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A Novel Bioflocculant Produced by *Cobetia marina* MCCC1113: Optimization of Fermentation Conditions by Response Surface Methodology and Evaluation of Flocculation Performance when Harvesting Microalgae

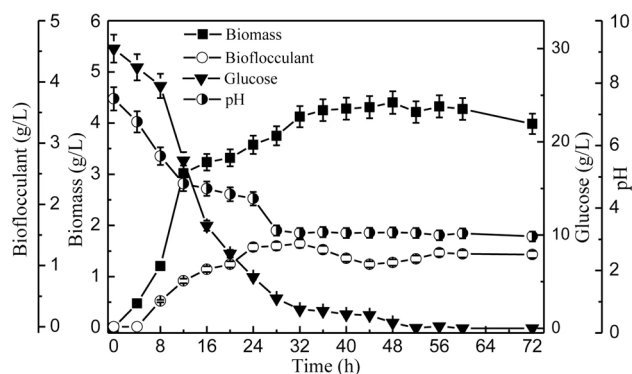SIYU ZENG[1], YINGHUA LU[2], XUESHAN PAN[2] and XUEPING LING[2]*

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Abstract

A preliminary study was carried out to optimize the culture medium conditions for producing a novel microbial flocculant from the marine bacterial species *Cobetia marina*. The optimal glucose, yeast extract, and glutamate contents were 30, 10, and 2 g/l, respectively, while the optimal initial pH of the culture medium was determined to be 8. Following response surface optimization, the maximum bioflocculant production level of 1.36 g/l was achieved, which was 43.40% higher than the original culture medium. Within 5 min, a 20.0% (v/v) dosage of the yielded bioflocculant applied to algal cultures resulted in the highest flocculating efficiency of 93.9% with *Spirulina platensis*. The bioflocculant from *C. marina* MCCC1113 may have promising application potential for highly productive microalgae collection, according to the findings of this study.

Introduction

Microalgal biomass is critical in biofuel research, with biomass harvesting as a critical step in achieving high biofuel efficiency. Compared to traditional techniques such as gravity filtration or centrifugation, the flocculation-based technique for algal cell harvesting is more convenient because a large amount of culture is available for treatment (Moradinejad et al. 2019). However, due to the problematic application, high cost (physical flocculation), and virulence (chemical flocculation) of the flocculation technique in microalgal biomass harvesting, the physical and chemical approaches of the flocculation technique are rendered inadequate (Cerff et al. 2012). Several studies have shown that incorporating bioflocculants or flocculant-producing microbes into microalgal cultures facilitates the bioflocculation process and increases the efficiency of microalgal harvesting for various species (Christenson and Sims 2011). Bioflocculants have received much attention in recent years as a promising alternative to chemical flocculants.

Bioflocculants are extracellular biopolymers that contain polysaccharides, proteins, glycoproteins, cellulose, lipids, nucleic acids, and glycolipids (Lian et al. 2008; Zheng et al. 2008). Environmental factors such as chemical or physical stress, substrate gradient, and life stages are known to cause the formation of bioflocculant (Chang an Su 2003). Nonetheless, using bioflocculants is fraught with difficulties in terms of flocculating activity, bioflocculant yield, and cultivation cost (Picciotto et al. 2021). These difficulties could be overcome by screening a large number of active bioflocculant-producing strains and optimizing their culture

conditions to obtain novel natural and environmentally friendly bioflocculants at high yields via the fermentation process (Yi et al. 2016). Bioflocculants derived from *Rhodococcus* species are useful in bioremediation processes such as cell separation during fermentation (Peng et al. 2007).

Marine bacteria are one of the most biotechnologically and economically valuable microorganisms, accounting for half of all bioactive secondary metabolites discovered (Bérdy 2005). *Cobetia marina* is a Gram-negative bacterium isolated from seawater along the coast. The type of marine bacteria can produce massive amounts of extracellular products (Shea et al. 1991; Romanenko et al. 2019). In 2012 a study discovered a bioflocculant activity of more than 90% with thermal stability produced by a marine bacterium of the genus *Cobetia*. The bacterial strain was isolated from sediment samples taken from Algoa Bay in South Africa's Eastern Cape Province. Ugbenyen et al. (2012) suggested that underutilized habitats, such as the marine environment, should be investigated as potential sources of novel bioactive compounds. However, this requires a comparative study involving the optimization of flocculation conditions to improve flocculating activity and bioflocculant production from *C. marina*.

In this context, the current study focused on optimizing the culture medium of a novel marine bacterial species, *C. marina* MCCC1113, to obtain a high yield of bioflocculant from these bacteria. The effects of the carbon source, nitrogen source, and the culture medium's initial pH were investigated. Response surface methodology (RSM) was used to optimize the fermentation conditions for bioflocculant production. In addition, the bioflocculant derived from *C. marina* MCCC1113 was used to harvest *Spirulina platensis*, *Chlorella vulgaris*, and *Haematococcus pluvialis*. The current study proposes a bio-friendly method for harvesting microalgae that will aid in developing greater competitiveness in the biofuel manufacturing industry in the future energy market.

Experimental

Materials and Methods

Bacterial culture. *C. marina* MCCC1113, a bioflocculant-producing strain isolated from west-Pacific Ocean hypobenthile sediment, was obtained from the Marine Culture Collection of China (MCCC). The strain was first cultured in an agar plate medium containing: tryptone 10 g/l, yeast extract 5 g/l, NaCl 30 g/l, and agar 20 g/l (Lei et al. 2015). The *C. marina* MCCC1113 inoculum from the agar plate culture was inoculated in a 100 ml Erlenmeyer flask with 30 ml of seed medium.

