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Optimization Using Response Surface Methodology (RSM) for production of Polyhydroxyalkanoate acid (PHA) in *Chlorella Vulgaris*

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ABSTRACT

The increasing demand for sustainable and biodegradable plastics has driven interest in polyhydroxyalkanoate (PHA) produced by microorganisms. In this study, the effects of nitrogen, phosphate, and iron concentrations on PHA production by *Chlorella vulgaris* were investigated and optimized using response surface methodology (RSM). A Box–Behnken experimental design was employed to evaluate the influence and interaction of the selected nutrients on PHA accumulation. The results showed that nutrient availability significantly affected both microalgal growth and intracellular PHA production. The highest PHA accumulation obtained in the experimental runs was 20.28 %CDW, achieved under nitrogen-deficient conditions (0 mM nitrogen, 0.051 mM phosphate, and 0.0082 mM iron). In addition, the highest biomass productivity reached 0.0345 g L^{-1} day^{-1}. These findings demonstrate that nutrient availability plays an important role in regulating PHA accumulation and highlight the potential of *C. vulgaris* as a promising microalgal source for sustainable biopolymer production.

1. Introduction

Microalgae have received more attention recently as a potential source of PHA. Microalgae can grow using sunlight, carbon dioxide, and basic nutrients, in contrast to bacteria, which often require substrates such as sugars or fatty acids. Consequently, this makes them more cost-effective and environmentally sustainable [1].

Microalgae can synthesize intracellular compounds, including lipids, carbohydrates, and PHA, dependent upon the species and environmental conditions [2]. The ability of many strains, including *Spirulina*, *H. pluvialis*, *Chlorella sp*., and *C. sorokiniana*, to synthesize PHA has been examined. Sudesh and Iwaata and Sharma et al. assert that these species are distinguished by their rapid growth, adaptability, and relatively high biomass productivity [3, 4].

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Microalgae with rapid growth rates can produce higher biomass in shorter cultivation periods, which is advantageous for large-scale production systems [5]. Additionally, species capable of redirecting carbon flux toward storage compounds under nitrogen or phosphorus limitation are more suitable candidates for PHA accumulation [6].

PHA buildup in microalgae is often affected by stressors such as light intensity or food deficiency. Under these conditions, microalgae tend to divert their carbon flux towards the synthesis of storage molecules such as PHA, rather than promoting cell development. They are suitable for biopolymer production due to their metabolic versatility, especially in extensive outdoor culture systems [4].

Despite the relatively lower PHA content in microalgae compared to bacteria, the sustainability advantages and the potential for utilizing waste materials offer microalgae a suitable platform for future bioplastic synthesis [5]. *C. vulgaris* is one of the most extensively studied microalgae due to its rapid growth rate, high photosynthetic efficiency, and ability to adapt to diverse cultivation conditions. It has been widely used in biotechnology for lipid and biomass production [7].

Under nitrogen limitation, *C. vulgaris* redirects carbon flux toward intracellular storage compounds, particularly lipids and carbohydrates. This stress-induced carbon accumulation behavior supports its inclusion in screening studies aimed at evaluating potential PHA accumulation under nutrient imbalance [8]. Based on the highest yield of PHA and the monomer diversity observed in *C. vulgaris* [9], further study was conducted to optimize the nutrient conditions to increase the polymer production in this research. Thus, RSM was introduced to evaluate the combined effects of nitrogen, phosphate and iron concentrations on PHA accumulation.

Although there have been many reports on PHA production in microalgae, optimization studies that systematically evaluate the combined effects of key nutrient factors such as nitrogen, phosphate, and iron remain limited in the literature. Most studies focus on demonstrating the occurrence of PHA synthesis or simple nutrient limitation rather than statistically optimizing medium composition to maximize yield using response surface methodology (RSM) [10]. This research therefore addresses a gap by conducting a comprehensive nutrient optimization for PHA accumulation in *C. vulgaris*, which has not been extensively explored in previous works.

In this study, nitrogen, phosphate, and iron were selected for optimization because these nutrients are known to affect the balance between biomass growth and PHA storage. Nitrogen and phosphate limitation have been associated with enhanced carbon storage as PHA under nutrient stress conditions [11]. Iron, although less commonly studied, has also been shown to impact PHA metabolism, particularly in combination with other nutrient factors [10].

However, existing research has typically evaluated these factors individually or qualitatively rather than through a systematic optimization approach. Therefore, this work fills a gap by applying RSM to quantitatively investigate and determine the optimum nutrient conditions for PHA production in *C. vulgaris*.

2. Methodology

2.1 Preparation of medium

The AF6 medium was used to cultivate the *C. vulgaris*. This medium is widely used in freshwater algal cultivation due to its balanced nutrient composition that can supports biomass growth under phototrophic conditions [12]. The media was prepared by dissolving analytical grade chemicals in distilled water. The pH of the AF6 medium was adjusted to 7.0 ± 0.2. All media were sterilized using autoclaving at 120°C for 15 minutes [13].

