Bioprocess and Biosystems Engineering (2020) 43:347–355
https://doi.org/10.1007/s00449-019-02230-6

RESEARCH PAPER

Induced cultivation pattern enhanced the phycoerythrin production in red alga *Porphyridium purpureum*

Yuanchao Xu[1] · Kailin Jiao[2] · Huichang Zhong[3] · Shengshan Wu[2] · Shih-Hsin Ho[4] · Xianhai Zeng[2,5] · Jinglong Li[1] · Xing Tang[2,4] · Yong Sun[2,4] · Lu Lin[2,4]

Received: 23 July 2019 / Accepted: 30 September 2019 / Published online: 12 October 2019

Abstract

Porphyridium purpureum is a rich source for producing phycoerythrin (PE); however, the PE content is greatly affected by culture conditions. Researchers have aimed to optimize the cultivation of *P. purpureum* for accumulation of PE. When traditional optimized culture conditions were used to cultivate *P. purpureum*, high PE contents were not usually achieved. In this study, an induced cultivation pattern was applied to *P. purpureum* for PE biosynthesis (i.e., an incremental approach by altering temperatures, light intensities, and nitrate concentrations). Results revealed that the induced pattern greatly improved the PE biosynthesis. The optimized PE content of 229 mg/L was achieved on the 12th cultivation day, which was a maximum PE content within one cultivation period and accounted for approximately 3.05% of the dry biomass. The induced cultivation pattern was highly suitable for PE synthesis in *P. purpureum*, which provided an important reference value to the large-scale production of PE.

Keywords *Porphyridium purpureum* · Induced cultivation pattern · Biomass · Phycoerythrin

Introduction

Phycoerythrin (PE) is a fluorescent protein that can be separated and purified from algae and laver, and is a new type of fluorescent marker dye that possesses a high market potential. PE is a natural dye that has high water solubility and is widely applied in the food and the cosmetics industries [1–4]. PE also has high fluorescence and photostability, which enhances its research value a lot in medical and molecular biology fields [2, 5–7]. In recent years, PE has been used as a photosensitizer in photodynamic therapies for tumor cells after showing significant effects in studies [8–10].

PE widely exists in Cyanophyta and Rhodophyta species, and can be extracted from algae such as *Porphyra haitanensis*, *Porphyridium purpureum*, *Synechococcus leopoliensis*, *Spirulina platensis,* and *Bangia fusco-purpurea.* The biomass productivity of *P. purpureum* is high, showing strong salt resistance and abundant production of high-value products in cells. The Phycobiliprotein (PB) content is high, in which PE is the most abundant [11–13]. However, under regular cultivation strategies, the productivity of PE in *P. purpureum* is limited, which prevents any further commercial-scale application [2, 13]. Therefore, to increase the content of PE in *P. purpureum* for larger scale production methods, culture methods have been the primary focus in research.

Jaime et al. [14] conducted semi-continuous cultivation of *P. purpureum* for PE, polyunsaturated fatty acid (PUFA) and extracellular polysaccharide (EPS) production, and highlighted that the production of these high-value products required different culture strategies. Fuentes-Grünewald

✉ Xianhai Zeng
xianhai.zeng@xmu.edu.cn

1 College of Biological Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan 250353, China

2 College of Energy, Xiamen University, Xiamen 361102, China

3 Xiamen Huison Biotech Co., Ltd., No. 1337, Tongji Rd, Tongji Industrial District, Xiamen 361100, China

4 State Key Laboratory of Urban Water Resource and Environment, School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150001, People's Republic of China

5 Fujian Engineering and Research Center of Clean and High-Valued Technologies for Biomass, Xiamen Key Laboratory of Clean and High-valued Utilization for Biomass, Xiamen University, Xiamen 361102, China

et al. [15] suggested that semi-continuous cultivation was more favorable for biomass accumulation in *P. purpureum* (47.04 mg/L/d) as well as extracellular polysaccharide (2.1 g/L). Kathiresan et al. [16] proposed that the concentrations of chlorides, nitrates and sulfates affected the synthesis of PE a lot. Wang and Freddy et al. [17, 18] also indicated that PE biosynthesis could be promoted in *P. purpureum* under optimal cultivation conditions [pH 8.0; light intensity of 7100.0 lx; ratio of inoculum to substrate 1:20; loaded liquid 100.3 mL; low temperature (20 °C); and low light (30 μmol/m^2s)].

