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Comparative study of the efficiency of removal of N and P of municipal wastewater and leached of vermicomposting with five strains of micro algae

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ABSTRACT

The use of microalgae for bioremediation of wastewater and organic leachates has been the subject of numerous investigations due to its capacity to remove significant amounts of nitrates and phosphates. In addition, to generate microalgal biomass with added value for its commercial interest. In this work we studied the removal of N and P municipal wastewater by five species of native microalgae, *Chlorella miniata*, *Coelastrella* sp., *Desmodesmus quadricauda, Neochloris oleoabundans, Verrucodesmus verrucosus*. The experiments in 16 L glass photo bioreactors were carried out in triplicate with 12:12 light: darkness, 25 ± 1°C Every three days, samples of 10 mL were taken in the cultures, to determine the concentration of N, P and cell density. The five-selected species demonstrated high removal efficiency of N (NH_4^+) between 87 and 100%; while there was a moderate efficiency of removal of P (PO_4^{-3}) between 18 and 69%. The ANOVA showed significant differences between the growth rates ($F = 6.85$, $p = 0.0001$). *V. verrucosus* was selected to evaluate its growth in alternative media and to be immobilized in alginate. The ANOVA test does not show significant differences between the free and immobilized cultures. Both technologies constitute an efficient alternative for bioremediation of municipal effluents.

Keywords: Wastewater; Nitrogen; Phycoremediation; Phosphate; Immobilization

1. Introduction

Bioremediation is the process by which organisms are used to purify pollutants, taking them from complex forms to simple forms and can be carried out in soil or water and may be less expensive than other technologies used to clean hazardous waste. For the treatment of wastewater and different effluents, phytoremediation has been used for some years, which can be defined as the use of aquatic plants and the phycoremediation with the use of macro or micro algae for the removal or biotransformation of pollutants [1–3].

The micro algae are a group of photosynthetic microorganisms with a high diversity and specializations; they are adapted to different environments and habitats [4–6], which makes it easier for them to grow in controlled systems producing environmental benefits such as CO_2 capture, treatment of contaminated effluents [7–9]. Likewise, the biomass produced can cover energy, food, drug and other bioprod-

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ucts demands and several species can outperform terrestrial crops in terms of biomass production [10–14].

Photosynthetic microorganisms are considered biorefineries; for its ability to synthesize bioproducts with high added value [10,12]. However, the limits for industrial production have been the high cost of nutrients; since approximately 80% of the total costs for the cultivation of micro algae are spent on nutrients and water [15]. In this context, the use of wastewater as a source of nutrients to grow micro algae can be a great alternative, due to the reduction of the cost of the environment and the removal of contaminating agents, also obtaining an environmental benefit and as well as biomass production of economic interest [16].

When micro algae grow in residual effluents or in organic leachates, an increase in pH occurs, which favors the precipitation of phosphorus and the volatilization of ammonium in crops. Moreover, they contribute to reduce the biochemical demand of oxygen, pathogens and N and P in comparison to other types of treatments [17,18]. To the consumption of nutrients, the forms of N that prefer micro algae are ammonium (NH_4^+) and nitrates (NO_3^-); as well as urea (NH_2CONH_2) and nitrogen dioxide NO_2^- can be used as sources of N [9,19,20]. Phosphorus is another essential nutrient for the growth of micro algae and they assimilate it preferentially as inorganic phosphate, being stored in the form of polyphosphate granules in the intracellular medium. This allows them to survive in the absence of this nutrient. Therefore, the growth rate of algae cannot respond immediately to changes in the external concentration of phosphorus, unlike the immediate responses to other resources such as ammonium, light, temperature [21,22].

Some research has been focused on studying cultures of these microorganisms either free or immobilized [19,23,24]; although in a free state, it has some limitations due to possible toxicity of the contaminants; as well as competition between native and exogenous populations and, in some cases, harvesting processes increase the costs and efficiency of obtaining biomass. While immobilized cell cultures provide microorganisms with protection against the toxic effect of the compounds present in the environment, and predation by other microorganisms; besides that, said methodology represents an energetic and functional saving [19,25].

