

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES
 JOURNAL OF
 ENVIRONMENTAL
 SCIENCES
www.jesc.ac.cn

Construction and application of the *Synechocystis* sp. PCC6803-*ftnA* in microbial contamination control in a coupled cultivation and wastewater treatment

Yalei Zhang¹, Chunmin Zhang^{1,2}, Xuefei Zhou^{1,*}, Zheng Shen¹, Fangchao Zhao¹, Jianfu Zhao¹

1. State Key Laboratory of Pollution Control and Resource Reuse, Key Laboratory of Yangtze Water Environment of Ministry of Education, Tongji University, Shanghai 200092, China. E-mail: zhangyalei@tongji.edu.cn

2. Water Conservancy Development Research Center, Taihu Basin Authority of Ministry of Water Resources, Shanghai 200434, China

ARTICLE INFO

Article history:

Received 26 July 2015

Revised 1 September 2015

Accepted 13 January 2016

Available online 1 April 2016

Keywords:

Synechocystis PCC6803-*ftnA*

Municipal wastewater treatment

Microbial contaminants

ABSTRACT

Inspired by iron fertilization experiments in HNLC (high-nitrate, low-chlorophyll) sea areas, we proposed the use of iron-rich engineered microalgae for microbial contaminant control in iron-free culture media. Based on the genome sequence and natural transformation system of *Synechocystis* sp. PCC6803, *ftnA* (encoding ferritin) was selected as our target gene and was cloned into wild-type *Synechocystis* sp. PCC6803. Tests at the molecular level confirmed the successful construction of the engineered *Synechocystis* sp. PCC6803-*ftnA*. After Fe³⁺-EDTA pulsing, the intracellular iron content of *Synechocystis* sp. PCC6803-*ftnA* was significantly enhanced, and the algae was used in the microbial contamination control system. In the coupled *Synechocystis* sp. PCC6803-*ftnA* production and municipal wastewater (MW, including *Scenedesmus obliquus* and *Bacillus*) treatment, *Synechocystis* sp. PCC6803-*ftnA* accounted for all of the microbial activity and significantly increased from 70% of the microbial community to 95%. These results revealed that while the stored iron in the *Synechocystis* sp. PCC6803-*ftnA* cells was used for growth and reproduction of this microalga in the MW, the growth of other microbes was inhibited because of the iron limitation, and these results provide a new method for microbial contamination control during a coupling process.

© 2016 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

Published by Elsevier B.V.

Introduction

In recent years, the potential applications for the development of microalgae for sustainable energy have been extensively studied, and microalgae have been suggested to be the fuel of the future (Zhang et al., 2012, 2014; Zhao et al., 2015a). Because of high photosynthetic yield, decrease in land competition for food production, and improved control of ground emissions, microalgae-based biofuels have been recognized as the “the third generation of biomass energy” and the “only current

renewable source of oil that could meet the global demand for transport fuels” (Aravantinou et al., 2013; Guo et al., 2013; Lam and Lee, 2012; Sacristan et al., 2013). For this to occur, algal biomass must be produced practically and economically. One of the methods to reduce the costs of algae mass cultivation is to combine wastewater treatment with algae biomass production, which was first suggested in the 1960s (Chai et al., 2013; Scott et al., 2010; Stephenson et al., 2010; Zhang et al., 2014).

The use of agricultural or municipal sewage in microalgae cultures can provide nutritional sources for algal growth and

* Corresponding author. E-mail: zhouxuefei@tongji.edu.cn (Xuefei Zhou).

significantly reduce culture costs (Zhu et al., 2013). However, wastewater also contains a numerous types of algae predators, miscellaneous algae, and bacteria, which in a large scale open-pond system, can affect the stability of the culture system (Sutherland et al., 2014; Zhong and Zhang, 2013). Few studies have been conducted on these non-target microbial research, and research on the measures to prevent or limit miscellaneous algae or bacterial intrusion is even scarcer. The problems of microbial contamination in a coupled system of algal production and wastewater treatment are of widespread concern and still need to be addressed.

In the 1920s, the North Pacific, Southern Ocean and Equatorial Pacific were known as key high-nutrient, low-chlorophyll (HNLC) regions in which biological productivity was lower than expected for the prevailing surface nutrient conditions (Edwards et al., 2004; Fujii et al., 2005). Recent large-scale iron experiments (Fujii et al., 2005; Mongin et al., 2011; Petrou et al., 2011; Torres and Ampuero, 2009) have shown that the relatively low productivity in these HNLC regions was due to low surface iron concentration because iron is one of the essential micronutrients for the growth of many types of phytoplankton and microalgae. Inspired by iron fertilization experiments in HNLC sea areas, we proposed that microalgae with excess iron cultured in an iron-free culture system would be able to use the stored excess iron for growth and reproduction. Thus, other organisms in the system would not grow well due to the lack of iron in the media. Constructing and using an iron-rich engineered microalgae could be a strategy for microbial contaminant control in a coupling system of algal production and municipal wastewater (MW, which is comparable to an iron-free culture medium) treatment. *Cyanobacteria* are autotrophic prokaryotes that perform oxygenic photosynthesis similar to higher plants (Parmar et al., 2011). *Synechocystis* sp. PCC6803 is a unicellular cyanobacterium, and its complete genome was first characterized in photosynthetic organisms. Moreover, this unicellular *Cyanobacterium* possesses many characteristics, including a natural DNA transformation system, simple cell structure, fast growth, strong adaptability, and an ability to obtain useful organic products with little expenditure of energy and resources (Abe et al., 2014; Markou et al., 2014; Van der Woude et al., 2014). Additionally, the products that are expressed in exogenous genes in *Cyanobacteria* cannot easily generate inclusion bodies, and most of the *Cyanobacteria* and their extracts are non-toxic to humans and animals (Savakis et al., 2013; Watanabe et al., 2012; Zhao et al., 2015b). Based on these characteristic, *Cyanobacteria* have been found to be the perfect model algae for studying genetic engineering.

