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Full Length Research Paper

Ammonia-nitrogen and orthophosphate removal by immobilized *Chlorella* sp. isolated from municipal wastewater for potential use in tertiary treatment

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***Chlorella* sp. isolated from municipal wastewater and entrapped in calcium alginate as algal sheets was employed to remove inorganic nutrients from domestic secondary effluents in parallel-plate bioreactor after starvation. The key factors affecting the nutrient removal efficiency, system stability and reuse efficiency of screens were discussed. The results show that cell density and starvation time significantly affected the nutrient uptake. A complete removal of NH_4^+-N and PO_4^{3-}-P was achieved within 4 h of treatment with the optimal cell density in the mixture of algal and 3 mm gel sheets after third cycle. Six cycles of wastewater treatment were accomplished in 18 days, achieving higher removal efficiency. NH_4^+-N removal efficiency was 81% after 1.25 h and 98.81% after 4 h, while PO_4^{3-}-P removal efficiency was 77.4% after 1.25 h and 100% after 4 h. It was confirmed that the immobilized *Chlorella* sp. has great potentialities in nutrient removal.**

Key words: Immobilized *Chlorella*, starvation, wastewater treatment, nitrogen removal, phosphorus removal.

INTRODUCTION

The domestic and agro-industrial wastewater, although after secondary treatments may continue to release large amounts of phosphorus and nitrogen. These nutrients are directly responsible for eutrophication of rivers, lakes and seas (Mallick, 2002; de-Bashan and Bashan, 2010; Sebnem and Ilgi, 2006). Microalgal or cyanobacterial cells have been widely used to remove the excessive nutrients and other contaminants because they have a high capacity of uptaking inorganic nutrients in tertiary wastewater treatment, while producing potentially valuable biomass (Martínez et al., 2000; James, 1998; Chevalier et al., 2000). Green algae such as *Chlorella* sp. and *Scenedesmus* sp. have been widely used in wastewater treatment as they grow fast and often naturally colonize the ponds, having high capabilities of nutrient removal. However, harvesting of biomass from the treated effluents is still a limiting factor for micro-algae utilization in wastewater purification (De la Noüe et al., 1992; James, 1998; de-Bashan and Bashan, 2010; Park et al., 2010).

A potentially alternative method to avoid the harvesting problem is application of mat-forming cyanobacteria or other green algal strains, which are either attached or immobilized for high nutrient removal capacity (James, 1998; Chevalier et al., 2000; Jiménez-Pérez et al., 2004; Endong et al., 2008). Efficient and rapid removal of nitrogen and phosphorus from wastewater can be consistently achieved by immobilized algae. Alginate is the natural polymer often used in algal systems (Robinson et al., 1989; Urrutia et al., 1995; Kaya et al., 1996; Lau et al., 1998; Susana et al., 2006), while alginate beads are used most frequently (Vichez and Vega, 1994; Tam and Wang, 2000; Inés et al., 2002; de-Bashan et al., 2004). Kaya et al. (1995, 1996) developed a novel immobilized algal system for wastewater biotreatments. In this process, *Scenedesmus* cells were immobilized on alginate screens

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for starvation removal cycles and its nutrient uptake rate was higher than alginate beads. Its major advantages are: (1) low consumption of light energy for cells; (2) high and rapid nutrient uptake and short retention time; (3) a small area requirement; (4) easy recovery of excessive biomass; (5) economic for screens reuse and convenient for continuous flow reactors (Kaya et al., 1995); (6) serves as a green technology without secondary pollution and the end products is easily converted to additional by-products such as fertilizers or biofuel. Kaya et al. (1995, 1996) studied the effect of intermittent CO_2 enrichment on nitrogen and phosphate removal during nutrient starvation and gel stability in this system. Endong et al. (2008) found that the cell density of mixture was the key factor influencing the nutrient removal. The nutrient removal efficiency by immobilized cells in beads could be increased by intrinsic and extrinsic factors, such as the algal age, density and the starvation of algae, photon energy availability, temperature, pH, CO_2 concentration, etc. They determine the interactions between algae, wastewater components, environmental factors and gel matrix. At the same time, different types of bioreactors should be used depending on their advantages, disadvantages and the aims (Martínez et al., 2000; James et al., 1998; Tam and Wang, 2000; Kaya et al., 1995). Optimization of these intrinsic and extrinsic factors in this new algal system could promote the nutrient removal, and reduce retention time and cost.

