ORIGINAL PAPER

Optimization of renewal regime for improvement of polysaccharides production from *Porphyridium cruentum* by uniform design

Liqin Sun · Changhai Wang · Cuihua Ma · Lei Shi

Abstract To probe the effects of renewal regime on the production of polysaccharides, *Porphyridium cruentum* was cultured semi-continuously in flat plate photobioreactor. Uniform design was used to optimize renewal conditions. Quadratic mathematic models related to productivity, total recovery yield of biomass and polysaccharides were set up to clarify the influence of individual factors and their interactions. According to the mathematic models, the optimal semi-continuous condition for total yield of polysaccharide was $NaNO_3$ 3.5 g/L, renewal rate 27%, renewal period 2.91 days. The optimal condition for polysaccharide output rate was $NaNO_3$ 0.5 g/L, renewal rate 5%, renewal period 7 days. With the optimal renewal regime, the maximal total recovery yields of polysaccharide achieved at 29.4 g, which was 1.57 times higher than that of batch cultivation. The maximum output rate of polysaccharide was 68.64 mg/L per day, which was 2.02 times higher than previous reported data.

Introduction

Marine microalgae are valuable sources for various purposes such as fuels, animal feeds, vitamins, pharmaceuticals, etc. [1]. In recent years, there has been a growing interest in researching microalga polysaccharide because they are easily to be cultivated in large quantity and stable quality. The unicellular marine microalga *Porphyridium cruentum* (*P. cruentum*) can synthesize and secrete sulfated polysaccharides to the cultural medium when the cells grow to its steady period. The extracellular polysaccharides from *P. cruentum* are highly sulfated acidic heteropolymers composed of xylose, galactose and glucose. The molecular weight is in the range of $2–7 \times 10^6$ Da [2]. Different from the polysaccharides extracted from plants or macroalgae, microalgae polysaccharides have some special properties for industrial uses such as in cosmetics, health food, anti-herpes, antiviral and anti-irritant medication [3–5].

Previous studies have shown that nutrient and light availability affected the production of cell biomass and extracellular polysaccharides [6–8]. Nitrogen determines the production of polysaccharides and influences the secretion of soluble polysaccharides [9]. At present, most researches on microalga cultivation are carried out in batch culture system. Nevertheless, the productivity of microalga biomass or polysaccharide is usually considerably higher in continuous or semi-continuous culture system [10, 11]. Due to the operational simplicity of semi-continuous culture, several parameters such as renewal rate and nutrient concentration were generally studied to control the productivity

L. Sun · C. Wang · C. Ma · L. Shi
Ocean School of Yantai University, 264005 Yantai,
Shandong, People's Republic of China
e-mail: sliqin2005@163.com

L. Sun · C. Wang
Marine Biochemical Engineering Lab, Dalian University
of Technology, 116024 Dalian, Liaoning,
People's Republic of China

C. Wang (✉)
Department of Bioscience and Bioengineering, Ocean School
of Yantai University, Dalian University of Technology,
Dalian, People's Republic of China
e-mail: chwang2001@sina.com

and biochemical composition [12, 13]. Although several data are available on the biochemical variability of *P. cruentum* controlled by changes of culture parameters like renewal rate, nutrient concentration in semi-continuous systems [10, 14], the previous research mostly focused on the single factor such as renewal rate or nutrients. The integrative factors and their interaction affecting the output rate and yield of exopolysaccharide still need to be studied.

In this work, we introduced the uniform design method to investigate the interaction between nutrition and renewal regime on extracellular polysaccharides production of *P. cruentum*. Culture parameters such as nitrogen concentration, renewal rate and renewal period were obtained by regression analysis for the maximum recovery yield and output rate of polysaccharides. The optimized method, culture technology, and the technical conditions achieved in this work could be an important reference for harvesting extracellular polysaccharide of *P. cruentum* in large-scale.