In this study, by taking advantage of the transformation system of *Synechocystis* sp. PCC6803, we engineered microalgae *Synechocystis* sp. PCC6803-*ftnA*. The high-iron-uptake microalgae were used for microbial contamination control in the coupling system of algal biomass production and MW treatment.

1. Materials and methods

1.1. *Synechocystis* sp. PCC6803-*ftnA* constructs

1.1.1. Chemicals and reagents

The chemicals, kits, primers, and reagents were purchased from common commercial vendors.

1.1.2. Cyanobacterial strains, growth conditions, and transformation

The *Synechocystis* sp. PCC6803 was obtained from The Pasteur Culture Collection (PCC; <http://www.pasteur.fr/ip/easysite/go/03b-000012-00g/collection-of-cyanobacteria-pcc>). The cells were grown on solid or liquid BG11 media, and the liquid cultures were grown in a shaking incubator (Zhang et al., 2012). The transformation of the *Synechocystis* sp. PCC6803 cells and the propagation of the transformants on selective media were performed as previously described (Abe et al., 2014), and the antibiotic, kanamycin (Km), was added at a concentration of 25 µg/mL.

1.1.3. Plasmids and gene constructs

pFUEL3d1 was the designed and constructed plasmid. The origin of replication for its propagation in *Escherichia coli* was coupled with ampicillin resistance. The plasmid also carries kanamycin resistance gene, as well as multiple cloning sites. We cloned *ftnA* into the multiple cloning sites to obtain the pFUEL3d1-*ftnA* construct. *ftnA* encodes ferritin, an iron storage protein (<http://www.ecogene.org/3.0/?q=gene/EG10921/proteinma>). Frequently, *Cyanobacteria* have limited iron. We examined the impact of additional iron storage capacity on the growth of the engineered *Cyanobacteria* to assess its advantage over the different wild-type strains of algae in a mixed culture. *ftnA* was chosen because of its function, which is to recruit and accumulate iron inside the cell. The *E. coli* sequence of the *ftnA* gene was optimized to fit the codon usage of *Synechocystis* sp. PCC6803, and the *ftnA* gene was synthesized by the GenScript Company, America. The *ftnA* gene was cloned in commercially available pUC57, a simple plasmid, amplified with suitable primers, and cleaved by restriction enzymes (NdeI and BamHI). The plasmids, pUC57 and pUC57-simple, were obtained from GenScript (GenScript Inc., USA). The DNA constructs were designed using the MacVector software (MacVector Inc., USA) and synthesized by GenScript Company, America. The primers used are shown in Table 1.

1.1.4. Construction of *Synechocystis* sp. PCC6803-*ftnA*

Synechocystis sp. PCC6803 is a unicellular *Cyanobacterium*, and its complete genome was first characterized in photosynthetic organisms. As shown in Fig. S1, wells #4, #5, and #6 were the agarose gel electrophoresis of the blue marker, the original (not restricted) pFUEL3d1 plasmid, and the restricted pFUEL3d1 plasmid, respectively. The plasmid pFUEL3d1 (4969 bp) was cut with the restriction enzymes NdeI and BamHI. The restriction should have yielded fragments of 4940 and 29 bp.

Table 1 – Tm and sequence of different primers.

Primer	Sequence (5'–3')	Tm (°C)
ftn-F1	CAAGGCGATTAAGTTGGGTAACG	56.2
ftn-mid-R1	AATCAAACAGACGCTGCATGTGCG	58.4
ftn-mid-F2	CGCACATGCAGCGTCTGTTGATT	58.4
ftn-R2	GAGCGGATAACAATTTACACAGG	56.3

ftn-F1/ftn-mid-R1 expected product size: 282 bp, Ta = 55°C; ftm-mid-F2/ftn-R2 expected product size: 444 bp, Ta = 55°C; ftm-F1/ftn-R2 expected product size: 702 bp, Tm for the overlapping fragment is 60.7°C, Ta = 55°C; expected size of the restricted fragment: ~500 bp.

Tm: melting temperature, Ta: annealing temperature.

The 29 bp fragment was too small to visualize on the 1.2% gel, but a change in the plasmid topology was observed. A circular plasmid runs faster than linearized DNA during gel electrophoresis. A circular plasmid can also be found in several topological states, e.g., super-coiled, coiled, and relaxed, and the plasmid is visualized on a gel as several (up to three) bands. These bands were observed in the first well (#5), whereas in the second well (#6) containing the restricted plasmid, we observed only one band that had a size that was between the sizes of the bands in the first well. The restriction worked well, and no remaining original-sized plasmids were observed.

To ligate the plasmid with the insert, the 29 bp fragment must be removed. Otherwise, the fragment can be ligated back into its original position instead of the insert. As shown in Fig. S1, wells #7, #8 and #9 were the agarose gel electrophoresis of the original (not restricted) *ftnA*-containing plasmid, the restricted *ftnA* and the blue marker, respectively. The insert was also restricted. In the third well (#7), we observed the same pattern of a circular plasmid in several topological states (several bands). In the next well, only a single band of the appropriate size (2710 bp) appeared. The two weak bands of 162 and 345 bp were the two portions of the *ftnA* gene.

