Enhanced phycocyanin production of *Arthrospira maxima* by addition of mineral elements and polypeptides using response surface methodology

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Introduction: As a pigment protein, phycocyanin has been widely used in the fields of food, nutraceutical and biotechnology due to its excellent biological activities of antioxidant. So far, *Arthrospira* has been generally considered as a great species for phycocyanin production.

Methods: In this study, independent and interactive effect of three factors, NaCl, Bainengsi (BS) and Bainengtai (BT) on microalgae growth and phycocyanin production were explored by response surface methodology (RSM). Using Box-Behnken design (BBD) method, the well-fitting quadratic models were established based on experimental results.

Results: Moreover, the maximum phycocyanin concentration reached 704.66 mg L^{-1} at the optimal condition (185 mM NaCl, 200 mg L^{-1} BS, and 20 mg L^{-1} BT), while the maximum phycocyanin content of 19.03% was obtained at another optimal condition (136 mM NaCl, 200 mg L^{-1} BS, and 50 mg L^{-1} BT). Compared to control, the concentration and content of phycocyanin were increased by 22.98% and 16.73%, respectively.

Discussion: Overall, this study demonstrated that addition of exogenous substances (BS, BT) into culture medium optimized by RSM was an effective approach to increase phycocyanin production, which paved a potential way to realizing high efficient production of algal biomass and bioactive substances.

1 Introduction

Microalgae have been extensively considered as renewable and sustainable resources for the production of various bioactive substances, such as phycobiliproteins (Manirafasha et al., 2016), carotenoids (Novoveská et al., 2019), polyunsaturated fatty acids (Lu et al., 2021), and polysaccharide (Sathasivam et al., 2019). Phycobiliprotein is a kind of pigment protein that widely exists in cyanobacteria, rhodophyta, cryptophyta, and glaucophyta (Lawrenz et al., 2011; Yu et al., 2017). In cyanobacteria, phycobiliprotein can account for 40% of the total soluble protein (Pandey et al., 2013). Generally, there are four types of phycobiliprotein, namely phycoerythrin, phycocyanin, phycoerythrocyanin, and allophycocyanin (Pagels et al., 2019). Phycocyanin shows great antioxidant and radical scavenging activity (Fratelli et al., 2021; Li, 2022). Besides, phycocyanin has other biological activities, like anti-inflammatory, anti-cancer, anti-diabetes, neuroprotective and hepatoprotective effects (Romay et al., 2003; Jiang et al., 2017; Prabakaran et al., 2020). Thereby, phycocyanin nowadays is widely used in food, nutraceutical, cosmetics, and pharmaceutical industries (Manirafasha et al., 2016; Khazi et al., 2020). Presently, the commercial price of phycocyanin varies enormously depending on its purity. It was reported that the price of food-grade phycoerythrin was approximately \$130 g^{-1} (Hsieh-Lo et al., 2019). Moreover, the market demands for phycoerythrin continuously increased in the past few years due to its natural and functional properties. Specifically, the global market size of phycocyanin was valued at \$155.3 million in 2020 and it is estimated to reach \$409.8 million by 2030 (Thevarajah et al., 2022).

Arthrospira, commonly known as *Spirulina*, is a multicellular and filamentous cyanobacteria (Soni et al., 2017). It is generally rich in protein, carbohydrates, phycocyanin, fatty acids, and chlorophyll (Anvar and Nowruzi, 2021). *Arthrospira* is well considered as an excellent source of the production of phycocyanin (CPC) (Nethravathy et al., 2019; Silva et al., 2020). To date, numerous studies have been conducted to improve the phycocyanin productivity and content of *Arthrospira*. The main strategies includes culture medium improvement, exogenous substances addition (Manirafasha et al., 2018), environmental condition optimization (Bachchhav et al., 2016), and changing of culture mode (Chen et al., 2006). The optimization of culture medium is always a basic work to enhance microalgal growth and phycocyanin production. Nitrogen is one of the primary nutrient requirements for algal cell growth. With the nitrogen (NaNO$_3$) concentration in the medium increased from 0.63 to 2.5 g L^{-1}, the biomass concentration of *Arthrospira* highly increased from 0.83 to 1.04 g L^{-1} (Can et al., 2017). In addition to nitrogen concentration, microalgae growth and phycocyanin production are also affected by type of nitrogen source. Mousavi et al. (2022) found that *Arthrospira* cultured in the medium containing (NH$_4$)$_2$SO$_4$ produced the largest amount

of phycocyanin (118 mg L^{-1}) while the medium with KNO$_3$ as the nitrogen source resulted in the highest biomass output (1.18 g L^{-1}). In addition, salinity of culture medium significantly influences algal cell growth and phycocyanin accumulation. The Zarrouk medium with 0.1 M NaCl considerably raised the biomass productivity from 139.5 mg L^{-1} d^{-1} at the normal condition (0.02 M NaCl) to 221.33 mg L^{-1} d^{-1}, and the total phycobiliprotein content increased from 11.08% to 15.63% (Abd El-Baky and El-Baroty, 2012). However, the impact of salinity on cell growth and phycobiliprotein production is highly related to NaCl concentration. The tremendously high NaCl concentration would result in very low biomass and phycobiliprotein productivity (Chen et al., 2016).

Besides, the addition of exogenous substances is also an efficient approach to promote the biomass and phycocyanin accumulation. The supplements of mineral elements, organic acids, and vitamins in culture medium have been reported to enhance biomass and phycocyanin production (Jung et al., 2014; Bachchhav et al., 2016; Mogany et al., 2018). Stepwise addition of selenium (Se) during the exponential growth phase of *Arthrospira. platensis* grown mixotrophically increased the phycocyanin production by 21.2% compared to control (Chen et al., 2006). The metabolic stress conditions induced by nutrient enrichment through additionally adding sodium glutamate and succinic acid strongly boosted the biomass production and phycocyanin concentration of *Arthrospira platensis* (Bachchhav et al., 2016). Hence, the addition of suitable substance in culture medium at an appropriate range is critical to efficient production of phycocyanin.