Materials and methods

Microorganism and the cultural conditions

Porphyridium cruentum was provided by the Institute of Oceanology, Chinese Academy of Sciences. The high-yield strain *P.C-03* was isolated, purified and cultivated in the optimum medium OMII [15]. The *P. cruentum* cells were grown in a newly designed flat-plate organic glass photobioreactor (FPPBR-50) (Fig. 1). The size was $50 \times 5 \times 60$ cm, and the actual cultural volume was 15 L. The temperature and pH were monitored by thermometer and pH meter installed on the top of the bioreactor. The temperature of culture system was controlled at 25 °C (± 1 °C) by air-conditioning in culture room. Light source was supplied by 15 W Philips cool-white lamps (F15T12/CW), and the light intensity was adjusted at 80 μE m^{-2} s^{-1}. The culture system was agitated by charging 1% CO_2 in compressed air at

10 L/min through an aeration tube extended to the bottom of the photobioreactor. The initial inoculation density was about 60×10^4 cells/mL, and the cultures were kept in this semi-continuous regime for 23 days.

Analytical determination

Cell numbers were counted six times under a microscope (OLYMPUS CH-2) using a bright-light hemacytometer. The value was mean $\pm$ SD. The biomass dry weight (DW) W and growth rate K were calculated by Eqs. 1 and 2, in which N_2 and N_1 were cell densities (cells/mL) at two different time points t_2 and t_1 (days).

$$W(\mathrm{g/L}) = 1.4054 \times \mathrm{OD}_{604} - 0.0727 \quad (r^2 = 0.9923) \quad (1)$$

$$K = \log_2(N_2/N_1)/(t_2 - t_1) \quad (2)$$

To determine the concentration of extracellular polysaccharide (EPS), the recovery suspension was centrifuged at 4,500 rpm for 10 min. Then, the top layer solution was subjected to the phenol-sulfuric acid test with glucose as the standard [16]. Each test was performed in triplicate. Value was the mean $\pm$ SD.

Total recovery yield (Y_{total}), productivity of cell biomass and extracellular polysaccharides produced by *P. cruentum* were calculated by Eqs. 3 and 4, in which C was the concentration of biomass and EPS of the renewal broth each time; n was the renewal times; V was the total culture volume (L); R was the renewal rate (%); P was the output rate; V_R was the total recovery volume (L) and t_g was the total culture time of *P. cruentum* (day).

$$Y_{\mathrm{total}} = \sum_{i=1}^{n-1} C_i VR + VC_n \quad (3)$$

$$P = \frac{Y_{\mathrm{total}}}{V_R t_g} \quad (4)$$

Nitrogen concentrations were measured by ultraviolet spectrophotometry [17].

Uniform design

The experiment for optimization of renewal regime was arranged as three factors, and each was at seven levels. The *UD* table U$_7$ (7^3) was applied to arrange the experiments (Table 1) [18]. Each trial was performed in triplicate. The semi-continuous regime was started at seventh day when the cell growth had reached the logarithmic middle-phase.

Statistical analysis

All experiments were performed in triplicate. The averages of total recovery yield, productivity of biomass and polysaccharides were taken as response values. The statistical
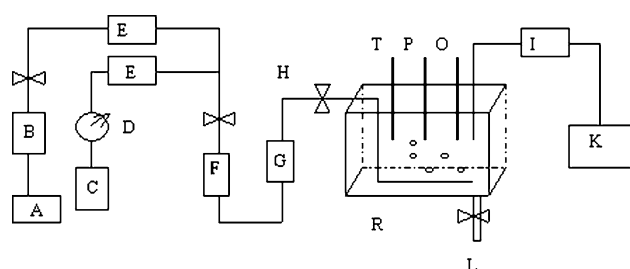

Fig. 1 Schematic drawing of flat-plate photobioreactor system employed by this study. *A* air compressor, *B* air reservoir, *C* CO$_2$ tank, *D* press meter, *E* flow meter, *F* oil and water seperator, *G* gas analyzer, *H* aeration tube (diameter 5 mm), *R* airlift flat-plate type photobioreactor, *I* Periplastic pump, *K* inlet reservoir, *L* sampling port, *T* thermometer, *P* pH probe, *O* DO probe

