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Application of *Chlorella* sp. and *Scenedesmus* sp. in the Bioconversion of Urban Leachates into Industrially Relevant Metabolites

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Abstract: This paper explores the ability of *Chlorella* sp. and *Scenedesmus* sp. to convert landfill leachates into usable metabolites. Different concentrations (0.5, 1, 5, and 10% *v*/*v*) of leachate coupled with an inorganic carbon source (Na_2CO_3, and $NaHCO_3$) were tested to improve biomass production, metabolites synthesis, and removal of NO_3 and PO_4. The result shows that both strains can effectively grow in media with up to 5% (*v*/*v*) leachate, while significantly reducing the concentrations of NO_3, and PO_4 (80 and 50%, respectively). The addition of $NaHCO_3$ as a carbon source improved the final concentration of biomass, lipids, carbohydrates, and the removal of NO_3 and PO_4 in both strains.

Keywords: *Chlorella* sp.; *Scenedesmus* sp.; lipids; waste reduction; nutrients removal

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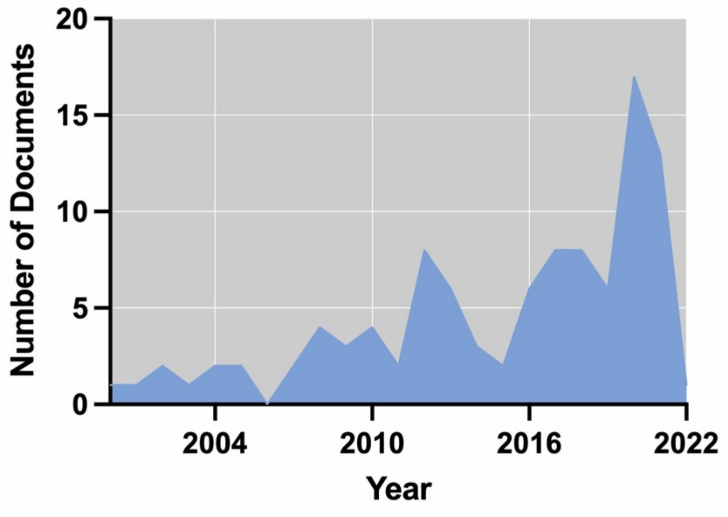
1. Introduction

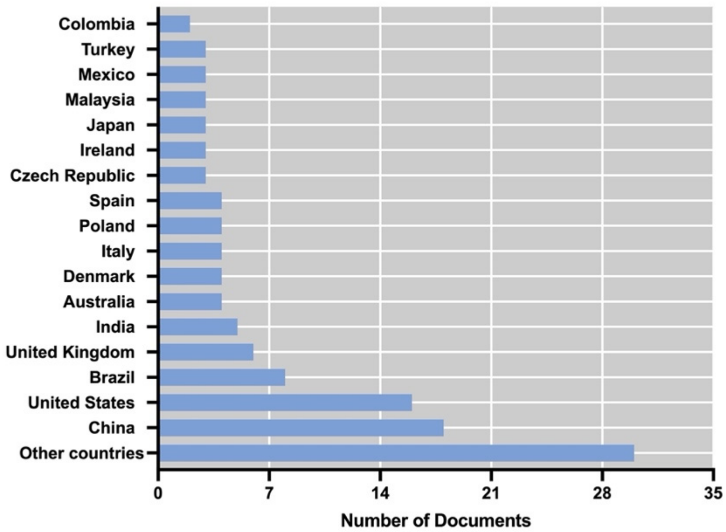
One of the primary wastes from landfills is leachate [1]. Landfill leachate is one of the most complex liquid wastes to treat due to its high content of recalcitrant organic compounds, salts, high concentration of ammonia nitrogen, nitrates, phosphates, and dissolved metals [2]. To date, there are different technologies (physical, chemical, and even biological) available for the treatment of this type of waste [3]. However, their low efficiency in reducing the pollutant capacity of this type of liquid waste and their low economic sustainability have led researchers worldwide to explore new technologies [4].

