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Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater

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ABSTRACT

Two species of microalgae growing as immobilized and free-cells were compared to test its ability to remove N and P in batch cultures of urban wastewater. The best microalgae-cell growth configuration was selected to be tested in bioreactor operated in semi-continuous mode. *Scenedesmus obliquus* showed a higher N and P uptake rate in urban wastewater than *Chlorella vulgaris*. When tested in semi-continuous mode and with the re-calcification of beads, *S. obliquus* was more effective in removing N and P for longer periods (181 h) than batch cultures; fecal coliforms removal was good (95%) although the final concentration was still unsuitable for discharge to natural water bodies. Protein and lipids content analysis suggest that, from a practical point of view, immobilized systems could facilitate the separation of the biomass from the treated wastewater although in terms of nutritional value of the biomass, immobilized systems do not represent an advantage over free-cell systems.

1. Introduction

Secondary effluents from wastewater treatment plants 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. Several types of unit processes exist for the removal of nutrients from wastewater but these are costly and produce high sludge content. Microalgae have been proposed as an alternative biological treatment to remove nutrients (Mallick, 2002). One of the limitations for the development of wastewater treatment systems based on microalgae is the harvest of the biomass at the end of the treatment process.

However, the immobilization of cells can represent an alternative for solving the problem as well as providing advantages such as an increase in the cell retention time within bioreactors and higher metabolic activity (Tam et al., 1994). Calcium alginate is commonly used for immobilizing microalgae and maintains the high viability of cells for extended periods of time. However, the matrix is vulnerable to the presence of chelating agents present in wastewater, such as phosphate and citrate, which affect the strength of the gel matrix and, ultimately, dissolves it (Jimenez-Perez et al., 2004). Nevertheless, this problem can be resolved with the re-calcification of alginate beads (Smidsrod and Skjak-Braek, 1990).

Studies on nutrient removal from urban wastewater by immobilized microalgae are limited and include studies on *Chlorella* immobilized in alginate (Lau et al., 1997); *Scenedesmus obliquus* immobilized in k-carrageenan (Chevalier and De la Noüe, 1985a) and *Scenedesmus intermedius* immobilized in calcium alginate (Jimenez-Perez et al., 2004). These studies have evaluated only the quality of the final effluent, and few have determined the nitrogen incorporation efficiency as protein, as well as the lipid content. Immobilized cells could be used as animal feed or as source of high-value chemicals if the biomass is harvest, along with nutrient removal from wastewater (Nuñez et al., 2001).

Therefore, in the present study the growth rates of *Chlorella vulgaris* and *S. obliquus* on urban wastewater were determined; their N–NH_4 and P–PO_4 removal capacity in free-cells and immobilized cells reactors and the protein and lipid content of each species was analyzed. The feasibility of semi-continuous cultures using immobilized cells in alginate beads with re-calcification to prolong the stability of the matrix was also evaluated.

2. Methods

2.1. Routine algal culture and acclimatization

Two species of microalgae were used: *S. obliquus*, isolated from a hypereutrophic soil and *C. vulgaris*, isolated from agricultural soil,

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both maintained as strains in the culture collection of the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE). For initial experiments, artificial wastewater with the following composition was prepared (mg L^{-1}): NaCl, 7 mg; $CaCl_2$, 4; $MgSO_4 \cdot 7H_2O$, 2; KH_2PO_4, 15 and NH_4Cl, 115. These concentrations were used simulating the mean values of the secondary effluent from the Universidad Autónoma de Baja California (UABC) wastewater treatment plant: $N–NH_4^+$: 32.5 mg L^{-1}; $N–NO^{-3}$: 2.0 mg L^{-1} and $P–PO_4^3$: 2.5 mg L^{-1}. Trace metals and vitamins were added following guidelines for "*f*/2" medium preparation (Guillard and Ryther, 1962). During acclimatization (1 month), the microalgae were transferred to fresh artificial wastewater every seven days. The artificial wastewater was only used for the acclimatization of cells and for direct comparison with real urban wastewater. For all other experiments, real secondary effluent from the UABC's wastewater treatment was used; due to its nature, there was large variation in the concentration of nutrients so percentages of removal were used to determine removal efficiency.

2.2. Preparation of immobilized algal beads

Once the microalgae were acclimatized, algal cells were harvested by centrifugation at 3500 rpm for 10 min. The cells were resuspended in 50 mL of distilled water to form a concentrated algal suspension with a cell density of 10×10^7 cells mL^{-1}. The algal suspension was then mixed with a 4% sodium alginate solution in 1:1 volume ratio to obtain a mixture of 2% algae–alginate suspension. The mixture was transferred to a 50 mL burette and drops were formed when "titrated" into a calcium chloride solution (2%). This method produced approximately 6500 uniform algal beads of approximately 2.5 mm diameter with an initial cells number of 3.5×10^5 cells $bead^{-1}$ for every 100 mL of the algae–alginate mixture. The beads were kept for hardening in the $CaCl_2$ solution for 4 h at 25 ± 2 °C, then rinsed with sterile saline solution (0.85% NaCl) and subsequently with distilled water. A concentration of 2.6 beads per ml of wastewater (equivalent to 1:25 bead:wastewater v/v) were placed in bioreactors made of transparent polyethylene terephthalate (PETE) containing 2.5 L of artificial wastewater or urban wastewater. The approximate volume of each bead was 0.01538 mL.