When we analyzed the sequence of the gene, we found that the *NdeI* enzyme cut the gene at the start of the gene and between 160 and 165 bp, and *BamHI* restricts the gene at its end. We purified these two bands by loading all of the reaction volume into the gel, cutting the two bands out, and purifying them on a column separately. The final concentrations of the *ftnA*-small and *ftnA*-big fragments were 24.3 and 69.7 ng/ μ L, respectively.

Using the natural transformation method, we mixed wild-type *Synechocystis* sp. PCC6803 and the *ftnA* vector, and the mixture was smeared onto a microporous membrane that was placed in a solid plate containing a certain concentration of Km. After approximately one week, the two pieces of the membrane containing the *ftnA* vector algae showed growth, and wild-type algae died under the Km stress. The mutant strains that were taken from Petri dish BG11 (25 μ g/mL Km) were inoculated with the new culture medium. This was repeated three times. Then, the strains were transferred to BG11 Petri dishes (50 μ g/mL Km), and the procedure was repeated three times. Genomic DNA of wild-type *Synechocystis* sp. PCC6803 and transgenic *Synechocystis* sp. PCC6803-*ftnA* were used as the templates, and the primers, *PsbA5f* (AACCGCCAGGTAAACTCTTCTCAAC) and *PsbA3r* (CTTTATCCGCCGGCACAGGTCTTC), were synthesized according to the upstream and downstream sequences of the ferritin iron storage protein gene and were used for PCR amplification. Fig. S2 shows confirmation at the molecular level that the *ftnA* gene was successfully integrated into the *Synechocystis* sp. PCC6803 genomic DNA.

1.1.5. PCR

Regular and colony PCR were carried out according to standard protocols (Abe et al., 2014; Savakis et al., 2013; Tillich et al., 2014).

1.2. Study of the application of *Synechocystis* sp. PCC6803-*ftnA* in microbial contaminant control

1.2.1. MW composition and pre-treatment

In the present study, municipal wastewater was obtained from a local conventional activated sludge plant. The water

was autoclaved at 121°C for 20 min and then stored at 1°C until use. The primary MW components and their respective concentrations (mg/L) were as follows: total phosphorus (TP) 2.0, total nitrogen (TN) 40.0, chemical oxygen demand (COD) 210.0, total suspended solid (TSS) 0.02, Al < 0.01, Ca 28.4, Cu < 0.01, Fe 0.026, K 9.8, Mg 4.5, Mn 0.07, Na 17.8, Ni < 0.01, and Zn < 0.01. The initial pH, which was adjusted using 1 mol/L hydrochloride or sodium hydroxide solution, was between 7.0–7.5.

1.2.2. Isolation and screening of microbial contaminants

We isolated *Scenedesmus obliquus* and *Bacillus* from the MW. Inoculations were performed under sterile conditions, and these contaminants were cultivated in autoclaved SE medium and LB medium, respectively. The cultures were then placed in a light incubator (GZX-300BS-III, CIMO Medical Instrument, Shanghai, China). The cultivation conditions were as follows: light intensity 27 μ mol photons/(m²-sec), light 14 hr/dark 10 hr, and temperature (25 \pm 0.5)°C.

1.2.3. Experimental design

The cultures of *Synechocystis* sp. PCC6803-*ftnA* were sampled at the end of the exponential growth phase. (1) The effects of different iron compounds on the iron uptake of *Synechocystis* sp. PCC6803-*ftnA* cells: Based on referenced studies and preliminary experiments, different iron compounds (Fe²⁺, Fe³⁺, Fe²⁺-EDTA, Fe³⁺-EDTA) at concentrations of 50 μ mol/L were added to a stirred cell suspension (BG11 medium, magnetic stirrer, 100 r/min). The fixation time was sampled, and we determined the iron content of the algal cells. (2) The effects of pulse mode on the iron uptake of *Synechocystis* sp. PCC6803-*ftnA* cells: To investigate the effects of pulse mode on the uptake of iron by *Synechocystis* sp. PCC6803-*ftnA*, three Fe³⁺-EDTA pulse modes were designed, as shown in Table 2. The fixation time was sampled, and we determined the iron content of the algal cells.

The optimal concentration of the pulsed iron compound and the pulse mode from Experiments (1) and (2) were applied in Experiment (3). (3) Investigation of microbial contaminant control: In this experiment, *S. obliquus* and *Bacillus* isolated from the MW (Section 1.2.2) were used as representative microbial contaminants. These contaminants were cultured in the MW with *Synechocystis* sp. PCC6803-*ftnA* that had taken up an adequate amount of iron. Approximately 10 mL of algal inocula were centrifuged (5074 \times g, 5 min at 4°C), and the pellets were washed twice with 15 mg/L NaHCO₃ solution. The inocula were then re-suspended and inoculated into 100 mL MW. Initially, the inoculation number of *Synechocystis* sp. PCC6803-*ftnA*: *S. obliquus*: *Bacillus* were 7:1.5:1.5. The cultivation conditions were as follows: light intensity 55–60 μ mol photons/(m²-sec), light 14 hr/dark 10 hr and temperature 30°C. Every 24 hr, samples were collected from each experimental

Table 2 – Three Fe³⁺-EDTA pulse modes (pH 8).

