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Enhancement of *Scenedesmus* sp. LX1 Biomass Production and Lipid Accumulation Using Iron in Artificial Wastewater and Domestic Secondary Effluent

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While coupling wastewater treatment with microalgal bioenergy production is very promising, new approaches are needed to enhance microalgal growth and lipid accumulation in wastewater. Therefore, this study investigated the effect of iron on the growth, nutrient removal, and lipid accumulation of *Scenedesmus* sp. LX1 in both artificial wastewater and domestic secondary effluents. When increasing the iron concentration from 0 to 2 mg/l in the artificial wastewater, the biomass production of *Scenedesmus* sp. LX1 increased from 0.17 to 0.54 g/l; the nitrogen and phosphorus removal efficiency increased from 15.7% and 80.6% to 97.0% and 99.2%, respectively; and the lipid content was enhanced 84.2%. The relationship between the carrying capacity/maximal population growth rate of *Scenedesmus* sp. LX1 and the initial iron concentration were also in accordance with the Monod model. Furthermore, when increasing the iron concentration to 2 mg/l in four different domestic secondary effluent samples, the lipid content and lipid production of *Scenedesmus* sp. LX1 was improved by 17.4-33.7% and 21.5-41.8%, respectively.

Keywords: Microalgae, biofuel, lipid, iron, *Scenedesmus* sp. LX1

Introduction

The current energy crisis represents one of the greatest challenges for human society in the 21st century. Some researchers consider microalgae-based biodiesel as a promising substitute for fossil fuel. When compared with biodiesel and bioethanol from terrestrial plants, microalgae-based biodiesel has several clear advantages: (1) a higher lipid productivity than oilseed crops due to a high lipid content and rapid growth [3, 19]; (2) the production of valuable chemicals [15]; (3) the ability of CO_2 fixation [3]; and (4) the possibility to couple bioenergy production with wastewater treatment [7]. However, microalgae-based biofuel production has not yet been commercialized on a large scale because of its high resource consumption. According to Yang's analysis, the production of 1 kg of biodiesel requires 3726 kg of water, 0.33 kg of nitrogen, and 0.71 kg of phosphate [21].

Coupling wastewater treatment with the production of microalgae-based biodiesel/biomass is an effective way to enhance inorganic nutrient removal by microalgae and reduce the cost of the growth medium for microalgal cultivation [4, 7, 17]. However, previous studies have indicated that most microalgae with a high lipid content cannot adapt

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to wastewater. For instance, *Dunaliella primolecta, Spirulina platensis, Phaeodactytuum ericornutum, Isochorysis* sp., *Nitzschia hantzschiana, Cyclotella hebeiana, Botryococcus braunii* 1049, and *Botryococcus braunii* 763 have all been found to be incapable of any significant growth in domestic secondary effluent [11].

Microalgae growth in wastewater is determined by several variables, including the availability of essential elements in the wastewater. Iron is one of the most important elements for the growth of almost all microorganisms as it is involved in vital processes, such as chlorophyll biosynthesis, nitrogen fixation, respiration, and radical scavenging [6]. Various recent studies reported that increasing the concentration of iron in the culture medium within a certain range significantly enhanced the lipid content and growth rate of certain microalgae strains [14, 16, 22]. When investigating the lipid accumulation properties of *Chlorella vulgaris*, Liu *et al.* found that increasing the iron concentration from 0 mol/l (0 mg/l) to 1.2×10^{-5} mol/l (0.67 mg/l) improved the lipid content in the microalgal biomass from 7.8% to 56.6% [14]. Pankowski *et al.* also reported that increasing the iron concentration from 6.25 nM (3.5×10^{-4} mg/l) to 300 nM (1.7×10^{-2} mg/l) increased the growth rate of *Fragilariopsis cylindrus* from 0.34 d^{-1} to 0.57 d^{-1} and that of *Fragilariopsis curta* from 0.14 d^{-1} to 0.28 d^{-1} [16].

However, despite such studies on the effects of iron in a typical culture medium, the feasibility of enhancing the growth, nutrient removal, and lipid accumulation performance of microalgae in domestic secondary effluent by the addition of iron has not yet been investigated or proposed. Furthermore, the iron demand differs according to different microalgal species.

