ENVIRONMENTAL ENGINEERING

Biomass production of Scenedesmus sp and removal of nitrogen and phosphorus in domestic wastewater

INGENIERÍA AMBIENTAL

Remoción de nitrógeno, fósforo y producción de biomasa de Scenedesmus sp en agua residual domestica

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Abstract
Domestic wastewater (DW) has been used as a substrate for both microalgae biomass production and nutrient removal. Biological treatment with photosynthetic microalgae provides aeration, reducing operating costs and the risk of volatilization of contaminants. It also provides oxygen to the bacteria for degradation of organic compounds. In this study, a microalga was isolated and identified as *Scenedesmus sp*. An experimental trial was performed using synthetic wastewater with different concentrations of N (40, 90 and 150 mg/L) and P (4, 15 and 50 mg/L). Each assay was inoculated with $1x10^6$ cells/ml under 16h of continuous light at 50-µmol m^{-2} s^{-1} $_{at}$ and 120 rpm for 7 days. Samples were taken at 0, 3, 5 and 7 days to determine the growth of microalgae and the concentration of nitrates, ammonium and phosphorus. The same treatment was carried out using real DW. Synthetic water of low and medium concentration had higher removal percentages. These were between 50 and 60 for nitrogen and 40 and 70 for phosphorus, with a maximum growth of $1x10^7$ cells /ml. For real DW, the removal was 65% for phosphorus and 80% for nitrogen. These results suggest *Scenedesmus sp* could be used to treat DW, enhancing nutrient removal and obtaining biomass for other purposes.

Keywords: *Biomass, microalgae, nutrients, wastewater.*

Resumen
Las aguas residuales domésticas se han utilizado como sustrato para la producción de biomasa de microalgas y eliminación de nutrientes. El tratamiento biológico con microalgas fotosintéticas proporciona aireación, reduciendo los costos de operación, el riesgo de volatilización de los contaminantes y proporciona oxígeno a las bacterias para la degradación de compuestos orgánicos. En este estudio se aisló una microalga a partir de aguas naturales y fue identificada como *Scenedesmus sp*. Se realizó un diseño experimental usando agua residual sintética con diferentes concentraciones de nitrógeno (40, 90 y 150 mg / L), fósforo (4, 15 y 50 mg / L) y la microalga aislada. Cada ensayo se inoculó con $1x10^6$ células/ml bajo 16 horas de iluminación a 50-µmol m^{-2} s^{-1} por 7 días a 120 rpm. Se tomaron muestras a 0, 3, 5 y 7 días para determinar el crecimiento de microalgas y la concentración de nitratos, amonio y fósforo. El mismo tratamiento se realizó utilizando agua residual domestica real. Las aguas sintéticas de baja y media concentración tuvieron mayores porcentajes de remoción, entre 50-60% para el nitrógeno y 40-70% para el fósforo, con un crecimiento máximo de $1x10^7$ células/ml. En el agua residual real, la remoción fue del 65% para el fósforo y el 80% para el nitrógeno. Estos resultados sugieren *Scenedesmus sp* podría ser utilizada para tratar agua residual doméstica, mejorando la eliminación de nutrientes y obteniendo biomasa para otros fines.

Palabras clave: *Aguas residuales, biomasa, microalgas, nutrientes.*

1. Introduction

Microalgae are photosynthetic microorganisms that have received special attention in recent years due to their potential for producing compounds of high biotechnological interest such as vitamins, proteins, cosmetics and health food (Harun et al., 2010). Microalgae have also been studied in relation to biofuel production since the oil content of their biomass can reach up to 80% of its dry weight (Caporgno et al., 2015; Chisti, 2007). It has been estimated that algal production can be as much as 3200-14600 gallons of lipid per acre per year. This is 130 times more than that produced by soybeans (Hu et al., 2008), which is the main crop used for the production of biofuels (Li. et al., 2011; Rawat et al., 2013). Another advantage of microalgae is that they do not represent competition as a food source and can capture CO2, so are an environmentally friendly option for biodiesel production (Honda et al., 2012).

There are numerous reports of the identification of microalgae strains able to store large amounts of lipid suitable for biodiesel production (Nwokoagbara et al., 2015). However, the main challenge for obtaining biofuels from these organisms is the high cost of production. This is due to the amount of water and nutrients needed during the growing process, often meaning that the energy produced is less than the energy expended (Kwietniewska & Tys, 2014). To improve the cost-effectiveness of the production of biodiesel from microalgae, the use of domestic wastewater (DW) has been proposed as a culture medium in order to integrate several processes: The production of biomass for biofuels, CO2 capture and water treatment (Ji et al., 2013; Razzak et al., 2013). The advantage of this approach is that at the same time that the microalgae remove the excess of nutrients in the wastewater, they produce biomass that can be used for the production of biodiesel (Caporgno et al., 2015). The success of this kind of treatment system is based on the ability of microalgae to effectively assimilate both organic carbon (Octavio et al., 2011) and inorganic nutrients such as N and P, maximising algal biomass and lipid production and achieving efficient nutrient removal (Dominguez et al., 2013).

