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ORIGINAL PAPER

Optimization of culturing conditions of *Porphyridium cruentum* using uniform design

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Abstract The red microalga *Porphyridium* contains many valuable compounds such as polysaccharides, polyunsaturated fatty acids, and phycoerythrin (PE). In this study, a uniform design method and regression analysis were used to investigate the effects of initial pH, light intensity, inoculation ratio, and liquid volume in flask on the optimal biomass, exopolysaccharides (EPS), and PE production of *Porphyridium cruentum* in a batch culture at laboratory scale. Using regression analysis, we obtained the models to clarify the effects of individual factors and their interactions on the biomass, EPS, and PE production of *P. cruentum*. The optimal condition for the biomass was the following: pH 5.0, light intensity 7098.0 lx, inoculation ratio 1:17.2, and liquid volume 100.0 ml; for EPS was pH 5.0, light intensity 4501.0 lx, inoculation ratio 1:20, and liquid volume 100.3 ml; while pH 8.0, light intensity 7100.0 lx, inoculation ratio 1:20, and liquid volume 100.3 ml was the best for PE production. The maximum biomass 3.27 g/l, EPS production 543.1 mg/l, and PE production 132.0 mg/l were demonstrated by confirmatory experiment to the optimum culture conditions in a reciprocal shaker. The statistical methods used in the present study are useful strategies for optimizing of culture conditions for other microalgae.

Keywords Biomass · Culture condition · Exopolysaccharide · Optimization · Phycoerythrin · *Porphyridium cruentum* · Uniform design

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Introduction

The red microalga *Porphyridium* is a source of biochemicals that possess nutritional and therapeutical value. These biochemicals include a high content of polysaccharides, long-chain polyunsaturated fatty acids, carotenoids such as zeaxanthin, and fluorescent phycobiliproteins. The polysaccharides of *Porphyridium* exhibited impressive antiviral activity (Huleihel et al. 2001, 2002; Huang et al. 2005). *Porphyridium cruentum* has been shown to contain four biliproteins with the following amounts (percentage): allophycocyanin (5%), *R*-phycoerythrin (11%), *b*-phycoerythrin (≈42%), and *B*-phycoerythrin (BPE) (≈42%) (Bermejo et al. 2001). Among the four biliproteins, BPE is particularly useful due to its high molar absorptivity and great fluorescence properties (Ayyagari et al. 1995). It has been also reported that BPE can be used as a pigment in the food, cosmetic, and pharmaceutical industries. The price of highly purified BPE in the market has been reported to be as high as US $50 per mg (Benavides and Rito-Palomares 2006; Dufossé et al. 2005). The high value makes it attractive. Therefore, it is worth developing an efficient method for the production of BPE. It is well known that culture conditions affect the biomass of *P. cruentum* as well as the quality of the exopolysaccharides (EPS) and phycoerythrin (PE) produced. There have been only a few reports about the effects of different culture conditions on growth, EPS, and PE production. The main conditions studied are light quality (You and Barnett 2004), high light intensity and low gas flow rates (Merchuk et al. 1998), pH, stirring and mineral nutrients in the flat plate glass reactors (Singh et al. 2000), aeration and agitation (Iqbal and Zafar 1993) and renewal rate of the culture volume (Fa'bregas et al. 1998). However, a systematic approach for the system has not been performed.

Uniform design (UD) is an experimental design strategy for the statistical optimization of a system. This design seeks experimental points to be uniformly scattered in the experimental domain. It is widely accepted, especially in situations where little is known of the theoretical function to be modeled. The reason of its high success rate is the economical and flexible experimental runs that determine many factors simultaneously. Therefore, UD has the advantage that it needs far fewer trials compared with the other statistical designs of experiments such as the orthogonal test (Fang 1998; Fang and Qin 2003). UD has been successfully applied in various fields such as chemistry, chemical engineering (Liang et al. 2000; Chen et al. 2003), and biotechnology (Cao et al. 2004, 2006; Wen et al. 2005; Chen et al. 2006). In this study, UD method and regression analysis were applied to optimize the culture conditions, including inoculation ratio, liquid volume, initial pH, and light intensity, for biomass, EPS, and PE production by *P. cruentum*.

