Effect of alginate structure and microalgae immobilization method on orthophosphate removal from wastewater

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Abstract Microalgae play an important role during the
tertiary treatment of municipal wastewater. Cell immobili-
zation techniques have been developed in order to improve
the quality of the treated wastewater and avoid wash out of
the biomass. Since cell immobilization method may affect
the nutrient removal efficiency, ten strains of microalgae
were immobilized in sodium alginate gel in different-
diameter circular screens, and orthophosphate removal
efficiency from municipal wastewater was studied. Results
indicate that the alginate immobilization screen size and
contact surface with wastewater affects the microalgae
synthesis activity and thus orthophosphate removal effi-
ciency. Increasing the contact surface by making smaller
alginate screens will increase the cation exchange rate and
reduce the orthophosphate concentration in the medium.
Among all microalgae treatments, *Scenedesmus rubescens*
MCCS 018, *Chlamydomonas* sp. MCCS 026, and *Chroo-
coccus dispersus* MCCS 006 had the highest PO_4^{3-}-P
removal efficiency of 68.8%, 71.9%, and 72.3% within
12 days.

Introduction

Wastewater treatment using microalgae has been studied
widely and shown to be effective in nutrient removal (e.g.,
Aslan and Kapdan 2006; de Bashan et al. 2008; Zhang et
al. 2008). The technology of using microalgae in wastewa-
ter treatment is based on natural ecosystems; thus it is not
environmentally dangerous and even if the biomass
produced is reused, causes no secondary pollution. Like-
wise, microalgae are highly effective at purifying the waste
by producing oxygen and removing heavy metals and
xenobiotic substances (Martínez et al. 2000).

Harvesting free cells from 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 (Rasoul-Amini et al. 2009; Muñoz
and Guieysse 2006; Mallick 2002). Biomass immobilization
is an efficient way of retaining biomass during wastewater
treatment (Nicolella et al. 2000), and microalgae immobili-
zation in polymeric material such as carrageenan, chitosan, or
alginate has been reported (Chevalier and De la Noüe 1985;
Lau et al. 1995; Robinson 1998). Alginate possesses the ideal
immobilization matrix specification (Martinsen et al. 1989;
Moreno-Garrido 2007; Moreira et al. 2006). Immobilization
also enhances nutrient removal (Mallick 2002; Tam and
Wong 2000; Jiménez-Pérez et al. 2004). Lau et al. (1997)
immobilized *Chlorella vulgaris* in carrageenan and alginate to
treat primary domestic wastewater. Free and immobilized
cells removed about 95% and 50% of ammonium and 99%
and 50% of phosphates from the wastewater in 3 days. Fierro
et al. (2008) immobilized *Scenedesmus* sp. cells in chitosan.

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Free-living cells removed 20% and 30% of nitrates and phosphates within 36 h while immobilized cells removed 70% of nitrate and 94% of phosphate within 12 h of treatment. The physical shape of the immobilization system's beads or screens should be considered as a technical parameter in this technologically oriented field (de Bashan and Bashan 2010). Kaya (1995) investigated the capacity of *Scenedesmus bicellularis* free and immobilized cells in alginate beads and screens to treat municipal wastewater. He found that higher nutrient removal efficiency was obtained by using immobilized cells on screens compared with free cells or bead-shaped alginate particles. Zhang et al. (2008) immobilized different cell density of *Scendesmus* sp. in calcium alginate screens of 2 or 3 mm thickness and measured nitrogen and phosphorus removal rates from artificial and real domestic secondary effluents. They included that cell density in gel is the key factor in the nutrient removal rather than gel thickness and that increasing cell density in the gel would decrease the removal efficiency.

The widely used microalgae cultures for nutrient removal are species of *Chlorella*, *Scenedesmus*, *Chlamydomonas,* and *Phormidium* (Aslan and Kapdan 2006; Mallick 2002). Alginate-immobilized *C. vulgaris* was more efficient than *Scenedesmus bijugatus* in removing N and P from wastewater (Megharaj et al. 1992). Shi et al. (2007) investigated nitrogen and phosphorus removal of *C. vulgaris* and *Scenedesmus rubescens* from secondary synthetic wastewater using the twin-layer immobilization system. These two algae removed ammonium, nitrate, and phosphate to less than 10% of their initial concentration within 9 days.

In this work, ten microalgae species were immobilized in sodium alginate gel in circular screens formed in different diameters, and orthophosphate removal efficiency from treated municipal wastewater of Shiraz were studied.

