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Hydrocarbon and Lipid Extraction from *Scenedesmus sp*. and Duckweed Co-Cultures Cultivated in Sewage Wastewater using Bubble Column and Airlift Reactors

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Fossil fuel depletion and the remediation of industrial wastewater can be solved simultaneously through cultivating microalgal biomass to produce biofuels. This study's objective focuses on the monoculture and co-culture of *Scenedesmus sp*. and *Lemna sp*. (Duckweed) for hydrocarbon and lipid recovery by employing a pilot plant study in a real industrial wastewater setting. This study aimed to quantify the extracted lipids and valuable hydrocarbons. When compared to Scenedesmus monoculture, the results showed that co-cultivation with duckweed significantly increased biomass productivity by 90 %, possibly due to an improved oxygen balance and synergistic nutrient uptake. Cultivation was performed in a 3-column bubble photobioreactor with controlled pH, light, growth medium, nitrogen and CO_2 aeration regulation. Harvested biomass underwent a combination of solid-liquid extraction techniques for the extraction of lipids and hydrocarbons. SEM revealed the surface morphology and efficient extraction of the algal biomass. The biomass TGA and DSC thermograms indicated that it was thermally stable up to 250 °C, with different decomposition patterns associated with different lipid fractions. FTIR spectra identified clustered functional groups around 1,000 – 1,300 cm^{-1} and ~1,460 cm^{-1} linked to long-chain hydrocarbons, esters, alkanes, and aromatic hydrocarbons. Alkanes, alkenes, and long-chain fatty acid methyl esters were identified by GC-MS as the dominant fractions in the extracts from the co-cultures (57.68 % FFA's, 10.84 % MAG's, 21.48 % DAG's and 7.85 % TAG's), showing a richer hydrocarbon profile and a higher yield of total hydrocarbons and lipid derivatives (27.6 % dry weight).

1. Introduction

The growing worldwide challenge of producing alternative fuels due to the limited, high-priced fossil fuel, also known as petroleum derived fuels, remains an urgent need. Multiple limitations in oil supply, availability, global warming and CO_2 emissions add to the demand for biofuels. Algal biomass gained significant attention as an alternative. Fossil fuel derived fuels are not only experiencing immense pressure of demand from the rapid urbanisation and population growth but further add to the negative global emissions of greenhouse gases causing environmental damage and climate change (Chen *et al*., 2018). In the United States, 18 million barrels of fuel are required per day (Lohman *et al*., 2013). These catastrophes necessitate the need for a more sustainable and renewable source of energy, i.e., biofuels. Biofuels provide several advantages, such as CO_2 neutrality, reduction in greenhouse gases and continuous biomass supply. Microalgae have gained significant attention as a renewable source for biofuel production. Biofuels are distinguished according to their physical characteristics, such as solids (biochar), liquids (bioethanol, vegetable oil and biodiesel) and gas (biogas, biosyngas and biohydrogen). First-generation biofuels (corn, soybean, sunflower, palm oil, rapeseed) and second-generation biofuels (Jatropha, Miscanthus and organic waste) still face challenges and compete with food supply or arable land. Third-generation biofuels produced from microalgae diminish these challenges since they require small land spaces and food competition is not experienced, while still possessing the ability to grow rapidly, high photosynthetic activity, high biomass accumulation and up to 100 times more lipid yields. They have the ability to grow heterotrophically (organic compounds only) or mixotrophically (organic compounds and CO_2). Microalgae's main storage lipids are neutral lipids (NL's) and triglycerols that can undergo

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transesterification to FAME's of C16 – C18 profiles, best suitable for biofuel production (Kona *et al*. 2022). Microalgae's great adaptation and manipulative characteristics make them easy to grow either on land or in closed systems, utilising various wastewaters, CO_2 enriched streams, waste organics or excess heat to gain nutrients and grow. Furthermore, this mechanism promotes economic feasibility, environmental sustainability, wastewater remediation and biofuel production. One of the major challenges associated with large-scale commercial algal biomass harvesting and lipid extraction is the cost factor as the extraction process is a power intensive and time consuming process (Marino *et al.*, 2021), Lipids are stored within the microalgal thick, rigid cell wall composed of complex carbohydrates and glycoproteins with mechanical strength and chemical resistance, hence adaptation to extract lipids efficiently in different microalgal species and quantify extracted lipids is vital (Sembada and Faizal, 2022). Similarly, Duckweed (Lemnaceae species) is also recognized as the fastest growing aquatic plant with high protein, carbohydrate and lipid contents (Takács *et al*., 2025). Although *Scenedesmus sp*. has proven itself for lipid extraction, significant bottlenecks and lack of pilot scale studies with real wastewater utilization remain. This study investigates the synergistic effects of co-culturing microalgae with aquatic macrophytes under real conditions. Previous co-culture systems analysed lab scale data in synthetic growth mediums (Hariskos and Posten, 2014). Furthermore, comparing hydrocarbon compositions between monocultures and co-cultures has been unexplored. Filling these gaps in a controlled environment with real sewage wastewater, with multi-platform characterization is one of few.