2.2 Cultivation conditions

The *C. vulgaris* was maintained in the respective medium in a plastic cell culture flask (25 cm^2, BioLite, Thermo Fisher Scientific, USA) without aeration. The culture was basically grown in a 250 mL Erlenmeyer flask containing 180 mL of sterile medium with 2% CO_2 containing air and the experimental cultures were conducted in a 200 mL flask with the standard medium until Day 15 [14]. The cultures were maintained under continuous illumination at an intensity of ±60 and 70 $\mu mol\ m^{-2}s^{-1}$ using white fluorescent lamps at 25 ± 2°C [15]. A photo meter (Apogee MQ-500-Full-Spectrum Quantum Meter, USA) was used to monitor the amount of light strength and the temperature, respectively [16].

Nitrogen was supplied as sodium nitrate ($NaNO_3$), phosphate as K_2HPO_4 and KH_2PO_4, and iron as ferric citrate (Fe-citrate) according to AF-6 medium formulation. The concentration of each nutrient was varied within the permissible range of the medium during RSM optimization [17].

2.3 Extraction of PHA

The PHA was directly extracted using the solvent chloroform. The dry cell weight was prepared by centrifuging 100 mL of the cultures for 10 minutes at 5000 rpm, the cultures were harvested. For complete digestion of all cell components, except PHA, the cell pellet was suspended in 10 mL of 4% sodium hypochlorite solution and incubated at 37 °C for 1–2 hours. During this time, lipids and proteins are degraded [18].

PHA granules extracted from the mixture were collected using a centrifuge at 3000 g x 30 min. The sediment was rinsed twice with 10 ml of distilled water by centrifugation under the same conditions. Then, the PHA granules in the sediment underwent undergo washing processes using 5 mL of ethanol. To produce dry PHA powder, the polymer granule was dissolved in boiling chloroform and evaporated using air drying [18].

2.4 Calculation of PHA content and productivity
2.4.1 PHA content

Polyhydroxyalkanoate (PHA) accumulation was expressed as the percentage of PHA relative to cell dry weight (CDW), which represents the intracellular polymer storage capacity of the microorganism. Expressing PHA as a percentage of biomass is commonly used in microbial and microalgal PHA studies to allow comparison between different cultivation conditions and strains [5]. The PHA content was calculated using the following equation:

$$\mathrm{PHA}(\%\mathrm{CDW}) = \frac{\text{PHA dry weight}}{\text{Cell dry weight}} \times 100\% \tag{1}$$

where PHA dry weight (gL^{-1}) represents the mass of polymer extracted or quantified from the biomass, while CDW (gL^{-1}) represents the total dry biomass obtained after drying. This approach provides a standardized representation of intracellular polymer accumulation in microbial cultures [19].

2.4.2 PHA productivity

PHA productivity represents the rate of polymer formation during the cultivation period and is commonly used to evaluate the efficiency of biopolymer production in microbial and microalgal systems [20]. In this study, PHA productivity was calculated based on the final PHA concentration obtained at the end of the cultivation period. Since the initial PHA content at the start of cultivation was negligible, the productivity was approximated using the following equation:

$$P_{PHA} = \frac{PHA_f}{t} \quad (2)$$

where P_{PHA} represents the PHA productivity (gL^{-1}day^{-1}), PHA_f represents the final PHA concentration (g/L), and t represents the cultivation time (days). This simplified calculation has been widely used in batch cultivation studies when the initial polymer concentration is assumed to be negligible compared to the final accumulated polymer [21].

2.4.3 Biomass Productivity

Biomass productivity is an important parameter used to evaluate the growth performance of microalgae during cultivation and reflects the efficiency of biomass formation under specific environmental and nutrient conditions [22]. In this study, biomass productivity was calculated based on the final cell dry weight obtained at the end of the cultivation period. Since the initial biomass concentration was relatively low compared to the final biomass produced during cultivation, biomass productivity was approximated using the following equation:

$$P_x = \frac{DCW}{t} \quad (3)$$

where P_x represents the biomass productivity (gL^{-1}day^{-1}), DCW represents the final cell dry weight concentration (gL^{-1}), and t represents the cultivation time (days). This simplified approach has been widely applied in batch microalgae cultivation studies when the initial biomass concentration is negligible relative to the final biomass concentration [8].

2.5 Optimization with response surface methodology

The optimum condition for PHA accumulation was determined using RSM with Design-Expert software (Version 11, Stat-Ease Inc., USA). Three independent variables were selected for this study, namely nitrogen, phosphate, and iron concentrations. A Box–Behnken design with three factors at three levels was employed to evaluate the effects of these variables on PHA accumulation in *C. vulgaris*. Each variable was tested at three levels (0%, 50%, and 100%), representing the lowest, intermediate, and highest nutrient concentrations in the culture medium. A total of 15 experimental runs were performed in triplicate. The experimental design matrix and nutrient concentrations used in each run are presented in Table 1.

