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Wastewater treatment using a microalgae consortium mainly composed of chlorella, in Mexico

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ABSTRACT

The bioremediation capacity of microalgae found in the wastewater channels in Irrigation District 03 (DR03) in Mexico was studied. The study evaluated the effect of pH, aeration, light intensity, and water source on the productivity, culture time, and composition of the microalgal biomass, predominantly made up of *Chlorella vulgaris* spp. Parameters such as Chemical Oxygen Demand (COD), total solids (TS), and turbidity were monitored to assess the efficiency and bioremediation capacity of the microbial consortium. The yield production of microalgal biomass was measured to evaluate the amount produced per unit volume of treated water. Two culture conditions were assessed: uncontrolled and controlled conditions of aeration and lighting. Under controlled culture conditions, a productivity of $0.031 \text{ g L}^{-1} \text{ d}^{-1}$ biomass with 17% lipidic fraction and 37.65% ash content was observed, along with a reduction in COD and turbidity by 57 and 85%, respectively. A biomass productivity of $0.024 \text{ g L}^{-1} \text{ d}^{-1}$ was obtained.

KEYWORDS

Biomass; bioremediation; microalgal biomass; wastewater treatment; wastewater treatment plants

Introduction

In urban areas of Mexico, about 230 cubic meters per second ($\text{m}^3 \text{s}^{-1}$) of wastewater are produced, but only 35% of it is treated (CONAGUA 2013). The Mezquital Valley in central Mexico receives an average flow of $60 \text{ m}^3 \text{ s}^{-1}$ of wastewater, which is used to irrigate 80,000 hectares of farmland, resulting in high crop yields. However, this situation poses risks to food security, public health, and environmental safety (Lesser et al. 2018).

In Mexico, thirty percent of wastewater treatment plants (WWTPs) operate using activated sludge, while 31% use stabilization ponds (CONAGUA 2013). These processes, including aeration, consume a lot of energy and account for 45–75% of the total treatment cost (Rosso, Stenstrom, and Larson 2008). Due to these methods' high costs and low efficiency, it is crucial to develop new, efficient, and cost-effective treatment systems.

In this context, treatment systems that use microalgae (MA) as bioremediation agents are being studied due to their ability to remove organic matter, heavy metals, and emerging contaminants. They also have the capacity to fix CO_2 and produce O_2 (Moghazy and Abdalla 2024; Raza, Rizwan, and Mujtaba 2024; Bahr et al. 2011; Pires et al. 2013).

Microalgae, when provided with enough nutrients, can grow on a wide range of substrates. In high COD wastewater systems, their growth has shown to be nonselective, allowing for the proliferation of numerous species, with around 50,000 known, although only about 23,000 have been studied (Aishvarya et al. 2015; Park, Craggs, and Shilton 2010).

The research and study of microalgae are primarily driven by their potential for producing food and biofuels, as well as their ability to help in bioremediation (Park and Craggs 2011; Ozcelik et al. 2024; Lee et al. 2024). Raza,

Rizwan, and Mujtaba (2024) showed that microalgae have the potential to treat wastewater even in contaminated environments, providing dual benefits of environmental remediation and bio-resource production.

Therefore, this research focused on a group of microalgae found in the canals of Irrigation District 03 (DR03). The microorganisms in the DR03 canals showed strong resilience to high levels of contamination, suggesting they could be used for environmental cleanup processes. The study examined various growth conditions using wastewater as a substrate and looked at different pH levels, light intensities, and aeration conditions. The researchers also measured the amount and makeup of the biomass produced.

Methodology

Sampling

Wastewater samples used for microbial culture were collected within the geographic location DR03, as shown in Figure 1. Samples were collected from 18 sites along the “Requena” canal, located at coordinates $20^{\circ}13'29.8''N$ $99^{\circ}05'23.2''W$, within the municipalities of Actopan, Francisco I. Madero, Progreso, Mixquiahuala, Tlahuelilpan, and Tula, Hidalgo State, Mexico.

Characterization of wastewater samples

Physicochemical analysis

The physicochemical characterization of the collected samples consisted of measuring different parameters *in situ* and using laboratory techniques.

The physicochemical variables of samples measured *in situ*, with a Hanna multiparameter meter model HI9829-00041, were temperature ($^{\circ}C$), Conductivity (CE, $\mu S.cm^{-1}$), and pH. On the other hand, turbidity (nephelometric method), total solids ($mg L^{-1}$, gravimetry method), and COD ($mg L^{-1}$, spectrophotometric method) were measured in the laboratory.