Materials and methods

Microorganism preparation

Microalgae were isolated during a screening program from soil samples collected from soil samples of paddy-fields of Fars province, south of Iran, from April to December 2004. Soil samples were suspended in a specific volume of distilled water. Surficial part (100 μL) was transferred to BG-11 solid culture medium (Borowitzka 1988), and Petri dishes were stored in a culture room under constant illumination (~25 μmol photons $m^{-2}s^{-1}$) with white fluorescent lamps at 25±2°C. After colonization, the isolation and purification were performed using plate agar method to obtain unialgal cultures (Ghasemi et al. 2003).

The algae were grown at room temperature in liquid BG-11 medium with shaking at 70 rpm. The taxonomic identification was done following the keys of Desikachary (1959) and John et al. (2003). In order to confirm and determine the species, the sequence of small subunit of rRNAs was studied using molecular markers. Genomic DNA of microalgal strains was prepared according to Rasoul-Amini et al. (2009). DNA fragments of ~800 and ~700 bp were amplified from genomic DNA of microalgal strains with polymerase chain reaction (PCR) by using universal primers against the 16S/18S rRNA genes, respectively. The forward 16S universal primer was 5′-CAGCCGCGGTAATAC-3′ and 5′-ACGGGCGGTGTG TAC-3′ using as the reverse primer (Billi et al. 2001). The universal eukaryotic primers 5′-GTCAGAGGTGAA ATTCTTGGATTTA-3′ as forward primer and 5′-AGGG CAGGGACGTAATCAACG-3′ as reverse primer, amplify a ~700-bp region of the 18S rRNA gene (Ghasemi et al. 2008). PCR amplifications were determined by 1% (*w/v*) agarose gel electrophoresis in Tris/Borate/EDTA buffer. PCR products were purified form agarose gel with the CoreBio PCR purification kit (Cat No. GE-100) and used as templates in sequencing reactions by CinnaGene Company. 16S/18S rRNA sequences were analyzed by using the BLAST program, and annotations of all microalgal strains were deposited in GeneBank (Table 1). Then, the isolated microalgae were kept in the liquid nitrogen and lyophilized in order to add into Microalgal Culture Collection of Shiraz University of Medical Science (MCCS) (Rasoul-Amini et al. 2010).

The so identified strains were *C. vulgaris* (MCCS 011, 013, 014, and 015), *Synechococcus* sp. (MCCS 034)*, Fischerella ambigua* (MCCS 004), *Oocystis* sp. (MCCS 033)*, Chroococcus dispersus* (MCCS 006)*, Chlamydomonas* sp. (MCCS 026), and *S. rubescens* (MCCS 018; Table 1).

Cell immobilization

After 10 days of cultivation, cell number was determined using Neubauer hemocytometer, and $6×10^7$ cells mL^{-1} of each strain were centrifuged at 2,000×*g* at 4°C for 25 min and washed twice in sterile saline (0.85% NaCl solution). The cells were suspended in 15 mL of saline and then mixed with 60 mL of 4% sodium alginate solution using magnetic stirrer for 15 min. Circular alginate screens were formed adding the algae–gel mixture to sterile 2% calcium chloride in a Petri dish for 1 h. For every replication of treatments, 20 mL of the alginate mixture was used. In the *C. vulgaris* MCCS 011, *C. vulgaris* MCCS 013, and *C. vulgaris* MCCS 014 treatments, this volume of gel formed one alginate screen with a height of 3–4 mm and mean diameter of 4.3 cm. For the other strains, 20 mL of the gel was divided and formed four smaller screens (5 mL of alginate mixture each) with a diameter of approximately 2.5 cm and 2–3 mm thickness. To investigate the effect of alginate, four blank alginate screens (5 mL of alginate gel each) were formed for

Table 1 The isolated microalgal strains and GenBank accession number and the number and volume of alginate gel used for immobilized screens in each replication of each treatment

Treatment	Accession number	No. of alginate screens	Volume of alginate gel in screen (mL)
Alginate		4	5
Oocystis sp. MCCS 033	GQ414516	4	5
C. vulgaris MCCS 011	EU374169	1	20
C. vulgaris MCCS 013	EU374171	1	20
C. vulgaris MCCS 014	EU375837	1	20
C. vulgaris MCCS 015	EU379938	4	5
Synechococcus sp. MCCS 034	GQ414517	4	5
Chlamydomonas sp. MCCS 026	FJ864686	4	5
F. ambigua MCCS 004	EU192145	4	5
C. dispersus MCCS 006	EU192147	4	5
S. rubescens MCCS 018	EU621364	4	5

every replication of alginate treatment (Table 1). Then, the screens were washed twice with sterile distilled water.