Table 1 Factors and levels in the uniform design experiments

Experiment No.	Factors		
	C_{NaNO_3} (g/L) (X_1)	Renewal rate (%) (X_2)	Renewal period (days) (X_3)
1	3 (1.5)	2 (10)	1 (1)
2	6 (3.0)	4 (25)	2 (2)
3	2 (1.0)	6 (40)	3 (3)
4	5 (2.5)	1 (5)	4 (4)
5	1 (0.5)	3 (20)	5 (5)
6	4 (2.0)	5 (30)	6 (6)
7	7 (3.5)	7 (50)	7 (7)

and stepwise regression analysis was performed with *UD* software (Uniform Design Version 3.0, developed by Wang Y. F., Jilin, China). The Tukey test ($p < 0.05$) was used to compare different treatments (SPSS for windows Version 11.5).

Results

The change of cultural parameters in semi-continuous cultivation

The renewal culture began on the seventh day because cells reached the highest growth activities in this period. The changes of cell density in different renewal regimes were shown in Fig. 2. The final cell density of batch culture was $(1{,}262.84 \pm 23.7) \times 10^4$ cells/mL. In semi-continuous culture system, the cell density reached equilibrium within each renewal period in experiments No. 1 and No. 2. In experiments No. 4, 5, 6 and 7, the cell density in the recovery suspensions exhibited periodic increase, the maximum was $(1{,}113.26 \pm 21.7) \times 10^4$ cells/mL on the 23th day of No. 6. The lowest cell density $(555.30 \pm 17.6) \times 10^4$ cells/mL was observed in experiment No. 3 due to its high renewal rate and short renewal period.
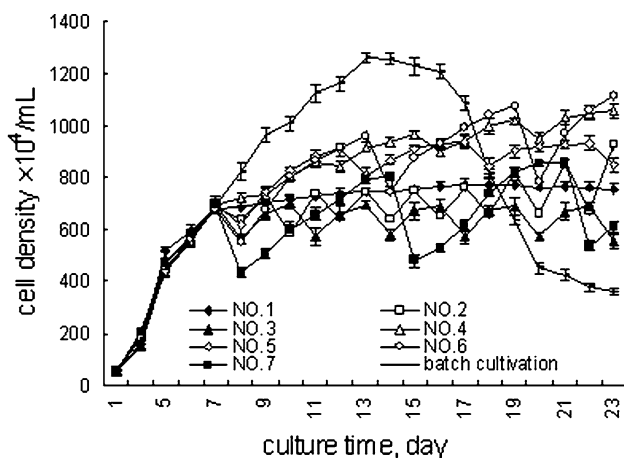
The renewal regimes also affected the production of extracellular polysaccharides in recovery suspensions. As shown in Fig. 3, in experiments No. 4, 5 and 6, the concentration of polysaccharides exhibited periodic increase within each renewal period. In experiment No. 4, the maximum polysaccharide yield was $1{,}108 \pm 13.2$ mg/L. The polysaccharide concentration decreased dramatically in experiment No. 3 because of high renewal rate and short renewal period. Meanwhile, the polysaccharide was only 252.92 ± 3.87 mg/L after the last renewal cycle. In batch culture, the highest polysaccharide concentration was 1.25 g/L at the 16th day. However, with the increase of polysaccharide secretion, the culture medium turned viscous, and the cells autolyzed. Therefore, the concentration of polysaccharide cannot be determined exactly.

The consumption of $NaNO_3$ in the cultural media was shown in Fig. 4. In experiment No. 2, a high average concentration of 2.0 ± 0.19 g/L was observed with a low standard deviation of 0.19, which indicated the establishment of a balance between the addition and consumption of nitrogen. The result could farther explain why cell density
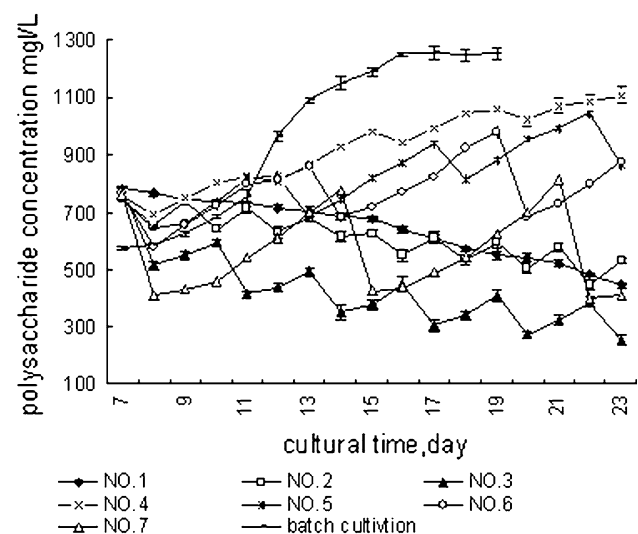