Preparation of the initial inoculum

Two inoculums were obtained: one under controlled aeration and photo-lighting conditions, and another one under restricted aeration and natural photo-lighting conditions. Previous reports have mentioned that different conditions can either promote or inhibit the growth of certain species of MA present, depending on the sample source. In this case, it refers to the microalgae present in the wastewater channels.

Under aeration and artificial lighting conditions, 1 liter of filtered wastewater was aerated using a 2W compressor and exposed to a continuous luminous intensity of 5280 lumens for

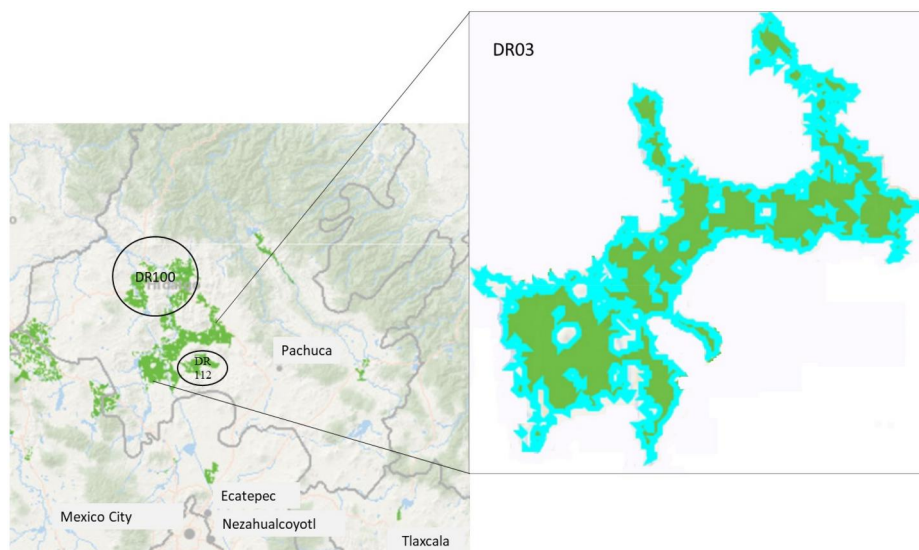


Figure 1. Geographical location of the Mezquital Valley, study area, wastewater sample collection. Figure taken from <http://sina.conagua.gob.mx/sina/tema.php?tema=distritosiego>.

72 h. These conditions led to the development of Inoculum 1 (I1).

On the other hand, when obtaining the inoculum under conditions of restricted aeration and natural lighting (I2), 1 L of wastewater was used. The operating conditions were as follows: constant stirring at 25–35 rpm with a paddle stirrer and a photoperiod of 14 h of light and 10 h of darkness.

Evaluation of microalgal growth conditions

Various initial pH conditions were tested on the substrates to assess how this parameter affects wastewater flow in the DR03 channels during the dry season (October–February) and the rainy season (March–September). The experiments involved using a 9:1 v/v wastewater-I1 ratio. The substrates were then adjusted to pH levels of 5, 7, and 9, with one control pH value (pH T) left unadjusted. 0.1 M HCl or NaOH solutions were used for pH adjustments. Additionally, experiments under restricted aeration conditions were conducted based on previous experiment results, ambient temperature, and a light-dark photoperiod of 14:10 h, reflecting the average annual environmental conditions. These experiments involved using aqueous substrate media in a 9:1 v/v wastewater-I2 ratio. Table 1 displays the specific conditions used in the different experiments.

Growth curves

The growth curves of the microalgae consortium were constructed by conducting daily direct counts every 12 h. The cell density (cells per milliliter, cells ml^{-1}) was quantified in triplicate using a Neubauer chamber with a depth of 0.1 mm until the beginning of the stationary phase. Samples from the different cultures were preserved with Lugol. Additionally, absorbance measurements were taken at 660 nm using a

Thermo Scientific GENESYS 10S Series UV-Visible Spectrophotometer (Aguilar 2016).

