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ORIGINAL ARTICLE

Uniform design for optimizing biomass and intracellular polysaccharide production from self-flocculating *Scenedesmus* sp.-BH

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Abstract Four microalgae species were isolated from freshwaters in Xinjiang, one of which was identified as *Scenedesmus* sp.-BH. Analysis of the specimens showed not only the characterization of self-flocculation with a flocculating rate of 92.29 % in 12 h, but relatively high biomass and intracellular polysaccharide (IPS) production (250.5 and 5.55 mg/L) under original BG11 medium. Uniform design was applied to optimize the nutritional conditions of *Scenedesmus* sp.-BH. The maximum biomass yield of 556.7 ±16.97 mg/L appeared at $NaNO_3$ 0.77 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.24 g/L, $MgSO_4{\cdot}7H_2O$ 0.24 g/L, ferric ammonium citrate 0.021 g/L, and $NaHCO_3$ 3.85 g/L, while a maximum IPS yield of 14.3±0.46 mg/L appeared at $NaNO_3$ 0.004 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.24 g/L, $MgSO_4{\cdot}7H_2O$ 0.21 g/L, ferric ammonium citrate 0.024 g/L, and $NaHCO_3$ 1.42 g/L. Both biomass and IPS production were over two-fold of those cultured under the original condition, which demonstrated that uniform design could successfully improve the production of biomass and IPS.

Keywords *Scenedesmus* sp.-BH · Medium components · Uniform design · Biomass · Intercellular polysaccharides

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Introduction

The Chlorophyta lives in the trunk status of the Plantae phylogenetic, but so far, studies of algal sulfated polysaccharides have usually focused on red and brown algae, while the interest in green algae has been relatively small. Green microalgae are important sources of sulfated polysaccharides. These polysaccharides containing hemi-ester sulfate groups in their sugar residues possess different therapeutic properties (Patel 2012), including strong anticoagulant (Shanmugam and Mody 2000; Mao et al. 2008), antioxidant (Costa et al. 2011; Ngo et al. 2011), and antimicrobial (Amaro et al. 2011) traits, and so on.

Several reports (Beardall et al. 2001; Giordano et al. 2002; Heraud et al. 2005; Sun and Wang 2009 Liu et al. 2010) previously revealed that microalgal cells showed dramatic reorganization of macromolecular composition in response to these changes in nutrient status. Optimizing nutritional conditions is essential to the production of microalgal polysaccharides, for example, sulfated polysaccharides for commercial mass production. In the past decade, considerable progress has been made toward understanding the effects of microalgae in response to nutritional conditions, especially carbon, nitrogen, phosphorus, and metal ion sources. Apart from molecular CO_2, bicarbonate ion was the preferred source of carbon for photosynthesis in certain marine algae (Nimer et al. 2008). Evidence by ÖSterlind (1951) reports that bicarbonate ion absorption is an active process in *Scenedesmus quadricauda* under certain conditions. The effects of nitrogen limitation that trigger the accumulation of polysaccharides were well documented (Sun et al. 2010; Wang et al. 2011; Yang et al. 2012) and generally, nitrate has been widely used as a form of inorganic nitrogen. The availability of bioactive trace metals such as magnesium and iron are required for biochemical processes, where they serve as cofactors for many enzymes and are crucial for catalytic activities (van Oijen et al. 2004). In addition, they affect the acquisition and

assimilation of macronutrients like C, N, and P (McKay et al. 2001).

The optimization of cultivation parameters, particularly nutritional and environmental factors, is very important. In recent years, due to economic conditions and the limitations of experimental design, it has become necessary to advance the application of uniform design, an effective and powerful approach for rapidly screening key factors from a multivariable system in order to optimize culture conditions (Wang et al. 2007; Sun et al. 2010; Hu et al. 2012).

The harvest is also an essential process in the mass production of microalgae. To decrease costs involved during the biomass harvesting step,the inherent characteristics and natural behaviors of microalgae should not be underestimated. Flocculation can be induced in different ways, including the use of Zn^{2+}, Fe^{3+}, Al^{3+}, other chemical flocculation and extreme pH, nutrient depletion, temperature changes, and so on (Salim et al. 2011). However, it should be stressed that any induced situation during culture growth to improve microalgae harvesting may also induce undesired changes in cell composition (González-Fernández and Ballesteros 2012). Thus, microalgal self-flocculation has attracted the attention of many researchers (Salim et al. 2011; Salim et al. 2012).

In the current study, one green microalga was identified among the isolates from Xinjiang freshwaters as *Scenedesmus* sp.-BH, which not only had the capacity for self-flocculation, but also higher biomass and IPS production under the original BG11 medium. Therefore, *Scenedesmus* sp.-BH with self-flocculation was chosen to optimize the nutritional conditions for maximizing biomass and IPS production with uniform design.

Materials and methods

Microorganisms and culture conditions

Microalgae used in this experiment were isolated from freshwaters in Xinjiang, China, using the spread plate method. They are preserved in the Key Laboratory for Green Processing of Chemical Engineering of Xinjiang Bingtuan. The basal culture medium BG11 consisted of the following components: 1.5 g/L of $NaNO_3$, 0.0524 g/L of $K_2HPO_4{\cdot}3H_2O$, 0.075 g/L of $MgSO_4{\cdot}7H_2O$, 0.036 g/L of $CaCl_2{\cdot}H_2O$, 0.006 g/L of citric acid, 0.006 g/L of ferric ammonium citrate, 0.001 g/L of $EDTANa_2$, 0.02 g/L of Na_2CO_3, 1.0 g/L $NaHCO_3$, and 1 mL/dL of trace elements solution. The trace elements solution contained the following components: 2.86 g/L of H_3BO_3, 1.86 g/L of $MnCl_4{\cdot}H_2O$, 0.22 g/L of $ZnSO_4{\cdot}7H_2O$, 0.39 g/L of $NaMoO_4{\cdot}2H_2O$, 0.08 g/L of $CuSO_4{\cdot}5H_2O$, and 0.05 g/L of $Co(NO_3)_2{\cdot}6H_2O$. The pH was adjusted to neutral by adding 1 mol/L HCl or NaOH. Cultures were incubated in 2-L Erlenmeyer flasks containing 1.5 L of culture medium at 24 ±2 °C under 4,000 lux continuous illumination. Algal stock culture with the absorbance value of 0.19±0.01 was inoculated to the medium to give a 10 % (v/v) concentration. Cultures were shaken by hand two or three times daily to avoid sticking.