The freshwater microalga *Scenedesmus* sp. LX1 was previously isolated from stored tap water containing 0.34 mg/l of nitrogen and 0.016 mg/l of phosphorus. This microalga was also found to grow well in secondary effluent, remove inorganic nutrients efficiently, and accumulate about 30% lipids in its dry biomass [10], making it a promising species for coupling microalgal biomass production and wastewater treatment.

Accordingly, this study investigated the growth, nutrient removal, and lipid/triacylglycerol (TAG) accumulation properties of *Scenedesmus* sp. LX1 with different initial iron concentrations, applied the Monod model to describe the relationship between the maximal microalgal population growth rate/carrying capacity and the initial iron concentration, and finally examined the influence of iron on *Scenedesmus* sp. LX1 in four different domestic secondary effluents.

Materials and Methods

Microalgal species used in this study

Scenedesmus sp. LX1 (Collection No. CGMCC 3036 in the China General Microbiological Culture Collection Center) was previously isolated [10] and preserved in a 50% BG11 medium.

Wastewater samples used in this study

Artificial wastewater and domestic secondary effluent were used for the microalgal cultivation in this study.

The artificial wastewater was based on a modified BG11 medium (mBG11) with similar nitrogen and phosphorus concentrations to typical domestic secondary effluent [23]. The initial dissolved total phosphorus and dissolved total nitrogen for every sample were 1.3 mg/l and 20 mg/l, respectively, and $K_2HPO_4 \cdot 3H_2O$ and $NaNO_3$ were used as the phosphorus and nitrogen sources, respectively. The initial iron concentration was controlled to investigate the effects of iron. The other elements in the artificial wastewater were the same as in the mBG11 medium.

Different domestic secondary effluents were obtained from four different wastewater treatment plants in Beijing (China), including the secondary effluent of a biological aerated filter (BAF) (sample A), two different secondary effluents of anaerobic-anoxic-oxic (A^2O) (sample B and C), and the secondary effluent of a Membrane Bio-Reactor (MBR) (sample D). The secondary effluents were all filtered through a 0.45 μm membrane and sterilized before the experiments. The water qualities of the domestic secondary effluents are shown in Table 1.

Table 1. Water quality of domestic secondary effluents from four wastewater treatment plants.

Samples	DTN (mg/l)	DTP (mg/l, before Fe addition)	DTP (mg/l, after Fe addition)	Fe (mg/l)
Sample A	24.99	0.94	0.94	0.76
Sample B	14.02	0.57	0.57	0.40
Sample C	12.89	0.23	0.23	0.46
Sample D	7.14	0.05	0.05	0.34

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Experiment set-up

To determine the effects of the initial iron concentration, 4 samples of artificial wastewater were prepared with initial iron concentrations of 0, 0.6, 1.2, 2, and 5 mg/l. When the iron concentration was less than 1.2 mg/l (iron concentration in mBG11 medium), iron was provided using ferric ammonium citrate. When the iron concentration exceeded 1.2 mg/l, 2.2 g/l $FeCl_3$ was added as a supplement.

To investigate the effects of iron in domestic secondary effluents, $FeCl_3$ was added to the secondary effluent samples to create an initial iron concentration of 2 mg/l. The domestic secondary effluent samples with and without iron addition were defined as the experimental group and control group, respectively. All the samples were numbered and sterilized before the experiments.

Scenedesmus sp. LX1 was cultivated in 200 ml of the mBG11 medium in 500 ml Erlenmeyer flasks placed in an artificial climate chamber (HPG-280H). 5 ml algal inocula were centrifuged (10,000 rpm for 10 min at 4°C) and the deposited algal cells washed twice using 15 mg/l of an $NaHCO_3$ solution. The inocula were then re-suspended in 5 ml of the $NaHCO_3$ solution and inoculated into each flask. The initial algae density was approximately 3×10^5 cells/ml. The cultivation conditions were as follows: light intensity 55 μmol photon/m/s, light/dark ratio of 14:10, and temperature of 25°C ± 1°C. The microalgal cultivation was conducted in triplicate (n = 3).