Materials and methods

Organism and culture conditions

Porphyridium cruentum was obtained from the Institute of Oceanology, Chinese Academy of Sciences (Qingdao, China).

The algal cells were grown in 500-ml Erlenmeyer flasks for 12 days containing 100–300 ml culture. Cells were cultivated in Koch medium, containing sterilized seawater which was enriched with nutrients (per liter): KH_2PO_4, 25 mg; KNO_3, 1,500 mg; $MgSO_4{\cdot}7H_2O$, 1,950 mg; Fect, 2.5 mg; NaCl, 12,500 mg; KCl, 400 mg; $MgCl_2{\cdot}6H_2O$, 1,250 mg; $CaCl_2{\cdot}2H_2O$, 600 mg; $NaHCO_3$, 100 mg; $Na_2B_4O_7{\cdot}10H_2O$, 25 mg, at 25°C under constant fluorescent light at an exposure intensity of 1,000–5,000 lx in a reciprocal shaker at 90 oscillations/min. Cultures were inoculated at 5% (1/20) to 25% (1/4) (v/v) of the seed culture.

Biomass determination

Biomass was measured on a dry weight basis. The cultures were harvested and the cells were washed twice with distilled water and dried at 60°C until constant weight was obtained.

Phycoerythrin determination

Phycoerythrin was extracted in distilled water from the pellets by freezing/thawing cycles. The extracts were centrifuged and PE concentration determined spectrophotometrically in the supernatant by measuring the absorbance of extracts at 565, 620, and 650 nm (Tetzuya 1988).

Exopolysaccharide determination

The EPS in the medium (soluble fraction) was determined by the phenol–sulfuric acid method (Zhang 1999) after centrifuging.

Uniform design

Based on the results of screening out with mono-factor experiments (Wang et al. 2004), the experiment for optimization of culture conditions was arranged four factors, i.e., initial pH (X_1), light intensity (X_2) (lx), inoculation ratio (X_3) (v/v), and liquid volume in flask (X_4) (ml) by design, each at nine levels. The UD table $U_9(9^4)$ was applied to arrange the experiments (Table 1), and each trial was performed in triplicate. The evaluated response Y was the content of PE or biomass or EPS.

Statistical analysis

All trials were carried out in triplicate and the average of the biomass, EPS, and PE yield was taken as response value. The statistical and stepwise regression analyses of the data were done using UD DPS software (Version 7.55 by Hangzhou Refine Information Tech. Co., Ltd, China).

Results and discussion

The design of the experiments and the results are shown in Table 2. The data obtained were analyzed using the DPS software. The equation relating the coefficients obtained for PE, biomass, and EPS production to the experimental variable is as follows:

$$\begin{aligned} Y_{\text{PEproduction}} &= 0.21608 - 0.0011633X_4 + 0.76761X_3^2 \\ &\quad + 0.0000024712X_4^2 + 0.00000078998X_1X_2 \\ &\quad - 0.035676X_1X_3 - 0.000000038378X_2X_4 \quad (1) \end{aligned}$$

$$\begin{aligned} Y_{\text{biomass}} &= 7.82663 - 0.089748X_1 - 0.050494X_4 \\ &\quad - 31.56174X_3^2 + 0.000085335X_4^2 \\ &\quad + 0.036789X_3X_4 \quad (2) \end{aligned}$$

$$\begin{aligned} Y_{\text{EPSproduction}} &= 719.15908 - 27.05941X_1 - 1.13986X_4 \\ &\quad + 100.58205X_1X_3 - 0.11557X_2X_3 \\ &\quad + 0.00017957X_2X_4 - 2.27495X_3X_4 \quad (3) \end{aligned}$$