Good results have been reported using wastewater for the production of biodiesel and biomass, but this production is influenced by the type of wastewater, nutrient concentration and kind of microalgae (He et al., 2013). Water bodies exposed to constant wastewater discharges are a good source of microalgae adapted to changing environments and are thus useful for the removal process, and biomass and biodiesel production (Arbib et al., 2014). The goal of this work was to isolate and identify a microalga from natural environments, assess its ability to remove nitrogen and phosphorus concentrations in different synthetic and real wastewaters, and relate this removal with biomass production in order to facilitate future exploitation for other uses (example: lipids, pigments, aquaculture, etc.).

2. Materials and methods

2.1 Sampling and isolation of microalgae

The microalga used in this study was isolated from the Porce II reservoir, located at 75° 09' 14" W and 6° 44' 57" N, in the department of Antioquia, Colombia. It is at an altitude of 870 MASL. The sampling site was chosen because it has high nutrient discharges, which facilitates the isolation of microalgae adapted to polluted environments that do not require pairing for selection. Samples were collected by phytoplankton dragging, stored in clear plastic containers and refrigerated until analysis.

The samples were centrifuged at 5000 rpm for 3 min. The concentrated biomass was diluted in sterile water and filtered through 0.45 μm filters. The filtrate was washed several times with sterile saline solution (0.9%) in order to eliminate bacteria. Serial dilutions were seeded in Bold Basal agar at 30°C. After the incubation time, microalgae with different morphotypes were picked out successively to isolate the different types of algae present in the sample. Each strain was cultured in 250 ml Erlenmeyer flasks with

sterile Bold Basal liquid medium for 10 days in an orbital shaker at 120 rpm with continuous light.

2.2 Assessment of species able to withstand high concentrations of nitrogen and phosphorus

The previously isolated microalgae were grown in sterile synthetic domestic wastewater with a known initial concentration of nitrogen and phosphorous, which was increased to 150 mg/L and 40 mg/L respectively. The tests were performed at 25°C under a light intensity of 50-µmol m^{-2} s^{-1}, with continuous agitation at 120 rpm. Microalgae growth was evaluated optically in order to select the microalgae species able to use high concentrations of nutrients. The best-adapted strain was cultivated three times to determine stable growth conditions.

2.3 Identification of the isolated microalgae

The identification was carried out at morphological and molecular levels. Morphological characteristics such as size, shape and relevant structures were evaluated. To obtain the genomic DNA, the microalga was lysed with liquid nitrogen and extraction was carried out using the ISOLATE Plant DNA Kit II (Bioline, Taunton, MA, USA) according to manufacturer instructions. A portion of gen 18S rRNA was amplified by PCR using the primer forward '-GGTGATCCTGCCAGTAGTCATATGCTTG-3 and primer reverse'-ATCCTTCCGCAGGTTCAC-CTACGGAAACC -3' (Yuan et al., 2011) with a reaction volume of 25 µl. PCR conditions were as follows: An initial cycle at 94 °C for 5 min, followed by 35 cycles at 95°C for 30s, 50°C for 1 min, 72°C for 1 min and, a final extension at 72°C for 10 min. The products were sequenced and identified using the BLAST (Basic Local Alignment Search Tool) program in the GenBank database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

2.4 Assay of nitrogen and phosphorus removal in synthetic domestic wastewater

In order to simulate different types of wastewater a 2^3 design was performed with combinations of nitrogen and phosphorus in accordance with previous reports of these elements in RDW (Wang & Lan, 2011; Li et al., 2011; Ruiz-Marín et al., 2010; Christenson & Sims, 2011). Synthetic domestic wastewater (SDW) was used as culture medium, with the following composition in g/L (Cho et al., 2011): $NaNO_3$ 1.5, K_2HPO_4 0.04, $MgSO_4$ $7H_2O$ 0.075, $CaCl_2$ $2H_2O$ 0.036, Na_2-EDTA 0.001, Na_2CO_3 0.02 mg and 1 mL of trace metals solution in mg/L: H3BO3 61.0, MnSO4-H_2O 169.0, $ZnSO_4$ $7H_2O$ 287, $CuSO_4$-$5H_2O$ 2.5 y $(NH_4)_6MoO_4$-$4H_2O$ 2.5. Water was supplemented with different concentrations and combinations of nitrogen and phosphorus (Table 1) in order to study the removal of these elements and their effect on cell growth.