The abovementioned studies have, to some extent, facilitated increases in PE synthesis by *P. purpureum*. However, due to the conditions (e.g., low temperature, low light, high pH, and high nutrition concentrations) used for cultivating *P. purpureum* in these studies, *P. purpureum* was subjected to environmental stress at the initial stage of culture, that affected the schizogamy of *P. purpureum* cells [12, 13, 19]. This manifested the low vitality of *P. purpureum* in its initial culture stage, which affected *P. purpureum* biomass accumulation and further PE biosynthesis.

By targeting at the abovementioned problem, in this study, an induced cultivation strategy was conducted to optimize the growth of *P. purpureum* and biosynthesis of PE. Briefly, the strategy was as follows: normal culture conditions for *P. purpureum* during the initial stage culture of *P. purpureum* cells; alteration of temperatures, light intensities, and nitrate concentrations to promote synthesis and accumulation of PE. This induced cultivation strategy greatly enhanced PE biosynthesis, which provides important reference values for the large-scale production of PE.

Materials and methods

Microalgae strain and culture medium

Porphyridium purpureum CoE1 The algal species used were purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology of the Chinese Academy of Sciences. The strain was further induced, screened, and stored in artificial seawater (ASW) with periodical refreshment by the author's research group [20].

Experimental design

Freddy et al. [18] used response surface methodology to confirm that cultivating *P. purpureum* at low light and low temperature optimizes PE production in nitrogen-replete medium. Therefore, in this experiment, temperature, light intensity, and potassium nitrate concentration were used as inducing factors, and three cultivation patterns (control pattern, specific pattern, and induced pattern) were designed. We compared three cultivation patterns on *P. purpureum* biomass production and PE biosynthesis. The details are as follows.

Control pattern

The algal species were inoculated in 2 L conical flasks containing 1 L ASW medium with 10% of the inoculum volume, and cultivated with continuous light in an illumination incubator. The temperature was controlled at 25 °C, the illumination intensity was set at 165 μmol/m^2s, and the sterile air ventilation rate was set at 1 L/min. The potassium nitrate concentrations were 1, 2, 4, and 6 g/L, corresponding to four experimental groups, respectively. Three 18-day parallel culture cycles were set up for each experimental group.

Specific pattern

The culture temperature was controlled at 20 °C and the illumination intensity was set at 55 μmol/m^2s. Other cultivation conditions were the same as those in the control pattern.

Induced pattern

The temperatures, illumination intensities, and nitrate concentrations were adjusted in 3 stages to induce PE biosynthesis by *P. purpureum*. First, the temperature was controlled at 25 °C and the illumination intensity was set at 165 μmol/m^2s, while the initial potassium nitrate concentrations in different experimental groups were all controlled at 1 g/L for culture for 2 days. The temperature was then adjusted to 23 °C and the light intensity to 110 μmol/m^2s while providing 0, 0.5, 1.5, and 2.5 g potassium nitrate, respectively, to different experimental groups, which were subsequently cultivated for 2 days. Finally, the temperature was adjusted to 20 °C and the illumination intensity to 55 μmol/m^2s while providing 0, 0.5, 1.5, and 2.5 g potassium nitrate, respectively, to that different experimental groups, which were cultivated until the 18th day. Other cultivation conditions were the same as those in the control group. All the tests were repeated for three times.

Sampling procedure

Preparation of the dry powder of P. purpureum The algal solution was collected and centrifuged at 8000 g for 10 min. Deionised water was then added to wash the cell pellet for 2–3 times. Later, the collected algae ware frozen and dried using a vacuum freeze drier (Bilon, FD-1000), while the collected dried algae cells was pulverized in a mortar and stored at − 20 °C for further analysis. The biomass of *P. purpureum,* total carbohydrate, total protein, and PE content was measured from culture samples every 48 h.