Identification of microalga

Nucleic acid extraction of algal cells was performed using the NuClean PlantGen DNA kit (Beijing ComWin Biotech Co., Ltd., China) according to the manufacturer's instructions. The 18S rDNA was amplified by polymerase chain reaction (PCR) using the universal Primer A (5′-GTCAGAGGTGAAATTC TTGGATTTA-3′) and Primer B (5′-AGGGCAGGGACGTA ATCAACG-3′) (Rasoul-Amini et al. 2009), and the ampliconic sequence was sent for analysis. The sequence obtained for the 18S rDNA gene was aligned to published sequences obtained from GenBank using Clustal X 1.83. A neighbor-joining tree was constructed from these data by the bootstrap method (1,000 replicates) using Mega 5.0 software.

Flocculating activity test

Samples of microalgal suspensions cultured in the basal BG11 medium were placed in an Erlenmeyer flask and the suspension was left to settle. During the settling period, an aliquot of the culture was withdrawn and then the optical density (OD) was measured at 680 nm at the same height in the Erlenmeyer flask to determine the flocculating rate. Similar to conventional tests, the flocculating rate was measured in the top part of the 2-L Erlenmeyer flask, where individual cells and formed flocs independently sink (Salim et al. 2011). The flocculating activity was expressed in the form of the flocculation rate, as calculated according to Eq. (1):

$$\text{Flocculating rate}(\%) = \frac{\text{OD}_{680}(t0)\text{-OD}_{680}(t)}{\text{OD}_{680}(t0)} \times 100, \qquad (1)$$

where OD_{680} (t_0) is the turbidity of sample taken at time zero and OD_{680} (t) is the turbidity of the sample taken at time t.

Growth analysis

Cultures were harvested by centrifugation at 5,000 rpm for 10 min and microalgal pellets were washed twice with distilled water and freeze-dried. The dry weight of microalgal biomass was determined gravimetrically and growth was determined regularly by measuring optical density at wavelength 680 nm (denoted as OD_{680nm}).

Determination of IPS production

After harvest, freeze-dried cells were analyzed for the determination of IPS production. The microalgal dried power was extracted with 0.1 mol/L sodium hydroxide solution at 90 °C for 2 h. After centrifugation, the supernatant was added at three-fold the volume of 95 % ethanol, and the mixture was kept for 16 h at 4 °C. The precipitate was dissolved in distilled water, then the solution was subjected to the Anthrone-sulfuric acid test with glucose as the standard (Yemm and Willis 1954). The IPS production was calculated using the following Eq. (2):

$$\text{IPS production}\,(\text{mg/L}) = (M \times C \times V \times 100)/W, \qquad (2)$$

where M is the biomass concentration of microalgae, mg/L; C is the concentration of crude polysaccharides calculated from the calibrated regression equation based on the absorbance at 620 nm, mg/mL; V is the diluted volume of the extraction solution, mL; and W is the mass of cultured microalgae, mg.

Uniform design

The experiment for the optimization of biomass and IPS production of *Scenedesmus* sp.-BH was arranged with five factors, each with twelve levels. The uniform design (UD) table $U_{12}{}^{*}(12^{10})$ was applied to arrange the experiments (Table 1), and each trial was performed in triplicate. The evaluated responses Y_1 and Y_2 were the content of biomass and IPS, respectively.

Statistical analysis

All trials were carried out in triplicate, and the average of the biomass and IPS production was taken as the response value. Statistical and stepwise regression analyses of the data were done using data processing system (DPS) software (Version 7.55 by Hangzhou Refine Information Tech. Co., Ltd, China).

Results and discussion

Screening of self-flocculating microalga

Characteristics of four microalgae

Four microalgae were isolated and purified from freshwaters in Northern Xinjiang, China. According to morphological characteristics, including cell size, shape, and chloroplast core protein number, they were named as *Scenedesmus* sp.-BH, *Schroederia* sp.-90, *Chlorella* sp.-YL and *Microcystis* sp.-W, respectively. Micrographs of the four microalgae are shown in Fig. 1. All cultures were unialgal and conducted in the basal BG11 medium.

To choose a microalga having relatively high biomass and IPS production, the biomass and IPS production of the four microalgae were investigated. Growth curves of the four microalgae monitored by the absorbance of microalgae suspension are shown in Fig. 2(a). We were only able to reveal the growth cycle in order to determine the harvest time of microalgae. As shown in Fig. 2(b), the maximum biomass and IPS production of *Scenedesmus* sp.-BH were approximately 250.5 and 5.55 mg/L more than the other three microalgae. Moreover, almost all the cells of this microalga formed small aggregates and settled at the bottom of the flask over time in the microalgal cultivation process. Therefore, tests of the flocculating rate from *Scenedesmus* sp.-BH were designed.

