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Optimising microalgae upscaling for sustainable biomass production using statistical design: A Box-Behnken, RSM and ANOVA-based approach

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ABSTRACT

Although microalgae have been widely studied for biofuel production, most optimisation studies focus on synthetic media or small scale trials, limiting real world applications. This study targets that research gap by characterising four industrial wastewater streams (sugar, dairy, brewery and sewage) and three high lipid algal strains (Chlorella vulgaris, Scenedesmus sp. and Spirulina platensis), identifying attractive lipid composition of 15.9 %, 28.55 % and 9.05 % respectively and identifying the best suitable wastewater and its adaptability. This is done by applying the Box-Behnken Design (BBD) with Response Surface Methodology (RSM) and Analysis of Variance (ANOVA) to optimise cultivation parameters (pH, light illumination period, wastewater growth medium ratio). The optimised conditions (pH 8.3, 18-hour light, 2:2 BBM-to-sewage ratio, 5 % CO_2, 7.5 L/min aeration) achieved 0.79/day at 1 L bench scale conditions, and 0.577/day at 30 L pilot reactor, validating the challenges experienced when upscaling. Additional enhancement from the nitrogen and CO_2 achieved 0,752/day. Critical parameter interactions, particularly between light illumination and growth medium composition, were identified ($p < 0.0001$). Unlike previous studies, this work optimised using real sewage wastewater, offering a practical and validated framework for decentralised wastewater treatment with biofuel production systems.

Introduction

Industrialization, urbanization, population growth and the over-reliance on fossil fuel resources have significantly contributed to the increase in greenhouse gases (GHGs), intensifying the global demand for freshwater, food, energy and technology. This resulted in the generation of excessive amounts of wastewater, a byproduct of consumption, manufacturing and production processes from various sources, including domestic, industrial, municipal, agricultural and stormwater [1]. The industrial sector significantly contributes to increased wastewater production, threatening the availability and quality. Notably, 359 billion liters of wastewater is generated globally per day. Numerous industrial sectors contribute to the large volumes of wastewater effluent; however, the quantity of wastewater generated does not correspond to the potential risk related to that wastewater source. Industrial wastewater is of major concern due to the composition and accumulation of suspended solids, organic and inorganic materials, nitrates, nitrites and soluble phosphorus present in water bodies [2]. The composition of wastewater is continuously changing with variations in strength, concentration, volumes and the presence of organic biodegradable materials, non-biodegradable substances, heavy metals, nitrates, nitrites, sulphates and other potential inhibitors that can severely affect the ecosystems and environment when released into the waterbodies. Furthermore, this poses severe threats to the environment and public health [3,4]. A study by Plohn et al [5] found that approximately 80 % of global wastewater is discharged without adequate wastewater treatment, posing hazardous implications for the ecosystems and human health. Nonetheless, population growth, urbanisation and industrialisation further challenge the present wastewater treatment and disposal capabilities globally [6]. Shortages of potable water further intensify the challenges, with an expected 20 – 30 % increase in global demand by 2050 [5]. Environmental concerns, especially water contamination, have intensified over the past three decades, emphasizing the urgent need for ecological action [7].

These current environmental challenges necessitate the urgent need to develop innovative solutions. Traditional wastewater treatment systems struggle with existing challenges, often being insufficient, energy intensive and costly. Hence, microalgae's potential makes it a promising

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candidate for sustainable energy production and wastewater treatment. Microalgae utilisation offers the dual benefit of cultivation in nutrient-rich wastewater, supporting rapid and healthy growth, but also facilitates pollutant removal essential for microalgae growth, whilst producing valuable algal biomass [8,9]. Wastewater provides a nutrient rich environment enabling microalgae's growth to thrive, hence a potential solution to the global wastewater crisis and biofuel production. Cultivating microalgae in wastewater supports prospects of hydrocarbon and lipid recovery, bioenergy generation and other high value products such as lipids, fats, carbohydrates and pigments [9–11].

