NUSANTARA BIOSCIENCE
Vol. 16, No. 2, pp. 263-276
November 2024

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n160213

Enhancing the production of phycocyanin biopigment from microalga *Arthrospira maxima* through medium manipulation utilizing Box-Behnken Design

MUHAMMAD SYAWALUDDIN HILMI YAHYA[1], MURNI HALIM[1,2], FADZLIE WONG FAIZAL WONG[1,2], HELMI WASOH[1,2], JOO SHUN TAN[3], MOHD SHAMZI MOHAMED[1,2,♥]

[1]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. 43400 UPM Serdang, Selangor, Malaysia. Tel.: +60-9769-4545, Fax.: +60-38946-7510, ♥email: m_shamzi@upm.edu.my

[2]Bioprocessing and Biomanufacturing Research Complex, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. 43400 UPM Serdang, Selangor, Malaysia

[3]School of Industrial Technology, Universiti Sains Malaysia. 11800 Gelugor, Pulau Pinang, Malaysia

Manuscript received: 21 March 2024. Revision accepted: 15 September 2024.

Abstract. *Yahya MSH, Halim M, Wong FWF, Wasoh H, Tan JS, Mohamed MS. 2024. Enhancing the production of phycocyanin biopigment from microalga* Arthrospira maxima *through medium manipulation utilizing Box Behnken Design. Nusantara Bioscience 16: 263-276.* Phycocyanin is among the valuable pigments produced by microalgae *Arthrospira* spp. possessing significant nutritional and coloring properties. It is widely used in food, nutraceutical, and biotechnology applications. Presently, *Arthrospira platensis* is a species very much established for producing phycocyanin commercially. Given the extensive research works and understanding of *A. platensis*, there exists a significant opportunity to explore lesser-studied but potentially valuable strains, such as *A. maxima*, specifically for pigment production capabilities. This study aims to optimize the phycocyanin production from the *A. maxima* by first considering vital media components for phycocyanin secretion by the microalgal cells, namely sodium nitrate, sodium bicarbonate, dipotassium phosphate, sodium chloride and a number of precursors. Upon identifying the most significant factors, their composition in the NRC production medium was manipulated using Response Surface Methodology (RSM). Initial screening using the Plackett-Burman Design revealed two macronutrients and a precursor that significantly affected the target response ($\rho>0.05$): sodium nitrate, dipotassium phosphate and glutamic acid. The three factors were further refined using the Box-Behnken Design (BBD), a variation of the RSM technique. In one BBD run, the highest phycocyanin yield was 224.86 mg/L, achieved using a recipe comprising 0.0125 M sodium nitrate, 0.375 mM dipotassium phosphate and 0.625 mM L-glutamic acid. This resulted in an increase of 37.85% improvement over the basal medium. BBD's validating recipe comprising 0.0125 M sodium nitrate, 0.375 mM dipotassium phosphate and 0.625 mM L-glutamic acid then produced 235.98 g/L of phycocyanin, which in turn has a 44.67% improvement of phycocyanin yield compared with an unoptimized NRC medium. This significant increase in phycocyanin yield demonstrates the potential of this research to enhance phycocyanin production for commercial use and further research. In conclusion, optimizing the composition of a medium can significantly increase phycocyanin production.

Keywords: *Arthrospira maxima*, Box-Behnken Design, medium formulation, phycocyanin, Plackett-Burman Design

INTRODUCTION

Arthrospira spp., a filamentous and multicellular cyanobacterium commonly referred to as blue-green algae, is a member of the Microcoleaceae family. *Arthrospira* spp. typically comprises unbranched filaments of cylindrical cells arranged in a spiral pattern when viewed under a light microscope and thrives in alkaline, brackish, saline waters within tropical and subtropical regions, favored by its optimal growth temperature at 35°C (Furmaniak et al. 2017). The *Arthrospira* genus encompasses more than 30 species, notably including the industrially important *A. platensis* (Fujisawa et al. 2010) with widespread applications in the food, feed and pharmaceutical industries. Recognized as a Generally Recognized As Safe (GRAS) microorganism, *Arthrospira* spp. has been acknowledged for its absence of known toxic effects, a designation endorsed by the FDA and ANISVA (Fleurence and Levine 2018). Notably, *Arthrospira* spp. has a distinctive pigment group called phycobiliproteins.

Phycobiliprotein is a highly fluorescent and brightly colored pigment made up of water-soluble protein and can usually be found in many types of cyanobacteria and red algae. They mainly act as antennae responsible for light harvesting. Owing to its water-soluble characteristic, phycobiliprotein is not located in the same place as other accessory pigments at the thylakoid membrane. Instead, it is usually located at the phycobilisome, with protein microbodies anchored at the thylakoid membrane (Stadnichuk and Tropin 2017). Among the phycobiliproteins derived from microalgae, phycoerythrin (PE) and allophycocyanin (AP) are present in small amounts, while phycocyanin (PC) stands out as the most abundant. PC, characterized by its intense blue, non-toxic, water-soluble pigment-protein complex, possesses great potential across various important applications (Cuellar-Bermudez et al. 2015; Pagels et al. 2019). Functioning as a blue fluorescent protein pigment, PC finds utility in

industries like colorant production, medicine and fluorescent markers. The single visible absorption for this strongly fluorescent pigment is between 615 and 620 nm, while the maximum fluorescence emission is around 650 nm. Notably, PC has a large Stokes shift and high quantum efficiency. Furthermore, the properties of PC, namely antioxidants, brightening, wound healing and antiacne, can be applied in the cosmetic industry (Ragusa et al. 2021). It also serves as a key ingredient in the development of "Lina blue" by Dainippon Ink & Chemicals (Sakura, Japan), widely employed as a colorant in chewing gum, ice sherbets, popsicles, candies, soft drinks, dairy products and wasabi (Roda-serrat et al. 2018). The global market value of phycocyanin nearly reached USD 112.3 million in 2018, with value projections expected to escalate to USD 409.8 million by 2030 (Thevarajah et al. 2022).

Cultivating *Arthrospira* spp. is an essential step in obtaining a variety of biochemical compounds, especially phycocyanin. Culturing conditions, duration of growth cycles, and environmental ability can influence the change in biochemical compound content (Manirafasha et al. 2018). The culturing conditions, particularly the medium composition, wield decisive influence over the growth phases of microalgae, thereby inducing changes in their composition and modulating the proportion of phycobiliproteins, including phycocyanin. Several studies revealed that macronutrients, especially nitrogen, phosphorous and carbon, have a significant impact on growth and biomass accumulation, thereby influencing phycocyanin production (Shanthi et al. 2018; Hao et al. 2019; Mirhosseini et al. 2022; Magwell et al. 2023). Additionally, nitrogen can influence the formation of phycobiliprotein and cell reserve in cyanobacteria (Liotenberg et al. 1996). Besides, salinity and trace metal can negatively impact phycocyanin content if the concentration exceeds the threshold limit (Zhou et al. 2018; Akbarnezhad et al. 2020; Markou et al. 2023). Furthermore, incorporating metabolic stressors into the medium components has demonstrated a potential to optimize phycocyanin production. Manipulating metabolites like Monosodium Glutamate (MSG), aspartate, succinic acid, and glycine significantly boosts phycocyanin yields, with MSG and glycine enhancing production by up to 30% and 22.5%, respectively (Kotinskyi et al. 2018; Manirafasha et al. 2018; Fekrat et al. 2021). These metabolites act as precursors or metabolic enhancers facilitating the biosynthetic pathways involved in phycocyanin production. Heme synthesis begins with the formation of the core molecule, aminolaevulinic acid (ALA), using either glutamate or succinyl coenzyme A as an immediate precursor, while heme itself is a precursor for phycocyanin (Manirafasha et al. 2016).