Table 1 Factors and levels of the uniform design experiment

Experiment No.	Factors				
	$NaNO_3$ (X_1)	$K_2HPO_4{\cdot}3H_2O$ (X_2)	$MgSO_4{\cdot}7H_2O$ (X_3)	$(NH_4)_3FeC_{12}H_{10}O_4$ (X_4)	$NaHCO_3$ (X_5)
1	1 (0)	3 (0.06)	4 (0.08)	9 (0.018)	12 (3.85)
2	2 (0.3)	6 (0.12)	8 (0.16)	5 (0.01)	11 (3.5)
3	3 (0.6)	9 (0.18)	12 (0.24)	1 (0.002)	10 (3.15)
4	4 (0.9)	12 (0.24)	3 (0.06)	10 (0.02)	9 (2.8)
5	5 (1.2)	2 (0.04)	7 (0.14)	6 (0.012)	8 (2.45)
6	6 (1.5)	5 (0.1)	11 (0.22)	2 (0.004)	7 (2.1)
7	7 (1.8)	8 (0.16)	2 (0.04)	11 (0.022)	6 (1.75)
8	8 (2.1)	11 (0.22)	6 (0.12)	7 (0.014)	5 (1.4)
9	9 (2.4)	1 (0.02)	10 (0.2)	3 (0.006)	4 (1.05)
10	10 (2.7)	4 (0.08)	1 (0.02)	12 (0.024)	3 (0.7)
11	11 (3)	7 (0.14)	5 (0.1)	8 (0.016)	2 (0.35)
12	12 (3.3)	10 (0.2)	9 (0.18)	4 (0.008)	1 (0)

Symbols X_1, X_2, X_3, X_4 , and X_5 represent the concentration of $NaNO_3$ (g/L), $K_2HPO_4{\cdot}3H_2O$ (g/L), $MgSO_4{\cdot}7H_2O$ (g/L), $(NH_4)_3FeC_{12}H_{10}O_4$ (g/L), and $NaHCO_3$ (g/L), respectively. Symbols 1–12 represent levels of each factor

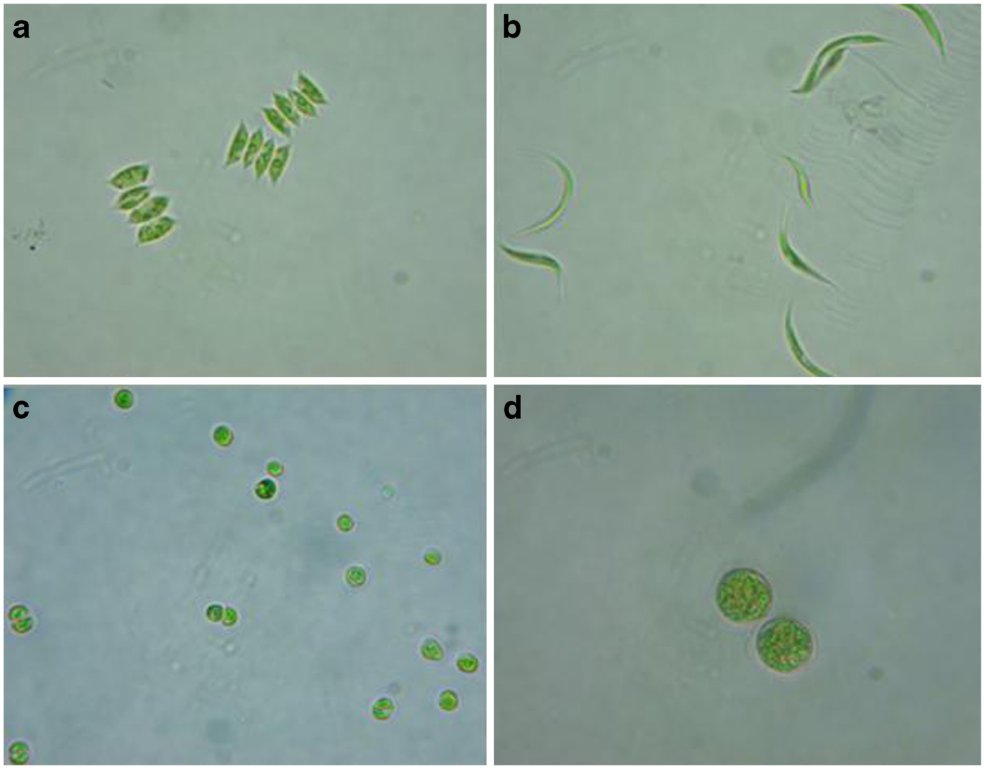

Fig. 1 Micrographs of four microalgae isolated from the freshwaters in Northern Xinjiang. (**a**) *Scenedesmus* sp.-BH, (**b**) *Schroederia* sp.-90, (**c**) *Chlorella* sp.-YL, (**d**) *Microcystis* sp.-W. Photomicrographs were obtained with a Primo Star microscope (*400), Carl Zeiss, Germany

Identification of microalga

Using PCR amplification and subsequent DNA sequencing, we determined almost the entire length of the 18S rDNA of the microalga. The length of the 18S rDNA sequence of BH in this study was 1,591 base pairs. The 18S rDNA sequence of BH showed 99 % sequence homology with *Scenedesmus obliquus* (FR865738.1). It was thus named *Scenedesmus* sp.-BH. As shown in Fig. 3, related sequences were downloaded and used to build a phylogenetic tree with Mega 5.0 software.