The phycoremedation technology confers an improvement of the physico chemical quality of the effluent, by means of a mechanism of low energetic cost, with entrance of the nutrients to the micro algal biomass and which can reach a high commercial value according to its chemical composition to be used [14,26,27].

In the present work, the capacity of ammonium and orthophosphates removal in municipal wastewater from micro algae isolated from some places of the Mexican Republic was evaluated.

2. Material and methods

2.1. Micro algae used and culture conditions

The micro algae selected in the present work were: *Chlorella miniata, Coelastrella* sp., *Desmodesmus quadricauda, Neochloris oleoabundans, Verrucodesmus verrucosus* (Table 1). These were collected with a Van Dorn-type bottle with a horizontal intake and were isolated using the micro pipette technique with a pointed tip [28]. These strains of micro algae belong to the micro algae culture collection of the Applied Phycology Laboratory of UAM Iztapalapa, México.

All micro algae were evaluated in batch cultures in triplicates and initiated with an inoculum of 20% (v/v) from exponential phase cultures of each micro algae species. The growth of each micro alga was maintained in 16 L glass photo bioreactors with aeration with aeration through injection pumps in a range of 0.6 vvm (volume of air per total volume of the bioreactor per minute) and at an irradiance of 50 mmol/m^2/s. Every third day, 10 mL were taken in each of the cultures to determine cell density and ammonium and phosphate content.

2.2. Culture media

The following media were selected: Foliar Fertilizer Bayfolan forte ©: Worm Humic Acid © and municipal wastewater. In Tables 2,3 the respective chemical composition.

Residual water: samples of treated municipal water were collected from an anaerobic reactor type UASB (Experimental Pilot Plant No. 9 of the UAM-I), where levels of ammonium concentration ranging from 20.5 to 40.8 mg/ L and of orthophosphates from 4.9 to 16.5 mg/L [24] have been reported. The samples were irradiated with UV light for a period of 48 h and for the assembly of the experiments this effluent treated at 100% (v/v) was used.

2.3. Immobilization in sodium alginate

For immobilization under sterile conditions, the micro encapsulation method with alginate was used, modified by Lukavsky (1986) [36]. For this, 1 g of sodium alginate was added in 30 mL of distilled water. Additionally, the free culture in exponential growth phase is centrifuged at 3500 rpm at 20°C (Fig. 1a) and the pellet mixed with the alginate. Place the material in syringes and drop it drop wise into a 2% calcium chloride solution. It is shaken gently during the process and then the spheres are washed with distilled water and placed in culture medium, all under aseptic conditions (Figs. 1b and 1c). Experiments were mounted on 16 L photo bioreactors with 14 L of municipal wastewater and one volume of two liters of immobilized micro algae.

2.4. Micro algae growth

The growth was determined by cell count for each micro algae, every third day, using the Neübauer camera in the optical microscope [28]

2.5. Nutrient removal analysis

For nutrient analyzes, 30 ml of the culture were taken every three days and then centrifuged. The phosphate analysis was performed by the ascorbic acid method [37] and N- [NH_4] by the method of Indophenol [37].

Table 1
Microalgae used in this study

Species	Taxonomic position	Location/year of isolation	Comments	Bibliographic references
Chlorella miniata	Phylum Chlorophyta Class: Trebouxiophyceae Order: Chlorellales Family: Chlorellaceae	Catemaco Lake, Veracruz, México, 2015	Cosmopolitan species	[29,30]
Coelastrella sp.	Phylum: Chlorophyta Class: Chlorophyceae Order: Sphaeropleales Family: Scenedesmaceae Subfamily:Coelastroideae	Chalchoapan Lake, Veracruz, México, 2010	It produces high beta-carotene quantities under stress conditions	[30,3132]
Desmodesmus quadricauda	Phylum: Chlorophyta Class: Chlorophyceae Order: Sphaeropleales Family: Scenedesmaceae Subfamily: Desmodesmoideae	Cantera Pumas, University City, México, 2016	Cosmopolitan species	[29,33]
Neochloris oleoabundans	Phylum Chlorophyta Class: Chlorophyceae Order: Sphaeropleales Family: Neochloridaceae	Organic garden soil, México City, 2010	Acumulates high quantities of lipids	[29,34]
Verrucodesmus verrucosus	Phylum Chlorophyta Class: Chlorophyceae Order: Sphaeropleales Family: Scenedesmaceae Subfamily: Scenedesmoidea	Temporal pools, México Estate, México, 2014	First record in the study area	[29,35]