Microalgae and cyanobacteria are highly diverse photosynthetic microorganisms found in diverse aquatic environments [5]. They are considered one of the novel biotechnological sources of different metabolites such as lipids, carotenoids, proteins, carbohydrates, bioplastics, auxins, mycosporine-like amino acids (MAA), and others [6]. One of the most exciting applications of algae is the removal of nutrients from polluted waters, or phytoremediation [7–13]. Phytoremediation using microalgae is not new since the first studies were carried out in San Obispo (California) by William J. Oswald's group in the mid-1950s. By employing this principle, it is possible to valorize certain effluents while reducing their impact and producing biomass with metabolites of industrial interest [14,15].

According to a SCOPUS search with the keywords "Landfill AND Leachate AND alga", during the last 22 years, about 102 scientific papers have been published (Figure 1a), especially in countries like China, United States, Brazil, and others (Figure 1b), which is few compared to other types of wastewaters treated with microalgae. The latter demonstrates the growing interest in searching for strains resistant to landfill leachates.



(**a**)



(**b**)

Figure 1. Evolution of the number of publications from 2000 to 2022 on the treatment of landfill leachate using microalgal biotechnology (**a**), and their countries of origin (**b**).

The application of algae and cyanobacteria to remove contaminants possesses limitations, such as the high energy input required for the harvesting of biomass [16–19] and the selection of the proper strain capable of withstanding the toxicity of leachate. Due to the unique chemical composition of this type of waste, a strain (or several strains) with high-growth capacity has not been isolated so far [20]. During the last few years, strains from different genera of microalgae and cyanobacteria such as *Chlamydomonas* sp. [21,22], *Chlorella* sp. [1,22–33], *Desmodesmus* sp. [23,34], *Picochlorum* sp. [35], *Scenedesmus* sp. [23,24,28,29,34], *Stigeoclonium* sp. [23], *Tetradesmus* sp. [26], *Microcystis* sp. [23], *Oscillatoria* sp. [23,28]; and even a consortium of microalgae [20] have been studied. Table 1 shows that the most reported concentration of leachate is 10% v/v [20,22,23,28–30,33]; however, some authors claim that higher concentrations of leachate (from 20%, up to undiluted leachate) can also be used by algae and cyanobacteria [24,26,32,36]. In a different approach, Kumari et al. [37] demonstrated that a microalgae-bacteria consortium (*Scenedesmus* sp. and *Paenibacillus* sp.) can effectively grow in a media with 20% v/v of leachate, thus removing toxic elements such as heavy metals and organic compounds. The objective of this study was to evaluate the viability of two strains of thermo-tolerant algae to grow in landfill leachate and their synthesis of usable metabolites such as carbohydrates and lipids, offering an alternative for the reduction of the hazardous potential of landfill leachate and the production of high value-added metabolites.

Table 1. Strains of algae and cyanobacteria cultured on different concentrations of landfill leachate.

Strain	Leachate Concentration (% *v/v*)	Biomass Concentration (g·dm^{-3})	Reference
Algal consortium	10	2.4	[20]
Chlamydomonas sp. SW15aRL	30	2.99	[21]
Ch. snowiae	10	n/a *	[22]
Chlorella sp.		8.2	[23]
		n/a *	[29]
		1.2	[30]
	15	0.34	[28]
Chlorella minutissima 26a	10	1.1	[25]
C. vulgaris	20	4	[26]
		n/a *	[24]
	5	0.45	[27]
	50	1.92	[32]
C. vulgaris FACHB-31	10	1.64	[31]
	100	2.13	[33]
C. pyrenoidosa	10	n/a *	[22]
C. pyrenoidosa (FACHB-9)	20	1.58	[1]
Desmodesmus sp.	7	1.3	[34]
Desmodesmus subspicatus (Brinkmann 1953/SAG)	20	n/a *	[24]
Microcystis sp.	10	8.1	[23]
Oscillatoria sp.		8.0	
	20	0.8	[28]
Picochlorum oculatum		1.9	[35]
Scenedesmus sp.	10	8.12	[23]
		n/a *	[29]
		0.16	[28]
S. obliquus	7	1.2	[34]
Stigeoclonium sp.	10	8.1	[23]
Tetradesmus obliquus	15	0.56	[26]

n/a *: Data non-available.