The growth velocity during the exponential phase and the doubling time were calculated using the equations proposed by Wood, Everroad, and Wingard (2005) and Satthong et al. (2019). Equation (1) was utilized for these calculations.

$$r = \frac{\ln(N_t/N_0)}{\Delta t} \quad (1)$$

Where: r = Population growth rate, N_0 = initial population size, N_t = final population size, Δt = time interval ($t_f - t_i$) in days. Time was expressed in days to calculate the doubling per day. The doubling time (T_2) was expressed in the same units as r and was calculated using Equation (2).

$$T_2 = \frac{0.6931}{r} \quad (2)$$

Where: T_2 is cell duplication, r is the population growth rate, and $0.6931 = \ln 2$.

Cultivation and processing of microalgae biomass

Harvest. The centrifugation was performed at 10,000 rpm for 5 min. Afterward, the supernatant was decanted and refrigerated for further analysis, while the biomass was collected and stored at 4 °C until analysis (Junior et al. 2020).

Determination of total lipids. 0.25–0.5 g of the different biomass samples were weighed on a dry basis, previously hydrolyzed with a solution of 4 M HCl in cellulose cartridges at a constant weight. Subsequently, Soxhlet extraction was carried out with petroleum ether for 20 extraction cycles. The extractions were performed in triplicate. The crude lipid content was calculated by difference (Chen et al. 2020).

Table 1. Culture media and culture conditions used in the experiments performed.

	Experiment 1 (E1)	Experiment 2 (E2)
Conditions	Aerobic with controlled agitation and photo lighting.	Restricted aeration conditions
Composition	90–10 %v/v wastewater-I1	90–10%v/v wastewater-I2.
Photoperiods	12:12 h of light: darkness	14:10 h of light: darkness
Luminous intensity	5280 lumen	Natural lighting
Temperature	Environment	Environment
Aeration	Air compressor 2 W	35 rpm mechanical agitation.
Grow time	192 h.	648 h.

W: watts; h: hours.

Table 2. Physicochemical parameters determined in the 18 DR03 wastewater samples, used as components of the microalgal growth substrates, $\pm SD$: standard deviation, $n = 18$.

Parameter	pH ($\pm SD$)	Conductivity ($\pm SD$) $\mu S \cdot cm^{-1}$	Temperature ($\pm SD$) $^{\circ}C$	Turbidity ($\pm SD$) NTU	COD ($\pm SD$) $mg L^{-1}$	ST ($\pm SD$) $mg L^{-1}$
Average value	8.36 \pm 0.77	1679 \pm 759	20.6 \pm 2	543 \pm 291	389 \pm 245	278 \pm 34

COD: chemical oxygen demand; TS: total solids.

Table 3. Heavy metal concentrations in untreated wastewater samples, SD : standard deviation, $n = 18$.

Sample	Cd $\mu g L^{-1} \pm SD$	Cr $\mu g L^{-1} \pm SD$	Pb $\mu g L^{-1} \pm SD$
Average	0.97 \pm 0.03	0.66 \pm 0.02	0.76 \pm 0.20
Minimum	0.66 \pm 0.01	0.20 \pm 0.01	0.28 \pm 0.01
Maximum	1.32 \pm 0.23	1.08 \pm 0.23	1.03 \pm 0.10

Monthly average allowable maximum limits in $mg L^{-1}$: Cd: 0.2, Hexavalent Cr: 1 y Pb: 0.5. Official Mexican standard NOM-001-SEMARNAT-1996, which establishes the maximum permissible limits of pollutants in wastewater discharges into national waters and assets.

Determination of total minerals

The porcelain capsules were heated to a constant weight at $500^{\circ}C$. Then, the samples weighing 1–2 g were put into the capsules (the exact weight depended on availability). Controlled combustion and progressive heating were conducted, starting at $500^{\circ}C$ and then following two heating ramps: the first at $550^{\circ}C$ and the second at $600^{\circ}C$ for 3 h. The remaining ash weight after combustion was measured and recorded (Ebert et al. 2019).

Determination of total nitrogen content

Five hundred milligrams of dry biomass was used for each sample using the Kjeldahl method. The digestion took place in a Buchi Switzerland Speed Digester K-436 at $350^{\circ}C$. Neutralization and distillation were carried out in a Buchi Switzerland Distillation Unit K-350. The distillates were titrated with HCl 0.1 N.

