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Original Research Paper

Removal of nitrogen and phosphorus from wastewater using microalgae free cells in bath culture system

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ABSTRACT

The effluents from wastewater contain nutrients (NH_4^+, NO_3^- and PO_4^{-3}) which have been identified as the main causes leading to eutrophication in natural waters. Therefore, the wastewater must receive suitable treatment before being discharged into water bodies. Microalgae play an effective role during urban wastewater treatment. In this work five strains of microalgae growing as free-cells were used and compared to test their ability to remove nitrogen-nitrate (NO_3^--N) and orthophosphate (PO_4^{3-}-P) in batch cultures of urban wastewater. The microalgae with the best cell growth configuration were selected, and introduced as a suitable strain for nutrient removal. Results indicate that *Chlorella* sp. (YG01) showed a higher N uptake rate (84.11%) and *Chlamydomonas* sp. (YG04) and *Chlamydomonas* sp. (YG05) showed a higher P uptake rate (100%) in urban wastewater than other species. Also during 2 weeks of each experiment, most of the N and P removal was occurred at the first 4 days.

1. Introduction

Because of the generation of a great volume of urban and industrial wastewaters in industrialized countries, and also risk of dumping these effluents into rivers, lakes or the sea, it is important to treat wastewaters to reduce contaminants to environment (Martinez et al., 2000). Inorganic substances (ammonium, nitrate and phosphate) which encourage vegetal growth, contributing to the eutrophication of the bodies of water containing the effluents, have been received more attention in the waste water treatment (Martinez et al., 2000). Therefore suitable treatment must be done for urban wastewater before being discharged into water bodies. Several types of processes are used for the removal of nutrients from wastewater such as mechanical (influx, removal of large objects, removal of sand and grit, primary sedimentation), chemical (disinfection) and biological (tricking bed filter, activated sludge) treatments but these are costly and produce high sludge content. As an alternative biological treatment, microalgae have been proposed to remove nutrients from wastewater (Ruiz-Marin et al., 2010) and also can be used for tertiary treatment of wastewater due to their capacity to assimilate nutrients (Larsdotter, 2006). As reported in several studies differences of nitrogen and phosphorus removal efficiencies depend on the media composition and environmental conditions such as the initial nutrient concentration, the light intensity, the nitrogen/phosphorus ratio, the light/dark cycle and algae species (Aslan and Kapdan, 2006).

Using microalgae in wastewater treatment has been studied widely and indicated to have positive effect in nutrient removal (Zamani et al., 2011). Microalgae are widespread in different locations such as soil, air and fresh water (Rasoul-Amini et al., 2011). The technology of using microalgae in wastewater treatment is based on natural ecosystems; therefore there is no danger for environment and even if the biomass produced is reused, causes no secondary pollution (Zamani et al., 2011). Nitrogen (N) and phosphorus (P) removal by microalgae in batch cultures of wastewater can be done using immobilized and free-cells techniques. In case the latter technique is used, harvesting free cells in effluent is necessary to improve the quality of the treated wastewater and avoid wash out of the biomass which potentially can be used in food and pharmacy industries and/or as biogas (Zamani et al., 2011).

In this research, we studied the kinetics of N and P elimination as well as simultaneous growth of five microalgae strains in the effluent from a secondary-sewage treatment, under constant conditions of stirring and temperature. The aim of this study was to determine the

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ability of different native strains of microalgae on the removal of N and P by evaluating NO_3^--N and PO_4^{3-}-P loss and biomass productivity.

2. Materials and methods

2.1. Microalgae isolation and cultivation

Five strains of isolated microalgae were used: two strains of *Chlamydomonas* sp., two strains of *Chlorella* sp. and one strain of *Oocystis* sp. Microalgae were isolated from agricultural soil samples of paddy-fields of Fars province, Iran, from May to December 2011, which were named YG01–YG05.

Soil samples were suspended in a specific volume of distilled water. The supernatant was transferred to BG-11 solid culture medium (Zamani et al., 2011), and Petri dishes were stored in a culture room under constant illumination (4150 lx) with white fluorescent lamps at 25 ± 2 °C. After colonization, the isolation and purification were performed using the plate agar method to obtain unialgal cultures (Zamani et al., 2011). The microalgal cells were grown at room temperature in liquid BG-11 medium with shaking at 130 rpm.