2. Microalgal lipids

Major fatty acids in microalgae are known to be 12 – 22 carbon atoms with between 0 – 6 double bonds. Esterifiable lipids are converted into FAMEs (the main component of biodiesel), affecting both the quantity and quality of biodiesel. Microalgal lipids are categorized into two types: (i) non-polar (NL's) namely acylglycerols, sterols, free fatty acids, wax and steryl esters, (ii) polar lipids namely phosphoglycerides, glycoslglcerides and sphingolipids. These lipids are different and play different roles in the structural components, metabolic and biological activities within the microbial cells. Nonetheless, these quantities of lipids vary according to species type, growth conditions and environmental conditions. For successful lipid extraction from the microalgal cell walls, specific microalgal pretreatment procedures must be considered. Various methods that allow lipids to be released into the extracting mixture include bead beating, microwave, ultrasonication, chemical methods, enzymatic disruption and many others.

2.1 Microalgal lipid extraction and quantification

Lipid extraction involves the separation of valuable lipids and fatty acids from the microalgal cellular matrix structure and water. Multiple quantification of lipids methods exists, commonly the gravimetric method using extraction solvents, Nile Red, Sulfo-Phospho-Vanillin Assay (SPV) and Thin Layer Chromatography (TLC).

2.1.1 Gravimetric analysis with organic solvent extraction (Bligh and Dryer 2:1 v/v chloroform and methanol)

The most common and widely used method to determine lipid content and validate other methods is the gravimetric method, consisting of lipid extraction using solvents following the basic chemistry concept of "like dissolves like", and lipid quantification using the weight of the sample after solvent evaporation (Tang *et al*., 2016). The solvent type used includes organic solvents, CO_2 solvents and ionic solvents. The microalgal lipid extraction mechanism occurs in 5 steps. Organic solvents seeped through the microalgal cell wall into the cytoplasm (step 1) to start the interaction within the complex lipid bodies (step 2). The non-polar organic solvents react with the NLs through Van de Waals forces, while the polar organic solvents react with polar lipids, generating strong hydrogen bonds that are strong enough to replace the lipid protein complex that prevents the non-polar organic solvents from accessing the lipids. As a result, an organic solvent lipid complex is produced (step 3), leading to the organic solvent lipid complex mixture diffusing out of the cell membrane (step 4) and the static organic solvent film (step 5), into the common bulk solvent mix.

2.1.2 Quantification of microalgal fatty acids

The biofuel potential components found in microalgal biomass are theoretically determined by the acyl chains of lipids, quantified by the total fatty acid constituent yield. Each fatty acid constituent differs in structural features (chain length, positioning, degree of saturation, branching of carbon chains) and other chemical groups (hydroxyl, epoxy, cyclo and keto). Lang *et al*., (2011) reported that the most common and abundant fatty acids found in microalgal lipid extract are palmitic acid (hexadecanoic, C16:0), stearic acid (octadecanoic, C18:0), oleic acid (octadecadienoic, C18:2) and linolenic acid (octadecatrienoic, C18:3), however other fatty acids C14, C20, C26 – C32 appears, but in relatively low concentrations. The quantification of microalgal fatty acids involves two main steps, namely: (i) FAME preparation and (ii) compositional analysis via gas chromatography.

Step 1: Transesterification or In Situ Transesterification
Algal triglycerides react with methanol and a catalyst (acid, base, or both) under heat to form FAMEs and glycerol. Common catalysts include NaOH, HCl, H_2SO_4, acetyl chloride, and methanol mixtures with H_2SO_4 and chloroform. Ratios are based on the fatty acid content post-lipid extraction.
In situ transesterification, FAMEs are produced directly from whole biomass without prior lipid extraction, providing a more accurate representation of total FAME content and biofuel potential.
Step 2: GC-MS Analysis
FAMEs are separated and quantified using gas chromatography-mass spectrometry (GC-MS) with a flame ionization detector (FID), employing temperature-specific columns.