Biomass concentration

The linear regression equations of dry cell weights and absorbance were obtained from Su et al. [21], and were used to measure the biomass of *P. purpureum*. Algal solution (3 mL) was collected and centrifuged at 4000 g for 15 min. The cell pellet was resuspended in fresh culture media and the absorbance of the solution was measured at 604 nm with an ultraviolet spectrophotometer (Shimadzu, UV-2600), which was substituted into Eq. (1) to obtain the *P. purpureum* biomass concentration:

$$Y = 2.4951 \times \mathrm{OD}_{604} - 0.5121 (R^2 = 0.997). \quad (1)$$

In the above equation, Y represents the biomass concentration (g/L) and OD represents the optical density of the substance at 604 nm.

Total carbohydrate concentration

The quantitative saccharification method [22] reported by the U.S. National Renewable Energy Laboratory (NREL) was adopted to measure the total carbohydrate content. Briefly, 3 mL 72% concentrated sulfuric acid (w/w) was added to the algal powder (3–5 mg) and the sample was incubated at 30 °C for 30 min for hydrolysis. The concentration of sulfuric acid decreased to 4% (w/w) and the solution was then hydrolyzed in a 1×10^5 Pa autoclave. Finally, a high-performance liquid chromatography (HPLC, Waters 2695) was used to measure the contents of glucose, xylose, and galactose in the hydrolysate to further calculate the total carbohydrate content [23].

Total protein concentration

The bicinchoninic acid method was used to determine the total protein content [24]. Three milliliters of the algal solution was collected and centrifuged at 8000 g for 5 min. The pellet was resuspended in 3 mL 0.1 M PBS (pH 7.0); then, 0.05 mm and 0.1 mm (volume ratio 1:1) diameter small magnetic beads were added and vibrated for 20 s on a high-speed vibration bead mill. Then, the samples were quickly placed on ice for cooling. The abovementioned steps were repeated for 4–5 times, and then, the supernatant was taken after centrifugation for 3 min at 8000 g. The BCA method was used to measure the total protein content on a microplate reader (Molecular Devices, SpectraMax® Prardigm®, Multi-mode Detection Platform). Standard curves were prepared based on the specifications of the BCA Protein Assay Kits (Thermo scientific).

PE concentration

PE was collected using a method developed by Chopin et al. [25] with appropriate modifications. Three milliliters of the algal solution was taken and centrifuged at 8000 g for 5 min; then, the pellet was resuspended in 3 mL 0.1 M PBS (pH 7.0). After repeated freezing and thawing, the sample was centrifuged for 5 min at 7500 g and the supernatant was collected. Absorbance at 455 nm, 564 nm, and 592 nm was measured using an ultraviolet spectrophotometer (Shimadzu, UV-2600).

The PE content was calculated using an equation proposed by Beer and Eshel [26]:

$$\mathrm{PE(g/L)} = \left[\left(\mathrm{OD}_{564} - \mathrm{OD}_{592}\right) - \left(\mathrm{OD}_{455} - \mathrm{OD}_{592}\right) \times 0.2\right] \times 0.12. \quad (2)$$

In the above equation, OD represents the optical density of the substance under a specific wavelength.

Results and discussion

Biomass concentration

In the lag phase of the cultivation (the first 2 days), the biomass accumulations in different patterns showed significant variance (Fig. 1): the average biomass growth rates in the control pattern (0.63 g/L/d) and the induced pattern (0.74 g/L/d) were greater than the specific pattern (0.31 g/L/d). After 2 days, *P. purpureum* showed differences in biomasses under different conditions; the biomass results suggested different growth tendencies for these (Fig. 1). In the control pattern, the nitrate concentrations used were 1, 2, 4 and 6 g/L, under which *P. purpureum* showed relatively strong salt resistance [11, 13]. It was shown that the biomass concentrations had a continuous increase until the 18th day, reaching 10.76, 11.45, 12.12, and 10.59 g/L, respectively (Fig. 1—■—). *P. purpureum* was relatively sensitive to the changes of temperatures and illumination intensities [12, 13, 27]. Under low temperature (20 °C) and low illumination (55 μmol/m^{2}s), the biomass concentration decreased from the 6th day and 10th day, respectively (Fig. 1—●—, 1 g/L KNO_3 and 2 g/L KNO_3), but when the initial nitrate concentrations reached 4 and 6 g/L, the biomass increased each day during the 18 days, having reached 8.47 and 8.72 g/L on the 18th day (Fig. 1—●—, 4 g/L KNO_3 and 6 g/L KNO_3).