In order to investigate the potential of *Chlorella* sp. which was isolated from municipal wastewater for nitrogen and phosphorus removal in tertiary wastewater treatment, it was applied in starvation removal cycle mode. On the other hand, the effect of algal age on removal of inorganic nutrients was previously studied by the authors by means of algal pre-harvesting flocculants in order to reduce centrifugation cost.

The purpose of this study was to investigate the optimal *Chlorella* cell concentration, starvation time, screen reuse efficiency and system stability. Moreover, on the basis of rehardening process, the evaluation of the removal efficiency of the same screens in longtime starvation removal cycle model for secondary effluent in Dalian Ecomomic and Technical Developing Zone was also done. As an economical tertiary treatment system, the immobilized cell technology was taken to accelerate nutrient uptake by improving the control process and increasing the treatment efficiency.

MATERIALS AND METHODS

Culture of *Chlorella* sp

Chlorella sp. isolated from municipal wastewater (Dalian, China) was conserved in the laboratory. The cell suspensions of *Chlorella* sp. were cultivated in artificial Dauta medium (Kaya et al., 1995). A free cell culture was grown in 3 L carboy at room temperature (about 20 ± 2°C) under a light intensity of 130 ± 6 µmol $m^{-2} s^{-1}$ from white circularity fluorescent light with 13:11 h light and dark photoperiod. Culture agitation and aeration were provided by filtered air bubbling. Microalgal growth in carboy was monitored in daily cell counts by using a hemacytometer of 0.1 mm depth to set up its daily survival curve. The cells were harvested by centrifugation (3450 rpm) at early stationary phase.

Immobilization of microalgae

The microalgal cells suspension and 6% sodium alginate solution were mixed together by 1:1 ratio, finally reaching 3% concentration of sodium alginate. In preparing the model, the mixture was hardened on stainless steel screen with 1 × 1 mm^2 mesh by 0.2 M calcium chloride solution for 1 h, finally resulting to 3 mm thick gel. After immobilization, the screens were washed with distilled water before submitting to ammonium and orthophosphate starvation. The calcium alginate was dissolved in 1.5% sodium citrate. In longtime starvation removal cycle model, the same screen was used for secondary effluent, its cell concentration in gel was 1.4 × 10^8 algae ml^{-1}, bioreactor cell density was 1.4 × 10^7 with five screens and 2.8 × 10^7 with ten screens separately.

Starvation stage and nutrient removal procedure

A complete treatment cycle consisted of immobilized cells starving in the growth photochamber for 48 h in air saturated at 100% relative humidity, followed by removal of nutrients from wastewater in darkness. The starvation photochamber was built with polyethylene. The humidity was controlled with a humidifier at 20 ± 2°C. The starvation time lasted for 24, 48, 60 h, under illumination with 100 ± 3 µmol $m^{-2} s^{-1}$ light intensity by fluorescents lamps and 13/11 h light/dark photoperiod. The parallel-plate bioreactor was taken for nutrient removal, which was installed with an air bubbling system without lamps. It was made with polyethylene and had a working volume of 350 ml, with a holding capacity of 5 or 10 parallel screens, 23 or 11.5 mm apart. The secondary domestic wastewater effluents used in for the test were collected from the wastewater treatment plant of Dalian Economic and Technical Developing Zone with 10:1 N/P ratio and pH 7.3. Before using it, filtration and sterilization (0.15 Mp, 20 min) was done. The experiments on NH_4^+-N and PO_4^{3-}-P were conducted in the parallel-plate bioreactor with immobilized starved microalgal cells. Every 5-ml wastewater sample was collected for analysis of residual NH_4^+-N and PO_4^{3-}-P at time of 0.25, 0.75, 1.25, 1.75, 2.25 and 4 h, respectively after it began to work. The samples of treated wastewater were stored at 4°C or frozen at -20°C before analyzing. Schematic presentation of treatment flow is shown in Figure 1.