	Pulse concentration (mol/L)	Frequency (n)	Pulse time (min)
Pulse 1	45	1	0
Pulse 2	22.5	2	0, 10
Pulse 3	15	3	0, 6, 12

group, and the amount of algae was determined. The algal growth, TN and TP of the MW were assayed.

1.2.4. Analytical methods

1.2.4.1. Biomass determination. The microalgae biomass was determined in triplicate by collecting 1 mL of the algae suspension, centrifuging at $5074\times g$ and 4°C for 10 min, and drying the pellet to a constant weight at 65°C . Triplicate blanks of each sample were treated in the same manner (Zhang et al., 2012). The cell numbers were counted under a microscope using a hemocytometer.

1.2.4.2. Lipid content determination. Total lipids were extracted with chloroform: methanol (1:1, V/V) and were quantified gravimetrically (Zhang et al., 2014).

1.2.4.3. TN, TP, and COD_{Cr} measurement. For the water quality measurement, the algal culture was centrifuged ($10,355\times g$ for 10 min at 4°C), and the supernatant that was obtained was subsequently filtered through a $0.45\text{-}\mu\text{m}$ membrane. The permeation was analyzed for TN and TP. All of the measurements were processed according to standard methods (Zhang et al., 2012).

1.2.4.4. Iron determination. For this assay, we quantified the intracellular iron content using a Varian spectrAA 220FS type flame atomic absorption spectrophotometer (Varian, America) based on the following method: Determination of iron, magnesium and manganese in foods (China, 2003).

The intracellular iron absorption rate (R_a , $\text{mg}/(\text{g}\cdot\text{min})$) and absorptivity (A , %) were calculated using Eqs. (1) and (2), respectively.

$$R_a = C_t - \frac{C_{t'}}{\Delta t} \quad (1)$$

$$A = \frac{1.6 \times 100 \times (C_t - C_{t'})}{56 \times S_p} \quad (2)$$

where, C_t (mg/g) is the concentration of intracellular iron at time t (min), $C_{t'}$ (mg/g) is the concentration of intracellular iron at time t' (min), Δt is $t' - t$, and S_p (mg/g) is the total content of the $\text{Fe}(\text{III})$ pulse at time t (min).

1.2.4.5. Reproducibility and statistical analyses. The reproducibility of the results was confirmed with multiple independent cultures. All of the experiments were carried out in triplicates, the measured values are expressed as the mean \pm standard deviation (SD) with $n = 3$.

2. Results and discussion

2.1. Effects of different iron compounds on iron uptake by algal cells

Firstly, we successfully constructed *ftnA* vector by integrating *ftnA* gene into pFUEL3d1 plasmid (Fig. S1). By adopting the natural transformation method, we were able to construct

transgenic *Synechocystis* sp. PCC6803-*ftnA* strain, which was confirmed by PCR (Fig. S2). We grew that transgenic *Synechocystis* in the BG11 Petri dish containing $50\ \mu\text{g}/\text{mL}$ Km. As we expected, *Synechocystis* sp. PCC6803-*ftnA* was able to survive under kanamycin stress, since the pFUEL3d1-*ftnA* plasmid carries Km resistance gene (Fig. 3). It demonstrated that homologous recombination occurred between the upstream and downstream fragment of the *ftnA* vector and the homologous sequences from the *Synechocystis* sp. PCC6803 chromosome to construct the exogenous *ftnA* gene that was integrated into the chromosome of *Synechocystis* sp. PCC6803. Fig. 1 also shows the differences in the iron uptake of *Synechocystis* sp. PCC6803-*ftnA* cells when four iron sources (Fe^{2+} , Fe^{3+} , $\text{Fe}^{2+}\text{-EDTA}$, and $\text{Fe}^{3+}\text{-EDTA}$) were pulsed separately. As shown in the figure, when Fe^{2+} and Fe^{3+} were used as the iron source, the iron content in the algal cells became constant after pulsing for 20 min. When $\text{Fe}^{2+}\text{-EDTA}$ and $\text{Fe}^{3+}\text{-EDTA}$ were used as the iron source and after pulsing for 30 min, the iron content in the algal cells became stable. However, when a chelate was used as the iron source, *Synechocystis* sp. PCC6803-*ftnA* required more pulsing time than when the ion form was used as the iron source. The iron content in the cells was higher when $\text{Fe}^{3+}\text{-EDTA}$ was used as the iron source.

2.2. Effects of the $\text{Fe}^{3+}\text{-EDTA}$ pulse mode on intracellular iron content

Iron has unique chemical properties; it can coordinate reactive oxygen species and participate in electron transfer and metabolism via redox reactions. Iron is an essential element for almost all microbes, plants, and animals. The iron content of the earth's crust is quite rich; however, Fe^{3+} solubility is low, especially in an aerobic environment. Ferritin is an essential protein for electron transfer during photosynthesis and respiration, and it directly participates in nitrate and nitrite reduction, fixation of nitrogen, chlorophyll synthesis and many other biosynthesis and degradation processes. Studies have shown that (Castruita et al., 2008; Sugie and Taniguchi, 2011) when iron penetrates into algal cells, it is not completely

