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Removal of nutrients and COD from wastewater using symbiotic co-culture of bacterium *Pseudomonas putida* and immobilized microalga *Chlorella vulgaris*

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ABSTRACT

Simultaneous removal of nutrients (ammonium and phosphate) and COD was investigated by the co-culture consortium of microalga *Chlorella vulgaris* and bacterium *Pseudomonas putida*. The co-culture system showed higher removal of both nutrients and COD than the each axenic culture, indicating that nutrients uptake capability of *C. vulgaris* was enhanced in the presence of *P. putida*. The best performance in the removal of nitrogen, phosphorus, and COD was obtained through the co-culture with suspended *P. putida* and immobilized *C. vulgaris*, demonstrating that the employment of immobilization of one species is more synergistic than suspended co-culture system in nutrients removal from wastewater.

Introduction

Nitrogen and phosphorus are the main elements for eutrophication of natural water bodies. Eutrophication became more common since the mid-20th century and considered as a severe environmental issue, which can trouble the balance of aquatic systems. Wastewaters containing nitrogen and phosphorus should not be discharged directly into water body before adequate treatment.

Conventional methods include many steps and are expensive for the removal of nutrients from wastewater. Anaerobic–anoxic–oxic (A^2O) process, the most commonly used biological nutrient removal (BNR) method, needs at least three bioreactors in series (such as anaerobic, anoxic, and aerobic) in which working conditions are contrastingly different and complicated [1]. Nitrogen removal is realized through nitrification and denitrification using aerobic and anoxic reactors, respectively. Phosphorus is removed through the coupling of anaerobic and aerobic reactors. And, the aerobic reactor serves for the removal of organics. Availability of COD is necessary for simultaneous removal of nitrogen and phosphorous because denitrifiers and phosphate-accumulating microorganisms need organic carbons to complete reactions [2]. Methanol is usually added in conventional BNR systems as a supplementary organic carbon source.

In this regard, the removal of nutrients from wastewater by using the co-culture of bacteria and microalgae is an alternative and renewable approach. If the growth and maintenance of two different microorganisms are compatible in a co-culture system, heterotrophic metabolism of aerobic bacteria and the capabilities of nutrient uptake and photosynthetic oxygenation of algae can be mutually symbiotic [3,4]. Photosynthetic microalgae can uptake nitrogen and phosphorous into their biomass as cell constituents and release exogenous oxygen to realize most of the aerobic bacterial requirements. On the other hand, heterotrophic bacteria can oxidize organic carbon (utilizing O_2 from microalgae) and produce CO_2 that can be consumed by microalgae as an autotrophic carbon source [5]. Sometimes, bacteria can stimulate algal growth by the release of vitamins and plant hormones [6], while microalgae can also release organic compounds that can be used by bacteria as an energy source [7]. Thus, the simultaneous removal of nitrogen, phosphorous, and organics can be achieved in a single reactor by selecting appropriate strains of bacteria and microalgae in co-culture system.

For the purpose of continuous operation of treatment reactor and miroalgal cells are to be utilized further (e.g., for biofuel production), the recovery of microalgal cells from the treated

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wastewater is necessary. If cells entrap in an immobilization matrix, microalgae can easily be separated from bacteria and harvested from the effluent stream. The main advantages of immobilization technique also include the prevention of biomass washout from the process, a greater degree of operational flexibility, and easy separation [8]. Alginate is a cost-effective matrix that can easily be used to entrap microbial cells in alginate beads. Cells were found evenly distributed within each bead, and cell viability is maintained successfully for an extended time [9]. Algal cells immobilized in alginate beads showed the same efficiency to remove nitrogen and phosphorous from wastewater as that by suspended cells.

Green microalga *Chlorella vulgaris* was selected for the purpose of nutrients removal and cell immobilization in this study because it has a fast growth rate and short reproduction time. *C. vulgaris* is often used for tertiary treatment of wastewater mainly for the removal of nitrogen and phosphorus [10]. Heterotrophic aerobic bacterium *Pseudomonas putida* is commonly present in activated sludge process and showing good performance in organics removal (or BOD removal). It is known that *P. putida* is co-culturable with *C. vulgaris* and can also increase the growth of *Chlorella* [11].

This study aimed to remove nitrogen, phosphorus, and COD simultaneously from synthetic wastewater by using the co-culture system of suspended *P. putida* and immobilized *C. vulgaris* in a single reactor. Alginate beads were used to immobilize *C. vulgaris*. It is the first study, to the best of our knowledge, in which the co-culture of immobilized algae and suspended bacteria is used for the wastewater treatment. One benefit of this immobilized culture is the convenience in separating *C. vulgaris* and *P. putida* after treatment. The performance of nutrients and COD removal was compared in different treatment systems, such as pure *P. putida* culture, suspended co-culture, co-immobilized culture, and co-culture with suspended *P. putida* and immobilized *C. vulgaris*. The change in the cell population of *C. vulgaris* and *P. putida* was compared, and several cycles of repeated treatment were monitored to test the stability of the co-culture combination with suspended *P. putida* and immobilized *C. vulgaris* in removing nutrients and COD.