Design of Experiments (DOE) is a systematic approach using statistical methods to explore relationships between manipulated and response variables, incorporating designs such as Plackett-Burman and Box-Behnken (Jankovic et al. 2021; Lee et al. 2022). The Plackett-Burman Design (PBD) employs an orthogonal array to screen and identify the significant factors affecting response variables. PBD is considered to be a very rugged test featuring $4 \cdot n$ number of experiment runs, where n is an integer and in each case, the maximum number of factors that can be studied is $4 \cdot n - 1$ (Das and Dewanjee 2018; Jankovic et al. 2021). On the other hand, the Box-Behnken Design (BBD), a component of Response Surface Methodology (RSM), optimizes conditions and examines variable interaction with efficiency and precision, albeit this can be obtained with lesser run number compared to other RSM composite designs (Gujral et al. 2018; Ait-Amir et al. 2020).

This study aims to enhance phycocyanin production from *Arthrospira maxima* through the innovative screening of medium components and precursors using a statistical design-of-experiment technique. Unlike previous research, this study employs a comprehensive approach to identify and optimize the critical factors influencing phycocyanin production. The novelty lies in the systematic application of the PBD to identify vital factors, followed by fine-tuning using the BBD design for optimization. This methodological advancement offers a more precise and efficient pathway to maximizing phycocyanin yields. The integration of metabolic stressors and innovative medium compositions highlights the unique approach of this study, paving the way for enhanced industrial applications of phycocyanin.

MATERIALS AND METHODS

The overall experimental design flow is outlined in Figure 1, which presents a step-by-step illustration of the methodological framework used in this study. The figure begins with inoculum preparation, followed by screening the significant macronutrients using Plackett-Burman Design, a steepest ascent experiment for finding coarse optimal region, Box-Behnken optimization, and concludes with a validation run. Each step highlights key processes and critical factors that contribute to the optimization of phycocyanin and biomass yield of *A. maxima*.

Cultivation of *Arthrospira maxima*

The *A. maxima* used in this study were sourced from the International Institute of Aquaculture and Aquatic Sciences (I-AQUAS), Universiti Putra Malaysia, Port Dickson, located in the state of Negeri Sembilan, Malaysia, and maintained in Zarrouk's medium (Zarrouk 1966) at pH 9, with slight modifications to the sodium chloride and sodium bicarbonate content. The composition of Zarrouk's medium (gram) per litres included: 16.8 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 23.0 g NaCl, 1.0 g K_2SO_4, 0.2 g $MgSO_4.7H_2O$, 0.08 g NaEDTA, 0.04 g $CaCl.2H_2O$, 0.01 g $FeSO_4.7H_2O$ and 1 mL trace elements solution containing of 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.222 g $ZnSO_4.7H_2O$, 0.39 g $Na_2MoO_4.2H_2O$, 0.079 g $CuSO_4.5H_2O$, 0.049 g $Co(NO_3)_2.6H_2O$. The culture was incubated at room temperature (26±2°C) under 16:8-hour light : dark cycles for 21 days, with manual agitation by hand shaking performed two or three times a day.

Screening of macronutrients and precursors

Nine variables were selected to ascertain the significant factor affecting phycocyanin production by *A. maxima*. They comprised six medium components (sodium nitrate, sodium bicarbonate, dipotassium phosphate, sodium chloride, potassium sulphate, and magnesium sulphate) alongside three precursors acting as metabolic stress (glutamic acid, succinic acid, and glycine). The screening was conducted to evaluate the significance of various nutrients on the biomass and phycocyanin yield of *A. maxima* using Nallayam Research Centre Media (NRCM) at pH 9, developed by the Nallayam Research Centre for cultivating Arthrospira species. (AlFadhly et al. 2022). This study utilized the 14-run Plackett-Burman Design for preliminary screening to identify key factors influencing phycocyanin formation among the nine factors.

Within the Plackett-Burman Design, each variable was assigned -1 for denoting the low level and +1 for the high level. A center point indicated as 0 was also incorporated to estimate for pure error and curvature. Table 1 shows the variable levels for each factor. The response functions under scrutiny included the dried cell weight of *A. maxima* (g/L) and the phycocyanin yield (mg/L). Design matrices for the screening experiment using the Placket-Burman design were generated from Design Expert version 11 (Stat-Ease Inc, USA). The outcomes derived from the Plackett-Burman Design were fitted to a first-order model represented by Equation 1. Subsequently, an Analysis of Variance (ANOVA) was applied to assess the significance of the fitted model for each response. Following the identification of significant factors, they were earmarked for further optimization.

$$Y = B_o + \sum_{i=1}^{n} B_i X_i$$

where Y signifies the predicted response, B_0 represents the model constant, X_i denotes the independent variable, and B_i illustrates the variable's linear coefficient.

Steepest ascent

The steepest ascent method, on the other hand, follows suit by attempting to guide the variables along the trajectory toward the optimal region, either by increasing or decreasing their effects. In this experiment, three factors (sodium nitrate, dipotassium phosphate, and glutamic acid) with the direction of perceived increasing/decreasing levels obtained from prior Plackett-Burman Design were further refined by varying their concentration. The phycocyanin yield and biomass of *A. maxima* gained at 21 days were compared for each run. Table 2 displays the design experiment of the steepest ascents.

Optimization using box Behnken Design

Based on the rough estimate of the possible optimal region of the three significant factors contributing to the maximum phycocyanin yield uncovered from the steepest ascent method, design levels were further fine-tuned with the Box-Behnken Design. The Box-Behnken Design also contains three levels: -1 denotes the low level, 0 for the center point and +1 for the high level. For this stage, 17 runs were generated by Box-Behnken Design using the design experiment software Design Expert version 11 (Stat-Ease Inc, USA). Table 3 illustrates the design matrix for each factor in the Box-Behnken Design. The obtained results following the cultivation experiments would be usually fitted to the second polynomial equation as follows:

$$Y = \alpha_0 + \beta_1 A + \beta_2 B + \beta_3 C + \gamma_1 AB + \gamma_2 AC + \gamma_3 BC + \omega_1 A^2 + \omega_2 B^2 + \omega_3 C^2$$

Where Y is the predicted response, α_0 is constant, β1, β2, and β3 are the linear coefficients, γ1, γ2, and γ3 represent the interaction coefficient between variables, ω1, ω2, and ω3 are the quadratic coefficients, whereas A, B, and *C* are the symbols for variables. ANOVA was used to analyze the statistically significant model, while contour and response surface plots were used to determine the interaction between variables.

Model validation run

The optimal medium formulation, as forecasted by the Box-Behnken Design, was validated in triplicate experiments. Here, *A. maxima* was cultivated in a modified NRC medium with three additional precursors (glutamic acid, succinic acid and glycine). The composition of optimum medium (gram) per liter contained the following components: 10.5 g $NaHCO_3$, 1.06 g $NaNO_3$, 0.065 g K_2HPO_4, 7.31 g NaCl, 0.523 g K_2SO_4, 1.11 g $MgSO_4.7H_2O$, 0.11 g L-glutamic acid, 0.531 g succinic acid, 0.075 g glycine and 0.01 g $FeSO_4.7H_2O$. The growth condition was retained by using a 500 mL conical flask at room temperature (26±2°C) under 16:8-hour light: dark cycles for 21 days. The culture was manually shaken two or three times a day. The final actual biomass and phycocyanin concentration responses were then compared with the regression model's predicted response.