Fig. 2 Growth curve of *P. cruentum* under different treatments

Fig. 3 Variety of polysaccharide concentration in recovery suspension under different treatments
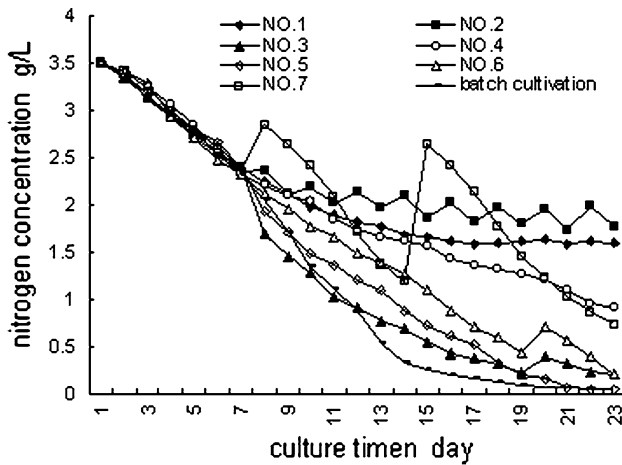
Fig. 4 Varieties of concentration of NaNO$_3$ in culture medium in different treatments

kept equilibrium and the highest growth rate was obtained in experiment No. 2 (Fig. 2; Table 2). In experiment No. 3, the concentration of NaNO$_3$ was at a lower average level of 0.78 g/L with a high standard deviation of 0.62. The results indicated that renewal regime affected the consumption-addition balance of nitrogen. Therefore, there would be an optimal renewal regime to maintain nitrogen concentration in culture liquid at stable level, and made cells growth steady.

Optimization on renewal regime for maximum recovery yield and productivity

According to Table 2, the average growth rate (K_{avg}) between different runs had significant difference ($p < 0.05$). The higher average growth rates were obtained in experiments No. 2 and 3, and both of which had short renewal period and high renewal rate. The total recovery yield of biomass and polysaccharides between different runs also had significant differences (ANOVA, $p < 0.01$), and their maximal value were obtained in experiments No. 2 with 63.9 and 27.7 g, respectively. Besides, the recovery

volume (V_R) in semi-continuous culture was larger than that in batch culture, and the larger V_R was observed in experiments No. 2 and 3 too (Table 2). The results indicated that the final recovery yield correlated with the K_{avg} and V_R, and recovery volume was the function of renewal rate and renewal period. Therefore, the optimum renewal regime was asked in order to obtain the maximal biomass and polysaccharide production. The output rate between different runs had slight significant difference ($p < 0.05$). The highest output rates of cell biomass and polysaccharides were obtained in experiments No. 6 and 4.

Equation 5 was established as the mathematic model for the optimization of three factors for maximum productivity. The quadratic equations relating to different indexes were expressed in terms of the values of all the independent variables and by neglecting the statistically insignificant terms. The mathematic expressions were established as Eqs. 6–10. The analysis of variance for the mathematical models was listed in Table 3.