2. Materials and Methods

2.1. Landfill Leachate

The leachate (mature leachate) was kindly supplied by "Parque Tecnológico Ambiental Guayabal" (Cúcuta, Norte de Santander) and its chemical composition (nitrates, phosphates, pH, conductivity, temperature, total dissolved solids, total suspended solids volatile suspended solids, salinity, BOD_5, and COD) was analyzed according to standard methods for the examination of water and wastewater [38].

2.2. Strains

Chlorella sp. (CHLO_UFPS010), and *Scenedesmus* sp. (SCEN_UFPS015) from INNOValgae collection (UFPS, Colombia) were used in this study. The strains were maintained in a 2 L glass flask containing 1.2 L of sterile Bold Basal [39]. Each flask was mixed through the injection of filtered air with 0.5% (*v*/*v*) CO_2 at a flow rate of 0.78 L min^{-1}(Resun, LP-100), 25 °C, and light-dark cycle of 12:12 h at 100 µmol m^{-2} s^{-1} for 30 days.

2.3. Experimental Design

The leachate was initially diluted (0.5, 1, 5, and 10% v/v). The concentration that enhanced the biomass production on both strains was later supplemented with different concentrations (0.8, 1.2, and 1.6 g·dm^{-3}) of either sodium carbonate (Na_2CO_3) or sodium bicarbonate ($NaHCO_3$) [40] prior to inoculation. As a control, both strains were cultured in a Bold Basal medium (BBM). The results were analyzed using a two-way ANOVA GraphPad Prism version 9.

All the strains were cultured (by triplicate) in a 2 L glass flask with a working volume of 1.2 L of sterile leachate. Each flask was mixed through the injection of filtered air at a flow rate of 0.78 L min^{-1} (Resun, LP-100) and a light-dark cycle of 12:12 h at 110 µmol m^{-2} s^{-1} for 30 days. The biomass produced was harvested by centrifugation (3400 rpm, 20 min, −20 °C) (Rotina 420-R, Hettich, Tuttlingen, Germany), washed thrice with distilled water, freeze-dried (FreeZone 4.5, Labconco, Kansas City, MO, USA), and stored (4 °C) until use. Finally, the different components of the biomass such as carbohydrates [41], lipids [42], proteins [43], carotenoids [44], and ash [45] were measured. The cells-free media was analyzed for their content of nitrates and phosphates.

3. Results

Leachate is known for its dark brown color, unpleasant odor, and high nitrogen but low phosphate concentration (Table 2). The measurement of BOD_5 and COD parameters is directly related to organic matter contamination and the age of a landfill, as this factor plays a fundamental role in the leachate composition [46].

Table 2. Characterization of landfill leachate.

Parameters	Units	Mean Value
Nitrates (NO_3)	mg·dm^{-3} NO_3	71 ± 0.04
Phosphates (PO_4)	mg·dm^{-3} PO_4	1.05 ± 0.07
pH	pH units	9.74 ± 0.1
Temperature	°C	25.10 ± 0.5
Conductivity	µS	35 ± 0.5
Total Dissolved Solids (TDS)	mg·dm^{-3}	2.31×10^{-5}
Salinity	mg·dm^{-3}	1.75×10^{-5}
Chemical Oxygen Demand (COD)	mg·dm^{-3}	630 ± 0.02
Biochemical Oxygen Demand (BOD_5)	mg·dm^{-3}	2.93 ± 0.05
Total solids (TS)	mg·dm^{-3}	20.73 ± 0.1
Total Suspended Solids (TSS)	mg·dm^{-3}	0.08 ± 0.01
Volatile Suspended Solids (VSS)	mg·dm^{-3}	0.04 ± 0.01

For the growth of microalgae, four concentrations of leachate were used: 0.5, 1, 5, and 10% v/v; however, according to the ANOVA analysis, the 10% v/v of leachate recorded the lowest biomass in comparison with the control (Figure 2). It was also possible to identify that a concentration of 5% favors biomass production in the two strains studied. According to the results, *Chlorella* sp. increased biomass production up to 0.6 g·dm^{-3}, which is due to the increase of nitrate and phosphate content in the medium; on the contrary, there was no significant difference in the biomass produced at different concentrations of leachate using *Scenedesmus* sp.

