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Effect of Iron Concentration on Growth, Protein Content and Total Phenolic Content of *Chlorella* sp. Cultured in Basal Medium

(Kesan Kepekatan Ferum terhadap Pertumbuhan, Kandungan Protein dan Jumlah Kandungan Fenolik *Chlorella* sp. yang dikultur dalam Media Basal)

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ABSTRACT

The aim of this study was to determine the effect of Fe^{3+} concentration (0.35, 4.89, 9.44 and 13.99 mg/L) on the growth, protein content and total phenolic content of Chlorella *sp. The* Chlorella *sp. cells were grown at 51% relative humidity, 25°C ± 2 under continuous illumination at 36 W irradiance supplied by day-light fluorescent lamp, and agitated by bubbling at a flow rate 2.7 m/s^2. Samples were collected every 2 days over 21 days of the cultivation period to estimate the growth of* Chlorella *sp. Protein and total phenolic content of samples were determined on phase 7^{th}, 14^{th}, and 21^{st} day of cultivation. Statistical analysis showed that there were significant differences ($p<0.05$) on growth, protein content and total phenolic content of* Chlorella *sp. at different iron concentrations. These differences could be related to specific differences in the cell metabolism. Protein content (8.34 mg/g dry weight), total phenolic content (8.70 mgGAE/g dry weight), cell number (1.03×10^7 cell/mL) and the specific growth rate (μ) of* Chlorella *sp. (1.85/day) were highest at the lowest Fe^{3+} concentration (0.35 mg/L).*

Keywords: Chlorella *sp.; growth; iron; protein content; total phenolic content*

ABSTRAK

Matlamat kajian ini adalah untuk mengenalpasti kesan kepekatan ferum (0.35, 4.89, 9.44 and 13.99 mg/L) ke atas pertumbuhan, kandungan protein dan jumlah kandungan fenolik Chlorella *sp.*. Chlorella *sp. hidup pada 51% kelembapan relatif, 25°C ± 2 di bawah pencahayaan yang berterusan dengan pemancaran 36 W (TIS 956-2533) yang dibekalkan oleh lampu fluoresen, dan dieram pada kadar pengaliran (2.7 m/s^2). Sampel diambil setiap 2 hari sepanjang 21 hari masa pengeraman untuk menganggar kadar pertumbuhan sel* Chlorella *sp. Kandungan protein dan jumlah fenolik dikenalpasti pada hari ke 7, 14 dan 21 sepanjang tempoh pengeraman. Analisis statistik menunjukkan bahawa terdapat perbezaan yang signifikan ($p<0.05$) pada pertumbuhan, kandungan protein dan jumlah kandungan fenolik* Chlorella *sp. pada kepekatan ferum yang berbeza. Perbezaan ini boleh dikaitkan dengan perbezaan spesifik dalam metabolisme sel. Kandungan protein* Chlorella *sp. (8.34 mg/g berat kering), jumlan kandungan fenolik (8.70 mgGAE/g berat kering), jumlah sel (1.03×10^7 sel/mL), dan kadar pertumbuhan spesifik (μ)* Chlorella *sp. (1.85/hari) adalah yang tertinggi pada kepekatan ferum yang terendah (0.35 mg/L).*

Kata kunci: Chlorella *sp.; ferum; jumlah kandungan fenolik; kandungan protein; pertumbuhan*

INTRODUCTION

Micro algal biomass is a valuable source for a wide range of fine chemicals, such as carotenoid pigments, vitamins, proteins, fatty acids, sterols, polysaccharides and other biologically active compounds, or potential health benefits. Micro algae like *Chlorella* have been suggested as a source of potential commercial application (e.g., food production). *Chlorella* is a highly valuable single cell freshwater green micro alga that is consumed by over 10 million people worldwide (Anderson 2005). It has survived for 2.5 billion years because of its extremely tough outer cell wall and its ability to quadruple in quantity every 20 h, making it the fastest growing plant on earth. It is the most extensive scientifically researched foodstuff and supplement in Japan. *Chlorella* sp. is ubiquitous in nature and has been isolated in diverse aquatic and aerial habitats and as symbionts in certain animals. Many members of this genus are highly adaptable to life under marginal conditions. Explosive growth rates under good conditions have earned it a reputation as an algal weed. In developing optimal process for *Chlorella* products, two major aspects are usually considered for improvement. One is the effects of environmental factors such as temperature, light intensity, pH, aeration and agitation, while another is the selection of a suitable nutrient medium. It is well known that the culture medium not only affects cell productivity, but also affects cell composition and yield of specific products (Imamoglu et al. 2007). Basal medium often used for culturing of *Chlorella* sp. It contains iron in the form of iron sulphate.