Determination of nutrients and cell viability

Ammonia and phosphorus were determined in three replicate samples according to Martı′nez et al. (2000). The content of chlorophyll a was analyzed by extraction of the immobilized cells with 2:1 (v/v) chloroform and methanol overnight. After centrifugation, the supernatant was determined for chlorophyll a content by chromatography at 665 nm.

Statistical analysis

The data of ammonia and phosphorus nutrient uptake and chloro-

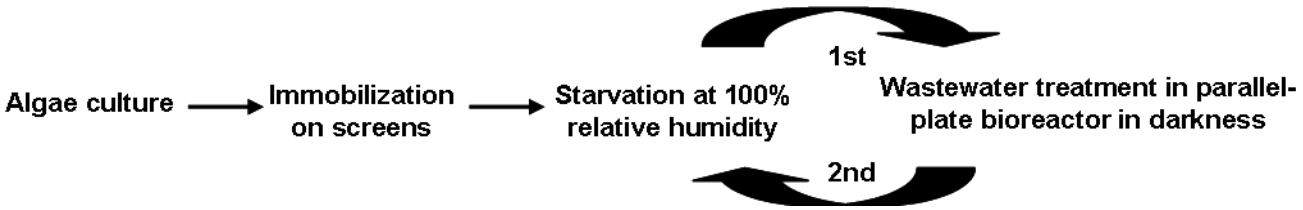

Figure 1. Schematic presentation of treatment flow

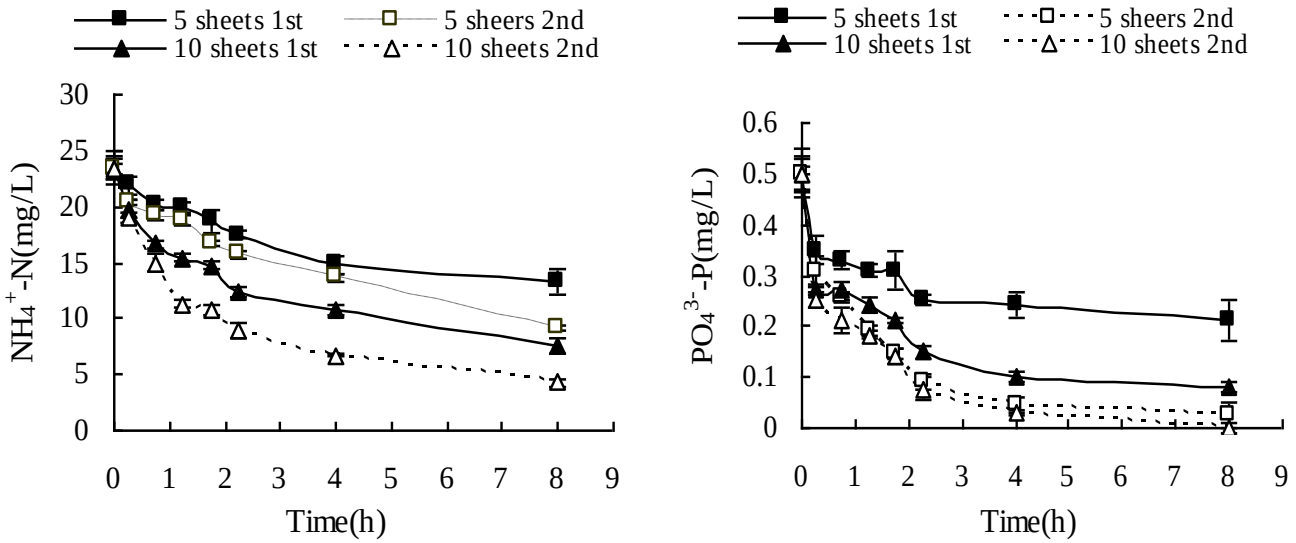

Figure 2. Residual concentrations of ammonium and orthophosphate in secondary effluent in bioreactor with different cell density of *Chlorella* sp. Real line represents the first trial after 48 h of nutrient starvation in air. Broken line represents the second trial after the first uptake followed by 48 h of nutrient starvation. ■: Cell density in bioreactor was 1.4 × 10^7algae/ml; ▲: cell density in bioreactor was 2.8 × 10^7algae/ml.

phyll a content were gotten using analysis of variance and multiple comparison tests in three trials.