Flocculating rate of *Scenedesmus* sp.-BH

The sedimentation of microalgal suspensions from *Scenedesmus* sp.-BH was monitored for 12 h and the absorbance (A_{680nm}) and flocculating rate of the microalgal cells

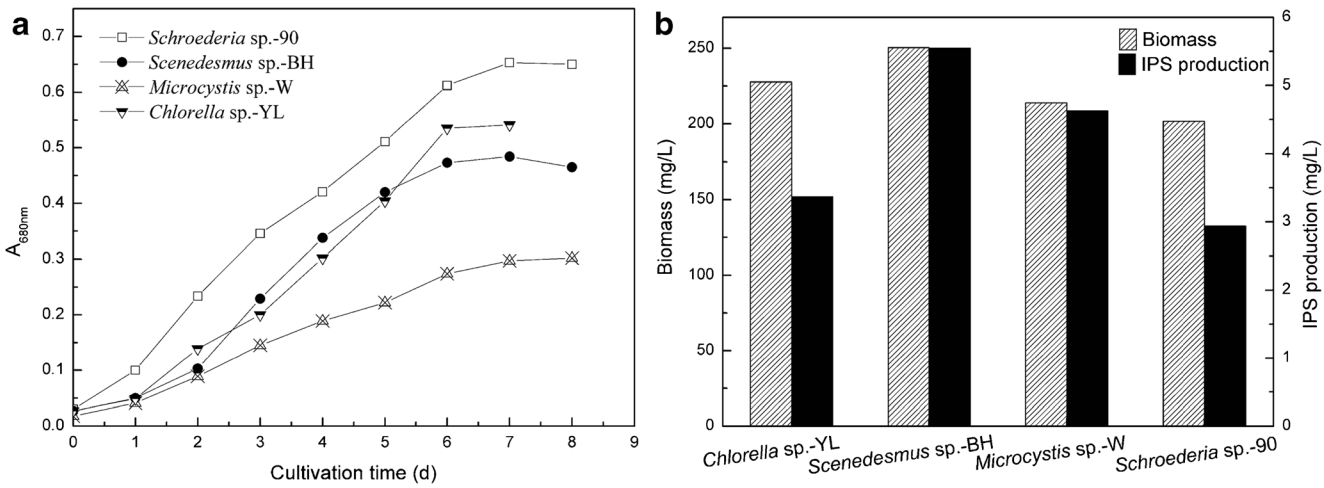

Fig. 2 Growth curves, maximum biomass (mg/L), and IPS production (mg/L) of the four green microalgae, *Chlorella* sp.-YL, *Scenedesmus* sp.-BH, *Schroederia* sp.-90, and *Microcystis* sp.-W. The culture medium was basal BG11 at 25±2 °C under 4,000 lux continuous illuminations. (**a**) represents the growth curves of four microalgae, (**b**) represents the maximum biomass (mg/L) and IPS production (mg/L) of four microalgae

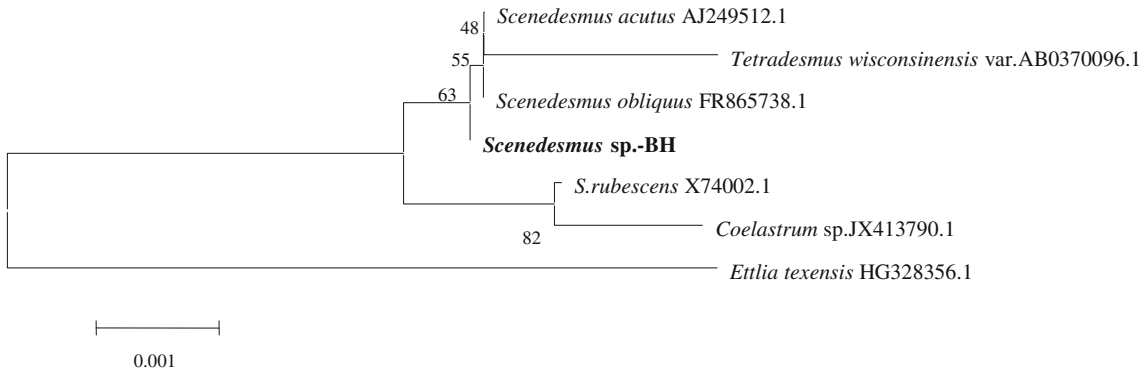

Fig. 3 Phylogenic analysis of the isolated *Scenedesmus* sp.-BH species based on the 18S rDNA sequence

were determined over time. During the settling period, dispersed microalgal cells aggregated together, formed larger flocs with a higher sedimentation rate, and settled at the bottom of the flask, resulting in an evident separation of cells from the culture broth. The A_{680nm} and flocculating rate of *Scenedesmus* sp.-BH over time are presented in Fig. 4. The value of A_{680nm} dramatically decreased in the initial two hours, and then continued to decrease more slowly. In contrast, the flocculating rate dramatically increased first, and then slowly increased. The flocculating rate and efficiency (per hour) could reach up to 92.29 % and 7.69 % after 12 h standing, respectively. Having found this microalga to have a self-flocculating property, we then studied the potential to improve its biomass and IPS production.

The self-flocculating phenomenon of green microalga *Scenedesmus* sp. has been reported in some previous studies. A new self-flocculating microalga *S. obliquus* AS-6-1 was characterized by Guo et al. (2013), who then used it as an extracting flocculating agent, which resulted in the fast flocculation of freely suspended cells of *S. obliquus* FSP-3 and *Chlorella vulgaris* CNW-11. As mentioned by Salim et al. (2011), although the flocculating microalgae *Ankistrodesmus falcatus*, *Scenedesmus obliquus*, and *Tetraselmis suecica* have the capacity to harvest the non-flocculating *Chlorella vulgaris*, *Chlorella vulgaris*, and *Neochloris oleoabunda*, respectively, the recovery efficiency is different. Salim et al. (2012) found that when adding the self-flocculating microalgae *Ettlia texensis*, *Ankistrodesmus falcatus*, and *Scenedesmus obliquus* to *Chlorella vulgaris* at a ratio of 0.25, the recovery of *C. vulgaris* increases from 25 % to, respectively, 40 %, 36 %, and 31 %. However, the flocculation rate of *Scenedesmus* sp. used in these studies was not calculated separately, and therefore, the results could not be compared with those in the current study.

