Improving protein production of indigenous microalga Chlorella vulgaris FSP-E by photobioreactor design and cultivation strategies

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Research Article

Improving protein production of indigenous microalga
*Chlorella vulgaris* FSP-E by photobioreactor design and cultivation strategies

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Fish meal is currently the major protein source for commercial aquaculture feed. Due to its unstable supply and increasing price, fish meal is becoming more expensive and its availability is expected to face significant challenges in the near future. Therefore, feasible alternatives to fish meal are urgently required. Microalgae have been recognized as the most promising candidates to replace fish meal because the protein composition of microalgae is similar to fish meal and the supply of microalgal-based proteins is sustainable. In this study, an indigenous microalga (*Chlorella vulgaris* FSP-E) with high protein content was selected, and its feasibility as an aquaculture protein source was explored. An innovative photobioreactor (PBR) utilizing cold cathode fluorescent lamps as an internal light source was designed to cultivate the FSP-E strain for protein production. This PBR could achieve a maximum biomass and protein productivity of 699 and 365 mg/L/day, respectively, under an optimum urea and iron concentration of 12.4 mM and 90 μM, respectively. In addition, amino acid analysis of the microalgal protein showed that up to 70% of the proteins in this microalgal strain consist of indispensable amino acids. Thus, *C. vulgaris* FSP-E appears to be a viable alternative protein source for the aquaculture industry.

**Keywords:** *Chlorella vulgaris* · Iron ions · Photobioreactor · Protein · Urea

1 Introduction

Due to increased awareness of what constitutes a healthy and balanced diet, the consumption of fish has increased in the past few decades, with fish accounting for about 17% of animal protein consumed worldwide [1]. The yield of the aquaculture sector is expected to surpass that of wild fisheries by 2020–2025 due to improvements in cultivation skills and the depletion of wild fish stocks [2, 3]. According to an estimation by the Food and Agriculture Organization (FAO) of the United Nations, fish feed represents over 50% of aquaculture cultivation costs [4] with protein being the most expensive dietary component.
Moreover, the protein feedstock used in commercial aquafeeds relies heavily on fish meal (FM) which is preferred due to its high protein content and balanced amino acids profile [5]. The price of FM is increasing due to the imbalance between supply and demand. In addition, the depletion of fishery resources is a key reason for the higher FM price and will become a critical factor determining the future FM supply [6].

Recently, microalgae have emerged as one of the most promising alternatives to fish meal thanks to their high protein content and high protein production rate (Henry 2012). In addition, algal biomass contains other valuable components, such as pigments (e.g. carotenoids), omega-3 fatty acids (e.g. DHA, EPA), vitamins and minerals, which further increase the nutritional value of algae-based feed [7, 8]. Studies have shown that serving algae as a feed additive enhances growth, feed utilization efficiency, physiological activity, stress response, starvation tolerance, disease resistance and carcass quality of cultured fish [9, 10]. However, one of the major challenges in using microalgae as aquafeed is lack of knowledge regarding the cultivation of protein-rich microalgae at a large scale for the production of proteins with high quality and low cost. It is known that light intensity and penetration efficiency are vital factors for growth of phototrophic microalgae because of the energy budget required for carrying out photosynthesis [11]. Therefore, it is necessary to develop high efficiency and cost-effective photobioreactors for the cultivation of candidate microalgal strains. Furthermore, medium composition is also important since cell growth rate and the biochemical composition of microalgae (e.g. proteins, lipids, and carbohydrates) are strongly dependent on medium composition. In particular, the concentration of the macronutrient nitrogen, is known to significantly affect microalgal growth and biomass composition due to its effects on protein and chlorophyll synthesis [12–14]. Moreover, iron is an essential mineral for cell growth, as iron plays a crucial role in photosynthetic and respiratory functions, chlorophyll biosynthesis and the reduction of inorganic nitrogen species. However, it is also known that an excess amount of iron induces the generation of radicals, leading to damage to DNA, protein, and the cell membrane [15, 16]. Moreover, it should be noted that algal protein content and its amino acid profile are closely linked to growth phase. For example, the protein content in microalgae usually decreases after nitrogen depletion, followed by an increase in carbohydrate or lipid content [17, 18]. Therefore, the timing of harvesting of microalgal cells for the purpose of protein production is important.