1) 40 mg N/L and 4 mg P/L	4) 40 mg N/L and15 mg P/L	7) 40 mg N/L and 40 mg P/L
2) 90 mg N/L and 4 mg P/L	5) 90 mg N/L and 15 mg P/L	8) 90 mg N/L and 40 mg P/L
3) 150 mg N/L and 4 mg P/L	6) 150 mg N/L and 9 mg P/L	9) 150 mg $_3$/L and 40 mg P/L

Table 1. *Combinations and concentrations of nitrogen and phosphorus.*

Assays were carried out in cylindrical glass vessels containing 250 ml of synthetic wastewater, and inoculated with 25 ml of microalgae culture at a concentration of $1x10^6$ cells/ml. All trials were performed in triplicate for a duration of 7 days. SDW without inoculation and SDW incubated in darkness were used as control. Samples were taken at 0, 3, 5 and 7 days of the incubation to determine the algal growth, nitrate, ammonium and phosphate concentration.

2.5 Assay of nitrogen and phosphorus removal in real domestic wastewater

To assess the ability of the microalgae to grow in real domestic wastewater (RDW) and remove N and P, a trial using RDW was carried out. The RDW was analyzed for certain physicochemical parameters, the results of which are shown in the table 2. The assays were performed in the same manner as those conducted with SDW.

Table 2. *Physicochemical characterization of real domestic wastewater.*

Parameters	Concentration
Conductivity	19.25 µs/cm
Turbidity	28.9 NTU
COD	62.370 mg / L
BOD_5	24 mg / / L
Alkalinity	181.2 mg / L
Nitrates	0.200 mg / L
Total Solids	234 mg / L
Ammonium	24,798 mg / L
Phosphorus	2,638 mg / L
Total Nitrogen	28,700 mg / L

2.6 Analytical methods

The cell count was determined using a Newbauer chamber. Microalga counts were made in triplicate at 0, 3, 5 and 7 days. The concentrations of nitrate (NO_3-), total phosphorus (TP) and ammonium (NH_4+) were determined by standard protocols APHA, (2012).

2.7 Statistical analysis

The results were analyzed using analysis of variance (ANOVA) and a Tukey test to analyze the relationship between different combinations and concentrations of the biomass of microalgae.

3. Results and discussion

3.1 Isolation and identification of microalgae

25 species of microalgae were found during the isolation process, but only one strain, molecularly identified as *Scenedesmus sp*., was able to grow efficiently at higher concentrations of nitrogen and phosphorus (150 mg/L and 40 mg/L respectively). Therefore, subsequent assays were performed with this strain. (Azim et al., 2002) reported that *Scenedesmus sp* is a microalga common in all types of freshwater bodies that plays an important role as a primary producer and contributes to the recovery of eutrophic waters. Several studies have been conducted with this species in wastewater treatment due to its high percentage of nutrient removal, growth rates and tolerance to changes in wastewater conditions (Ruiz-Marín et al., 2010; Zhang et al., 2008; Martínez et al., 2000).

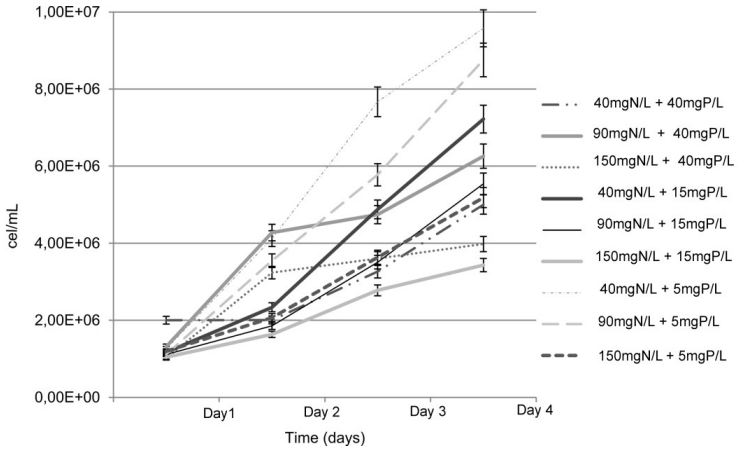

Figure 1. *Algal growth in synthetic domestic wastewater at different concentrations of nitrogen and phosphorus.*