Table 1. Assigned levels for each factor in the Plackett-Burman Design

Factors	Symbol	Actual level of coded factors	
		-1 (M)	+1(M)
Sodium bicarbonate	A	0.05	0.2
Sodium nitrate	B	0.02	0.05
Sodium chloride	C	0.05	0.2
Dipotassium phosphate	D	0.001	0.01
Potassium sulphate	E	0.00	0.006
Magnesium sulphate	F	0.001	0.008
Glutamic acid	G	0.001	0.005
Glycine	H	0.001	0.003
Succinic acid	I	0.001	0.008

Table 2. Experimental design for the steepest ascent to uncover optimal region of phycocyanin production

Run	Factors		
	Sodium nitrate (M)	Dipotassium phosphate (mM)	L-glutamic acid (mM)
1	0.030	2.00	2.00
2	0.025	1.50	1.50
3	0.020	1.00	1.00
4	0.015	0.75	0.75
5	0.010	0.50	0.50
6	0.005	0.25	0.25

Table 3. Assigned levels for each factor in Box-Behnken Design

Factors	Actual level of coded factors		
	-1 (mM)	0 (mM)	+1 (mM)
Sodium nitrate	7.5	10	12.5
Dipotassium phosphate	0.375	0.5	0.625
L-glutamic acid	0.375	0.5	0.625

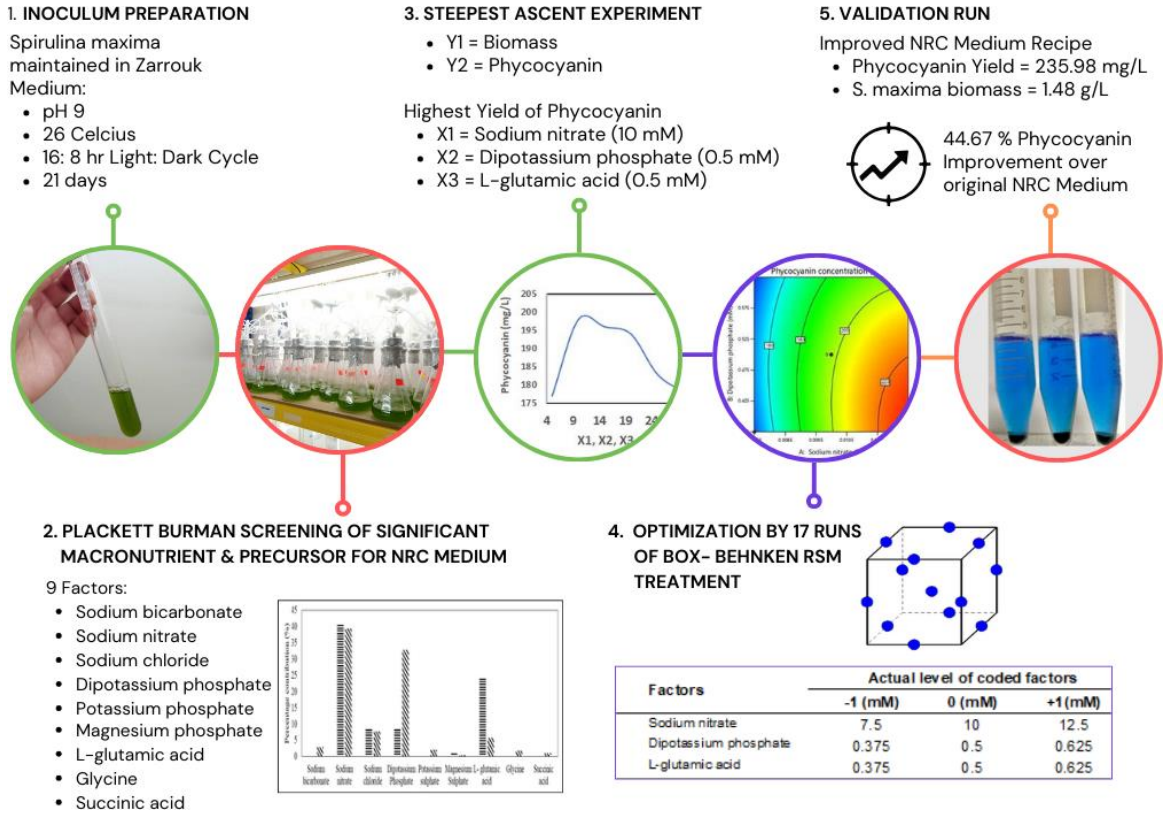

Figure 1. Overview of experimental design

Biomass density

Cell density was determined by the gravimetric method involving repeated weighing and oven drying of filter paper that entrapped the microalgal biomass until constant weight was achieved (Moheimani et al. 2013). Specifically, a 10 mL sample of *A. maxima* was filtered using pre-weighed glass microfiber filter paper with the aid of a vacuum pump. The filter paper containing *A. maxima* cells was then dried for 48 hours in an oven at 70°C until it reached a constant weight. The filter was weighed using an electronic balance (Ohaus PA413, China).

Extraction and detection of phycocyanin concentration

The extraction of phycocyanin was based on a particular method with a slight modification (Moraes et al. 2011). Next, 10 mL of samples were centrifuged at 3,500 RPM for 15 minutes (Kubota 2010, Japan) to separate the supernatant (spent medium) and settled pellet (microalgal cells). The pellet was washed with distilled water to remove any excess medium and dissolved salt. Then, 1 mL of 0.1 M phosphate buffer containing 0.1 M EDTA was added and left overnight at 5 ºC in the freezer. After that, 2 mL 0.1 M of phosphate buffer containing 0.1 M EDTA and 100 µg/mL of lysozyme was added, and the sample was left for four days at room temperature. The solution was measured using a spectrophotometer at 620 nm and 652 nm. The estimated concentration of phycocyanin was calculated based on the following equation:

$$\text{Phycocyanin (mg/L)} = \frac{OD_{620} - 0.474\,OD_{650}\,x1000}{5.34}\ x\ \frac{V_{ext}}{V_{cul}}$$

Where V_{ext} and V_{cul} denote the volume of extraction and volume of culture, respectively.

RESULTS AND DISCUSSION

Screening of macronutrients and precursors via Plackett-Burman Design

Screening plays an important role in any optimization process, which is primarily aimed at identifying significant factors influencing the response while discarding variables that prove to be trivial. This study employed a screening approach utilizing the Plackett-Burman Design (PBD) to pinpoint the key variables that create favorable conditions for enhancing phycocyanin production in *A. maxima*. The PBD was chosen owing to its saturated design, which led to a simple and reduced run (Lee et al. 2022). From the nine variables considered, six were typically found as part of media components: A) Sodium bicarbonate, B) Sodium nitrate, C) Sodium chloride, D) dipotassium phosphate, E) Potassium sulphate, and F) Magnesium sulphate, while the other three variables were those presenting metabolic stress affecting phycocyanin production: G) L-glutamic acid, H) Glycine and I) Succinic acid. Table 4 shows the PBD matrix comprising 14 experimental runs, including two added center points (Run 13 and 14), with the corresponding resulting biomass and phycocyanin yields meticulously documented.

From Table 4, the biomass of *A. maxima* for each run ranges from 0.55 g/L to 1.10 g/L, with the highest observed in Run 5 (1.10 g/L), followed by Run 12 (1.0 g/L) and Run 11 (0.95 g/L). The highest biomass in Run 5 is a notable improvement compared to the original NRC media, which yielded biomass levels between 0.5 g/L and 0.7 g/L, as reported by AlFadhly et al. (2022). A study by de Castro et al. (2015) also demonstrated that manipulating sodium bicarbonate, sodium nitrate, and irradiance using a central composite rotational design can achieve biomass levels ranging from 1.0 g/L to 3.2 g/L. Meanwhile, the highest phycocyanin concentrations were observed in Run 12 (160.3 ± 10.8 mg/L), Run 7 (105.4 ± 4.33 mg/L), and Run 5 (99.1 ± 4.71 mg/L). The phycocyanin concentration obtained in Run 12 exceeds the original NRC media, which yielded around 50 mg/g (AlFadhly et al. 2022). Furthermore, the study by Manirafasha et al. (2018) showed that adding a precursor can increase phycocyanin concentration by 13% to 24%.