This study aims to establish selective microalgae strains possessing high lipid and hydrocarbon content suitable for biofuel production, particularly focusing on culturing and screening of the strains and various industrial wastewater. Advancing our understanding in this area, innovative technologies that promote sustainable water management, resource recovery and the reduction of GHG's can be developed effectively. Furthermore, this study will identify specific industrial wastewater mediums that support microalgae growth requirements while producing a substantial amount of biomass, as this bottleneck poses significant challenges when it comes to upscaling and optimizing. The variation of the growth medium ratio with Bolds Basal Medium (BBM) and sewage wastewater was a novel technique aimed at enhancing growth rate and biomass production. Utilizing the statistical experimental methodology allowed all the basics and critical parameters to be perfected according to the setup and operating conditions, with the aim of yielding maximum algae growth. Conducting a design of experiment analysis provided detailed, tailored information while also reducing experimental time and cost, hence selecting this widely used technique to optimize factors for the growth of *Scenedesmus sp*. At the bench scale level, simple cultivation parameters with simple factorial plans were selected as the starting point. Concluding from the DOE analysis revealed the significant factors influencing the output responses, as well as which factors hold potential threats as seen by the nonlinear data in the RSM and contour plots. This study selected the DOE as it was necessary to know all the statistical analysis, ANOVA and linear regressions to set the foundation of the cultivation process. Box Behnken Design utilized 3 factor levels for this design with fewer runs as compared to Central Composite Design, hence making it cost effective. Many other studies utilized the Box-Behnken and design for algae growth and biomass optimization. Although several studies have applied statistical optimisation to microalgal cultivation, most rely on solely synthetic media or solely wastewater, remaining limited to laboratory-scale experiments which do not adequately capture the complexity of real wastewater streams or the challenges of scale-up (Table 1).

Statistical optimisation approaches, particularly the Box–Behnken Design, have been applied to improve algal growth and lipid productivity. For instance, Scenedesmus sp. achieved lipid yields up to 315 mg/L, Arthrospira maxima produced 235.98 mg/L of phycocyanin, and Chlorella vulgaris recorded growth rates of 0.84/day with biomass productivity of 147 mg/L/day under optimised conditions. Similarly, Scenedesmus sp. produced up to 32.7 % lipid dry weight, confirming the strong applicability of BBD and response surface methodologies in algal cultivation studies. Lipid productivities across strains vary widely, ranging from 35.1 mg/L/day in Scenedesmus quadricauda to 142 mg/L/day in Nannochloropsis oculata NCTU-3 (Javad et al., 2019). More recent research highlights Chlorella sorokiniana as a promising strain for integrated wastewater treatment and biofuel generation. Studies demonstrated that iron and zinc supplementation significantly enhanced its biomass productivity and lipid accumulation (Mohseni et al., 2021). Furthermore, the use of hematite iron oxide nanoparticles improved microalgae growth, nutrient removal, and bioproduct yields (Gao et al., 2023). Such studies strengthen the argument for combining real wastewater streams with nutrient enrichment strategies to improve the scalability and efficiency of microalgal cultivation systems.

Table 1
Summary of studies and key findings that utilised BBD for algal growth optimisation.

Literature source	Objective/Methodology	Design factors	Findings
[12]	Lipid production from *Scenedesmus sp*.	BBB (3 level – 3 factors)	315 mg/L maximum lipids
[13]	Arthrospira maxima phycocyanin yields	BBB (3 level – 3 factors)	235,98 mg/L phycocyanin
[14]	*Chlorella vulgaris* multi-response optimisation	BBB (3 level – 3 factors)	0,84/day growth rate, 147 mg/L/day biomass productivity
[15]	Lipid maximisation for *Scenedesmus sp* for biodiesel	BBB (3 level – 3 factors)	32,7 % lipids dry weight $R^2 = 0,9864$

This is the first study to combine wastewater sources to evaluate the enhancement of nutrients on growth rate and biomass yields. Few studies have systematically compared industrial wastewaters for microalgal productivity while integrating Box–Behnken Design (BBD) optimisation with pilot-scale validation. This gap limits both scientific understanding and practical implementation of wastewater-to-biofuel systems. This study aims to fill that gap through the following objectives (i) Characterise four wastewater streams (sugar, dairy, brewery, sewage) and three microalgal strains to identify the most promising strain–medium combination for lipid yield and biomass productivity, (ii) Apply BBD and ANOVA to optimise key cultivation parameters (pH, illumination cycles, growth medium ratio) at bench scale and (iii) Validate scalability by transferring optimised conditions to a 30 L pilot reactor and evaluating hydrodynamic and nutrient effects.