Municipal wastewater source and experimental condition

Wastewater from secondary effluent of Shiraz, Iran, wastewater refinery was collected, filtered, and then sterilized by autoclaving. Immobilized microalgae in alginate screens (ten strains/species) and also blank alginate screens were added to 500 mL of autoclaved wastewater in Erlenmayer flasks for every replication. The same volume of wastewater was added to flasks with no alginate screens and no microalgae as the Blank treatment. Then, the flasks were placed in culture room at 20±1°C and irradiance of approx. 25 μmol photons $m^{-2}s^{-1}$. All treatments and blank wastewater were in three replicates (12 treatments in total).

The experiment lasted 12 days. Using a sterile pipette, 10 mL of the wastewater was taken every 4 days for PO_4^{3-}-P measurement according to the Murphy–Riley method based on standard methods for the examination of water and wastewater (American Public Health Association 2005). The results were analyzed using F test at $P \leq 0.05$.

Results

The concentration of PO_4^{3-}-P and percent of changes during every 4 days, 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 PO_4^{3-}-P concentration). The trend of changes in measured data is shown in Fig. 1.

Table 2 Orthophosphate concentration in the treatments during the experiment (mg L^{-1})

Treatment	PO_4^{3-}-P Concentration±SD			
	1	4	8	12[a]
Blank	1.37±0.05	1.10±0.01	1.03±0.04	1.03±0.05
Alginate	1.37±0.05	1.88±0.06	1.85±0.13	1.99±0.43
C. vulgaris MCCS 011	1.37±0.05	0.85±0.01	0.63±0.05	0.43±0.07
C. vulgaris MCCS 013	1.37±0.05	1.16±0.09	0.64±0.05	0.44±0.05
C. vulgaris MCCS 014	1.37±0.05	1.14±0.25	0.82±0.08	0.62±0.04
C. vulgaris MCCS 015	1.37±0.05	1.51±0.11	0.77±0.03	0.44±0.03
F. ambigua MCCS 004	1.37±0.05	1.61±0.05	0.97±0.09	0.65±0.26
Oocystis sp. MCCS 033	1.37±0.05	1.38±0.06	0.71±0.05	0.50±0.01
S. rubescens MCCS 018	1.37±0.05	1.62±0.17	0.84±0.13	0.43±0.07
C. dispersus MCCS 006	1.37±0.05	1.56±0.27	0.62±0.11	0.39±0.10
Synechococcus sp. MCCS 034	1.37±0.05	1.58±0.06	0.93±0.10	0.45±0.06
Chlamydomonas sp. MCCS 026	1.37±0.05	1.51±0.01	0.71±0.04	0.38±0.02

[a] Elapsed times (days)

Table 3 Percent of changes in PO_4^{3-}-P concentration of treatments in different periods of time (%)

Treatment	Percent of changes (%)			
	1–4	4–8	8–12	1–12[a]
Blank	19.57	6.13	0.68	25.02
Alginate	−37.73	1.58	−7.72	−46.01
C. vulgaris MCCS 011	37.92	26.23	30.83	68.32
C. vulgaris MCCS 013	14.75	44.91	31.31	67.74
C. vulgaris MCCS 014	16.40	27.98	25.27	55.01
C. vulgaris MCCS 015	−10.34	49.02	42.65	67.74
F. ambigua MCCS 004	−18.09	39.85	32.85	52.30
Oocystis sp. MCCS 033	−1.15	48.43	29.71	63.34
S. rubescens MCCS 018	−18.89	48.32	49.03	68.68
C. dispersus MCCS 006	−13.84	60.09	39.13	72.35
Synechococcus sp. MCCS 034	−15.68	41.32	51.08	66.79
Chlamydomonas sp. MCCS 026	−10.26	52.70	46.14	71.91

[a] Elapsed times (days)

The initial concentration of orthophosphate in the wastewater is 1.37 mg L^{-1} and decreased in almost all treatments to the minimum value of 0.38 mg L^{-1} in Chlamydomonas sp. MCCS 026 and 0.39 mg L^{-1} in C. dispersus MCCS 006 with the approximate removal efficiency of 72% over 12 days. A continuous increase and decrease in orthophosphate concentration occurred in Alginate and Blank, respectively, resulting in addition of 46% of PO_4^{3-}-P to and removal of 25% from the medium. In all treatments, except C. vulgaris MCCS 011, MCCS 013, and MCCS 014, orthophosphate concentration increases during the first 4 days and then decreases. In the C. vulgaris, MCCS 011, MCCS 013, and MCCS 014 treatments depletion of orthophosphate occurred over the whole period of the experiment.