In this study, photobioreactors (PBRs) equipped with an internal or external light source were utilized to grow an indigenous protein-rich microalga (Chlorella vulgaris FSP-E) photoautotrophically using CO2 as the sole carbon source. The PBR was illuminated by cold cathode fluorescent lamp (internal light source) or external irradiation of fluorescent lamp (TL5) at the same light intensity. For medium improvement, the effects of nitrogen source concentration on microalgal growth and protein production were explored. Finally, microalgal cultivation was conducted at different ferrous ion concentrations to investigate the effect of the concentration of this trace element on C. vulgaris FSP-E growth and cellular protein content.

2 Methods and materials

2.1 Microalgal culture and medium composition

The microalgal strain used in this study was isolated from a freshwater area located in southern Taiwan (Ho et al. 2013 [35]). The microalgae was identified as Chlorella vulgaris based on its morphology and 23S plastid rDNA sequence matching (NCBI accession number HE978273), and was named as Chlorella vulgaris FSP-E. C. vulgaris FSP-E was grown on a modified version of Basal medium [19, 20] consisting of (g/L): KH2PO4, 1.25; MgSO4·7H2O, 1.0; CaCl2·2H2O, 0.1106; FeSO4·7H2O, 0.0498; EDTA·2Na, 0.5; H3BO3, 0.1142; ZnSO4·7H2O, 0.0882; MnCl2·4H2O, 0.0144; Na2MoO4·2H2O, 0.0119; CuSO4·5H2O, 0.0157; Co(NO3)2·6H2O, 0.0049; and Urea, 0.56.

2.2 Photobioreactor design and operation

The conventional PBR was a glass vessel with a working volume of 1 L illuminated with TL5 fluorescent lamps (14 W, Philips, Taiwan) mounted on both sides of the PBR as the external light source (Supporting information, Fig. 1A). Agitation of the culture was performed using a magnetic stirrer on the bottom of the vessel. The light intensity was set at 150 μmol/m²/s, which was found to be the preferred light intensity to grow C. vulgaris FSP-E for protein production with the lowest energy consumption. For the modified PBR, the same 1 L glass vessel and same agitation system were used. Three sets of cold cathode fluorescent lamps (CCFLs) were vertically inserted into the PBR from the top to achieve a light intensity of ca. 150 μmol/m²/s with uniform light distribution (Supporting information, Fig. 1B). The microalgae were grown at room temperature (27 ± 1°C), pH 6.8, and 300 rpm agitation. The microalgae were pre-cultured and inoculated into the PBR with an inoculum size of 0.02 g/L. Filtered 2% CO2 (concentration was adjusted by mixing with air) continuously aerated the culture medium at a rate of 200 mL min⁻¹ or 0.2vvm (volume gas per volume of the medium per min) and was the sole carbon source supplied to the PBR.

2.3 Cultivation of Chlorella vulgaris FSP-E at different light intensities

The C. vulgaris cells were cultivated in the modified PBR described in Section 2.2 at three different light intensity (70, 150, or 300 μmol/m²/s) under whole-day illumination.
The initial urea concentration and Fe²⁺ concentration was 9.3 mM and 180 μM, respectively. Samples were regularly taken from the microalgal culture to monitor cell growth and protein content.

2.4 Cultivation of *Chlorella vulgaris* FSP-E at different urea concentrations

The urea concentration of modified basal medium was adjusted to examine the effects of urea concentration on microalgal cell growth and protein production in the modified PBR. Including the original urea concentration of modified Basal medium, six different urea concentrations (3.1, 6.2, 9.3, 12.4, 15.5, 18.6 mM) were tested. The microalgal culture was exposed to whole-day illumination with a light intensity of 150 μmol/m²/s. The Fe²⁺ concentration in the medium was 180 μM. Samples were regularly taken from the microalgal culture to monitor cell growth and protein content.