The final inocula for use at a dosage of 10% (v/v) were prepared in 50 ml Erlenmeyer flasks containing 10 ml of the seed medium and incubated for 36 h inside a rotary shaker at 150 rpm and 28°C. The seed medium for the strain contained 10 g/l tryptone, 5 g/l yeast extract, and 30 g/l NaCl. Then, 10 ml of the seed culture was inoculated into 50 ml of minimal medium, and the flasks were shaken on a rotary shaker at 150 rpm, 28°C for 48 h. At 8 h intervals, samples (3×50 ml (three shaking flasks) at a time) were taken, and cell mass, bioflocculant yield, and glucose consumption were measured. All values presented in this article result from at least three independent experiments. The minimal medium contained 10 g/l of glucose, 5 g/l of tryptone, and 1 g/l of yeast extract, all diluted in a mixture of artificial seawater (80% (v/v) and distilled water (20% (v/v). The artificial seawater had previously been prepared by mixing as follows: NaCl 24 g/l, $MgCl_2 \cdot 6H_2O$ 11 g/l, Na_2SO_4 4 g/l, $CaCl_2 \cdot 6H_2O$ 2 g/l, KCl 0.7 g/l, KBr 0.1 g/l, H_3BO_3 0.003 g/l, $Na_2SiO_3 \cdot 9H_2O$ 0.005 g/l, $SrCl_2 \cdot 6H_2O$ 0.004 g/l, NaF 0.003 g/l, NH_4NO_3 0.002 g/l in water. The optimal medium contained 30 g/l of glucose, 10 g/l of yeast extract, and 2 g/l of glutamate, all diluted in a mixture of artificial seawater (80% (v/v) and distilled water (20% (v/v).

After preparing the medium in distilled water and adjusting the initial pH to 8.0 with 2 M HCl or 2 M NaOH, it was subjected to a 20-minute disinfection process at 121°C.

Microalgal culture. The microalga *C. vulgaris* was procured from the Algal Culture Collection, Ji'nan University, China. The alga was cultured for 7–12 days in BG-11 medium (Al-Rikabey and Al-Mayah 2018) at 28°C, 2.5% CO_2 atmosphere, 12-h light/12-h dark photoperiod, and the illumination intensity of 300 μmol photons/m²s (Sun et al. 2015).

Arthrospira (*S.*) *platensis* was obtained from the IHB (Institute of Hydrobiology), CAS (Wuhan, China) and pre-cultured in the Zarrouk medium, which had previously been prepared according to the method described by (Xie et al. 2020). In the PBR (photobioreactor), which served as the glass vessel, all precultures and production cultures were grown in Zarrouk medium (1 l). The production cultures were incubated for 10–12 days at 28°C under persistent lighting (300 μmol photons/m²s), with the external light source being T5 ESSENTIAL white LED lamps (21 W; Philips Co., China) mounted on both sides of the PBR. The pH of the culture was kept constant at 9 by continuously injecting 2.5% CO_2 at a rate of 0.2 v/v (Xie et al. 2020).

H. pluvialis was cultured in Bold's basal medium with a few modifications (the addition of 1.0 ppm of thiamine, 2.5 ppm of biotin, and 1.5 ppm of vitamin B_{12}) inside a PBR at 23°C for 7 days with a continuous injection of 2.5% CO_2 at an aeration rate of 0.2 v/v (Ma et al. 2020).

Dry cell weight and sugar consumption determination. The bacterial broth was centrifuged at $9,000 \times g$ for 20 min at 4°C, and the pellet containing insoluble materials was washed with distilled water to remove any remaining medium salts. The pellet containing the bacterial cells was then resuspended in 10 ml distilled water and centrifuged as previously described. The pellet was dried at 60°C for 24 h until it reached a constant weight. Each sample was obtained in threes. The supernatant was also kept for further study. The glucose concentration was determined using the dinitrosalicylic acid method (Miller 1959).

Production and purification of bioflocculant. The bioflocculant was purified using a modified version of the method described by Wang et al. (2013). With gentle shaking, the supernatant obtained at the end of this procedure was gradually added to a 2-fold volume of cold ethanol and then left undisturbed overnight at 4°C. The mixture was centrifuged at $9,000 \times g$ for 20 min at 4°C the next day, and the supernatant was discarded while the pellet was lyophilized to yield the crude bioflocculant. The crude bioflocculant was then dissolved in distilled water, and 2% CPC (cetylpyridinium chloride) was added to the aqueous solution while stirring. A few hours later, the centrifuged precipitate and the CPC complex were dissolved in NaCl (0.5 M). The precipitate obtained after adding a 2-fold volume of cold ethanol was washed with ethanol and then lyophilized to obtain the purified bioflocculant. All procedures were carried out in triplicate.

Determination of the flocculation efficiency of bioflocculant. *C. marina* MCCC1113 was inoculated in 150 ml of the optimal medium and cultured for 32 h at 28°C and 150 rpm until it reached the stationary phase. As previously stated, the bioflocculant was purified. Fifty mg/l of purified bioflocculant was added to 200 ml of microalgal fermentation culture containing 2.5 ml of $CaCl_2$ solution, followed by gentle mixing for 30 s at room temperature and then leaving the mixture undisturbed for 15 min. Meanwhile, a control experiment was set up without the bioflocculant. An aliquot of culture was taken from the upper phase at one-third height and subjected to turbidity decline analysis. The flocculation efficiency was calculated using the equation reported by Lei et al. (2015), which is provided below:

$$\text{Flocculation efficiency (\%)} = \frac{A - B}{A} \times 100 \qquad (1)$$

In the above equation, A and B denote the optical density of the microalgal culture sample at 550 nm prior to and after flocculation, respectively. All measurements were undertaken in triplicate.

Flocculation experiment of supernatant of the fermentation broth. Concentration (*H. pluvialis*) and dilution were used to keep the concentration of the three types of algae within a specific range (OD value of about 1). (*S. platensis* and *C. vulgaris*). *C. marina* MCCC1113 fermented for 24 h in minimal medium. The fermentation liquid supernatant and the algal liquid were mixed in a 1:10 volume ratio, and 0.5 mm $CaCl_2$ was added as a coagulant aid. Shake the bacteria gently for 20 s and set aside for 5 min before removing the liquid supernatant.