2.3. Batch cultures

Stock suspensions of *C. vulgaris* and *S. obliquus* were cultivated in 3 L bioreactors containing 2.5 L of artificial wastewater at 25 ± 1 °C and light intensity of 135 μE m^{-2} s^{-1}. The bioreactors were aerated to keep free-cells and immobilized beads in suspension and in completely mixed conditions. The following configurations were set up: free-cells in artificial wastewater (AWF); free-cells in urban wastewater (UWF); immobilized cells in artificial wastewater (AWI); immobilized cells in urban wastewater (UWI); a control (urban wastewater without microalgae). All experiments were run three times.

At 6 h intervals, 150 mL of wastewater were collected for analysis during 48 h runs. $N–NH_4$, $N–NO_3$, $P–PO_4$ and pH concentrations were determined according to standard methods (APHA, 1995). The number of algal cells in the beads were determined with particle counter (Beckman Coulter Multisizer 3) after dissolving one bead in (0.25 M) $Na_2HPO_4 \cdot 7H_2O$ solution (pH 7.0) and 0.5 mL of lugol. The determination of chlorophyll *a* in free and immobilized cells was carried out according to Parsons et al. (1984). The cultures were filtered through a glass fiber filter (Osmonics GF/C) of 2.4 cm in diameter. Filters were plunged into tubes containing 5 mL of 90% acetone and kept in darkness at 4 °C during 24 h for pigment extraction; subsequently, chlorophyll *a* concentration was determined spectrophotometrically.

For the lipids and protein analysis (expressed in percentage based on the dry organic weight), five alginate beads were dissolved and subsequently filtered through a GF-C glass fiber filter (2.5 cm diameter), previously rinsed with distilled water, and incinerated at 470 °C for 4 h. After filtering, the samples were stored at −20 °C as recommend by Cordero-Esquivel et al. (1993) and proteins and lipids extraction was done directly on this algal concentrate obtained from filtration. Proteins were extracted with NaOH, as proposed by Correa-Reyes (1996) and then analyzed according to Lowry et al. (1951) with bovine albumin (98%) as standard. Lipids were extracted following the methodology of Bligh and Dyer (1959) and their quantification was estimated by the method of Pande et al. (1963) using tripalmitin (99%) as standard. For the determination of ash-free dry weights, five beads were dissolved and filtered through a GF-C glass fiber filter as previously described. The samples were dried at 120 °C and put to constant weight for 2 h in a conventional oven and then in a muffle furnace at 450 °C for 3 h.

2.4. Semi-continuous cultures

Once the best microalgae-growth configuration for urban wastewater treatment was established, this was chosen to be used in bioreactors running under the same conditions as batch cultures but in semi-continuous mode. Moreover, photosynthesis–irradiance curves suggested an optimum light intensity of 200 μE m^{-2} s^{-1} to be used. The beads were initially incubated for 48 h in wastewater without solution replacement (pre-cycle). Later the solution was decanted for analysis and fresh wastewater was added for further incubation for 35 h (subsequent cycles). This procedure was repeated for six consecutive runs and samples were collected every 24 h and at the end of each cycle (35 h) and analyzed for nutrients, chlorophyll, protein and lipids content as explained earlier. Two series of reactors were set, one with artificial wastewater (AWI) and another with urban wastewater (UWI). Experiments were run three times.

3. Statistical analyses

For all statistical analyses, STATISTICA 5.0 software was used. The mean, confidence interval and standard deviation values of the triplicates for each treatment were calculated. The effects caused by two substrates (artificial wastewater and urban wastewater) on the growth of both types of microalgae and nutrients removal cultivated in free and immobilized state were evaluated by analysis of covariance (ANCOVA) at $P \leqslant 0.05$. The Tukey Test at $P \leqslant 0.05$ was applied when results showed significant differences.

4. Results and discussion

4.1. Algal growth under batch culture conditions

Free and immobilized cells growth for both types of microalgae followed an exponential model (Fig. 1A and B). The lag phase in free-cell cultures was shorter for *S. obliquus* (8 h) than *C. vulgaris* (20 h) for both AWF and UWF (Fig. 1A), suggesting that *S. obliquus* had a greater adaptation and viability than *C. vulgaris* in urban wastewater, as reported by Martinez et al. (2000). In the present study, both species of microalgae immobilized in alginate showed growth immediately after the beads were added in the medium (Fig. 1B) unlike other studies where immobilized cells showed a longer lag period compared with free-cells (Chevalier and De la Noüe, 1985a; Lau et al., 1997).

Growth rates for free-cell cultures of *C. vulgaris* cultivated in AWF and UWF did not show significant differences ($P = 0.975$)