Table 2
Bayfolan forte culture medium

Compound	% (p/v)	Compound	% (p/v)
Total nitrogen (N)	11.470	Phosphoric oxide (P_2O_5)	8.000
Potassium oxide (K_2O)	6.000	Boron (B)	0.036
Copper (Cu)	0.040	Iron (Fe)	0,050
Molybdenum (Mo)	0.005	Zinc (Zn)	0.080
Thiamine hydrochloride	0.004	Sulfur (S)	0.230
Calcium oxide (CaO)	0.025	Cobalt (Co)	0.002

To 1 L of distilled water was added one milliliter of Bayfoland Forte.

Table 3
Worm humic acid medium AC-H

Compound	Content (ppm)	Compound	Content (ppm)
Organic matter	60.12	Fulvic and humic acids	17.80
Total nitrogen (N)	8.68	Assimilable potassium (K_2O)	3.27
Assimilable phosphorus (P_2O_5)	2.5	Calcium	7.21
Natural phytoregulators	600	Magnesium	0.48
Sulfur	3100	Iron	1500
Zinc	70	Manganese	1500
Copper	152	Boron	30
Molybdenum	50	Cobalt	2

To 1 L of sterilized water were added five ml of worm humic acid.

2.6 Statistical analysis

To compare the results, a one-way analysis of variance (ANOVA) was performed. The results were analyzed using the NCSS 2000 software and the Tukey significance tests at 0.05.

3. Results

With the cell growth data of the five species, a one-way nonparametric Kruskal Wallis ANOVA test was performed, giving a value of $p = 0.003 < \alpha = 0.05$ and an analysis of multiple comparisons, thus demonstrating significant differences in *Verrucodesmus verrucosus* and *Coelastrella* sp. to the rest of the species in cultivation. Fig. 3 allows to identify in a more detailed way the distribution of the data and the median in comparison.

Td = Time in days, the samples were obtained from cultures at 10 and 23 days of the start of experiment.

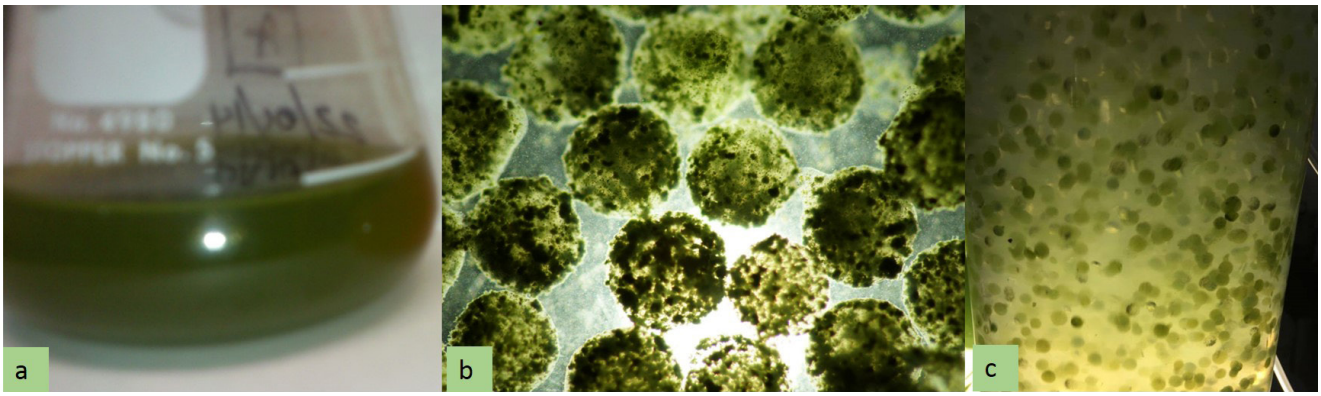

Fig. 1. (a) Free cultivation of micro algae, (b) *Verrucodesmus verrucosus* cells immobilized in alginate beads, under magnifying glass, (c) Cultivation immobilized in alginate beads.