For these three types of microalgae, a volume ratio optimization experiment was performed, and the flocculation efficiency was determined when the volume ratio of supernatant and the algal liquid was 5%, 10%, 15%, and 20%, respectively. The optical density at 680 nm (OD_{680}) after flocculation was used to calculate the efficiency of flocculation using the equation (1) provided above. A and B in the equation represent the OD_{680} of the microalgal culture before and after adding supernatant, respectively. In the same process, optical density (OD_{680}) was measured in a control experiment without bioflocculant. All measurements were taken in triplicate.

Plackett-Burman design. Plackett-Burman experiments were carried out with the Design-Expert 8.0 (Stat-Ease Inc., USA) software to identify the independent factors that were significantly associated with the fermentation of the bioflocculant produced by *C. marina* MCCC1113.

A 5-factor-2-level block design with glucose, yeast extract, glutamate, culture duration, and pH of the culture medium as independent factors (Table I) was created. According to the Plackett-Burman design, glucose, yeast extract, and glutamate concentrations were significant independent factors. Therefore, the standard RSM was used to evaluate the main operating parameters of the culture medium [glucose (A), yeast extract (B), and glutamate dosage (C)] during the fermentation process.

Statistical analyses. The 3-level-3-factor Box-Behnken Design (BBD) and a standard RSM were used to evaluate the main operating variables [glucose (A), yeast extract (B), and glutamate dosage (C)] in the culture medium during the fermentation process. At the same time, a model was built concurrently based

Table I
Effect analysis of independent variables in Plackett-Burman design.

Factor	Level		p	Significant
	−1	1		
Glucose	25	35	0.0022	++
Yeast extract	5	15	0.004	++
Glutamate	1	3	0.025	+
Culture time	28	36	0.85	−
pH	7	8	0.861	−

++ – extremely significant influence on the results of the experiment ($p < 0.01$)

+ – significant influence on the results of the experiment ($p < 0.05$)

on the BBD equation (Jeganathan et al. 2014). The following parameters were set based on preliminary experimental findings: glucose dosage 25–35 g/l, yeast extract 5–15 g/l, and glutamate 1–3 g/l. The current study included 17 trials in which the independent factors were investigated at three levels, namely the low (–1), medium (0), and high (+1) levels. The bioflocculant yield achieved in the simulated trials was defined as the response parameter (Y). All experiments were carried out in triplicate.

The second-order polynomial equation was used to analyze the RSM-based experimental data with Design-Expert 8.0. To evaluate the analytical model qualitatively, diagnostic analysis and ANOVA were used. The response parameter (Y) was fitted using a polynomial quadratic version of the second-order model:

$$Y = \beta_0 + \sum \beta_i X_i + \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \qquad (2)$$

where Y denotes the predicted response, β_0 denotes the intercept, β_i and β_{ii} denote the linear and quadratic coefficients, respectively, X_i and X_j denote the input influencing parameters of Y, respectively, and β_{ij} denotes the linear interaction between the regression factors X_i and X_j.

Data were analyzed by the analysis of variance (ANOVA) for the response surface model. A significance level of $p < 0.05$ was used. The mean values are from three replicates.

Results

Effect of the initial concentration of carbon source on cell growth and bioflocculant production. *Dynamic courses of the bioflocculant yield.* Figure 1a depicts time courses of bioflocculant yield at various initial glucose concentrations. The bioflocculant began to accumulate at the start of the exponential phase and reached a peak at 32 h, after which the accumulation rate slowed in the late stationary phase. The biomass content of the bioflocculant peaked at 32 h at initial glucose levels of 40 and 50 g/l, after which it declined. The maximum bioflocculant yield of 0.92 g/l was obtained after 32 h of culture at an initial glucose level of 30 g/l. After which, the yield stabilized at the beginning of the stationary phase. This bioflocculant yield was approximately 13.04–18.48% greater than that obtained with other initial glucose concentrations.

Dynamic courses of biomass accumulation at different initial concentrations of glucose. The biomass yields at various initial glucose concentrations are shown in Fig. 1b. Higher initial glucose concentrations slowed bacterial growth in the early hours of cultivation (before 12 h). Nonetheless, the experimental groups entered the exponential growth phase quickly, and bacterial cell growth in the different groups was similar and at comparable levels during the exponential phase of the culture. Higher initial glucose concentrations also