The removal of nitrate and phosphate is presented in Figure 3. According to the ANOVA analysis, there is a significant difference in the removal of NO_3 and PO_4, in both *Chlorella* sp and *Scenedesmus* sp. using 5% v/v of leachate. *Chlorella* sp. was able to remove 81.87% and 56.78% of NO_3 and PO_4 respectively. In the case of *Scenedesmus* sp. this strain was able to remove 90% and 54% of NO_3 and PO_4 respectively.

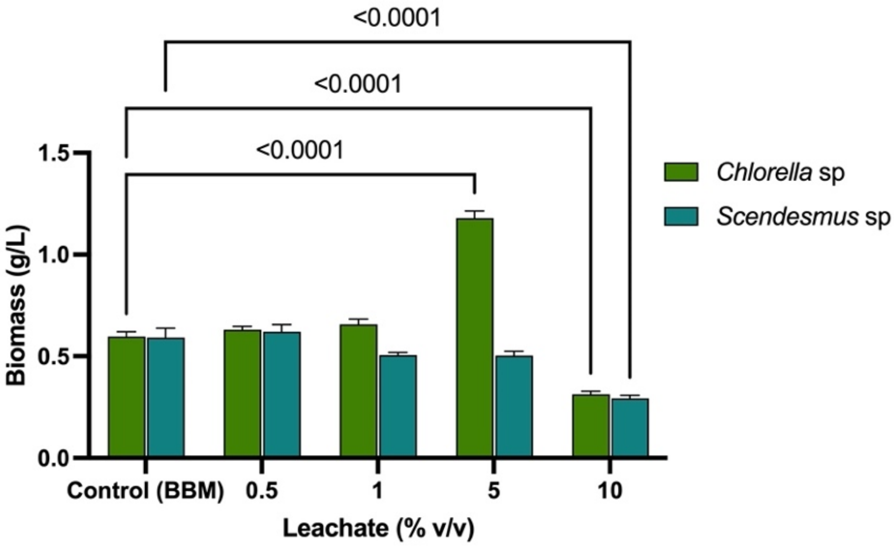


Figure 2. Biomass produced under different concentrations of leachate.

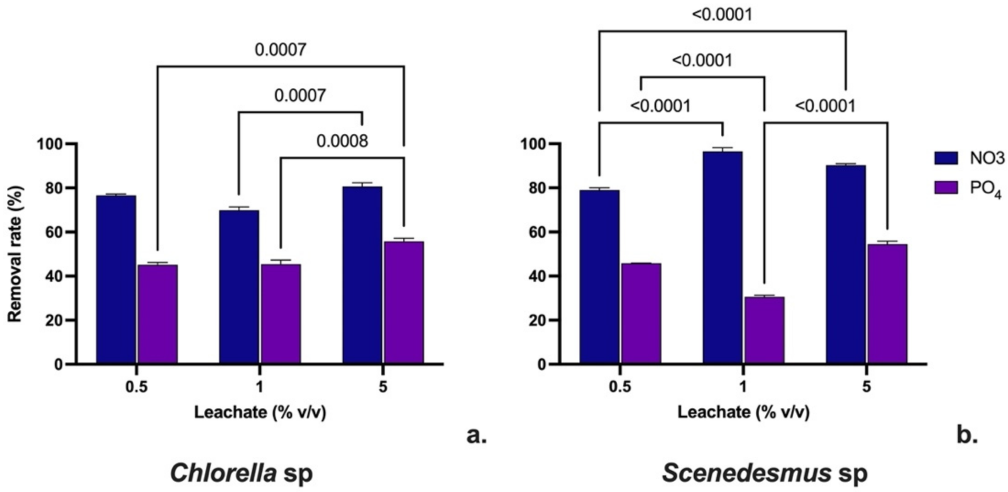


Figure 3. Removal of N and P in leachates by *Chlorella* sp. (**a**) and *Scenedesmus* sp. (**b**).