When the initial culture conditions (25 °C, 165 μmol/m^{2}s, 1 g/L KNO_3) were altered to special culture conditions (20 °C, 55 μmol/m^{2}s light and the KNO_3 at 1, 2, 4 and 6 g/L), the biomass showed a tendency of first rapid growth followed by a gradual slow growth, which, on the 18th day, reached 9.80, 10.63, 10.25, and 9.92 g/L, respectively

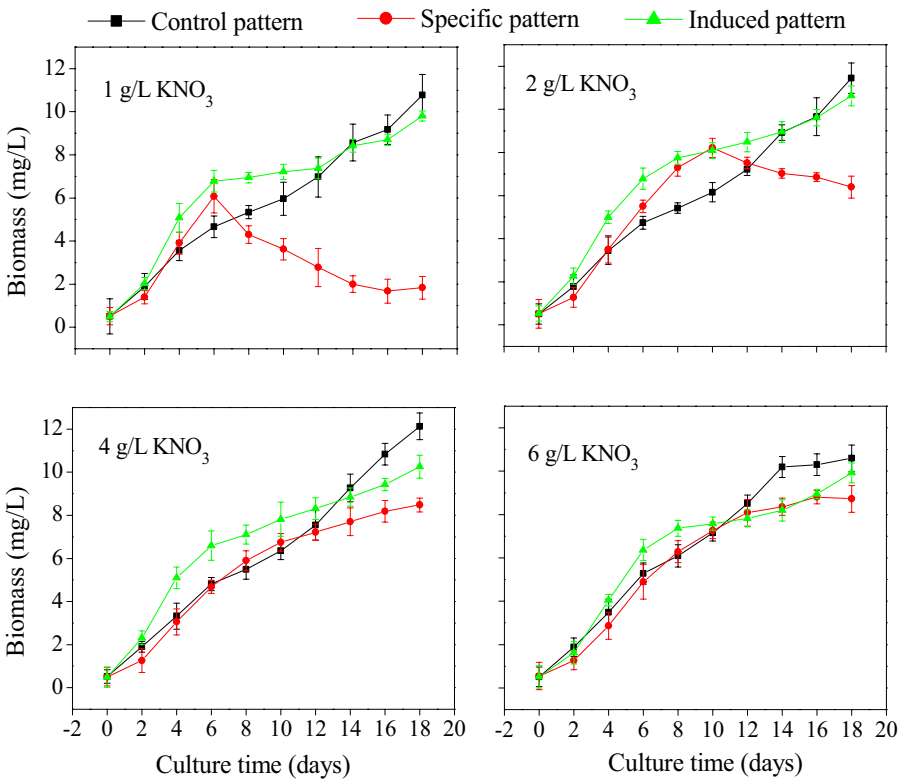

Fig. 1 Effects of three culturing patterns on the *P. purpureum* biomass accumulation in—■— Control pattern (25 °C, 165 μmol/m^{2}s),—●— Specific pattern (20 °C, 55 μmol/m^{2}s) and—▲— Induced pattern (25 °C → 20 °C, 165 μmol/m^{2}s → 55 μmol/m^{2}s), respectively. The data were collected as means of 3 repeated trials

Table 1 Effects of different cultivation patterns on *P. purpureum* biomass concentrations after 18 day cultivation

Cultivation pattern	Maximum biomass (g/L)	Rangeability (g/L)	Growth rate (g/L/d)
Control pattern	12.12 ± 0.12	11.6 ± 0.11	0.644 ± 0.011
Specific pattern	8.72 ± 0.17	8.18 ± 0.14	0.454 ± 0.014
Induced pattern	10.63 ± 0.26	10.11 ± 0.24	0.561 ± 0.024

Control pattern (25 °C, 165 μmol/m^{2}s), Specific pattern (20 °C, 55 μmol/m^{2}s), Induced pattern (25 °C → 20 °C, 165 μmol/m^{2}s → 55 μmol/m^{2}s). All the tests were conducted in three replicates

(Fig. 1—▲—). This indicated that *P. purpureum* had been adapted to the changes in the environment and was able to grow smoothly.