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_1^2 + b_5 x_1 x_2 + b_6 x_1 x_3 + b_7 x_2^2 + b_8 x_2 x_3 + b_9 x_3^2 \tag{5}$$

$$K_{avg} = 0.126 + 0.0001 x_1 + 0.991 x_2 - 0.04 x_3 + 0.004 x_1 x_3 - 1.12 x_2^2 \tag{6}$$

$$Y_{c-total} = 36.4 + 233 x_2 - 6.26 x_3 - 0.424 x_1^2 + 1.28 x_1 x_3 - 354 x_2^2 \tag{7}$$

$$Y_{EPS-total} = 15.28 + 84.32 x_2 + 1.70 x_1 x_2 + 0.154 x_1 x_3 - 168 x_2^2 - 0.091 x_3^2 \tag{8}$$

$$P_{biomass} = 22.4 + 26.2 x_1 + 7.47 x_3 - 5.17 x_1^2 - 1.11 x_1 x_3 - 86.6 x_2^2 \tag{9}$$

$$P_{EPS} = 19.81 - 1.99 x_1 + 8.4 x_3 + 10.59 x_1 x_2 - 53.41 x_2^2 - 16.14 x_2 x_3 \tag{10}$$

All mathematic expressions were found to have high correlation ($R^2 > 0.99$) (Table 3), which suggested that the

Table 2 Experiment of *UD* U$_7$ (7^3) for the optimization of recovery yield of biomass, EPS and growth rate mean ± SD

Runs	Factors and levels			Response					
	X_1 g/L	X_2%	X_3 day	K_{avg} (per day)	V_R (L)	$Y_{c-total}$ (g)	$Y_{EPS-total}$ (g)	$P_{biomass}$ (mg/L per day)	P_{EPS} (mg/L per day)
No. 1	1.5	10	1	0.18 ± 0.016	39	50.7 ± 1.04	22.3 ± 2.14	54.4 ± 1.78	24.9 ± 1.33
No. 2	3	25	2	0.25 ± 0.012	48.6	63.9 ± 0.69	27.7 ± 0.96	57.1 ± 4.47	26.8 ± 0.45
No. 3	1	40	3	0.24 ± 0.004	51.0	57.8 ± 2.31	22.4 ± 1.46	49.3 ± 2.33	19.1 ± 0.75
No. 4	2.5	5	4	0.06 ± 0.032	18.0	32.4 ± 1.75	19.3 ± 5.11	75.2 ± 0.91	46.7 ± 1.11
No. 5	0.5	20	5	0.09 ± 0.04	27.0	40.5 ± 0.79	23.1 ± 4.23	65.2 ± 4.56	43.8 ± 2.25
No. 6	2	30	6	0.14 ± 0.04	28.5	50.6 ± 4.75	24.9 ± 2.14	77.2 ± 2.71	38.0 ± 4.67
No. 7	3.5	50	7	0.17 ± 0.007	37.5	46.6 ± 3.78	17.7 ± 1.11	54.1 ± 1.53	20.5 ± 2.43
Batch cultivation				0.13 ± 0.003	15	28.1 ± 2.17	12.6 ± 3.23	81.4 ± 5.11	36.6 ± 3.01

Table 3 Variance analysis by stepwise regression on mathematic models

Index	Correlation coefficient R^2	Ratio of equal square F t	T test for regression coefficient				
			t_1	t_2	t_3	t_4	t_5
K_{avg}	0.9992	240.7[a]	1.52[b]	19.90[a]	−18.40[a]	1.82[b]	−15.17[a]
$Y_{c\text{-total}}$	0.9998	1,083[a]	42.38[a]	−29.77[a]	−4.027[b]	12.07[b]	−31.67[a]
$Y_{EPS\text{-total}}$	0.9999	10,630[a]	136.9[a]	16.27[a]	16.58[a]	−164[a]	−40.04[a]
$P_{biomass}$	0.9967	260.88[a]	6.45[b]	8.15[b]	−6.07[b]	−2.64[b]	−16.90[a]
P_{EPS}	0.9988	264.3[a]	−1.43[b]	20.83[a]	1.84[b]	−3.54[b]	−14.76[a]

Stylebook capability $N = 7$, significance $\alpha = 0.05$, critical value $F(0.05, 5, 1) = 230.2$

[a] Variables had significant difference

[b] Variables have no significant difference

model was suitable for evaluating the semi-continuous system. Generally, the calculated F value should be greater than that of the critical value, if the model was a good prediction for experimental results. Here, all of the computed F values for the equations were greater than that of the critical values, and the regression coefficients of equations were statistically significant (Table 3). Therefore, the equations were authentic.