Analytical methods

The algal density was determined by measuring the optical density of the algal culture at 650 nm (OD_{650}) every two days. According to a previous study [11], the relationship between the algal density (D, cells/ml) and the OD_{650} can be expressed as follows (Eq. (1)):

$$D = 9.52 \times 10^6 OD_{650} + 70957 \text{ , } R = 0.997 \quad (1)$$

The suspended solid method was used to determine the algal biomass dry weight (DW), where 40 ml of the culture was filtered using a pre-weighed 0.45 μm membrane filter. The filter was then dried at 105°C to maintain a constant weight.

Microalgal growth under certain environmental conditions can be described using the logistic model (Eq. (2)) [11]:

$$N = \frac{K}{1 + e^{a - rt}} \quad (2)$$

where N (cells/ml) is the algal density at time t (d), K (cells/ml) is the carrying capacity (maximum algal density reached in the culture), a is a constant in the logistic model that indicates the relative position from the origin, and r (d^{-1}) is the intrinsic growth rate.

This equation can be transformed into a linear form:

$$\ln\left(\frac{k}{n} - 1\right) = a - rt \quad (3)$$

A regression line can then be obtained where the slope is equal to the intrinsic growth rate (r).

The following equation shows the population growth rate dN/dt in cells (ml day)$^{-1}$ in the logistic model:

$$\frac{dN}{dt} = dN\left(\frac{K - N}{K}\right) \quad (4)$$

The population growth rate is at its maximum value, R_{max} [cells (ml day)$^{-1}$], when N reaches half of K. R_{max} is expressed in Eq. (5).

$$R_{max} = \frac{rK}{4} \quad (5)$$

The total lipids in the algal cells were extracted using a chloroform/methanol solution (1/1, v/v) and quantified gravimetrically [2]. After measuring the amount of total lipids, the dried lipids were dissolved in 0.8 ml of isopropyl alcohol. The TAGs were then determined using an enzymatic colorimetric method based on a commercial kit from Beijing BHKT Clinical Reagent Co. Ltd., No. 2400076 [11].

The algal culture was filtered through a 0.45 μm membrane filter to measure the water quality. The filtrate was then used to determine the dissolved total phosphorous concentration (DTP) according to Chinese standard testing methods. Plus, the dissolved total nitrogen concentration (DTN) was measured using a total organic carbon analyzer (TOC-V_{CPH}, SHIMADZU), while the iron concentration was determined via inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Thermo IRIS).

Results and Discussion

Effects of iron concentration on growth of *Scenedesmus* sp. LX1

The growth curves of *Scenedesmus* sp. LX1 in the artificial wastewater at different initial dissolved iron concentrations are shown in Fig. 1. After 16 days of cultivation,

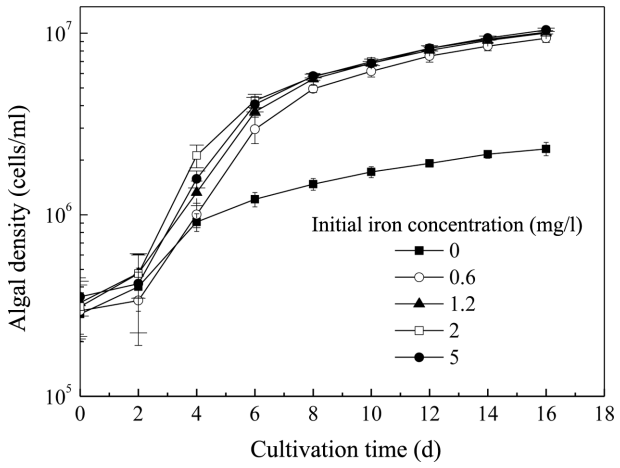

Fig. 1. Growth curves of *Scenedesmus* sp. LX1 in artificial wastewater with different initial dissolved iron concentrations.