As a micronutrient, iron is required in only small concentration. However iron is one of the most important chemical nutrients required for growth of *Chlorella* sp. In recent years, the importance of iron in microalgae growth has been studied by many researchers. Behrenfeld et al. (2006) confirmed that iron had a key function in regulating phytoplankton biomass. Most *Chlorella* sp. strains are tolerant to a broad range of salt concentrations and pH values, so that the composition of the medium is normally not critical. Moreover, studies on the growth of *Chlorella pyrenoidosa* at various concentrations of macronutrients (NO_3^-, K, Mg, S, P, Cl and micronutrients (Fe, Cu, Zn, Mn, B and Mo) showed that the mineral (NO_3, Mg, K, P, S, Fe, Zn, Mn, Cl) is required for autotrophic and heterotrophic (with sugar) growth (Borowitzka & Borowitzka 1988). It is evident that *Chlorella pyrenoidosa* requires more Mn, Fe, Zn and nitrate and less Mg and K for autotrophic growth than for heterotrophic growth. However, essential trace metals such as copper, zinc and cobalt are toxic at high concentrations, and iron forms insoluble hydrous ferric oxide precipitates that are largely unavailable to aquatic algae (Rich & Morel 1990). In addition these ferric precipitates adsorb other essential metals and lower their availability.

This study was carried out to determine the effect of iron on the growth, protein content and total phenolic content of *Chlorella* sp. for large volume batch culturing technique cultivation. The objective of this research was to determine the effect of iron concentration on the growth, protein content and total phenolic content of *Chlorella* sp. cultured in Basal medium.

Materials and Methods

THE ALGA AND CULTURE CONDITIONS

The green microalga, *Chlorella* sp. was obtained from the Biotechnology laboratory of Burapha University of Thailand. This alga was maintained in Basal medium (Yuan et al. 2002) consisting of (per litre): 1.25 g KNO_3; 1.25 g KH_2PO_4; 1 g $MgSO_4.7H_2O$; 0.50 g EDTA; 0.1142 g H_3BO_3; 0.1110 g $CaCl_2.2H_2O$; 0.0882 g $ZnSO_4.7H_2O$; 0.0498 g $FeSO_4.7H_2O$; 0.0157 g $CuSO_4.5H_2O$; 0.0142 g $MnCl_2.4H_2O$; 0.0071 g MoO_3; 0.0049 g $Co(NO_3)_2.6H_2O$. Under continuos illumination 36 W irradiance (TIS 956-2533) supplied by day-light fluorescent lamp, the relative humidity (RH) was 51% and agitated bubbling flow rate was 2.7 m/s^2. 7-day old culture (500 mL, approximately 1 × 10^6 cell/mL) was inoculated into 5 L medium. Different of Fe^{3+}concentrations (0.35, 4.89, 9.44 (control) and 13.99 mg/L) were added to 6L bottles, each containing 5 L medium. The Fe^{3+} concentration on 9.44 mg/L was a control treatment because it was a baseline concentration (Yuan et al. 2002). The medium pH was adjusted to pH 6.1 prior to autoclave at 121°C for 20 min. The effect of different Fe^{3+} concentration was investigated for 21 days of cultivation period.

DETERMINATION OF *Chlorella* sp. GROWTH

The cell numbers of *Chlorella* sp. were counted under a microscope by using haemacytometer and cell counter every two days over a period 20 days. The specific growth rate (μ) of the cells was calculated in logarithmic phase from 8th to 12th of cultivation day, as $\mu = \ln X_2 - \ln X_1 / dt$, where X_2 is the cell number in 12th cultivation day, X_1 is the cell number in 8th cultivation day and dt is the time required for the increase in number from X_1 to X_2 (Imamoglu et al. 2007).

DETERMINATION OF PROTEIN CONTENT

Protein content was estimated by Bradford assay (Bradford 1976). *Chlorella* sp. (0.1 g dry weight) was crushed with 5 mL of 0.85% NaCl and transferred into tube. After the tube was centrifuged 3000 rpm for 20 minutes at 4°C to remove residual cell and filter debris, the supernatant 80 μL, 720 μL NaCl 0.85% and 200 μL Biorad dye reagent was taken and put in eppendorf tube. After 5 min of incubation at room temperature for 5 minutes, the absorbance at 595 nm was measured. Bovine serum albumin (0-800 μg/mL) was used for the standard curve.

