, carbon and nitrogen sources, the carbon-to-nitrogen (C/N) ratio, and fermentation time. Temperature directly influences both bacterial metabolism and polymer properties; deviations from the optimal range can impair enzyme activity and reduce PHA accumulation (Trego *et al*., 2024). Fermentation time is another crucial parameter, as prolonged cultivation generally enhances PHA yield; however, once cells reach the stationary phase, further extension may result in diminishing returns due to nutrient depletion and by-product accumulation (Reis *et al*., 2011). Carbon provides the primary energy source for bacterial growth and PHA formation, whereas nitrogen regulates the biosynthetic process. Under nitrogen-limiting conditions, cells redirect metabolism from biomass production toward polymer accumulation, a recognized strategy to enhance PHA yield. Consequently, the C/N ratio serves as a regulatory balance between growth and polymer synthesis. Under nitrogen-limiting and carbon-excess conditions, cells divert resources from biomass production toward PHA accumulation, making high C/N ratios particularly favorable for maximizing yield (Johnson *et al*., 2010; Dash *et al*., 2019). Considering the complex interplay among these factors, a statistical optimization approach, such as response surface methodology (RSM), was employed to systematically evaluate their combined effects and identify optimal conditions for maximizing PHA production. RSM enables efficient modeling of factor interactions and reduces the number of experimental trials compared with traditional one-factor-at-a-time approaches, thereby providing a more comprehensive understanding of how environmental variables influence polymer biosynthesis.

Among the many bacteria capable of producing PHAs, *Priestia* spp. have emerged as promising candidates due to their adaptability to diverse environmental conditions and their ability to accumulate substantial amounts of PHA (Pillai *et al*., 2017; Cal *et al*., 2025). Although formerly classified under the genus *Bacillus*, phylogenomic analyses have led to its reassignment into the newly defined genus *Priestia*, along with other members of the *Bacillus subtilis* group (Gupta *et al*., 2020). This reclassification highlights the importance of strain-specific studies, particularly as *P. aryabhattai* has demonstrated the ability to produce both PHB and PHBV when cultivated on low-cost substrates and under nitrogen-limited conditions (Pillai *et al*., 2020). This study

investigates the effects of temperature, fermentation time, and carbon-to-nitrogen (C/N) ratio on the growth and PHA biosynthesis of *P. aryabhattai* ML113, a bacterial strain isolated from bean-cultivated soil in Me Linh, Hanoi (Nguyen *et al*., 2025). The objective is to identify and optimize the cultivation conditions that enhance PHA accumulation, thereby supporting the development of sustainable biopolymer production systems.

MATERIALS AND METHODS

Microorganisms

The strain *P. aryabhattai* ML113, isolated from bean-cultivated soil in Me Linh commune, Hanoi, belongs to the microbial strain collection of the Laboratory of Bioactive Compounds from Microorganisms, Institute of Biology.

PHA biosynthesis by *P. aryabhattai* ML113

A single isolated colony of *P. aryabhattai* ML113, obtained after activation, was inoculated into a test tube containing 5 mL of LB medium and incubated at 37°C with shaking at 200 rpm for 24 hours. The seed culture of *P. aryabhattai* ML113 was transferred into a 1 L Erlenmeyer flask containing 250 mL of sterile HT medium composed of (g/L): glucose 30, peptone 5, $Na_2MoO_4 \cdot 2H_2O$ 0.005, $MnSO_4$ 0.005, $FeSO_4 \cdot 7H_2O$ 0.01, $CaCl_2 \cdot 2H_2O$ 0.02, NaCl 30, $MgSO_4 \cdot 7H_2O$ 0.2, $KH_2PO_4 \cdot 2H_2O$ 0.2, and K_2HPO_4 1.6. The medium was adjusted to pH 7.0 before sterilization. Fermentation was conducted at 37°C with agitation at 200 rpm for 72 hours. Upon completion, the culture broth was centrifuged at 8000 rpm for 10 minutes to collect the cell biomass, and the supernatant was removed.

Measurement of cell biomass

The harvested cell biomass was washed thoroughly with distilled water to remove residual medium components, then dried in a hot air oven at 50°C until a constant weight was obtained. The resulting dry biomass was weighed to determine the dry cell weight (DCW), which served as a measure of total cell mass.