Heavy metals determination

Both treated and untreated wastewater samples were processed using the methods outlined in NMX-AA-051-SCFI-2016. They were passed through a $0.45 \mu m$ pore size membrane, where 5 ml of each sample was mixed with 5 ml of concentrated nitric acid in TeflonTM tubes. These mixtures were then digested in an autoclave at $100^{\circ}C$ and 15 psi for 1 h, and the digested mixtures were completed at 25 ml. All samples were quantified using reference targets and calibration curves ranging from 0 to $100 \mu g L^{-1}$ for each metal. Additionally, microalgal biomass samples were calcined at $600^{\circ}C$ for 2 h, and the resulting ashes were mineralized with 5 ml of HNO_3 . The

solutions were diluted to 10 ml with deionized water. Finally, the determination of Pb, Cr, and Cd was carried out using a Perkin-Elmer (USA) Optima 8300 plasma source emission spectrophotometer in a nitric matrix.

Results

Physicochemical parameters and quantification of heavy metals in DR03 wastewater samples

Table 2 summarizes the results of the physicochemical characterization of the 18 wastewater samples collected. The average pH values ranged from 7.9 to 9.1, indicating the presence of alkaline compounds such as hydroxides, carbonates, and ammonia, among other compounds. The EC values indicate a high content of ionic solutes, associated with the presence of strong electrolytes and high sodium concentrations. Turbidity, COD, and total solids suggest a high presence of organic and inorganic contaminants. Pearson's standard deviation (s) and relative standard deviation were utilized as absolute and relative dispersion measures, respectively.

Table 3 summarizes the concentrations of heavy metals that were determined and quantified in the samples. The quantification of heavy metals was also carried out in the effluents treated with MA to evaluate their bioaccumulative capacity of heavy metal ions.

The concentrations of the heavy metals studied in the water treated with MA were lower than the detection limit of the method ($LOD = 0.001 \mu g L^{-1}$). The concentrations found in the biomass samples were, on average, $90 \pm 0.50 \mu g kg^{-1}$ for Cd, $250 \pm 0.10 \mu g kg^{-1}$ for Cr, and $120 \pm 0.60 \mu g kg^{-1}$ for Pb. These results indicate a high accumulation capacity of heavy metals by MA, which has been reported in several studies. This is attributed to the high efficiency of these consortia in utilizing nutrients present in different substrates where they grow (Aditya et al. 2022).

Analysis of the growth of the microalgal consortium in wastewater

The pH level is crucial in determining the rate and quantity of microalgal biomass, as well as the preference for the growth of specific microalgal species. This is due to the chemical species associated with nutrients and the availability of carbonates, nitrates, phosphates, and other anions. Altering the availability of nutrients in the environment can favor the growth of certain microalgal species while inhibiting others or restricting the growth of all or some species (Powell 2013).

The Species Distribution Diagrams (SDD) were obtained using the Medusa 3.0 program to illustrate the relationship between pH and the prevalence of four inorganic ions in wastewater matrices. The diagrams show the predominant chemical species of these ions at different pH levels, which could explain their availability as nutrients for microalgae.

At pH 9, the predominant chemical species are $(\text{H}_2\text{CO}_3)^-$, $(\text{HPO}_4)^{2-}$, $(\text{NO}_3)^-$, and $(\text{SO}_4)^{2-}$. Interestingly, the best biomass growth rate and productivity were observed at an initial pH of 9. This suggests that the most available inorganic species for the evaluated microalgal consortium are present in an alkaline medium with a pH of 9 (see Figure 2).

The initial pH values were 5 and 7, and it was observed that the pH value increased toward alkaline levels, reaching close to 9. These results indicate that the MA consortium, which was studied, is well-suited to the typical alkaline conditions of the wastewater in the DR03 channels. Additionally, the MA consortium even promotes alkaline water. In all cases evaluated, maximum growth was achieved after 96 h of adaptation (see Figure 3).

Growth curves

In Figure 4(a), the microalgal growth curve is depicted under conditions of restricted aeration and natural light. The initial pH was kept at 8.6 instead of the usual 9. The graph shows the different phases of microalgal growth: adaptation (0–192 h), exponential growth (192–408 h), and stationary phase (408–504 h), followed by the decline phase.

The graph in Figure 4(b) illustrates the growth behavior of the microalgal consortium under controlled aeration and photo-lighting conditions with 12:12 cycles. During the initial 72 h, the microalgae underwent an adaptation period. Subsequently, accelerated growth was observed between 72 and 120 h. After 120 h, the microalgae entered the senescence stage. It is worth noting that under aeration and photo-lighting conditions, the microalgae reached cell counts of more than 8 cells ml^{-1} within 96 h of cultivation. In contrast, under conditions of restricted aeration and natural lighting, the growth rate was lower, with a maximum cell count of 4 cells ml^{-1} at 384 h of cultivation.