Scenedesmus sp. LX1 grew into the stationary stage. The maximum algal densities at the initial dissolved iron concentrations of 0, 0.6, 1.2, 2, and 5 mg/l were 2.3×10^6, 9.4×10^6, 10.0×10^6, 10.1×10^6, and 10.4×10^6 cells/ml, respectively. Plus, under the above iron concentrations, the maximum population growth rates of *Scenedesmus* sp. LX1 in the artificial wastewater were 0.16×10^6, 0.87×10^6, 1.05×10^6, 1.00×10^6, and 1.05×10^6 cells (ml d)$^{-1}$, respectively. Thus, the addition of iron significantly enhanced both the maximum biomass production and the growth rate of *Scenedesmus* sp. LX1. However, the enhancement was limited when the iron reached a certain concentration.

The maximal algal cell dry weights (DWs) of *Scenedesmus* sp. LX1 at the different initial dissolved iron concentrations are shown in Table 2. The maximal algal cell DW after 16 days of cultivation was only 0.17 g/l when no iron was added. When the iron concentration increased from 0 mg/l to 0.6 mg/l, the maximal algal cell DW was enhanced 219%, reaching 0.55 g/l. As phosphorous is an important limiting resource for the large-scale production of microalgal biofuel, increasing the utilization efficiency of phosphorous has become a key research focus [20]. In the current experiment, the biomass yield per unit of phosphorus was increased 157.4%, resulting in a biomass yield of 425.6 kg-biomass/kg-P, which is nearly twice the biomass yield (160 kg-biomass/kg-P) obtained in the previous study by Wu [20]. However, further increases in the iron concentration exhibited no significant effect on the maximal algal cell DW ($p > 0.05$) or biomass yield per unit of phosphorus (data not shown).

Other studies have identified nitrogen and phosphorus as the most important factors affecting microalgal growth [9]. Yet, when coupling microalgal biomass production and wastewater treatment, nitrogen and phosphorus do not increase the biomass production. Therefore, other enhancement approaches are needed.

The carrying capacity (K) and maximal population growth rate (R_{max}) of *Scenedesmus* sp. LX1 with the different dissolved iron concentrations were obtained by conducting a linear regression analysis (Eq. (3)) of the growth data and using Eq. (5). The results are shown in Table 2.

The Monod model (Eqs. (6) and (7)) was used to describe the relationship between K, R_{max}, and the initial dissolved iron concentration:

$$K = \frac{K' \cdot S_{Fe}}{K_{S,Fe} + S_{Fe}} \tag{6}$$

$$R_{max} = \frac{R'_{max} \cdot S_{Fe}}{K'_{S,Fe} + S_{Fe}} \tag{7}$$

where S_{Fe} (mg/l) is the initial iron concentration in the artificial wastewater, $K_{S,Fe}$ (mg/l) is the half-saturation constant for the maximum biomass, $K_{S,Fe}$ (mg/l) is the half-saturation constant for the population growth rate, K' (cells/ml) is the maximum value of K at the saturated iron concentration, and R'_{max} (cells (ml d)$^{-1}$) is the maximum value of R_{max} at the saturated iron concentration.

Datafit 9.0 was used to examine the fitting efficiency between the experimental data and the Monod model and to obtain the parameter values. As a result, the following Monod parameters were obtained: $K' = 1.31 \times 10^7$ cells/ml,

Table 2. Maximal algal cell dry weight, carrying capacity (K), and maximum population growth rate (R_{max}) of *Scenedesmus* sp. LX1 with different initial dissolved iron concentrations ($p < 0.01$).

Initial iron concentration (mg/l)	0	0.6	1.2	2	5
Maximal algal cell dry weight (g/l)	0.17 ± 0.01	0.55 ± 0.02	0.58 ± 0.03	0.54 ± 0.01	0.54 ± 0.01
K ± S.D. (10^6 cells/ml)	2.80 ± 0.78	9.76 ± 0.83	10.62 ± 0.34	11.69 ± 0.27	12.69 ± 0.85
R_{max} ± S.D. (10^6 cells (ml d)$^{-1}$)	0.16 ± 0.01	0.87 ± 0.07	1.05 ± 0.04	1.00 ± 0.03	1.05 ± 0.04