Response surface methodology (RSM) is a set of mathematical and statistical techniques that are based on fitting a polynomial equation to experimental data (Bezerra et al., 2008). Currently, the RSM has been used to optimize the culture process of microalgae. To maximize the biomass production of *Chlorella* sp. as biofuel production stock, the optimal concentration of nitrogen and glycerol (0.114 g L^{-1} and 2.70 g L^{-1}) were determined by the RSM, and the highest biomass reached 2.41 g L^{-1} at this condition (Skorupskaite et al., 2015). Verma et al. (2020) performed the culture experiments to improved medium using the RSM, and obtained the highest biomass productivity of 0.86 g L^{-1} d^{-1} at the optimal conditions. Undoubtedly, RSM is an efficient and effective means to optimize the culture medium.

There are commercial products of mineral element complexes and peptide complexes, commonly called Bainengsi (BS) and Bainengtai (BT), respectively, in China. BS contains many mineral elements including Si, Mo, Se, Ni, Bo, K and so on. BT is composed of enzymatic hydrolysates of high-quality proteins sourced from plants. BS and BT have been used in agriculture and feed industries to enhance the plant and animal growth. In the present study, we consider BS and BT as the additional supplements for *Arthrospira maxima* culture. The main aim of the research is to evaluate the independent and

interaction effect of three factors (BS, BT, and NaCl concentrations) on biomass and phycocyanin production of *Arthrospira maxima*, and to determine the optimal addition amounts of these substances using RSM to maximize the productivity and content of phycocyanin.

2 Materials and methods

2.1 Microalgae strain

Arthrospira maxima (FACHB-438) was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), China. For stock culture preparation, the microalgal cells were cultivated in Zarrouk medium (Zarrouk, 1996) at 31°C and light intensity of 80 μ mol m^{-2} s^{-1}.

2.2 Exogenous substances

Two types of exogenous substances (BS and BT) were added into culture medium to explore their impacts on the growth and phycocyanin production of Arthrospira maxima. BS and BT were purchased from Jiangsu Rishengchang Biotechnology Company, China. BS is the mineral element complexes and composed of various inorganic microelements. Based on element analysis *via* the method of inductively coupled plasma optical emission spectrometer (ICP-OES), the contents of Na, K, Cl, Fe, Mn, Mg, Mo, Ba, S, B, Al, Ti, Zn, Rb, Ni and Ir were 34.24, 13.35, 7.36, 1.37, 0.84, 0.44, 0.23, 0.21, 0.15, 0.12, 0.11, 0.09, 0.06, 0.04, 0.03 and 0.01 mg kg^{-1}, respectively. BT is a mixture of amino acids, polypeptide and proteins, and generally recognized as polypeptides complexes. According to the formula provided by the company, it contains 5.1% amino acids (<180 Da), 34.2% small peptides (180~6000 Da), 31.6% polypeptide (6000~30000 Da) and 29.1% proteins (>30000 Da), respectively. The major types of amino acids were arginine, phenylalanine, valine, lysine, leucine, isoleucine, threonine and methionine. The commercial prices of BS and BT are $4.83 kg^{-1} and $4.14 kg^{-1}, respectively.

2.3 Experiments design

RSM using the Box-Behnken Design (BBD) method was employed to optimize the culture medium with the supplement of BS and BT. The effects of three independent variables (concentrations of NaCl, BS and BT) at different ranges on biomass concentration and phycocyanin production were assessed. For this, a three-factor three-level experimental design was employed (Table 1). Ranges of variables were chosen based on our preliminary studies. According to single-factor experiments, the three factors had a positive effect on biomass and phycocyanin accumulation at low concentration,

TABLE 1 Independent variables (NaCl, BS and BT concentration) and their levels used in the Box-Behnken design.

Factors	Units	Coded symbols	Range of variables (Levels)		
			-1	0	1
NaCl	mM	A	10	115	220
BS	mg L^{-1}	B	200	300	400
BT	mg L^{-1}	C	20	50	80

and showed an inhibitory effect at high concentration. The optimal concentration of each factor was selected as the central point of the BBD. The quantitative relationship between dependent [Y$_1$: biomass concentration, Y$_2$: phycocyanin concentration (CPC$_{conc}$) and Y$_3$: phycocyanin content (CPC$_{cont}$)] and independent variables (A: NaCl, B: BS and C: BT) was determined. The experimental design, mathematical model and statistical analysis were carried out using Design-Export software (13.0.1.0).

According to BBD, the total experiments included 17 groups (Table 2). The medium of each experimental group differed by varying addition amounts of NaCl, BS, and BT into Zarrouk medium. All media were autoclaved at 121°C for 20 min after the initial pH was adjusted to 9.5 with 1 M NaOH. Microalgae cultivated at logarithmic growth phase were inoculated into 250 mL flasks with 100 mL culture medium at 10% (v/v) inoculation rate after the medium cooled to room temperature. *Arthrospira maxima* were cultivated at 31°C in shake flasks with rotation rate of 120 rpm, and illuminated by 6 light tubes at light intensity of 144 μ mol m^{-2} s^{-1} for 16 days. Then, microalgae cells were harvested by filtration to measure the biomass concentration, CPC$_{conc}$ and **CPC$_{cont}$**. Each experiment was conducted in triplicate. Taking the maximum value of CPC$_{conc}$ and **CPC$_{cont}$** as targets, respectively, validation experiments were conducted based on the optimal conditions predicted by the model.

2.4 Analytical methods

2.4.1 Biomass concentration and productivity

Biomass concentration was determined by dry weight method. In brief, algal cells were harvested using 500 mesh screens, then washed with deionized water 2 times, and transferred to a pre-dried weighing disc, followed by oven drying at 75°C until constant weight. The biomass concentration (X) and productivity (P$_{biomass}$) (Skorupskaite et al., 2015) were calculated using the following equations:

$$X(g\,L^{-1}) = \frac{m_1 - m_0}{V_{cul}} \qquad (1)$$

where m$_1$ represents the weight (g) of dried weighing disc containing the sample, and m$_0$ (g) represents the weight of pre-dried empty weighing disc. V$_{cul}$ (L) is the sampling volume.

TABLE 2 Experimental data and predicted value based on established models of CPC$_{conc}$ and CPC$_{cont.}$.