Table 1
Experimental design of RSM in different nutrients

Run	Nitrogen (mM)	Phosphate (mM)	Iron (mM)
1	1.650	0.051	0.0082
2	0.825	0.102	0.0082
3	1.650	0.051	0.0000
4	1.650	0.102	0.0041
5	0.825	0.000	0.0000
6	1.650	0.000	0.0041
7	0.825	0.051	0.0041
8	0.825	0.102	0.0000
9	0.825	0.051	0.0041
10	0.000	0.102	0.0041
11	0.000	0.000	0.0041
12	0.825	0.000	0.0082
13	0.825	0.051	0.0041
14	0.000	0.051	0.0082
15	0.000	0.051	0.0000

3. Results

3.1 Growth profile of C. vulgaris

The growth profiles of microalgae cultivated under different nutrient conditions are presented in Fig. 1(a) and Fig. 1(b). In general, most experimental runs exhibited a typical microalgal growth pattern consisting of lag, exponential, and stationary phases [23]. During the initial cultivation period (day 0–3), the cultures showed relatively slow growth, indicating an adaptation phase in which the cells acclimatized to the culture environment.

Rapid biomass accumulation was observed between day 3 and day 10, where OD_{750} increased significantly, indicating active cell division and metabolic activity [22]. After approximately day 10, the cultures gradually entered the stationary phase, where the increase in OD_{750} became slower. This trend is commonly observed in microalgal cultivation as nutrient depletion and metabolic by-products begin to limit further cellular growth [23].

Variations in growth behaviour among the experimental runs were evident, suggesting that nutrient composition significantly influenced microalgal growth performance. Certain nutrient combinations supported rapid biomass accumulation, while others resulted in slower growth rates. For instance, run 6 exhibited negligible growth throughout the cultivation period, indicating that the nutrient composition under this condition was not favourable for microalgal growth. Similar observations have been reported in previous studies where inappropriate nutrient balance can inhibit microalgal metabolism and biomass formation [4].

Overall, the observed growth patterns confirm that nutrient availability plays a critical role in regulating microalgal growth and metabolic activity during cultivation. To improve the clarity of visualization, the growth curves were divided into two figures due to the large number of experimental runs. Fig. 1(a) and Fig. 1(b) show the growth curves of *C. vulgaris* under different nutrient conditions. The data are presented as standard deviation (n = 3).

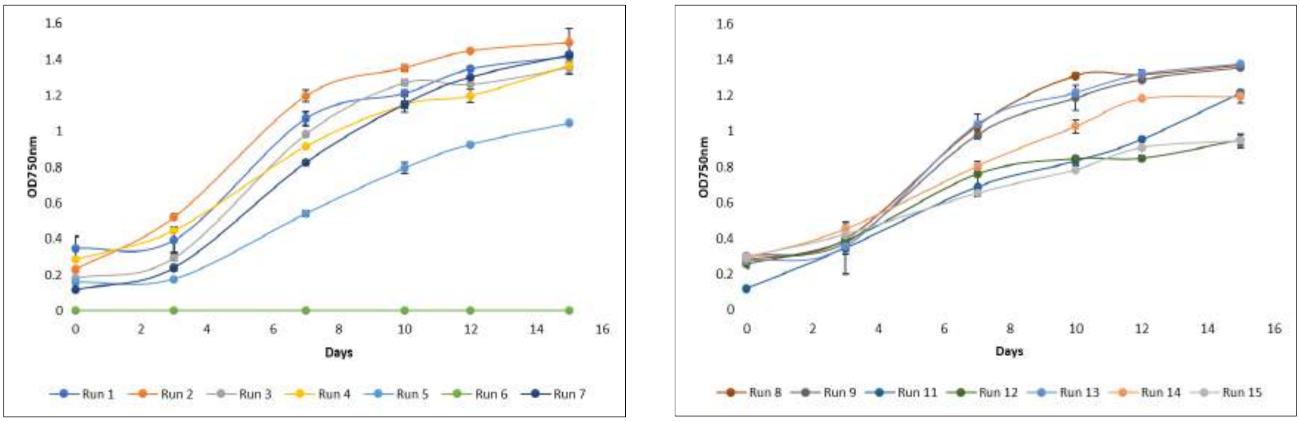

Fig. 1. Growth curve of *C. vulgaris* under different concentration of nutrients (a) Experiments run 1 to 7 (b) Experiments run 8 to 15

3.2 Biomass productivity

The biomass productivity calculated for each experimental run is presented in Fig. 2. Biomass productivity ranged from approximately 0.004 to 0.034 g L^{-1} day^{-1}, indicating substantial variation among the tested nutrient conditions. Higher biomass productivity values for *C. vulgaris* have been reported in previous studies. For instance, previous study reported biomass productivity of approximately 0.31 g L^{-1} day^{-1} without CO_2 supplementation and 0.45 g L^{-1} day^{-1} with 4% CO_2 supply [24].