Extraction of PHA

Dried cell biomass was subjected to PHA extraction using a modified version of the method described by Hahn *et al.* (2014). Briefly, the biomass was treated with a mixture of sodium hypochlorite and chloroform at a ratio of 1:100 (w/v) and incubated at 60°C for 2 hours with gentle agitation (100 rpm). After incubation, the aqueous phase containing cell debris was discarded, and the chloroform layer, which carried the dissolved PHA, was transferred into a glass Petri dish. Methanol and water (7:3, v/v) were added to the extract to precipitate PHA, followed by centrifugation at 12,000 rpm for 15 minutes. The resulting pellet was washed twice with 95% ethanol and air-dried overnight at room temperature. The purified PHA was weighed, and residual biomass was calculated by subtracting the PHA weight from the initial DCW. PHA content was expressed as a percentage of DCW. All experiments were conducted in triplicate.

Experimental design

Effects of single factors on the growth and PHA production of P. aryabhattai ML113

Temperature: Growth and PHA accumulation of *P. aryabhattai* ML113 were evaluated at 25°C, 30°C, 35°C, 40°C, and

45°C. Fermentation was carried out in HT medium with an initial C/N ratio of 15:1 at a shaking speed of 200 rpm for 72 hours.

Fermentation time: To monitor the time-dependent dynamics of biomass formation and PHA synthesis, fermentation was carried out in HT medium with an initial C/N ratio of 15:1 at 35°C. Samples were collected at 24-hour intervals throughout a total incubation period of 96 hours for analysis.

C/N Ratio: HT medium was used to evaluate the impact of the carbon-to-nitrogen ratio at a fixed temperature of 35°C. The molar C/N ratio was adjusted to 15:1, 23:1, and 28:1 by altering the concentrations of glucose and peptone. After 72 hours of incubation, cell biomass and PHA content were determined.

Optimization of PHA production of P. aryabhattai ML113 using response surface methodology

The optimization of PHA production of *P. aryabhattai* ML113 was performed using response surface methodology (RSM) with a Box–Behnken design (BBD). Three key process variables—temperature (°C), fermentation time (h), and C/N molar ratio—were investigated at three coded levels (–1, 0, +1). The experimental design comprised 15 runs, including 3 replicates at the center point, which provided an estimate of experimental error and ensured model accuracy. Design-Expert software (version 13, Stat-Ease Corp., Minneapolis, MN, USA) was used to generate the design matrix, fit a second-order polynomial regression model, and perform analysis of variance (ANOVA) to evaluate the significance of linear, quadratic, and interaction terms. Non-significant terms ($p > 0.05$) were excluded in the final reduced model, while significant factors ($p < 0.05$) were retained. The model adequacy was confirmed using R^2, adjusted R^2, predicted R^2, lack-of-fit tests, and adequate precision metrics.

The model is represented as: $Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$, where: Y is the predicted response (PHA concentration), β_0 is the intercept term, β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients, β_{12}, β_{13}, β_{23} are the interaction coefficients, and A, B, C are the coded values of the independent variables (temperature, time, and C/N ratio, respectively).

RESULTS AND DISCUSSION

Influence of temperature on the growth and PHA production of *P. aryabhattai* ML113

Temperature is a crucial environmental factor that influences microbial growth and the metabolic pathways associated with PHA biosynthesis. In this study, *P. aryabhattai* ML113 exhibited temperature-dependent patterns of both biomass and PHA accumulation (Figure 1). The strain was capable of producing PHA across the tested range (25-45°C), but a maximum yields were obtained at 35°C, where biomass (1.562 g/L) and PHA accumulation (0.612 g/L; 39.18% content) reached their highest levels. At suboptimal temperatures (25°C), reduced metabolism led to minimal growth and PHA content (14.43%), whereas higher temperature stress (45°C) resulted in lower polymer accumulation (20.6%) despite relatively high biomass, indicating possible enzyme inhibition or accelerated polymer degradation. These findings establish 35°C as the optimal cultivation temperature for *P. aryabhattai* ML113 and align with previous

reports that low temperatures constrain PHA biosynthesis (Trego *et al*., 2024). Similar optimal temperatures for PHA production have been reported in related strains. For instance, *Bacillus aryabhattai* PHB10 exhibited a maximum biomass of 4.36 g/L and accumulated 74.89% PHA (3.26 g/L) at 31°C (Pillai *et al*., 2017), while *Bacillus* sp. C1 synthesized 1.09 g/L of PHA, accounting for 49.2% of cell dry weight, at an optimal temperature of 37°C (Dash *et al*., 2019). The ability of ML113 to maintain polymer production across a relatively broad temperature window (25 - 40°C) highlights its thermal adaptability, which is particularly advantageous for scaling up fermentation processes under industrial conditions where temperature fluctuations are common.