The maximum biomasses concentrations in different cultivation patterns showed differences among each other (Table 1). Within the same cultivation time (18 days), the maximum biomass concentrations reached 12.12, 8.72, and 10.63 g/L in the control, specific, and induced pattern, respectively. It suggested that the control pattern offered greater support for *P. purpureum* biomass accumulation than other patterns employed.

The metabolism of *P. purpureum* is easily affected by the growth environment [13, 14, 18, 28]. In normal culture conditions (Fig. 1—■—), algal cells have normal metabolism, high growth rates, rapid division and reproductive rates, so the biomass is high. In specific culture conditions (Fig. 1—●—), algal cells were subjected to high environmental stress under continuous low temperature, low light, and high concentration of nitrogen, resulting in low vitality and weak cell division and reproductive ability, so biomass accumulation was small. In the induction culture conditions (Fig. 1—▲—), algal cells were cultivated in a slow transition from normal temperature, normal light intensity, and normal nitrogen concentration to low temperature, low light, and high nitrogen, which enabled *P. purpureum* to have fast growth rates and rapid division and reproduction rates. After transition, *P. purpureum* was possibly able to resist environmental stress and accumulate biomass normally under induction. Therefore, the biomass in the induced pattern was closer to that of the normal cultivation than the specific pattern.

PE content

The differences of PE concentration in *P. purpureum* in different cultivation patterns are shown in Fig. 2. In the lag phase (first 2 days) of the cultivation, *P. purpureum* synthesized a large amount of phycobiliprotein (phycoerythrin, phycocyanin, and allophycocyanin) to capture more light; light transformation in chloroplasts supports the cell schizogamy [13, 29]. The PE contents in these three patterns differed from one another. When the nitrate concentration was 4 g/L, the PE contents in control and specific pattern reached the maximum values of 147 and