The lower productivity observed in the present study is likely due to differences in cultivation conditions, as the previous study employed a photobioreactor (PBR) system, whereas the current study was conducted in conical flasks with a lower CO_2 concentration (2%). Photobioreactor systems generally provide better light distribution, mixing, and gas transfer, which can enhance microalgal growth and biomass production [24]. Thus, the biomass productivity obtained in this study is still comparable with values reported in previous studies.

Among the experimental runs, the Run 13 exhibited the highest biomass productivity, suggesting that the nutrient composition under this condition provided favourable conditions for microalgal growth. Adequate nutrient availability, particularly nitrogen and phosphorus, is essential for supporting protein synthesis, nucleic acid formation, and cellular metabolism, which ultimately contribute to biomass accumulation [8].

Conversely, the Run 6 showed the lowest biomass productivity, which is consistent with the minimal growth observed in the growth profile. This indicates that the nutrient combination used in this run may have limited essential metabolic processes required for cell proliferation. Nutrient imbalance or limitation can significantly reduce microalgal growth rates and biomass production [22]. In addition to biomass productivity, PHA content and productivity were evaluated to assess the biopolymer production potential of the culture. The biomass productivity in different experiments run as shown in Fig. 2, and the data are presented as mean ± standard error (n = 3).

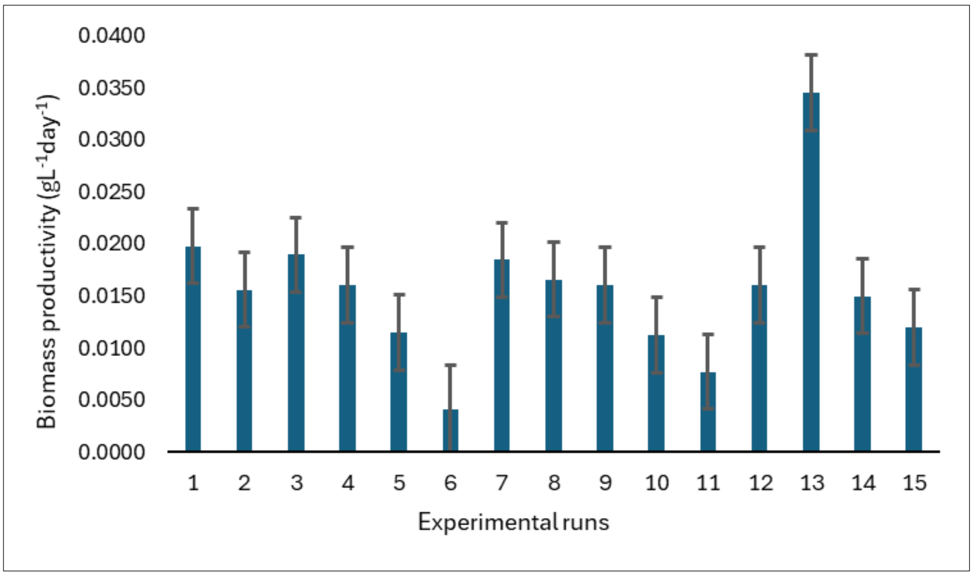

Fig. 2. Biomass productivity different experiments run

3.3 PHA accumulation in C. vulgaris

The biomass productivity, PHA accumulation, and PHA productivity obtained under different experimental runs are summarized in Table 2. The results reveal that PHA accumulation (%CDW) is not directly proportional to biomass productivity. For instance, the Run 14 exhibited the highest PHA accumulation (20.28 % CDW) despite having lower biomass productivity than Run 13, which produced the highest biomass productivity (0.0345 g L^{-1} day^{-1}). This observation suggests a metabolic shift in which cellular carbon is redirected toward intracellular storage compounds rather than biomass formation [4].

Although Run 13 exhibited only moderate PHA accumulation (11.39 % CDW), it achieved the highest PHA productivity (0.0039 g L^{-1} day^{-1}) due to its significantly higher biomass productivity. This indicates that overall polymer productivity depends on both biomass formation and intracellular PHA content, highlighting the importance of balancing growth conditions and metabolic stress to maximize biopolymer production [23].

Under nutrient imbalance or limitation, particularly nitrogen deficiency, microalgal cells often redirect photosynthetically fixed carbon toward storage metabolites such as lipids, carbohydrates, or PHA. This metabolic response occurs because cell division becomes restricted while carbon fixation may continue, leading to the accumulation of intracellular carbon reserves [23]. As a result, conditions that strongly support cellular growth may not necessarily promote the highest intracellular polymer accumulation [4]. The PHA accumulation obtained in this study is comparable with previously reported values for microalgae cultivated under nutrient-limited conditions, as summarized in Table 2.