For better comparison, orthophosphate removal rate was calculated by the following equation:

$$R_P = \frac{C_m - C_f}{t_m - t_f} \quad (1)$$

Where R_P is PO_4^{3-}-P removal rate (mg L^{-1} day^{-1}), C_m is the maximum P concentration (mg L^{-1}) during the experiment, C_f is the final P concentration (mg L^{-1}), t_m is the time of maximum P concentration in the medium, and t_f is the final day of the experiment. Results are given in Table 4. In Blank, C. vulgaris MCCS 011, MCCS 013, and MCCS 014 treatments, the duration of orthophosphate depletion is 12 days. Within this period, more orthophosphate is removed in the treatments containing microalgae in comparison with the Blank treatment. Among these four treatments, the highest and lowest removal rates are by C. vulgaris MCCS 011 (0.078 mg L^{-1} day^{-1}) and Blank (0.028 mg L^{-1} day^{-1}), respectively. In the treatment containing blank alginate screens, there is an increase in PO_4^{3-}-P

concentration with the mean rate of 0.052 mg L^{-1}day^{-1}. In other microalgal treatments, the PO_4^{3-}-P removal period is 8 days, and the removal rate varies from the lowest 0.110 mg L^{-1}day^{-1} for Oocystis sp. MCCS 033 to the highest 0.149 mg L^{-1}day^{-1} for S. rubescens MCCS 018. The differences between the Microalgae treatments and Blank are statistically significant at the 5% level (Table 5).

Discussion

Wastewater samples were collected from secondary effluent of the wastewater treatment plant, before chlorination, so autoclaving was necessary to eliminate bacteria and pathogens. According to Hernandez et al. (2006), wastewater autoclaving causes the precipitation of orthophosphate. Since the concentration of calcium compounds in Shiraz wastewater is high, calcium phosphate complexes formed by autoclaving and remained in the samples.

The 25% of decrease in orthophosphate concentration in Blank treatment during the experimental time is due to the continual precipitation of orthophosphate.

In Alginate treatment, the 38% of increase in orthophosphate concentration occurred during the first period (days 1–4), and the rest of the total increase (8%) occurred in 8 days. The PO_4^{3-}-P concentration increased as a result of alginate presence in the medium. As described, the alginate screens were formed by adding the gel into calcium chloride solution (2%), and the screen was hardened as a result of cation exchange between the sodium ions in the structure of alginate (CyberColloids 2010) and calcium ions of the solution. Since the screen hardening process lasted 1 h, the internal sodium ions had not been exchanged by calcium ions and the inner
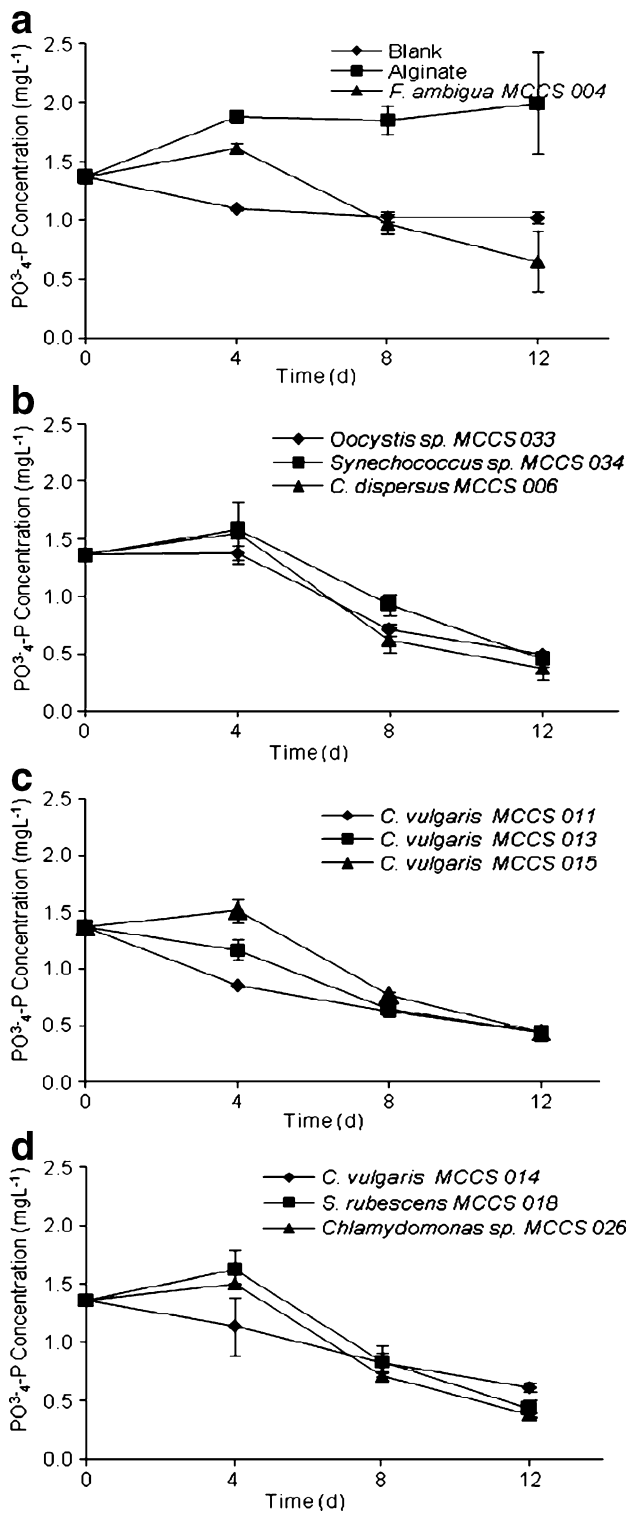
Fig. 1 Orthophosphate concentrations of treatments in wastewater; **a** Blank, Alginate, *F. ambigua* MCCS 004; **b** *Oocystis* sp. MCCS 033, *Synechococcus* sp. MCCS 034, *C. dispersus* MCCS 006; **c** *C. vulgaris* MCCS 011, *C. vulgaris* MCCS 013, *C. vulgaris* MCCS 015; **d** *C. vulgaris* MCCS 014, *S. rubescens* MCCS 018, *Chlamydomonas* sp. MCCS 026. Error bars=mean±SD, *n*=3