Std	Run	Variables						Responses					
		A: NaCl		B: BS		C: BT		CPC$_{conc}$			CPC$_{cont}$		
		mM		mg L^{-1}		mg L^{-1}		mg L^{-1}			%		
		Actual	Coded	Actual	Coded	Actual	Coded	Actual	Predicted	Residual	Actual	Predicted	Residual
1	16	10	-1	200	-1	50	-1	528.22	527.94	0.28	17.60	17.44	0.16
2	3	220	1	200	1	50	-1	646.22	646.62	-0.40	18.08	18.09	-0.01
3	2	10	-1	400	-1	50	1	588.98	588.58	0.40	17.47	17.46	0.01
4	12	220	1	400	1	50	1	550.09	550.37	-0.28	16.93	17.09	-0.16
5	7	10	-1	300	-1	20	0	564.06	556.32	7.74	16.93	17.05	-0.12
6	10	220	1	300	1	20	0	631.03	622.6	8.43	16.61	16.56	0.05
7	14	10	-1	300	-1	80	0	491.91	500.34	-8.43	16.08	16.13	-0.05
8	9	220	1	300	1	80	0	506.79	514.53	-7.74	17.02	16.9	0.12
9	11	115	0	200	0	20	-1	677.91	685.94	-8.03	17.86	17.9	-0.04
10	4	115	0	400	0	20	1	639.75	647.89	-8.14	17.68	17.57	0.10
11	6	115	0	200	0	80	-1	591.82	583.68	8.14	17.67	17.77	-0.10
12	8	115	0	400	0	80	1	594.14	586.11	8.03	17.14	17.11	0.04
13	13	115	0	300	0	50	0	605.94	609.17	-3.24	17.87	18.25	-0.38
14	17	115	0	300	0	50	0	620.54	609.17	11.37	18.48	18.25	0.23
15	15	115	0	300	0	50	0	602.18	609.17	-6.99	18.08	18.25	-0.16
16	5	115	0	300	0	50	0	615.48	609.17	6.31	18.35	18.25	0.10
17	1	115	0	300	0	50	0	601.73	609.17	-7.45	18.45	18.25	0.20

$$P_{biomass} \ (g \ L^{-1} \ d^{-1}) = \frac{X_1 - X_0}{t_1 - t_0} \qquad (2)$$

where X_1 and X_0 represent the biomass concentration (g L^{-1}) on days t_1 and t_0, respectively.

2.4.2 Determination of phycocyanin

Phycocyanin was extracted from *Arthrospira maxima* by using modified wet extraction method (Khazi et al., 2018). Specifically, 5 mL culture was filtered with 500 mesh screens to obtain wet biomass. It was washed by deionized water, re-suspended in 20 mL of 100 mM sodium phosphate buffer (pH 7), and subjected a freeze of -20°C for 12h. Thereafter, the sample was centrifuged at 8000×g for 10 min and the blue supernatant was collected. The CPC$_{conc}$ in the supernatant was determined by measuring the absorbance at 620 and 652 nm using ultraviolet-visible spectrophotometer (UV-5200) (Mogany et al., 2018; Novoveská et al., 2019). The concentration and the content of CPC were calculated as follows:

$$CPC_{conc} \ (mg \ L^{-1})$$
$$= \frac{(A_{620nm} - 0.474 \times A_{652nm}) \times 1000 \times V_{extr}}{5.34 \times V_{cul}} \qquad (3)$$

Where CPC$_{conc}$ was the concentration of CPC in the solution of microalgae, A_{620} and A_{652} were optical density of extracting solution at 620 and 652 nm, respectively. V_{extr} (L) and

V_{cul} (L) were the volume of extracting solution and culture sample, respectively.

$$CPC_{cont} \ (\%) = \frac{CPC_{conc}}{10X} \qquad (4)$$

where CPC$_{conc}$ was the concentration of CPC (mg L^{-1}) obtained from Eq. (3), and X was the biomass concentration (g L^{-1}) obtained from Eq. (1).

The gross volumetric productivity of CPC was calculated using Eq. (5).

$$P_{CPC}(mg \ L^{-1}d^{-1}) = \frac{CPC_{conc\_end}}{t} \qquad (5)$$

where CPC$_{conc\_end}$ was the final CPC concentration (mg L^{-1}), and t was the total duration of the cultivation (d).

2.5 Statistical analysis

After performing all experiments, the quadratic second-order equation with interaction terms was used to model the relationship between dependent and independent variables:

$$y_i = \beta_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=2}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \qquad (6)$$

where y_i, and β_0 represent response variable and interception coefficient, respectively, and b_i, b_{ii}, and b_{ij} are regression

coefficients, n is the number of studied variables, x_i and x_j represent independent variables.

The statistical differences among the experiments were assessed by the analysis of variance (ANOVA) *via* the Design-Export software (13.0.1.0). The main effects and interaction effects of each factor were analyzed. All analyses are significant at $p<0.05$.

3 Results

3.1 Model fitting and statistical analysis

In order to achieve the maximal biomass concentration and phycocyanin accumulation, the relationship between the three independent and dependent variables was analyzed. The response surface models were identified for the coded factors and the quadratic second-order equations with interaction terms were used for data fitting. The final model equations and the variance analysis for the responses were shown in Table 3. The significance of the models was specified by low p-values ($p<0.05$) (Nemanja et al., 2019). The p-values of the fitting models of biomass concentration, phycocyanin concentration and content were 0.0007,<0.0001 and 0.001, respectively, indicating that all generated models were significant. Meanwhile, the high values of R^2 (>0.9481) represented that all models were well fitting. For each model, the adequate precision was more than 4, which was desirable for good discrimination and indicated an adequate signal for the models to be used to navigate design space. Moreover, the low variation coefficients (1.33 to 2.28%) and the insignificant lack of fit ($p>0.05$) implied a high experimental reliability and a good correlation between the responses and the independent variables.

3.2 Microalgal growth

The biomass concentration and productivity of *Arthrospira maxima* under various cultivation conditions were summarized in Table 4. Compared with the control, the growth performance of most experimental groups decreased after adding three

exogenous substances synchronously. There were only four treatments (run 3, 4, 10 and 11) in which the biomass concentrations and productivities were higher than that of the control. Among them, the run 10 showed the best microalgae growth, attaining the highest biomass concentration of 3.8 g L^{-1} and productivity of 0.22 g L^{-1} d^{-1}. The common feature of these four treatments was the addition of middle-high concentration of NaCl (115 and 220 mM) and low-middle concentration of BT (20 and 50 mg L^{-1}). Moreover, the perturbation plot revealed that the biomass was sensitive to NaCl and BT (Figure 1A). Hence, it can be speculated that adding a relative high concentration of NaCl and a small amount of BT can help to increase the biomass concentration. 3D response surfaces and contour plots showed more details about the relationships between the three variables and responses (Figure 2). The effect of exogenous substances at different concentration levels on the biomass concentration was shown in Figures 2A–C. The curvatures indicated that there was a strong interactive effect between the NaCl and BS concentration, as well as the NaCl and BT concentration with the low p-values of 0.0026 and 0.090, respectively (Table S1). Overall, the biomass concentration first increased and then declined with the increase of NaCl concentration at the fixed concentration of BT (50 mg L^{-1}). However, the point of trend change depended on the BS concentration. For the combined impact of NaCl and BT, the maximal biomass concentration was achieved at the high NaCl and low BT concentration. The interaction between BS and BT was not significant for the growth of *Arthrospira maxima* ($p>0.05$, Table S1).