Note: a, b, c
■ Glucose = 30 g/L
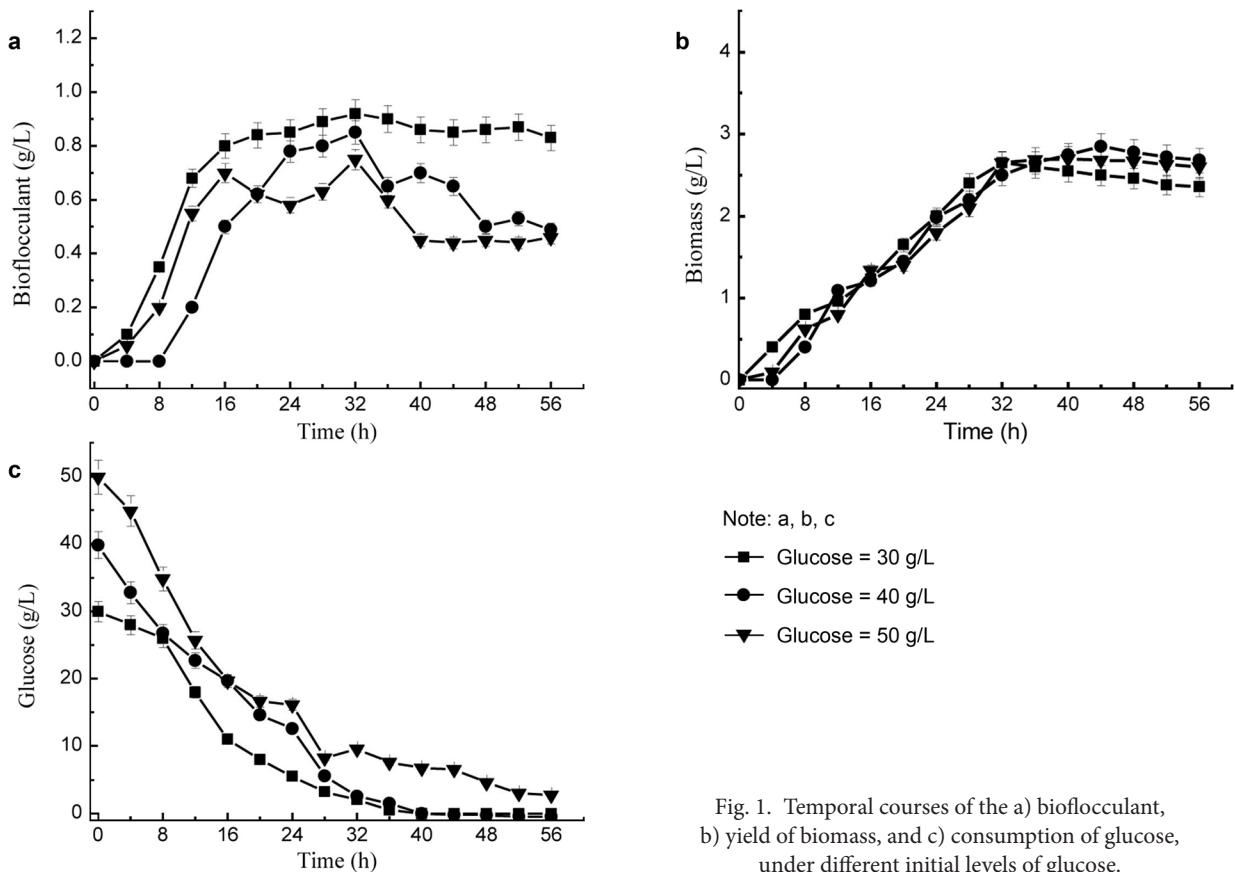● Glucose = 40 g/L
▼ Glucose = 50 g/L

Fig. 1. Temporal courses of the a) bioflocculant, b) yield of biomass, and c) consumption of glucose, under different initial levels of glucose.

resulted in slower bacterial growth in the stationary phase of culture, despite a high biomass yield. At initial glucose concentrations of 30 g/l and 40 g/l, the weight of dry biomass peaked at 32 h and 48 h, respectively, and a decline in biomass yield was observed after these time points. The biomass yield was maximum at 48 h at an initial glucose concentration of 50 g/l, after which the biomass continued accumulating due to sufficient glucose retention in the medium for growth. Despite rapid bacterial growth at low initial glucose levels during the early cultivation stage, a high biomass yield of 2.85 g/l at an initial glucose level of 40 g/l was obtained.

Dynamic courses of glucose consumption at different initial concentrations of glucose. The trend of glucose consumption, as shown in Fig. 1c, was consistent across all groups, demonstrating a gradual decrease from the initial value to around 3 g/l. The glucose uptake was rapid in the early hours of culture (24 h), but it decelerated later in the culture. At initial glucose levels of 30 g/l and 40 g/l, total glucose consumption was observed in 32 h, with a residual content of less than 2 g/l. Compared to the other groups at an initial glucose level of 50 g/l, there was a greater concentration of residual glucose, approximately 10 g/l, in the medium at 32 h, which was gradually consumed until the end.

Effect of different nitrogen sources on biomass and bioflocculant production. Based on the literature (Liu and Cheng 2010; Cosa et al. 2011; Nontembiso et al. 2011) and our preliminary experiment, six types of nitrogen sources (A/YE – yeast extract, B/Glu – glutamate, C – tryptone, D – ammonium nitrate, E – ammonia chloride, F – urea; the control group was cultured in minimal medium) were first tested individually to determine the optimal single nitrogen source in the flask culture. Whereafter, the chosen optimal single nitrogen source was tested as the proper nitrogen source mix based on the different weight ratios as a complex nitrogen source was studied for improving biomass and bioflocculant yield of *C. marina* MCCC1113 from the flask culture to the scale-up bioreactor culture.

Effect of using a single nitrogen source on biomass and bioflocculant production. Six nitrogen sources were evaluated for biomass and bioflocculant production after 36 h of growth in a minimal medium (Fig. 2a). Both yeast extract and glutamate aided cell growth and bioflocculant production. Yeast extract produced the most bioflocculant (0.98 g/l), while glutamate produced the most biomass (2.6 g/l), with both values significantly higher than those obtained in the other groups which used tryptone, NH_4NO_3, NH_4Cl, or urea as the nitrogen source.

Effects of using a combination of nitrogen sources on biomass and bioflocculant production. Based on the results shown in Fig. 2a, the combination of yeast extract and glutamate in various weight ratios ranging
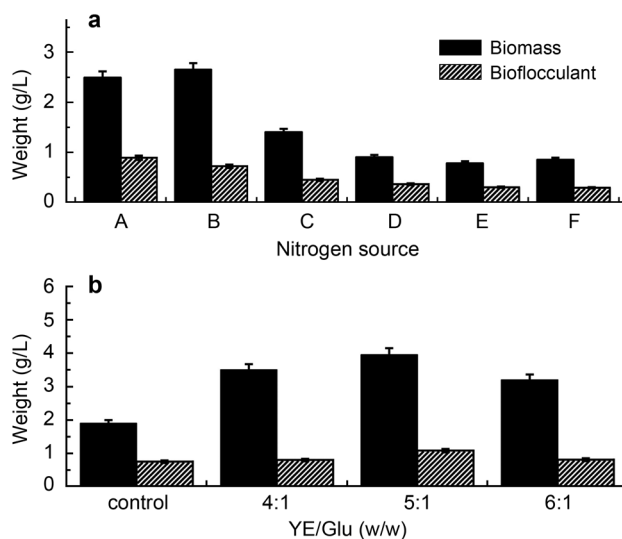