The biomass production using Na_2CO_3 and $NaHCO_3$ at different concentrations (0.8, 1.2, and 1.6 g·dm^{-3}), and the removal of NO_3 and PO_4 can be found in Figure 4. In the case of *Chlorella* sp. $NaHCO_3$ enhanced the biomass concentration up to 1.23 g·dm^{-3} (using 1.6 g·dm^{-3} of $NaHCO_3$), which according to the ANOVA analysis is higher than the other concentrations evaluated. On the other hand, different concentrations of Na_2CO_3 did not increase the final concentration of biomass compared to the control. In the case of *Scenedesmus* sp., 0.8 g·dm^{-3} of $NaHCO_3$ was enough to increase the biomass concentration up to 0.77 g·dm^{-3}. Higher concentrations of both $NaHCO_3$ and Na_2CO_3 reduced the final content of biomass in this alga. Another important result is the removal of NO_3 and PO_4 using different carbon sources. According to the ANOVA analysis, there is not much difference in the removal of either NO_3 or PO_4 when both strains were grown using $NaHCO_3$ in comparison with the control (alga grown in 5% v/v leachate). The only significant difference recorded was found in the removal of NO_3 when *Chlorella* sp. was grown using different concentrations of $NaHCO_3$.

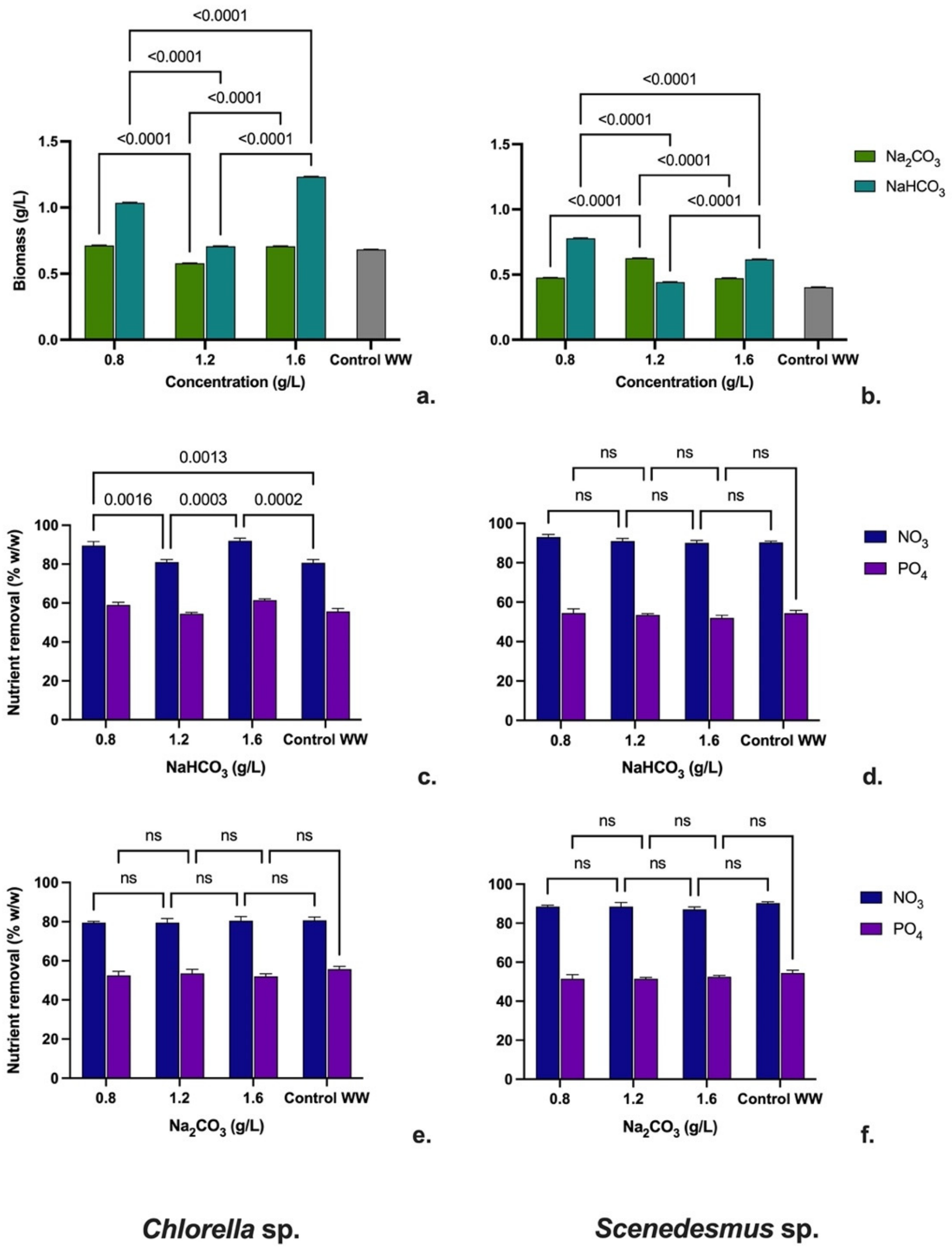


Figure 4. Biomass concentration, removal of NO_3 and PO_4 under different carbon sources coupled with 5% (*v*/*v*) landfill leachate for *Chlorella* sp. (**a**,**c**,**e**), and *Scenedesmus* sp. (**b**,**d**,**f**).

