Trophic Mode Conversion and Nitrogen Deprivation of Microalgae for High Ammonium
Removal from Synthetic Wastewater

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Abstract

In this study, a well-controlled three-stage process was proposed for high ammonium removal from synthetic wastewater using selected promising microalgal strain UMN266. Three trophic modes (photoautotrophy, heterotrophy, and mixotrophy), two N sufficiency conditions (N sufficient and N deprived), two inoculum modes (photoautotrophic and heterotrophic), and different $\text{NH}_4^+\text{-N}$ concentrations were

compared to investigate the effect of trophic mode conversion and N deprivation on high $\text{NH}_4^+\text{-N}$ removal by UMN266. Results showed that photoautotrophic inoculum with trophic mode conversion from heterotrophy to photoautotrophy and N deprivation in Stage 2 turned was the optimum plan for $\text{NH}_4^+\text{-N}$ removal, and average removal rates were 12.4 mg/L/d and 19.1 mg/L/d with initial $\text{NH}_4^+\text{-N}$ of 80 mg/L and 160 mg/L in Stage 3. Mechanism investigations based on algal biomass carbon (C) and N content, cellular composition, and starch content confirmed the above optimum plan and potential of UMN266 as bioethanol feedstock.

Keywords: Trophic mode conversion, Nitrogen deprivation, Ammonium removal, Microalgae, Nitrogen starvation.

1. Introduction

Ammonium ($\text{NH}_4^+\text{-N}$) removal is one of the most challenging tasks in worldwide wastewater treatment, since various agro-industrial sections generate volumes of sewage with high $\text{NH}_4^+\text{-N}$ concentrations, such as livestock wastewater, landfill leachate, and fertilizer wastewater, causing eutrophication, odor, groundwater contamination, soil degradation, etc. (Wiszniewski et al., 2006; Cai et al., 2013). $\text{NH}_4^+\text{-N}$ could be removed through multiple physicochemical and biological ways, among which biological methods have been of great interest and widely studied in the past few decades (Parker and Others, 1975; Lau et al., 1995; Posadas et al., 2013). Activated sludge is the most widely adopted biological treatment process, removing $\text{NH}_4^+\text{-N}$ through nitrification-denitrification, requiring high cost in aeration, but with $\text{NH}_4^+\text{-N}$ removal seldom satisfactory for direct discharge (Godos et al., 2009). To overcome this,

microalgae-based systems were introduced (Ogbonna et al., 2000; Ji et al., 2012). As the most readily uptake form of nitrogen source for microalgae (Cai et al., 2013), $\text{NH}_4^+\text{-N}$ could be better removed in microalgae-based systems than in bacteria-based activated sludge systems (Posadas et al., 2013). Principles of $\text{NH}_4^+\text{-N}$ removal in these systems are 1) O_2 produced through algal photosynthesis facilitates nitrification-denitrification; 2) pH increase caused by photosynthesis stimulates ammonia (NH_3) volatilization; 3) intensified NH_3 volatilization with system agitation (known as air-stripping effect); 4) uptake of $\text{NH}_4^+\text{-N}$ by microalgal cells. Assimilation of $\text{NH}_4^+\text{-N}$ by algal cells constitutes only a fraction of overall $\text{NH}_4^+\text{-N}$ removal in such systems (Voltolina et al., 1998; Godos et al., 2009), leading to volume NH_3 discharge and possible air pollution. To monitor the net $\text{NH}_4^+\text{-N}$ removal by algal assimilation, a well-controlled process using synthetic wastewater needs to be adopted to rule out influences of nitrification-denitrification, elevated pH, and NH_3 volatilization.

Effects of nitrogen starvation/deprivation on microalgal growth have been widely studied, mostly with the purpose of biofuel production (Hsieh and Wu, 2009; Wang et al., 2013; Griffiths et al., 2014). Generally, under N starvation/deprivation, synthesis of N-rich constituents necessary for cell growth and division is restrained significantly, leading to reduced or even ceased algal growth. Meanwhile, metabolic pathway of carbon assimilation diverts from protein synthesis to lipid or carbohydrate production as carbon and energy storage (Griffiths et al., 2014). While previous research focused on how N starvation/deprivation affects biomass composition and algal growth, few studied the response of algal growth and nutrient uptake after recovering from N

starvation/deprivation. Tendency to uptake more $\text{NH}_4^+\text{-N}$ after recovery from N starvation/deprivation has been reported among higher plants and marine phytoplankton (Dortch et al., 1982; Lee and Rudge, 1986), but not yet among freshwater microalgae.

Studies showed that higher inoculation of microalgae into wastewater favors $\text{NH}_4^+\text{-N}$ removal, owing much to better shock load adjustment (Lau et al., 1995). To achieve higher inoculation, more algal biomass should be readily obtained. Among the three major modes of microalgae cultivation, i.e., photoautotrophy (with light as energy source and inorganic carbon as carbon source), heterotrophy (with organic compounds as both energy source and carbon source), and mixotrophy (able to use both light and organic compounds as energy source, and both inorganic and organic carbon as carbon source), heterotrophy and mixotrophy generally display much higher biomass yield than traditional photoautotrophy (Wang et al., 2014). Thus, application of heterotrophically or mixotrophically cultivated microalgae as “seeds” should enhance $\text{NH}_4^+\text{-N}$ removal.