Equation 6 was the quadratic function of average growth rate (K_{avg}) of microalga cells in semi-continuous culture. t test for the regression coefficient of variable revealed that renewal rate (X_2) and renewal period (X_3) had more significant effect ($p < 0.05$) on growth rate than the nitrogen concentration (X_1). The t test results of variable coefficients in Eq. 8 showed that all three factors (X_1, X_2, X_3) had significant effect ($p < 0.05$) on total recovery yield of polysaccharides ($Y_{EPS\text{-total}}$). t test for Eq. 7 showed that only the renewal rate (X_2) and the renewal period (X_3) had a significant effect on total recovery yield of biomass ($Y_{c\text{-total}}$), but the interaction between nitrogen concentration (X_1) and renewal period (X_3) had no significant effect (Table 3). In Eq. 9, none of three factors was found significant ($p > 0.05$), but square of renewal rate (X_2) had a significant effect on the productivity of biomass. Similarly, only renewal period (X_3) and interaction of X_2 and X_3 (X_2X_3) had a significant impact on productivity of polysaccharides in Eq. 10.

Based on the model evaluation, the optimal test variables and anticipated maximum indexes were calculated by UD software with gridding attempt method (Table 4). Experimental confirmations were carried out with each set of calculated optimal renewal regimes, and the culture time was 23 days. Regularity for change of cell growth, EPS production and nitrogen consumption in the process of culture were similar to the above results showed in Figs. 2, 3, 4. The final experimental results were in good agreement with predicted maximum indexes (Table 4), which also proved the credibility of the mathematic models.

Discussion

The concentration of nutrient salts and light attenuation were the key limiting factors for cell growth and secondary metabolites synthesis in batch culture. The semi-continuous culture could change the nitrogen concentration and light utilization efficiency, accordingly overcame the restrictions and kept microalgal cells growing steadily. Renewal rate, renewal period, nitrogen concentration, and their interaction had significant effects on the cell growth and recovery yield (Table 3). In our previous study on growth dynamics of *P. cruentum* [19], we have validated that light attenuation was the function of incident light intensity (I_0), cell density, and light-path of photobioreactor (LP). With increase of cell density, the degree of light attenuation increased. In this study, renewal rate correlated with nitrogen concentration and cell density in the culture media, so it affected cell

Table 4 The optimized renewal regime and concentration of NaNO₃ for maximum index

Index	Optimum renewal regime			Anticipated maximum index	Experimental result
	C_{NaNO_3} (g/L) (X_1)	Renewal rate % (X_2)	Renewal period (days) (X_3)		
$Y_{c\text{-total}}$ (g)	1.50	33	1.0	69.5 ± 4.43	68.7 ± 3.41
$Y_{EPS\text{-total}}$ (g)	3.5	27	2.91	28.16 ± 0.28	29.44 ± 1.12
$P_{biomass}$ (mg/L per day)	1.78	5	7	90.9 ± 19.50	85.6 ± 9.87
P_{EPS} (mg/L per day)	0.5	5	7	72.11 ± 7.66	68.64 ± 4.04
K_{avg} (per day)	3.5	44	1.0	0.32 ± 0.06	0.297 ± 0.04

growth rate and polysaccharide production. Renewal period determined recovery time and consumption rate of nitrogen, and nitrogen concentration influenced the growth of cells and the production of polysaccharides [9]. Therefore, an optimal renewal regime is needed for obtaining the maximum value of different index.

If the cell growth period, namely, the special time required for the cell density updated to the initial level before renewal, was longer than the renewal period, cell density in the recovery suspension could be increased by applying a longer renewal period. It was understandable that the highest and the lowest concentration of biomass were observed in experiments No. 6 and 3, respectively (Fig. 2). As shown in Fig. 3, the concentration of polysaccharides in the recovery suspension increased with addition of renewal degrees in experiments No. 4, 5 and 6, which elucidated that low renewal rate and long renewal period would facilitate the synthesis of polysaccharides. Nitrogen concentration remained steady when supply and consumption was at equilibrium. However, if the supply did not keep up with consumption, the nitrogen concentration decreased. In experiment No. 4, the average nitrogen concentration was 1.56 ± 0.43 g/L in culture liquid. Accordingly, the concentration of polysaccharides reached the highest value (Figs. 3, 4). The result could agree well with our previous work about culture medium optimization, which showed that the highest polysaccharides production from *P. cruentum* was obtained at $NaNO_3$ concentration of 1.5 g/L.