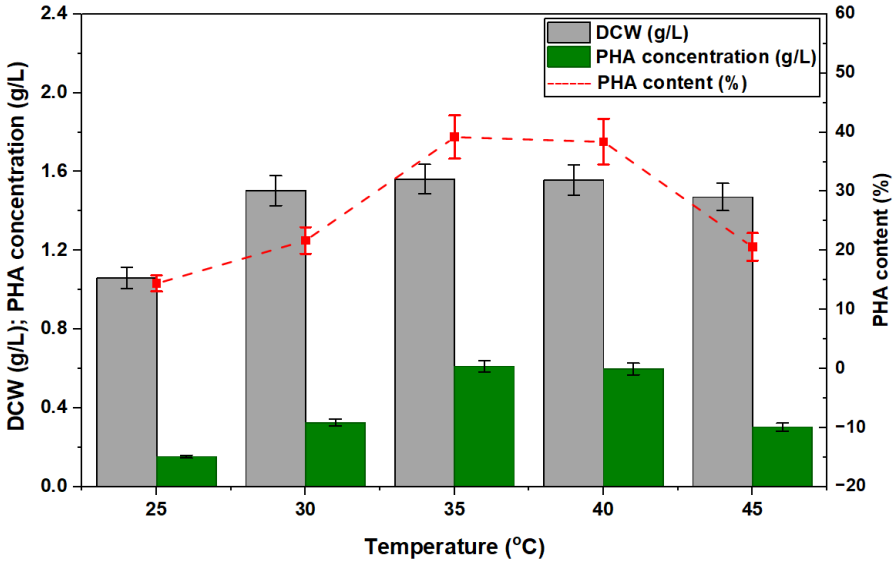

Figure 1. Effect of temperature on dry cell weight (DCW), PHA concentration, and PHA content of *P. aryabhattai* ML113 after 72 hours of fermentation.

Influence of fermentation time on the growth and PHA production of P. aryabhattai ML113

Cultivation time is a critical parameter that affects both biomass accumulation and PHA biosynthesis. In this study, *P. aryabhattai* ML113 was cultivated in HT medium, and its fermentation performance was assessed at 24, 48, 72, and 96 hours, as presented in Figure 2. Both DCW and PHA concentrations increased over time, peaking at 72 hours with values of 1.562 g/L DCW, 0.612 g/L PHA, and 39.18% PHA content. A marked decrease in PHA content was observed after 96 hours (20.15%), suggesting that extended incubation does not enhance production efficiency and may even reduce polymer accumulation. This decline is likely due to the mobilization of intracellular PHA as a carbon and energy source once external nutrients become limited, a phenomenon commonly reported in PHA-producing bacteria. These findings are consistent with the optimal fermentation duration reported for PHA biosynthesis in *B. megaterium* and *Ralstonia eutropha*. According to Patil *et al.* (2024), the cell biomass of *R. eutropha* increased from 1.86 g/L at 24 hours to 6.65 g/L at 72 hours, corresponding to the highest PHA yield (3.84 g/L) and polymer content (63.9%).

Extending the incubation to 96 hours caused a partial decrease in PHA content (57.8%), likely due to polymer depolymerization as a survival strategy under nutrient-depleted conditions. Similarly, *B. megaterium* exhibited approximately 4 g/L dry cell biomass and synthesized about 2.8 g/L PHA at 72 hours, further supporting 72 hours as the optimal fermentation time for maximal PHA accumulation.