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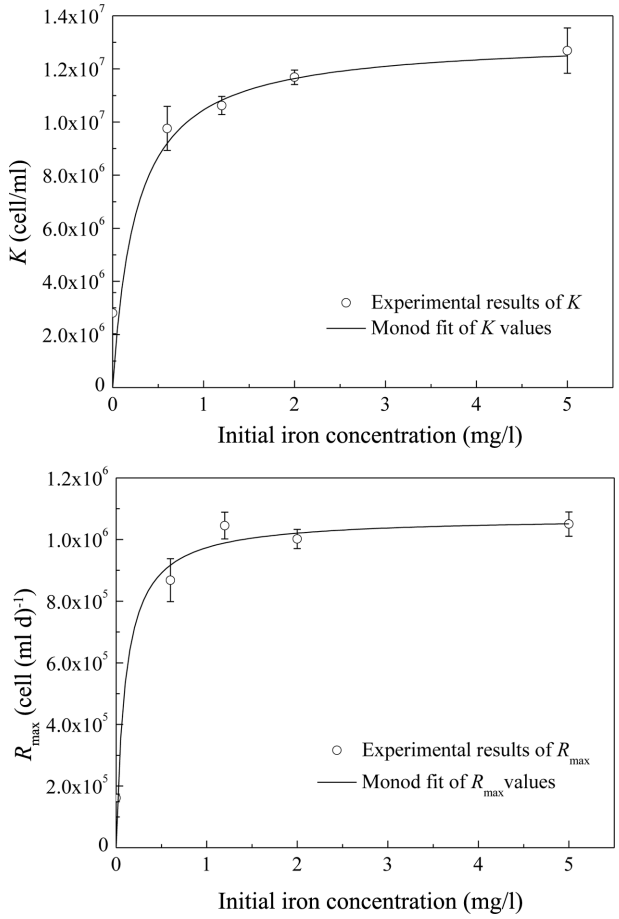

Fig. 2. Carrying capacity (*K*) and maximal population growth rate (R_{max}) of *Scenedesmus* sp. LX1 with different initial dissolved iron concentrations in artificial wastewater.

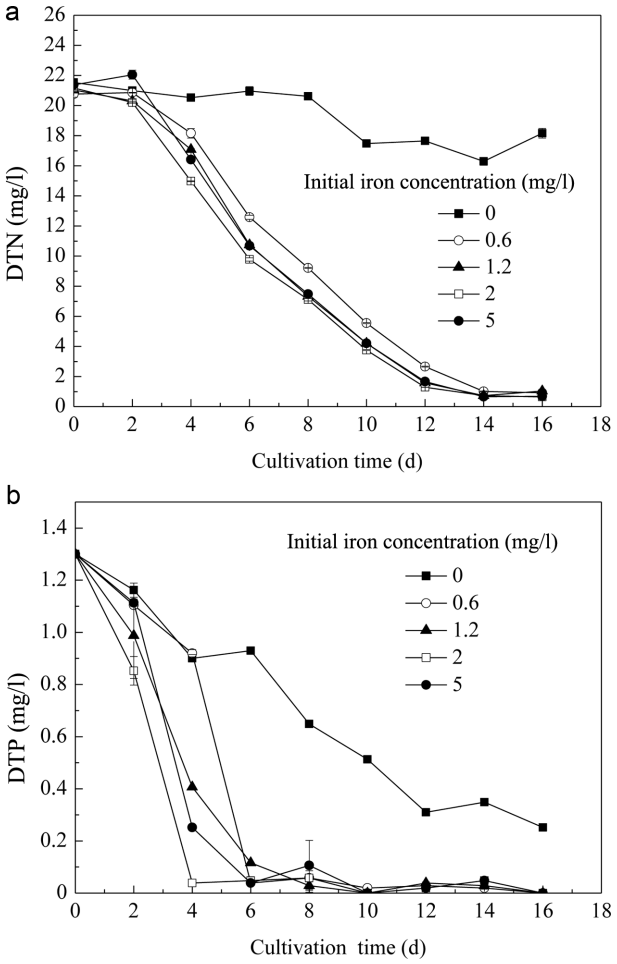

Fig. 3. Changes in DTN (a) and DTP (b) concentration in artificial wastewater with different initial dissolved iron concentrations.