The population growth rate correlated with the doubling time in the exponential phase of wastewater under the two evaluated conditions (Table 4). Under controlled conditions, a higher growth rate was observed, with a correlation coefficient (r) of 0.37 and a shorter doubling time (T_2) of 1.89 h. Meanwhile, under ambient conditions, without aeration and sunlight, the values were $r = 0.23$ and $T_2 = 3.07$ h.

Microalgal growth correlation between cell density and absorbance

To evaluate the spectrophotometric method to monitor the culture of the experiments, The correlation expressed as a correlation coefficient was evaluated ($R^2 = 0.991$) calculated from optical density data at 660 nm against expressed cell concentration (cell ml^{-1}) $\times 10^6$ (see Figure 5). It was considered only up to the stationary phase; Due to, later growth stages (decline) interference increases due to the presence of similar adsorbent species; as well as the formation of cellular agglomerates. The spectrophotometric method made it possible to accurately determine the growth parameters as a function of absorbance.

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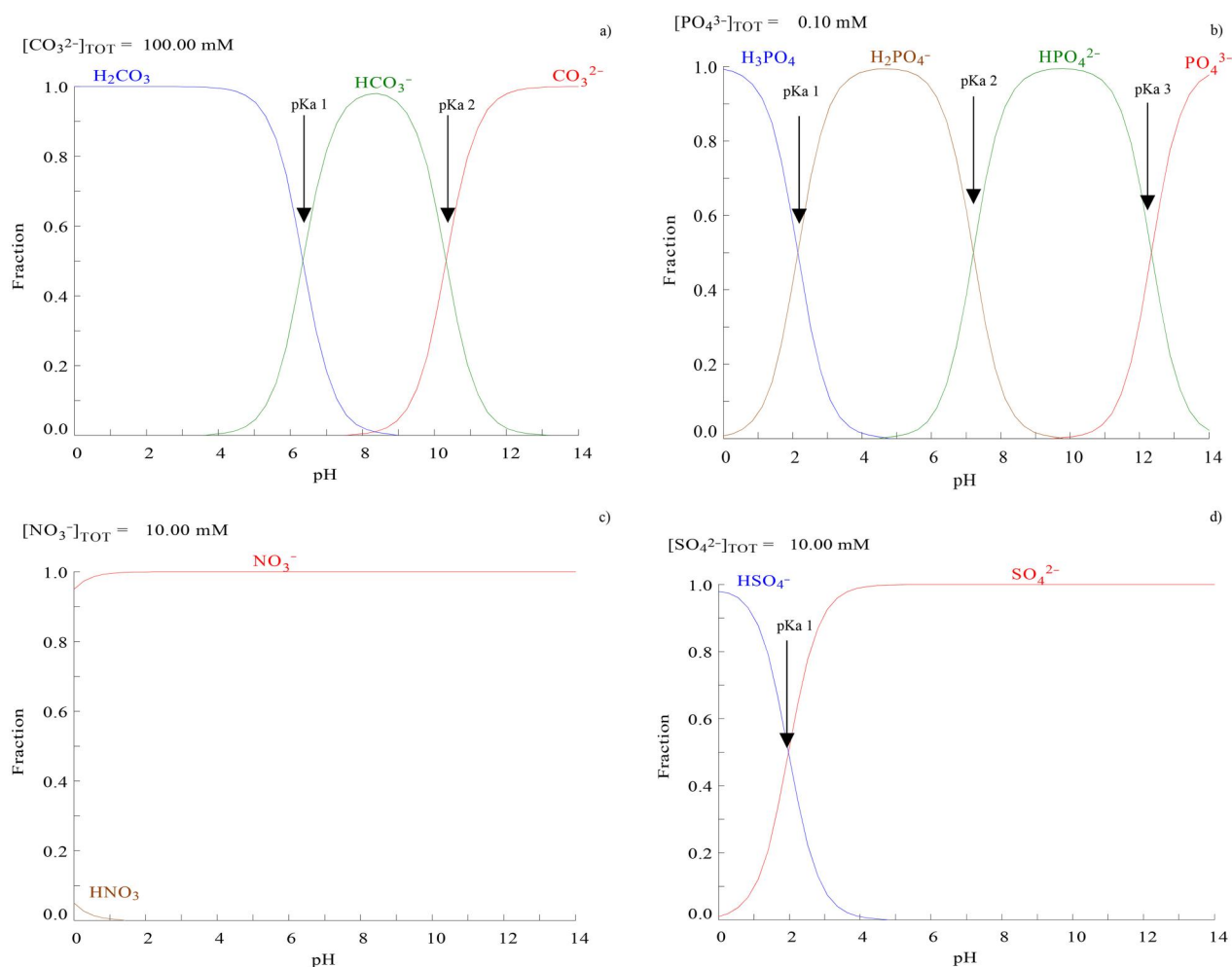