2.5 Cultivation of *Chlorella vulgaris* FSP-E at different Fe²⁺ concentrations

To examine the effect of Fe²⁺ concentration on growth and protein production of *C. vulgaris* FSP-E, four different Fe²⁺ concentrations (22.5, 45, 90, and 180 μM) were supplemented into the modified basal medium, representing 0.125-, 0.25-, 0.5-, and one-fold of the original Fe²⁺ concentration (180 μM) in the modified basal medium. *C. vulgaris* FSP-E was cultivated using the modified photobioreactor as described earlier under whole-day illumination with a light intensity of 150 μmol/m²/s. The nitrogen source (i.e. urea concentration) was fixed at 12.4 mM. Samples were regularly taken from the microalgal culture to monitor cell growth and protein content.

2.6 Determination of microalgae cell concentration

Liquid samples were regularly collected from the culture broth and optical density was measured at 688 nm (denoted as OD₆₈₈) using UV/VIS spectrophotometer (model U-2001, Hitachi, Tokyo, Japan) after appropriate dilution with deionized water. To obtain dry cell weight (DCW), 4 mL of liquid sample was centrifuged, dried and the resulting biomass was placed on a weighing paper. The biomass and paper was weighed on an electronic balance (model FD-720, Kett, Tokyo, Japan) and the difference in weight between the dried empty paper and the dried biomass-loaded paper constituted the dry cell weight of the microalgae.

2.7 Determination of residual urea concentration

The urea concentration of the culture medium was analyzed with a published urease protocol [21]. Microalgae samples were collected and filtered with 0.22 μm filter membrane. 100 μL of each sample was added into 0.5 mL urease solution and the mixture was placed in a 37°C water bath for 10 min. After that, the reaction was stopped with 1.0 mL phenol nitroprusside (enzyme terminator). Then, 1.0 mL alkaline hypochlorite (chromogenic agent) was mixed with the reaction mixture, which was incubated at room temperature for 30 min. The optical density (at 570 nm) of the final mixture was measured and the value was converted to urea concentration via appropriate calibration.

2.8 Determination of light intensity

The light intensity on the wall of the photobioreactor was measured with an LI-250 light meter with a LI-190SA pyranometer sensor (LI-COR, Inc., Lincoln, Nebraska, USA). This light meter gives a unit of μmol/m²/s for the measured light intensity.

2.9 Determination of protein content and amino acid profile

The protein content and amino acid profile were determined as described previously [22]. The microalgal biomass was centrifuged and washed by deionized water. After being lyophilized, a fixed amount of biomass (25–30 mg) was hydrolyzed with 1 mL of 6 N HCl in vacuum-sealed glass tubes. The hydrolysate was dried by vacuum pump at 55°C. The concentrate was re-dissolved with 2 mL NaN₃ solution and appropriately diluted for amino acid analysis. Samples were analyzed by ion chromatography (ICS-5000, Amino Acid Analyzer, DIONEX, USA equipped with an electrochemical detector). Samples were injected into a 25 cm-long AminoPac PA10 Analytical column (BioLC, Dionex, USA) with an internal diameter of 2 mm. The mobile phases were created by designated proportions of four solutions including: (i) 10 mM sodium hydroxide; (ii) 250 mM sodium hydroxide; (iii) 25 mM NaOH in 1 M sodium acetate; and (iv) 100 mM acetic acid. The injection sample volume was 5 μL and column temperature was controlled at 30°C. Amino acid concentration was estimated by interpolating concentration versus peak area based on standard curves. The protein content was estimated by summation of the content of the 20 amino acids analyzed.

3 Results and discussion

3.1 Effects of photobioreactor type on microalgal growth and protein production

Conventional PBRs usually suffer the drawback of short light penetration distance. When the cell density of phototrophic microalgae cultures becomes higher, light intensity inside the PBR rapidly decreases due to self-
shading effects arising from high biomass concentration [11]. To improve the light utilization of conventional PBRs, a modified PBR equipped with a set of three immersed internal light sources was designed for microalgal cultivation (Supporting information, Fig. 1). Cold cathode fluorescent lamps (CCFLs), featuring low heat emissions, were chosen as the light source to reduce the overheating problems associated with conventional light sources (such as fluorescent lamps). CCFLs have been widely used in diverse fields, such as car navigation system displays, notebook computers, PDAs, liquid crystal monitors, and widescreen LCD TVs [23]. Recently, a CCFL was applied to plant cultivation to make use of advantageous attributes such as low heat emission, low energy consumption, long service life, small diameter, and uniform light intensity [23–25].