DETERMINATION OF TOTAL PHENOLIC CONTENT

Total phenolic content was estimated by the Folin-Ciocalteau method. Sample (100 μL) was extracted in 900 μL of distillated water and 50 μL Folin-ciocalteau reagent, after 30 min of incubation at room temperature, 200 μL 10% Na_2CO_3 was added. After 1.5 h of incubation at room temperature, the absorbance at 730 nm was measured. Gallic acid (0.025, 0.05, 0.1 and 0.2 mg/mL) was used for the standard calibration curve. The results were expressed as gallic acid aquivalent (GAE/g dry weight) of *Chlorella* sp, and calculated as mean value ± SD (n = 3).

Statistical analysis included one-way analysis of variance (ANOVA) and Two-way analysis of variance. The significant data using Duncan New Multiple Range test at 95% confidence interval, and statistical evaluation was carried out using SPSS 15 software.

Results

GROWTH

The effect of 4 different concentrations of Fe^{3+} on the growth of *Chlorella* sp. was investigated for 20 days of cultivation period. $FeSO_4.7H_2O$ was a chemical that used as source of Fe^{3+} in Basal medium. The original concentration of $FeSO_4.7H_2O$ was 0.0498 g/L. The experiments were performed under the same growth conditions. The effect of iron concentration can be clearly seen by monitoring the cell number of *Chlorella* sp. (see Figure 1).

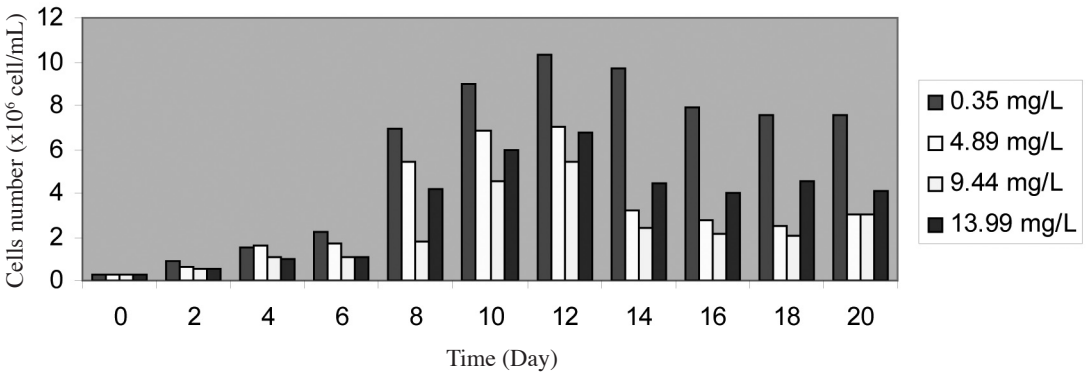

FIGURE 1. Cells number of *Chlorella* sp. (× 10^6 cell/mL) sp. cultured in different of Fe^{3+} concentration (mg/L)

Based on Figure 1, the cell number reached the maximum value (1.03 × 10^7 cell/mL) in the lowest of Fe^{3+} concentration (0.35 mg/L). On the other hand, at concentration 4.89 mg/L of Fe^{3+} the cell number increased to about 7.05 × 10^6 cell/mL. It was significantly higher compared to control treatment (p<0.05). In 0 day cultivation, there was no significant different compared to control treatment. In the end of cultivation of *Chlorella* sp. (20 day) the cell number was higher (7.58 × 10^6 cell/mL) in the lowest of Fe^{3+} concentration.

The specific growth rate (μ) was calculated from the 8th to 12th cultivation day in logarithmic phase. The specific growth rate of *Chlorella* sp. reached the maximum value in 0.35 mg/L of Fe^{3+} concentration (1.85/day), it was significantly higher than control treatment (p<0.05).

Table 1 shows the specific growth rate (μ) of *Chlorella* sp. from the 8th day to 12th cultivation day in logarithmic phase at different of Fe^{3+} concentration.

TABLE 1. The specific growth rate (μ) of *Chlorella* sp. during logarithmic phase

Concentration of Fe^{3+} (mg/L)	The specific growth rate (μ)
0.35	1.85±
4.89	1.53±
9.44	1.56±
13.99	1.56±

The morphology of *Chlorella* sp. in every concentration of Fe^{3+} in Basal medium at 14th of cultivation day as shown in Figure 2.