Fig. 2. a) Effects of the N source alone on the biomass and bioflocculant production, b) effect of the ratio of combined N source on the biomass and bioflocculant production.
A/YE – yeast extract, B/Glu – glutamate, C – tryptone, D – ammonium nitrate, E – ammonia chloride, F – urea. The control group was cultured in a minimal medium.

from 4:1 to 6:1 was considered for use as the nitrogen source in the following experiment. As shown in Fig. 2b, the 5:1 (w/w) ratio of yeast extract and glutamate produced the highest values for biomass and bioflocculant, which were 3.05 g/l and 1.06 g/l, respectively. Both values were higher than the control group. The minimal medium culture was used as the control group. Furthermore, biomass and bioflocculant production were higher in the culture using a single nitrogen source. Therefore, this combined nitrogen source medium was chosen as the best medium to use in subsequent experiments.

Effect of initial pH on biomass and bioflocculant production. Fig. 3a and 3b depicts the variations in biomass, pH value, and bioflocculant yields at various initial pH values. Each group was cultured for 32 h in the optimal medium inside a rotary shaker at 28°C and 150 rpm. The final pH value stabilized at around 4 ± 0.5, as shown in Fig. 3a. Furthermore, a pH of 8 resulted in the highest biomass (3.54 g/l) and bioflocculant (1.15 g/l) production (Fig. 3b). *C. marina* MCCC1113 produced bioflocculant in the pH range of 4–10, and a weakly basic medium was more conducive to this bioflocculant production.

Using RSM for optimization. A 5-factor 2-level block design with glucose, yeast extract, glutamate, culture duration, and pH of the culture medium as independent factors (Table I) was created. According to the Plackett-Burman design, the concentrations of glucose, yeast extract, and glutamate were significant independent factors. Therefore, the standard RSM was used to evaluate the main operating parameters of the culture

Table II
Box-Behnken design arrangement and responses.

RUN	Glucose (g/l)	Yeast extract (g/l)	Glutamate (g/l)	Bioflocculant (g/l)
1	30	10	2	1.30
2	30	10	2	1.19
3	30	5	1	0.9
4	35	5	2	0.65
5	30	15	3	0.95
6	30	10	2	0.89
7	35	10	3	0.79
8	25	15	2	0.57
9	30	5	3	0.87
10	25	10	1	0.32
11	25	5	2	0.54
12	35	10	1	0.79
13	30	10	2	1.22
14	30	10	2	1.28
15	30	15	1	0.92
16	25	10	3	0.58
17	35	15	2	0.65

Fig. 3. The change curve of pH for the a) biomass and b) bioflocculant.

medium [glucose (A), yeast extract (B), and glutamate dosage (C)] during the fermentation process. Finally, a model was built using the Box-Behnken Design equation (Table II).

The response parameter (Y) was fitted using a quadratic polynomial version of the second-order model provided below:

$$Y = 1.05 + 0.11A + 0.016B + 0.032C - 0.003AB - 0.065AC + 0.015BC - 0.37A^2 \quad (3)$$

where Y denotes the yield of the bioflocculant, and A, B, and C denote the contents (g/l) of glucose, yeast extract, and glutamate, respectively.

Box-Behnken design and seventeen groups of different research parameter combinations of experimental data fitting yielded the prediction model equation (3). The quadratic coefficient is negative in equation (3), and the parabolic surface represented by it opens downward, indicating that the equation has a maximum value.
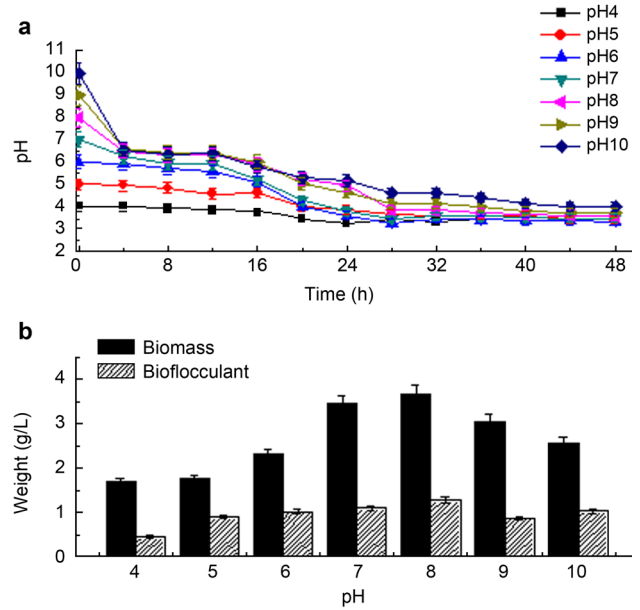
In the ANOVA results for the quadratic polynomial equation shown in Table III, the model had low p-values and high F-values ($p < 0.001$).

The correlations between the response and the designed levels of each factor were visualized by expressing the fitted polynomial equation as 3D surface plots, as were the inter-parameter interactions. The three-dimensional response surface plot and two-dimensional contour plot (Fig. 4a–4f) can intuitively represent the degree of influence of various influencing factors (yeast extract, glucose, glutamate) on the response value (bioflocculant). The mutual effect of yeast extract and glucose is shown in Fig. 4a–4b, which shows that the surface effect of yeast extract and glucose is close to the peak when the yeast extract is 10 g/l and the glucose is 30 g/l. The contour line is oval, indicating that the factors interact significantly. Similarly, Fig. 4c–4d depict the interaction of glutamate and glucose. The response surface diagrams are convex with downward openings, and the contour line is oval, indicating that the factors interact significantly. In contrast, Fig. 4e–4f show the interaction of glutamate and yeast

Table III
ANOVA for response surface quadratic model.