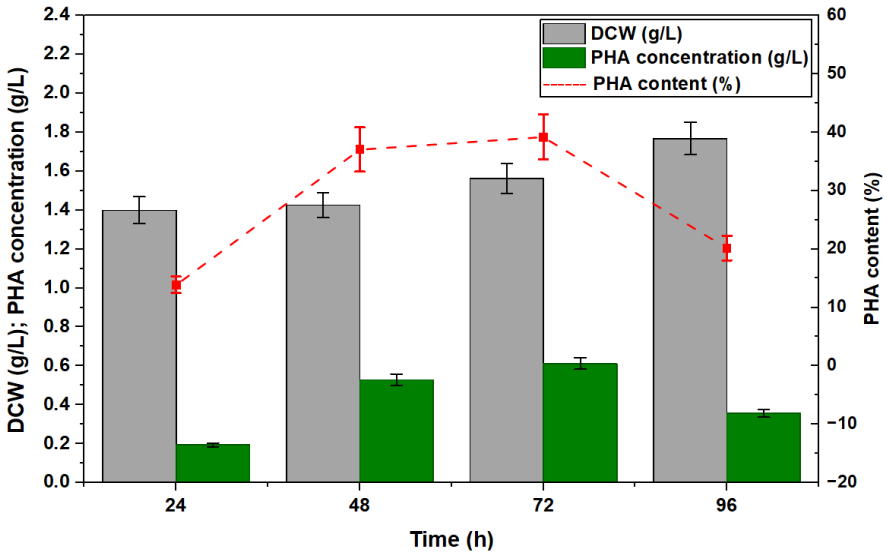

Figure 2. Effect of fermentation time on dry cell weight (DCW), PHA concentration, and PHA content of *P. aryabhattai* ML113.

Influence of C/N ratio on the growth and PHA production of P. aryabhattai ML113

The carbon-to-nitrogen (C/N) ratio is a key regulatory factor affecting bacterial growth and PHA accumulation. In this study, *P. aryabhattai* ML113 was cultivated in HT medium containing glucose and peptone, with the C/N ratio adjusted to 15:1, 23:1, and 28:1 (mol/mol). Biomass and PHA production were evaluated after 72 hours of incubation at 35°C. As illustrated in Figure 3, an increase in the C/N ratio from 15:1 to 28:1 resulted in a gradual enhancement of both cell growth and PHA accumulation. At a C/N ratio of 15:1, the DCW was 1.562 g/L, the PHA concentration was 0.612 g/L, and PHA content reached 39.18%. At 23:1, DCW increased to 1.938 g/L with 0.882 g/L PHA and 45.51% content. The highest values were obtained at a 28:1 ratio, where DCW reached 2.167 g/L, PHA concentration was 1.16 g/L, and the PHA content peaked at 53.53%. These results confirm that nitrogen limitation, under carbon-excess conditions, promotes a metabolic shift from growth to PHA storage. At low C/N ratios, nitrogen remains sufficient to support biomass synthesis, limiting PHA accumulation. In contrast, high C/N ratios restrict nitrogen availability, triggering carbon overflow and the accumulation of polymers. However, the increasing trend in both biomass and PHA in this study suggests that *P. aryabhattai* ML113 tolerates moderate nitrogen limitation without compromising growth. Similar findings have been reported in other PHA-producing bacteria. For instance, studies in *Cupriavidus necator* and *Bacillus* species

also demonstrated enhanced PHA yields at elevated C/N ratios, typically in the range of 20:1 to 30:1, depending on the carbon and nitrogen source (Ahn *et al*., 2015; Romero Sanchez *et al*., 2025). Fine-tuning this ratio is essential for maximizing PHA yield in large-scale fermentation processes.

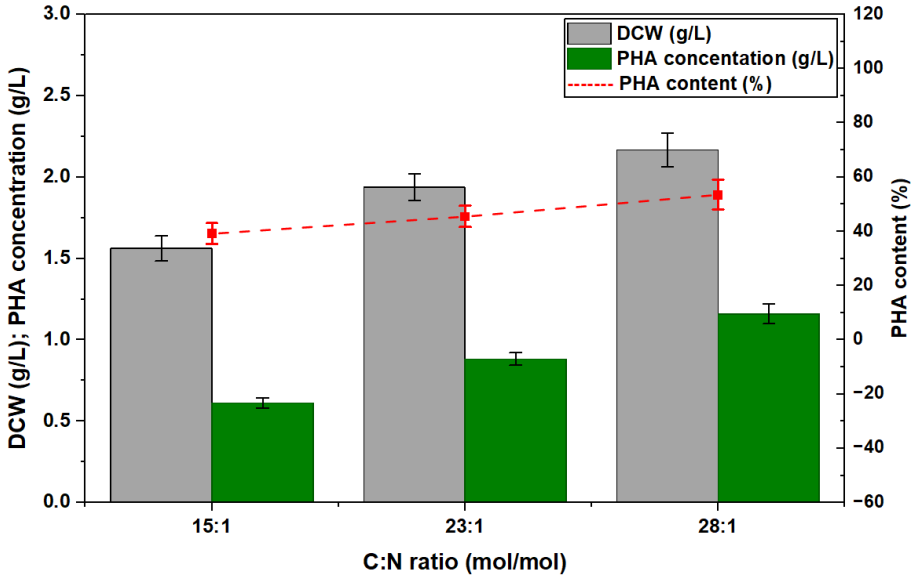

Figure 3. Effect of C:N ratio on dry cell weight (DCW), PHA concentration, and PHA content of *P. aryabhattai* ML113.