$K_{S,Fe}$ = 0.26 mg/l, R'_{max} = 1.07×10^6 cells (ml d)$^{-1}$, and $K'_{S,Fe}$ = 0.10 mg/l ($p < 0.01$).

The Monod model employing the initial iron concentration as a growth-limiting substrate was calculated using Eqs. (8) and (9) and the above Monod parameters. The resulting fitted curves for K and R_{max} are shown in Fig. 2; the curves fit the experimental data to a high degree (R^2 = 0.87; 0.95).

$$K = 1.31 \times 10^7 \times \frac{S_{Fe}}{0.26 + S_{Fe}} \quad (8)$$

$$R_{max} = 1.07 \times 10^6 \times \frac{S_{Fe}}{0.10 + S_{Fe}} \quad (9)$$

According to the above results, to achieve a high biomass production of *Scenedesmus* sp. LX1, the initial concentration of iron should be higher than 2 mg/l under an initial nitrogen and phosphorus concentration of 20 mg/l and 1.3 mg/l, respectively.

Effects of iron concentration on nitrogen and phosphorus removal

The changes in the DTN (a) and DTP (b) concentration in the artificial wastewater with the different initial dissolved iron concentrations are shown in Figs. 3a and 3b. Phosphorus was rapidly removed from the artificial wastewater during the initial growth stage of *Scenedesmus* sp. LX1 with a removal efficiency of 30.7%, 29.2%, 68.7%, 97.0%, and 80.1% on the fourth day. On the same day, the residual nitrogen concentrations were 20.5, 18.2, 17.1, 15.0, and 16.4 mg/l, thereby resulting in a removal efficiency of 4.7%, 12.5%, 18.8%, 29.2%, and 23.3%, respectively. These results indicated that the addition of iron significantly enhanced the nitrogen and phosphorus removal efficiency

of the microalgae. After 16 days of cultivation, the nitrogen and phosphorus removal efficiency exceeded 95%, except in the group with no added iron, representing a nitrogen and phosphorus removal efficiency of 16% and 73%, respectively. Therefore, the addition of iron at certain concentrations was found to be necessary for the efficient removal of nitrogen and phosphorus from the wastewater when using *Scenedesmus* sp. LX1.

Effects of iron concentration on lipid accumulation of *Scenedesmus* sp. LX1

After 16 days of cultivation, the lipid content of the microalgal biomass and lipid TAG content of *Scenedesmus* sp. LX1 with different initial iron concentrations in the artificial wastewater were determined and the results are shown in Fig. 4.

At the iron concentrations ranging from 0 mg/l to 5 mg/l, the lipid TAG content was approximately 12% (w/w), and no significant difference ($p > 0.05$) was found.

However, when increasing the initial iron concentration, the lipid content of the microalgal biomass increased significantly ($p < 0.01$). When compared with the group without added iron, the lipid content per unit of microalgal biomass increased 16.9%, 84.2%, 84.2%, and 62.5% at the iron concentrations of 0.6, 1.2, 2, and 5 mg/l, respectively. Similar results have also been reported by other researchers. Yeesang *et al.* found that the lipid content of the microalgal strains TRG, KB, SK, and PSU, which are all green microalgae species isolated from lakes and freshwater ponds in southern Thailand, increased significantly when increasing the iron concentration from 0 mM (0 mg/l) to 0.037 mM (2.06 mg/l) [22]. The cellular mechanisms for the photosynthesis of microalgal cells are indispensable to lipid accumulation [5]. Thus, increasing the iron in the artificial wastewater may have increased the amount of chlorophyll in the microalgal cells [8] and also affected the activity of some essential enzymes related to photosynthesis, such as superoxide dismutase, peroxidase, and catalase [1]. This may be the main explanation for the positive effect of iron on the lipid accumulation of *Scenedesmus* sp. LX1.