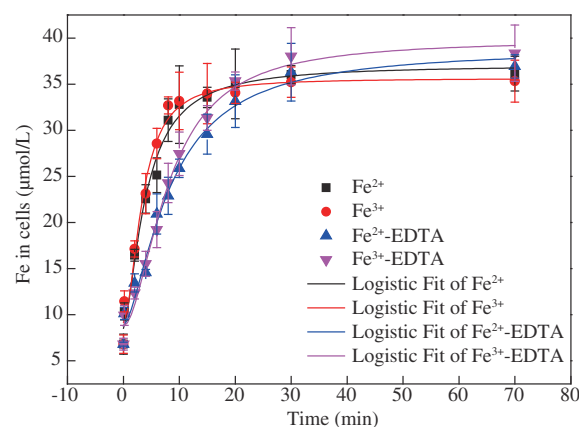


Fig. 1 – Effects of different iron sources on the iron uptake by *Synechocystis* sp. PCC6803-*ftnA* cells.

utilized immediately by the microalgae. Some iron is stored for regulating the metabolism of the algal cells. *ftnA*, is an exogenous gene that can be transformed into *Synechocystis* sp. PCC6803 in algal cells, and it can greatly improve iron uptake. Fe^{3+} -EDTA-pulsed wild type *Synechocystis* sp. PCC6803 and transgenic *Synechocystis* sp. PCC6803-*ftnA* were compared for their intracellular iron content, as shown in Fig. 2. The intracellular iron content of the two algal cells became stable after pulsing for 30 min. However, the intracellular iron content in *Synechocystis* sp. PCC6803-*ftnA* was higher than in *Synechocystis* sp. PCC6803, and the difference was significant. After 70 min, the intracellular iron content of the two species was 38.35 and 17.93 $\mu\text{mol/L}$, respectively, and the content in the transgenic algae was 2.14 times the content of the wild-type.

We studied the iron uptake of algal cells using three types of Fe^{3+} -EDTA pulse modes (Fig. 3). Overall, the iron uptake amount in the algal cells with Pulse 2 and Pulse 3 was higher than with Pulse 1; Pulse 2 and Pulse 3 were stable 30 min after the first pulse. There was no significant difference in the uptake efficiency; thus, we used Pulse 2 for the following experiments.

2.3. Application research of *Synechocystis* sp. PCC6803-*ftnA* in microbial contaminant control

Exploring a novel method of microbial contamination control in a coupling system of microalgae production and waste water treatment was the purpose of this study. MW was used in the experiment, and its iron content was approximately 0.3 mol/L , which can be regarded as an iron-free culture environment. *S. obliquus* and *Bacillus* were isolated from MW and were used as the representative miscellaneous algae and miscellaneous bacteria, respectively.

Iron is one of the nutritional elements necessary for microalgal growth, and it has an important role in electron transport and enzymatic reactions during photosynthesis, respiration, nitrogen fixation, protein and nucleic acid synthesis and other physiological metabolic processes (Fujii et al., 2005). Studies have shown that when microalgae in the environment have an iron deficiency, the iron that is stored

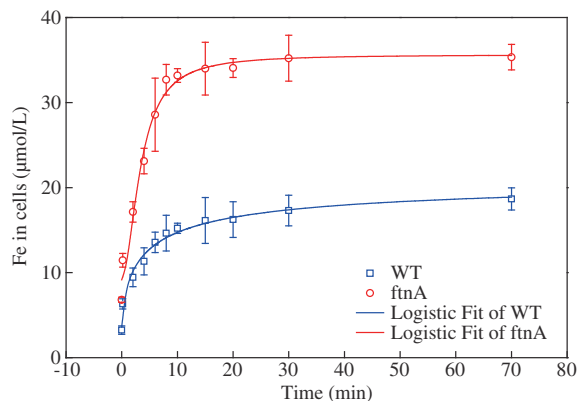


Fig. 2 – Comparison of the intracellular iron content between *Synechocystis* sp. PCC6803 and *Synechocystis* sp. PCC6803-*ftnA*. WT: wild type *Synechocystis* sp. PCC6803; *ftnA*: *Synechocystis* sp. PCC6803-*ftnA*.

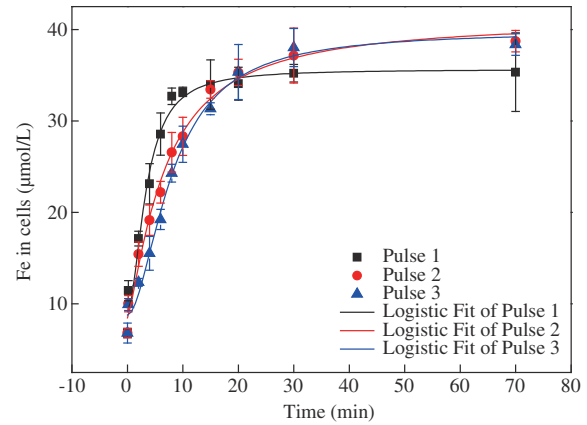


Fig. 3 – Effect of the pulse Fe^{3+} -EDTA mode on the intracellular Fe content and absorptivity of intracellular iron by *Scenedesmus obliquus*.

in ferritin can be used for algal growth (Fujii et al., 2005; Lis et al., 2014; Zha et al., 2012). Fig. 4 displayed the biomass changes between four experimental groups and four control groups. The biomass of the experimental group was significantly higher than the control group. These results revealed that *Synechocystis* sp. PCC6803-*ftnA* can utilize the iron that is stored in its cells for growth and reproduction in an iron-free culture. However, the wild-type *Synechocystis* sp. PCC6803, *S. obliquus* and *Bacillus* did not have excess iron storage, and the low-iron environment was detrimental to cell growth. Thus, the iron deficiency limited the conventional microbial cells. The lipid content of the microorganisms in the experimental group and the control group after a 10-day incubation is shown in Fig. 5. Overall, the total lipid content of the four experimental groups was significantly higher than in the four