*C. vulgaris* FSP-E were grown in conventional and modified PBRs. Table 1 compares the performance with regard to cell growth and protein production of microalgae obtained from the two types of PBRs. The modified PBR with internal light source (immersed CCFLs) resulted in a 15% higher growth rate, as well as 26% higher biomass productivity and 24% higher protein productivity when compared with the conventional PBR. The presence of internal light sources in the modified PBR promoted cell growth and protein productivity, probably due to better light penetration and distribution with the internal light sources. However, the protein content was similar regardless of the type of PBR. Therefore, in the rest of the experiments in this study, the modified PBR with internal light sources was used for the cultivation of *C. vulgaris* FSP-E.

### 3.2 Effects of light intensity on microalgal growth and protein production

Light intensity is usually the most critical factor influencing photoautotrophic growth of microalgae. The composition of microalgal cells, in particular microalgal pigments, known to be dependent on the supply of light energy [17, 34]. Therefore, this study commenced with the selection of the most suitable light intensity for the growth and protein production of *C. vulgaris* FSP-E. Three light intensities (namely, 70, 150, 300 μmol/m²/s) were examined. As indicated in Table 2, when the microalgae were grown under illumination at a light intensity of 150 μmol/m²/s, the protein content and protein productivity reached the highest at 50% and 302 mg/L/day, respectively, whereas the final biomass concentration obtained from illumination at 300 μmol/m²/s was slightly higher than that for 150 μmol/m²/s. Since the main focus of this study was to optimize the microalgal protein production of *C. vulgaris* FSP-E, the light intensity of 150 μmol/m²/s was chosen for the rest of the experiments described in this work.

### 3.3 Effects of nitrogen concentration on microalgal growth and protein production

Nitrogen source is one of the most important components in the medium. As nitrogen is the pivotal element for the synthesis of proteins, nucleic acids and photosynthetic pigments, it affects growth and protein production of microalgae [26]. It has also been shown that the type and concentration of nitrogen source significantly affect the growth and biochemical composition of algae [27, 28]. In this study, urea was used as the nitrogen source for economic reasons, and the effect of urea concentration on cell growth and protein production of *C. vulgaris* FSP-E was investigated. Urea concentration was chosen in the range of 0.5 to 5.0 μM.

<table>
<thead>
<tr>
<th>Light intensity (μmol/m²/s)</th>
<th>Biomass production a) (g/L)</th>
<th>Biomass Productivity b) (mg/L/day)</th>
<th>Protein content (%)</th>
<th>Protein productivity c) (mg/L/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>3.65 ± 0.31</td>
<td>339 ± 4</td>
<td>42.9 ± 2.6</td>
<td>145.5 ± 1.4</td>
</tr>
<tr>
<td>150</td>
<td>4.13 ± 0.12</td>
<td>604 ± 2</td>
<td>50.0 ± 1.3</td>
<td>302.0 ± 2.1</td>
</tr>
<tr>
<td>300</td>
<td>4.23 ± 0.22</td>
<td>512 ± 3</td>
<td>41.1 ± 0.8</td>
<td>210.6 ± 3.0</td>
</tr>
</tbody>
</table>