The mathematic models revealed the effects of single factors and their interaction on growth rate, total recovery yield and output rate. From the quadratic equations, it was found that the property and significant difference of individual variable varied with the target product. For instance, the total recovery yield of biomass ($Y_{c\text{-total}}$) correlated with renewal rate (X_2), renewal period (X_3), and the interaction between nitrogen concentration and renewal period (X_1X_3), but the $Y_{EPS\text{-total}}$ was the function of renewal rate (X_2), X_1X_2, and X_1X_3. The maximum average growth rate and total recovery yield of biomass were achieved under renewal condition of short renewal period and high renewal rate (Table 4), which can be explained by that short renewal period and high renewal rate could keep the nitrogen concentration of culture system in a normal level, the same time, it could reduce light attenuation degree. Thus, the growth rate and biomass were increased. However, extension of renewal period was in favor of the increase of total recovery yield of polysaccharides (Table 4). The maximum output rate of both biomass and polysaccharides were observed at low renewal rate and long renewal period. The optimal concentration of $NaNO_3$ for maximum productivity of polysaccharides was 0.5 g/L, which was lower than that for maximal productivity of cell

biomass (1.78 g/L) (Table 4). The results indicated that the deficiency of nitrogen would stimulate synthesis of polysaccharide in some microalgal cells [20, 21]. Besides, different microalga species and different culture system have dissimilar optimal renewal regime. For instance, the optimum renewal rate for *Chlorella* spp. cultured in column photobioreactor was 33%, whereas the optimum renewal rate for *Phaeodactylum tricornutum* in 250 mL Erlenmeyer flasks was 18.2% [22, 23].

According to the experimental results (Table 4), the average production of biomass and polysaccharide were calculated as 4.58 and 1.96 g/L, respectively, in semi-continuous culture system (the average production was the ratio of total yield to volume of photobioreactor 15 L). However, only biomass production of 2.22 g/L and EPS production of 1.25 g/L were achieved in batch culture system. The results confirmed that semi-continuous culture mode was more efficient for increasing the production. In this study, the maximal output rate of polysaccharide was 68.64 mg/L per day, which was 2.02 times higher than the one cultivated in 20 cm LP flat plate photobioreactor 15 L. The polysaccharide concentration of *P. cruentum* cultured in batch or semi-continuous mode were mostly in the ranged of 500–950 mg/L [1, 6, 14]. Although some researchers reported higher polysaccharide concentration [10, 24], they cultured the alga in 80 mL glass tubes. The difference maybe caused by the quantitative method. Generally, the total recovery yield and output rate are the function of renewal rate and renewal period, also affected by nutrients. The high polysaccharide concentration would not always imply high total recovery yield and high output rate. For example, the highest exopolysaccharide concentration was 1.10 g/L in experiment No. 4 (Fig. 3), but the maximal total recovery yield of EPS was harvested in experiment No. 2, not in No. 4 (Table 2). Similarly, the highest polysaccharide concentration was 1.25 g/L in batch culture. However, the total recovery yield was 4.5-fold and the output rate was 7.8-fold lower than that in semi-continuous culture with optimal renewal regime.

The established mathematic models were credible and efficient in the determination of optimal renewal regime for growth rate, total recovery yield and productivity. The optimal renewal condition was objective dependent and could be designed accordingly.