Table 4. Plackett-Burman Design matrix of nine variables with observed and predicted biomass and phycocyanin yield by *Arthrospira maxima*

Run	A	B	C	D	E	F	G	H	I	Biomass (g/L)		Phycocyanin @ Day 21 (mg/L)	
										Obs	Pred	Obs	Pred
1	+1 (0.2)	+1 (0.05)	-1 (0.05)	+1 (0.01)	+1 (0.006)	+1 (0.008)	-1 (0.001)	-1 (0.001)	-1 (0.001)	0.65±0.05	0.71	29.8±0.61	44.9
2	-1 (0.05)	+1 (0.05)	+1 (0.2)	-1 (0.001)	+1 (0.006)	+1 (0.008)	+1 (0.005)	-1 (0.001)	-1 (0.001)	0.70±0.12	0.64	74.8±2.02	49.2
3	+1 (0.2)	-1 (0.02)	+1 (0.2)	+1 (0.01)	-1 (0)	+1 (0.008)	+1 (0.005)	+1 (0.003)	-1 (0.001)	0.85±0.1	0.76	51.9±2.21	53.6
4	-1 (0.05)	+1 (0.05)	-1 (0.05)	+1 (0.01)	+1 (0.006)	-1 (0.001)	+1 (0.005)	+1 (0.003)	+1 (0.008)	0.55±0.03	0.54	13.0±3.82	25.4
5	-1 (0.05)	-1 (0.02)	+1 (0.2)	-1 (0.001)	+1 (0.006)	+1 (0.008)	-1 (0.001)	+1 (0.003)	+1 (0.008)	1.10±0.14	1.03	99.1±4.71	119.5
6	-1 (0.05)	-1 (0.02)	-1 (0.05)	+1 (0.01)	-1 (0)	+1 (0.008)	+1 (0.005)	-1 (0.001)	+1 (0.008)	0.65±0.06	0.76	92.5±2.52	76.3
7	+1 (0.2)	-1 (0.02)	- (0.05)	-1 (0.001)	+1 (0.006)	-1 (0.001)	+1 (0.005)	+1 (0.003)	-1 (0.001)	0.70±0	0.86	105.4±4.33	122.6
8	+1 (0.2)	+1 (0.05)	-1 (0.05)	-1 (0.001)	-1 (0)	+1 (0.008)	-1 (0.001)	+1 (0.003)	+1 (0.008)	0.75±0.08	0.81	95.6±2.96	91.3
9	+1 (0.2)	+1 (0.05)	+1 (0.2)	-1 (0.001)	-1 (0)	-1 (0.001)	+1 (0.005)	-1 (0.001)	+1 (0.008)	0.65±0.09	0.64	32.6±3.85	49.2
10	-1 (0.05)	+1 (0.05)	+1 (0.2)	+1 (0.01)	-1 (0)	-1 (0.001)	-1 (0.001)	+1 (0.003)	-1 (0.001)	0.65±0.03	0.71	30.6±4.72	22.3
11	+1 (0.2)	-1 (0.02)	+1 (0.2)	+1 (0.01)	+1 (0.006)	-1 (0.001)	-1 (0.001)	-1 (0.001)	+1 (0.008)	0.95±0.05	0.93	71.9±1.93	73.2
12	-1 (0.05)	-1 (0.02)	-1 (0.05)	-1 (0.001)	-1 (0)	-1 (0.001)	-1 (0.001)	-1 (0.001)	-1 (0.001)	1.00±0.06	1.03	160.3±10.8	142.1
13	0 (0.125)	0 (0.035)	0 (0.125)	0 (0.0055)	0 (0.003)	0 (0.0045)	0 (0.003)	0 (0.002)	0 (0.0045)	0.90±0.13	0.77	80.9±9.21	72.5
14	0 (0.125)	0 (0.035)	0 (0.125)	0 (0.0055)	0 (0.003)	0 (0.0045)	0 (0.003)	0 (0.002)	0 (0.0045)	0.90±0.09	0.77	76.1±5.51	72.5

Note: Medium constituents expressed in unit molar (M): (A) Sodium bicarbonate; B. Sodium nitrate; C. Sodium chloride; D. dipotassium phosphate; E. Potassium sulphate; F. Magnesium sulphate; G. L-glutamic acid; H. Glycine and I. Succinic acid. Only real variables are shown; dummy variables in PBD were excluded

The full regression model ANOVA results of each factor affecting dried biomass, as well as phycocyanin yield in *A. maxima*, are recorded in Table 5. In the case of biomass, this table unequivocally illustrates that only sodium nitrate was deemed a significant medium factor where a crucial criterion necessitated a p-value of less than 0.05 ($p<0.05$) for significance. Notwithstanding, the entire model for biomass was considered insignificant, as indicated by a p-value of 0.2251. Meanwhile, in the case of variables influencing phycocyanin, the whole model, in turn, demonstrated a statistical significance, as indicated by a p-value of less than 0.05 (0.0389). Within this model, two variables stood out as statistically significant: sodium nitrate (0.0069) and dipotassium phosphate (0.0095), highlighting their pronounced influence on phycocyanin yield. However, it is important to note that despite the model's overall significance in terms of p-value and a favorable R^2 value (0.9398), the predicted R^2 (pred-R^2) value was shown as negative (-0.7596). This negative value could signify an overfitting of the model, suggesting that it may struggle to predict outcomes for new observations effectively. Thus, the backward elimination technique was systematically employed to address this issue. The backward elimination technique begins by selecting all variables and removing those with the highest insignificance. This technique was repeated until updated ANOVA produced an insignificant lack of fit, as well as the model p-value turned significant and improved the predicted R^2.

Employing the backward elimination technique resulted in revised regression models, with the new ANOVA outlined in Table 6 for both experimental responses. The updated table for dry biomass reflected the model's newfound significance, characterized by a p-value of 0.0022 with a 0.22% likelihood that the F-value (10.15) was attributable to random noise. Notably, two variables now emerged as statistically significant: A) sodium nitrate ($p=0.015$) and G) L-glutamic acid ($p=0.073$), signifying their notable influence on the dried cell weight of *A. maxima*. Besides, this model has an R^2 of 0.8186, which indicates its ability to explain around 81.86% of its variability. On the other hand, the model for phycocyanin yield has a significant p-value (0.0007) and a 0.07% chance that the F-value (13.58) occurs due to noise. This model also retained two significant variables: sodium nitrate (0.0007) and dipotassium phosphate (0.0014). Furthermore, this model has 0.8579 of R^2, which indicates that this model can explain 85.79% of the variation. In addition, the revision also has a well-adjusted R^2 (0.7947) and predicted R^2 (0.6059). The ANOVA result generated a first-order equation in terms of coded variables shown in the following equations for modelling biomass and phycocyanin yield.

$$Y_{biomass} = +1.14 - 7.22 * B + 0.67 * C - 11.11 * D - 41.67 * G$$

$$Y_{phycocyanin} = + 193.53 - 1693.65 * B - 150.7 * C - 5149.43 * D - 4876.14 * G$$

In the screening step, significant variables were determined based on statistically significant ($p\text{-value}<0.05$) and percentage contribution. The percentage contribution for each variable is tabulated in Figure 2. Four variables, sodium nitrate, sodium chloride, dipotassium phosphate, and L-glutamic acid, were found to have the potential to impact the models significantly. In contrast, other variables were disregarded due to their negligible influence on enhancing the regression models and their minimal contributions. The first variable chosen was sodium nitrate, which has a significant p-value (0.0015 and 0.0007) and high percentage contribution, around 37% to 40% for biomass and phycocyanin yield, respectively. Both models described that lower concentrations of sodium nitrate can produce high biomass and phycocyanin yields. Next, to be included was sodium chloride, which has almost the same percentage contribution of biomass and phycocyanin, around 8.6% concentration, but was statistically barely insignificant in both models.

Additionally, dipotassium phosphate exhibited a substantial percentage contribution, contributing significantly, particularly in phycocyanin yield (31.4%) and moderately for biomass (8.64%). However, this variable was only statistically significant to the phycocyanin yield model. Notably, a reduction in the concentration of dipotassium phosphate appeared to enhance biomass and phycocyanin yield. The final factor selected was that of L-glutamic acid which contributed a high percentage in biomass (24.05%) but was moderate in phycocyanin yield (5.79%). The model showed that low-concentration L-glutamic acid increased phycocyanin yield and biomass. As for the ANOVA table, this variable was only significant in the biomass model since it registered a p-value of 0.0874 for phycocyanin production.