Optimization of PHA production of P. aryabhattai ML113 using RSM

To maximize PHA production of *P. aryabhattai* ML113, a statistical optimization strategy was implemented using RSM with a BBD. Three key process parameters—temperature (A, °C), fermentation time (B, h), and the carbon-to-nitrogen (C/N) molar ratio (C)—were selected based on preliminary single-factor experiments. Each factor was evaluated at three coded levels (–1, 0, +1) (Table 1). The experimental design comprised 15 runs, including three replicates at the center point to estimate pure error and enhance model reliability (Table 2).

Table 1. Experimental factors and coded levels used in the Box–Behnken design for optimization of PHA production by *P. aryabhattai* ML113.

Factor	Name	Unit	Coded values		
			-1	0	+1
A	Temperature	°C	30	35	40
B	Time	h	65	70	75
C	C/N ratio	mol/mol	25	30	35

Table 2. Box–Behnken design matrix with experimental and predicted PHA concentration values for optimization of PHA production by *P. aryabhattai* ML113.

Run	Temperature (°C)	Time (h)	C/N ratio (mol/mol)	Observed PHA (g/L)	Predicted PHA (g/L)
1	30	65	30	0.8538	0.8940
2	30	75	30	0.9375	0.9492
3	40	65	30	0.6241	0.5356
4	40	75	30	0.7003	0.6699
5	30	70	25	0.7486	0.7250
6	30	70	35	1.0118	0.9908
7	40	70	25	0.3532	0.3968
8	40	70	35	0.6874	0.6811
9	35	65	25	0.5969	0.6491
10	35	65	35	1.2502	1.2415
11	35	75	25	0.9454	1.0612
12	35	75	35	1.0202	1.0189
13	35	70	30	1.3421	1.2886
14	35	70	30	1.2794	1.2886
15	35	70	30	1.2976	1.2886

The analysis of variance (ANOVA) for the quadratic regression model (Table 3) demonstrated that the model was highly significant (F-value = 246.33; $p < 0.0001$), confirming a strong correlation between the selected factors and PHA concentration. The lack-of-fit test was not significant ($p = 0.7398$), indicating that the model adequately represents the experimental data without systematic error or unexplained variation. Among the linear terms, temperature (A) and C/N ratio (C) exerted the most substantial effects ($p = 0.0001$ and 0.0002, respectively), while fermentation time (B) had a smaller but statistically significant influence ($p = 0.0215$). Interaction effects were limited; only BC (time × C/N) was significant ($p = 0.0006$), whereas AB and AC interactions were not significant ($p > 0.05$). All three quadratic terms (A^2, B^2, and C^2) were highly significant ($p < 0.0001$), demonstrating a strong curvature in the response surface and indicating that the maximum PHA production lies within the experimental domain rather than at the boundaries.

Fit statistics (Table 4) reinforced the model's robustness: the model exhibited a high R^2 of 0.9977, with adjusted R^2 (0.9937) and predicted R^2 (0.9892) values in close agreement. The small difference of 0.0045 (< 0.2) between adjusted and predicted R^2 satisfies widely accepted criteria for RSM models, indicating excellent consistency between internal model fit and external predictive accuracy. This close alignment confirms that the quadratic model is not overfit and retains strong predictive power for new observations. Moreover, the adequate precision ratio (44.60), far

exceeding the recommended minimum of 4, demonstrates a high signal-to-noise ratio. The low coefficient of variation (C.V. 3.15%) and standard deviation (0.0287) further attest to the precision and reproducibility of the experiments. Together, these findings demonstrate that the quadratic regression model is statistically robust, highly predictive, and well-suited for navigating the design space to identify optimal conditions for PHA production. The dominance of quadratic effects over linear effects highlights the necessity of RSM for multi-factor optimization, as opposed to one-factor-at-a-time approaches.

Table 3. Analysis of variance for the quadratic model describing PHA concentration by *P. aryabhattai* ML113.