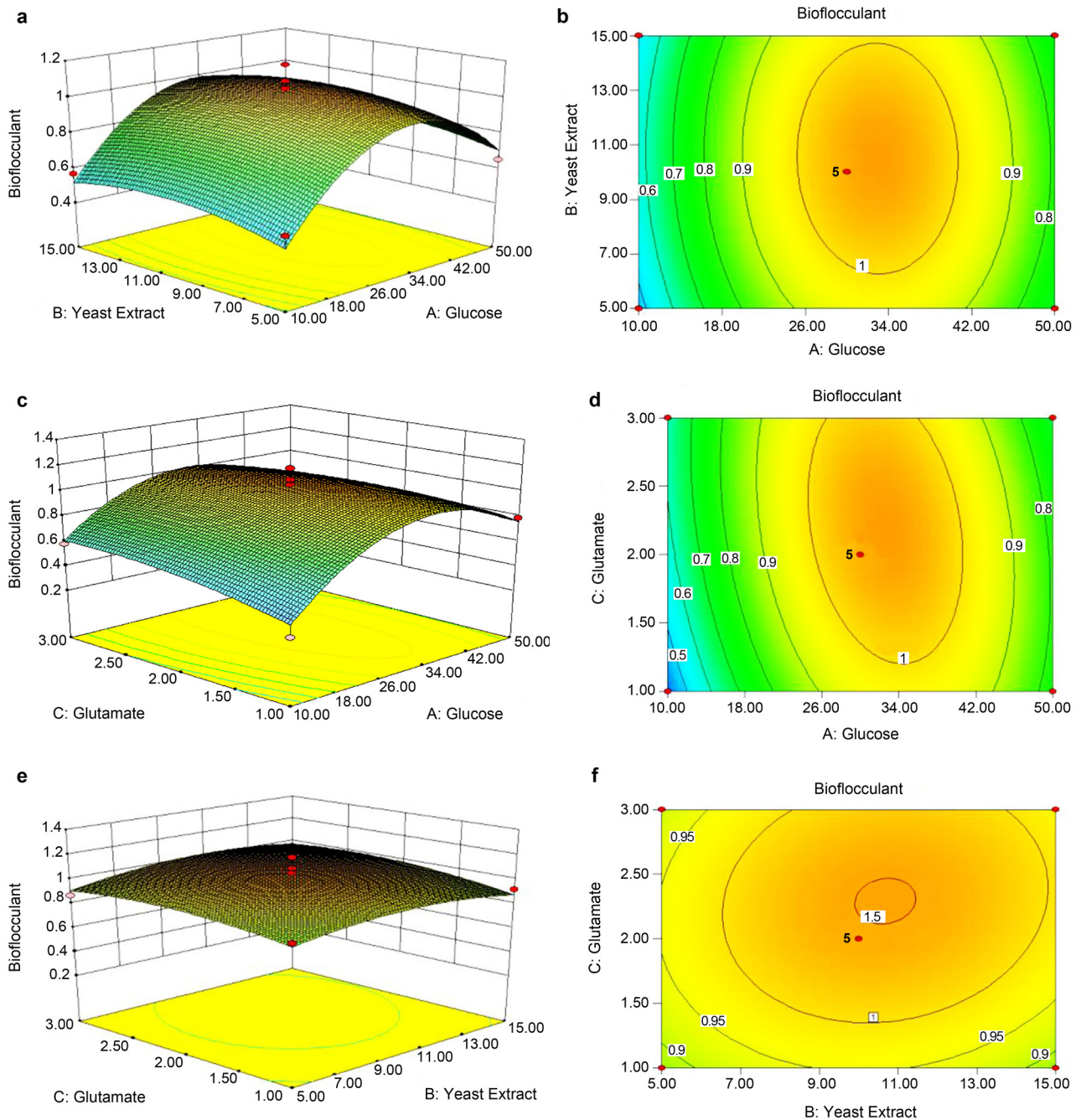| Source	Sum of square	DF	Mean square	F-value	Prob $> F$
Model	0.76	9	0.084	7.02	0.0088
Residual	0.084	7	0.012		
Lack of fit	0.039	3	0.013	1.16	0.4282
Pure error	0.045	4			
Cor total	0.84	16			
$R^2 = 0.9803$	$R^2_{adj} = 0.7721$	$R^2_{pred} = 0.1744$	CV = 13.53	Adeq. precision = 7.526	

Fig. 4. RSM and contour plots illustrating the interplaying effects of various factors on bioflocculant production; a–b) glucose (g/l) and yeast extract (g/l), c–d) glucose (g/l) and glutamate (g/l), e–f) yeast extract (g/l) and glutamate (g/l).

extract. The 2D contour lines resemble circles, indicating that the interaction between factors is insignificant and has little influence on bioflocculant yield.

The optimal fermentation conditions for *C. marina* MCCC1113 were determined using the "numerical optimization" function of Design-Expert 8.0 based on equation (3). The predicted yield of bioflocculant for 30 g/l glucose, 10 g/l yeast extract, and 2 g/l glutamate was 1.28 g/l. Glucose was the most critical factor in the fermentation process out of the three.

Verification of the optimal condition. The optimal concentration determined by RSM was used in subse-

quent experiments, the results of which are shown in Fig. 5. The bioflocculant yield of *C. marina* MCCC1113 was 1.36 g/l, which was close to the RSM-predicted bioflocculant yield of 1.28 g/l. The biomass increased as the glucose was consumed, reaching a maximum of 4.4 g/l when the glucose was depleted. The pH of the culture broth decreased as the cells grew exponentially and then stabilized after 28 h of culture.