Constituents such as proteins, nucleic acids, and chlorophylls, are the N pools of microalgal cells, most of which are photosynthesis related therefore light dependent. Our hypothesis is that the trophic mode conversion from heterotrophy or mixotrophy to photoautotrophy would trigger more $\text{NH}_4^+\text{-N}$ uptake, since exclusive photoautotrophic growth would contribute to the synthesis of more light dependent constituents. This paper therefore, aims to investigate high concentrations of $\text{NH}_4^+\text{-N}$ removal in a well-controlled three-stage process using synthetic wastewater. Stage 1 aims to heterotrophically or mixotrophically accumulate as much as possible algal biomass, followed by 48 h N deprivation in Stage 2, and photoautotrophic cultivation with high

$\text{NH}_4^+\text{-N}$ concentrations in Stage 3. One indigenous freshwater strain tolerant of high $\text{NH}_4^+\text{-N}$ concentration and could grow well in heterotrophy was selected and identified out of ten microalgal strains. Three trophic modes, two N sufficiency conditions in Stage 2, and different $\text{NH}_4^+\text{-N}$ concentrations in Stage 3 were compared, to investigate the effects of trophic mode conversion, N deprivation and $\text{NH}_4^+\text{-N}$ concentrations on $\text{NH}_4^+\text{-N}$ removal thus $\text{NH}_4^+\text{-N}$ -rich wastewater treatment. Photoautotrophic and heterotrophic modes of inoculation were also compared to investigate the effect of inoculum trophic mode on $\text{NH}_4^+\text{-N}$ removal. This paper could serve as a first detailed study about the influence of N deprivation and trophic mode conversion on $\text{NH}_4^+\text{-N}$ removal by locally isolated freshwater microalgae, offering useful information for $\text{NH}_4^+\text{-N}$ -rich wastewater treatment.

2. Material and Methods

2.1. Algal strains and growth medium

10 strains were used in the study, 8 of which were isolated in local water bodies, named as UMN230, UMN244, UMN258, UMN266, UMN268, UMN270, UMN271, UMN285 (Zhou et al., 2011); 2 other strains, UTEX78 and UTEX2714, were purchased from the Culture Collection of Algae at the University of Texas.

Algal strains were cultivated in autoclaved synthetic wastewater according to Perez-Garcia et al. (2010) with some modifications (mg/L): NaCl, 7; CaCl_2 , 4; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 2; K_2HPO_4 , 21.7; KH_2PO_4 , 8.5; Na_2HPO_4 , 25; NH_4Cl , 63 for photoautotrophic stock culture and 315 for heterotrophic stock culture; H_3BO_3 , 0.57; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 0.25; $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 1.1; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.25; $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 0.08;

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.015, and Na_2EDTA , 2.5. HEPES was added as buffer, on the basis of 5 mM for photoautotrophic stock culture (Shi et al., 2007). pH was controlled at 7.0. 10 g/L glucose was added as organic carbon source for heterotrophic stock culture. For heterotrophic stock culture, algal cells in photoautotrophic stock culture were first inoculated onto glucose supplied sterile agar plates, cultivated in dark for 3-4 days, after then microalgal cells on the agar plates were inoculated onto another batch of glucose supplied sterile agar plates, and cultivated in dark for 3-4 days. This step was repeated 3 times to accomplish full adaptation from photoautotrophic to heterotrophic cultivation. Then, algal cells on the plates were inoculated into autoclaved heterotrophic synthetic wastewater, cultivated in dark and kept as heterotrophic stock culture. The stock culture was transferred to fresh autoclaved heterotrophic synthetic wastewater every 3 days, and to prevent bacterial contamination, microscopic examination was conducted before each transfer.

Algal strains were inoculated 10% ($V_{\text{inoculation}}/V_{\text{wastewater}}$) into 100 mL synthetic wastewater in 250 mL Erlenmeyer flasks. The flasks were placed on a shaker with 100 rpm rotation speed. The cultures were kept at $25 \pm 2^\circ\text{C}$ under continuous cool-white fluorescent light with intensity of $70 \mu\text{mol}/(\text{m}^2 \cdot \text{s})$.

2.2. Synthetic wastewater

To rule out influences of nitrification-denitrification, elevated pH, and NH_3 volatilization on $\text{NH}_4^+\text{-N}$ removal, synthetic wastewater was autoclaved throughout this study. The recipe was based on growth medium with some modifications (mg/L): NH_4Cl 252, 504, 755 to get $\text{NH}_4^+\text{-N}$ concentrations of 80, 160, and 240 mg/L; a mixture

of 190 mg/L NaHCO_3 and Na_2CO_3 (C concentration) was added when inorganic carbon sources were included. HEPES was added as buffer and pH was controlled at 7.0.

2.3. Strain selection and identification

2.3.1. Strain selection

10 strains of microalgae were heterotrophically cultivated in 250 mL flasks with 100 mL synthetic wastewater. Strains with significantly higher biomass yield were chosen for further selection using a two-step strategy. In the first step, strains were heterotrophically cultivated for 3 days; then heterotrophically cultivated but deprived of N for 48 h in the second step. Algal biomass was collected through centrifuge and was washed once with N deprived synthetic wastewater before transferred to the second step. Optimum strain based mainly on biomass productivity after step-2 was selected for further investigation. All cultures were carried out in duplicate. The optimum strain was kept in both photoautotrophic and heterotrophic stock cultures.