To summarise the findings, three out of nine factors were chosen for the following Box-Behnken optimization: sodium nitrate, dipotassium phosphate, and L-glutamic acid. This decision was driven by their high to moderate percentage contributions in both models and their statistical significance in at least one of the ANOVA models.

Approaching the coarse optimal region via the steepest ascent experiment

The three NRC medium constituents that were deemed significant from the Plackett-Burman (PBD) exercise would first be subjected to the steepest ascent experiment. This experiment aimed to pinpoint the near-optimal concentration region for each variable. Based on the factors effect graph (Figure 3), the trending profile indicated that all variables should be decreased in concentration, aligning perfectly with the design of the steepest ascent experiment. This process was done until no further improvement was met. The obtained optimum concentration would then serve as the center point for a more fine-tuned optimization utilizing Box-Behnken Design (BBD). The result of this experiment is tabulated in Table 7. Upon inspection, the result showed that Run 5 has the highest phycocyanin yield (198.062±7.874 mg/L). The biomass yield remained relatively consistent across all runs, hovering around 0.9 g/L to 1.0 g/L. Consequently, Run 5 was chosen as the center point for BBD.

Table 5. ANOVA of the full regression model for A. Biomass density and B. Phycocyanin yield by *Arthrospira maxima*

Sources	Sum of Squares	df	Mean Square	F-value	p-value
Model A	0.2900	9	0.0322	2.26	0.2251
A-Sodium bicarbonate	0.0008	1	0.0008	0.0583	0.8210
B-Sodium nitrate	0.1408	1	0.1408	9.86	0.0349
C-Sodium chloride	0.0300	1	0.0300	2.10	0.2209
D-Dipotassium Phosphate	0.0300	1	0.0300	2.10	0.2209
E-Potassium sulphate	0.0008	1	0.0008	0.0583	0.8210
F-Magnesium Sulphate	0.0033	1	0.0033	0.2333	0.6543
G-L-glutamic acid	0.0833	1	0.0833	5.83	0.0731
H- Glycine	0.0000	1	0.0000	0.0000	1.0000
J- Succinic acid	0.0008	1	0.0008	0.0583	0.8210
Residual	0.0571	4	0.0143		
Lack of Fit	0.0571	3	0.0190		
Pure Error	0.0000	1	0.0000		
Cor Total	0.3471	13			
Model Summary: R^2 = 0.8354; C.V.% = 15.21; Adj-R^2 =0.465; Pred-R^2 = -1.8896					
Model B	18473.11	9	2052.57	6.94	0.0389
A-Sodium bicarbonate	573.39	1	573.39	1.94	0.2362
B-Sodium nitrate	7744.82	1	7744.82	26.19	0.0069
C-Sodium chloride	1532.87	1	1532.87	5.18	0.0851
D-Dipotassium phosphate	6443.53	1	6443.53	21.79	0.0095
E-Potassium sulphate	401.98	1	401.98	1.36	0.3084
F-Magnesium Sulphate	74.56	1	74.56	0.2521	0.6420
G-L-glutamic acid	1141.28	1	1141.28	3.86	0.1209
H- Glycine	367.13	1	367.13	1.24	0.3276
J- Succinic acid	193.54	1	193.54	0.6544	0.4639
Residual	1182.97	4	295.74		
Lack of Fit	1171.92	3	390.64	35.35	0.1229
Pure Error	11.05	1	11.05		
Cor Total	19656.08	13			
Model Summary: R^2 = 0.9398; C.V.% = 23.73; Adj-R^2 =0.8044; Pred-R^2 = -0.7596					

Table 6. ANOVA of a reduced regression model for A. Biomass density and B. Phycocyanin yield by *Arthrospira maxima*

Sources	Sum of Squares	df	Mean Square	F-value	p-value
Model A	0.2842	4	0.0710	10.15	0.0022
B-Sodium nitrate	0.1408	1	0.1408	20.13	0.0015
C-Sodium chloride	0.0300	1	0.0300	4.29	0.0683
D-Dipotassium phosphate	0.0300	1	0.0300	4.29	0.0683
G-L-glutamic acid	0.0833	1	0.0833	11.91	0.0073
Residual	0.0630	9	0.0070		
Lack of Fit	0.0630	8	0.0079		
Pure Error	0.0000	1	0.0000		
Cor Total	0.3471	13			
Model Summary: R^2 = 0.8186; C.V.% = 10.65; Adj-R^2 =0.7380; Pred-R^2 = 0.6131					
Model B	16862.51	4	4215.63	13.58	0.0007
B-Sodium nitrate	7744.82	1	7744.82	24.95	0.0007
C-Sodium chloride	1532.87	1	1532.87	4.94	0.0534
D-Dipotassium phosphate	6443.53	1	6443.53	20.76	0.0014
G-L-glutamic acid	1141.28	1	1141.28	3.68	0.0874
Residual	2793.57	9	310.40		
Lack of Fit	2782.52	8	347.81	31.47	0.1370
Pure Error	11.05	1	11.05		
Cor Total	19656.08	13			
Model Summary: R^2 = 0.8579; C.V.% = 24.31; Adj-R^2 =0.7947; Pred-R^2 = 0.6059					

Table 7. Steepest ascent experimental design with recorded biomass density and phycocyanin yield

Run	Variable level			Final biomass density (g/L)	Phycocyanin yield (mg/L)
	Sodium nitrate (mM)	Dipotassium phosphate (mM)	L-glutamic acid (mM)		
1	30	2.0	2.0	1.05	178.927 ± 4.683
2	25	1.5	1.5	0.9	183.798 ± 0.931
3	20	1.0	1.0	0.95	193.872 ± 0.391
4	15	0.75	0.75	0.9	195.962 ± 0.27
5	10	0.5	0.5	1.0	198.062 ± 7.874
6	5	0.25	0.25	1.0	77.125 ± 1.183

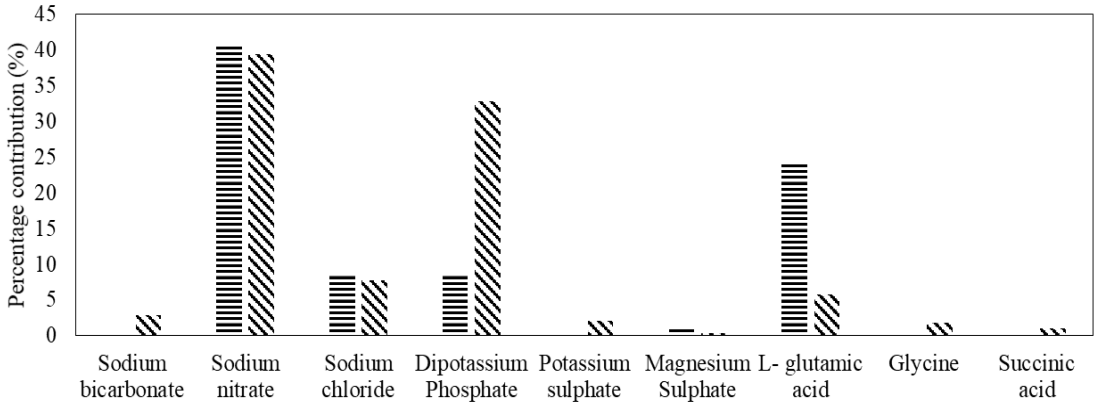

Figure 2. Percentage contribution for each medium variable for (▤) biomass and (▨) phycocyanin yield

Optimization of medium components and precursors using Box-Behnken Design

Table 8 documents the 17 runs from Box Behnken Design; the midpoint values used in this BBD were based on Run 5, as previously determined through the steepest ascent experiment. Meanwhile, the -1 and +1 BBD levels were subsequently adjusted accordingly by the Design Expert software, as shown in Table 3. Table 8 indicates the updated BBD's design matrix, including the observed and predicted biomass and phycocyanin yield.