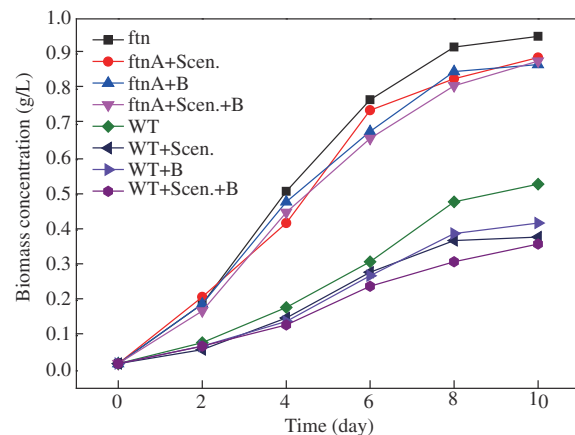


Fig. 4 – Changes in the biomass for different treatment groups and the control group. *ftnA*, *ftnA* + *Scen.*, *ftnA* + *Scen.* + *B*, et al. stand for the biological species in the culture system at the initial time. *ftnA*: *Synechocystis* sp. PCC6803-*ftnA*; WT: *Synechocystis* sp. PCC6803; *Scen.*: *Scenedesmus obliquus* isolated from MW; *B*: *Bacillus* isolated from MW.

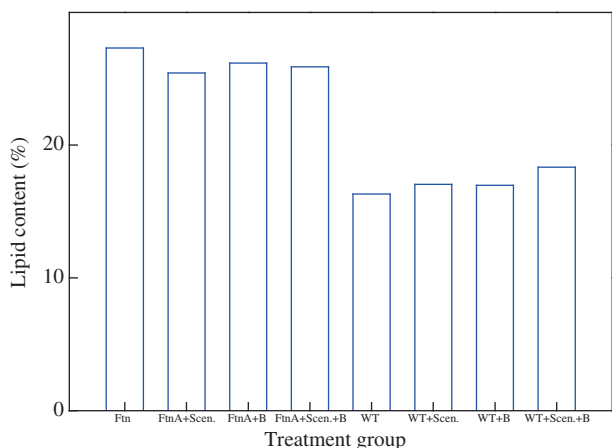


Fig. 5 – Changes in the lipid content for different treatment groups and the control group.

control groups, which indicates that iron is not only conducive to increased microbial biomass, but it also promotes the accumulation of fat in microbial cells.

Two cases of WT + Scen. + B. in the experimental group and ftnA + Scen. + B. in the control group were further studied. At the initial stage of the culture, the inoculation number of *Synechocystis* sp. PCC6803-ftnA: *Synechocystis* sp. PCC6803: bacteria in the culture system were approximately 7:1.5:1.5, respectively. After 10 days of incubation, changes in the proportion of each microbe in the culture system were observed, as shown in Fig. 6. Few changes in the proportion of each microbe in the control group were observed. However, the proportion in the three experimental groups varied significantly. Initially, *Synechocystis* sp. PCC6803-ftnA accounted for 70% all of the microorganisms but increased significantly to 95% to become the dominant species in the system. The corresponding biomass and TN/TP removal efficiency were 0.7 g/L and >99%,

respectively. The proportions of *S. obliquus* and bacteria decreased, which showed that *Synechocystis* sp. PCC6803-ftnA used iron that it stored by itself to grow well in the low-iron culture medium. The other miscellaneous algae and bacteria grew poorly because of the iron limitation. We thus achieved non-target microbial control during the entire process of the culture.

Iron primarily exists in the form of dissolved iron, colloidal iron, and iron particles in water. Among these forms, dissolved Fe^{2+} and Fe^{3+} are bio-available, and the other forms must transform into dissolved iron to be available. Some research on iron uptake by microalgae has been performed. Castruita et al. (2008) found that when Fe enters an algal cell, some of the iron can be stored in ferritin or a similar protein and is used by the algal cell immediately. Under low-iron or iron deficient conditions, these proteins can be used by microalgae to adjust metabolic processes when exogenous bio-available iron is not sufficient. The capacity to take up iron varies in different algae species. *Diatom*, *Trichodesmium*, and *Microcystis aeruginosa* have the capacity to take up and store iron. As an exogenous gene for an iron storage protein, ftnA can be recombined with *Synechocystis* sp. PCC6803, which increases this microbe's capacity to store iron compared with the wild type. Hopkinson and Morel (2009) found that most microalgae possess iron carriers and the ability to chelate iron. However, instead of directly using chelated iron or an iron carrier to maintain photosynthesis and respiration, microalgae reduce iron to zero-iron. Zero-iron is then absorbed into the algal cell. Fujii et al. (2010) found that *M. aeruginosa* can secrete a type of extracellular superoxide that is an iron reducing agent, which can reduce the organic or inorganic state of Fe^{3+} to promote the absorption of iron. When there is a strong iron chelating agent, unchelated Fe^{2+} is produced by the photochemical effects of Fe^{2+} and is absorbed by the microalgae.