3. Materials and Methodology

Scenedesmus sp. obtained from Nelson Mandela University (NMU) and Duckweed from local ponds in Durban were cultivated in sewage wastewater collected from a treatment plant in Durban, South Africa. Raw effluent was treated to 24 h UV sterilization before cultivation to eliminate microbial contamination. Cultivation took place in a 3-column, automatically pH controlled bubble column under operating conditions pH of 8.3, 18 h light illumination, 3:1 sewage to BBM growth medium ratio, 7.5 L/h flow rate of 50 % air and 50 % nitrogen, 5 % CO_2 sparging, in a 1 m long column, with 33 cm diameter. On completion of the cultivation cycle, the biomass was harvested using settling and filtration, followed by immediate solvent extraction. Lipid extracts were analysed using FTIR (Perkin Elmer Spectrum Two), TGA (TA Instruments SDT Q600) and GC-MS (Shimadzu QP2020 NX). Leftover biomass was observed using SEM analysis (Tescan MIRA3 RISE) and XRD.

3.1 Extraction of algal lipids and hydrocarbons

Lipid and hydrocarbon extraction from harvested algal biomass was conducted using a solid-liquid solvent extraction method, based on the modified Bligh and Dyer technique. Approximately 10.00 g of wet biomass (equivalent to ~1.00 g dry weight) was homogenized with 20 mL of a chloroform: methanol mixture (2:1, v/v) to ensure efficient extraction of both polar and non-polar lipids, based on the modified Bligh and Dyer method Guckert *et al*. (1988) and (Bligh and Dyer, 1959). To enhance cell disruption, solvent penetration and lipid solubilizaiton, the mixture was vortexed for 10 min to ensure effective disruption of the cell matrix and dissolution of intracellular lipids. The homogenate was then centrifuged at 4,000 rpm for 10 min to separate the organic solvent layer. The lower organic phase (supernatant) containing the lipid extract was carefully collected. To maximize lipid recovery, the residue was re-extracted with an additional 10 mL solvent mixture and centrifuged again. The combined organic extracts were combined and washed with a saline solution (0.9 % NaCl) using a 1:1 ratio to remove impurities and phase-separate the layers. The organic layer was separated, transferred to pre-weighed glass vials and dried over anhydrous sodium sulfate to remove any residual water, until a constant weight was achieved. The total lipid content was determined gravimetrically by weighing the extracted lipids and expressed as a percentage of the dry biomass weight.

3.2 Transesterification of Extracted Lipids

The crude lipid extracts were subjected to acid-catalyzed transesterification to convert triglycerides into fatty acid methyl esters (FAMEs) for biofuel characterization. A 1:10 (w/v) ratio of lipid to methanol was used, where methanol was pre-mixed with 1 % (v/v) concentrated sulfuric acid (H_2SO_4) as the catalyst. The reaction was carried out in sealed glass tubes at 60 °C in a water bath for 90 min with occasional mixing. After cooling to room temperature, the reaction mixture was diluted with 5 mL of hexane and 5 mL of distilled water to facilitate the separation of the FAMEs. The upper hexane layer containing the methyl esters was carefully collected and dried over anhydrous sodium sulfate, and stored in GC-MS vials for analysis.

3.3 FAME analysis

The extracted fatty acid methyl esters (FAMEs) and hydrocarbon fractions were analysed using GC-MS equipped with a flame ionization detector (FID) 75954 – 30 using splitless µL injections into a 30 m (Silica) SH-I-5Sil MS capillary column optimized for lipid profiling. Individual FAME components were identified by comparing retention times and mass spectra with commercial standards, including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linolenic acid (C18:3). Quantification was performed using internal standards such as methyl pentadecanoate (C15:0) and methyl heptadecanoate (C17:0), and results were expressed as a percentage of dry biomass. Simultaneously, hydrocarbon compounds including alkanes (C10–C32), alkenes, and aromatic hydrocarbons were identified based on spectral comparison with the NIST reference library. Quantitative analysis of hydrocarbon content was conducted using internal standards such as n-eicosane (C20) or squalene. The total hydrocarbon yield was likewise reported relative to the dry biomass weight and compared across monoculture and co-culture systems to evaluate compositional differences.