2.3.2. Strain identification

Genomic DNA of algal cells was extracted using Qiagen DNeasy® Plant Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's instruction with minor modification. Around 100 mg algal paste was placed in a 2.0 mL centrifuge tube after being washed with autoclaved distilled water for 3 times. 200 μL AP1 Buffer and 4 μL RNase A was added and vortexed for 2 min, and the mixture was incubated for 15 min at 65 °C to lyse the cells (mixed 2-3 times during incubation). 130 μL AP3 Buffer was added to the lysate and mixed, then incubated on ice for 10 min to precipitate proteins, polysaccharides, and detergents, and was centrifuged at 13,000 rpm for 5 min. Next, the

lysate was transferred to a QIA shredder spin column, and centrifuged at 13,000 rpm for 2 min. The supernatant was transferred to a new 2.0 mL centrifuge tube, to which 1.5 volumes of AW1 Buffer was added and mixed. This mixture was pipetted to the DNeasy mini spin column, centrifuged at 7,000 rpm for 1 min, then placed in a new 2.0 mL tube with 500 μ L AW2 Buffer added and centrifuged at 7,000 rpm for 1 min. The filtrate was discarded and another 500 μ L AW2 Buffer was added and centrifuged at 13,000 rpm for 2 min to dry the membrane. The column was placed in a new 2.0 mL centrifuge tube with 100 μ L AE Buffer added onto the membrane and incubated at room temperature for 10 min, then centrifuged at 7,000 rpm for 1 min to elute the DNA.

Polymerase chain reaction (PCR) amplification was adopted to amplify the extracted 18S rRNA gene. Three sets of primers (Nozaki et al., 1995; Grzebyk et al., 1998; Auinger et al., 2008) were used to narrow down the identification possibilities (see Table 1). 2 μ L extracted DNA was used as genomic DNA template and added to a PCR tube containing 12.5 μ L mixture of PCR Buffer, dNTP, and Taq polymerase (TaKaRa, Otsu, Japan), 1.25 μ L of each primer, and 8 μ L distilled water, with a total volume of 25 μ L. The mixture was denatured at 94 °C for 5 min. The PCR program was run for 30 cycles (1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C), and 10 min of final extension at 72 °C. PCR product was purified with QiAquick gel extraction kit (Qiagen, Germantown, MD, USA), then sent to Biomedical Genomics Center at University of Minnesota (Saint Paul, MN, USA) for DNA sequencing. The resulting 18S rRNA gene sequences were aligned and compared with the nucleotide sequences of

some known microorganisms in GenBank database of the National Center for Biotechnology Information using Basic Local Alignment Search Tool (BLAST).

2.4. Three-stage process design

The experiment was carried out in three stages. In Stage 1, selected strain was heterotrophically or mixotrophically (with light and CO₂ generated by heterotrophy as inorganic carbon source) cultivated for 3 days; then heterotrophically or mixotrophically cultivated but deprived of N for 48 h in Stage 2; then finally photoautotrophically cultivated in inorganic carbon sources supplied synthetic wastewater for 5 days in the last stage. From Stage 2, photoautotrophic cultures were added as control, transferred into Stage 3 with similar biomass densities as heterotrophic and mixotrophic cultures. For all trophic modes, cultures without N deprivation were used as control. Algal biomass was collected through centrifuge then washed once with target wastewater before transferred to the next stage. All cultures were carried out in triplicate in 250 mL flasks with 100 mL synthetic wastewater. To investigate the influence of inoculum trophic mode on NH₄⁺-N removal, photoautotrophic and heterotrophic inoculum were compared in two separate batches. For photoautotrophic inoculum (photoautotrophic mode of inoculation), microalgal cells in photoautotrophic stock culture were inoculated; while for heterotrophic inoculum (heterotrophic mode of inoculation), microalgal cells in heterotrophic stock culture were inoculated into experimenting cultures. Table 2 outlines the experimental design.

2.5. Growth and chemical analysis

2.5.1. Determination of algal growth

Algal growth was measured daily by total volatile suspended solids (TVSS), representing biomass concentration based on dry weight. 4 mL of algae suspension was used from the flasks according to standard method (APHA, 1995).

2.5.2. Nutrient analysis

1.5 mL of algae suspension was collected daily from each flask for nutrient consumption analysis. The samples were centrifuged at 10,000 rpm for 5 min, then the supernatants were properly diluted and analyzed for NH_4^+ -N concentration, following Hach DR 5000 Spectrophotometer Manual (Hach, 2008). Removal rate (%) was expressed by dividing the difference between the first day and the selected day concentrations by the first day concentration, and then multiplied by 100.

2.5.3. C and N contents in algal biomass

Analysis of C and N contents in algal cells was carried out with an Exeter Analytical CE-440 Elemental Analyzer (Chelmsford, MA). 1.5-2 mg freeze dried algal biomass was used for each sample.

2.5.4. Starch content analysis

Starch content in algal biomass was analyzed with Starch (GO/P) Assay Kit (Sigma-Aldrich, MO, USA). 50-100 mg freeze dried biomass was used for each sample.

2.6. TEM analysis of microalgae

Centrifuged microalgae paste was sent to Imaging Center at University of Minnesota (Saint Paul, MN, USA) for Transmission Electron Microscopy (TEM) analysis. About 100 mg wet biomass was required for each sample. All images were taken at magnification of 11,500 times under 1.00 s exposure.