The concentration of the different metabolites analyzed are presented in Figure 5. In the case of carbohydrates, different concentrations of $NaHCO_3$, and Na_2CO_3 increased the final concentration in *Chlorella* sp. Most studies using leachate as a source of nutrients for algal production mainly report the concentration of carbohydrates and lipids. However, other metabolites such as proteins and carotenoids must be measured and reported. To the best of the author's knowledge, this is the first study that reports the effect of leachate on the concentration of carbohydrates, lipids, proteins, and carotenoids. The highest concentration of carbohydrates (28% *w*/*w*) was achieved using 1.2 $g \cdot dm^{-3}$ of $NaHCO_3$. In the case of *Scenedesmus* sp., only $NaHCO_3$ enhanced the concentration of carbohydrates (27% *w*/*w*) over the control (23% *w*/*w*). In the synthesis of total proteins, both $NaHCO_3$ and Na_2CO_3 reduced their concentration in *Chlorella* sp. On the other hand, 1.6 $g \cdot dm^{-3}$ of $NaHCO_3$ increased the concentration of up to 46% *w*/*w* of proteins in *Scenedesmus* sp. In the case of lipids, 1.2 $g \cdot dm^{-3}$ of Na_2CO_3 increased its concentration (8%) in *Chlorella* sp. in comparison to the control. In *Scenedesmus* sp. 1.2 $g \cdot dm^{-3}$ of $NaHCO_3$ increased the concentration by up to 8% w/w. The concentration of total carotenoids was increased up to 8% (w/w) in *Chlorella* sp. (0.8 g·dm^{-3} of Na_2CO_3); in contrast, none of the evaluated carbon sources enhanced the concentration of total carotenoids in *Scenedesmus* sp. Finally, the concentration of total ashes increased from 11 to 15% w/w in all the treatments for both algae.