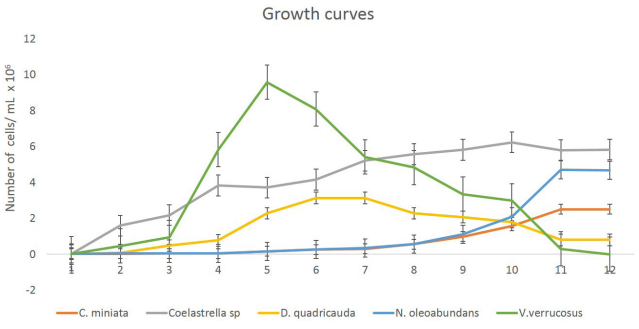

Fig. 2. Micro algae growth (cells/ml × 106) *Chlorella miniata, Coelastrella sp., Desmodesmus quadricauda, Neochloris oleoabundas, Verrucodesmus verrucosus* in treated wastewater.

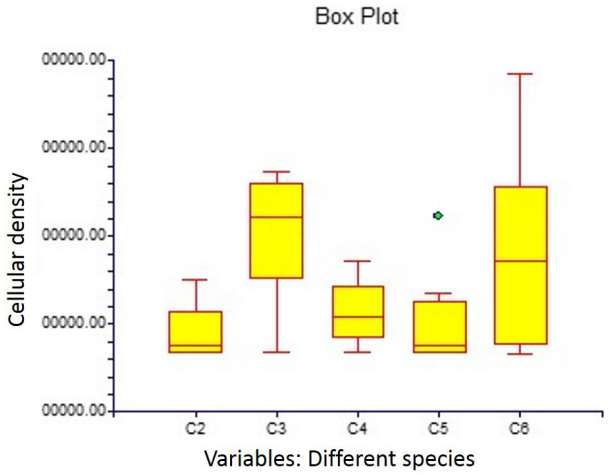

Fig. 3. Cell density differences between the five species in culture C2 *Chlorella miniata*.; C3 *Coelastrella* sp.; C4 *Desmodesmus quadricauda*; C5 *Neochloris oleoabundas*; C6 *Verrucodesmus verrucosus*.

V. verrucosus reached its maximum exponential growth and biomass at day 9 (Figs. 2, 3) showing significant differences ($p < 0.05$); it shows a removal of N-(NH_4) of 87% and a removal of P-(PO_4^{-3}) of 30% (Table 4).

Table 4
N and P (%) removal by the microalgae *Chlorella miniata, Coelastrella sp., Desmodesmus quadricauda, Neochloris oleoabundans, Verrucodesmus verrucosus* growing in treated wastewater

Microalgae	Removal (%) (Td_{10})		Removal (%) (Td_{23})	
	N	P	N	P
Chlorella miniata	50	16	87	30
Coelastrella sp	77	24	97	34
Desmodesmus quadricauda	98	16	100	18
Neochloris oleoabundans	56	58	97	69
Verrucodesmus verrucosus	87	30	98	37

One way non parametric Kruskal Wallis ANOVA test shows a value of $p = 0.004 < \alpha = 0.05$ for *V. verrucosus*, because of this Ho is rejected, this means that all medians are equals (Fig. 5). Turkey test showed that the greatest difference was found between worm humic acid and wastewater.

Regarding the removal in the three culture media, they presented significant differences ($p < 0.05$); in the presence of fertilizer, 100% and 85% removal of N and P were achieved, respectively. In the worm humic acid, the micro alga reached a removal of N up to 83%, and a cell density 2.3×10^6 in municipal wastewater of 98% and a maximum cell density of 9.5×10^6.

The efficiency in the removal of phosphates and ammonium did not present significant differences ($p > 0.005$) between both systems.

4. Discussion

The five species of native micro algae selected for this study (Table 1) showed removal of ammonium in a range of 87 to 100%; however, in the case of orthophosphates it ranges from 18 to 69% (Table 4).