From this dataset, the fitness to a second-order polynomial by Design Expert software resulted in two sets of second-order polynomial equations, as expressed in terms of coded value for biomass density and phycocyanin yield as follows.

$$\text{Biomass (g/L)} = 11.08 - 1031*A - 10.22*B - 8.92*C + 320*AB + 320*AC + 2.67*10^{-15}*BC + 36800*A^2 + 6.72*B^2 + 5.12*C^2$$

$$\text{Phycocyanin yield (mg/L)} = -16.57 + 48213.69*A + 478.84*B - 784.01*C - 22675.12*AB + 38342.17*AC + 247.88*BC - 2.09*10^6*A^2 - 445.05*B^2 + 259.17*C^2 \quad \text{Equation (7)}$$

In these equations, A represents sodium nitrate, B signifies dipotassium phosphate, and C stands for L-glutamic acid. The first equation illustratred that the interaction terms AB and AC, alongside all quadratic terms, contributed synergistically to biomass accumulation. Conversely, the linear terms in A, B, and C negatively influenced biomass accumulation. Non-existent BC's interaction term can be translated to the term that does not exhibit any discernible positive or negative effect on biomass accumulation. In the next equation describing phycocyanin yield, antagonistic effects are evident in the linear term of C, with a coefficient of -784.01, the interaction term AB (-22,675.12), and the quadratic terms A^2 ($-2.09*10^6$) and B^2 (-445.05). Conversely, other terms such as the linear terms of A (48,213.69) and B (478.84), as well as the interaction terms AC (38,342.17) and BC (247.88), demonstrated a synergistic effect on phycocyanin yield.

Graphical explanations of the mutual effect between interacting terms are represented by the contour plots and response surface plots shown in Figure 4, which first display the terms that influence biomass. Subsequent Figure 5 shows the terms affecting phycocyanin yield. All figures describing biomass interestingly indicated that an inverted relationship existed between factors and biomass density, with the optimal region lying somewhere at the corners of the surface plot, particularly highlighted in Figures 4.A and B with yellow to reddish heatmap. On the other hand, figures describing the phycocyanin yield eventually revealed the interaction that existed between terms with the projection of elliptical contours, with only Figure 4.C showing a somewhat nearly flat surface with no discernible optimum.

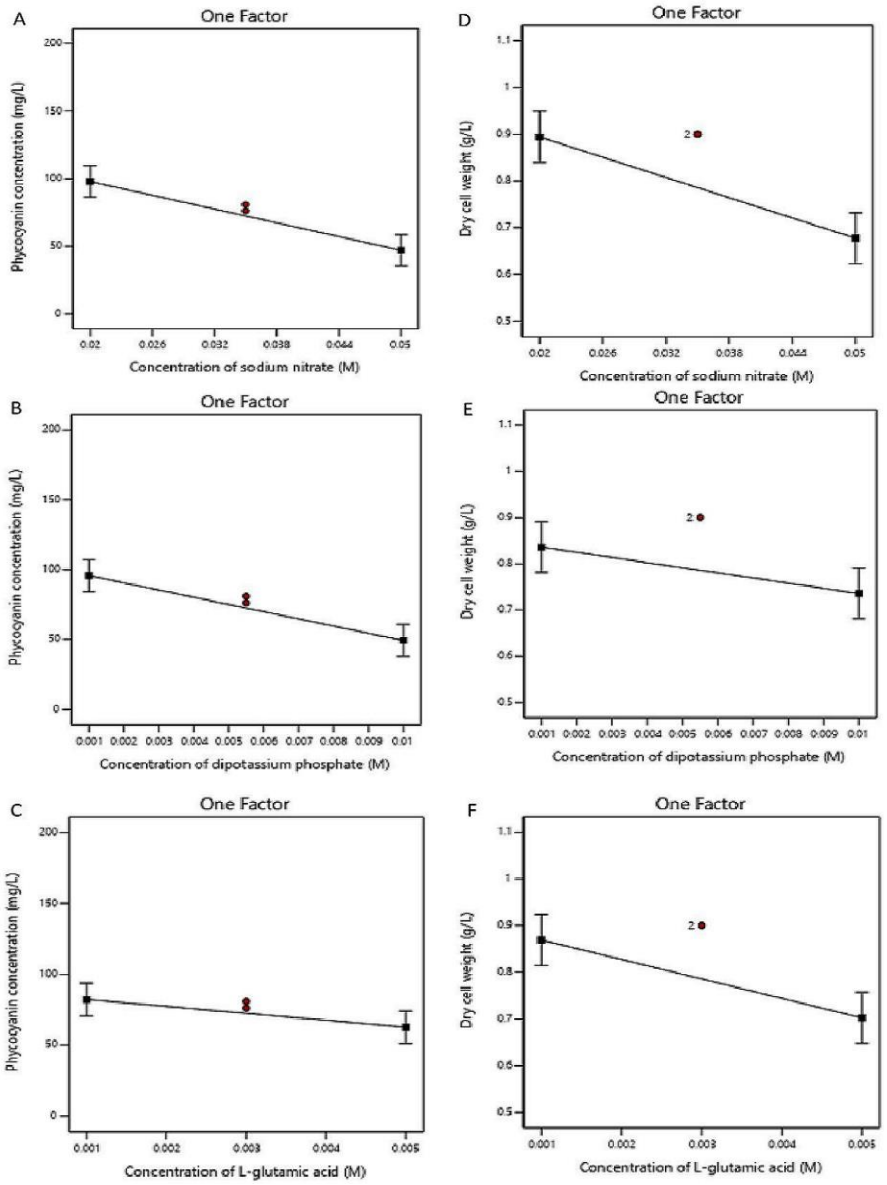

Figure 3. One-factor effect graph on A-C. The *A. maxima* phycocyanin yield and D-F. Biomass density

Table 8. BBD design matrix with observed and predicted values of biomass density and phycocyanin yield by *Arthrospira maxima*

Run	A: $NaNO_3$ (M)	B: K_2HPO_4 (mM)	C: L-Glu (mM)	Biomass (g/L)		Phycocyanin Yield (mg/L)	
				Obs	Pred	Obs	Pred
1	-1 (0.0075)	-1 (0.375)	0 (0.5)	1.45±0.1	1.44	148.81±3.87	150.85
2	+1 (0.0125)	-1 (0.375)	0 (0.5)	1.35±0.09	1.40	224.86±7.4	220.34
3	-1 (0.0075)	+1 (0.625)	0 (0.5)	1.20±0.14	1.15	138.00±1.7	142.53
4	+1 (0.0125)	+1 (0.625)	0 (0.5)	1.55±0.13	1.56	200.65±11.84	198.61
5	-1 (0.0075)	0 (0.5)	-1 (0.375)	1.45±0.06	1.46	169.87±3.4	163.36
6	+1 (0.0125)	0 (0.5)	-1 (0.375)	1.40±0.08	1.34	206.85±3.93	206.90
7	-1 (0.0075)	0 (0.5)	+1 (0.625)	0.90±0.12	0.956	139.23±4.49	139.17
8	+1 (0.0125)	0 (0.5)	+1 (0.625)	1.45±0.13	1.44	214.69±13.42	221.20
9	0 (0.01)	-1 (0.375)	-1 (0.375)	1.35±0.13	1.36	201.09±8.14	205.55
10	0 (0.01)	+1 (0.625)	-1 (0.375)	1.25±0.11	1.29	172.81±3.73	174.80
11	0 (0.01)	-1 (0.375)	+1 (0.625)	1.20±0.11	1.16	186.86±4.53	184.87
12	0 (0.01)	+1 (0.625)	+1 (0.625)	1.10±0.12	1.09	190.05±3.89	185.58
13	0 (0.01)	0 (0.5)	0 (0.5)	1.10±0.17	1.04	197.63±3.17	197.14
14	0 (0.01)	0 (0.5)	0 (0.5)	1.00±0.1	1.04	203.68±0.88	197.14
15	0 (0.01)	0 (0.5)	0 (0.5)	1.00±0.1	1.04	194.28±7.22	197.14
16	0 (0.01)	0 (0.5)	0 (0.5)	1.10±0.2	1.04	196.79±1.99	197.14
17	0 (0.01)	0 (0.5)	0 (0.5)	1.00±0.26	1.04	193.31±9.79	197.14