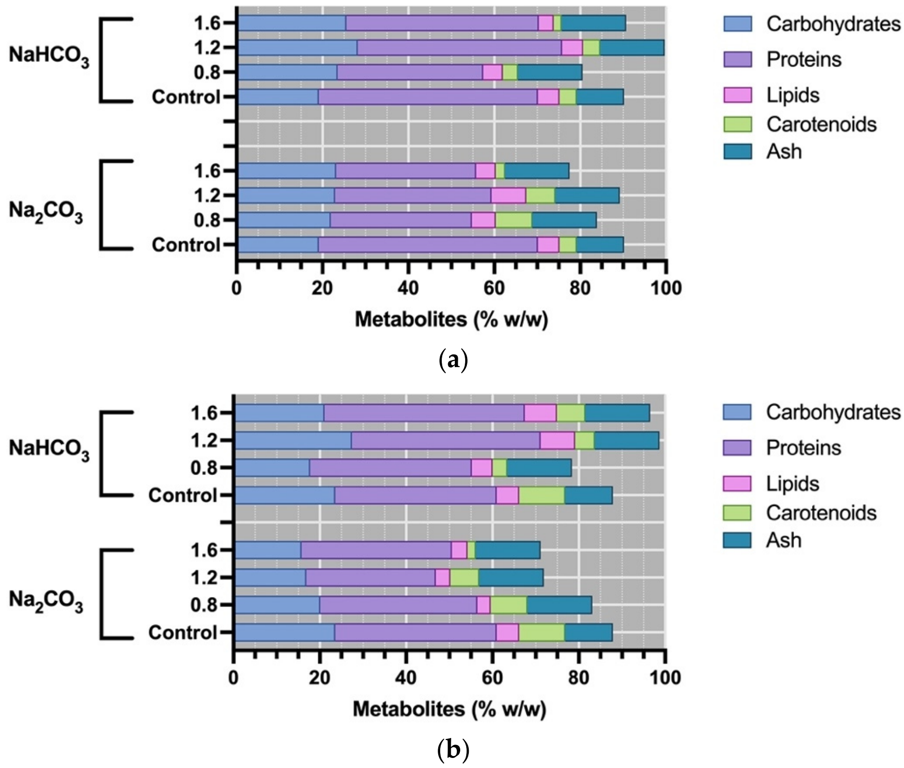


Figure 5. Production of metabolites of industrial interest at different carbonate and bicarbonate concentrations in *Chlorella* sp. (**a**) and *Scenedesmus* sp. (**b**).

4. Discussion

Sustainable production of feedstocks for different commercial purposes such as fuels, plastics, dyes, and others [5] is one of the main pillars to improve the sustainability of microalgae and cyanobacteria biomass-based bioprocesses [47]. The physicochemical characterization of the leachate shows that it has a pH of 9.74, which would allow optimal growth of most microalgae and cyanobacteria [37]. According to Kurniawan et al. [48], leachates less than one-year-old (young leachates) contain high COD concentrations (>15 g·dm^{-3}). On the contrary, leachates from stabilized landfills (older than five years) contain shallow COD concentration (<3 g·dm^{-3}), which is comparable to the COD observed in Table 2 with a value of 2.93 g·dm^{-3}, thus indicating that the leachate employed corresponds to old landfill leachate. During the last 20 years, different researchers have evaluated the ability of different strains to grow in culture media enriched with landfill leachate; however, different results have proved that microalgae could not withstand the toxicity of the medium due to the high concentrations of contaminants, causing inhibition in their growth due to a deficiency in their metabolization process [23].

High nitrogen concentrations ensure efficient phosphorus removal in the leachate [47]. According to Nordin et al. [28], some algae adapted to leachate can remove high concentrations of Nitrate and Phosphate. In their case, strains belonging to *Chlorella*, *Scenedesmus*, and *Oscillatoria* sp. isolated from Jeram sanitary landfill (Malaysia) can remove up to 380 mg·dm^{-3} of NO_3. In another research, Porto et al. [26] used 5% of leachate and removed up to 65% of NO_3 and 31% of PO_4 using *C. vulgaris*. On the other hand, when *Tetradesmus obliquus* was grown in medium with 15% v/v of leachate, only 56% of NO_3 and 29% of PO_4 present could be removed. However, the medium of T. obliquus contained

3 times more leachate, so the concentrations removed by the two strains are relatively close. Other results such as those presented by Paskuliakova et al. [21] demonstrate the ability of *Chlamydomonas* sp. SW15aRL to remove 97% of the PO_4 present in a system with 30% leachate. On the other hand, Chang et al. [36] were able to remove 99% of NH_4 and 100% of PO_4 by growing *C. vulgaris* in a culture medium with 50% leachate. Other works such as Chang et al. [32] evaluated the growth of *C. vulgaris* in undiluted leachate. Their results show that it is possible to remove up to 96% of $NH_4^+NO_3$ and 100% of PO_4 present in undiluted leachate. It is noteworthy that Chang et al. [32] designed a scalable membrane-based tubular photobioreactor (SM-PBR), which allowed reducing the contact of the cells with the culture medium, thus reducing the toxic effect of the leachate on the cells.

The addition of a carbon source is one of the most important parts of algal production, since this microorganism is considered a sustainable carbon sink. CO_2 is the most frequent source. In the photoautotrophic culture, the CO_2 reacts with the different salts in the media producing carbonate ions (HCO_3^-). These ions can flow through the algal membrane and be used in the chloroplast [47]. Therefore, supplementing the leachate with different concentrations of sodium carbonate or sodium bicarbonate will eventually improve the growth of the microalgae and reduce the polluting capacity of the leachate. According to the results reported in this work, the addition of $NaHCO_3$, especially in *Chlorella* sp. significantly increased the final biomass concentration; however, the removal of NO_3 and PO_4 did not increase in the same way as the biomass. This may be due to the lighting conditions inside the culture flask since high biomass concentrations and the characteristic color of the leachate (present in dilutions of 5% v/v) may reduce the ability of light to penetrate the flask and reduce photosynthetic capacity.