a) Final biomass concentration  
b) Maximum biomass productivity  
c) Maximum protein productivity
growth and protein production of \textit{C. vulgaris} FSP-E was investigated. The FSP-E strain was cultivated on modified Basal medium containing different urea concentrations (from 3.1 to 18.6 mM). As shown in Table 3, the specific cell growth rate increased from 0.848 to 1.219 day\(^{-1}\) when the urea concentration was increased from 3.1 to 12.4 mM. Meanwhile, with an urea concentration of 12.4 mM, the final biomass production reached 5.62 g/L after cultivation for nine days. This represents a biomass productivity of 613 mg/L/day, which was the highest among all the urea concentrations examined. When the urea concentration was further increased to 18.6 mM, the cultivation time that was required for complete consumption of urea was prolonged from nine to 12 days. The specific growth rate of the microalgae decreased slightly when the urea concentration was higher than 12.4 mM, while the final biomass production increased to 7.37 g/L when 18.6 mM of urea was used. However, the biomass productivity resulting from using urea at concentrations of 15.5 and 18.6 mM was still 18\% lower than that obtained from using 12.4 mM urea (Table 3). Since urea is an organic nitrogen source, it might be difficult for microalgae to assimilate urea when its concentration is too high, leading to a negative effect on cell growth kinetics. This is similar to the effect of substrate inhibition. Therefore, taking all factors into account, the optimal urea (as nitrogen source) concentration for cell growth seems to be 12.4 mM. Previous studies also observed the existence of an optimal nitrogen source concentration for microalgal growth [29]. Increasing the concentration of nitrogen seems to promote microalgal growth, whereas negative effects on cell growth may occur when the nitrogen concentration exceeds the optimal value.

Protein content and protein productivity obtained using different urea concentrations are shown in Table 3. When 3.1 mM urea was used, the biomass production and protein content were both low, probably due to insufficient nitrogen. When the urea concentration was increased to 9.3–18.6 mM, protein content increased dramatically from 22 to 49–57\% per dry cell weight. The highest protein content (55.7\%) and productivity (319.2 mg/L/day) occurred when urea concentration was 6.2 mM. Evaluation of the data shown in Table 3 suggests that when the urea concentration was 6.2 mM or higher, protein productivity is similar. An explanation for this could be that the nitrogen concentration affects both cell growth and protein synthesis, but the effects of some specific nitrogen source concentrations could occur in opposite directions for these two parameters. For example, when using an urea concentration of 9.3 and 12.4 mM, the biomass productivity was higher but the protein content was lower (Table 3).

### 3.4 Evolution of amino acid profiles of \textit{C. vulgaris} FSP-E during transient growth

As a consequence of cell growth, the nitrogen source is gradually consumed. The availability of a nitrogen source in the culture medium has a significant impact on the biochemical composition of microalgae. For example, nitrogen depletion and starvation can induce lipid or carbohydrate accumulation [26, 27, 30]. To examine the effect of residual nitrogen concentration on protein content in the biomass of \textit{C. vulgaris} FSP-E, the protein content of the microalgae were determined during the course of cell growth. Figure 1 shows the time course of protein content during the growth of \textit{C. vulgaris} FSP-E on medium containing different urea concentrations. All cultures show a similar trend in protein content profile in response to the residual nitrogen concentration. The protein content in the microalgae initially increase in proportion to the percentage of nitrogen source utilized until it reaches a maximum value when 90\% of the nitrogen source has been consumed. After this point, the nitrogen source is considered to be depleted and prolonged nitrogen starvation results in a significant decrease in protein content. Similar findings regarding the correlation between protein content in microalgae and the residual nitrogen source