Table 2
Comparison of PHA accumulation reported for microalgae in previous studies

Microalgae species	Conditions	PHA (%CDW)	Reference
Chlorella fusca	Carbon source optimization	14-17.3	Cassuriaga et al., [28]
Nostoc muscorum	Carbon source optimization	0.68	Bhati and Mallick, [29]
Nostoc muscorum	Carbon source optimization + Nitrogen deficiency	0.78	
Nostoc muscorum	Carbon source optimization + Phosphorus deficiency	0.71	
Scenedesmus sp.	Nutrient optimization	0.82-29.92	Gabriela García et al.,[10]
Stigeoclonium sp. B23	Light + Carbon source	0.12	Mourão et al., [30]
Chlorella pyrenoidosa	Carbon dioxide source	0.27	Das et al., [31]
C. vulgaris (this study)	Nutrient optimization	**20.28**	**This study**

The PHA accumulation obtained in this study was also compared with previously reported values for microalgae, as summarized in Table 2. The results show that the PHA accumulation achieved in *C. vulgaris* (20.28 %CDW) is comparable with values reported in previous studies, which generally range from low levels below 1% of CDW to higher values exceeding 20% of CDW depending on the microalgal species and cultivation conditions. For example, *Chlorella fusca* has been reported to accumulate between 14–17.3% of CDW under carbon source optimization conditions, while *Scenedesmus* sp. has shown PHA accumulation up to 29.925% of CDW under nutrient limitation conditions.

Table 3
Biomass productivity, PHA accumulation (%CDW), and PHA productivity of
C. vulgaris under different nutrient conditions

Run	Nitrogen (mM)	Phosphate (mM)	Iron (mM)	Biomass ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ day^{-1}$)	PHA (% CDW)	PHA productivity ($g\ L^{-1}\ day^{-1}$)
1	1.650	0.051	0.0082	0.0198	0.0198	16.35	0.0032
2	0.825	0.102	0.0082	0.0156	0.0156	15.02	0.0023
3	1.650	0.051	0.0000	0.0190	0.0190	14.12	0.0026
4	1.650	0.102	0.0041	0.0161	0.0161	10.94	0.0017
5	0.825	0.000	0.0000	0.0115	0.0115	7.07	0.0008
6	1.650	0.000	0.0041	0.0042	0.0000	0.00	0.0000
7	0.825	0.051	0.0041	0.0185	0.0185	12.39	0.0022
8	0.825	0.102	0.0000	0.0166	0.0166	8.95	0.0014
9	0.825	0.051	0.0041	0.0161	0.0161	10.95	0.0017
10	0.000	0.102	0.0041	0.0113	0.0113	9.47	0.0010
11	0.000	0.000	0.0041	0.0077	0.0077	8.57	0.0006
12	0.825	0.000	0.0082	0.0161	0.0161	5.19	0.0008
13	0.825	0.051	0.0041	0.0345	**0.0345**	**11.39**	**0.0039**
14	0.000	0.051	0.0082	0.0150	**0.0150**	**20.28**	**0.0030**
15	0.000	0.051	0.0000	0.0120	0.0120	15.66	0.0018

These variations indicate that PHA accumulation in microalgae is strongly influenced by nutrient availability and cultivation conditions. Similar growth storage trade-offs have been widely reported in microalgae cultivated under nutrient stress conditions [8]. Therefore, RSM was employed to systematically evaluate the effects and interactions of nitrogen, phosphate, and iron concentrations on PHA production. Table 3 shows the biomass productivity, PHA accumulation (%CDW), and PHA productivity of *C. vulgaris*.

3.3 Statistical analysis in RSM

RSM was applied to analyse the effects of nitrogen, phosphate, and iron concentrations on PHA accumulation in *C. vulgaris*. The experimental design matrix is shown in Table 4. The experimental design consisted of 15 runs generated using a Box–Behnken design with the response of PHA accumulation in *C. vulgaris* as shown in Table 5. The model has three replicated points as the centre point. Also, Table 5 was used to develop a mathematical model using analysis of variance (ANOVA) to predict the optimum values for the three factors in order to achieve the optimum amount of PHA accumulation.

Table 4
Factors and levels in the Box-Behnken design

Parameter	Symbol	-1	0	+1
Nitrogen	A	0 mM	1.650 mM	0.825 mM
Phosphorus	B	0 mM	0.051 mM	0.102 mM
Iron	C	0 mM	0.0041 mM	0.0082 mM

Table 5
Design matric using Box-Behnken design and experimental results

Run	Nitrogen (mM)		Phosphate (mM)		Iron (mM)		PHA (% CDW)
	A	Level	B	Level	C	Level	
1	1.650	0	0.051	0	0.0082	+1	16.35
2	0.825	+1	0.102	+1	0.0082	+1	15.02
3	1.650	0	0.051	0	0.0000	-1	14.12
4	1.650	0	0.102	+1	0.0041	0	10.94
5	0.825	+1	0.000	-1	0.0000	0	7.07
6	1.650	0	0.000	-1	0.0041	0	0.00
7[a]	0.825	+1	0.051	0	0.0041	0	12.39
8	0.825	+1	0.102	+1	0.0000	-1	8.95
9[a]	0.825	+1	0.051	0	0.0041	0	10.95
10	0.000	-1	0.102	+1	0.0041	0	9.47
11	0.000	-1	0.000	-1	0.0041	0	8.57
12	0.825	+1	0.000	-1	0.0082	+1	5.19
13[a]	0.825	+1	0.051	0	0.0041	0	11.39
14	0.000	-1	0.051	0	0.0082	+1	20.28
15	0.000	-1	0.051	0	0.0000	-1	15.66

[a]Centre points

The statistical significance and adequacy of the quadratic model for PHA production were evaluated using analysis of variance (ANOVA), as presented in Table 6. The model exhibited a high F-value of 82.97 with a p-value of <0.0001, indicating that the regression model was statistically significant. This suggests that the selected variables significantly influenced PHA accumulation in *C. vulgaris*.