PROTEIN CONTENT (mg/g DRY WEIGHT)

Analysis of protein content 7 day after cultivation showed that the highest amount of protein content was obtained from a treatment using concentration of 0.35 mg/L Fe^{3+}. It was significantly higher (p<0.05) compared to control treatment (9.44 mg/L). On the contrary, control treatment has shown as the lowest result. Fourteen days after cultivation the amount of protein contents was ranging from 3.37 – 6.44 mg/g dry weight. The highest value of protein content was observed from a treatment using 0.35 mg/L Fe^{3+} and was significantly higher (p<0.05) compared to control treatment. The lowest result of protein content was obtained from control treatment (Table 2). Analysis of protein content 21 day after cultivation showed that the highest amount of protein was yielded by a treatment using concentration of 0.35 mg/L Fe^{3+}. It was significantly higher (p<0.05) compared to control treatment (9.44 mg/L). The lowest result was observed from control treatment (Table 2).

TOTAL PHENOLIC CONTENT (mgGAE/g DRY WEIGHT)

Analysis of total phenolic content 7 days after cultivation showed that the highest amount of total phenolic content was obtained from a treatment using concentration of 0.35 mg/L Fe^{3+}. It was significantly higher (p<0.05) compared to control treatment (9.44 mg/L). On the contrary, the lowest amount of total phenolic content was observed from a treatment using 4.89 mgGAE/g dry weight. It was significantly lower compared to control treatment. After 14 days incubated in various concentration of Fe^{3+} in basal medium, amount of total phenolic contents was ranging from 7.14 – 8.04 mgGAE/g dry weight. The highest value of phenolic content was observed from a treatment using 13.99 mg/L Fe^{3+} and was significantly higher (p<0.05) compared to control treatment. The lowest result of total phenolic content was obtained from a treatment using 4.89 mg/L $Fe^{3+,}$ and was not significantly lower compared to control treatment (Table 3).

Analysis of total phenolic content 21 day after cultivation showed that the highest amount of total phenolic content was yielded by a treatment using concentration of 0.35 mg/L Fe^{3+}. It was significantly higher (p<0.05) compared to control treatment (9.44 mg/L). The lowest result was observed from a treatment using 13.99 mg/L Fe^{3+}, and was not significantly lower compared to control treatment (Table 3).

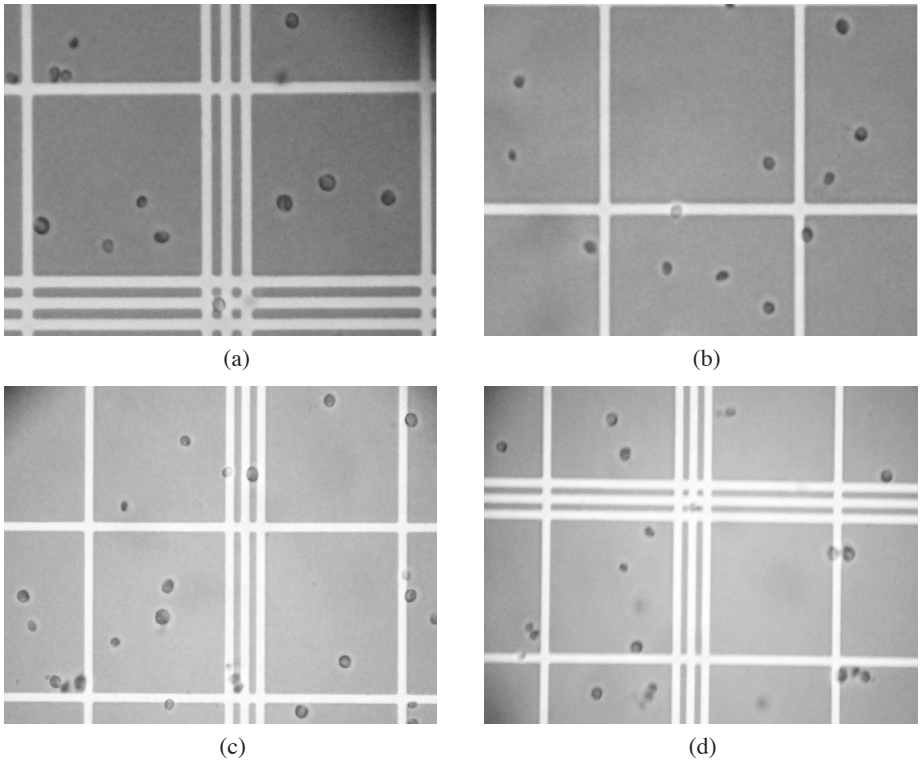

FIGURE 2. The morphology of *Chlorella* sp. under microscope (×100) in Basal medium at different concentration of Fe^{3+} (a) 0.35 mg/L, (b) 4.89 mg/L, (c) 9.44 mg/L,(d) 13.99 mg/L