4. Results and Discussion

4.1 FTIR analysis

The FTIR spectra below (Figure 1a and b) cover the typical range up to 4,000 cm^{-1}, highlighting key functional groups relevant to biodiesel characterization. Prominent peaks around 2,950–2,850 cm^{-1} correspond to C–H stretching vibrations, indicative of alkanes saturated hydrocarbons contributing to the fuel's energy content. Peaks near ~1,700–1,740 cm^{-1} are associated with C=O stretching, confirming the presence of carbonyl compounds such as esters and ketones, key indicators of biodiesel components like fatty acid methyl esters (FAMEs). The *Scenedesmus sp*. monoculture exhibits broader and more intense C–H stretching peaks, likely due to a higher diversity of bio-compounds. In the 1,050–1,250 cm^{-1} region, the observed C–O stretching bands suggest the presence of alcohols, esters, and ethers, i.e. oxygenated compounds beneficial for combustion efficiency. The co-culture of Duckweed and Scenedesmus displays similar spectral features but with more pronounced peaks around ~1,700 cm^{-1}, implying a higher concentration of esters or ketones. Enhanced absorbance in the 1,000–1,300 cm^{-1} region further indicates a richer profile of oxygenated functional groups, potentially due to greater bio-oil or lipid derivative content. Subtle variations near ~2,600 cm^{-1} may reflect differences in hydrocarbon composition and their interaction with other biomolecules. Comparative analysis reveals that the monoculture contains relatively higher concentrations of carbonyl and C–O functional groups, underscoring its suitability for biodiesel production. In contrast, the co-culture blend demonstrates a broader spectrum of oxygenated compounds, suggesting an elevated presence of esters and alcohols, which could enhance combustion properties. Additionally, absorbance near ~1,460 cm^{-1} corresponds to CH_2 and CH_3 bending vibrations, reinforcing the presence of hydrocarbon chains. The strong O–H stretching band around ~3,400 cm^{-1}, observed more distinctly in the co-culture, indicates alcohols, phenols, and other bio-related compounds that may offer advantages in fuel properties. The co-culture regime with a higher composition of esters and carbonyls suggests a more FAME and biodiesel ready compound.

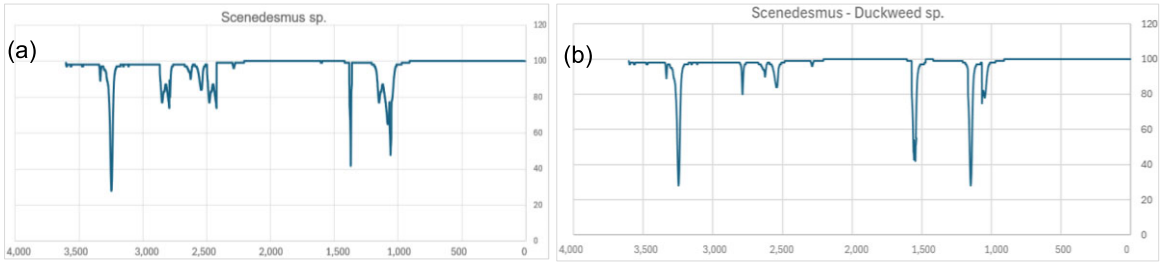

Figure 1: FTIR chromatograph of extract culture from (a) Scenedesmus monoculture; (b) Scenedesmus-Duckweed co-culture

4.2 GC-MS

When compared to a standard mix of 22 analytical grade lipids, the monoculture extract was composed of 23.71 % FFAs, 21.58 % MAGs, 29.54 % DAGs and 19.12 % TAGs. The co-culture extract consisted of 57.68 % FFAs, 10.84 % MAGs, 21.48 % DAGs and 7.85 % TAGs. While traditional transesterification methods for biodiesel production prefer TGA-rich extract, the co-culture presents a distinct characteristic for direct esterification, since FFAs are ideal for acid-catalysed esterification. Bypassing transesterification makes larger-scale applications easier and more cost-effective. The high FFA's show great activity of lipid production in the co-culture system, suggesting a successful, robust and adaptable microbial consortium.