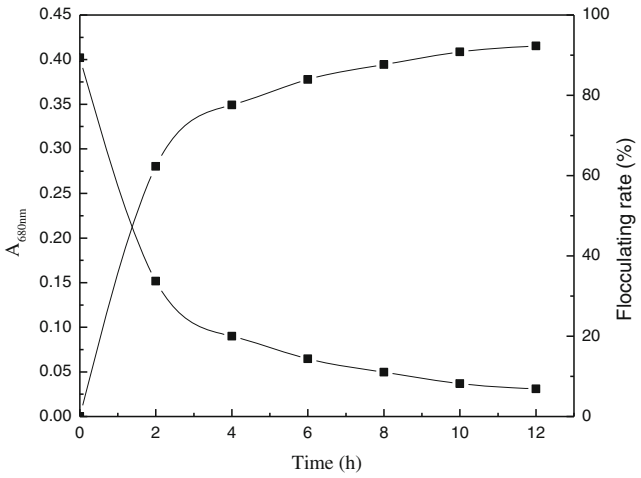

Fig. 4 Variation tendency of A_{680nm} and flocculating rate (%) over time for the self-flocculating *Scenedesmus* sp.-BH

Optimization of nutrients conditions for *Scenedesmus* sp.-BH with uniform design

Different microalgal species and strains vary greatly in terms of nutrient requirements (González-Fernández and Ballesteros 2012). There are many nutritional factors controlling the cell growth and IPS content, such as carbon, nitrogen source, and other essential macro- and micronutrients like magnesium and iron. When factors and levels influencing experimental results were greater in number, uniform design—which needs a smaller number of trials compared with orthogonal design—was a better choice. Therefore, uniform design was used for investigating the impact of the dosage of $NaNO_3$, $K_2HPO_4{\cdot}3H_2O$, $MgSO_4{\cdot}7H_2O$, ferric ammonium citrate, and $NaHCO_3$ in basal BG11 medium on the biomass and IPS production from *Scenedesmus* sp.-BH.

Optimization of biomass production

Values of responses (biomass and IPS production) at different experimental combinations are given in Table 2. There were a total of 12 runs for optimizing five individual parameters in the *UD* table $U_{12}{}^{*}(12^{10})$, which was applied to the biomass and IPS production. As shown in Fig. 5, the growth tendency

Table 2 Application of *UD* $U_{12}^{*}(12^{10})$ for the optimization of biomass (mg/L) and IPS production (mg/L)

Experiment No.	Factors					Responses	
	X_1	X_2	X_3	X_4	X_5	Biomass (Y_1)	IPS (Y_2)
1	1	3	4	9	12	169.9±4.43	9.4±0.31
2	2	6	8	5	11	400.9±6.08	11.1±0.49
3	3	9	12	1	10	415.8±3.06	7.0±0.25
4	4	12	3	10	9	341.2±8.34	11.4±0.53
5	5	2	7	6	8	288.4±2.40	6.3±0.01
6	6	5	11	2	7	224.4±1.46	6.9±0.02
7	7	8	2	11	6	220.3±2.26	5.2±0.06
8	8	11	6	7	5	219.9±0.90	5.2±0.22
9	9	1	10	3	4	178.6±7.31	3.5±0.01
10	10	4	1	12	3	143.6±0.05	3.4±0.03
11	11	7	5	8	2	106.9±3.87	1.8±0.06
12	12	10	9	4	1	63.2±0.28	0.7±0.02

and the maximum A_{680nm} of *Scenedesmus* sp.-BH are significantly different in different combinations.

The maximum value of biomass (415.8 mg/L) was recorded under the experimental condition of $NaNO_3$ 0.60 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.18 g/L, $MgSO_4{\cdot}7H_2O$ 0.24 g/L, ferric ammonium citrate 0.002 g/L, and $NaHCO_3$ 3.15 g/L. By applying quadratic polynomial stepwise regression to the experimental data, Eq. (3) was established as the mathematic model for the optimization of five factors for maximum biomass production.

$$Y\text{biomass} = 102.00 - 283.55X_2X_5 + X_3X_5. \quad (3)$$

The model's adequacy was checked by an F-test and with the determination coefficient R. The analysis of variance on biomass production showed that the model was highly

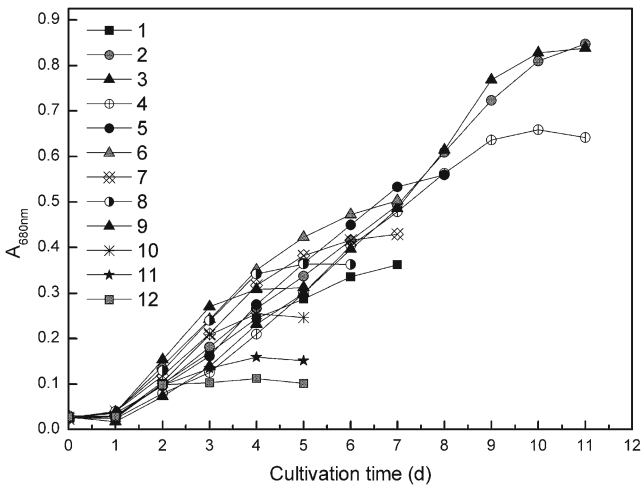

Fig. 5 Growth curves of *Scenedesmus* sp.-BH under different treatments with $U_{12}^{*}(12^{10})$ uniform design

Table 3 Results of the stepwise regression analysis for the optimization of biomass

	Parameters		
	Partial correlation coefficient	t-test	Significance p
$X_2{*}X_5$	0.78121	3.75416	0.00376
$X_3{*}X_5$	0.74506	3.35107	0.00735

significant ($p<0.001$) with an F-value of 27.1. The determination coefficient ($R=0.9261$) was given by an analysis of variance (ANOVA) of the quadratic regression model. The value of the adjusted determination coefficient ($R=0.9089$) also confirmed that the model was highly significant. Therefore, the model was found to be adequate for prediction within the range of experimental variables.