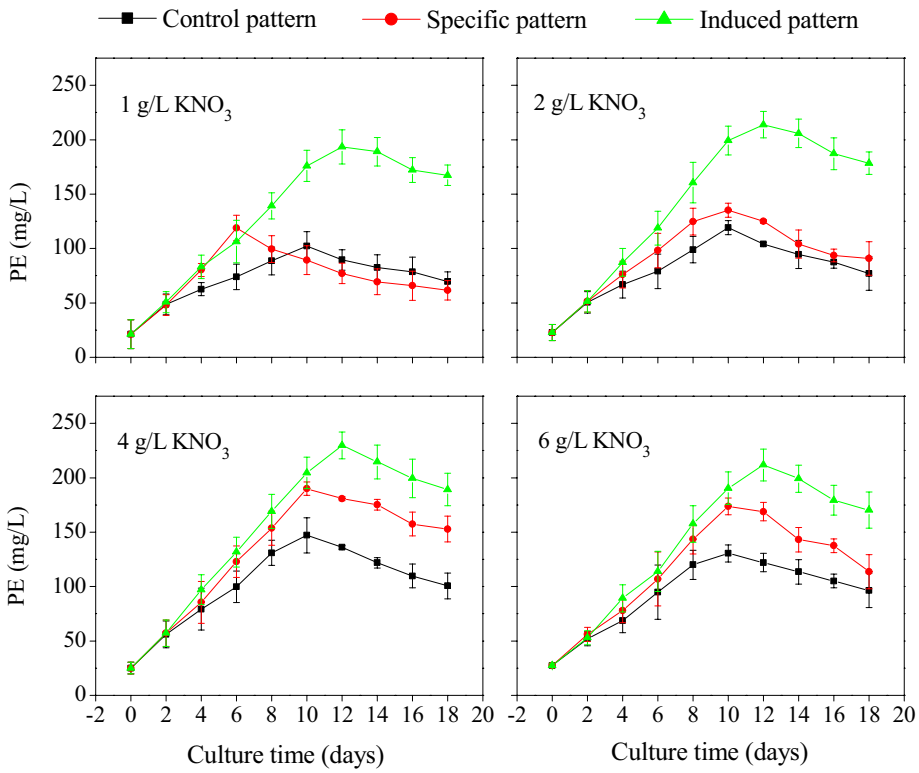

Fig. 2 Effects of different cultivation patterns on PE synthesis in *P. purpureum* (—■— Control pattern: 25 °C, 165 μmol/m^{2}s;—●— Specific pattern: 20 °C, 55 μmol/m^{2}s;—▲— Induced pattern: 25 °C → 20 °C, 165 μmol/m^{2}s → 55 μmol/m^{2}s). All the tests were conducted in three replicates

Table 2 Maximum PE content obtained from three cultivation patterns

Cultivation pattern	Maximum PE content (mg/L)	Rangeability (mg/L)	Growth rate (mg/L/d)
Control pattern	147 ± 6	121 ± 5	12.1 ± 0.5
Specific pattern	190 ± 10	167 ± 9	16.7 ± 0.9
Induced pattern	229 ± 11	204 ± 10	17.0 ± 0.8

Control pattern (25 °C, 165 μmol/m^{2}s), Specific pattern (20 °C, 55 μmol/m^{2}s), Induced pattern (25 °C → 20 °C, 165 μmol/m^{2}s → 55 μmol/m^{2}s). All the tests were conducted in three replicates

Table 3 PE yields in different experimental groups

Species	Culture pattern	Biomass (g/L)	PE content (mg/L)	PE yield (% d.w.)	References
Mastocarpus stellatus	Wild	N/A	N/A	0.20	Nguyen et al. [30]
Grateloupia turuturu	Wild	N/A	N/A	0.52	Mathilde et al. [31]
Polysiphonia urceolata	Wild	N/A	N/A	0.89	Liu et al. [32]
Porphyridium cruentum	Specific culture	3.07	73.98	2.41	Fuentes et al. [33]
Porphyridium cruentum	Specific culture	3.29	123.0	3.74	Wang et al. [17]
Pseudanabaena sp.	Specific culture	0.92	39.2	4.26	Sanjiv et al. [12]
Palmaria palmata	Wild	N/A	N/A	1.23	Justine et al. [34]
Rhodosorus marinus	Specific culture	2.00	89.0	4.45	Dupre et al. [35]
Porphyridium marinum	Specific culture	2.00	79.0	3.95	Nesrine et al. [36]
Porphyridium purpureum	Induced culture	7.51	229.0	3.05	This study

190 mg/L, respectively, on the 10th day. The PE content in induced pattern reached the maximum value of 229 mg/L (to the culture) on the 12th day, which was much higher than the other two patterns applied (Table 2), and it was accounted for approximately 3.05% of the dry biomass (Table 3). This indicated that the induced pattern is the

most appropriate strategy for the PE synthesis by *P. purpureum*. Combined with Fig. 1, the biomass concentrations in these three patterns generally showed continuous increases, whereas the PE contents showed an initial increase, followed by a decrease that was independent to biomass concentration curves (Fig. 2). To achieve the maximum PE content, the optimal cultivation time for *P. purpureum* in the control and specific pattern was 10 days, whereas 12 days for the induced pattern. In the previous research, 18 days will be necessary for the cultivation of *P. purpureum* [8, 19, 21]. This study reduces the cultivation time to 12 days for PE production, which will greatly enhance the economy and technical efficiency in industrial cultivation.

The specific pattern (Fig. 2—●—) facilitated an increase in PE synthesis in *P. purpureum*. *P. purpureum* was subjected to environmental stress due to these conditions (e.g., low temperature, low light, and high nutrient concentrations). This manifested the low vitality of *P. purpureum* in its initial culture stage. In the induced pattern (Fig. 2—▲—), the high growth rates of *P. purpureum* in its initial culture stage indicate resistance to the stress factors. In the middle and later culture stages, *P. purpureum* accumulated more PE. In summary, the induced cultivation pattern was more suitable for the synthesis of PE in *P. purpureum* than the other cultivation patterns tested.