Previous studies compared similar factors on different reactor setups. Not many studies considered the natural qualities of the wastewater source, type of light source, or the various parameters in small scale to pilot plant studies. This study advances knowledge in the field by utilising real sewage wastewater from industry (not synthetic water) coupled with the application of BBD optimisation beyond the laboratory scale, making the applicability to real life more valid. This study investigated blue light illumination based on previous studies demonstrating its efficiency in enhancing chlorophyll activity and photosynthetic rates in *Scenedesmus sp,* as well as creating a more sterile cultivation environment. This study investigated the effect of alternative growth mediums and alternative air supply options (nitrogen + air), which is beneficial for economic viability, overall process costing and techno-economic analysis to design the lowest costing microalgal biorefinery approach that manages sewage wastewater effectively, providing long term solutions based on. The novelty and new knowledge of this paper targets novel application and analysis of Design of Experiments, Box-Behnken Design and Analysis of Variance with *Scenedesmus sp*., growth rate and in conjunction optimising biomass production. This method of statistical analysis is innovative in the field as it provides suitable models for different apparatus set ups and cultivation reactors. Furthermore, the BBM utilised the BBD method to capture growth rates by analysing the factors affecting growth rates, i.e. pH, light illumination and growth medium ratio. This baseline experimental matrix of 17 runs found the comprehensive outline for further upscaling. Analysis of this model and response factors evaluated the microalgae while energy accuracy and reliability, displaying the overall potential of biofuel production. An indepth analysis of the response surface plots and quadratic modelling concluded that solid, practical results were obtained at each step of this modelling. The regression model was evaluated using several statistical plots. Performing characterisation on the algal biomass and the various wastewater sources displayed each strain's capabilities and current wastewater compositions for future prospects and study needs.

Microalgae

Microalgae's strong ability to thrive and grow in various wastewater sources under diverse environmental conditions with low energy requirements appears feasible and sustainable on a global scale. Their potential to transform a variety of wastewater effluent nutrients into high-value compounds is primarily attractive, whilst various toxins are being removed through biosorption, bioaccumulation and biodegradation processes. Heavy metals are among the most common components of wastewater, causing toxicity and harming aquatic animals and plants. Their ability to resist decomposition results in heavy metals entering the food chain, posing major challenges to plants and animals. Microalgae have been identified as the most effective biomaterial for heavy metal absorption and accumulation, with greater potential than other types of sorbents. Aquatic habitats often contain microalgae and have attracted much attention, due to their high absorption capacity, low cost and natural occurrence around the world. Although algae remediation using wastewater studies started in the early 1960s, it still remains a sustainable bioeconomic approach. However, a novel process for large-scale applications remains to be developed. Microalgae's ability to utilise nitrogen, phosphorus, inorganic and organic carbon to grow aids in biomass production, decreasing N, P and chemical oxygen demand (COD) present in industrial wastewater sources, reduction of greenhouse gases and bioenergy production [16] Specific strains are capable of heavy metal removal via biosorption. Although industrial wastewater supports microalgal growth, the high metal concentrations and organic toxins, deficient carbon levels and low N and P compositions in industrial wastewater make cultivation of microalgae challenging [17,18]. Certain pollutants, mainly organic pollutants are degradable with the help of microorganisms; although some persistent organic pollutants (POP's) accumulate and exert toxic traits. According to a study by Chinnasamy *et al.* [19] combined removal of N, P, heavy metals and toxic substances using algae is achievable using industrial wastewater. These specific nutrients present in wastewater are vital and play a crucial role in microalgae growth, to ensure the formation of useful bioproducts [3,20]