The results of the regression analysis for biomass listed in Table 3 indicated that the interaction between $K_2HPO_4{\cdot}3H_2O$, $NaHCO_3$, $MgSO_4{\cdot}7H_2O$, and $NaHCO_3$ had a significant influence on biomass ($p<0.01$). The model predicted the maximum biomass yield of 579.8 mg/L that appeared at $NaNO_3$ 0.77 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.24 g/L, $MgSO_4{\cdot}7H_2O$ 0.24 g/L, ferric ammonium citrate 0.021 g/L, and $NaHCO_3$ 3.85 g/L.

Optimization of IPS production

The maximum value of IPS production (11.4 mg/L) was recorded under the experimental condition of $NaNO_3$ 0.90 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.24 g/L, $MgSO_4{\cdot}7H_2O$ 0.060 g/L, ferric ammonium citrate 0.020 g/L, and $NaHCO_3$ 2.8 g/L. By applying quadratic polynomial stepwise regression to the experimental data, Eq. (4) was established as the mathematic model for the optimization of five factors for maximum IPS production.

$$Y\text{IPS prosuction} = 9.60 - 2.23X_1 - 4105.97X_4^2 - 5.69X_1X_2 + 1277.25X_2X_4. \quad (4)$$

The goodness of fit of the model was also examined using the determination coefficient ($R=0.9688$), which implied that the sample variation of more than 96 % was attributed to the variables. The test statistic F value for the overall regression

Table 4 Results of the stepwise regression analysis for the optimization of IPS production

	Parameters		
	Partial correlation coefficient	t-test	Significance p
X_1	−0.84258	4.1392	0.00326
$X_4{*}X_4$	−0.49938	1.525	0.16577
$X_1{*}X_2$	−0.53602	1.67988	0.13149
$X_2{*}X_4$	0.74971	2.99738	0.01714

Table 5 The anticipated and experimental indices in optimum medium components

Index	Optimum medium components					Anticipated maximum index	Experimental result
	X_1	X_2	X_3	X_4	X_5		
Biomass (mg/L)	0.770	0.240	0.240	0.021	3.848	579.8	556.7±16.97
IPS (mg/L)	0.004	0.240	0.212	0.024	1.424	14.6	14.3±0.46

was significant at the upper 5 % level, which further supported that the second-order model was very adequate in approximating the response surface of the experimental design. The regression analysis for IPS production presented in Table 4 showed that the X_1 linear coefficient, which constituted the most significant term, i.e., $NaNO_3$, had a very significant effect on IPS production ($p<0.01$); $K_2HPO_4{\cdot}3H_2O$, and ferric ammonium citrate had a significant influence on IPS production ($p<0.05$). However, the interaction between $NaNO_3$, $K_2HPO_4{\cdot}3H_2O$, and quadratic ferric ammonium citrate had an influence on IPS production, but did not show a significant effect.

The model predicted that a maximum IPS yield of 14.6 mg/L appeared at $NaNO_3$ 0.0039 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.24 g/L, $MgSO_4{\cdot}7H_2O$ 0.21 g/L, ferric ammonium citrate 0.024 g/L, and $NaHCO_3$ 1.42 g/L. This predicted value was higher than any from the twelve experiments.

Verification of the models

In order to validate the optimal values, three dependent replicates were performed from the optimum nutritional conditions obtained by DPS software, listed in Table 5. Colors of the cultivation broth were significantly different, with the original BG11 medium appearing green, while the broths cultured in optimum nutritional conditions for biomass and IPS production were dark green and yellow, respectively, due to the different chlorophyll concentrations that each growth mode had. As shown in Fig. 6, growth curves of the *Scenedesmus* sp.-BH cultured in three types of culture medium were dramatically different, indicating that the five nutritional factors considered in this experiment could markedly impact the growth of *Scenedesmus* sp.-BH.

The final experimental results were in good agreement with predicted maximum indices (Table 5), which also proved the credibility of the mathematic models. The biomass and IPS production of *Scenedesmus* sp.-BH cultured in the optimum medium was over two-fold of the initial BG11 medium, which is commonly used for the cultivation of blue and green freshwater microalgae.

The experiments in our study present a comprehensive study on the optimization of critical medium components for the production of biomass and IPS from *Scenedesmus* sp.-BH. The mathematical models obtained from uniform design software reveal the effects of single factors and their interaction with the biomass and IPS production of *Scenedesmus* sp.-BH. From quadratic equations, we found that the properties and significant differences between individual variables vary with the target product. For instance, the biomass correlates with the interaction of $K_2HPO_4{\cdot}3H_2O$ and $NaHCO_3(X_2X_5)$, $MgSO_4{\cdot}7H_2O$ and $NaHCO_3(X_3X_5)$ concentration, but the IPS production is the function of $NaNO_3$ concentration (X_1), the interaction of $K_2HPO_4{\cdot}3H_2O$, and ferric ammonium citrate (X_2X_4) concentration. The maximum output of both biomass and IPS production is observed at high $K_2HPO_4{\cdot}3H_2O$, $MgSO_4{\cdot}7H_2O$, and ferric ammonium citrate concentration. The optimal concentration of $NaNO_3$ for the maximum production of IPS is 0.004 g/L, which is lower than that for maximal biomass (0.77 g/L) (Table 5).