RESULTS AND DISCUSSION

Effect of *Chlorella* cell concentration in bioreactor on nitrogen and phosphorus uptake

As shown in Figure 2, the cell concentration in parallel-plate wastewater treatment bioreactor (PPR) was 1.4 × 10^7 algae/ml with five sheets and 2.8 × 10^7 algae/ml with 10 sheets. The removal efficiency of NH_4^+-N and PO_4^{3-}-P in PPR was 2.8 × 10^7 > 1.4×10^7 algae/ml in the successive trials. In the second treatment, the removal efficiencies of ammonium and orthophosphate were 60.7 and 84% with five sheets, and 81.6 and 100% with ten sheets after 8 h.

The different removal efficiencies of ammonium and orthophosphate by cells (Figure 2) indicated that the nutrient removal rate was dependent on cell density which determines the substrate diffusion. The *Chlorella* cells occupied most parts of the useful volume of beads, and the exit route for molecules is longer (Vichez and Vega, 1994). However, it was shown that such simple inorganic nutrient ions such as nitrate, ammonium and phosphate would be freely available to the immobilized algae as their free counterparts (Lau et al., 1997). The initial cell density of *Chlorella* sp. is critical for removing ammonia and phosphate. Higher removal efficiency can be achieved by properly increasing cell density or thickness in gel, while lower cell concentrations would reduce the nutrient removal efficiency. On the other hand, higher cell concentrations in gel or thickness were not effective or necessary because it may reduce light penetration in the bioreactor. Meanwhile, it resulted in self-shading and limits the growth and activities of algal cells.

Effect of starvation times on nitrogen and phosphorus removal efficiency

In the literature, the nitrogen and phosphorus removal efficiencies varied depending on medium composition and

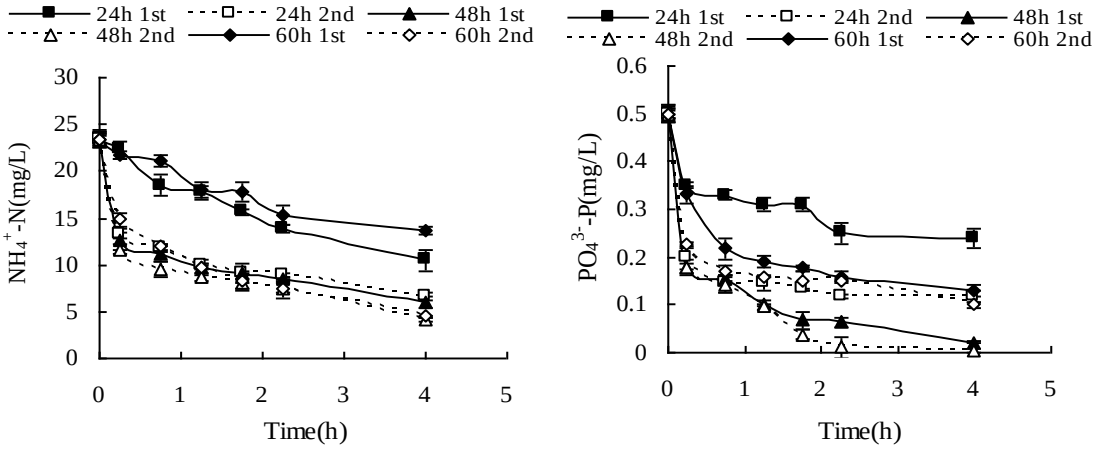

Figure 3. Residual concentrations of ammonium and orthophosphate under different starvation time of *Chlorella* sp. in starvation-treatment cycles. Real line represents the first trial after 24 h (■), 48 h (▲), 60 h (◆) of nutrient starvation in air. Broken line represents the second trial after the first uptake followed by 24 h (□), 48 h (△), 60 h (◇) of nutrient starvation.

environmental conditions such as initial nutrient concentration, light intensity, nitrogen/phosphorus ratio, light/dark cycle or algae species (Sebnem and Ilgi, 2006) and starvation. As shown in Figure 3, the results indicated that the largest removal efficiency of NH_4^+-N was 82.4% after 4 h incubation with cells starved for 48 h; 99% of PO_4^{3-}-P was removed after 2.25 h incubation with cells starved for 48 h in second treatment cycle. 71.8 and 80.9% of NH_4^+-N were obtained after 4 h incubation with cells starved for 24 and 60 h, 76.4 and 80% of PO_4^{3-}-P were removed after 4 h with cells starved for 24 and 60 h, individually in the second trial. The optimal starvation time for ammonia and phosphate removal was 48 h ($p<0.05$). Longer starvation time (60 h) caused damage to cells, and it evidently decreased the nutrient uptake than 48 h starvation.