References

1. You T, Barnett SM (2004) Effect of light quality on production of extracellular polysaccharides and growth rate of *Porphyridium cruentum*. Biochem Eng J 19:251–258

2. Geresh S, Adin I, Yarmolinsky E, Karpasas M (2002) Characterization of the extracellular polysaccharides of *Porphyridium sp.*: molecular weight determination and rheological properties. Carbohydr Polym 150:183–189

3. Mahmoud A, Vladimir Z, Jacov T, Arad SS (2002) Activity of *Porphyridium sp* polysaccharide against herpes simplex viruses in vitro and in vivo. Biochem Biophys Meth 50:189–200

4. Liu XC, Vonshak A (2004) Modified responses of *Porphyridium sp* cells with an inxreased polysaccharide level to stress. Guangdong Plant 2:166–173

5. Gu NY, Liu YF (2002) Bioactive substances of *Porphyridium* and their applications. J China Mar Medicat 6:43–48

6. Wang J, Chen BL, Rao XZ (2007) Optimization of culturing conditions of *Porphyridium cruentum* using uniform design. World J Microbiol Biotechnol 6:117–123

7. Joung HY, Sung JK, 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

8. Chen BL, Liang SZ, Wang J (2004) Optimization cultural conditions of *Porphyridium cruentum* in the stirred photobioreactor. J Fujian Normal Univ Nat Sci Edition 2:91–96

9. Arad MS, Brown SY, Dubinsky O (1992) Effect of nitrate and sulfate starvation on polysaccharide production in *Rhodella reticulate*. Bioresour Technol 42:141–143

10. Fabregas J, Garcia D, Morales E et al (1998) Renewal rate of semicontinuous cultures of the microalga *Porphyridium cruentum* modifies phycorythrin, exopolysaccharide and fatty acid productivity. J Ferment Bioeng 86:477–481

11. Zhu YF, Lin X, Xu TC et al (2004) Effects of light intensity, concentration of nitrogen and renewal rate in semicontinuous cultures on growth and recovery yield of *Nanochloropsis oculate* (Droop) Hibberd. J Fish Sci China 11:159–164

12. Otero A, Fabregas J (1997) Changes in the nutrient composition of *Tetraselmis suecica* cultured semicontinuously with different nutrient concentrations and renewal rate. Aquaculture 159:111–123

13. Otero A, Garcia D, Morales ED et al (1997) Manipulation of the biochemical composition of the eicosapentaenoic acid rich microalga *Isochrysis galbana* in semixontinuous cultures. Biotechnol Appl Biochem 26:171–177

14. Singh S, Arad SM, Richmond A (2000) Extracellular polysaccharide production in outdoor mass cultures of *Porphyridium sp* in flat plate reactors. J Applied Phycol 12:269–275

15. Sun LQ, Lin J, Wang CH (2004) Application of uniform design in the media optimization of *Porphyridium cruentum*. J Mar Sci Bull 23:23–29

16. Zhang WJ (2003) Saccharide complex biochemical research technology. Zhejiang University Press, Hangzhou, pp 11–12

17. State Environmental Protection Administration (1989) Inspect and analysis methods of water and waste water. China Environmental Science Press, Beijing, China, pp 278–280

18. Zeng SJ (2005) Uniform design and its application. Medicine Technology Press, Beijing, p 122

19. Shi L (2008) Optimization of culture conditions for *Porphyridium cruentum* in the flat plate photobioreactors. Master Dissertation submitted to Yantai University, pp 31–33

20. Maria A, Guzman M, Claudia C et al (2007) Effects of fertilizer-based culture media on the production of exocellular polysaccharides and cellular superoxide dismutase by *Phaeodactylum tricornutum* (Bohlin). J Appl Phycol 19:33–41

21. Otero A, Dominguez A, Lamela T et al (1998) Steady-states of semicontinuous cultures of a marine datom: effect of saturating nutrient concentrations. J Exp Mar Biol Ecol 227:233–234

22. Zhang DL, He PM, Zhou HQ (1999) Experiment on different culture methods of *Chlorella spp*. J Shanghai Fish Univ 8:1–5

23. Zhu YF, Wu SJ, Guo XQ (2000) Effects of nitrogen, phosphorus concentration and renewal rate on recovery yields of *Phaeodactylum tricornutum* in semicontinuous cultures. Mar Sci 24:35–38

24. Hernandez CJ, Cass-Valdez M, Ortega-Garcia S (2000) Effect of climatic variation on the relative abundance of red alga *Gelidium robustus* in Baja Califormia Sur, Mexico. J Appl phycol 177:177–188