4.3 TGA/DSC analysis

TGA (mass loss % as temperature increases) assessed the thermal behavior and decomposition of monoculture versus co-culture, as it was essential to understand and evaluate its suitability for biofuel applications. TGA curve revealed distinct thermal degradation, highlighting the key differences in moisture content, volatile compounds and char formation (Figure 2a and b). Between 50 – 200 °C, a minor loss was observed due to the evaporation of free and bound water. *Lemna sp*. displayed slightly higher weight loss during this phase, indicating and aligning with the initial higher moisture content during the characterization steps. Between 200 – 500 ºC, major significant decomposition took place due to the breakdown of major biochemical components such as hemicellulose (200 – 300 ºC), cellulose (300 – 400 ºC) and lipid and proteins (~ 250 – 500 ºC). Hence, the presence of proteins and lipids in *Lemna sp*. led to a more pronounced degradation rate as compared to *Scenedesmus sp*. Beyond 500 ºC (500 – 900 ºC), the biomass consisted of fixed carbon and inorganic ash. The residual mass of *Scenedesmus sp*. was slightly higher, indicating a higher char formation, which has implications

for biofuel production. DSC thermograms reveal the critical thermal transitions in the biomass. The stronger endothermic peak (± 100 ºC) corresponded to the moisture content, exothermic peak (200 – 500 ºC) indicates the decomposition of organic matter, with *Lemna sp*. showing a more intense exothermic response, suggesting a higher response during pyrolysis. The exothermic peaks confirm that the co-culture releases more energy during decomposition, making it suitable for thermal conversion and higher energy release.

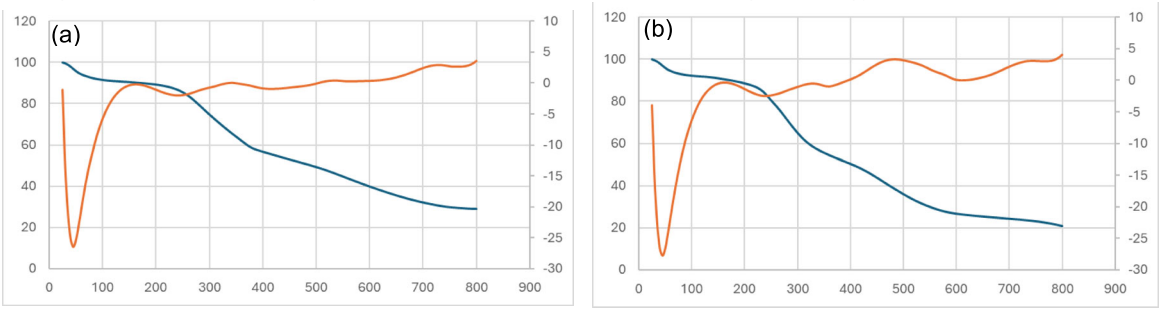

Figure 2: TGA and DSC curves of lipid extracted from (a) monoculture; (b) co-culture

4.4 XRD analysis of monoculture biomass versus co-culture biomass

The XRD pattern (Figure 3a) indicates Scenedesmus monoculture exhibits moderate crystallinity, characterized by broad peaks associated with semi-crystalline cellulose and structural polysaccharides. These features suggest an organized cellular matrix with a notable amorphous component. The co-culture biomass follows a comparable trend, with noticeable peak shifts and increased peak intensities. This variation indicates mutual influence between the two species on biomass structure and composition, potentially enhancing heterogeneity in cell wall structure, which may improve lipid accessibility. The intensified peaks in the co-culture (Figure 3b) suggest increased crystallinity and higher biomass density, implying enhanced biomass productivity and potential for downstream lipid and hydrocarbon extraction. Higher crystallinity usually impedes lipid extraction, but the higher amorphous areas appear more permeable for solvent diffusion. The shared synergies and increased nutrient uptake from wastewater improved lipid and hydrocarbon generation. Because the co-culture system combines higher biomass with better extractability, it seems more beneficial for biofuel applications.