<table>
<thead>
<tr>
<th>Urea concentration (mM)</th>
<th>Cultivation time(^a) (days)</th>
<th>Biomass production(^b) (g/L)</th>
<th>Protein content (% of dry cell weight)</th>
<th>Biomass productivity(^c) (mg/L/day)</th>
<th>Protein productivity(^d) (mg/L/day)</th>
<th>Specific growth rate (day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>3.93</td>
<td>2.04 ± 0.06</td>
<td>22.5 ± 0.7</td>
<td>380 ± 6</td>
<td>85.5 ± 1.6</td>
<td>0.848 ± 0.025</td>
</tr>
<tr>
<td>6.2</td>
<td>3.93</td>
<td>3.06 ± 0.09</td>
<td>55.7 ± 1.7</td>
<td>573 ± 2</td>
<td>319.2 ± 3.6</td>
<td>1.060 ± 0.031</td>
</tr>
<tr>
<td>9.3</td>
<td>7.13</td>
<td>4.13 ± 0.12</td>
<td>50.0 ± 1.3</td>
<td>604 ± 2</td>
<td>302.0 ± 2.1</td>
<td>1.110 ± 0.033</td>
</tr>
<tr>
<td>12.4</td>
<td>8.87</td>
<td>5.62 ± 0.17</td>
<td>49.1 ± 1.5</td>
<td>613 ± 4</td>
<td>301.0 ± 3.0</td>
<td>1.219 ± 0.037</td>
</tr>
<tr>
<td>15.5</td>
<td>10.27</td>
<td>6.02 ± 0.18</td>
<td>56.6 ± 1.7</td>
<td>516 ± 5</td>
<td>297.2 ± 0.8</td>
<td>0.983 ± 0.029</td>
</tr>
<tr>
<td>18.6</td>
<td>12.05</td>
<td>7.37 ± 0.22</td>
<td>56.9 ± 1.7</td>
<td>522 ± 1</td>
<td>297.0 ± 2.9</td>
<td>0.986 ± 0.030</td>
</tr>
</tbody>
</table>

\(a\) The cultivation time required to reach complete consumption of urea
\(b\) Final biomass concentration
\(c\) Maximum biomass productivity
\(d\) Maximum protein productivity
concentration in the medium have been reported in related studies [26, 31]. Under nitrogen starvation conditions, microalgae tend to convert the carbon from photosynthesis into storage compounds, such as lipids or carbohydrates, resulting in an increase in lipid or carbohydrate content [17, 18]. In addition, during the nitrogen deficiency period the process of autophagy may be initiated to degrade cellular components, such as organelles and non-growth-essential proteins, while the basic elements will be reused to build other indispensable proteins. There-

Figure 1. Protein content of C. vulgaris FSP-E at different growth phases under different initial urea concentrations. The initial Fe²⁺ concentration was 180 μM. The light intensity was 150 μmol/m²/s. All cultures show a similar trend in their protein content profile in response to the residual nitrogen concentration. The protein content in the microalgae initially increases in proportion to the percentage of nitrogen source consumption until 90% of the nitrogen source was consumed and the protein content reached a maximum value.
fore, a decrease in overall protein content in microalgae after nitrogen depletion is expected [26].

From Fig. 1 it can be seen that nitrogen depletion is unfavorable for the accumulation of protein in microalgal cells. However, it is of interest to understand whether the amino acid profile of the microalgal protein also varies with the concentration of residual nitrogen source. Figure 2 provides a detailed description of the nitrogen source-dependent variation in the amino acid profiles of algal protein during different cultivation stages in a batch culture. As seen in Fig. 2, the content of each amino acid versus the percentage of nitrogen source consumption seems to show a similar trend to that of total protein content (Fig. 1). The content of most amino acid species increased in proportion to the level of nitrogen consumption until reaching highest peak value at a nitrogen utilization percentage of 90%. After nitrogen depletion (defined as over 90% nitrogen utilized), the content of each amino acid decreased with time (Fig. 2).