Starvation of algal cells was conducted to promote quick depletion of the dissolved nutrients in wastewater, especially ammonium and orthophosphate ions, which are the major causes of aquatic plant blooms. With massive algal growth in open ponds, removal of NH_4^+-N and PO_4^{3-}-P usually takes three to six days to obtain over 90% of efficiency depending on the concentration of the inoculum (Kaya, 1995). The results indicated that the removal of nutrients from wastewater was more evident than conventional biological tertiary wastewater treatment (free cells or bead-shaped alginate particles).

Removal of nitrogen and phosphorus from secondary effluents during the starvation-treatment cycles

It is well known that the nutrient removal efficiency of immobilized microalgae not only depends on cell concentrations, but also on starvation (Hernandez et al., 2006). The removal efficiency of ammonia and orthophosphate at each cycle is shown in Figure 4. It was clearly observed that higher removal efficiency of NH_4^+-N and PO_4^{3-}-P occurred in 18 days in secondary effluents. Successive starvation-treatment cycle improved inorganic nutrient removal efficiency in 1st to 3rd cycles because of cell growth and proliferation. The removal efficiency of ammonia and orthophosphate in the 4th cycle was stable due to the balance of positive and negative factors affecting nutrients removal efficiency. The positive factors include cell growth and proliferation in gel, while negative factors include light limitation and substrate diffusion. Municipal sewage contains various organic compounds such as volatile acids, non-volatile soluble acids, fatty acids, amino acids and carbohydrates, many of which are known to be used by algae for mixotrophic and heterotropic growths (Mallick and Rai, 1994). Thus, under light-limited conditions, mixotrophic growth has a great potential for production of biomass and elimination of wastewater nutrients.

In the 5 to 6th cycles, the cell removal efficiency decreased because of many negative factors and longtime starvation. In many previous studies, *Chlorella* sp. was immobilized by carrageen in finite volume of the bead, which evidently contributed to maximum cell-holding capacity. When algal cells multiply, they occupy more space within the beads. Their massive proliferation probably weakens the polymeric linkage of gel matrix, which in turn reduces the gel strength in holding the cells within the matrix. Once the space of beads is fully occupied, the maximum cell-holding capacity can be achieved. The continuous growth and proliferation of algal cells accelerate weakening of polymeric linkage of the bead matrix as a consequence of population expansion,

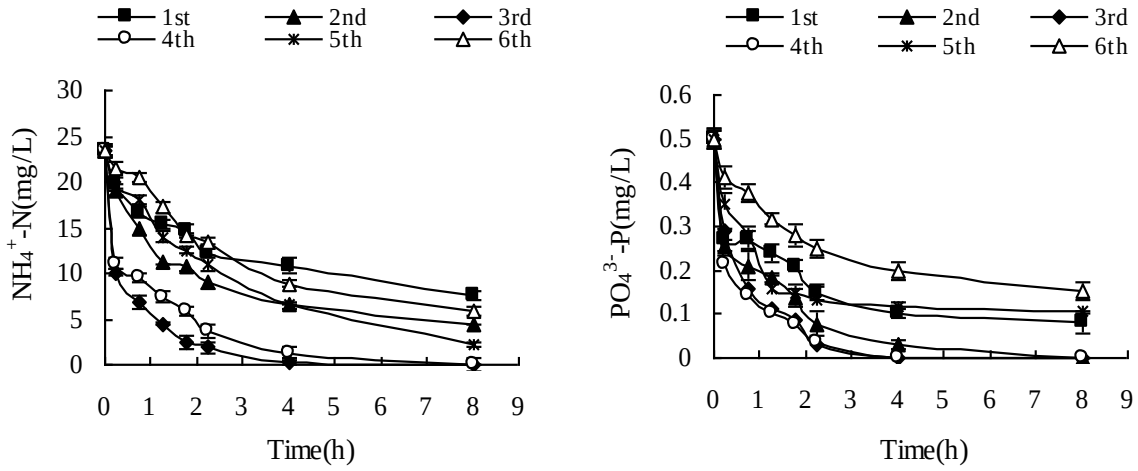

Figure 4. Residual concentrations of ammonium and phosphate in 48 h starvation-treatment cycles in secondary effluents.