The desired extracted products from microalgae are dependent on the environment in which the algae are grown, nutrient compositions and biomass yields. Nutrient composition plays a vital role in microalgae cultivation. Nutrient deprived or excessive environments affect the quality and quantity of biomass generated. Carbon dioxide together with light is utilised during photosynthesis, multiplying and producing valuable biomass. A study by Christi [21] stated that 1.83 tons of carbon dioxide is required to produce 1 ton of biomass; hence the role of algae and carbon fixation. Nitrogen concentration strongly influences microalgal growth and biochemical compositions, protein, lipid and carbohydrate synthesis [22,23]. According to Xu *et al.* and Prochazkovci *et al.* [24,25] nitrate is preferred over ammonium salts present in cultivation mediums for stability. It was also established that ammonia concentrations exceeding 25 μm are toxic to microalgal growth. Nitrogen depletion/deficiency, however, enhances lipid production but lowers biomass generation [26,27]. Phosphorus emerges as another critical factor affecting microalgal growth, hence impacting lipid production, fatty acid composition yield and the vital cellular process of microalgae [27,28]. Phosphorus is essential for synthesising phospholipids, DNA, RNA and ATP which are critical pathways for metabolic pathways [29]. Phosphorus should be less than 1% in algal biomass and 0.03 – 0.06 % in the cultivation medium for optimum growth [25,28,30]. Microalgae utilises phosphorus in the forms of polyphosphate or orthophosphate, to enhance microalgae growth and the nutritional composition of biomass [25]. Therefore, microalgae are continuously gaining attention and being recognised as a promising energy source due to their efficient use of wastewater, higher biomass yields in comparison to traditional conventional crops and their ability to grow on non-agricultural land. According to a study by Zuka et al [31], repurposing 50 % of industrial wastewater (approximately 88 trillion gallons) for microalgal cultivation could yield approximately 247 million tons of microalgal biomass and 37 million tons of microalgal lipids, with an oil content of 15 g per 100 g of dry algae.

The treatment of industrial wastewater faces multifaceted challenges that demand strategic solutions and are highly energy-intensive. Regulatory compliances also stand as a barrier, necessitating innovative wastewater treatment technologies; ensuring stringent environmental standards are met. The diverse range of contaminants present in industrial wastewater poses a significant threat to the environmental well-being, but by reviewing the composition and characterisation of wastewater sources, industries can mitigate this complexity and offer a targeted eco-friendly solution addressing wastewater management. Scalable treatment systems with energy-efficient technologies contribute to effective and responsible industrial wastewater treatment. Microalgae's ability to absorb and metabolise various pollutants aligns with this targeted eco-friendly and robust approach to addressing large volumes and contaminant diversity of industrial wastewater. Implementing a microalgal industrial remediation solution not only meets regulatory requirements but also contributes to a cleaner and more sustainable future [3,32].

For large-scale cultivation, strain selection, cultivation conditions and process feasibility are crucial to achieving the desired traits in the final product. However, significant challenges remain, including cell genetics, culture conditions, recovery efficiency and economic and technological barriers, which hinder the large-scale production of biodiesel from microalgae. Therefore, the selection of the most appropriate microalgae strain is crucial for an economically viable and energy-efficient process [21,33]. The selection of microalgal strains for biofuel production is guided by their potential for large-scale cultivation and overall feasibility. Key criteria for selecting these strains include their ability to produce high lipid content, dominance over other wild strains in open pond cultivation, minimal nutrient requirements, adaptability to diverse conditions, tolerance to a wide range of temperatures, and rapid growth rates with efficient productivity cycles. These attributes are essential for optimizing the biofuel potential of microalgae in industrial applications.

Microalgae utilisation for biofuel production is solely distinguished its growth rate and high lipid content, making them suitable to produce methyl esters through transesterification. Microalgae lipid content can comprise of up to 75 % of their biomass, depending on the selected strain and the cultivation technique [34,35]. Menegazzo and Fonseca [36] reported that when specific microalgal strains ae cultivated under the optimised conditions, the oil produced can surpass those of the most well productive plant-based vegetable oils – potentially achieving 47 000 – 141 000 litres per hectare annually. Microalgal lipid yields can exceed those of conventional plants by 20 times, but are influenced by factors such as growth mediums, hydrodynamics, fluid flow, biotic factors, abiotic factors and environmental conditions (e.g., light, temperature, salinity, nutrient availability), and extraction methods used [36,37]. *Chlorella sp., Nannochloropsis sp.,* and *Scenedesmus sp.* was highlighted as promising species for biofuel production due to their lipid content, rapid growth rates, high organic content and easy adaptability (Table 2). Optimum cultivation conditions ensure maximum lipid and biomass yields. Additionally, to make this process viable, selected harvesting and lipid extraction methods are essential. Therefore, selecting

Table 2
Microalgae species and their attainable lipid productivity (mg/L/day) (Javed et al., 2019).