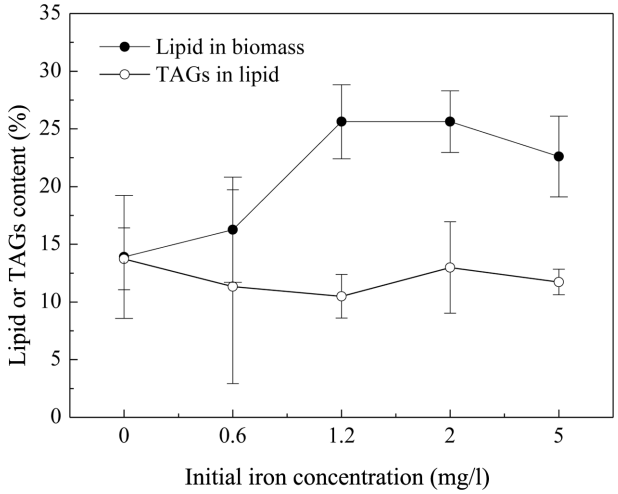

Fig. 4. Lipid content in microalgal biomass (dry weight) and lipid TAG content (dry weight) of *Scenedesmus* sp. LX1 after 16-day cultivation with different initial dissolved iron concentrations in artificial wastewater.

In a previous study, Li *et al.* also investigated various approaches to improve the lipid accumulation of *Scenedesmus* sp. LX1 and found that EMA concentrations of 0.5, 1.0, and 2.0 mg/l increased the lipid TAG content 34.8%, 78.3%, and 79.1%, respectively, when compared with the control group without EMA treatment. Yet, the EMA treatment had an insignificant effect on the lipid content [12].

Conversely, in the present study, the addition of iron significantly enhanced the lipid content, yet had an insignificant effect on the TAG content. Thus, to improve both the lipid and TAG content of the microalgal biomass, the combined effects of iron and EMA should be examined in further studies.

In the present study, iron significantly enhanced the growth, nutrient removal, and lipid accumulation of *Scenedesmus* sp. LX1 in the artificial wastewater. However, contradictory results regarding the effect of nitrogen and phosphorus on microalgal growth and lipid accumulations have been reported in previous studies, where a high biomass production and high lipid content could not be obtained simultaneously [13, 18]. Therefore, the results of this study offer a promising solution for the contradiction between biomass production and lipid accumulation using a low-cost reagent used in traditional wastewater treatment ($FeCl_3$).

Effects of iron on biomass production and lipid accumulation of *Scenedesmus* sp. LX1 in domestic secondary effluent

This study also investigated the feasibility of using iron in domestic secondary effluents. Due to water quality differences, the maximum algal densities and maximum algal cell dry weights varied significantly in the different domestic secondary effluent samples. Yet, the addition of iron did

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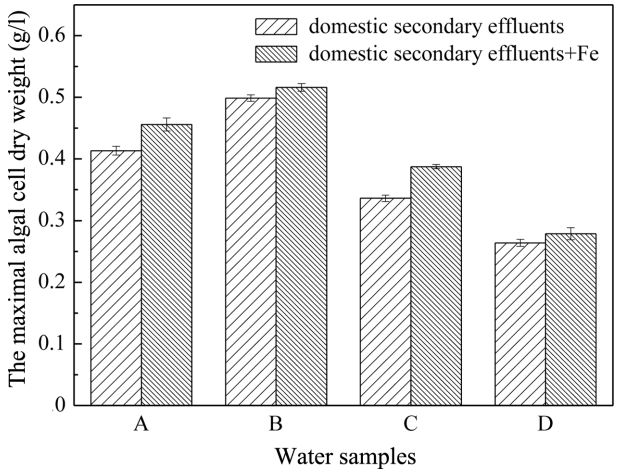

Fig. 5. Maximal algal cell dry weight of *Scenedesmus* sp. LX1 in domestic secondary effluents with (up to 2 mg/l) and without iron addition.

enhance the maximal algal cell dry weight of the experimental groups (Fig. 5). When compared with the control groups, the maximal algal cell dry weight of the experimental groups was increased 10.3%, 3.5%, 15.2%, and 5.7% with wastewater samples A, B, C, and D, respectively ($p < 0.05$). The effect of iron on enhancing the biomass production in the wastewater samples was perhaps limited as the initial iron concentrations in the wastewater samples were sufficient for the growth of *Scenedesmus* sp. LX1. As shown in Fig. 2, when the concentration of iron was higher than 0.5 mg/l, the enhancement effect on biomass production became less and less effective.