3.3 Phycocyanin concentration

Likewise, the relationships between the phycocyanin concentration and three independent variables were analyzed. The ANOVA results of the model showed that the single factors (A, C), interaction terms (AB, AC), and secondary terms (A^2, B^2) had significant effects on phycocyanin concentration and the p-values of these factors were all less than 0.05 (Table S2). The phycocyanin concentration was more sensitive to NaCl concentration than BS and BT concentrations (Figure 1B),

TABLE 3 Analysis of variance for the response surface models.

Source	Modified Equations with Significant Terms	p-value	R^2	Adj.R^2	SD	Lack of Fit
Biomass concentration	$4.26877+0.011369A-0.006224B-0.021286C-0.000017AB-0.000044AC+0.000025BC-0.000014A^2+0.000012B^2+0.000119C^2$	0.0007	0.9539	0.8947	0.0769	0.08
CPC$_{conc}$	$706.87395+2.64811A-1.22795B-1.17036C-0.003735AB-0.004133AC+0.003373BC-0.00491A^2+0.002333B^2-0.007333C^2$	<0.0001	0.9797	0.9536	10.74	0.20
CPC$_{cont}$	$16.26259+0.020213A-0.004221B+0.076443C-0.000024AB+0.0001AC-0.000028BC-0.000075A^2+0.000009915B^2-0.000844C^2$	0.001	0.9481	0.8813	0.2338	0.69

Biomass concentration; CPC$_{conc}$, phycocyanin concentration; CPC$_{cont}$, phycocyanin content; A, NaCl concentration; B, CPC$_{conc}$; C, CPC$_{cont}$; R^2, determination coefficient; Adj.R^2, adjusted R^2; SD, standard deviation; CV, coefficient of variation.

TABLE 4 Comparison of biomass concentration and productivity among the different treatments.

Medium types	Treatment	Addition ratio of exogenous substances			Biomass concentration ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ d^{-1}$)
		NaCl(mM)	BS ($mg\ L^{-1}$)	BT ($mg\ L^{-1}$)		
Control medium	Run 0	0	0	0	3.52	0.20
Experimental medium	Run 1	115	300	50	3.26	0.19
	Run 2	10	400	50	3.38	0.20
	Run 3	220	200	50	3.58	0.21
	Run 4	115	400	20	3.62	0.21
	Run 5	115	300	50	3.35	0.19
	Run 6	115	200	80	3.35	0.19
	Run 7	10	300	20	3.33	0.19
	Run 8	115	400	80	3.47	0.20
	Run 9	220	300	80	2.98	0.17
	Run 10	220	300	20	3.80	0.22
	Run 11	115	200	20	3.80	0.22
	Run 12	220	400	50	3.25	0.19
	Run 13	115	300	50	3.39	0.20
	Run 14	10	300	80	3.06	0.18
	Run 15	115	300	50	3.33	0.19
	Run 16	10	200	50	3.00	0.17
	Run 17	115	300	50	3.36	0.19

which was confirmed by the linear model coefficients (Table 3). The interactive effect of NaCl and BS concentration was similar to that on biomass concentration (Figures 2A, D). Specifically, the effect of NaCl concentration on CPC_{conc} was dependent on the BS concentration. The high CPC_{conc} were obtained at the extreme low BS concentration (200 mg L^{-1}) and NaCl concentration ranged from 125-220 mM (Figure 2E). The interactive effect of NaCl and BT concentration was significant but not strong. The CPC_{conc} increased first and then decreased with the increase of NaCl concentration. The high concentration of BT reduced the CPC_{conc}. A combination of a high concentration of NaCl (100-200 mM) and a low concentration of BT (20-25 mg L^{-1}) was beneficial to the CPC accumulation. The interaction between BS and BT is not significant (p>0.05, Table S2). Both low concentrations of BT and BS is beneficial to CPC synthesis.

3.4 Phycocyanian content

It was observed that a trend of first dramatically increasing and then decreasing phycocyanin content as the NaCl
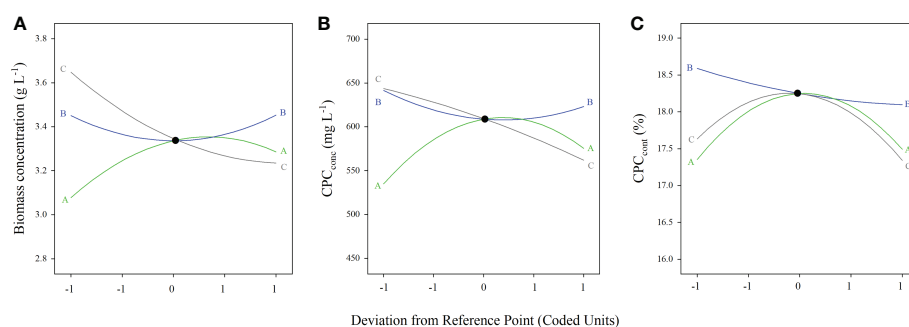