Regarding the removal efficiency of NH_4, the following order was obtained: *Desmodesmus quadricauda*> *Verrucodesmus verrucosus* > *Coelastrella* sp. > *Neochloris oleoabundans* > *Chlorella miniata* and in terms of the efficiency of

removal of PO_4 the following order was obtained: *Neochloris oleoabundans* > *Verrucodesmus verrucosus* > *Coelastrella* sp. > *Chlorella miniata*. > *Desmodesmus quadricauda*

In the particular case of *Desmodesmus quadricauda* the consumption of ammonium was efficient, since after 10 days of the experiment 98% had already been consumed. As for phosphorus, only 18% was consumed (Table 4).

The maximum cell density obtained for *Neochloris oleoabundans* in this study, 4.6×10^6 cells / mL (Fig. 2), can be considered significant if it is compared with the result reported by Band-Schmidt (1997) [38] who, using conventional means, obtained a maximum density of 6.67×10^6 cell/L in 17 d using MIII medium and 6.77×10^6 cell/L in 15 d using medium F/2. Also, Elvira-Antonio et al. [39] report a maximum cell density in a treatment period of 8 days, being 28.1×10^6 cell/mL in the culture using artificial residual water. On the other hand, Olguín [40] when using dilutions of anaerobic effluents from porcine waste, with the species *Neochloris oleoabundans*, obtained removals close to 98%. *N. oleoabundans* is one of the most studied micro algae for the production of biofuels, due to its capacity to store a large quantity of lipids [10,14]. Therefore, if it grows in a wastewater effluent, it is promising since it lowers production costs and generates an environmental benefit.

In relation to the cultivation of *Chlorella miniata*, several species of this genus have been used in numerous studies of bioremediation, feeding and obtaining bioproducts [11,41,42]. Elvira-Antonio et al. [39] reported a removal for *Chlorella vulgaris* cultures fed with 30, 15 and 10 mg/L NH_4 + of 77.4%, 70.4% and 52.6% from artificial wastewater, in 6 days of treatment. Caporgno et al. [42] cultivated *Chlorella kessleri* with urban wastewater, where the maximum efficiency of elimination of nitrogen and phosphorus was around 96% and 99% respectively, during the 11-d batch cultures. Most species of this genus are recommended for treatment of effluents contaminated by their high capacity to adapt to them, but in this study, we obtained values of ammonium removal of 87% and phosphorus of 30% (Table 4), which were the lowest removal levels of the five species under study.

In works with *Coelastrella* sp. for the treatment of swine wastewater, removal values of 90% to 100% of the ammonia content are reported [43]. And when compared with a conventional medium such as BG11, the species grew faster in all the wastewater samples than in the BG11 medium. In our case, the alga obtained its maximum density on day 19 with 6.2×10^6 cel/mL in the municipal wastewater achieving removal values of up to 97% for ammonium and 34% for phosphates (Table 4). And it is the one that after the species *V. verrucosus* obtains maximum amount of algal biomass (Figs. 2 and 3). So, it is a species with high potential to be used in bioremediation processes.

In summary, the best growth obtained with municipal wastewater was obtained in the following order: *Verrucodesmus verrucosus* > *Coelastrella* sp. > *Neochloris oleoabundans* > *Desmodesmus quadricauda* > *Chlorella miniata*.

The species *Verrucodesmus verrucosus* showed to adapt to the worm humic acid and to the wastewater (Table 5, Figs. 4, 5) both free and immobilized (Table 6). This suggests that the micro alga has the potential to grow in alternative growing media and serve as a biological mechanism for the treatment of effluents, avoiding or modulating mainly eutrophication and other types of contamination. The difference in the removal times is linked to the initial concentrations of the culture media (Table 4). On the other hand, it was observed that it is not necessary to have incubation times that are too long since around 14 d the reactor can be harvested. This species has been little studied for bioremediation processes and is an alga with high potential, regardless of the effluent (Table 5) [44] and whether working in free or immobilized systems (Table 6).