It shows that Fe²⁺ concentration has more impact on microalgae growth than on protein content or amino acid profile over the concentration range of 22.5–90 μM.
(Phe); valine (Val); threonine (Thr); tryptophan (Try); isoleucine (Ile); leucine (Leu); methionine (Met); isocine (Lys); histidine (His); and arginine (Arg). Dispensable amino acids (DAAs) include: glycine (Gly); alanine (Ala); serine (Ser); cysteine (Cys); proline (Pro); tyrosine (Tyr); aspartate (Asp); glutamate (Glu); asparagine (Asp); and glutamine (Gln). As indicated in Fig. 2, along with the increase in nitrogen utilization percentage (prior to nitrogen starvation), the content of each amino acid also increases. Previous studies have investigated nitrogen partitioning in algal cells and changes to the algal nitrogen storage pool under nitrogen starvation [14, 31]. Under nitrogen-sufficient condition, algal cells will assimilate environmental nitrogen and accumulate it in the form of various nitrogen-containing compounds, either inorganic forms (nitrate and ammonium) or organic forms (amino acids, peptides, protein, RNA, and pigments). When nitrogen depletion occurs, the intermediate nitrogen reservoir (nitrate, ammonium, amino acids, and peptides) is directed to synthesize proteins in order to support growth. Meanwhile, the cells degrade non-growth related protein by autophagy [14]. Hence, the decline of protein content is observed when nitrogen starvation occurs. In addition, variation to the amino acid profile may be observed due to nitrogen redistribution in algal cells. As shown in Fig. 3A, the relative amount of IAAAs slightly decreased from 58 to 52% along with the consumption of urea. However, variation in the relative IAA content was not significant. In contrast, when nitrogen utilization percentage reached 90%, the biomass productivity and protein content reached their maximum levels, leading to maximal protein productivity. Taking this into consideration, the optimal harvesting time, for the purpose of using microalgal biomass as protein source in feeds, would be at 90% nitrogen utilization (or just before nitrogen starvation occurs).

### 3.5 Effects of Fe\textsuperscript{2+} concentration on microalgal growth and protein production

Iron is an essential mineral for phytoplankton growth. Many studies point out that iron limitation is a key factor linked to species diversity and productivity in oceanic algal communities [15, 32]. Algae exploit iron for photosynthetic and respiratory functions, the reduction of inorganic nitrogen species (e.g. nitrate), and the biosynthesis of chlorophyll. To investigate the effect of Fe\textsuperscript{2+} concentration on growth and protein production of *Chlorella vulgaris* FSP-E, the microalga was grown on modified Basal medium containing different iron concentrations (namely, 180, 90, 45 and 22.5 μM) using the modified PBR described in Section 2.2. As shown in Table 4, biomass productivity slightly increased as the Fe\textsuperscript{2+} concentration increased from 22.5 to 90 μM, reaching the highest biomass productivity (699 mg/L/day) at 90 μM. However, when the iron concentration was further increased from 90 to 180 μM, both the specic cell growth rate and biomass productivity decreased significantly. Protein content and productivity were similar at Fe\textsuperscript{2+} concentrations of 22.5–90 μM, but became markedly lower when the Fe\textsuperscript{2+} concentration was too high (i.e. at 180 μM). These results suggest that a sufficient amount of Fe\textsuperscript{2+} ions in the medium is required for efficient cell growth and protein production, whereas performance declines when iron concentrations are in excess. Similar findings were obtained in previous work [16, 33], showed that a Fe\textsuperscript{2+} concentration suitable for the growth of *C. vulgaris* is within the range of 90–200 μM. Those reports also pointed out that excess iron in the medium probably decreases the growth rate of *C. vulgaris* because of the induction of oxidative stress [16]. The highest protein productivity observed was 365.6 mg/L/day, which was obtained when using a Fe\textsuperscript{2+} concentration of 90 μM (Table 4). As shown in Table 5, although strain FSP-E does not have the highest protein content among the strains tested, the maximum protein productivity reported in the present work (365.6 mg/L/day) is significantly higher than those reported in the literature. Also, the FSP-E strain was able to grow in outdoor open systems (such as open pond and raceway pond) (data not shown), and this robustness indicates FSP-E’s potential for future commercialization.

Although the variation in Fe\textsuperscript{2+} concentration from 22.5 to 180 μM did not significantly affect the total protein content (49–53%) of *C. vulgaris* FSP-E (Table 4), when the Fe\textsuperscript{2+} concentration was too high (i.e. at 180 μM), there was a significant difference in the amino acid profile of the pro-

#### Table 4. The effect of Fe\textsuperscript{2+} concentration on cell growth and protein production of *C. vulgaris* FSP-E at an initial urea concentration of 12.4 mM and a light intensity of 150 μmol/m\textsuperscript{2}/s