The relationship between the independent variables and the response (PHA accumulation) was described using a second-order polynomial regression model. The coded values of A, B, and C represent the actual concentrations of nitrogen, phosphate, and iron concentrations, respectively. Based on the experimental data, the regression equation for predicting PHA accumulation was obtained as follows:

$$\begin{aligned}\mathrm{PHA}(\%\mathrm{CDW}) = {} & 11.81167 - 8.11768A + 202.41013B - 1670.32520C \\ & +59.655538AB - 176.64449AC + 9505.02151BC \\ & +2.36058A^2 - 2283.09625B^2 + 2.03401 \times 10^5 C^2 \end{aligned} \quad (4)$$

The relationship between predicted and actual responses of the total PHA accumulation in *C. vulgaris* as shown in Figure 3. The correlation of coefficient (R^2) of the developed model is 0.99 that indicates 99% of the total variation in PHA contents was attributed to the experimental studied. The value of adjusted correlation of coefficient (Adj R^2 = 0.98) is also high to advocated for a high significance of the model.

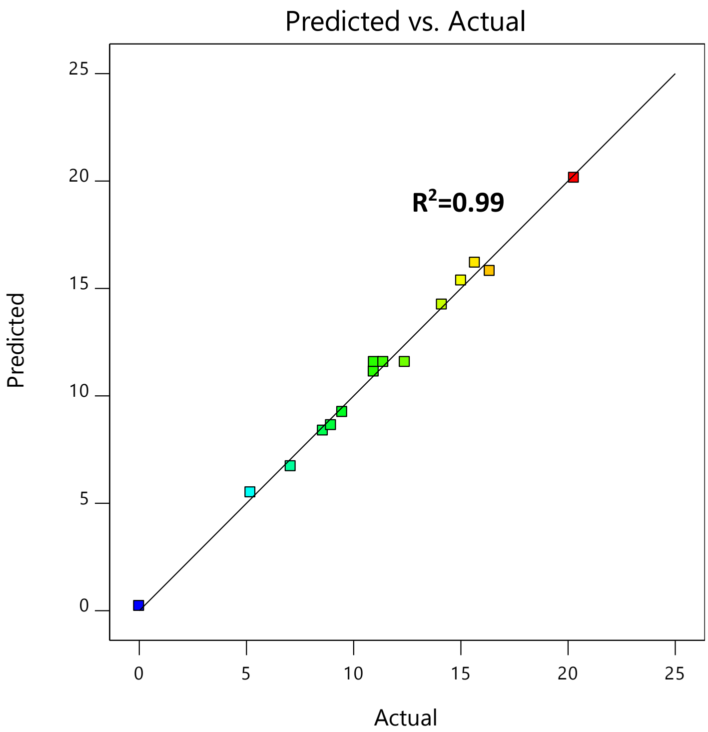

Fig. 3. Relationship between predicted and actual responses of the total PHA accumulation in *C. vulgaris* (note: red and blue coloured points stand for the highest and the lowest contents of PHA respectively)

According to Jung et al., the P value is the likelihood of observing the observed F value if the null hypothesis is true, whereas the model F value is a test for comparing model variance with error variance. Additionally, authors claimed that the model's relevance is expressed by the larger F value. Additionally, the model terms are significant if the P value is less than 0.05. The P value is used to determine the significance of the corresponding coefficient [26].

Among the linear factors, phosphate (B) showed the highest influence on PHA production with the largest F-value (150.20, p <0.05) (Table 6), followed by nitrogen (A) and iron (C). Phosphate plays a crucial role in cellular metabolism as it is involved in ATP generation, nucleic acid synthesis, and energy transfer reactions within microalgal cells. Changes in phosphate availability can therefore significantly influence metabolic pathways related to biomass formation and storage compound accumulation [4].

Nitrogen also exhibited a significant effect on PHA production ($p < 0.05$) as shown in Table 6. Nitrogen availability is known to regulate microalgal growth and carbon metabolism, where nitrogen limitation often triggers the redirection of carbon flux toward intracellular storage compounds such as lipids and PHA. Under nitrogen-deficient conditions, cellular growth slows while photosynthetic carbon fixation may continue, leading to the accumulation of carbon storage polymers [23].