Figure 2. Species distribution diagram (a) CO_2 in aqueous medium, (b) PO_4^{3-} , (c) NO_3^- , and (d) SO_4^{2-} ; $\text{pKa} = -\log K_a$.

formation of cellular agglomerates. The spectrophotometric method accurately determined the growth parameters based on absorbance.

Assessment of bioremediation capacity and production of biomass MA

The levels of COD, total solids, and turbidity were measured at the beginning and end of the experiments. Table 5 outlines the findings and depicts the percentage of removal. A significant reduction in COD was noticed when the aeration conditions were restricted. This means that when the entry of CO_2 was limited, the microorganisms present in the wastewater utilized the organic and inorganic matter as their primary carbon source (Oswald and Gotaas 1957). This suggests that microorganisms can easily adapt to the environment's composition and conditions, utilizing pollutants as a source of carbon. On the other hand, when aeration conditions were applied, air with a

specific concentration of CO_2 was introduced. This increased the concentration of inorganic carbon species that are more accessible to the microorganisms, leading them to consume carbonates before addressing the organic pollutants in the wastewater.

Proximal composition of MA biomass

Table 6 summarizes the data on the components of the microalgal biomass, as well as yield and cultivation time.

The proximate analysis of biomasses in this study suggests that producing biodiesel from microalgae could be cost-effective and highly efficient. According to Qiao et al. (2022), subjecting microalgae to combined salinity stress and myo-inositol treatment can result in lipid overproduction and nutrient removal from wastewater, integrating microalgae cultivation with wastewater treatment.