3. Results and Discussion

3.1. Strain selection and identification

3.1.1 Heterotrophic growth of 10 strains and two-step selection out of top 4 candidates

8 locally isolated and 2 UTEX purchased strains were compared in this study. The two UTEX strains were respectively *Scenedesmus obliquus* and *Chlorella vulgaris*, both being widely reported for growing in wastewaters (Ji et al., 2012; Batista et al., 2015). A majority of indigenous strains were adopted since local strains are usually more subject to environmental stress (Zhou et al., 2011). All 10 strains could grow both photoautotrophically (stock culture) and heterotrophically (Fig.1a-b) in synthetic wastewater, as was consistent with previous results (Perez-Garcia et al., 2010). Since most NH_4^+ -N-rich wastewaters also contain loads of organic pollutants (Wiszniewski et al., 2006), the ability of heterotrophic rather than traditional photoautotrophic growth is advantageous for wastewater purification (Wang et al., 2014). Light was not introduced in strain selection since many NH_4^+ -N-rich sewage are dark colored and with poor light penetration (Zhou et al., 2012). Calculated from the approximate molecular formula of microalgal biomass, i.e. $\text{CO}_{0.48}\text{H}_{1.83}\text{N}_{0.11}\text{P}_{0.01}$ (Chisti, 2007), and based on lab results (data not shown due to figure limits), residual glucose and nutrient levels were far above 0, indicating each strain's capacity to heterotrophically accumulate biomass in synthetic wastewater (Fig. 1a-b). 4 strains of higher biomass concentrations, i.e. UMN266, UMN268, UMN285, and UTEX2714 were chosen for further selection.

Fig. 1c-f displays heterotrophic growth of the 4 top candidates through N sufficient (NS) and N deprived (ND) steps, NS cultures were conducted as control. Synthetic

wastewater as above was introduced throughout cultivation for NS cultures. For ND cultures, same medium was adopted for the first 3 days, then converted to ND synthetic wastewater for 48 h N deprivation. Compared with Fig. 1a-b, biomass concentrations were higher for all top 4 strains, and UMN268 and UMN285 had longer exponential phases, caused probably by higher inoculation in the second round of selection (Lau et al., 1995). For all 4 strains, growth of microalgae became stagnant or even reduced after N deprivation, as in accordance with previous studies (Hsieh and Wu, 2009; Wang et al., 2013; Griffiths et al., 2014). However, for UMN268 and UTEX2714, biomass concentrations dropped to half of their peak values, indicating poor adjustment to N deprivation. Since availability of high biomass after N deprivation was the principle for optimum strain selection, these 2 strains were eliminated for further study. For UMN266 and UMN285, biomass concentrations were comparable and did not drop much after N deprivation. However, based on our lab experiences, UMN285 was prone to bacterial contamination when heterotrophically cultivated, making it hard to determine the exact removal of $\text{NH}_4^+\text{-N}$ by microalgae. Therefore, UMN266, isolated from Kaller Lake, Minnesota, was selected as the promising strain for further investigation.

3.1.2. Identification of UMN266

Comparison among UMN266 18s rRNA gene sequences and GenBank strains is shown in Table 3. Combined with morphological observation, UMN266 was identified as a strain of *Chlorella sorokiniana*. Taxonomic diagram of UMN266 according to NCBI's Taxonomy Report is displayed in Fig. S1. As a species from *Chlorella* genus,

Chlorella sorokiniana was reported as a robust strain capable of mixotrophic, heterotrophic, and photoautotrophic growth (Ogbonna et al., 2000; Chen et al., 2011) and could grow well in various wastewater streams (Godos et al., 2009; Zhou et al., 2011), selection results of this study is in accordance with previous research.

3.2. Growth and $\text{NH}_4^+\text{-N}$ removal of UMN266 in three-stage treatment process

To rule out influences of pH on algal growth and $\text{NH}_4^+\text{-N}$ removal, HEPES was added as buffer, with pH adjusted to 7.0 before inoculation, and kept 6.4-7.0 in Stage 1 and Stage 2, and 7.0-7.5 in Stage 3 (data not shown). To rule out the effect of rotate mixing on $\text{NH}_4^+\text{-N}$ removal, $\text{NH}_4^+\text{-N}$ in synthetic wastewater without UMN266 inoculation was measured, changes of $\text{NH}_4^+\text{-N}$ concentration throughout the three stages were depicted as the difference between wastewater with and without algal cells.

3.2.1. Photoautotrophic inoculum batch

Growth of UMN266 in three-stage process with photoautotrophic inoculum is displayed in Fig. 2a-c. NS cultures were conducted as control. Despite of strong illumination ($300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), NS,M and NS,H cultures in Stage 1 and Stage 2 showed little differences, indicating mutual shading caused by high cell densities (Chisti, 2007). Biomass of NS,P and ND,P cultures displayed no obvious differences and both dropped slightly in Stage 2, caused probably by environmental shock (Lau et al., 1995). In Stage 3, all cultures were transferred to high $\text{NH}_4^+\text{-N}$ (80 mg/L and 160 mg/L initially, autoclave caused deviation from initial values) synthetic wastewater for photoautotrophic cultivation. Initial 80 mg/L $\text{NH}_4^+\text{-N}$ was considered as high since 45 mg/L is generally regarded as toxic for photosynthetic microorganisms (Abeliovich and