Table 1 Factors and levels of the uniform design experiment

Experiment no.	Factors			
	Initial pH (X_1)	Light intensity (X_2)	Inoculation ratio (X_3)	Liquid volume (X_4)
1	1(5.0)	2(1,500)	4(1:8.0)	7(250.0)
2	2(5.5)	4(2,500)	8(1:4.4)	5(200.0)
3	3(6.0)	6(3,500)	3(1:10.0)	3(150.0)
4	4(6.5)	8(4,500)	7(1:5.0)	1(100.0)
5	5(7.0)	1(1,000)	2(1:13.3)	8(275.0)
6	6(7.5)	3(2,000)	6(1:5.7)	6(225.0)
7	7(8.0)	5(3,000)	1(1:20)	4(175.0)
8	8(8.5)	7(4,000)	5(1:6.7)	2(125.0)
9	9(9.0)	9(5,000)	9(1:4.0)	9(300.0)

Symbols X_1, X_2, X_3, and X_4 represent factors of pH, light intensity (lx), inoculation ratio (v/v), and liquid volume (ml), respectively. Symbols *1–9* represent the levels of each factor

In Eq. 1, Y is the response (PE), X_1 is initial pH, X_2 is light intensity, X_3 is inoculation ratio, and X_4 is liquid volume; with $R = 0.99384$, F-value = 26.8000, standard error $S = 0.00509$, significance $p = 0.0364$. In Eq. 2, Y is the response (biomass), X_1 the coded value of initial pH, X_3 the code value of inoculation ratio and X_4 the code value of liquid volume; with $R = 0.9886$, F-value = 25.7717, standard error $S = 0.2011$, significance $p = 0.0114$. Equation 2 showed that the values of X_2 (light intensity) do not appear in the mathematical expression of the experimental design, meaning that a different value of X_2 in these ranges (1,000–5,000 lx) had little influence on biomass. In Eq. 3, Y is the response (EPS production), X_1, X_2, X_3, and X_4 are code variables of initial pH, light intensity, inoculation ratio, and liquid volume, respectively, with $R = 0.99364$, F–value = 25.9409, standard error $S = 17.3345$, significance $p = 0.0376$.

The mathematical model was expressed in terms of the values of all the independent variables and by neglecting the statistically insignificant terms. The closer the value of R is to 1, the better the correlation between the obtained and the predicted values. The values of R were 0.99384 for PE production, 0.9886 for biomass, and 0.99364 for EPS production, which suggested that the experimental data be in good agreement with predicted values. The F-value is a ratio of the mean square from regression to the mean square from the real error. Generally, the calculated F-value should be several times greater than the tabulated F-value, if the model is a good prediction of the experimental results and the estimated factor effects are real. Here, the computed F-values of 26.8000 for PE production, 25.7717 for biomass, and 25.9409 for EPS revealing that this regression was statistically significant ($p < 0.05$) at the 95% confidence level.

The results of the regression analysis listed in Tables 3–5. Table 3 indicated that X_4 linear coefficient, which constitutes the most significant term, i.e., liquid volume, had a significant effect on PE production ($p < 0.05$). The interaction between initial pH and inoculation ratio, pH and light intensity, light intensity and liquid volume had influence on PE, but they did not show a significant effect. Table 4 shows that X_4 linear coefficient, which constitutes the most significant term, i.e., liquid volume, had a significant effect on biomass ($p < 0.01$). However, X_1 linear coefficient, i.e., pH, did not have significant effect on biomass ($p > 0.1$). The interaction between inoculation ratio and liquid volume had influence on biomass, but did not show a significant effect. Table 5 illustrated that X_4 linear coefficient, which constitutes the most significant term, i.e., liquid

Table 2 Application of UD $U_9(9^4)$ for optimization of biomass (g/l), EPS (mg/l), and PE production (mg/l)