The first mutual effect between sodium nitrate and dipotassium phosphate is illustrated in Figures 4.A and 5.A. From these figures, the highest biomass was obtained by increasing sodium nitrate concentration from 0.01 M to 0.0125 M and increasing the concentration of dipotassium phosphate to 0.625 M while maintaining L-glutamic acid at 0.5 mM. However, this trend did not hold for phycocyanin yield, as the highest levels were reached when the concentration of dipotassium phosphate was decreased from 0.5 mM to 0.375 mM, coupled with an increase in sodium nitrate concentration from 0.01 M to 0.0125 M while maintaining L-glutamic acid at 0.5 mM. This figure showed that the interaction between sodium nitrate and dipotassium was different for biomass and phycocyanin yield. This phenomenon can be related to the dependence of phycocyanin and biomass on nitrogen available in the medium, where nitrogen starvation can cause stalemate or reduction in phycocyanin due to nitrogen uptake being prioritized in microalgae growth (Markou et al. 2014; Nur et al. 2019). In contrast, increasing phosphorus concentrations can promote biomass accumulation up to a certain point, but phosphorus limitation can lead to alterations in biochemical composition, particularly in photosynthetic pigments (Benavente-Valdés et al. 2016; Fattore et al. 2021).

Next, the interaction between sodium nitrate and L-glutamic acid while keeping dipotassium phosphate constant is depicted in Figures 4.B and 5.B. It was evident that adjusting the sodium nitrate concentration from 0.01 M to 0.0125 M and simultaneously altering the L-glutamic acid concentration from 0.5 mM to 0.625 mM, with dipotassium phosphate held at a constant 0.5 mM resulted in the highest biomass and phycocyanin yield. The interdependency between sodium nitrate and L-glutamic acid concerning biomass and phycocyanin production was shown throughout this interaction. This dependency was attributed to the microalgae's capacity to convert organic nitrogen sources, such as L-glutamic acid, into a nitrogen source through deamination facilitated by periplasmic amino acid oxidase (Kumar and Bera 2020).

Lastly, the combined effect of dipotassium phosphate and L-glutamic acid is illustrated in Figures 4.C and 5.C. The highest biomass and phycocyanin yield were achieved when L-glutamic acid and dipotassium phosphate concentrations were reduced from 0.5 mM to 0.375 mM, while sodium nitrate was maintained at 0.01 M. However, no significant interaction between dipotassium phosphate and L-glutamic acid was observed, especially when the concentration of sodium nitrate was altered, either by means of increasing or decreasing. Statistical analysis further supported this observation, where the p-value for the interaction between dipotassium phosphate and L-glutamic acid was insignificant.

Validation of predicted best outcome simulated from regression model

The BBD result was run through the software's optimization module, which predicted that the maximum phycocyanin yield and biomass by *A. maxima* could be achieved using concentration as follows: 0.0125 M of sodium nitrate, 0.375 mM of dipotassium phosphate, and 0.625 mM of L-glutamic acid. Other essential nutrients included 0.125 M of sodium bicarbonate, 0.125 M of sodium chloride, 0.003 M of potassium sulphate, 0.0045 M of magnesium sulphate, 0.001 M of glycine, and 0.0045 M of succinic acid. This model has a desirability of 0.992 and was predicted to obtain 1.48 g/L of biomass and 238.342 mg/L of phycocyanin yield. Meanwhile, the actual observed result produced 1.45 g/L of biomass and 235.976 mg/L of phycocyanin yield. A 95% confidence interval was employed to compare the observed and predicted values to validate the model's reliability and accuracy. As detailed in Table 9, the results affirmed the model's trustworthiness, showcasing a remarkable proximity between the observed and predicted outcomes. Since the aim of this study placed more emphasis on biopigment phycocyanin than the final biomass, by comparing the pre-and post-optimization conditions, a separate cultivation of *A. maxima* that employed the unoptimized basal NRC medium yielded about 163.26 mg/L of phycocyanin, which effectively put this new formulation to affect a 44.67% improvement over the original medium.

Nitrogen plays a crucial role in the growth and metabolite production. It is also an important building block for the protein backbone of phycobiliproteins, including phycocyanin. Numerous previous studies have emphasized the significant impact of nitrogen concentration, especially when sodium nitrate is employed, on both biomass and phycocyanin yield (de Castro et al. 2015; Mirhosseini et al. 2021). This assertion is reinforced by the findings from the Plackett-Burman Design (PBD), which highlight the substantial contribution of nitrogen to biomass (40.57%) and phycocyanin yield (39.4%). Banayan et al. (2022) also found that nitrogen sources play a vital role in phycocyanin production in PBD. Although earlier research has suggested an optimal sodium nitrate concentration of 0.03 M, L-glutamic acid as a secondary nitrogen and carbon source may alter this concentration (Shanthi et al. 2018). The inclusion of L-glutamic acid in the medium, serving as a source of glutamate, a precursor in various essential pathways such as the phycobilin cycle and Tricarboxylic Acid (TA) cycle, has the potential to enhance biomass and phycocyanin yield (Fekrat et al. 2021; Musifa et al. 2023). This statement is backed by the finding in this experiment where the use of L-glutamic acid influenced biomass especially. In addition, a study showed that using 0.05g/L of L-glutamic acid can enhance biomass (Shanthi et al. 2018).

Table 9. Statistical analysis using a 95% confidence interval of the validation experiment

Response	Predict	Observed	Std Dev	SE Pred	95% PI Low	95% PI High
Phycocyanin yield	238.34 mg/L	235.98 mg/L	1.6575	1.172	252.04	222.256
Biomass	1.48 g/L	1.4 g/L	0.0566	0.04	1.948	0.932