Preliminary determination of the flocculating efficiency of bioflocculant produced from *C. marina* MCCC1113. In this part of the experiment, we attempted to observe the interaction between supernatant
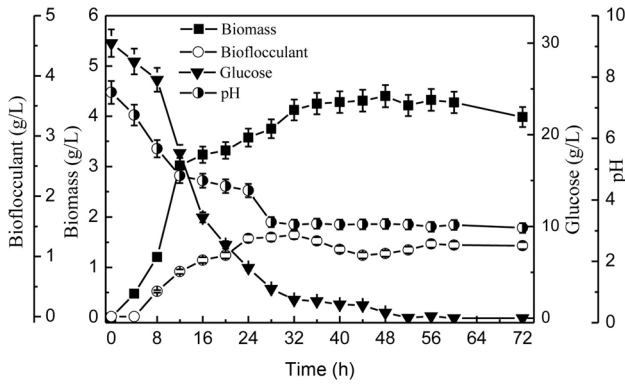
Fig. 5. Temporal courses of cellular growth, bioflocculant production, and pH for *C. marina* MCCC1113 cultivated in the optimal medium inside a rotary shaker at 150 rpm and 28°C for 72 h.

and microalgae by conducting a preliminary study on the flocculation efficiency of the supernatant broth on three different types of microalgae rather than using crude or purified bioflocculant.

The results presented in Table IV suggested that the bioflocculant produced from *C. marina* MCCC1113 offered good flocculation efficiency for *S. platensis* (69.4%) within 5 min. At the same time, the flocculation efficiency of the bioflocculant for *H. pluvialis* in 5 min was 32.1%, and the flocculation efficiency of *C. vulgaris* negligible.

Table IV
Flocculation efficiency of bioflocculant produced
by *C. marina* on various microalgae.

Flocculation time (min)	Group	*H. pluvialis*	*C. vulgaris*	*S. platensis*
		FE (%)		
5	Treatment	32.1	–	69.4
	Control	26.4	–	32.9

FE – flocculating efficiency
Flocculation efficiency was measured by the optical density at 680 nm (OD_{680}) after flocculation. A control experiment without bioflocculant was done in the same process and optical density (OD_{680}) was measured. All measurements were carried out in triplicates.

Table V
Determination of the optimum ratio
of bioflocculant/microalgae (v/v).

FE (%) of different algae	Amount of BF/algae added (%)				
	0	5	10	15	20
H. pluvialis	20	34.8	36.7	47.2	63
S. platensis	32.9	86.4	87.8	89.4	93.9
C. vulgaris	29.1	48.1	53.2	53.1	51.1

BF – bioflocculant, FE – flocculating efficiency
The bacteria culture supernatant was added to the algal cultures at proportions of 0.0% (v/v), 5.0% (v/v), 10.0% (v/v), 15.0% (v/v), 20.0% (v/v), 5 mM $CaCl_2$ was added in every case. The original culture medium instead of fermentation medium as a control. All analyses were obtained in triplicate.

Effect the bioflocculant/microalgae (v/v) ratio. In a flocculation test, the effect of the bioflocculant-to-microalgal culture broth ratio was evaluated, and the results are shown in Table V. The main goal was to see how the bioflocculant/microalgae affected the bioflocculation efficiency of three common algae. The bioflocculant to microalgal culture broth ratio was 20.0% (v/v), resulting in the highest flocculating efficiency of 93.9% with *S. platensis* after 5 min. However, in the case of the other microalgae tested, this ratio was ineffective.

Discussion

The primary goal of this study was to determine the best culture conditions for *C. marina* MCCC1113 bioflocculant production and the flocculating efficiency of the produced bioflocculant for different microalgae. Changing the carbon source in the culture medium was found to have a significant impact on cellular growth and bioflocculant production. Glucose is a common carbon source used by a wide range of microorganisms (Colonia et al. 2021). Carbon sources in the culture medium required for bioflocculant production have been well reported to differ for different microorganisms (Gong et al. 2008; Liu et al. 2010; Cosa et al. 2011). Glucose was one of the most preferred and least expensive organic carbon sources, and its use in the production of bioflocculants has been documented (Lachhwani et al. 2005; Xia et al. 2008; Liu et al. 2010). Therefore, the glucose content of the culture medium was optimized first in this study.

According to the analysis of the dynamics depicted in Fig. 1a–1c, cell growth and bioflocculant accumulation were somewhat concurrent and could be linked to the glucose content of the medium. The bioflocculant accumulated with the growth of the bacteria and reached a peak at 32 h, after which the accumulation rate slowed in the late stationary phase. Different initial glucose concentrations had little effect on cell growth, whereas higher initial glucose concentrations resulted in higher levels of residual glucose in the medium. Lower initial glucose levels favored bioflocculant accumulation. The adequate initial glucose concentration for good cell growth and bioflocculant accumulation was 30 g/l of glucose, which was then chosen as the initial glucose concentration in the subsequent experiment for nitrogen source optimization in the medium.

In terms of the impact of nitrogen sources on bioflocculant production, it has been reported that nitrogen sources are an essential nutrient that boosts bioflocculant production, and microorganisms can produce bioflocculant using either organic or inorganic nitrogen sources (Liu et al. 2010; Cosa et al. 2011). Tryptone was favorable for bioflocculant production of *Chryseobacte-*

rium daeguense W6 (Liu et al. 2010). Nontembiso et al. (2011) reported that *Bacillus* sp. Gilbert produced bioflocculant using ammonium chloride, resulting in more than 90% flocculating activity. In the current study, the microorganism commonly utilized all the tested nitrogen sources as the sole nitrogen source in fermentation culture. *C. marina* MCCC1113 could effectively use yeast extract or glutamate as the sole nitrogen source in the culture medium. When yeast extract was used as the sole nitrogen source in the culture medium, bioflocculant production was the highest, and glutamate was used as the nitrogen source, yielding the highest biomass (Fig. 2a). Organic nitrogen sources accelerated the accumulation of flocculants more than inorganic nitrogen sources, with yeast extract and glutamate showing significant promotion. Therefore, the next step was to evaluate the combination of yeast extract and glutamate as a nitrogen source. The 5:1 (w/w) yeast extract to glutamate ratio used as a nitrogen source at a concentration of 10 g/l resulted in the highest biomass and bioflocculant production (Fig. 2b), which were 84.2% and 42.8% higher, respectively, than the original culture medium values. Therefore, these nutrient concentrations were chosen for preparing the optimized medium.