Azov, 1976). To accelerate microalgal growth thus $\text{NH}_4^+\text{-N}$ removal, exogenous inorganic carbon was supplied. Dissolved salts instead of CO_2 enriched aeration were adopted to rule out air-stripping effect (Chi et al., 2011). ND,M and ND,H cultures displayed higher biomass than corresponding NS cultures, with significantly higher growth rates in the first two days of Stage 3, while little difference in biomass concentrations between NS,P and ND,P cultures was observed, indicating recovery from N deprivation could only accelerate algal growth with trophic mode conversion. Slightly higher biomass of ND,M and ND,H cultures in $\text{NH}_4^+\text{-N}$ 160 mg/L group than in 80 mg/L group was observed, showing UMN266 can survive as high as 160 mg/L $\text{NH}_4^+\text{-N}$ and favors higher $\text{NH}_4^+\text{-N}$ for algal growth..

$\text{NH}_4^+\text{-N}$ removal profile by UM 266 is depicted in Fig.2d-f. NS cultures were conducted as control. N sufficiency was confirmed in all NS cultures in Stage 1 and Stage 2, during which $\text{NH}_4^+\text{-N}$ removal in NS,M cultures was slightly higher than NS,H cultures, proving high illumination could stimulate $\text{NH}_4^+\text{-N}$ uptake through photosynthesis related pathways (Oh-Hama et al., 1965). ND,M and ND,H cultures displayed much faster $\text{NH}_4^+\text{-N}$ removal than corresponding NS cultures in the first 2 days of Stage 3, then slowed down to similar removal rates later. $\text{NH}_4^+\text{-N}$ removal in ND,P culture was only slightly higher than NS,P culture. 160 mg/L groups displayed better $\text{NH}_4^+\text{-N}$ removal than 80 mg/L groups. These were all consistent with algal growth shown in Fig. 2b-c, since $\text{NH}_4^+\text{-N}$ could be directly used for generating light dependent constituents through photosynthesis (Cai et al., 2013). Luxurious uptake of $\text{NH}_4^+\text{-N}$ by microalgae after N starvation/deprivation has been reported by Dortch et al.

(1982), observing enhanced $\text{NH}_4^+\text{-N}$ uptake by strains of marine algae. The ability of UMN266, a freshwater microalgal strain, to uptake more $\text{NH}_4^+\text{-N}$ after N deprivation, implied its potential for efficient $\text{NH}_4^+\text{-N}$ removal. Highest removal rate of 12 mg/L/d and 23.6 mg/L/d was observed in 80 mg/L and 160 mg/L $\text{NH}_4^+\text{-N}$ groups, respectively. Treatment of synthetic and real $\text{NH}_4^+\text{-N}$ -rich wastewater ($\text{NH}_4^+\text{-N}>45$ mg/L) by microalgae has been widely researched before. Removal rates higher than this study have been reported, but mostly under favorable conditions such as alkaline environment, air/ CO_2 enriched aeration, or combined (Lau et al., 1995; Perez-Garcia et al., 2010; Ji et al., 2012). With agitation accounts for as much as 45% of total $\text{NH}_4^+\text{-N}$ removal, and $\text{NH}_4^+\text{-N}$ loss through NH_3 volatilization over 35% when pH is 9.0, and over 80% when pH is 10.0, the amount of $\text{NH}_4^+\text{-N}$ actually uptake and removed by algal cells was quite limited. Among the scarce pH controlled around neutral studies, highest $\text{NH}_4^+\text{-N}$ removal rate was about 13 mg/L/d, and the effect of agitation was still not ruled out (Zhou et al., 2012). Therefore, $\text{NH}_4^+\text{-N}$ removal in this paper was considerably superior to previous studies, and the proposed three-stage process could serve as an effective means for $\text{NH}_4^+\text{-N}$ -rich wastewater treatment. Considering the modest advantage of ND,H culture over ND,M culture in $\text{NH}_4^+\text{-N}$ removal, and easier control of heterotrophic culture over mixotrophic culture, trophic mode conversion from heterotrophy to photoautotrophy with N deprivation in Stage 2 should be selected as the optimum plan. Average removal rates of ND,H cultures were 12.4 mg/L/d and 19.1 mg/L/d with initial $\text{NH}_4^+\text{-N}$ of 80 mg/L and 160 mg/L in Stage 3. For future applications, real wastewater with different C/N ratios could be adopted in different

stages. For example, both organic carbon and $\text{NH}_4^+\text{-N}$ -rich animal wastewater (Godos et al., 2009) could be used in Stage 1, followed by high organic carbon but low $\text{NH}_4^+\text{-N}$ paper mill wastewater (Cai et al., 2013) in Stage 2, and low carbon but high $\text{NH}_4^+\text{-N}$ wastewater from steel-making plant in Stage 3 (Yun et al., 1997). Such application of different real wastewater streams, however, was not conducted in this manuscript, and should be included in further research.

3.2.2. *Heterotrophic inoculum batch*

Growth of UMN266 in three-stage process with heterotrophic inoculum is displayed in Fig. 3a-c. NS cultures were conducted as control. Different from photoautotrophic inoculum batch, much higher biomass in NS,M than NS,H cultures were observed in Stage 1 and Stage 2, this however, was contrary to the results of Ogbonna et al. (2000), which was probably because nutrient levels in previous research were not adequate for both photoautotrophy and heterotrophy in mixotrophic cultures. In Stage 3, although UMN266 could tolerate as high as 240 mg/L initial $\text{NH}_4^+\text{-N}$ (Fig. 3b-c and Fig. S2), algal growth could compare with photoautotrophic inoculum batch. For NS,P and ND,P cultures in Stage 3, long lag phases were observed, indicating severe environmental shock.