Materials and methods

Microorganisms and cultivation conditions

The freshwater green microalga *C. vulgaris* AG 30007 was obtained from Korea Biological Resource Center (Daejeon, Korea). *C. vulgaris* was inoculated in a modified BG-11 medium [12] using 250-mL flask and incubated at 25 °C and 50 μmol m^{-2} s^{-1} of light intensity (24 h). In order to obtain quicker growth, a bubble-column photobioreactor [13] was used with the supply of 5% (v/v) CO_2 at 0.1 vvm and 100 μmol m^{-2} s^{-1} light using fluorescent tubes prior to wastewater treatment. The gram-negative aerobic bacterium *P. putida* ATCC 17514 was grown in liquid nutrient broth at 30 °C before using for wastewater treatment.

Chlorella was selected because they are widely used for the studies on the treatment of wastewaters, easily found actually in municipal wastewater treatment systems, and has been proved to efficiently assimilate nitrogen and phosphorus from variety sources of wastewaters. *P. putida* was selected because it is an aerobic and culturable bacteria species which is popularly involved in activated sludge processes for the treatment of wastewater.

Synthetic municipal wastewater

Synthetic municipal wastewater (SMW) was prepared in order to investigate the removal performance of nitrogen, phosphorous, and organic carbon. The composition of SMW included: NH_4Cl 190, K_2HPO_4 40, KH_2PO_4 15, $NaHCO_3$ 275, and glucose 450 mg L^{-1}. The initial values of inorganic nutrients such as nitrogen and phosphorous were 50 and 10 mg L^{-1}, respectively, representing the medium strength municipal wastewater. The initial level of COD was adjusted at 490 mg L^{-1} using glucose. The concentrations of inorganic carbon and alkalinity were measured as 50 and 200 mg L^{-1}, respectively. Prepared SMW was autoclaved at 121 °C to avoid contamination prior to use.

The compositions of municipal wastewater are very diverse depending upon wastewater sources. The concentrations of nitrogen, phosphorus, and other components are not always similar in real municipal wastewaters. The composition of SMW used in this study was based upon Tchobanoglous and Burton [14], where the composition of real wastewater generally ranges from low-strength to high-strength. The medium-strength wastewater was selected in this study to depict the characteristics of averaged values of influents in several local municipal wastewater treatment plants. Glucose is generally employed as an organic carbon in artificial wastewaters because it is well biodegradable and results in a stable COD or BOD values. Organic constituents and nutrients are supposed to be assimilated into the biomass of bacteria and microalgae.

Cell immobilization

Microorganisms were immobilized using the method described by Gonzalez and Bashan [6]. Briefly, 5 mL of the suspension of *C. vulgaris* was mixed with 20 mL of sterilized 2% alginate solution and gently stirred for 15 min. The solution was dripped from a sterile syringe into a 2% $CaCl_2$ solution with slow stirring. The beads (4 mm in diameter) formed were left for 1 h at 20 °C for curing and then washed in sterile saline solution (0.85% of NaCl). *P. putida* was immobilized in the similar manner. For the co-immobilization of *C. vulgaris* and *P. putida*, the same concentration of each microorganism was mixed, but the volume of each microbial culture was reduced to 2.5 mL before adding the alginate solution.

Experimental scheme

After the separate growth of *C. vulgaris* and *P. putida*, each was centrifuged (3000 rpm for 3 min) and then washed three times with 0.85% sterilized NaCl solution. The initial concentrations of *C. vulgaris* and *P. putida* were determined as 5×10^6 cells mL^{-1} and 2.5×10^6 cells mL^{-1}, respectively. The inoculum ratio of immobilized microalgae to bacteria was 20 (Fig. 2), which was based on the preliminary study [11] showing that a stable co-culture consortium and a good nutrient removal performance are maintained under the conditions with higher microalgae population.

Experiments were performed in 250-mL Erlenmeyer flasks containing 200 mL working volume of wastewater in a shaking incubator. During the treatment of SMW, the continuous illumination of 50 μmol m^{-2} s^{-1} (24:0 light/dark) was provided via white fluorescent lamps. The temperature was controlled at 25 °C. External aeration or CO_2 was not supplied into the reactors; the mixing was solely driven by flask-shaking. Four different treatment systems were set up: (i) pure culture of suspended *P. putida*, (ii) suspended co-culture of *P. putida* and *C. vulgaris*, (iii) co-immobilized culture, and (iv) co-culture of immobilized *C. vulgaris* and suspended *P. putida.* Experiments have been performed at least three times for the verification and data are presented with *p*-values less than 0.05 through statistical analysis of variance (ANOVA) in SPSS ver. 22.0.