The pH of the culture medium is an important factor in the production of bioflocculant (Yokoh et al. 1996). Some studies have found a link between the medium initial pH and the production of bioflocculant by some microorganisms. *Bacillus licheniformis* X14 (Cosa et al. 2011) optimally produced bioflocculant in an alkaline pH environment, whereas *Serratia ficaria* (Gong et al. 2008) optimally produced bioflocculant in an acidic environment. Under neutral pH conditions, *Halomonas* sp. OKOH (Mabinya et al. 2011) produced a high yield of bioflocculant. According to Fig. 3a–3b, *C. marina* MCCC1113 produced bioflocculant in the pH range of 4–10, with the highest biomass and bioflocculant production at pH 8. This bioflocculant production was aided by a weakly basic medium. It is consistent with the findings of Liu et al. (2010) and Ugbenyen et al. (2014). pH affects bacterial adhesion during the late fermentation stage and influences biomass growth throughout the culture time. It is thought that pH value may influence hydrophobicity and bioflocculation (Juarez Tomás et al. 2002).

ANOVA (Table III) was used to optimize the culture conditions based on the Plackett-Burman design (Table I) and the BBD design (Table II). When the R^2 values were high, the experimental and predicted values matched very well. The analysis of variance (ANOVA) was used to evaluate the experimental data for better goodness (Table III), and the p-value revealed the model's significance ($p < 0.05$). The lack of fit (1.16) also implies that the result is not statistically significant based on the pure error. A detailed model is needed to fit the data if the p-value of lack of fit is significant (< 0.05). The R^2 value for an effective model should be close to 1, indicating an adequate model (Yuan et al. 2016).

Furthermore, R^2 (0.9803) indicates the suitability of the experimental results. The adjusted R^2 (R^2_{adj}) of 0.7721 and the Adeq. precision of 7.526 further validates the significance of the model. The R^2_{adj} (0.7721) was 77.21%, indicating that the independent factors contributed to the overall variation in the fermentation course, while the model could only interpret 22.79% of the overall variation. The prediction results suggested that the model could adequately describe correlations of the bioflocculant with the significant parameters. The established polynomial model is adequate for the results, and it implies a relationship between bioflocculant yield and factors (Garcia et al. 2020).

The RSM numerical optimization method was used in this study to determine the optimal component in the culture medium. Meanwhile, the upper and lower limits of the variable range are incorporated into the optimization procedure. The graphical representation of the regression equation produces a three-dimensional response surface plot, commonly used to show relationships between experimental and response levels of variables (Haider and Pakshirajan 2007). The steeper slope in the glucose graph (Fig. 4a–4b) implied that when compared to increasing the yeast extract content, increasing the glucose level would better facilitate bioflocculant production promotion. Glucose content had a more pronounced effect than glutamate (Fig. 4c–4d). The effects of yeast extract and glutamate content on bioflocculant production were similar (Fig. 4e–4f). The response surface diagrams are convex with downward openings, and the contour line is oval, indicating that the factors interact significantly.

Furthermore, ANOVA and other related tests were used to calculate the value of the equation for the RSM suitability assessment. The medium components for the bioflocculant's maximum predicted yield were 30 g/l of glucose, 10 g/l of yeast extract, and 2 g/l of glutamate, with an initial pH range of 7 to 8. The highest bioflocculant production value was 1.38 g/l, representing a 43.40% increase. The predicted conditions were experimentally acceptable and remarkably reproducible, laying the groundwork for future algae collection research.

The temporal courses of cellular growth, bioflocculant production, and pH for *C. marina* MCCC1113 cultured in the optimized medium determined by RSM optimization are depicted in Fig. 5. It was discovered that *C. marina* MCCC1113 bioflocculant production could be divided into two stages. Before 32 h of culture, the first stage was characterized by rapid cellular growth and bioflocculant synthesis, accompanied by rapid glucose consumption and a sharp drop in the pH of the culture medium. At 32 h, the glucose

was nearly depleted, and the highest bioflocculant yield had been achieved. After 32 h, the second stage of fermentation began, with a gradual increase in biomass but slight improvement in the bioflocculant production. After 36 h, the increase had stabilized. Furthermore, the pH of the culture medium remained constant at this stage. These phenomena could be attributed to a lack of the N and energy sources required for cellular growth (Li et al. 2020). A fed-batch culture with glucose may be appropriate for enhancing cell growth and bioflocculant production.

The preliminary flocculation efficiency of the *C. marina* MCCC1113 bioflocculant revealed effective in harvesting *S. platensis* (Table IV). The supernatant's ability to bioflocculate efficiently in a short period demonstrated that the bioflocculant produced by *C. marina* MCCC1113 has commercial potential. The highest flocculation efficiency of 93.9% within 5 min was achieved with a bioflocculant to microalgal broth culture ratio of 20.0% (v/v) for *S. platensis* cultures (Table V). This value was higher than that obtained for the *C. vulgaris* and *H. pluvialis* cultures tested in this study. Different bioflocculants may have different flocculation efficiencies for different microalgae. Another novel aspect of this section of the study is that even though many bioflocculants produced by marine bacteria have been reported for decades (Chang and Su 2003; Cosa, et al. 2011), there have been few bioflocculants produced by *C. marina* MCCC1113 that can perform effective bioflocculation on microalgae. The primary goal of this section of the paper was to show that the novel bioflocculant has the potential to improve flocculation capacity in a short time under controlled conditions.

Conclusion

The current study describes a new bioflocculant derived from *C. marina* MCCC1113. The optimal culture conditions for the production of this bioflocculant were determined, including the carbon source, nitrogen source, and pH, and the highest bioflocculant yield of 1.36 g/l was obtained. The novel bioflocculant was effective at flocculating *S. platensis*. Because microbial flocculants can be used in various fields in the future, this study lays the groundwork for relevant, comprehensive studies on the development and application of microbial bioflocculants.

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