$\text{NH}_4^+\text{-N}$ removal by UMN266 is displayed in Fig. 3d-f. NS cultures were conducted as control. In Stage 1 and Stage 2, greater difference of $\text{NH}_4^+\text{-N}$ removal between NS,M and NS,H cultures was observed than in photoautotrophic inoculum batch, caused probably by the transition from heterotrophy in stock culture to mixotrophy, since many light dependent compounds need to be generated (Oh-Hama et al., 1965). For all

$\text{NH}_4^+\text{-N}$ concentrations, $\text{NH}_4^+\text{-N}$ removal in Stage 3 was not as efficient as in photoautotrophic inoculum batch, as it was with algal biomass results. However, although biomass in ND cultures was no higher than NS cultures, $\text{NH}_4^+\text{-N}$ removal in ND,M and ND,H cultures were still significantly higher than corresponding NS cultures, indicating N deprivation alone could stimulate more $\text{NH}_4^+\text{-N}$ uptake for M and H cultures. ND,P and NS,P cultures displayed little difference in $\text{NH}_4^+\text{-N}$ removal, as was consistent with algal growth results.

Comparing results from photoautotrophic and heterotrophic inoculum batches, it revealed that three-stage treatment process with photoautotrophic inoculum served as a better plan in terms of both algal growth and $\text{NH}_4^+\text{-N}$ removal. This is also more feasible in real $\text{NH}_4^+\text{-N}$ -rich wastewater treatment, since photoautotrophic stock cultures require less control and are not as likely to be contaminated by other microorganisms (Chisti, 2007). For more efficient $\text{NH}_4^+\text{-N}$ removal, Stage 3 can be shortened to 2 days, since removal rates in ND cultures were highest in the first 2 days of Stage 3.

3.3. Mechanism investigation of the three-stage treatment process

Since the aim of Stage 1 was just to accumulate as much algal biomass as possible, and NS,P and ND,P cultures only appeared from Stage 2, mechanism investigations were carried out comparing cultures of three trophic modes and two N sufficiency conditions by the end of Stage 2 and 2 days after Stage 3, since the latter date was when $\text{NH}_4^+\text{-N}$ removal was highest. Also, since all $\text{NH}_4^+\text{-N}$ concentration groups displayed similar removal trends, only cultures with initial 80 mg/L $\text{NH}_4^+\text{-N}$ were compared.

3.3.1. C and N contents of algal biomass

C and N contents of UMN266 biomass in Stage 2 and Stage 3 are shown in Fig. 4. For photoautotrophic inoculum batch, C content of algal biomass in Stage 2 did not vary much between NS and ND cultures (Fig. 4a), this could be explained that C content of energy storage (starch and lipid) caused by N deprivation was similar to that of algal biomass (Chisti, 2007). In Stage 2, N content of ND cultures were largely reduced due to N deprivation (Fig. 4c). In Stage 3, all cultures and especially ND,H culture displayed increase in N content. This was probably because generation of N-rich photosynthesis related constituents (enzymes, proteins, chloroplasts, etc.) was most suppressed in heterotrophy, and after N deprivation, the gap between self-owned and actual need of such constituents in Stage 3 was further extended, leading to enhanced driving force of N uptake and increased algal N content. This was consistent with NH_4^+ -N removal (Fig. 2e), confirming trophic mode conversion from heterotrophy to photoautotrophy with N deprivation in Stage 2 as the optimum plan for NH_4^+ -N removal.

For heterotrophic inoculum batch (Fig. 4b, Fig. 4d), similar trends were observed, only that both C and N contents were slightly lower than photoautotrophic batch. Much higher content of chlorophylls (about 74% C) contributed to higher C content of algal cells in photoautotrophic inoculum batch (Chisti, 2007). The suppressed photosynthetic pathways in heterotrophic inoculum batch restrained N-rich constituent synthesis, which lead to lower N content than photoautotrophic inoculum batch (Oh-Hama et al., 1965).

3.3.2. Cellular composition change

For photoautotrophic inoculum batch (Fig. S3-S5), UMN266 has relatively high starch content, evidenced by the shiny surfaced starch granules. Not as many lipid

bodies (in uniform grey) were observed, which was reasonable since anabolism of starch and lipid as energy storage share the pathway in algal cells (Yu et al., 2011). For both NS and ND cultures, energy storage were higher in Stage 2 than in Stage 3, and higher contents were observed in ND cultures, explained by the fact that microalgae could accumulate more starch/lipid under stressed conditions (Griffiths et al., 2014). For both NS and ND cultures, considerably more chloroplasts (strap-shaped) were observed in Stage 3 than in Stage 2, indicating enhanced photosynthesis since photoautotrophy became exclusive (Oh-Hama et al., 1965). ND culture displayed much higher chloroplast content than NS culture, implying recovery from N deprivation could contribute to generation of more N assimilating enzyme-rich chloroplasts (Fischer and Klein, 1988), which lead to more N uptake and higher NH_4^+ -N removal, as was in consistence with results of this study.