Source	Sum of squares	df	Mean square	F-value	p-value	Remark
Model	1.83	9	0.2033	246.33	<0.0001	significant
A-Temperature	0.1017	1	0.1017	123.22	0.0001	
B-Time	0.0090	1	0.0090	10.88	0.0215	
C-C/N ratio	0.0757	1	0.0757	91.67	0.0002	
AB	0.0008	1	0.0008	0.95	0.3749	
AC	0.0000	1	0.0000	0.05	0.8289	
BC	0.0504	1	0.0504	61.03	0.0006	
A^2	1.30	1	1.30	1573.87	<0.0001	
B^2	0.1040	1	0.1040	125.99	<0.0001	
C^2	0.2495	1	0.2495	302.33	<0.0001	
Residual	0.0041	5	0.0008			
Lack of Fit	0.0001	1	0.0001	0.13	0.7398	not significant
Pure Error	0.0040	4	0.0010			
Cor Total	1.83	14				

Table 4. Fit statistics for the quadratic regression model describing PHA concentration.

Parameter	Value	Parameter	Value
Standard deviation	0.0287	Adjusted R^2	0.9937
Mean	0.9119	Predicted R^2	0.9892
Coefficient of variation (C.V.%)	3.15	Adeq Precision	44.60
R^2	0.9977		

Based on the ANOVA results and fit statistics, the final quadratic regression equation predicting PHA concentration (Y) as a function of temperature (A), fermentation time (B), and C/N ratio (C) is presented below:

Coded equation $Y = 1.28863 - 0.15945A + 0.04738B + 0.13753C + 0.01978AB + 0.00463AC - 0.15870BC - 0.41035A^2 - 0.11610B^2 - 0.17985C^2$

Actual equation $Y = -59.6196 + 1.05615A + 0.82239B + 0.89704C + 0.00079AB + 0.00019AC - 0.00635BC - 0.01641A^2 - 0.00464B^2 - 0.00719C^2$

where Y = predicted PHA concentration (g/L); A = Temperature (°C); B = Fermentation time (h); C = C/N ratio (mol/mol).

To visually interpret the interaction effects among the studied variables and validate the fitted quadratic regression model, three-dimensional (3D) response surface plots were generated. These plots provide an intuitive understanding of how each pair of factors—temperature, fermentation time, and C/N ratio—affects PHA concentration while holding the third factor at its center level. By examining the curvature of these surfaces, the regions of maximum predicted PHA production can be readily identified, complementing the statistical analysis presented in Tables 3 and 4. The interactive effects of each pair of factors on PHA biosynthesis can be clearly observed in Figure 4. PHA concentration increases as temperature rises to approximately 34°C and fermentation time approaches 72 h, after which further increases lead to a slight decline in predicted yield (Figure 4a). This concave surface corresponds to the strong significance of the quadratic terms A^2 and B^2 identified in ANOVA, confirming that the optimum lies within the experimental range. With fermentation time fixed at 70 h, both temperature and C/N ratio exert strong influences on PHA accumulation (Figure 4b). The highest PHA concentration is achieved at moderate temperature (~34°C) combined with a C/N ratio of around 30 mol/mol, whereas deviations toward higher or lower values of either factor reduce production. These findings are consistent with ANOVA results, where both temperature and C/N ratio exhibited high statistical significance. At a constant temperature of 34°C, the combined effects of fermentation time and C/N ratio reveal a clear interactive pattern (Figure 4c). The optimal PHA yield is predicted at intermediate levels of both variables, while deviation in either direction diminishes accumulation. This agrees with the significant BC interaction term revealed by ANOVA, emphasizing the necessity of balancing fermentation time and C/N ratio to achieve maximum PHA productivity.

Based on the quadratic regression model, the RSM software generated 100 predicted solutions for maximizing PHA production. To verify the predictive reliability of the RSM model, the software-generated statistical summary for each solution is shown in Table 5, illustrating predicted mean values, confidence intervals, and tolerance intervals for PHA concentration. Based on these predictions, nine representative solutions were randomly selected for experimental validation (Table 6). Observed PHA concentrations were compared with predicted values to evaluate the accuracy of the model.