<table>
<thead>
<tr>
<th>Fe\textsuperscript{2+} concentration (μM)</th>
<th>Biomass production(\textsuperscript{a})) (g/L)</th>
<th>Protein content (\textsuperscript{b})) (%)</th>
<th>Biomass productivity(\textsuperscript{b})) (mg/L/day)</th>
<th>Protein productivity(\textsuperscript{b})) (mg/L/day)</th>
<th>Specific growth rate (\textsuperscript{c})) (day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.5</td>
<td>3.04 ± 0.09</td>
<td>53.4 ± 1.6</td>
<td>658 ± 3</td>
<td>351.2 ± 1.5</td>
<td>1.665 ± 0.049</td>
</tr>
<tr>
<td>45</td>
<td>3.04 ± 0.10</td>
<td>51.7 ± 1.5</td>
<td>670 ± 2</td>
<td>346.4 ± 2.4</td>
<td>1.853 ± 0.055</td>
</tr>
<tr>
<td>90</td>
<td>3.17 ± 0.10</td>
<td>52.3 ± 1.6</td>
<td>699 ± 1</td>
<td>365.6 ± 1.9</td>
<td>1.859 ± 0.056</td>
</tr>
<tr>
<td>180</td>
<td>3.27 ± 0.11</td>
<td>49.1 ± 1.4</td>
<td>613 ± 3</td>
<td>301.0 ± 0.9</td>
<td>1.219 ± 0.037</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a})\) Final biomass concentration  
\(\textsuperscript{b})\) Maximum biomass productivity  
\(\textsuperscript{c})\) Maximum protein productivity
tein collected from *C. vulgaris* FSP-E cells at 90% nitrogen consumption. As shown in Fig. 3B, when Fe²⁺ concentration was too high (at 180 μM), the relative indispensable amino acid (IAA) content out of total protein was as low as 52%. In contrast, when Fe²⁺ concentration was adjusted to 90 μM or lower, the relative IAA content increased to almost 70%. It was also observed that the content of isoleucine, leucine, histidine and phenylalanine increased, whereas glycine, cysteine and tyrosine decreased. However, the total content of aromatic amino acids (i.e. phenylalanine and tyrosine) was similar for all iron concentrations. From these results, it can be seen that variation in iron concentration in the medium may lead to substantial changes in amino acid composition but has no obvious influence on overall protein content. In contrast, Fe²⁺ concentration has more impact on microalgal growth than on protein content or amino acid profile over the concentration range of 22.5–90 μM. In conclusion, high protein content and the diversification of amino acids strengthens *C. vulgaris* FSP-E candidature as an alternative to fish meal. As far as protein production is concerned, the optimal Fe²⁺ concentration is 90 μM, since it resulted in the highest protein productivity.

### 4 Conclusions

The effect of various cultivation conditions on the growth and protein production of *C. vulgaris* FSP-E were investigated. The highest protein production achieved was 365 mg/L/day when *C. vulgaris* FSP-E was cultured with modified Basal medium containing 12.4 mM urea and 90 μM Fe²⁺ in a modified PBR with an internal light source consisting of cold cathode fluorescent lamps. The nitrogen source availability strongly affected the protein content and the maximum protein productivity occurred when 90% nitrogen source was consumed. An excess amount of Fe²⁺ in the medium caused considerable variation in the amino acid profile of the microalgal protein. Under optimal conditions, the microalgal protein consisted of over 70% indispensable amino acids out of total protein, which makes microalgal protein suitable for use as aquafeed.

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The authors declare no financial or commercial conflict of interest.

### 5 References


<table>
<thead>
<tr>
<th>Strains</th>
<th>Operation mode</th>
<th>Working volume (L)</th>
<th>Cultivation conditions</th>
<th>Protein content (% w/w)</th>
<th>Protein productivity (mg L⁻¹ day⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmodesmus sp.</td>
<td>Batch</td>
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<td>fluorescent lamps</td>
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<td>34.6</td>
<td>111.4</td>
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<tr>
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<td>53.8</td>
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<td>21.7</td>
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<td>46.7</td>
<td>18.6</td>
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<td>106.4</td>
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<td>cold cathode fluorescent lamps</td>
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<td>52.3</td>
<td>365.6</td>
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²) N.A.: not available
³) internal illumination


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