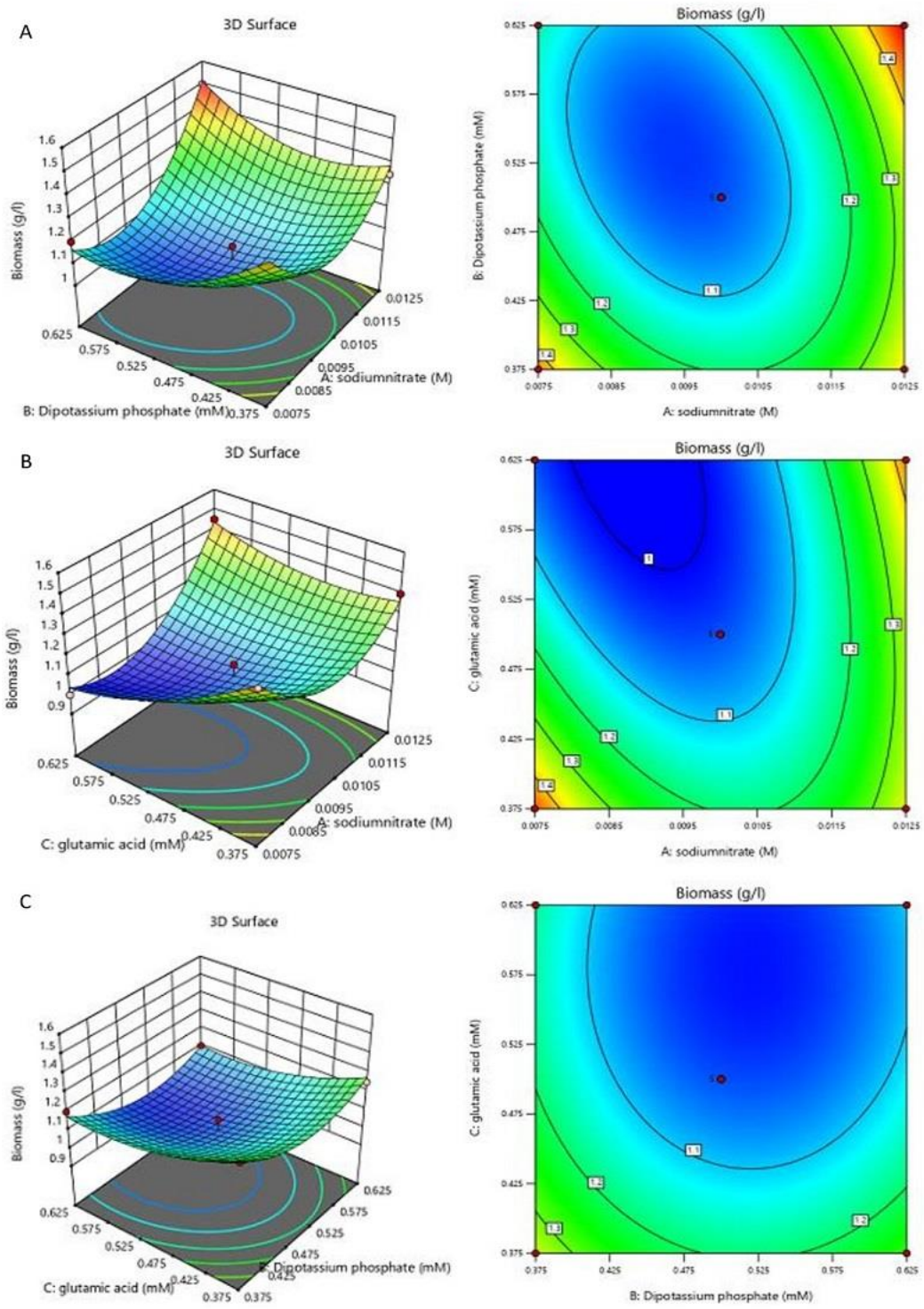

Figure 4. The mutual effect of A. Sodium nitrate and dipotassium phosphate, B. Sodium nitrate and L-glutamic acid, C. Dipotassium phosphate and L-glutamic acid on biomass by *Arthrospira maxima* as represented by contour and response surface plots

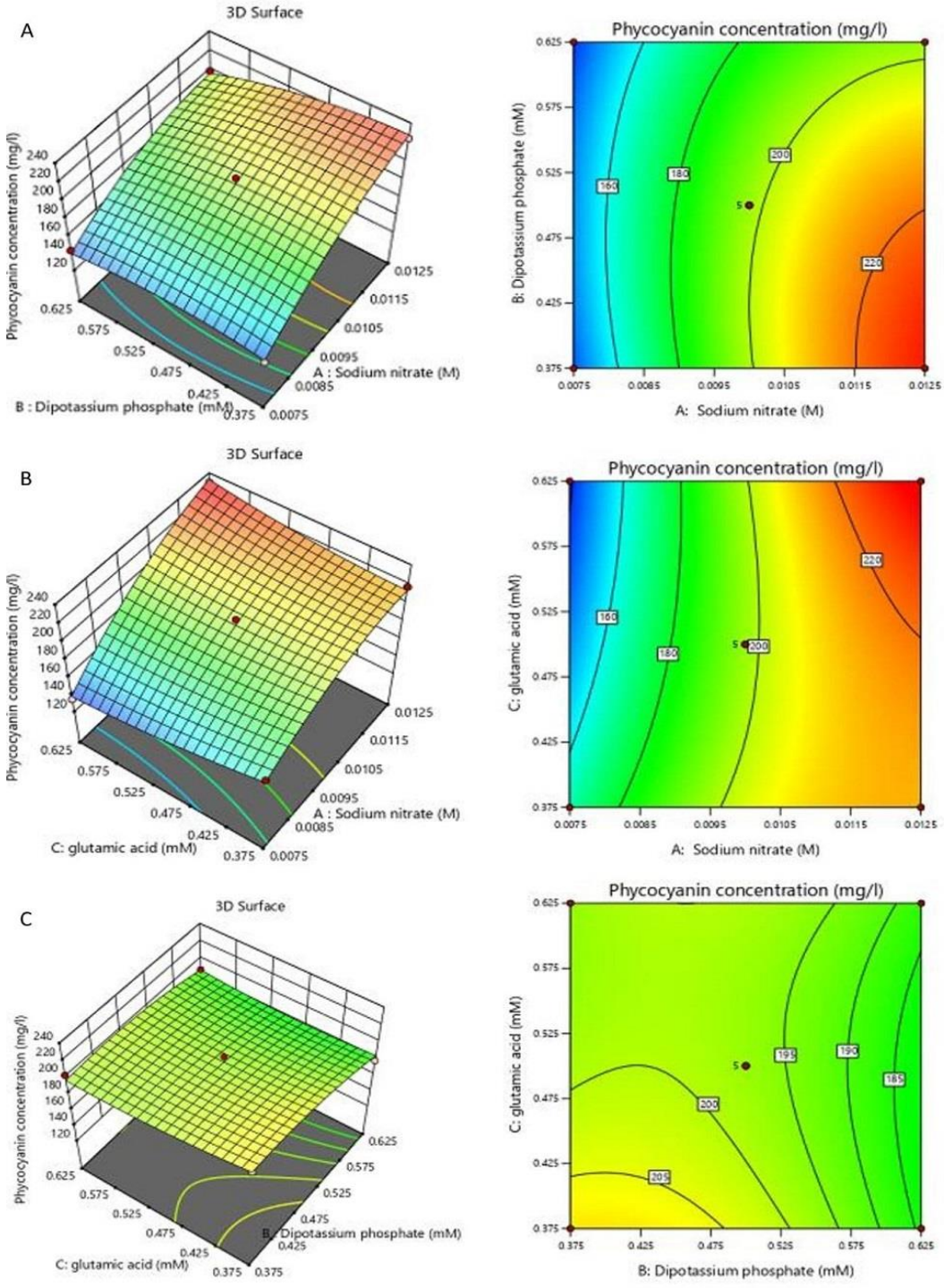

Figure 5. Mutual effect of A. Sodium nitrate and dipotassium phosphate, B. Sodium nitrate and L-glutamic acid, C. Dipotassium phosphate and L-glutamic acid on phycocyanin yield by *Arthrospira maxima* as represented by contour and response surface plots

Finally, phosphorus is a vital macronutrient that supports photosynthesis in producing various biological molecules such as phospholipids, Adenosine Triphosphate (ATP), and nucleic acids. Typically, phosphorus is sourced from dipotassium phosphate, which is frequently employed in culture media. While alterations in dipotassium phosphate concentration may not significantly impact biomass, it can induce changes in the biochemical composition. This statement is supported by Markou et al. (2012a, b), stating that altering phosphate concentration has little or no effect on biomass production. In addition, both studies discovered that a low concentration of dipotassium

phosphate (0.04 g/L to 0.05 g/L) can maintain optimal biomass production without loss. This finding is consistent with the results of this research, where dipotassium phosphate was significant for phycocyanin production but not for biomass production in the PBD experiments. Meanwhile, the use of low concentrations of dipotassium phosphate in this study exhibited high phycocyanin concentrations. The study by Hao et al. (2019) presented similar results when using low concentrations of dipotassium phosphate. This phenomenon is likely due to a resource allocation shift toward photosynthesis to generate essential nutrients (Senatore et al. 2023). However, there is a lack of studies on how dipotassium phosphate affects phycocyanin production, especially by *Arthrospira* sp. nutrients.

This study revealed that, out of nine screening media compositions investigated through the Plackett-Burman Design, only three, namely sodium nitrate, potassium dihydrogen phosphate, and L-glutamic acid, exerted significant influence on either biomass or phycocyanin yield. Through optimization using the steepest ascents followed by the Box-Behnken Design, the highest phycocyanin yield was achieved at 235.976 mg/L and biomass at 1.45 g/L. This was accomplished by employing the formulated NRC medium, which contained 0.0125 M of sodium nitrate, 0.375 mM of potassium dihydrogen phosphate, and 0.625 mM of L-glutamic acid. In conclusion, this study emphasizes the vital role of media composition in maintaining biomass and enhancing phycocyanin yield.

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