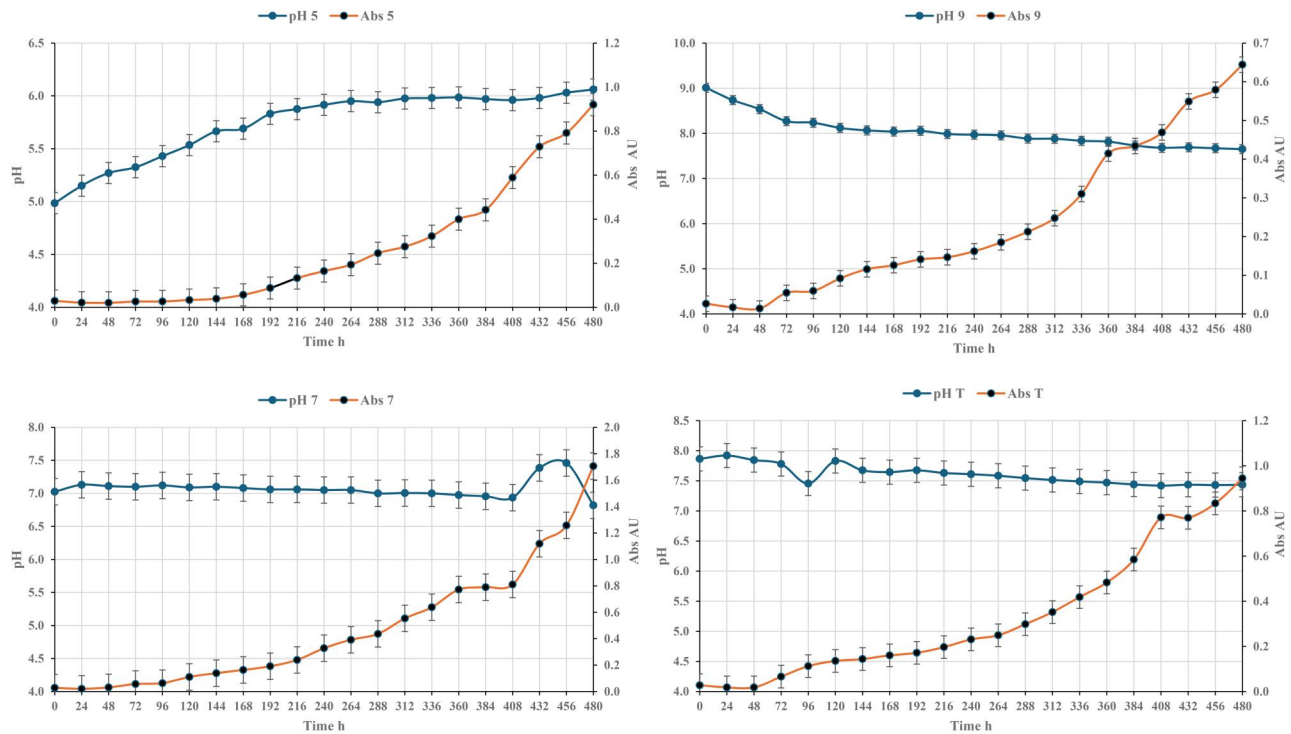


Figure 3. Variation in pH and cell concentration ($\text{cell ml}^{-1} \times 10^6$): (a) pH 5, (b) pH 7, (c) pH 9, and (d) pH T.

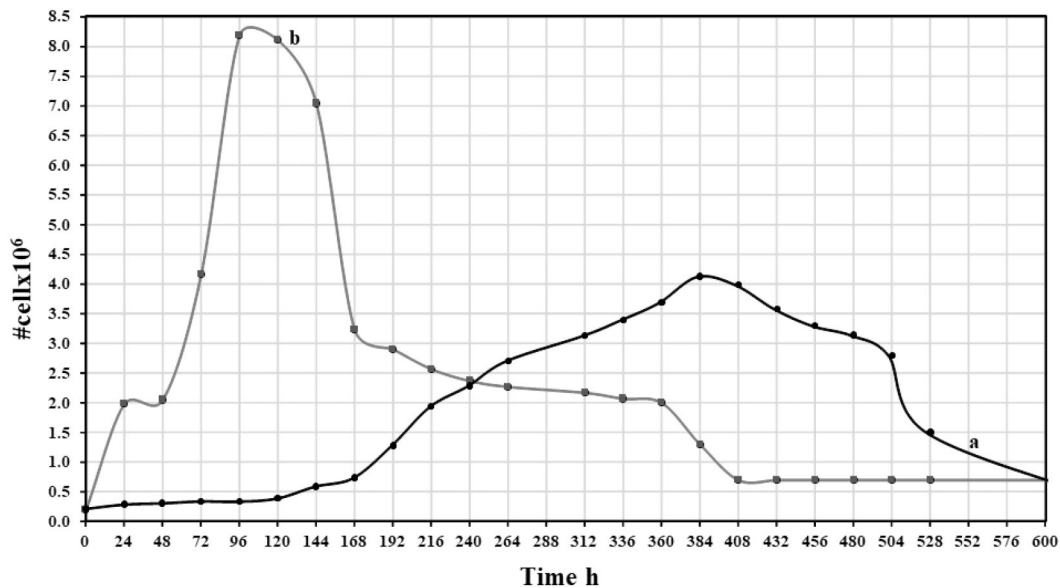


Figure 4. Growth curves: (a) restricted aeration and (b) controlled aeration.

Table 4. Growth speed and doubling time were obtained in the exponential phase of the MA under two conditions of grow evaluated, *SD*: standard deviation, *n* = 5.

	Controlled conditions at pH 9	Ambient conditions
Population growth rate (<i>r</i>) ($\pm SD$)	0.37 (0.01)	0.23(0.01)
Doubling time (<i>T</i> ₂) (cell division/h) ($\pm SD$)	1.89 (0.05)	3.07(0.01)

Additionally, the use of activated sludge with microalgae has been studied for removing nitrogen and phosphorus from wastewater in pulp and paper mills. This approach yielded a lipid

productivity of $\sim 41 \text{ mg L}^{-1} \text{ day}$, with a significant percentage of balanced saturated and unsaturated fatty acids. These findings offer a promising way to combine wastewater treatment with biomass

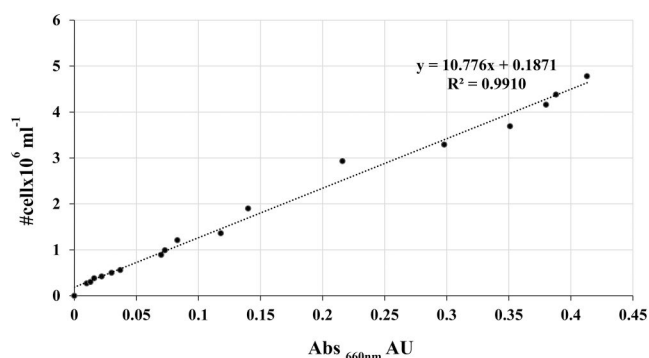


Figure 5. Correlation between optical density to 660 nm (AU) vs. cell concentration (cell ml^{-1}) $\times 10^6$.