2.2. Identification and characterization

The taxonomic identification was done following the keys of Desikachary (1959) and John et al. (2002). In order to determine and confirm the species, the sequence of small subunit of 18S rRNA was studied using molecular markers. Genomic DNA of microalgae strains was prepared according to Rasoul-Amini et al. (2009). DNA fragment of ~700 was amplified from genomic DNA of microalgae strains with polymerase chain reaction (PCR) by using universal primers against the 18 S rRNA genes. The universal eukaryotic primers 5′-GTCAGAGGTGAAATTCTTGGATTTA-3′ as forward primer and 5′-AGGGCAGGGACGTAATCAACG-3′ as reverse primer, amplified a ~700-bp region of the 18S rRNA genes (Zamani et al., 2011). PCR amplifications were determined by 1% (w/v) agarose gel electrophoresis in Tris/Borate/EDTA buffer. A single band of amplified DNA product of ~700-bp was recorded. PCR products were purified form agarose gel and used as templates in sequencing reactions by CinnaGen company. 18S rRNA sequences were an alyzed using the BLAST program, and published in the NCBI databases under the specific accession numbers (Table 1). The isolated microalgae were kept in the liquid nitrogen and lyophilized in order to be added into Microalgal Culture Collection (MCCS) of Shiraz University of Medical Science (Zamani et al., 2011).

2.3. Preparation of municipal wastewater source and experimental condition

Wastewater was collected from secondary effluent of Shiraz, Iran, wastewater refinery. Filtered, and then sterilized by autoclave. Approximately 3.5 ml from each free cells of microalgae were added to 100 ml of autoclaved wastewater in 250 ml Erlenmeyer flasks for each replication (pH=9.0). The same volume of wastewater was added to flasks with no cells of microalgae as the blank treatment. Then, the flasks were placed in culture room at 25 ± 2 °C and constant illumination (4150 lx). All treatments and blank wastewater were conducted in three replicates (18 treatments in total). The experiment lasted 14 days. Using microalgae growth curve, achieving high biomass productivity in microalgae for 14 days was shown. Using a sterile pipette, 2 ml of the wastewater was taken every 4 days for PO_4^{3-}-P and NO_3^--N measurement according to the Ultraviolet Spectrophotometric Screening method for nitrogen with different concentration of KNO_3 as standard and Ascorbic Acid method for phosphorous with different concentration of K_2HPO_4 as standard based on standard methods for the examination of water and wastewater (Greenberg et al., 1998).

Table 1
The published sequences of 18S rRNA of microalgae in the NCBI with their length and accession numbers.

Microalgae	Accession number	Length (base pair)
Chlorella sp. (YG01)	KC456059	613
Chlorella sp. (YG02)	KC456060	620
Oocystis sp. (YG03)	KC456061	336
Chlamydomonas sp.(YG04)	KC456062	502
Chlamydomonas sp. (YG05)	KC456063	560

2.4. Determination of chlorophyll α and β carotene

In the first and last day of experiment 3 ml of the microalgae suspension was taken for Chlorophyll α concentration determination according to the method described by Eijckelhoff and Dekker (1997). Suspension of microalgae were centrifuged at 2500 rpm at 4 °C for 10 min and then rinsed with acetone (80%), then centrifuged again and analyzed using the mentioned method.

Beta-carotene concentration determined according to the n-hexane method (Eijckelhoff and Dekker, 1997). In the first and last day of experiment 1 ml of the microalgae suspension was taken and centrifuged at 3000 rpm at 4 °C for 5 min and then rinsed with ethanol/n-hexane (2/1) and distilled water respectively and analyzed with n-hexane method (Eijckelhoff and Dekker, 1997).

2.5. Statistical analyses

For all statistical analyses Paired-Samples *T* Test, SPSS Statistics Software Version 19.0 was used. The mean, confidence interval, *P* value and standard deviation values of the triplicates for each treatment were calculated. The effects caused by urban wastewater on the growth of several types of microalgae and nutrients removal cultivated in free state were evaluated and statistical significance of all treatments removal were evaluated.

3. Results and discussion

3.1. Identification of microalgae

The identified microalgal strains, based on chemotaxonomic and 18S rRNA data, cultivated in sterile BG-11 medium for the purpose of this study, consisted of *Chlorella* sp. (YG01), *Chlorella* sp. (YG02), *Oocystis* sp. (YG03), *Chlamydomonas* sp. (YG04) and *Chlamydomonas* sp. (YG05). The lengths of the 18S rRNA region of five species of microalgae and their specific accession numbers are shown in Table 1.

3.2. Removal of nitrogen-nitrate under batch culture conditions

The concentration of NO_3^--N and percent of changes during different periods of time, and the whole period of the experiment are given in Tables 2 and 3 (positive sign indicates the decrease and negative sign indicates increase in NO_3^--N concentration). The changes in measured data are shown in Fig. 1.

The initial concentration of NO_3^--N in the wastewater was 190.7 mg L^{-1} and decreased in almost all treatments to the minimum value of 30.30 mg L^{-1} in *Chlorella* sp. (YG01) with the approximate removal efficiency of 84.11% over 14 days ($P < 0.05$). A continuous increase and decrease in NO_3^--N concentration occurred in blank and samples, respectively, resulting in addition of removal of 84.11% from