Microalgal specie	Lipid productivity per day (mg/L/day)
Chlorella vulgaris	170
Scenedesmus quadricauda	35.1
Scenedesmus sp.	40.8 – 53.9
Nannochloropsis sp.	37.6 – 90
Nannochloropsis oculata	84 – 142
Nannochloropsis oculata NCTU-3	142

the most suitable microalgae strain in terms of biomass generation and lipid content is crucial.

Chlorella vulgaris, Scenedesmus sp., and *Spirulina platensis* were selected for cultivation and lipid and hydrocarbon extraction based on their known growth rate capability, biosorption capabilities, environmental adaptability and their efficiency in producing lipids suitable for biofuels. These particular strains have demonstrated strong potential in both biofuel production and wastewater treatment.

The *Chlorella species* in particular, *Chlorella vulgaris* is one of the most extensively researched microalgal strains with proven high lipid productivity. Ahorsu *et al* [38] found *Chlorella vulgaris* to demonstrate lipids yields of 60 – 70 % of its dry biomass, making it ideal for biodiesel production. Additionally, *Chlorella vulgaris* demonstrated effective nutrient removal, achieving 85 % total phosphorus (TP) and 89 % total nitrogen (TN) removal efficiency in wastewater (Wang *et al.*, 2015), and effective removal of metal ions such as Fe^{2+} and Mn^{2+} with 100 % efficiency and up to 98 % of Mg^{2+} and Al^{3+} [39]. *Chlorella vulgaris* biomass productivity was previously recorded to achieve 0.040 g/L/day when using air biosequestration, highlighting the potential and suitability of *Chlorella vulgaris* for biofuel applications [3,40].

Scenedesmus sp., known for its colonial growth form, also offers high lipid productivity and wastewater treatment efficiency. It has been reported to remove 87 % of TN, 83 % of TP and 92 % of suspended solids [41,42]. Additionally, *Scenedesmus quadricuda* achieved 92 % TN and 71 % phosphate removal from dairy wastewater, which is particularly nutrient-rich [4]. In terms of lipid yield, *Scenedesmus obliquus* recorded a biomass productivity of 14 g/L/day using 18 % CO_2 biosequestration, with lipid content ranging between 12 % and 14 % [17]. Furthermore, *Scenedesmus obliquus* produced lipids at a growth rate of approximately 0.009 g/L/day when cultivated under air biosequestration, making it attractive for biodiesel production.

Spirulina platensis is generally well recognised for its high protein composition (60 – 70 % of dry biomass weight), also possesses potential for biofuel production due to its lipid content (10 – 15 % of dry biomass weight). According to Rodolfi *et al.* [43], *Spirulina* has the potential to yield 20 to 40 liters of biodiesel for every ton of dry algal biomass under optimum cultivation conditions. Li and Zhou further added that *Spirulina* growth productivity reached 30 g/m/day, hence supporting its rapid growth rate [44]. *Spirulina* further demonstrates the ability to effectively remove pollutants from wastewater, achieving 95 % of ammonia and 85 % of phosphorus removal, aiding in the overall aim of biofuel production and wastewater treatment [45]. Although other strains such as *Botryococcus braunii* and *Nannochloropsis sp.*, also have the capability of producing lipids, their growth rates and longer cultivation times hinder their scalability. *Botryococcus braunii* has the potential to produce lipid content of 75 % but the slower growth cycle and low biomass generation makes it less feasible compared to *Chlorella vulgaris* and *Scenedesmus sp* [46]. Likewise, *Nannochloropsis oculate* can yield lipids up to 142 mg/L/day while its overall growth rates and productivity are not attractive [47].

In conclusion, the selection of *Chlorella vulgaris, Scenedesmus sp.*, and *Spirulina platensis* as optimal candidates for biodiesel production is well-supported by their unique and complementary strengths. *Chlorella vulgaris* is particularly notable for its high lipid content and adaptability to various wastewater conditions, making it ideal for large-scale biodiesel production. *Scenedesmus sp.* thrives in nutrient-deficient environments while accumulating significant hydrocarbons, offering a sustainable option for biofuel generation. Its robustness to climatic variations and its production of substantial lipid quantities, make it a resilient and productive option. They have been predominantly used in successful wastewater treatment, with the added advantage that modifications to their growth and stress conditions can further enhance specific desired traits, such as lipid accumulation. *Spirulina platensis* contributes rapid growth rates and efficient nutrient removal, enhancing wastewater treatment alongside biomass production. These strains provide a robust and comprehensive approach to maximizing lipid yield, enhancing biomass productivity, and delivering significant environmental benefits, making them highly suited for advancing biodiesel production and wastewater remediation in industrial settings. Additionally, the potential for co-culturing these specific strains present an exciting opportunity for future research, further supporting their selection for biodiesel production and underscoring the importance of continued investigation into their individual and combined capabilities.