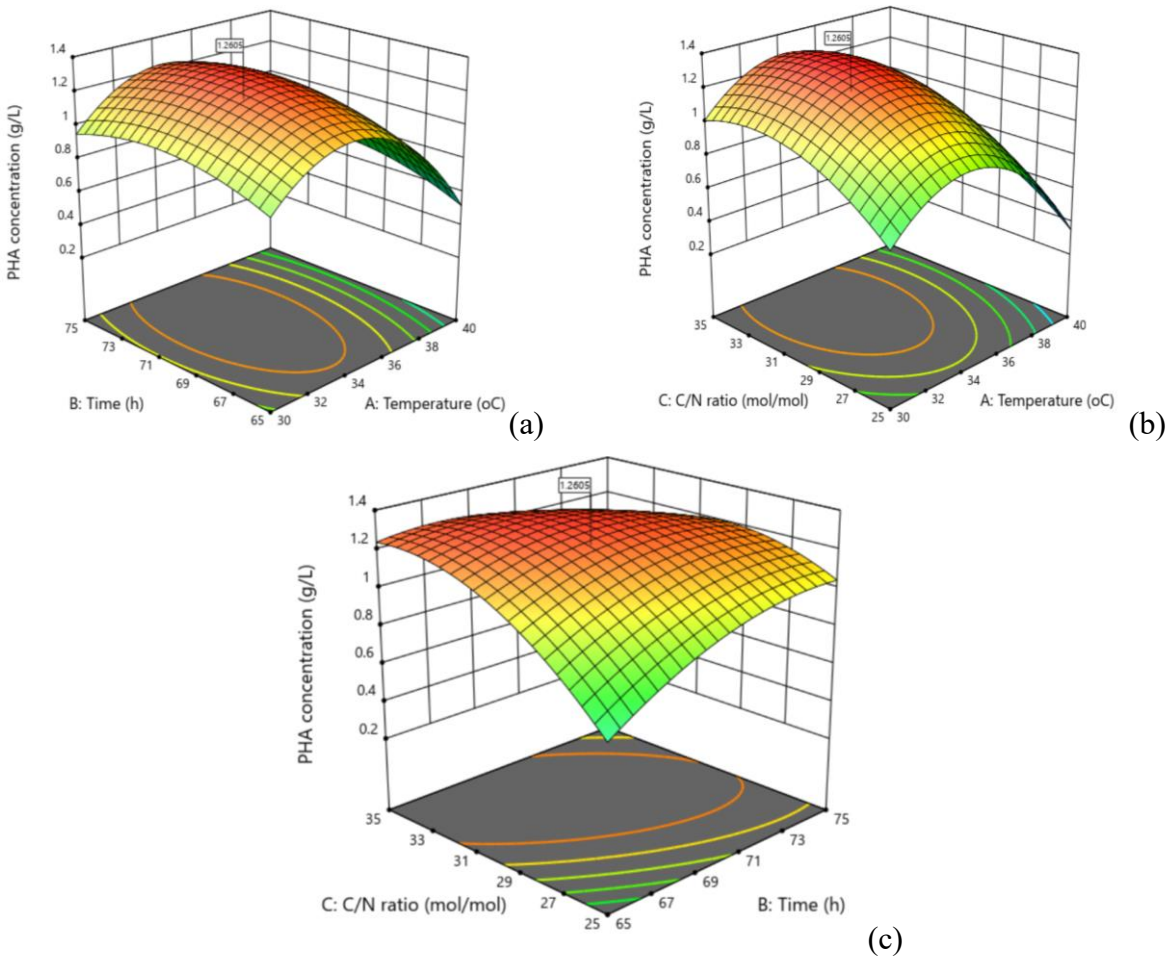

Figure 4. Three-dimensional (3D) response surface plots illustrating the combined effects of temperature (A), fermentation time (B), and C/N ratio (C) on PHA concentration by *P. aryabhattai* ML113. (a) Effect of temperature and time at fixed C/N ratio (30 mol/mol); (b) Effect of temperature and C/N ratio at fixed fermentation time (70 h); (c) Effect of time and C/N ratio at fixed temperature (35°C). The plots demonstrate the curvature of the design.

Table 5. Statistical summary of one predicted solution (Solution 1 of 100) generated by the RSM model, showing predicted mean, standard deviation, standard error, 95% confidence intervals (CI), and 99% tolerance intervals (TI) for PHA concentration.

Response	Predicted mean	Predicted median	Std dev	SE mean	95% CI low for mean	95% CI high for mean	95% TI low for 99% Pop	95% TI high for 99% Pop
PHA concentration	1.2605	1.2605	0.0287	0.0132	1.2264	1.2946	1.0792	1.4418

Pop refers to the population, indicating that the tolerance interval is expected to contain 99% of the population values with 95% confidence.

Table 6. Selected combinations of temperature, fermentation time, and C/N ratio from the 100 predicted solutions generated by RSM, including both predicted and experimentally observed PHA concentrations.