TABLE 2. Protein content (mg/g dry weight) of *Chlorella* sp. in different of Fe^{3+} concentration

Concentration of Fe^{3+} (mg/L)	Protein content (Mean ± SD) (mg/g dry weight)		
	Time (Day)		
	7	14	21
0.35	5.48±0.22^{e}	6.44±0.30^{g}	8.34±0.11^{h}
4.89	3.24±0.35^{a}	5.09±0.516^{d}	5.79±0.76^{f}
9.44	3.10±0.11^{a}	3.37±0.50^{ab}	5.15±0.15^{d}
13.99	3.62±0.21^{b}	4.25±0.12^{c}	5.23±0.80^{de}

A concentration 9.44 mg/L was expressed as control treatment
Common letter indicated no significant among treatment at 0.05 of level confidence

TABLE 3. Total phenolic content (mgGAE/g dry weight) of *Chlorella* sp. in different of Fe^{3+} concentration

Concentration of Fe^{3+} (mg/L)	Total phenolic content (Mean ± SD) (mgGAE/g dry weight)		
	Time (Day)		
	7	14	21
0.35	8.70±0.27^{g}	7.74±0.21^{g}	6.34±0.14^{c}
4.89	6.65±0.29^{cd}	7.14±0.30^{ef}	5.68±0.19^{b}
9.44	7.06±0.16^{ef}	7.24±0.21^{f}	5.80±0.85^{b}
13.99	6.86±0.18^{de}	8.04±0.93^{g}	5.62±0.15^{b}

A concentration 9.44 mg/L was expressed as control treatment
Common letter indicated no significant among treatment at 0.05 of level confidence

Discussion

This paper reports the effect of different concentration of Fe^{3+} on growth, protein content and total phenolic content of *Chlorella* sp. The results present here using *Chlorella* sp. cells in cultured under laboratory condition in large volume batch culturing technique, indicated that excess iron could be responsible for the decrease in *Chlorella* sp. growth. In contrast with lower concentration of iron (0.35 mg/L) could increased the cell growth (1.03×10^7 cell/mL) and specific growth rate (1.85/day) of *Chlorella* sp. The data showed that the lowest concentration of iron Fe^{3+} (0.35 mg/L) had significantly increased on growth of *Chlorella* sp. cell compared to control. Probably, this is caused by the the excess of iron in higher concentration of Fe^{3+} in Basal medium. Excess of iron could be toxic on algal growth. Estevez et al. (2001) described that the iron supply at concentrations lower than 90 μM could be considered limiting for algal growth. However, Kolber et al. (1994) pointed out that in their field experiments in the equatorial Pacific, 2 days following iron enrichment, photosynthetic energy conversion efficiency began to decline. It was also indicated that some algal cultures showed deleterious effects if exceeding an iron threshold (14–28 μM) in unpolluted freshwater. On the other hand, the basal medium contained cobalt, where the cobalt indicated the inhibition of cell growth. (Khotamasi & Khotamasi 2005) demonstrated that cobalt was employed as a possible competitive inhibitor of iron-uptake because of its similar size. Study on *Pseudomonas aeruginosa* also indicated that cobalt interference in iron-uptake could inhibit growth. Iron mixed with cobalt inhibit growth of *Chlorella* sp. by interfering with its iron-uptake mechanism.

The phenolic content was extracted with 80% acidified methanol using Folin-Ciocalteau method. The highest amount of phenolic content (8.70 mgGAE/g dry weight) was obtained in the lowest of Fe^{3+} concentration. In this study, the amount of phenolic content of *Chlorella* sp. had similar value with Li et al. (2007). According to Li et al. (2007), the total phenolic of *Chlorella* sp. was ranging from 0.97-14.35 mgGAE/g dry weight). For microalgae, there has been very limited information on their phenolic content.

Conclusions

The addition of Fe^{3+} concentration above baseline concentration (9.44 mg/L) showed decrease in all parameters (protein content, total phenolic content, cell number, and the specific growth rate). It seems not necessary to use Fe^{3+} concentration above 9.44 mg/L. Thus, the biochemical compositions of *Chlorella* sp. give interesting qualities, which can be applied in food the industry. However, prior to commercialization, *Chlorella* sp. must be analyzed for the presence of toxic compounds to prove its harmlessness.

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