screens layer had cation exchange capacity. By adding the screens into the wastewater, the cation exchange process continued and caused the re-solution of the precipitates, accumulation of Ca^{2+} into the alginate matrix, abandonment of PO_4^{3-}-P, and finally increase of orthophosphate concentration in the medium. Since there is no microalga in alginate screens of Alginate treatment to consume orthophosphate, the cation exchange between the precipitates and alginate matrix continued until the 12th day resulting in an increase of PO_4^{3-}-P concentration.

Inorganic nutrient ions such as phosphate would be available to the immobilized algae as freely as to their free counterparts (Chevalier et al. 2000). Jiménez-Pérez et al. (2004) measured the amount of P entrapped by 1,500 beads without algae during the experiment and concluded that the Ca–alginate matrix accumulated a maximum of ca. 100 mg P per culture in the first 10 h. In this work, as the P content of alginate screens was not measured, the effect of alginate in orthophosphate removal cannot be discussed.

In most of the microalgae treatments, PO_4^{3-}-P concentration increased in the first period, but it is 50 to 70% less than the increase of orthophosphate in Alginate treatment. In spite of the difference in immobilization method, the existence of microalgae caused this variation. While the precipitates were re-solved by alginate, microalgae biosorbed the orthophosphate, therefore the orthophosphate concentration in the microalgae treatments is less than alginate treatment.

According to Table 3, there is a difference in P removal efficiency of microalgae treatments in the first period. In the three treatments of *C. vulgaris* MCCS 011, MCCS 013, and MCCS 014, PO_4^{3-}-P concentration decreased during the first 4 days whereas, in other treatments, it increased. This difference is related to the immobilization method and contact surface of the screens with the wastewater. In the *C. vulgaris* MCSS 011, MCCS 013, and MCCS 014 treatments, 20 mL of alginate gel formed one circular screen with an approximate diameter of 4.3 cm and thickness of 3–4 mm while, in other immobilized microalgae treatments and also blank alginate treatment, this volume of gel formed four smaller and thinner screens with a mean diameter of 2.5 cm. The contact surface of alginate screens with the media is calculated according to the following equation:

$$A = 2n\pi(r^2 + rh) \qquad (2)$$

Table 4 Period of reduction and removal rate of orthophosphate from wastewater in treatments