This study was carried out as a small-scale laboratory experiment. The results from the research provide theoretical guidance for practical application. However, the biomass of

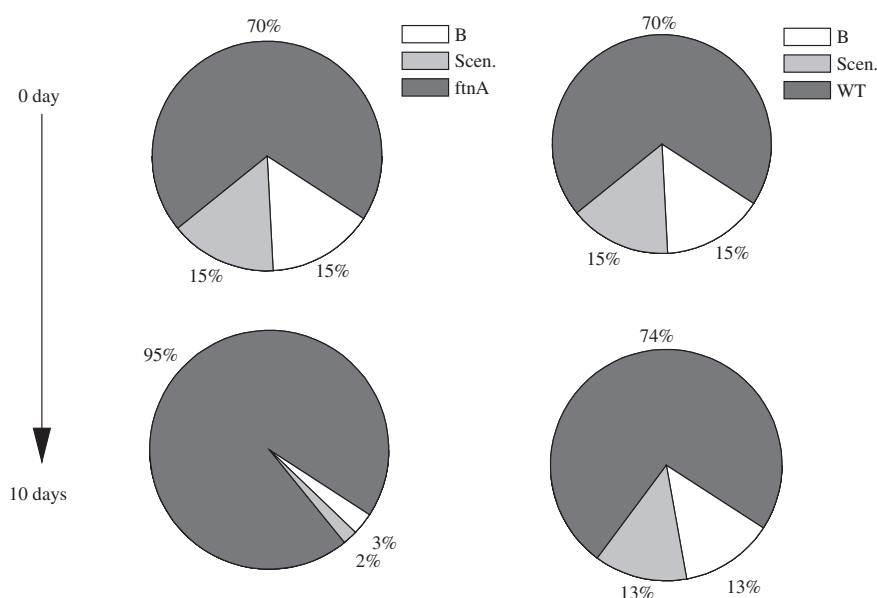


Fig. 6 – Changes in the microbial composition of the culture system.

the *Synechocystis* sp. PCC6803-ftnA cultured in MW was only approximately 0.7 g/L. According to a preliminary study by the authors, supplementing proper nutrients could be a strategy for enhancing algal biomass growth in the coupling system. Thus, it is desirable to identify the added elements that significantly affect the growth, nutrient uptake and lipid accumulation properties of *Synechocystis* sp. PCC6803-ftnA. Constant adjustments and further optimization in practical applications will be subjects of future research.

3. Conclusions

The engineered *Synechocystis* sp. PCC6803-ftnA was completely constructed. Compared with the wild type, the iron uptake ability of *Synechocystis* sp. PCC6803-ftnA under Fe-EDTA pulse conditions was significantly enhanced, and the exogenous genes reached a good expression level. In the coupling system of *Synechocystis* sp. PCC6803-ftnA production and MW treatment, the stored iron in the algal cells was used for growth and reproduction. *Synechocystis* sp. PCC6803-ftnA gradually became the dominant species, and the growth of other microbes was inhibited; these results provide a new method for microbial contamination control during a coupling process.

Acknowledgments

This work was supported by the National Key Technologies R&D Program of China (No. 2012BAJ25B02). We also very much appreciate the critical and constructive comments of the anonymous reviewers who have improved this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jes.2016.01.021>.