The parabolic evolution of PE concentration has been demonstrated in *P. purpureum* [13]; these results are similar to the results of our experiment. It has been shown that synthesis of PE in *P. purpureum* is greater under low temperature, low light, and high nitrogen conditions [18]. This is evident when comparing results from the special pattern with the normal pattern. The induced pattern had greater PE content than the other two patterns. This may be due to the vitality of the algae cells during the lag phase. With the constant change of temperature, light intensity and nitrogen source, the synthesis and accumulation of phycoglobin gradually increased. Therefore, *P. purpureum* biomass accumulated with a large amount of PE.

Total protein content

To further study PE synthesis, the total protein contents in different patterns were measured and the results are shown in Fig. 3. In the lag phase of the cultivation, the changes in total protein contents in the three patterns were similar, showing a general continuous growth increase (Fig. 3). After 2 days of cultivation, in the control pattern, the total protein content steadily increased. In the specific pattern, the total protein content showed a different trend to the control pattern; when the nitrate concentrations were 1 and 2 g/L, the total protein concentration decreased from the 8th day and 12th day, respectively, but when the initial nitrate concentrations reached 4 and 6 g/L, the total protein concentration increased each day during the 18 days (Fig. 3—●—). In addition, in the induced pattern, the total protein content showed

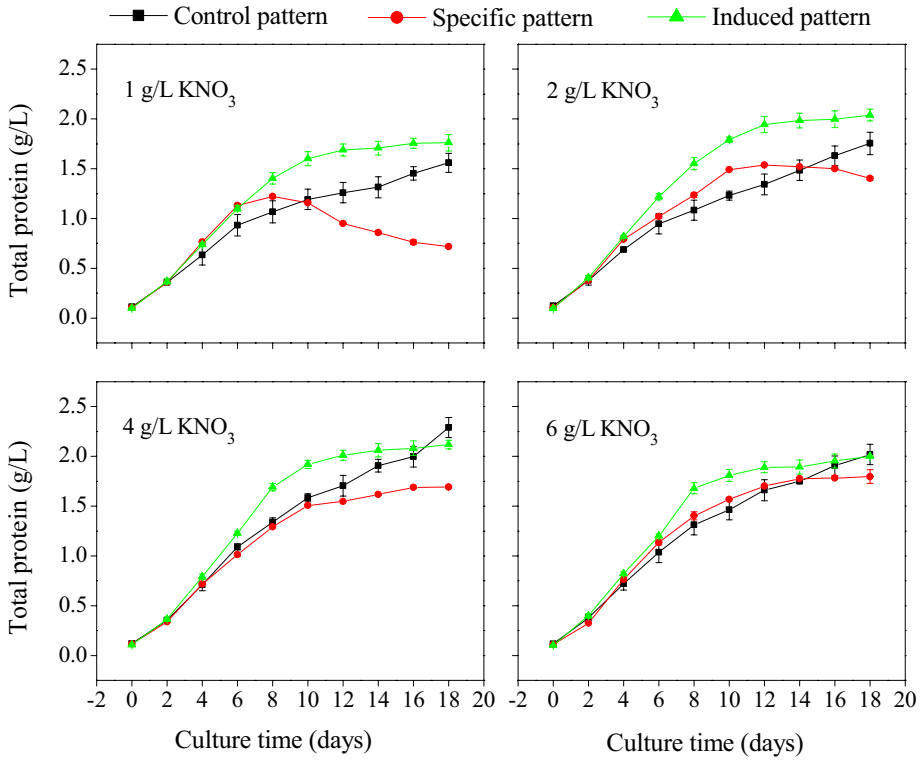

Fig. 3 Effects of three cultivation patterns on the total protein content in *P. purpureum* (—■— Control pattern: 25 °C, 165 μmol/m^{2}s;—●— Specific pattern: 20 °C, 55 μmol/m^{2}s;—▲— Induced pattern: 25 °C → 20 °C, 165 μmol/m^{2}s → 55 μmol/m^{2}s). All the tests were conducted in three replicates

Table 4 Proportions of PE, total carbohydrates and total protein contents in *P. purpureum* after 10 day cultivation with different strategies