3.2 Microalgal growth during removal of phosphorus and nitrogen in SDW

Scenedesmus sp growth during removal of phosphorus and nitrogen is shown in Figure 1. The best results were obtained using low phosphorus concentration and medium and low nitrogen concentrations, with estimated growth of 1.0 x10^7 cell/ml up to the seventh day. On the other hand, high concentrations of nitrogen and phosphorus

reduced the growth of the microalga by up to 60%. Compared with other studies, the final biomass produced in this work was low (Chinnasamy et al., 2010; Cho et al., 2011; Yujie et al., 2011; Xue et al., 2010; Wang et al., 2010). However, the removals were similar, which indicates that the removal percentage per cell was much higher than in the other studies.

Many environmental parameters such as pH, temperature and light can influence the algal population to increase (Stevenson et al., 1996). One factor influencing the ability to assimilate nutrients is the shortage of CO_2 or carbonates to perform photosynthesis. In this study, CO_2 was not supplied during incubation time; which could represent a limiting factor for the growth of microalgae and explain the low biomass production. Several reports have indicated that the lack of CO_2 influences algal growth (Stevenson et al., 1996).

3.3 Removal of phosphorus and nitrogen in SDW

Removal percentages of inorganic nutrients in synthetic wastewater are shown in Table 3. Statistical analysis indicated dependence between N and P ($p < 0.05$). This suggests that the presence of one nutrient was needed to remove the other, and that the concentration of both nutrients influenced the capacity to obtain higher removal percentages. All combinations have the same tendency, therefore the concentration of both nutrients influenced the obtaining of higher percentages of removal. The best removals of nitrogen (50-65%) and phosphorus (40-70%) were obtained with combinations containing low phosphorus concentration and medium and low nitrogen concentration. With Tukey's test, the concentrations of 40 and 90 mg N / L and 5-15 mg P / L showed no significant difference ($p > 0.05$), reflecting the similarity of the removal percentages (Table 3). High phosphorus concentration (40 mg/L) affected the removal of both nutrients. At this level, removal percentages of phosphorus were 8-13% and of nitrogen 13-26%. With the combination of 40 mg N/L and 40 mg P/L, negative removal percentages were obtained, indicating that at the end of the process the nutrient concentration were higher than at the initial stage. Given that this combination also affected the growth of microalga (Figure 1), this increase in nutrient concentration might be due to the release of compounds resulting from the algae death caused by the inhibitory effect of high concentrations of nutrients, which reduced their metabolism and returned the nutrient content of the cells to the medium by cell lysis.

Table 3. *Ratios of removal at different concentrations of phosphorus and nitrogen.*

mg PO_3 / L	mg NO_3 / L	% P removal	% N removal
40	40	-2.30 (2.91) *	-19.70 (5.29)
40	90	8.80 (5.26)	13.59 (4.97)
40	150	11.98 (4.01)	26.67 (6.73)
15	40	50.92 (2.02)	19.62 (10.44)
15	**90**	**42.36 (5.84)**	13.50 (3.8)
15	150	12.95 (4.51)	35.33 (19.98)
5	**40**	**69.23 (12.63)**	**66.59 (20.08)**
5	**90**	**26.12 (4.26)**	**53.00 (10.37)**
5	150	30.48 (4.12)	18.39 (3.32)

** Represents the coefficient of variance*

The ammonium concentration was not detected (<5mg NH_3+/L) at the third day of assay in all combinations. This suggests that ammonium could have allowed the growth of *Scenedesmus sp*, but inhibited the use of nitrates by microalga (Stewart, 1974), and that nitrates only began to be removed when the ammonium concentration was low.

DW possesses a high variation in the stoichiometric ratio N: P. This can affect microalgae metabolism and growth. *Scenedesmus sp* has a great advantage over other microalgae due to its tolerance to different types of wastewater (Hall, 2009). Its physiological range of N: P is from < 5, under severe N-limitation, to > 100, under severe P-limitation (Geider & La Roche, 2002). The combinations with better removal rates and biomass production were within this range. With high concentration of phosphorus, the N: P ratio was 1:4. This indicates a deficiency of nitrogen, and thus a limiting factor for growth, in accordance with other studies. Additional research obtained similar results with *Scenedesmus sp* in domestic wastewater, with percentages of phosphorus and nitrogen removal

of 30-70% respectively (Ruiz-Marín et al., 2010; Zhang et al., 2008) and a retention time of between 9 and 15 days. In this study, we tried to simulate the retention time of a real wastewater plant treatment process (about 7 days), so the removal percentage was expected to be greater if retention days were increased.