For heterotrophic inoculum batch (Fig. S6-S8), starch was also the major form of energy storage for UMN266, as similar to photoautotrophic inoculum batch. In Stage 2 however, advantage of ND over NS culture regarding energy storage was not significant. This was probably because in heterotrophic inoculum, pathway of starch/lipid anabolism was already active and later N deprivation could not make much difference (Griffiths, 1965). Chloroplasts were observed in Stage 3 for both cultures, but not as many as in photoautotrophic inoculum batch. It was possible that photosynthetic pathways have been long suppressed in heterotrophic inoculum (Oh-Hama et al., 1965), and algal cells could not quickly adjust to the conversion in Stage 3.

Since so far there is no mature technique to stain all the proteins in microalgal cells, protein content could only be indirectly displayed through the relative cellular amount of lipid and carbohydrate shown in TEM images. For further theoretical research, proteomics data should be collected to locate the proteins/enzymes related to $\text{NH}_4^+\text{-N}$ uptake by this indigenous UMN266, and such study has been conducted by other fellow workers of the lab and will be elaborated in another manuscript.

3.3.3. *Starch content*

Observed as major form of energy storage, starch contents of UMN266 under different inoculum modes, cultivation modes and N sufficiency conditions in Stage 2 and Stage 3 were quantified in Table 4. For photoautotrophic inoculum batch, starch contents of ND,M and ND,H cultures in Stage 2 were significantly higher than corresponding NS cultures, and all M and H cultures had much higher starch contents than P cultures, proving accumulation of starch under high C/N ratio (Cheng et al., 2015). In Stage 3, starch contents of NS,M and NS,H cultures were comparable as in Stage 2, while starch contents of corresponding ND cultures dropped considerably, indicating for ND,M and ND,H cultures, pathway of starch accumulation in Stage 3 was probably shunted to photosynthetic pathways such as chloroplast generation (Fig. 5), which lead to more $\text{NH}_4^+\text{-N}$ removal (Fig. 2). Although starch contents decreased in Stage 3 for ND,M and ND,H cultures, they were still considered advantageous as potential feedstock for bioethanol production (Tanadul et al., 2014). In such scenario, algal biomass could be harvested after three-stage $\text{NH}_4^+\text{-N}$ -rich wastewater treatment

process for bioethanol production, saving the cost of biomass disposal and gaining interests with bioethanol production at the same time.

For heterotrophic inoculum batch, ND,M and ND,H cultures were not superior to corresponding NS cultures in terms of starch content in Stage 2, as in accordance with Fig. 6. An exception was observed for ND,P culture, whose extra high starch content could be explained by a most stressed environment (photoautotrophy with few chloroplasts) due to inoculum trophic mode (Fig. S6). For M and H cultures, no significant differences of starch content were observed between Stage 2 and Stage 3, whereas for P cultures, starch content in Stage 3 was much lower. This was probably because for heterotrophically inoculated M and H cultures, dominance of starch synthesis pathway over photosynthetic pathways has long existed and could not transit soon in Stage 3. However, for P cultures, the already activated photosynthesis was further stimulated in Stage 3, which contributed to more related constituents therefore less starch generation (Oh-Hama et al., 1965). ND,P culture displayed more chloroplasts than NS,P culture in Stage 3 (Fig. S6), resulting more reduction of starch content.

4. Conclusions

Indigenous UMN266, identified as *Chlorella sorokiniana*, was selected out of ten candidates based on heterotrophic growth with/without N supply. A three-stage process was proposed to investigate the effects of trophic mode conversion and N deprivation on NH_4^+ -N removal by UMN266. Photoautotrophic inoculum with trophic mode conversion from heterotrophy to photoautotrophy and N deprivation in Stage 2 was the optimum plan for NH_4^+ -N removal, with average removal rates of 12.4 mg/L/d and 19.1

mg/L/d when initial $\text{NH}_4^+\text{-N}$ was 80 mg/L and 160 mg/L. Mechanism investigations confirmed the optimum plan and potential of UMN266 as bioethanol feedstock.

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Table 1 Primers used in PCR amplification and sequencing

Primer set No.	Name of primer	Sequence of primer (5'-3')
1	EK82f (forward)	GAAACTGCGAATGGCTC
	Proto5r (reverse)	GACGGGCGGTGTGTAC
2	16S1N (forward)	TCCTGCCAGTAGTCATATGC
	16S2N (reverse)	TGATCCTCT/CGCAGGTTCAC
3	rbcL 1-20 (forward)	ATGGTTCCACAAACAGAAAC
	rbcL 803-782b (reverse)	TCGTGCATAATAATAGGTACAC

Table 2 Comparison of two independent batches of the three-stage process

Batch No.		A	B
Culture conditions	Stage 1* (3 days)	NS,M NS,H	NS,M NS,H
	Stage 2* (48 h)	NS,M ND,M	NS,M ND,M
		NS,H ND,H	NS,H ND,H
		NS,P ND,P	NS,P ND,P
	Stage 3* (5 days)	NS,P	NS,P
Trophic mode of inoculum		Photoautotrophic	Heterotrophic
Light intensity ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)		300	300
Initial $\text{NH}_4^+\text{-N}$ in Stage 3 (mg/L)		80, 160	80, 160, 240
Inorganic carbon sources in Stage 3		Added	Added

*NS,M=N sufficient and mixotrophic; *NS,H=N sufficient and heterotrophic;

*ND,M=N deprived and mixotrophic; *ND,H=N deprived and heterotrophic;