FIGURE 1
Perturbation plots of the dependent variables. **(A)** biomass concentration, **(B)** CPC_{conc}, and **(C)** CPC_{cont}. **(A)** NaCl concentration; **(B)** BS concentration; **(C)** BT concentration.
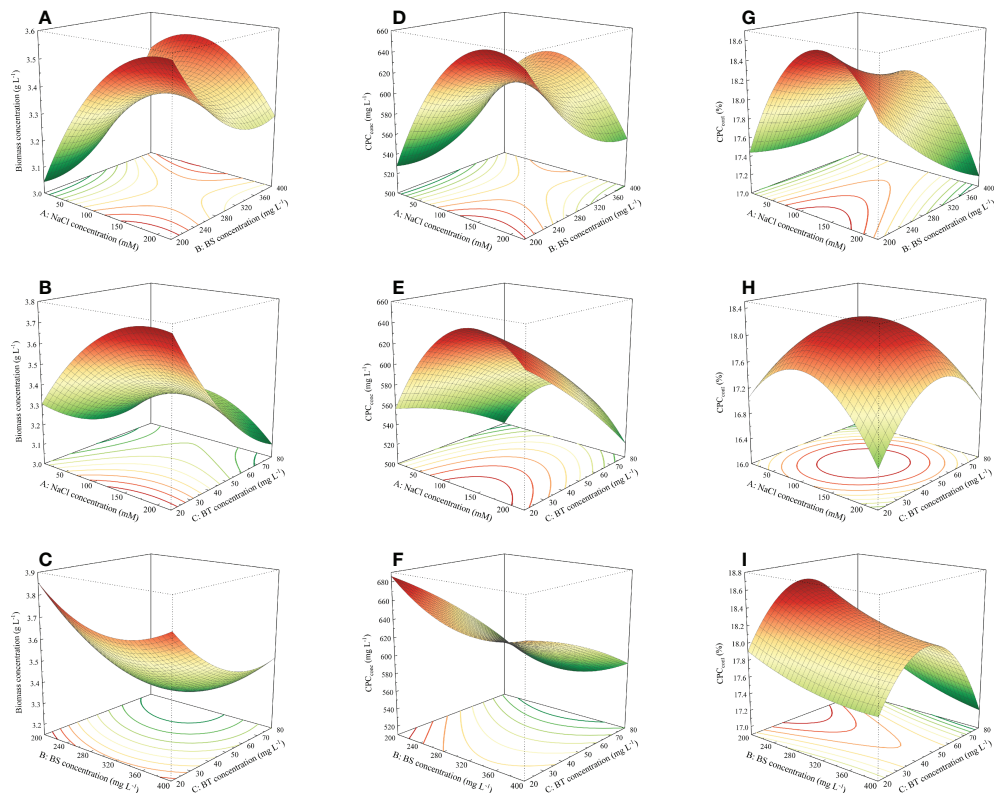
FIGURE 2
Response surface plots visualizing the interactive effects among the NaCl, BS and BT concentration for responses. **(A–C)** biomass concentration, **(D–F)** CPC$_{conc}$, and **(G–I)** CPC$_{cont}$.

concentration increased from 50 mM to 220 mM (Figure 2G). The maximum CPC$_{conc}$ was found when the NaCl concentration ranged from 100 to 180 mM. The variation in BT concentration, however, had slight impact on the content of phycocyanin when the NaCl concentration was ranged from about 10-40 mM. The perturbation plot demonstrated that the CPC$_{cont}$ was sensitive to NaCl and BT concentrations (Figure 1C), and their interaction was significant ($p<0.05$, Table S3). 3D model presented that CPC$_{cont}$ was low at the extreme concentrations of NaCl and BT. When the concentration of NaCl or BT is constant, the CPC$_{cont}$ significantly increased first and then declined sharply with the increase of another factor. The sensitivity of CPC$_{cont}$ to BT was also reflected in the interaction between BS and BT (Figure 2I). As the increase of BT concentration from 20 to 80 mg L^{-1}, the CPC$_{cont}$ increased first and then decreased, and the optimum concentration of BT was about 50 mg L^{-1}. With the variation in BS concentration, the CPC$_{cont}$ did not change obviously, indicating that the CPC$_{cont}$ was not sensitive to BS, which was consistent with the results shown in the perturbation plots (Figure 1C).