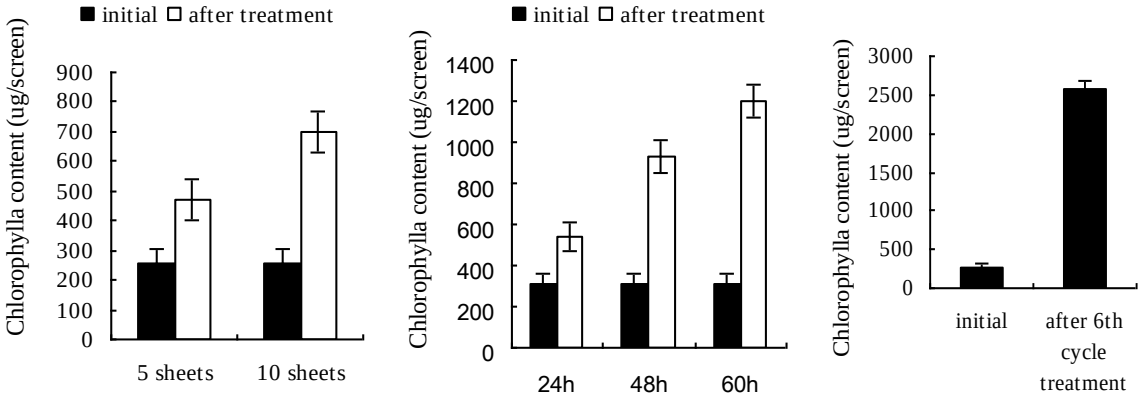

Figure 5. Chlorophyll a content of immobilized *Chlorella* sp. cells of different cell concentrations (left), starvation time (middle) and six treatment cycle (right).

and finally lead to extensive leakage at the periphery of the carrageen beads (Lau et al., 1998). After the forth cycle, rehardening of the screens is theoretically necessary. Although, the screens were strengthened, leakage of cells was unavoidable because the cyclic operation time was extended. The experiment was terminated after the sixth cycle and cell leakage was not observed. The analysis of chlorophyll a content showed that the immobilized cells were still viable after all starvation-treatment cycles. The elimination of orthophosphate was in the same tendency as ammonia (Figure 4).

Cell viability

The photosynthetic pigment content of micro-algal cells depends on nitrogen availability. It is a particularly attractive choice because the pigments are specific markers for autotrophic plankton and they are severely affected by cellular death rate (Kaya et al., 1995). The algal physiological station indirectly indicates the nutrient removal efficiency. The levels of chlorophyll a (Figure 5) before and after 24, 48, 60 h period of nutrient starvation cycle indicate that the viability of cells was not affected by the cyclic operation of nutrient starvation. After the sixth cycle in the second effluents, the contents of chlorophyll a also increased. This may be due to the following: (1) cell growth and multiplication (Tam and Wang, 2000; Kaya et al., 1995); (2) decreased incident light by immobilization, self-shading of light and screen effect by gel, thus increasing the synthesis of pigments (Vichez and Vega, 1994); (3) immobilization promoting algal anabolism and physiological activity, so finally increasing removal efficiency of inorganic nutrients (Yan et al., 1995).

Conclusions

The present study demonstrated that immobilization of *Chlorella* sp. on screens for starvation-wastewater treatment would be a promising method for the tertiary treatment of wastewaters. Furthermore, cell density in Photobioreactor was one factor affecting nutrient removal efficiency. Nutrient removal efficiency can be improved by starvation. The system stability and reuse efficiency of screens were maintained throughout 18 days with 6 cycles. Although, alginate sheet immobilized algae system and starvation treatment were more efficient in removing ammonia and orthophosphate from secondary effluents, further studies are needed before it is applied in large scale wastewater treatment.

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