Table 5
Removal of N and P (%) by the microalgae *V. verrucosus* in different culture media

Culture medium	Initial nutrient concentrations		% removal Td_{10}		% removal Td_{23}	
	N (NH_4)	P (PO_4^{-3})	N	P	N	P
Bayfoland forte	11.47	8.0	72	15	100	85
Worm Humic Acid	8.7	2.5	75	34	83	57
Wastewater	22.0	8.0	77	30	98	37

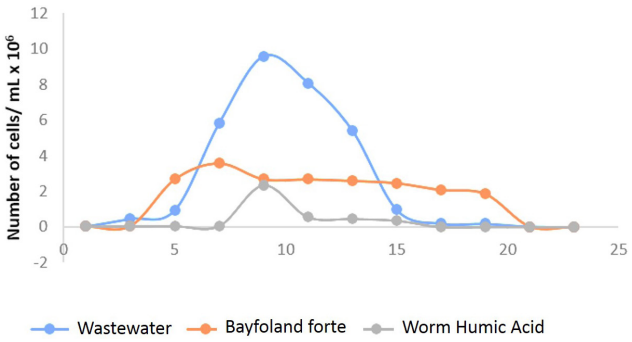

Fig. 4. Growth of *Verrucodesmus verrucosus* (cells/mL × 10^6) in wastewater, Bayfoland forte and Worm Humic Acid.

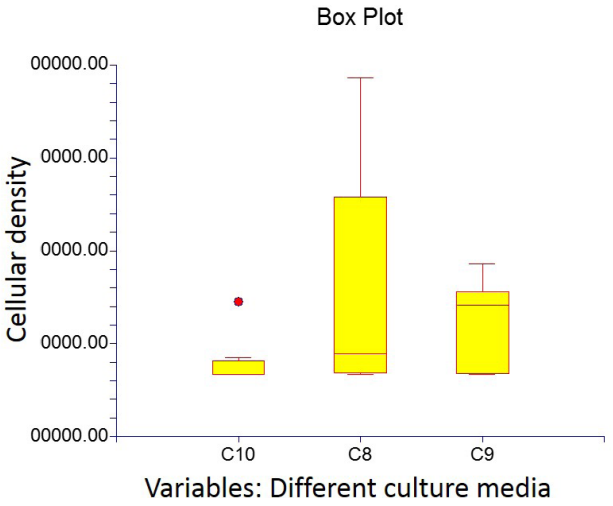

Fig. 5. Differential growth (cells / mL × 10^6) of *V. verrucosus* in wastewater (C8), Bayfoland forte (C9) and worm humic acid (C10).

Table 6
N and P removal (%) using wastewater in free and immobilized microalgae cultures

Culture	Removal (%) Td_{10}		Removal (%) Td_{23}	
	N	P	N	P
Free	77	30	98	37
Immobilized	68	24	99	49

The cultures of micro algae in residual water or in media elaborated from residual water give us results of very different yields in the literature; since there are different variables that are linked to cell growth. First, the species; they can be local species, (as in this case); species from international collections or even genetically modified species. Another important aspect is the characteristics of the culture medium, in terms of its nature: artificial, commercial, natural, wastewater effluents, leachates and others; and the third aspect the culture conditions: type of culture (autotrophic, heterotrophic, mixotrophic), type of light (artificial or natural), light cycle, light intensity, reactor volume, reactor type, mass transfer (agitation, gasification, of gasification), temperature, pH, CO_2 concentration and O_2 concentration. Therefore, it is very difficult to establish points of comparison. However, micro algae of the Chlorophyte class are among the most reported in literature for this type of treatment [7,9,10].

The harvesting process to separate the algal biomass from the already treated water is sometimes difficult due to the very nature of the algae. The most economical method could be sedimentation; this process reduces operating costs, depends on the type of species, size, cell motility density and if flocculants are added to accelerate the process, the clean effluent can be contaminated. [10,45,46]. So, by immobilizing the cells in sodium alginate, we make the harvesting process friendlier and easier, leaving the effluent clean and recovering the biomass to produce value-added products or to be used in a new bioremediation process; however, it should be considered that its efficiency may be greater in laboratory conditions with respect to larger-scale crops.

Comparing this work with those of Li et al. [15] and Lynch et al. [47] we can affirm that micro algae isolated from bodies of water or natural environment could adapt better and present good results in the removal of nutrients from contaminated effluents, under specific conditions. In addition to this, exotic species are not introduced and knowledge about the potential for the use of our resources is increased. Therefore, the ability of native micro algae to remove N and P from municipal wastewater and other effluents is demonstrated. And this efficiency does not diminish when working with an immobilized system, thus facilitating the harvesting of the biomass and release of the effluent, being this an efficient system of bioremediation.

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