3.5 Optimization and experimental validation

The comparison between the actual experimental and the predicted data using the quadratic regression model was shown in Figure 3. The strong linear relationship indicated that there was a good agreement between the predicted and experimental results, demonstrating this model can be used in prediction and optimization. According to the model predication, the maximum CPC$_{conc}$ was achieved when the concentrations of NaCl, BS and BT were 185 mM, 200 mg L^{-1} and 20 mg L^{-1}, respectively. However, it should be noted that the CPC$_{cont}$ was not maximum at this condition. The optimal condition estimated for obtaining the maximum CPC$_{cont}$ were 136 mM NaCl, 200 mg L^{-1} of BS and 50 mg L^{-1} of BT. Subsequently, the validation experiments were performed at the optimal conditions. The experimental results (CPC$_{conc}$ of 704.66 mg L^{-1}, CPC$_{cont}$ of 19.03%) were very close to the predicted data (CPC$_{conc}$ of 710.13 mg L^{-1}, CPC$_{cont}$ of 18.63%) at the respective optimal condition (Figure 4).
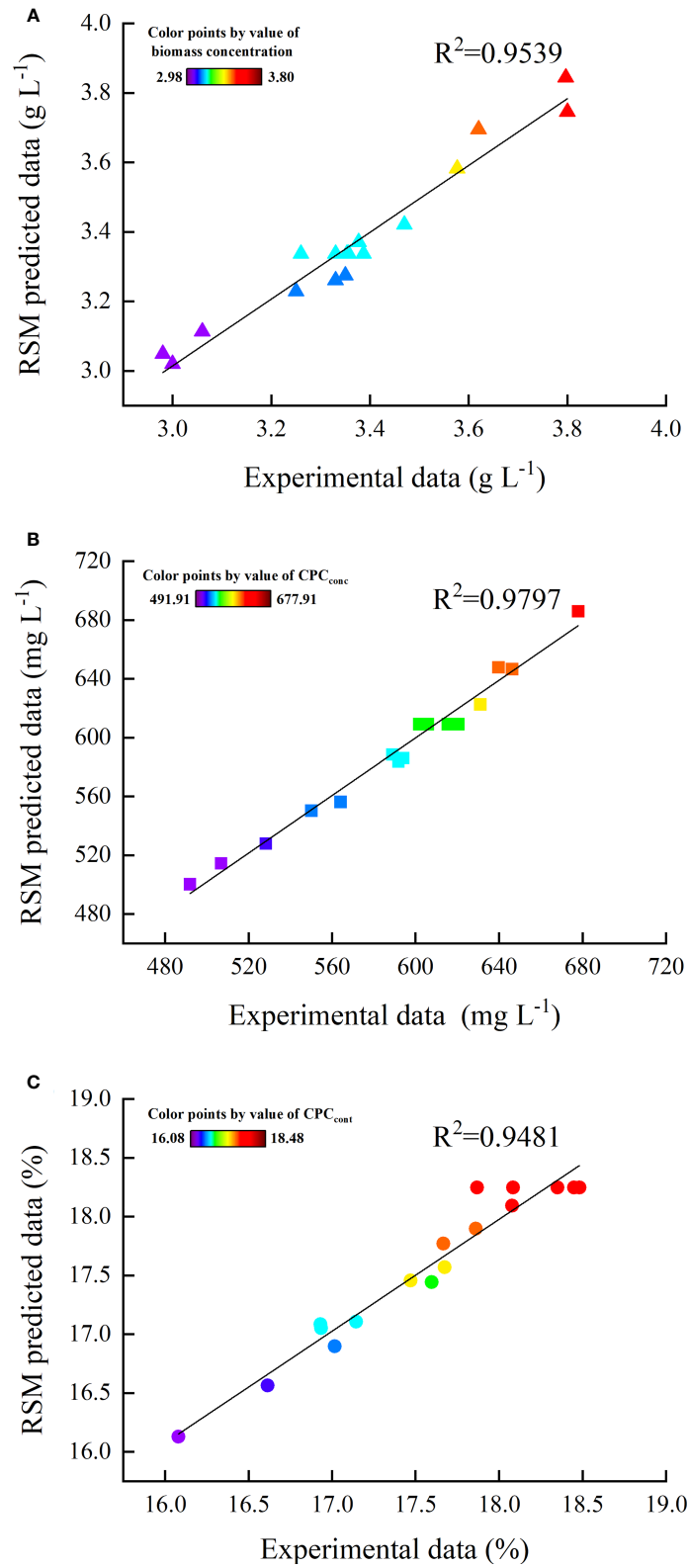
FIGURE 3
Comparison between experimental and predicted values of **(A)** biomass concentration, **(B)** CPC_{conc}, and **(C)** CPC_{cont}.
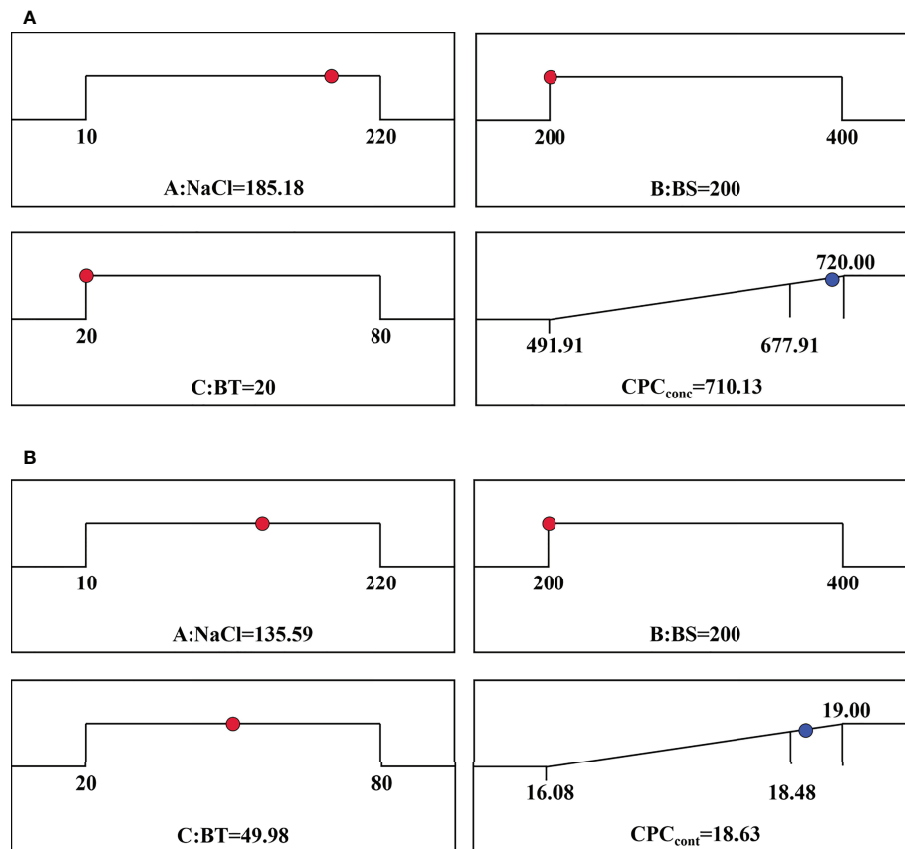
FIGURE 4
Optimal conditions predicted by the models for **(A)** CPC$_{conc}$ and **(B)** CPC$_{cont}$.

4 Discussion

Three factors (NaCl, BS, and BT) were chosen in this study to evaluate their impacts on the growth and phycocyanin production of *Arthrospira maxima*. The RSM model of algal biomass revealed that two of the factors, NaCl and BT, had a more substantial impact in comparison with BS concentration. The results of microalgae growth indicated that higher NaCl concentrations (100-220 mM) were advantageous for microalgae growth, which was basically consistent with previous studies (Abd El-Baky and El-Baroty, 2012). They reported that the medium containing 100 mM of NaCl can dramatically increase the growth of *Arthrospira maxima* as compared to control (20 mM NaCl), and the corresponding specific growth rate increased from 0.089 to 0.097 d^{-1}. Nevertheless, cell proliferation of *Arthrospira platensis* was sharply slowed down when the concentration of NaCl raised above 500 mM because of more severe detrimental effect of salt stress (Chen and Tang, 2012). The content of chlorophyll and carotenoid in algal cell was also decreased, which consequentially resulted in declining of photosynthetic rate. The culture experiment results