Considering different algal strains, cultivation methods, and cultivation scales, the required optimum culture conditions are quite different, while the effects of culture conditions on biomass and intracellular polysaccharides of algae are basically similar. The activities of some key enzymes of algal cells, such as high ATP-citrate lyase, can promote microalgae to shift from sugar storage sugar to lipids (Bellou and Aggelis 2013) and sequentially decrease the content of

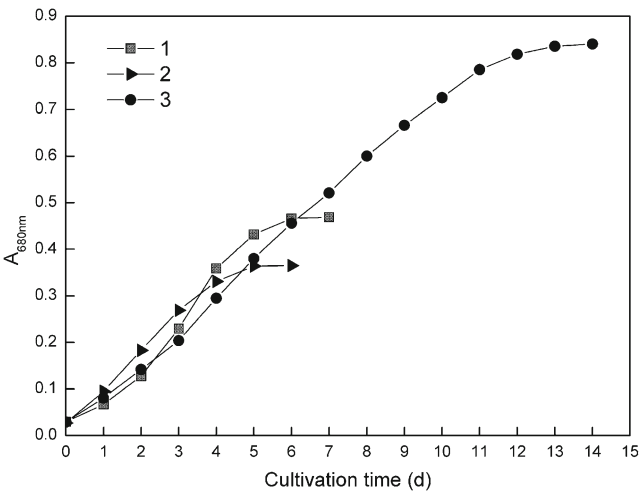

Fig. 6 Growth curves of the *Scenedesmus* sp.-BH cultured in different culture conditions. 1 presents the *Scenedesmus* sp.-BH cultured in basal BG11 medium as the control; 2 presents the *Scenedesmus* sp.-BH cultured in optimum medium components for biomass; 3 presents the *Scenedesmus* sp.-BH cultured in optimum medium components for IPS production

polysaccharides. Phosphorus plays a significant role in most cellular processes, especially those involved in the formation and transformation process of energy metabolism. Liang et al. (2013) found that different phosphorus levels could significantly influence the lipid, carbohydrate, and protein content of freshwater microalga *Chlorella* sp. Yingying and Changhai found that both the growth and biochemical compositions (such as chlorophyll, protein, and polysaccharide) in *Isochrysis galbana* can be affected by phosphorus. The interrelationships between phosphorus availability and the basic physiological reactions such as growth, reproduction, and biochemical composition have also been reported in other microalgae (Fabregas et al. 2000; Yim et al. 2003). In our experiments, the range of $K_2HPO_4{\cdot}3H_2O$ concentration from 0.065 g/L to 0.24 g/L was beneficial for cell growth, and intracellular polysaccharide biosynthesis. The bicarbonate ion was another main source of carbon for the growth and metabolism of microalgae. The results obtained in our study showed that the interaction of $K_2HPO_4{\cdot}3H_2O$ and $NaHCO_3$, $MgSO_4{\cdot}7H_2O$ and $NaHCO_3$ concentration had an important effect on the growth of *Scenedesmus* sp.-BH, with a biomass increase from 250.5 mg/L to 556.7 mg/L.

The nitrogen source also influences the intracellular polysaccharide biosynthesis of microalgae. Wang et al.(2011) reported that the bound polysaccharide and total polysaccharide content of *Microcystis aeruginosa* cultured in 5 % N of standard BG-11 media were significantly higher than those of *M. aeruginosa* cultured in all other nitrogen concentration media (10 %, 25 % and 100 %). Yang et al. (2012) also found that bound polysaccharides of *M. aeruginosa* cultured in 0.26 mg/L nitrogen concentration were obviously higher than those cultured in 2.55 and 25.47 mg/L at all experimental temperatures and light intensities. In our experiments, when $NaNO_3$ concentration decreased from 1.5 g/L to 0.004 g/L, it significantly influenced the production of intracellular polysaccharides of *Scenedesmus* sp.-BH ($p<0.001$).

Iron, one of the structural elements of organic components, plays an essential role in photosynthesis and biomacromolecule synthesis of microalgae. As van Oijen et al. (2004) had already reported, iron stress appears primarily to affect photosynthesis in the Antarctic diatom *Chaetoceros brevis*, causing a reduction in the diurnal production of water-extractable carbohydrates. Liu et al. (2008) found that adding $FeCl_3$ into the media not only prolonged the period of the exponential growth phase and increased the final cell density of *Chlorella vulgaris*, but also greatly accumulated the lipid only when the iron concentration in the initial medium reached a certain value. In our study, the $NaNO_3$ concentration and the interaction of $K_2HPO_4{\cdot}3H_2O$ and ferric ammonium citrate concentration showed a significant effect on the IPS production of *Scenedesmus* sp.-BH, with an overall content increase from 5.55 mg/L to 14.3 mg/L.

Conclusions

A new self-flocculating microalga, identified as *Scenedesmus* sp.-BH, was isolated from freshwaters in Xinjiang, China. We found that this microalga contained a higher biomass and rate of IPS production compared with the other three microalgae in our lab. The biomass and IPS production of *Scenedesmus* sp.-BH cultured in optimized medium with uniform design could reach up to 556.7±16.97 and 14.3±0.46 mg/L, respectively, which were more than two-fold greater than those obtained from the original culture medium. The results obtained in this study showed that uniform design combining stepwise regression analysis was a powerful method for the optimization of culture conditions from microalgae.

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References

Amaro HM, Guedes AC, Malcata FX (2011) Antimicrobial activities of microalgae: an invited review. Science against Microbial Pathogens: Communicating Current Research and Technological Advances. Formatex Microbiology Book Series, Nova Science Publisher 3: 1272–1284

Beardall J, Berman T, Heraud P, Omo Kadiri M, Light BR, Patterson G, Roberts S, Sulzberger B, Sahan E, Uehlinger U (2001) A comparison of methods for detection of phosphate limitation in microalgae. Aquat Sci 63:107–121

Bellou S, Aggelis G (2013) Biochemical activities in *Chlorella* sp. and *Nannochloropsis salina* during lipid and sugar synthesis in a lab-scale open pond simulating reactor. J Biotechnol 164:318–329