Experiment no.	Factors				Response		
	Initial pH (X_1)	Light intensity (X_2)	Inoculation ratio (X_3)	Liquid volume (X_4)	Biomass	PE	EPS
1	1(5.0)	2(1,500)	4(1:8.0)	7(250.0)	0.63	58.8	343.5
2	2(5.5)	4(2,500)	8(1:4.4)	5(200.0)	0.83	71.2	380.6
3	3(6.0)	6(3,500)	3(1:10.0)	3(150.0)	1.99	82.4	458.2
4	4(6.5)	8(4,500)	7(1:5.0)	1(100.0)	2.40	112.0	500.4
5	5(7.0)	1(1,000)	2(1:13.3)	8(275.0)	0.47	66.0	254.7
6	6(7.5)	3(2,000)	6(1:5.7)	6(225.0)	0.46	48.0	352.2
7	7(8.0)	5(3,000)	1(1:20)	4(175.0)	1.02	72.0	409.1
8	8(8.5)	7(4,000)	5(1:6.7)	2(125.0)	2.19	91.5	442.7
9	9(9.0)	9(5,000)	9(1:4.0)	9(300.0)	0.34	35.2	314.2

Symbols X_1, X_2, X_3, and X_4 represent factors of pH, light intensity (lx), inoculation ratio (v/v), and liquid volume (ml), respectively. Symbols *1–9* represent the levels of each factor

Table 3 Results of the stepwise regression analysis for optimization of PE production

	Parameters		
	Significance p	t-test	Coefficient
X_4	0.04131	3.43746	–0.92479
X_3^2	0.14566	1.95430	0.81013
X_4^2	0.0382	3.53415	0.92843
X_1X_2	0.46597	0.83298	0.50751
X_1X_3	0.11389	2.21195	–0.84252
X_2X_4	0.28355	1.30305	–0.67761

Table 4 Results of the stepwise regression analysis for optimization of biomass

	Parameters		
	Significance p	t-test	Coefficient
X_1	0.21900	1.45641	–0.64358
X_4	0.00907	4.73562	0.93915
X_3^2	0.12487	1.93657	–0.74537
X_4^2	0.02082	3.70088	0.90572
X_3X_4	0.15613	1.74387	0.70951

Table 5 Results of the stepwise regression analysis for optimization of EPS production

	Parameters		
	Significance p	t-test	Coefficient
X_1	0.26314	1.37386	–0.6980
X_4	0.04484	3.32642	–0.92028
X_1X_3	0.50774	0.74995	0.46850
X_2X_3	0.39110	0.99977	–0.57726
X_2X_4	0.16150	1.84952	0.79438
X_3X_4	0.55935	0.65474	–0.42013

volume, had a significant effect on EPS production ($p < 0.05$). The interaction between initial pH and inoculation ratio, light intensity and inoculation ratio, light intensity and liquid volume, inoculation ratio and liquid volume had influence on EPS, but did not show a significant effect. On the basis of condition optimization evaluated from the model, the optimal values of the test variables obtained by DPS software are as follows: (1) for high PE production, the optimum combination was initial pH 8.0, light intensity 7100.0 lx, inoculation ratio 1:20, and liquid volume 100.3 ml. (2) for high biomass, the combination was initial pH 5.0, light intensity 7098.0 lx, inoculation ratio 1:17.2, and liquid volume 100.0 ml. (3) for high EPS, the optimum culture condition was initial pH 5.0, light intensity 4501.0 lx, inoculation ratio 1:20, and liquid volume 100.3 ml. Under these conditions, the predicted PE production, biomass, and EPS were 123.0 mg/l, 3.29 g/l, and 538.4 mg/l, respectively. After obtained the optimal culture conditions by statistical designs, we tested its feasibility. The arrangement and result of confirmatory trials was as follow, the maximal predicted value of PE yield, biomass, and EPS were 123.0 mg/l, 3.29g/l, and 538.4 mg/l, respectively, where the corresponding experimental response were 132.0 mg/l for PE, 3.27 g/l for biomass, and 543.1 mg/l for EPS. The experimental values of PE production, biomass, and EPS were in good agreement with those of the predicted value. The optimal culture conditions produced 133.6% increase in PE production, 140.4% increase in biomass and 98.9% increase in EPS production when compared with that achieved in unoptimized flask cultures as light intensity 3,000 lx, inoculation ratio 1:6.7, and liquid volume 200 ml and pH normal.