Iron was also identified as a significant factor influencing PHA production ($p < 0.05$) in Table 6. Iron is an essential micronutrient involved in photosynthetic electron transport, respiration, and several enzymatic reactions in microalgae. Variations in iron availability can therefore affect photosynthetic efficiency and metabolic activity, indirectly influencing the synthesis of intracellular storage compounds [27].

The interaction effect between nitrogen and phosphate (AB) and between phosphate and iron (BC) were statistically significant, indicating that the combined effects of these nutrients play an important role in regulating PHA accumulation. This suggests that the balance between macronutrients and micronutrients influences the metabolic pathways responsible for carbon storage in microalgae [4].

The quadratic terms (A^2, B^2, and C^2) were also significant, indicating the presence of curvature in the response surface and confirming that the relationship between nutrient concentrations and PHA production is not purely linear. This behaviour is common in microalgal cultivation systems where both nutrient limitation and nutrient excess can affect metabolic regulation and polymer accumulation [23].

Furthermore, the lack of fit was not significant as shown in Table 6, indicating that the model adequately describes the relationship between the selected variables and the response. A non-significant lack of fit suggests that the model predictions are in good agreement with the experimental data. Table 6 shows the ANOVA for the quadratic model of PHA production.

Table 6
ANOVA for the quadratic model of PHA production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	344.66	9	38.30	82.97	< 0.0001	**significant**
Nitrogen (A)	19.75	1	19.75	42.79	0.0012	
Phosphate (B)	69.33	1	69.33	150.20	< 0.0001	
Iron (C)	15.24	1	15.24	33.01	0.0022	
AB	25.20	1	25.20	54.60	0.0007	
AC	1.43	1	1.43	3.09	0.1389	
BC	15.80	1	15.80	34.23	0.0021	
A^2	9.53	1	9.53	20.65	0.0061	
B^2	130.20	1	130.20	282.10	< 0.0001	
C^2	43.17	1	43.17	93.52	0.0002	
Residual	2.31	5	0.4615			
Lack of Fit	1.22	3	0.4062	0.7460	0.6162	**not significant**
Pure Error	1.09	2	0.5445			
Cor Total	346.97	14				

The interaction effects of nutrient factors on PHA accumulation are illustrated using three-dimensional response surface plots in Fig. 4(a–c). These plots show the combined effects of nitrogen, phosphate, and iron concentrations on PHA accumulation in *C. vulgaris*. As shown in Fig. 4(a), PHA accumulation increased with increasing phosphate concentration at moderate nitrogen levels.

However, further increase in nitrogen concentration resulted in lower PHA accumulation, indicating that excessive nitrogen levels may reduce intracellular polymer accumulation [23].

A similar trend was observed for the interaction between nitrogen and iron in Fig. 4(b), where PHA accumulation increased at moderate nitrogen concentrations but decreased when nitrogen levels became higher. This behavior suggests that nitrogen availability influences the balance between cellular growth and storage compound synthesis [4]. The interaction between phosphate and iron is shown in Fig. 4(c). PHA accumulation increased with increasing phosphate concentration at moderate iron levels but decreased at higher iron concentrations, suggesting that nutrient balance plays an important role in regulating carbon storage metabolism in microalgae [4].