3.4 Removal of phosphorus and nitrogen in real RDW

The removal of phosphorus in RDW was 65 ± 12% and for nitrogen was 80 ± 9%, higher than what was obtained with SDW. The presence of other microorganisms in DW could have influenced nutrient removal and ammonium transformation into nitrates, as evidenced by the increase in nitrate concentration at the end of the trial. The greatest growth of cells was obtained with RDW (2.55x 1010 cells/ml). This might be due to the presence of other micronutrients present in the RDW that stimulated the growth of microalga. RDW was not treated or sterilized, in order to evaluate the influence of microorganisms and economize the removal process. Favorable results were obtained that support the use of the community of *Scenedesmus sp*. for the treatment of actual ARD.

3.5 Evaluation of microalgae-microorganism interaction in the removal of nitrogen and phosphorus.

In wastewater, a microbial flora exists that can influence microalgae metabolic activity. To assess the effect of this flora on N and P removal, a trial incubated in darkness was carried out in order to inhibit metabolism of the algae. Figures 2 and 3 show the effect of other microorganisms on nutrient removal.

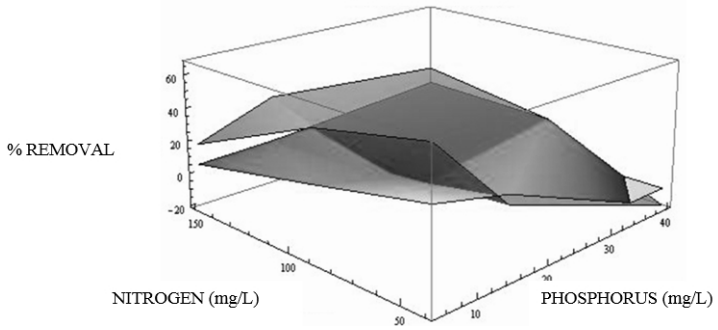

Figure 2. *Nitrogen removal by darkness trial (bottom sheet) and lighted trial (top sheet).*

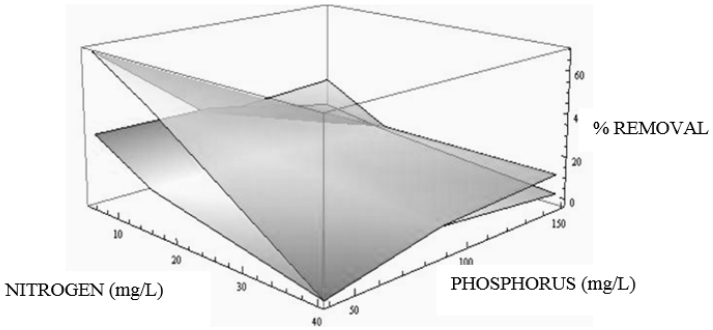

Figure 3. *Phosphorus removal by darkness trial (bottom sheet) and lighted trial (top sheet).*

The percentage of phosphorous removal was higher in the assays performed without inhi-bition of microalgae, which suggests that the microorganisms present in the wastewater had no inhibitory effect on

algae. This was also found by determining the algal biomass, which was not affected during the trial. In assays performed in darkness P removal averaged 30% (Figure 3), indicating that approximately 40% of the total removal of P was produced by the action of the microflora naturally present in the water. However, part of this removal can also be explained by the initial activity of microalgae added. An increase in biomass was not observed in determining the algal growth during this test, but this biomass could be active during the first days.

The study of the interaction, competition and cooperation between other microorganisms and microalgae has been analyzed in different studies. (de-Bashan et al., 2005), found that the biomass of Chlorella vulgaris, as well as the ammonia removal, increased in the presence of Azospirillum brasilense. Nagadomi et al. (2000) and Sawayama et al. (1998) found that certain species of bacteria are able to raise the removal of phosphorus by 90% in the presence of microalgae.

4. Conclusions

The best removal percentages of phosphorus and nitrogen were in RDW with a percentage removal of 65 ± 12% and 80 ± 9% respectively, being higher than that obtained with SDW. Among the different concentrations of N and P in SDW, the highest removal percentages were obtained with medium and low concentration of those nutrients, with removal percentages between 50 and 65%, and 40 and 70% respectively. The maximum algal growth was lower than in other studies, but with similar removal of nitrogen and phosphorus, indicating good removal percentages per cell. *Scenedesmus sp*. proved to have few requirements in terms of maintenance and biomass production, and was shown to be efficient in treating moderate wastewater loads. This research attempted to simulate a real and economic way to culture microalgae, and seek options for nutrient removal and production of biomass that can be used for other activities like aquaculture or lipids.

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