demonstrated that the addition of BT into medium was beneficial to microalgae growth. BT can be considered as nitrogen source as the primary component of BT is polypeptide. Nitrogen source is essential for cell growth and involved in the synthesis of amino acids, purines, pyrimidines, and other compounds used in cellular metabolism (Li et al., 2018). Hence, the increase of nitrogen concentration in a certain range can efficiently encourage the growth of microalgae (Mousavi et al., 2022). As the nitrogen concentration (NaNO$_3$) increased from 0.625 to 2.5 g L^{-1}, the biomass of *Arthrospira platensis* enhanced by 1.25 times at the culture temperature of 35°C (Can et al., 2017). In addition to nitrogen concentration, the type of nitrogen source was also highly affected the microalgae growth. Costa et al. (2001) observed that the optimal order of the nitrogen type for the growth of *Arthrospira platensis* was sodium nitrate, ammonium nitrate, acid ammonium phosphate, urea, ammonium sulphate, and ammonium chloride, at the same concentration of 10 mM. Interestingly, this order greatly changed when the nitrogen source concentration increased up to 30 and 50 mM, indicating the effect of nitrogen source on microalgae growth

depended on its concentration. Besides, exogenous addition of organic nitrogen source into culture medium is a effective approach to enhance microalgae growth. Shanthi et al. (2018) found that the biomass productivity of *Arthrospira platensis* increased to 175, 153 and 144 mg L^{-1} d^{-1} by addition of L-Arginine, L-Asparagine and L-Glutamine, respectively. These growth rates were significantly higher than 114 mg L^{-1} d^{-1} obtained in the control group. And, these findings also confirmed that the type of nitrogen source (structure of amino acids) influenced the microalgae growth. Manirafasha et al. (2018) examined the effect of various concentration of sodium glutamate additionally added into Zarrouk medium on the growth of *Arthrospira platensis*, and found that the biomass concentration at 4 mM concentration of sodium glutamate reached 2.54 g L^{-1} that is much higher than ~1.4 g L^{-1} obtained in the control. In the present study, overall, algal biomass was gradually increased as the BT concentration increased, which is probably due to the beneficial impact of multiple amino acids degraded from polypeptide. In contrast, there was a little influence of BS on microalgae growth (p>0.05), which was possibly attributed to the sufficient minerals already existed in the Zarrouk medium. Further increased in minerals concentration did not allow the increase of microalgae growth.

NaCl had a huge impact on phycocyanin accumulation, as seen by the 3D surface plots and contour plots (Figures 2D–F). The phycocyanin concentration peaked when the NaCl concentration was 136 mM. Enhanced production of phycocyanin by NaCl is mostly related to the growth performance of microalgae and the reflection of phycocyanin synthesis metabolism to salt stress. On one hand, the increase in phycocyanin concentration caused by the ongoing accumulation of biomass in the region of 100-200 mM NaCl concentration. On the other hand, the phycocyanin concentration was associated with intracellular phycocyanin content. The increase of phycocyanin content can facilitate the production of phycocyanin. Phycocyanin can be generated in greater numbers like some antioxidant enzyme to resist damage from reactive oxygen radicals caused by environmental stresses (Singh et al., 2018; Gauthier et al., 2020). But an excessive concentration of NaCl will inhibit the synthesis of phycocyanin. Electron transport is hampered by extreme salt stress, which could halt the photosystem II activity and cause the amount of phycocyanin to decrease (Kirst, 1990).

However, the adaptability and domestication of *Arthrospira* can be used to overcome the challenge of high concentrations of NaCl impeding microalgae growth and phycocyanin. (Tredici et al., 1986; Hagemann, 2011). Liu et al. (2016) domesticated two strains of *Arthrospira platensis* (SP843 and SP972) using 200-800 mM of NaCl, and observed that they grew well in the medium containing 200 and 400 mM of NaCl, respectively. Meanwhile, the concentrations of phycocyanin increased by 180% and 157%, respectively, in comparison to the control. After salt acclimation, *Arthrospira* has a better capacity to produce phycocyanin at high

salt concentration. Thereby, *Arthrospira* can be domesticated to increase its tolerance to salt stress, which can help *Arthrospira* culture in locations where excessive salinity makes it difficult.

As a nitrogen source, BT was conducive to the microalgae growth and phycocyanin production. In addition to affecting the growth of *Arthrospira*, the nitrogen source also affected the synthesis of phycocyanin. Nitrogen deficiency will limit the production of proteins associated with photosynthetic activity such as phycocyanin (Li et al., 2018). Thus, the increase of nitrogen concentration will improve the production of phycocyanin. The types of nitrogen sources also had an impact on phycocyanin accumulation. Gladfelter et al. (2022) discovered that compared to nitrate, the reduced nitrogen forms (ammonium/ammonia and urea) can much more promote algae growth and phycocyanin accumulation. In addition, in a research on improving the sources of nitrogen in the culture medium, the phycocyanin content was increased by 23% after optimizing the medium that included soda ash production wastes, NaNO$_3$, KCl and KH$_2$PO$_4$ (Xie and Wang, 2017).

In this work, the addition of BS into the medium did not exert a obvious positive effect to the phycocyanin production. This result slightly differed from the findings of the other previous study. Mostly researches showed that the addition of mineral components can promote the phycocyanin accumulation. Compared to the control (Zarrouk medium), phycocyanin concentration increased by 1.6 times when the modified Zarrouk medium containing more Ca^{2+} from oyster shell and soil extract was used (Jung et al., 2014). A low concentration (<0.3 µg mL^{-1}) of Ni increased the content of phycocyanin in Microcystis aeruginosa (Liu et al., 2005). It was reported that the dual effects of Se on promoting growth at low concentrations while also being toxic at high concentrations to *Arthrospira* (Chen et al., 2007). The addition of Se increased the phycocyanin concentration of *Arthrospira maxima* from 226 to 274 mg L^{-1} which was 21.2% higher than that in the control group (Chen et al., 2006). Most studies on the influence of mineral elements on phycocyanin presently focused on a single factor, such as Ni, Ca and Fe (Sloth et al., 2006; Velu et al., 2019; Rawat et al., 2022; Yang et al., 2022), and showed a good effect on phycocyanin. *Arthrospira* requires a small number of mineral components to grow. It is possibly that the conventional Zarrouk medium has enough minerals, to support the cell growth and phycocyanin production. Thus, the extra-addition of BS, a complex of different mineral elements, cannot generate significant positive impact, even showed inhibitory effect on phycocyanin production at a high concentration because of the resulted toxic of some metal elements.

In order to optimize the *Arthrospira* medium, we established the predicted models of algal biomass and phycocyanin production using response surface method. The maximum concentration (704.66 mg L^{-1}) and content (19.03%) of phycocyanin were successfully achieved using the ideal conditions predicted by this model. These values were 22.98% and 16.73% higher than those of the control group.