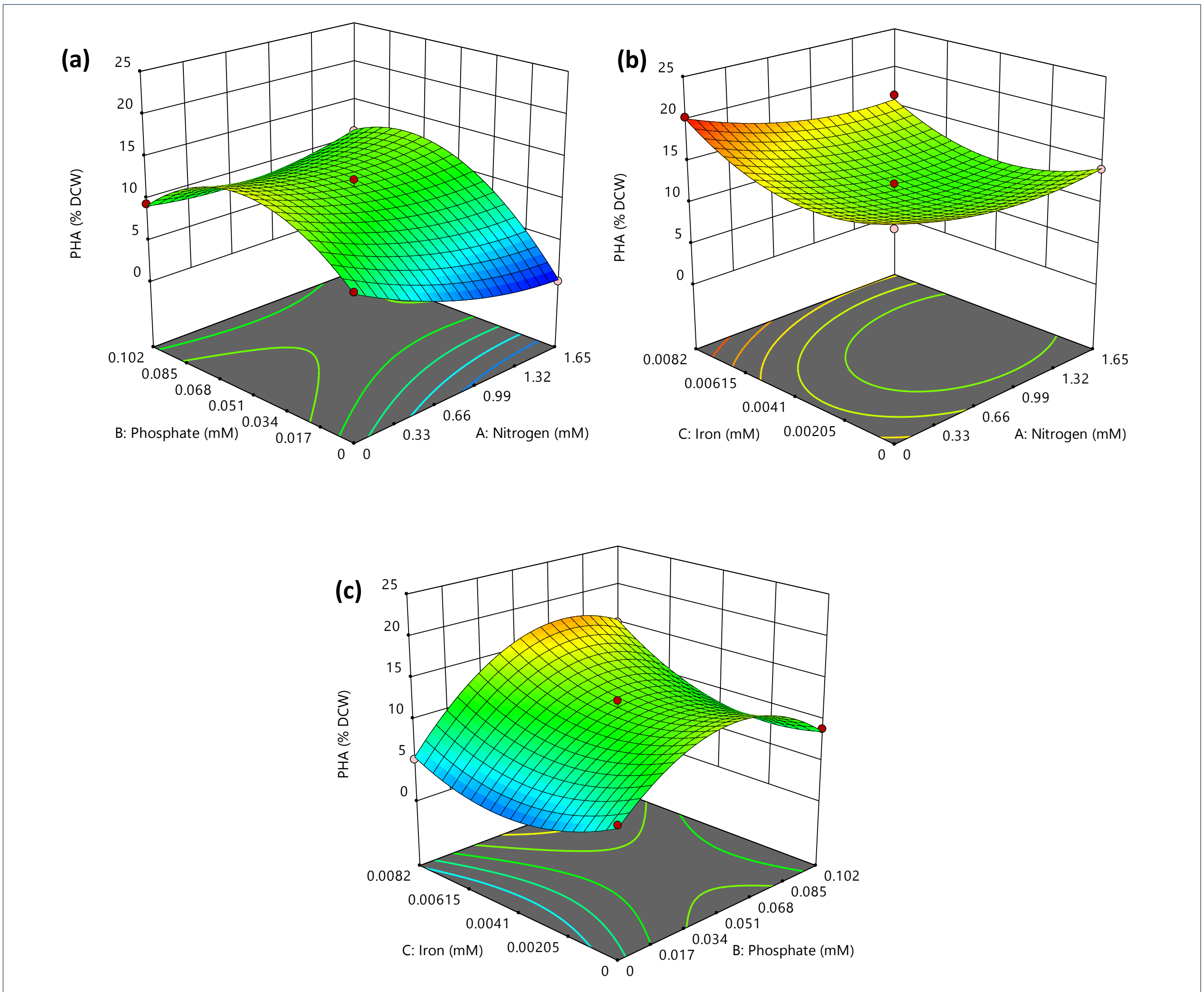

Fig. 4. Three-dimensional (3D) response surface plot showing the effects of all factors on the contents of PHA accumulation: (a) effects of reaction nitrogen and phosphate (AB); (b) effects of reaction nitrogen and iron (AC); and (c) effects of phosphate and iron (BC)

3.4 Optimization analysis

Numerical optimization using RSM was employed to determine the optimum conditions for maximizing PHA accumulation in C. vulgaris based on the developed regression model. The optimization was performed using the numerical optimization function in Design-Expert software (Stat-Ease Inc., USA).

The results suggested that the optimum conditions for PHA production were achieved at a nitrogen concentration of 1.537 mM, phosphate concentration of 0.00941 mM, and iron concentration of 0.00810 mM. Under these conditions, the predicted PHA accumulation was 5.82 %CDW, with an overall desirability value of 1.000, indicating a highly favorable optimization solution within the experimental design space.

However, the experimental results obtained in this study showed that higher PHA accumulation could occur under certain nutrient conditions, particularly under nitrogen-limited environments. Nutrient limitation has been widely reported to trigger the accumulation of intracellular storage compounds in microalgae, as cellular growth becomes restricted while photosynthetic carbon fixation may continue, resulting in the redirection of carbon toward storage metabolites such as PHA [4].

4. Conclusions

In this study, the effects of nitrogen, phosphate, and iron concentrations on PHA production by *Chlorella vulgaris* were successfully evaluated using response surface methodology. The results demonstrated that nutrient availability significantly influenced both microalgal growth and intracellular PHA accumulation. The highest PHA accumulation obtained in the experimental runs was 20.28 %CDW, indicating that *C. vulgaris* has considerable potential for biopolymer production under suitable nutrient conditions (nitrogen 0mM; phosphate 0.051mM; and iron 0.0082mM). The response surface analysis further revealed that the interaction between nutrient factors plays an important role in regulating PHA production. Overall, this study highlights the importance of nutrient optimization in enhancing PHA production and demonstrates the applicability of response surface methodology as an effective tool for optimizing microalgal biopolymer production.

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Conflict of Interest Statement
The authors declare that there is no conflict of interest regarding the publication of this paper. No financial support, grants, or other forms of compensation were received that could have influenced the outcomes of this work. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author Contributions Statement
Author A conducted the experiments and collected the experimental data. Author B supervised the study and critically revised the manuscript. Author C performed the response surface methodology (RSM) analysis and assisted in data interpretation. Author D contributed to the interpretation of PHA polymer data and provided technical expertise on polymer characterization. Author E provided guidance in manuscript preparation an. All authors read and approved the final manuscript.

Data Availability Statement
All data generated or analysed during this study are included in this published article. Additional datasets are available from the corresponding author upon reasonable request. Where applicable, publicly available datasets used in the study are cited in the references

Ethics Statement

This study was conducted in accordance with the ethical standards of the institutional and/or national research committee. Ethical approval was obtained where required, and informed consent was obtained from all participants involved in the research

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