KNO_3 (g/L)	PE			Total protein			Total carbohydrate		
	Control pattern (%)	Specific pattern (%)	Induced pattern (%)	Control pattern (%)	Specific pattern (%)	Induced pattern (%)	Control pattern (%)	Specific pattern (%)	Induced pattern (%)
1	1.7	–	2.4	5.0	–	7.1	37.7	–	44.7
2	1.9	1.6	2.5	6.2	6.4	8.7	35.1	40.1	40.1
4	2.3	2.8	2.6	6.9	7.5	9.8	33.8	37.3	38.5
6	1.8	2.3	2.5	5.7	7.6	8.8	30.5	34.5	36.5

Control pattern (25 °C, 165 μmol/m^2s), Specific pattern (20 °C, 55 μmol/m^2s), Induced pattern (25 °C → 20 °C, 165 μmol/m^2s → 55 μmol/m^2s). All the tests were conducted in three replicates

a rapid increase, followed by a steady increase. Overall, the changes of total protein contents in the three patterns showed a general steady increase (other than the specific pattern under 1 g/L and 2 g/L of nitrate). The trends of total protein and PE changes were different, as total protein content increased with an increase of biomass, which suggested that PE synthesis and accumulation were independent from total protein accumulation.

When *P. purpureum* accumulates biomass, cells are constantly dividing to produce new algal cells. In the new algal cells, organelles and cytoplasm are produced in large quantities, and protein is the main component of the cell membrane and cytoplasm, so the total protein content increases with the increase in biomass. However, PE accumulation first increases and then decreases, because PE only exists in the algal phycobilisome and participates in photosynthesis. When cells entered the lag and logarithmic phases, PE is produced in large quantities for increased rates of photosynthesis and high growth rates. When cells enter the stationary and death phases, photosynthesis weakens and PE might be degraded and used to synthesize other metabolites, such as polysaccharides and lipids.

P. purpureum productivity

P. purpureum not only produces PE, but also produces many other important substances such as polysaccharides, monosaccharides, carotene, and chlorophyll [11–13]. As shown in Table 4, the proportions of other metabolites were observed during on the 10th day cultivation when high PE content was in a high level.

Table 4 suggested that during the high PE content period, the total carbohydrate and total protein contents were greater than the PE content. When the nitrate concentration was 1 g/L, the total carbohydrate content in the induced pattern reached the maximum percentage of 44.7%; and when the nitrate concentration was 4 g/L, the total protein content in the induced pattern reached the maximum percentage of 9.8%. This indicated that when the content of PE was at its highest value, there were also large amounts of other metabolites produced by *P. purpureum*. This suggested that co-production of PE and other high added value products, such as polysaccharides of *P. purpureum*, would greatly enhance the feasibility and economy efficiency of *P. purpureum* microalgae industry [30].

Photosynthesis provides energy for *P. purpureum* growth, and *P. purpureum* uses this energy to metabolize nutrients and synthesize various high-value products, such as polyunsaturated fatty acids, extracellular lipopolysaccharides, and carotenoids [37]. Therefore, when *P. purpureum* synthesizes a large amount of PE, this indicates that the algal cells are undergoing a high rate of division and reproduction, and at the same time, they are likely to produce greater amounts of high-value products through their metabolic processes.

Conclusion

Biorefinery demonstration proves to be very important to the microalgae industry. Cultivation, one of the most important downstream processes, is proven to be critical, which greatly affects the ecological technical and economy efficiency. The biomass accumulation and PE synthesis by *P. purpureum* varied greatly under different growth patterns, providing operation, and induction information for specific applications. This study has also found that PE content initially increased and then decreased, which suggests that reducing the cultivation period from 18 to 12 days is appropriate, which would enhance the production efficiency. Moreover, *P. purpureum* also produces rich carbohydrates and other proteins around such timepoint, which will contribute to the great progress in industrial economy.

Acknowledgements We are grateful for funding supported by the special fund for Fujian Ocean High Tech Industry Development (No. FJHJF-L-2018-1), China, the Natural Science Foundation of Fujian Province of China (No. 2019J06005), and the Energy development

Foundation of the College of Energy, Xiamen University (Grant No. 2017NYFZ02).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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