Table 5. Characterization data of substrates after microalgal growth, *SD*: standard deviation, $n = 3$.

		COD ($\pm SD$) (mg L^{-1})	TS ($\pm SD$) (mg L^{-1})	Turbidity ($\pm SD$) (nephelometric turbidity units, NTU)
E1	Initial	326.00 (54)	1216.51 (66)	124.00 (2)
	Final	225.00 (21)	922.54 (7)	18.42 (1)
	% Removal	30.98 (2)	24.17 (5)	85.16 (0.5)
E2	Initial	200.00 (36)	184.00 (5)	81.00 (1)
	Final	114.00 (10)	74.00 (2)	59.10 (5)
	%Removal	57.00 (5)	40.21.(4)	72.96 (2)

Table 6. Proximal composition, yield, and productivity of microalgal biomass collected under two evaluated conditions, *SD*: standard deviation, $n = 5$.

Parameter	E1 ($\pm SD$)	E2 ($\pm SD$)
Total lipids %	17.75 (1.15)	7.5 (2.70)
Total minerals expressed as ashes %	37.65 (3.01)	33.12 (0.35)
Total nitrogen content %	20.77 (2.34)	18.72 (2.66)
Yield $\text{g L}^{-1} \text{d}^{-1}$	0.41 (2.62)	0.30 (1.05)
Productivity $\text{g L}^{-1} \text{d}^{-1}$	0.031 (3.45)	0.024 (2.36)

production for long-term sustainability (Su et al. 2022; Talapatra et al. 2023).

Studies by Radmehr et al. (2023) have demonstrated that a mixture of microalgae and activated sludge resulted in five times higher lipid content compared to activated sludge alone in batch experiments. Efficient sedimentation plays a crucial role in using microalgae as biodiesel feedstock, as it can reduce the cost of harvesting, which accounts for 30% of the total cost of producing biodiesel from microalgae (Grima et al. 2003).

The direct transesterification (DT) method has shown improved recovery percentages of fatty acid methyl esters (FAMES) from microalgae compared to the conventional oil-extraction-transesterification (OET) method (Loh et al. 2021). Microalgae biomass production could serve as a viable energy source, but direct combustion is not recommended due to the generation of greenhouse gases (Sturm and Lamer 2011).

While the lipid content of the generated biomass was found to be 17.75%, modifications to the process could potentially improve the production of FAME, providing a promising alternative for biodiesel generation (Li et al. 2008).

Overall, the research demonstrates the potential of lipid production from microalgae biomass for aerobic wastewater treatment and biodiesel generation, reducing energy use, carbon emissions, and environmental impacts (Geng et al. 2022; Velasquez-Orta 2013; Revellame 2013; Mu et al. 2014; Brennan and Owende 2010).

Microalgae biomass is a valuable raw material with diverse applications and properties, including the production of high-value compounds and its use in various industries (Wo and Hamza 2024; Yap et al. 2021; Udaiyappan et al. 2017; Deviram et al. 2020). Future research should focus on optimizing microalgae production to achieve higher FAME yields and improve the feasibility of sustainably generating biodiesel.

Conclusions

The analyzed samples exhibit consistent physico-chemical characteristics, which are important for standardizing the bioremediation process and designing a bioreactor for growing and remediating DR03 wastewater. The presence of heavy metals indicates that any developed processes

should be capable of reducing their concentration or withstanding high levels of them.

The study of the growth system helped determine that a pH of 9 is best for cultivating the MA consortium in this study, as it promotes the highest production of biomass in the wastewater. pH significantly impacts the availability of dissolved nutrients, which in turn affects biomass growth. Both substrates showed a significant reduction in COD, and the analysis of the microalgal biomasses revealed promising crude lipid percentages.

The determined kinetic parameters provide a basis for designing photobioreactors for scaling up the process to an industrial level while also using wastewater as a substrate for cultivating microalgal biomass for biofuel production.

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Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

The manuscript was not submitted to any other journals. Original work: a research article.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

Not applicable.

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