As the addition of iron showed a limited effect on enhancing microalgal growth in the secondary wastewater samples, the effects of iron on nutrient removal were also insignificant (Data not shown). Notwithstanding, the addtion of iron had a significant stimulatory effect on the lipid accumulation of *Scenedesmus* sp. LX1 in all the wastewater samples.

The lipid content and total lipid production of *Scenedesmus* sp. LX1 in the different wastewater samples with (experimental groups) and without (control groups) the addition of iron are shown in Table 3 and Fig. 6, respectively. The addition of iron increased the lipid content in the experimental groups by 21.5%, 17.4%, 23.1%, and 33.7% when compared with the control groups for wastewater samples A, B, C, and D, respectively ($p < 0.05$). Plus, the lipid content of the microalgal biomass in the different wastewater samples ranged from 13% to 30%. In a previous study, Li *et al.* found that after 10 days cultivation in a secondary domestic effluent with an initial total phosphorus and total nitrogen of 0.5 mg/l and 15.5 mg/l, respectively, the lipid content of *Scenedesmus* sp. LX1 remained constant at around 30% [11]. This lipid content is comparable with the current results.

Table 3. Relatively enhanced percentage of lipid content of *Scenedesmus* sp. LX1 after 16-day cultivation in domestic secondary effluents with iron addition.

Wastewater samples	A	B	C	D
Relatively enhanced percentage (%)	21.5	17.4	23.1	33.7

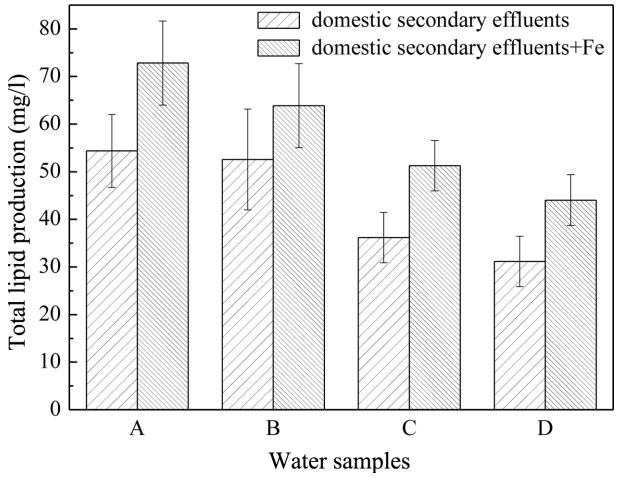

Fig. 6. Total lipid production of *Scenedesmus* sp. LX1 after 16-day cultivation in domestic secondary effluents with (up to 2 mg/l) and without iron addition.

Furthermore, the synchronous increase in the algal cell dry weight and the lipid content with the addition of iron significantly increased the total lipid production in the experimental groups by 34.0%, 21.5%, 41.8%, and 41.3% when compared with the control groups for wastewater samples A, B, C, and D, respectively. Meanwhile, similar to the artificial wastewater results, the added iron did not have any significant effect on the lipid TAG content of *Scenedesmus* sp. LX1 in the domestic wastewater samples (Data not shown).

In conclusions, the relationship between the carrying capacity (K)/maximal population growth rate (R_{max}) of *Scenedesmus* sp. LX1 and the initial iron concentration matched well with the Monod model. The addition of iron was proven to be essential for efficient nutrient removal by *Scenedesmus* sp. LX1. When increasing the iron concentration in artificial wastewater from 0 mg/l to 2 mg/l, the lipid

content of *Scenedesmus* sp. LX1 increased 84.2%. Plus, increasing the iron concentration to 2 mg/l in different domestic secondary effluent samples also significantly enhanced the biomass production and lipid accumulation of *Scenedesmus* sp. LX1, thereby significantly increasing the total lipid production. Nonetheless, further research is needed to clarify the mechanism of the positive effects of iron on lipid accumulation by microalgal cells.

Acknowledgments

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