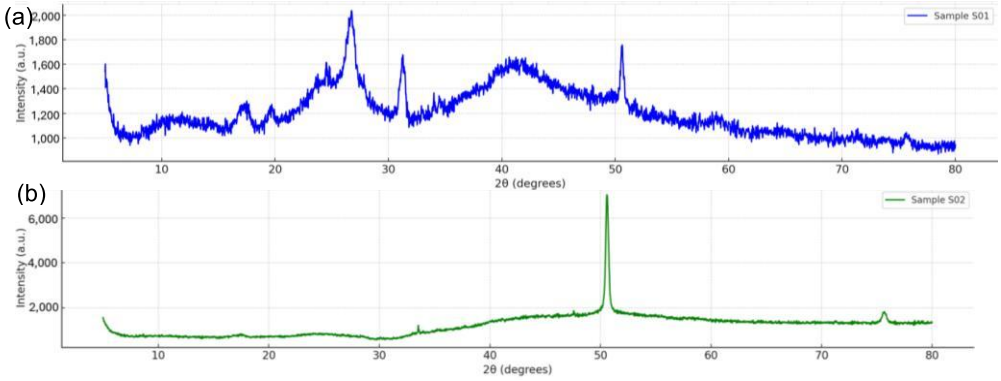

Figure 3: XRD patterns of biomass (a) monoculture and (b) co-culture

4.5 SEM analysis of biomass prior and after extraction

The structural composition and extraction effectiveness of *Scenedesmus sp*. biomass monoculture and Scenedesmus-Duckweed sp. co-culture systems were crucially studied. The monoculture sample had compact cellular arrangements with distinct and intact cell walls before lipid/hydrocarbon extraction (Figure 4a and b). *Scenedesmus sp*. robust cell wall composition, which is primarily composed of cellulose and spore-like materials, and a relatively small surface area, favored the resistance to solvent penetration. Complete cellular lysis was not consistently attained, but post-extraction images showed moderate cell wall disruption with apparent surface indentations and localized ruptures. The co-culture system showed more morphological changes. A considerably larger and more porous biomass matrix was seen in Figure 4c, along with widespread cellular disruption and collapse in the tissues of the duckweed and algae (Figure 4d). Full cell wall rupture and extensive extrusion of cellular contents were observed. Increased solvent permeability and diffusion were caused by the co-culture's heterogeneous composition of duckweed fronds and root structures. Improved

solvent biomass interactions and more efficient lipid mobilization were possible by the larger surface area and decreased structural cohesiveness. The co-culture possessed complementary growth traits that have promoted greater biomass accessibility, which would have enhanced the effectiveness of lipid and hydrocarbon recovery.

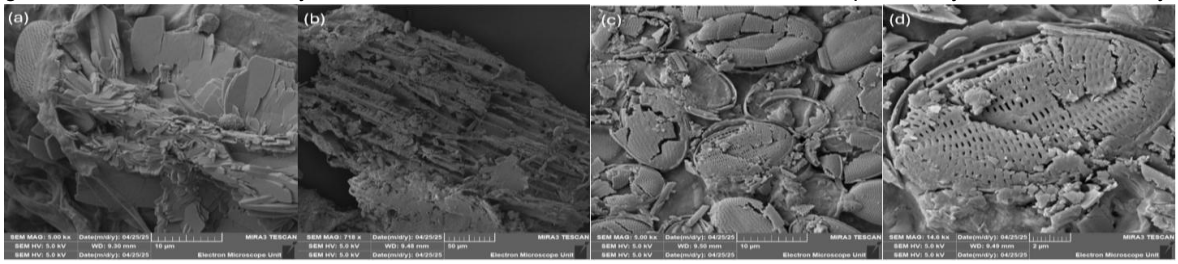

Figure 4: Scanning Electron Micrographs of biomass samples before and after lipid/hydrocarbon extraction: (a) Scenedesmus sp. before; (b) Scenedesmus sp. after; (c) co-culture before; (d) co-culture after

5. Conclusion

This study demonstrated that co-culturing not only boosted lipid/hydrocarbon yields but also aided in extraction and harvesting due to structural advantages. *Scenedesmus*-Duckweed sp. performed better with a richer biofuel profile, offering greater functional enhancements for a wider range of bioproducts, biofuel performances and emission profiles. However, either extract does not display extreme deviations or contaminants, making it safe and suitable for engines/biofuel production. *Scenedesmus sp*. monoculture has a closer composition to petrodiesel, better for conservative blending. The co-culture demonstrated efficiency with higher FFAs, highlighting the potential of integrated cultivation approaches in improving downstream bioprocessing. Utilizing real wastewater further aligns with SDGs, economic practices and reducing global waste. This form of wastewater remediation targets dual benefits for both the environment and the energy sector.

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