REFERENCES

- Abe, K., Miyake, K., Nakamura, M., Kojima, K., Ferri, S., Ikebukuro, K., et al., 2014. Engineering of a green-light inducible gene expression system in *Synechocystis* sp. PCC6803. *Microb. Biotechnol.* 7, 177–183.
- Aravantinou, A.F., Theodorakopoulos, M.A., Manariotis, I.D., 2013. Selection of microalgae for wastewater treatment and potential lipids production. *Bioresour. Technol.* 147, 130–134.
- Castruita, M., Shaked, Y., Elmegreen, L.A., Stiefel, E.I., Morel, F., 2008. Availability of iron from iron-storage proteins to marine phytoplankton. *Limnol. Oceanogr.* 53, 890–899.
- Chai, C., Yin, X.D., Ge, W., Wang, J.W., 2013. Effects of nitrogen and phosphorus concentrations on the bioaccumulation of polybrominated diphenyl ethers by *Prorocentrum donghaiense*. *J. Environ. Sci.* 25 (2), 376–385.
- China, N.S.F.T., 2003. Determination of iron, magnesium and manganese in foods (GB 1990–12396).
- Edwards, A.M., Platt, T., Sathyendranath, S., 2004. The high-nutrient, low-chlorophyll regime of the ocean: Limits on biomass and nitrate before and after iron enrichment. *Ecol. Model.* 171, 103–125.
- Fujii, M., Rose, A.L., Omura, T., Waite, T.D., 2010. Effect of Fe(II) and Fe(III) transformation kinetics on iron acquisition by a toxic strain of *Microcystis aeruginosa*. *Environ. Sci. Technol.* 44, 1980–1986.
- Fujii, M., Yoshie, N., Yamanaka, Y., Chai, F., 2005. Simulated biogeochemical responses to iron enrichments in three high nutrient, low chlorophyll (HNLC) regions. *Prog. Oceanogr.* 64, 307–324.
- Guo, Z., Liu, Y., Guo, H.Y., Yan, S., Mu, J., 2013. Microalgae cultivation using an aquaculture wastewater as growth medium for biomass and biofuel production. *J. Environ. Sci.* 25, S85–S88.
- Hopkinson, B.M., Morel, F., 2009. The role of siderophores in iron acquisition by photosynthetic marine microorganisms. *Biometals* 22, 659–669.
- Lam, M.K., Lee, K.T., 2012. Microalgae biofuels: A critical review of issues, problems and the way forward. *Biotechnol. Adv.* 30, 673–690.
- Lis, H., Shaked, Y., Kranzler, C., Keren, N., Morel, F.M., 2014. Iron bioavailability to phytoplankton: An empirical approach. *ISME J.* 9, 1003–1013.
- Markou, G., Vandamme, D., Muylaert, K., 2014. Microalgal and cyanobacterial cultivation: The supply of nutrients. *Water Res.* 65, 186–202.
- Mongin, M., Matear, R., Chamberlain, M., 2011. Simulation of chlorophyll and iron supplies in the Sub Antarctic Zone South of Australia. *Deep-Sea Res. II Top. Stud. Oceanogr.* 58, 2126–2134.
- Parmar, A., Singh, N.K., Pandey, A., Gnansounou, E., Madamwar, D., 2011. Cyanobacteria and microalgae: A positive prospect for biofuels. *Bioresour. Technol.* 102, 10163–10172.
- Petrou, K., Hassler, C.S., Doblin, M.A., Shelly, K., Schoemann, V., van den Enden, R., et al., 2011. Iron-limitation and high light stress on phytoplankton populations from the Australian Sub-Antarctic Zone (SAZ). *Deep-Sea Res. II Top. Stud. Oceanogr.* 58, 2200–2211.
- Sacristan, D.A.M., Luna-Pabello, V.M., Cadena, E., Ortiz, E., 2013. Green microalga *Scenedesmus acutus* grown on municipal wastewater to couple nutrient removal with lipid accumulation for biodiesel production. *Bioresour. Technol.* 146, 744–748.
- Savakis, P.E., Angermayr, S.A., Hellingwerf, K.J., 2013. Synthesis of 2,3-butanediol by *Synechocystis* sp. PCC6803 via heterologous expression of a catabolic pathway from lactic acid- and enterobacteria. *Metab. Eng.* 20, 121–130.
- Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., et al., 2010. Biodiesel from algae: Challenges and prospects. *Curr. Opin. Biotechnol.* 21, 277–286.
- Stephenson, A.L., Kazamia, E., Dennis, J.S., Howe, C.J., Scott, S.A., Smith, A.G., 2010. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: A comparison of raceways and air-lift tubular bioreactors. *Energy Fuel* 24, 4062–4077.
- Sugie, K., Taniguchi, A., 2011. Continuous supply of bioavailable iron for marine diatoms from steelmaking slag. *ISIJ Int.* 51, 513–520.
- Sutherland, D.L., Turnbull, M.H., Broady, P.A., Craggs, R.J., 2014. Effects of two different nutrient loads on microalgal production, nutrient removal and photosynthetic efficiency in pilot-scale wastewater high rate algal ponds. *Water Res.* 66, 53–62.
- Tillich, U.M., Wolter, N., Franke, P., Duhring, U., Frohme, M., 2014. Screening and genetic characterization of thermo-tolerant *Synechocystis* sp. PCC6803 strains created by adaptive evolution. *BMC Biotechnol.* 14, 66,1.
- Torres, R., Ampuero, P., 2009. Strong CO₂ outgassing from high nutrient low chlorophyll coastal waters off central Chile (30S): The role of dissolved iron. *Estuar. Coast. Shelf Sci.* 83, 126–132.
- Van der Woude, A.D., Angermayr, S.A., Puthan, V.V., Osnato, A., Hellingwerf, K.J., 2014. Carbon sink removal: Increased

- photosynthetic production of lactic acid by *Synechocystis* sp. PCC6803 in a glycogen storage mutant. *J. Biotechnol.* 184, 100–102.
- Watanabe, S., Shiwa, Y., Itaya, M., Yoshikawa, H., 2012. Complete sequence of the first chimera genome constructed by cloning the whole genome of *Synechocystis* strain PCC6803 into the *Bacillus subtilis* 168 genome. *J. Bacteriol.* 194, 7007.
- Zha, S.H., Xu, X.D., Hu, H.H., 2012. A high sensitivity iron-dependent bioreporter used to measure iron bioavailability in freshwaters. *FEMS Microbiol. Lett.* 334, 135–142.
- Zhang, C.M., Zhang, Y.L., Zhuang, B.L., Zhou, X.F., 2014. Strategic enhancement of algal biomass, nutrient uptake and lipid through statistical optimization of nutrient supplementation in coupling *Scenedesmus obliquus*-like microalgae cultivation and municipal wastewater treatment. *Bioresour. Technol.* 171, 71–79.
- Zhang, Y.L., Su, H.Y., Zhong, Y.N., Zhang, C.M., Shen, Z., Sang, W.J., et al., 2012. The effect of bacterial contamination on the heterotrophic cultivation of *Chlorella pyrenoidosa* in wastewater from the production of soybean products. *Water Res.* 46, 5509–5516.
- Zhao, F., Su, Y., Tan, X., Chu, H., Zhang, Y., Yang, L., et al., 2015a. Effect of temperature on extracellular organic matter (EOM) of *Chlorella pyrenoidosa* and effect of EOM on irreversible membrane fouling. *Colloids Surf. B Biointerfaces* 136, 431–439.
- Zhao, F., Tan, X., Zhang, Y., Chu, H., Yang, L., Zhou, X., 2015b. Effect of temperature on the conversion ratio of glucose to *Chlorella pyrenoidosa* cells: Reducing the cost of cultivation. *Algal Res.* 12, 431–435.
- Zhong, Y.N., Zhang, Y.L., 2013. Influence of contaminative bacteria during cultivation of *Chlorella pyrenoidosa* in soybean processing wastewater. *Adv. Mater. Res.* 666, 27–31.
- Zhu, L., Wang, Z., Shu, Q., Takala, J., Hiltunen, E., Feng, P., et al., 2013. Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. *Water Resour.* 47, 4294–4302.