At present, there have been many studies on improving the production of phycocyanin, and the corresponding results are summarized in Table 5. It can be seen that the phycocyanin concentration varied greatly, which ranged from 100-1500 mg L^{-1}, with most of it concentrated in the range of 200-500 mg L^{-1}. Also, phycocyanin content had a large range that from ~5% to ~20%. This great variation in concentration and content of CPC was probably caused by the different culture condition employed in the different literature, such as algal strains, culture medium, reactor type, culture mode, temperature and light intensity. In the current study, the maximum CPC$_{conc}$ and CPC$_{cont}$ reached 704.66 mg L^{-1} and 19.03%, respectively, and these values were comparable to or even higher than the most results obtained in the previous studies. It is noteworthy, in the study conducted by Chaiklahan et al. (2022), that the phycocyanin content reached 19.88% that is slightly higher than our result (19.03%), whereas the phycocyanin productivity (123.66 mg L^{-1} d^{-1}) is much greater than 44.04 mg L^{-1} d^{-1} achieved by our study. The main reason was that they used the semi-continuous culture mode, in which the regular addition of fresh medium greatly ensured the nutrients required for the growth of microalgae, achieving a high biomass productivity (0.62 g L^{-1} d^{-1}) and consequent high phycocyanin productivity. In contrast, the batch mode was employed in the present study and the resulted biomass productivity was only 0.23 g L^{-1} d^{-1}. Surprisingly, the extreme high level of phycocyanin concentrations (>1000 mg L^{-1}) was attained by Zeng et al. (2012) and Xie et al. (2015). In the former

study, the intermittent supply of CO_2 provided sufficient carbon source to microalgae growth, thus achieving a very high biomass concentration (7.26 g L^{-1}). Xie et al. (2015) employed a feed-batch mode which is more efficient in terms of biomass accumulation compared to the batch mode, leading to a high biomass concentration (6.78 g L^{-1}) and consequent high phycocyanin concentration (1034.8 mg L^{-1}).

It should be noted that the conecntration of NaCl in the culture medium was increased and additional BS and BT were added after optimizaiton, which will increase the produce cost. Thereby, we briefly conducted a economic analysis as it is very important and essential. At the optimal condition achieving the maxiumum phycocyanin concentration, the phycocyanin production will additionally increase 0.13 g L^{-1} compared to the control. However, the extra additon of these substances in 1 L culture medium brought extra cost (~$0.03) that was calculated through multiplying the additional quantities of subtansces (NaCl, BS and BT) by their commerical prices. In terms of the market price of food-grade phycocyanin ($0.5 g^{-1}) (Güroy et al., 2017), the extra production of phycocyanin per liter (0.13 g) valued approximately $0.07, which exceeded the input cost ($0.03). Therefore, it would be cost-effective and economically feasible for the phycocyanin production after optimizaiton.

Overall, a high level of phycocyanin production was achieved by optimization of culture medium added by BT and BS based on RSM. However, this study used the mode of batch

TABLE 5 Summary of algal growth and phycocyanin production of *Arthrospira* reported in the literature and this study.

Number	Species	Special culture condition	Culture mode	Biomass concentration (g L^{-1})	Biomass productivity (g L^{-1} d^{-1})	CPC concentration (mg L^{-1})	CPC content (%)	Phycocyanin productivity (mg L^{-1} d^{-1})	Reference
1	*Arthrospira platensis*	$(NH_4)_2SO_4$ as nitrogen source	batch	1.04	0.07*	NA	11.3%	NA	Mousavi et al., 2022
2	*Arthrospira platensis*	addition of sodium glutamate	batch	8.00	1.14*	452.32*	5.65%	64.62*	Manirafasha et al., 2018
3	*Arthrospira platensis*	addition of succinic acid	batch	5.57	0.80*	288.86*	5.18%	41.27*	
4	*Arthrospira platensis*	addition of NaAC	batch	1.65	NA	226.05*	13.7%	NA	Chen et al., 2006
5	*Arthrospira platensis*	cultivation using blue light	batch	0.97*	0.19*	128.63	13.2%	25.73*	Chini Zittelli et al., 2022
6	*Arthrospira platensis*	intermittent CO_2 supply	batch	7.26*	0.40*	1220	16.8%	67.8	Zeng et al., 2012
7	*Arthrospira platensis*	synergistic effects of light intensity, photoperiod and cell density	semi-continuous	NA	0.62	NA	19.88%	123.66	Chaiklahan et al., 2022
8	*Arthrospira platensis*	optimization of light intensity and initial biomass concentration	feed-batch	6.78	0.588	1034.8	16.1%	94.8	Xie et al., 2015
9	*Arthrospira maxima*	NaCl stress and addition of BS and BT	batch	3.63	0.23	704.66	19.03%	44.04	This study

*It indicated that the values were calculated based on the relative data reported in the articles.
NA, the data is unavailable.

culture. Moreover, during the process, there was no additional carbon (such as CO_2) supplied into the medium. Therefore, more work could be performed in the next step to further increase the phycocyanin productivity by using feed-batch or semi-continuous mode, as well as providing additional carbon source based on the optimized medium.

5 Conclusions

In this study, the forecasting models has been established based on RSM, aiming to optimize culture conditions to enhance algal biomass and phycocyanin accumulation. According to the models, it was observed that NaCl was the most important factor to the responses. Meanwhile, the interactive effect of NaCl and BT had the greatest effect on phycocyanin production. The maximum values of phycocyanin concentration and content (704.66 mg L^{-1} and 19.03%) were reached at the respective optimal conditions determined by the models. Hence, BS and BT can be potentially considered as the great exogenous additives to promote *Arthrospira maxima* growth and phycocyanin synthesis. In the next step, semi-continuous or feed-batch mode as well as the supply of CO_2 can be used to further increase the productivity of algal biomass and phycocyanin.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

TY: conceptualization, methodology, data curation, formal analysis, visualization, and writing-original draft. JH: conceptualization, methodology, data curation, formal analysis, investigation, supervision and writing-review and editing, funding acquisition. BS: investigation, data curation, formal analysis. LW: methodology, data curation, formal analysis.

A-HZ: conceptualization, writing-review and editing, funding acquisition. D-FZ: funding acquisition, project administration. YZ: project administration, resources. GM: project administration, resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

YZ and GM were employed by Jiangsu Coast Development Group Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1057201/full#supplementary-material

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