Costa MSSP, Costa LS, Cordeiro SL, Almeida-Lima J, Dantas-Santos N, Magalhães KD, Sabry DA, Albuquerque IRL, Pereira MR, Leite EL, Rocha HAO (2011) Evaluating the possible anticoagulant and antioxidant effects of sulfated polysaccharides from the tropical green alga Caulerpa cupressoides var. flabellata. J Appl Phycol 24: 1159–1167

Fabregas J, Dominguez A, Regueiro M, Maseda A, Otero A (2000) Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis*. Appl Microbiol Biotechnol 53:530–535

Giordano M, Kansiz M, Heraud P, Beardall J, Wood B, McNaughton D (2002) Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pools in the marine microalga *Chaetoceros muellerii* (Bacillariophyceae). J Phycol 37:271–279

González-Fernández C, Ballesteros M (2012) Microalgae autoflocculation: an alternative to high-energy consuming harvesting methods. J Appl Phycol. doi:10.1007/s10811-012-9957-3

Guo SL, Zhao XQ, Wan C, Huang ZY, Yang YL, Asraful Alam M, Ho SH, Bai FW, Chang JS (2013) Characterization of flocculating agent from the self-flocculating microalga *Scenedesmus obliquus* AS-6-1 for efficient biomass harvest. Bioresource Technol. doi:10.1016/j.biortech.2013.01.120

Heraud P, Wood BR, Tobin MJ, Beardall J, McNaughton D (2005) Mapping of nutrient-induced biochemical changes in living algal cells using synchrotron infrared microspectroscopy. FEMS Microbiol Lett 249:219–225

Hu T, Zeng H, Chen Z, Huang X, Yang Y, Wang G (2012) The optimization of regeneration tissue culture system of three chilli peppers cultivars based on the uniform design and the mathematical model equation. Acta Biol Hung 63:372–388

Liang K, Zhang Q, Gu M, Cong W (2013) Effect of phosphorus on lipid accumulation in freshwater microalga *Chlorella* sp. J Appl Phycol 2: 311–318

Liu Y, Wang W, Zhang M, Xing P, Yang Z (2010) PSII-efficiency, polysaccharide production, and phenotypic plasticity of *Scenedesmus obliquus* in response to changes in metabolic carbon flux. Biochem Syst Ecol 38:292–299

Liu ZY, Wang GC, Zhou BC (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technol 99:4717–4722

Mao WJ, Fang F, Li HY, Qi XH, Sun HH, Chen Y, Guo SD (2008) Heparinoid-active two sulfated polysaccharides isolated from marine green algae *Monostroma nitidum*. Carbohyd Polym 74:834–839

McKay RML, Twiss MR, Nalewajko C (2001) Trace metal constraints on carbon, nitrogen and phosphorus acquisition and assimilation by phytoplankton/Algal Adaptation to Environmental Stresses. Springer, Berlin Heidelberg, pp 111–134

Ngo DH, Wijesekara I, Vo TS, Van Ta Q, Kim SK (2011) Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. Food res int 44:523–529

Nimer NA, Iglesias-Rodriguez MD, Merrett MJ (2008) Bicarbonate utilization by marine phytoplankton species. J Phycol 33:625–631

ÖSterlind S (1951) Inorganic carbon sources of green algae. III. Measurements of photosynthesis in *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. Physiol Plantarum 4:242–254

Patel S (2012) Therapeutic importance of sulfated polysaccharides from seaweeds: updating the recent findings. 3. Biotech 2:171–185

Rasoul-Amini S, Ghasemi Y, Morowvat MH, Mohagheghzadeh A (2009) PCR amplification of 18S rRNA, single cell protein production and fatty acid evaluation of some naturally isolated microalgae. Food Chem 116:129–136

Salim S, Bosma R, Vermuë MH, Wijffels RH (2011) Harvesting of microalgae by bio-flocculation. J Appl Phycol 23:849–855

Salim S, Vermuë MH, Wijffels RH (2012) Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. Bioressource Technol 118:49–55

Shanmugam M, Mody KH (2000) Heparinoid-active sulphated polysaccharides from marine algae as potential blood anticoagulant agents. Curr Sci 79:1672–1683

Sun Y, Wang C (2009) The optimal growth conditions for the biomass production of *Isochrysis galbana* and the effects that phosphorus, Zn^{2+}, CO_2, and light intensity have on the biochemical composition of *Isochrysis galbana* and the activity of extracellular CA. Biotechnol Bioproc Eng 14:225–231

Sun L, Wang C, Ma C, Shi L (2010) Optimization of renewal regime for improvement of polysaccharides production from *Porphyridium cruentum* by uniform design. Bioproc Biosyst Eng 33:309–315

van Oijen T, van Leeuwe MA, Gieskes WWC, de Baar HJW (2004) Effects of iron limitation on photosynthesis and carbohydrate metabolism in the Antarctic diatom *Chaetoceros brevis* (Bacillario phyceae). Eur J Phycol 39:161–171

Wang J, Chen B, Rao X, Huang J, Li M (2007) Optimization of culturing conditions of *Porphyridium cruentum* using uniform design. World J Microb Biot 23:1345–1350

Wang W, Liu Y, Geng L, Yang Z, Chen Y (2011) The effect of nitrogen concentration and its interaction with light intensity on population dynamics and polysaccharide content of *Microcystis aeruginosa*. J Freshwater Ecol 26:241–248

Yang Z, Geng L, Wang W, Zhang J (2012) Combined effects of temperature, light intensity, and nitrogen concentration on the growth and polysaccharide content of *Microcystis aeruginosa* in batch culture. Biochem Syst Ecol 41:130–135

Yemm EW, Willis AJ (1954) The estimation of carbohydrate in plant extracts by anthrone. Biochem J 57:508–514

Yim JH, Kim SJ, Ahn SH, Lee HK (2003) Optimal conditions for the production of sulfated polysaccharide by marine microalga *Gyrodinium impudicum* strain KG03. Biomol Eng 20:273–280