Treatment	Duration of decrease (d)	Removal rate (mg L^{-1} day^{-1})
Blank	12	0.028
Alginate	12	−0.052
C. vulgaris MCCS 011	12	0.078
C. vulgaris MCCS 013	12	0.077
C. vulgaris MCCS 014	12	0.062
C. vulgaris MCCS 015	8	0.134
F. ambigua MCCS 004	8	0.120
Oocystis sp. MCCS 033	8	0.110
S. rubescens MCCS 018	8	0.149
C. dispersus MCCS 006	8	0.146
Synechococcus sp. MCCS 034	8	0.141
Chlamydomonas sp. MCCS 026	8	0.141

Where A is the surface area (cm^2), r is the radius (cm), h the thickness of screen (cm), and n number of screens in the flask. The contact surface of alginate screens in the *C. vulgaris* MCCS 011, MCCS 013, and MCCS 014 treatments is 123.74 cm^2, and in the other treatments, it is 175.70 cm^2.

The smaller contact surface of alginate screens in the *C. vulgaris* MCCS 011, MCCS 013, and MCCS 014 treatments means a smaller cation exchange at the surface of the screens, so that, within 4 days, the precipitate re-solution process had been completed; meanwhile, the microalgae had biosorbed the PO_4^{3-}-P from the medium. In the other treatments, the increase of PO_4^{3-}-P concentration continued after day 4 because of the higher contact surface of the alginate screens, and the P removal occurred later.

Table 5 Statistical analysis (F test) results of orthophosphate concentration in alginate and microalgae treatments compared with Blank treatment (Wastewater flasks containing no microalgae or alginate screen)

Microalgae	P value
Alginate	0.1957
C. vulgaris MCCS 011	0.0829
C. vulgaris MCCS 013	0.0686
C. vulgaris MCCS 014	0.1311
C. vulgaris MCCS 015	0.0469
F. ambigua MCCS 004	0.0724
Oocystis sp. MCCS 033	0.0620
S. rubescens MCCS 018	0.0394
C. dispersus MCCS 006	0.0334
Synechococcus sp. MCCS 034	0.0476
Chlamydomonas sp. MCCS 026	0.0399

Kaya (1995) reported that immobilization of microalgae in alginate screens enhanced the nutrient removal efficiency compared with free or immobilized cells in alginate beads. Since increasing the number of beads per volume of effluent reduced light penetration and enhanced self-shading effects, the beads settled to the bottom of the reactor (Tam and Wong 2000; Abdel Hameed 2006). The light penetration in algal culture is affected directly by incident light and inversely by the depth and density of the algal culture (Zhang et al. 2008). Cell colonies located near the surface of the bead shade the internal ones, which produces a limitation of photosynthesis, thus decreasing nutrient uptake rate (Kaya 1995; Jiménez-Pérez et al. 2004). Microalgae immobilized in alginate screens receive more light thus algal anabolism and physiological activity would increase. Zhang et al. (2008) concluded that cell density in gel was the key factor affecting nutrient removal efficiency when algal sheet thickness was 2–3 mm.

Based on our results and comparison of orthophosphate removal rates in treatments, it can be concluded that the reduced thickness and increased contact surface area of the alginate screens prevents the self-shading effects of cells thus, increasing algal activity. Cells occupy a significant fraction of the useful volume of the matrix, and the cell density in the surface of immobilized matrix is high (Kuhn et al. 1991), and so the exit route for molecules is longer (Vichez and Vega 1994). Thus, as the surface area increases, more microalgae cells can contribute to nutrient removal and enhance the removal efficiency and rate.

The rates obtained here are markedly lower than those previously published as the initial cell density and immobilization method differed. Lau et al. (1997) obtained removal rates with *C. vulgaris* of 0.42 and 0.41 mg P L^{-1} day^{-1} for free and immobilized cells.

In the *S. rubescens* MCCS 018, *Chlamydomonas* sp. MCCS 026, *C. dispersus* MCCS 006, and *C. vulgaris* MCCS 011 treatments orthophosphate removal was statistically significantly different compared with the Blank treatment. Therefore, these strains have potential to be used as purifying agents in tertiary wastewater treatment in photobioreactors or high-rate algal ponds and beside that, produce a biomass which is enriched with proteins, fatty acids, etc., which might be used in the agriculture, pharmaceutical, and food industries.

Further research on initial cell concentration, immobilization method (different beads or screens diameter and/or thickness; hardening period, the solution, and its concentration), percent of alginate used for immobilization, and also initial nutrient concentration and environmental aspects should be done to determine the most effective immobilization shape and optimize the best removal conditions.

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