*NS,P=N sufficient and photoautotrophic; *ND,P=N deprived and photoautotrophic

Table 3 Homology among 18s rRNA gene sequences of UMN266 and GenBank

strains				
Primer set No.	rDNA length (bp)	Database strain	GenBank Accession No.	Proximity (%)
1	1197	<i>Chlorella sorokiniana</i> (UTEX2714)	LK021940.1	94
		<i>Chlorella vulgaris</i> (CCAP211/79)	FR865683.1	94
		<i>Micractinium</i> sp. (CCAP248/2)	FR865695.1	94
		<i>Chlorella luteoviridis</i> (CCAP211/5B)	FR865678.1	94
2	292	<i>Micractinium</i> sp. (CCAP248/2)	FR865695.1	98
		<i>Chlorella sorokiniana</i> (UTEX2714)	LK021940.1	96
3	776	<i>Chlorella sorokiniana</i> (isolate 1230)	KJ742376.1	99
		<i>Chlorella sorokiniana</i>	KJ397925.1	99
		<i>Chlorella</i> sp. (ArM0029B)	KF554427.1	94
		<i>Chlorella variabilis</i> (isolate NC64A)	KJ718922.1	92

Table 4 Starch content of UMN266 in different stages, cultures, and modes of inoculum

Starch content (%)		Stage 2	Stage 3
Photoautotrophic inoculum batch	NS,M	32.4±0.1	32.3±0.6
	NS,H	22.1±0.0	17.6±0.4
	NS,P	5.2±0.0	6.5±0.2
	ND,M	45.0±0.6	34.6±1.7
	ND,H	38.4±0.2	22.1±0.6
	ND,P	4.7±0.6	7.0±0.7
Heterotrophic inoculum batch	NS,M	27.1±1.1	28.7±2.1
	NS,H	33.2±0.6	31.8±0.2
	NS,P	26.5±0.4	12.7±0.8
	ND,M	22.8±0.2	25.8±0.3
	ND,H	22.9±0.3	19.1±0.0
	ND,P	41.4±0.1	20.1±0.2

Figure Caption

Fig. 1. Growth of 10 strains (a) UMN266, UMN244, UMN268, UMN271, UMN270; (b) UTEX78, UMN230, UMN285, UTEX2714, UMN258 under heterotrophic mode, and growth of the top 4 candidates (c) UMN266; (d) UMN268; (e) UTEX2714; (f) UMN285 through NS and ND stages in synthetic wastewater. In the legend, NS: nitrogen sufficient, ND, nitrogen deprived. NS cultures were conducted as control.

Fig. 2. Growth and $\text{NH}_4^+\text{-N}$ removal of UMN266 in three-stage treatment process with photoautotrophic inoculum. (a) TVSS in Stage 1 and Stage 2; (b) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (c) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L; (d) $\text{NH}_4^+\text{-N}$ removal in Stage 1 and Stage 2; (e) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (f) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L. NS cultures were conducted as control.

Fig. 3. Growth and $\text{NH}_4^+\text{-N}$ removal of UMN266 in three-stage treatment process with heterotrophic inoculum. (a) TVSS in Stage 1 and Stage 2; (b) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (c) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L; (d) $\text{NH}_4^+\text{-N}$ removal in Stage 1 and Stage 2; (e) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (f)

NH_4^+ -N removal in Stage 3, with initial NH_4^+ -N concentration of 160 mg/L. NS cultures were conducted as control.

Fig. 4. C and N contents of UMN266 biomass. (a) C content in photoautotrophic inoculum batch; (b) C content in heterotrophic inoculum batch; (c) N content in photoautotrophic inoculum batch; (d) N content in heterotrophic inoculum batch.

Figure. 1. Growth of 10 strains (a) UMN266, UMN244, UMN268, UMN271, UMN270; (b) UTEX78, UMN230, UMN285, UTEX2714, UMN258 under heterotrophic mode, and growth of the top 4 candidates (c) UMN266; (d) UMN268; (e) UTEX2714; (f) UMN285 through NS and ND stages in synthetic wastewater. In the legend, NS: nitrogen sufficient, ND, nitrogen deprived.

NS cultures were conducted as control.

Figure. 2. Growth and $\text{NH}_4^+\text{-N}$ removal of UMN266 in three-stage treatment process with photoautotrophic inoculum. (a) TVSS in Stage 1 and Stage 2; (b) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (c) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L; (d) $\text{NH}_4^+\text{-N}$ removal in Stage 1 and Stage 2; (e) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (f) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L. **NS cultures were conducted as control.**

Figure 3. Growth and $\text{NH}_4^+\text{-N}$ removal of UMN266 in three-stage treatment process with heterotrophic inoculum. (a) TVSS in Stage 1 and Stage 2; (b) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (c) TVSS in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L; (d) $\text{NH}_4^+\text{-N}$ removal in Stage 1 and Stage 2; (e) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 80 mg/L; (f) $\text{NH}_4^+\text{-N}$ removal in Stage 3, with initial $\text{NH}_4^+\text{-N}$ concentration of 160 mg/L. NS cultures were conducted as control.

Figure 4. C and N contents of UMN266 biomass. (a) C content in photoautotrophic inoculum batch; (b) C content in heterotrophic inoculum batch; (c) N content in photoautotrophic inoculum batch; (d) N content in heterotrophic inoculum batch.