On the effect of pH on the growth of *Porphyridium*, few studies are available. Practically, the range of pH used for the cultures is very wide since it goes from 5.0 to 8.3 (Jones et al. 1963; Nuutila et al. 1997). The pH optimum of 8.0 estimated for PE production is close to the one adopted by Singh et al. (1998), who obtained the optimum growth conditions of pH 7.5, stirring and mineral nutrients in the flat plate glass reactors for EPS production. The highest eicosapentaenoic acid production per cell was achieved at pH 7.6 with 41.8 g/l NaCl and 12.1 mM nitrate in the production medium and using a growth temperature of 8°C (Nuutila et al. 1997). The pH optimum of 5.0 found by this study for biomass and EPS was different from those reported by Singh et al. (1998) and Nuutila et al. (1997).

Porphyridium cruentum grow over a wide range of light intensity, but light level and spectrum greatly control the cell growth. The growth rate in continuous light was faster than in a dark–light cycle (Jones et al. 1963). Light quality is a key factor for the growth and polysaccharide production. Blue and red light could be used to improve the efficiency of photosynthesis and increase the production of extracellular polysaccharide (You and Barnett 2004). The optimum light intensity found by this study (7100.0 lx for PE production) is close to the one adopted by Merchuk et al. (1998), who indicated that higher productivities of biomass and polysaccharide were achieved in the air-lift reactor under high light intensity and low gas flow rates. Muller-Feuga et al. (2003) obtained the light sources delivered a continuous 206 $\mu E/m^2/s$ average photon flux density to cultures reaching concentrations of 3 g/l in batch and 0.7 g/l in chemostat.

Liquid volume in flask is closely related to light intensity, carbon dioxide supply and some rheological properties

during the microalga cultivation processes. When algal cells are grown in photobioreactors, the light decreases exponentially with distance from the irradiated side of the photobioreactor. In the case of dense cultures, this attenuation can be very sharp. The algae near the front are exposed to high photon flux density, which allows a high growth rate. At the core of the reactor, the cells receive less light as a result of mutual shading and thus grow slowly. The greater the light penetrates into the culture, the stronger the effect would be (Merchuk et al. 1998). Liquid volume in flask strongly affected the EPS and PE production, decreased the liquid volume which increased with the light penetration into the liquid, allowed *P. cruentum* cells exposed to high light intensity, and thus led to higher EPS and PE production. Singh et al. (2000) indicated that the highest concentrations of cell mass and polysaccharides were obtained with the narrowest (1.3 cm) light path in a flat plate photobioreactor.

Concerning the optimal inoculation ratio determined, a relatively low value is required in the present study. Higher inoculation rapidly consumed the medium nutrients and overall resulted in less cell growth, EPS and PE production compared to lower inoculation. Sarada et al. (2002) showed that inoculum volume and light intensity, sodium nitrate and inoculum volume had significant effect on yield of biomass of *Haematococcus pluvialis*. The former showed a positive effect while the latter one possessed a negative effect.

Conclusions

Culture conditions are traditionally optimized by the one-at-a-time strategy, i.e., varying one factor while keeping the others constant. Although this strategy is simple and easy as it does not need statistical analysis, it involves a relatively large number of experiments and the interactions among the factors are often neglected. The results obtained in this study showed that UD combining regression analysis is a powerful method for the optimization of the culture conditions for biomass, EPS, and PE production, as it not only provide information about the interactions among the factors, but also help to calculate the optimal conditions by using the smallest number of experiments. The models proved to be efficient in defining optimal conditions for biomass, PE, and EPS production of *P. cruentum*. The successful application of this optimization technique in this study implies that this method is worthy applying to other culture systems for the production of bioactive and biomass of algae.

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