Different researchers have documented the efficiency of adding inorganic carbonaceous salts to algal cultures. White et al. [33] reported how the addition of $NaHCO_3$ significantly increased biomass and pigment concentrations. Other authors such as Pancha et al. [49] found that 0.6 $g \cdot dm^{-3}$ of $NaHCO_3$ boosts up to a 23% higher concentration on a strain of *Scenedesmus* sp. This specific result is similar to the final concentration achieved in this research. Na_2CO_3 has also been highlighted as having its positive effects in terms of high biomass production and lipid increase. Duan et al. [50] reported that 20 $mg \cdot dm^{-3}$ of Na_2CO_3 increased two-fold the final concentration of *S. obliquus.* Finally, a study found that the controlled addition of Na_2CO_3 coupled with NO_3 in a specific C/N ratio can dramatically increase the concentration of biomass and hydrocarbons in *B. braunii* [40].

When considering algal production in landfill leachates, the first metabolites that come to mind are lipids and carbohydrates. Both metabolites are important for their application as raw material to produce biodiesel and bioethanol [5]. Since most of the studied wastewaters cannot be employed to produce food or feed products, the energetic focus is the next step towards the sustainability of the algal production system. Therefore, most studies focus heavily on the effect of the leachate concentration on the synthesis of lipids and carbohydrates [51–56]. The work of Pancha et al. [49] proved that nitrate depletion together with stepwise addition of $NaHCO_3$ enhances the synthesis of lipids. These results are in accordance with the work of Li et al. [57], who found that a high concentration of $NaHCO_3$ in *Chlorella* sp. for example, 160 Mm (13.33 $g \cdot dm^{-3}$), stimulated lipid accumulation, although it inhibits cell growth.

According to Cuellár-García et al. [58,59], the type and concentration of the carbon source are particular to every single strain. Some are adapted to high carbonaceous environments, while others do not tolerate high levels of this ion, which in turn may synthesize more or carbohydrates, lipids, or even proteins. In a recent study, Vijay et al. [60] found that the addition of $NaHCO_3$ substantially increased the protein content in *S. obtusus*. Since the first two are the goal for many studies, the last may hinder the thermal efficiency of the biomass produced [61–65]. Carotenoids are considered high-value metabolites of great interest as nutraceuticals [60] which may not have value since the biomass was produced on leachate. Therefore, these low-cost carotenoids can be exploited as cheap colorants or dyes for nonhuman products such as plastics or fabrics, and the colorant-free biomass can be used for biofuels or even bioplastics production.

5. Conclusions

The results obtained in this research show the capacity of landfill leachate as a source of nutrients to produce algal biomass with specific metabolites. In this case, both strains were able to grow in media with up to 5% (v/v) leachate with a high concentration of biomass and removal of 80% (w/w) of NO_3, and 50% (w/w) of PO_4. However, higher levels of leachate (10% v/v) proved to be toxic for the strains. Regarding the carbon source, the results show that *Chlorella* sp. and *Scenedesmus* sp were able to increase the final concentration of biomass using both carbon sources, with sodium bicarbonate ($NaHCO_3$) being the carbon source that achieved the most significant increase in final biomass content (up to 1.23 $g{\cdot}dm^{-3}$, with a control of 0.68 $g{\cdot}dm^{-3}$) in *Chlorella* sp. On the other hand, the most significant production of metabolites such as carotenoids occurred when Na_2CO_3 was used in both strains at a concentration of 0.8 $g{\cdot}dm^{-3}$. In the case of lipids, $NaHCO_3$ in *Scenedesmus* sp obtained the highest values at 1.2 $g{\cdot}dm^{-3}$, while in *Chlorella* sp. Na_2CO_3 at 1.2 $g{\cdot}dm^{-3}$ achieved the highest lipid production. Also, the highest production was observed for obtaining carbohydrates and proteins when using $NaHCO_3$ at 1.2 $g{\cdot}dm^{-3}$. For carbohydrates, both strains used $NaHCO_3$ at that concentration, while for proteins, only *Scenedesmus* sp. achieved the highest value.

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