Run ID	Temperature (°C)	Time (h)	C/N ratio (mol/mol)	Predicted PHA (g/L)	Observed PHA (g/L)	Rank	Selection status
1	32,78	69.08	29.80	1.2605	1.2634	12	
12	35,07	73.47	30.10	1.2642	1.2757	1	
15	33.55	73.19	29.66	1.2766	1.1902	1	
43	32.66	71.04	31.79	1.2900	1.3763	1	
62	35.26	66.92	31.99	1.2704	1.2669	1	
79	34.19	66.03	33.59	1.2913	1.3416	1	
90	34.01	71.90	29.85	1.3014	1.4473	1	Selected
94	33.70	66.56	33.80	1.3010	1.3354	1	
99	32.70	71.46	32.89	1.2678	1.2780	1	

The comparison between predicted and observed PHA concentrations for the nine selected solutions (Table 6) demonstrates that the RSM model achieved high predictive accuracy across most experimental conditions. The majority of observed values closely matched predictions, with deviations generally within the 95% confidence intervals estimated by the model. However, larger discrepancies were noted for certain points, particularly Run 15 and Run 90, where the observed values were lower or higher than predicted by 6–11%. These differences likely reflect the limitations of quadratic response surface models when predicting outcomes at points further from the central region of the design space or near experimental boundaries. Overall, the validation confirms that the RSM approach is reliable for guiding process optimization of PHA production in *P. aryabhattai* ML113, while emphasizing the importance of experimental confirmation for outlier conditions. In this study, optimization of fermentation parameters using RSM improved PHA production by *P. aryabhattai* ML113 by approximately 1.25-fold compared with traditional one-factor-at-a-time (OFAT) experiments. This result highlights the power of RSM in modeling complex biological systems, where second-order regression equations capture curvature and significant factor interactions—particularly the interaction between fermentation time and C/N ratio—allowing the identification of true optimal conditions located within the design space rather than at its boundaries. The statistical significance of all quadratic terms (A^2, B^2, C^2) in the ANOVA analysis and the concave response surfaces observed in 3D plots, with predicted maxima around 34°C, 70 h, and a C/N ratio of 30, further validate the robustness of the fitted model.

The improvement observed in this work is consistent with previous studies demonstrating the utility of RSM for PHA production optimization. For example, *Burkholderia* sp. ISTR5 cultivated on lignin-rich industrial wastewater achieved a 42.5%

increase in PHA yield through a Box–Behnken design, demonstrating that RSM can be applied successfully even to complex and recalcitrant substrates (Morya *et al*., 2021). Similarly, *B. megaterium* pPSPHAR1/1, optimized via RSM-based media formulation, produced 764.2 mg PHA per gram of cell dry weight, corresponding to a 1.35-fold improvement over initial OFAT conditions (Pham *et al*., 2022). A recent study with *Klebsiella* sp. MK3 also reported high PHA titers of 4.37 mg/mL from wood waste after RSM optimization, illustrating its value for valorizing lignocellulosic biomass (Kumar *et al*., 2025). These studies confirm that the 1.25-fold increase achieved in the present work is well within the range of improvements commonly obtained through RSM, which typically vary from approximately 1.2-fold enhancements to gains exceeding fourfold, depending on the microbial strain, substrate, and process complexity. Beyond yield improvement, RSM offers distinct advantages in both experimental efficiency and predictive accuracy: fewer experimental runs are required to map factor interactions, random error is minimized through center-point replication, and model validation is reinforced through targeted confirmation experiments. The close agreement between predicted and observed PHA concentrations in this study underscores the robustness of the model and its applicability for scaling up *P. aryabhattai* ML113 cultivation and integrating cost-effective substrates into sustainable PHA production workflows.

Taken together, this study demonstrated a systematic optimization of PHA production by *P. aryabhattai* ML113 through sequential OFAT screening followed by RSM modeling. OFAT experiments identified temperature, fermentation time, and C/N ratio as key variables, with the highest PHA concentration reaching 1.16 g/L at 35°C, 72 h, and a C/N ratio of 28/1. Using a central composite design in RSM, a statistically robust quadratic model was developed, revealing significant quadratic effects for all factors and a strong interaction between fermentation time and C/N ratio. Under optimized conditions (approximately 34°C, 72 h, C/N ratio 30), the PHA concentration increased to 1.4473 g/L, representing a 1.25-fold improvement over the OFAT result. The close agreement between predicted and observed values validates the model's reliability and highlights the effectiveness of integrating OFAT and RSM for improving microbial PHA production and supporting process scale-up.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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