Materials and methods

3.1. Wastewater characterisation

This study considered four various types of industrial wastewater, namely dairy, sugar, brewery and sewage wastewater obtained from industries located across Durban, South Africa. Wastewater samples were collected bi-weekly between 10:00 am and 11:00 am throughout 2024, ensuring consistency by sampling at designated time intervals. The effluents were collected directly from the final sampling points before environmental discharge. Repeated samples were obtained during each collection to verify parameter consistency and assess the stability of the industrial processes. The wastewater was collected using sterile 25-liter bottles for transportation to the laboratory. Thereafter, all samples were filtered and immediately stored in airtight 25-liter containers. The samples were preserved at 4°C to ensure their integrity for subsequent analysis. This methodological approach ensured accurate and timely analysis of wastewater samples, providing a reliable basis for assessing their suitability for algal cultivation and subsequent bioresource recovery. Physical, chemical and biological characterisation was performed for each wastewater source.

3.1.1. Physical characterisation

The key physical parameters of the wastewater source included pH, electrical conductivity (EC), temperature, total suspended solids (TSS) and dissolved oxygen (DO) and were measured using a YSI MPS 556 multi-parameter system at the Institute of Water and Wastewater Technology (IWWT), DUT. Total suspended solids were determined by measuring 100 mL of effluent poured into a pre-weighted beaker, followed by oven drying at 105°C until a constant weight was achieved. Total solids (TS) were determined by drying well-homogenized samples at 103°C for 24 hours. The volatile solids (VS) content was measured by incinerating the dried samples at 550°C for one hour in a furnace.

3.1.2. Chemical characterisation

Nitrogen concentrations including ammonia (NH_3), nitrite (NO_2) and nitrate (NO_3), along with phosphorus (P) and chlorine (Cl) concentrations, were measured according to the methods outlined by APHA, AWWA, and WEF (1998), a Gallery^{TM} Discrete water analyzer and a chlorine pocket meter as described by Mutanda *et al.* (2011), respectively.

3.1.3. Biological characterisation

Chemical oxygen demand (COD) was determined using a MODEL spectrophotometer. The samples were digested by mixing 2.5 mL of wastewater with 1.5 mL of $K_2Cr_2O_7$ and 3.5 mL of sulfuric acid reagent, then placed in a digester at 150 °C for 2 hours. After digestion, the samples were allowed to cool to room temperature before being shaken and settled to facilitate precipitation. The absorbance of the digested samples was then measured using the spectrophotometer to determine the COD values.

Biological Oxygen Demand (BOD) was assessed using 300 mL of well-mixed wastewater effluent. The initial dissolved oxygen (DO) concentration in each bottle was measured with a dissolved oxygen meter at the beginning of the incubation period. The standard incubation period was set at 20 ± 1 °C for 5 days. Following this period, the final DO concentration in each bottle was recorded. The BOD values reflect the change in oxygen levels, indicating the oxygen consumed per

liter of sample (mg/L). Blank bottles filled with distilled water were utilized to account for any changes in oxygen levels during incubation (APHA, AWWA, WEF, 1998). BOD was then calculated using the appropriate equation, with the dilution factor indicating how many times the original sample was diluted.

$$BOD = \frac{Initial\ dissolved\ oxygen - Final\ dissolved\ oxygen}{Dilution\ factor} \quad (1)$$

3.2. Microalgae biomass characterisation

Proximate, ultimate and compositional analyses were used to understand the microalgae composition and capabilities. Proximate analysis is important when considering fuel production, i.e. moisture content, ash content, volatile matter and fixed carbon content. This will help to understand the combustion behaviour of the fuel, whereas the ash content significantly affects the combustion rate. Ultimate analysis/elemental analysis (C, H, N, S and O composition of the sample) was carried out using an elemental analyser. Establishing the elemental ratios H:C, N:C and O:C play a crucial role in fuel efficiency and quality. Lastly, biochemical analysis established the lipid, carbohydrate and protein content present in the biomass. Experimental procedures used to determine physical properties of the algal biomass incorporated established standard methods and protocols that are widely used for biomass characterisation in various organisations such as ASTM (American Society for Testing and Materials) and AOAC (Association of Official Analytical Chemists), as seen below:

Proximate/physical characterisation

Moisture content (E1756): Five grams of wet algal biomass is weighed in a pre-weighed dish. The sample is then dried in an oven at 105°C until a constant weight is achieved. The moisture content is determined by calculating the weight loss during the drying period.

$$Moisture\ content\ (\%) = \frac{Initial\ biomass\ weight - dry\ weight}{Initial\ biomass\ weight} \times 100 \quad (2)$$

Ash Content (ASTM E1755): Five grams of the dried algal biomass are weighed in a pre-weighed dish. The sample is then incinerated in a furnace at 550 – 600°C until a constant weight is reached. The ash content is determined by calculating the remaining weight after incineration.

$$Ash\ content\ (\%) = \frac{Weight\ of\ ash}{Initial\ weight\ of\ dried\ sample} \times 100 \quad (3)$$

Volatile matter (ASTM E872): The dried algae sample is heated at 950°C for 7 minutes in a covered crucible. The volatile matter is determined by calculating the weight loss during the heating period.

$$Volataile\ matter\ (\%) = \frac{Weight\ loss}{Initial\ dried\ weight} \times 100 \quad (4)$$

Fixed carbon content

$$Fixed\ carbon\ (\%) = 100 - Sum\ of\ (Moisture\ content + Ash + Volatile\ matter)\% \quad (5)$$

Ultimate characterisation

Lipid extraction

Total lipid extraction was performed according to the method by Folch *et al.* (1957), where a 2:1 (v/v) mixture of chloroform and methanol was used with microwave assisted cell disruption [48]. 20 mL of solvent is added to 1 g of dried biomass and digested in a 1000-watt microwave digester at 100°C for 10 minutes. The solvent mixture containing the extracted lipids was allowed to settle in a liquid-liquid extraction separating funnel. The lower layer was removed and filtered using Whatman paper. The solvent was evaporated in a 60°C oven. The extracted crude microalgal lipids are then measured gravimetrically and lipid yields were calculated using the equation by Talukder *et al.* (2012) as follows:

$$Total\ lipid\ yield\ (\%) = \frac{Extracted\ lipid}{Total\ lipid\ in\ biomass} \times 100 \quad (6)$$

Protein analysis in whole algae and residual biomass

The protein content of the algal biomass was estimated according to Lowry *et al.* (1951) with reagents prepared as described by Lopez *et al.* (2010). A lysis buffer solution (25 mL per 100 mg of dried biomass) was added to the ground sample, which was then vortexed for 2 minutes. The supernatant was collected after centrifugation at 3000 g (gravimetric force) for 10 minutes. This process was repeated, and the supernatants from both rounds were combined. Thereafter, 0.5 mL of Sodium Dodecyl Sulfate (SDS) solution was added to 0.5 mL of the combined supernatant and vortexed. The mixture was combined with 5 mL of reagent-C, vortexed, and after 10 minutes, 0.5 mL of Folin reagent was added and left to stand for 30 minutes. Absorbance was measured at 750 nm using a HACH DR 300 spectrophotometer. The protein concentration was determined using a calibration curve, with the yield calculated as follows:

$$Protein\ yield\ (\%) = \frac{CVD}{m} \times 100 \quad (7)$$

Carbohydrate analysis in the microalgae biomass

The total carbohydrate content present in the algal biomass was determined using the sulfuric acid method as described by DuBois *et al.* (1956). A 100 mg sample of dried cell biomass was mixed with 2 % (v/v) diluted sulfuric acid and hydrolyzed in an autoclave at 121°C for 30 minutes. After hydrolysis, the mixture was neutralized by adding 1 M H_2SO_4 until effervescence ceased. The supernatant was collected by centrifugation at 1509 g for 10 minutes. From the supernatant, 0.1 mL was transferred into a test tube and diluted to 1 mL. Then, 1 mL of 5 % (w/v) phenol solution and 5 mL of 96 % H_2SO_4 were added. The mixture was incubated in a water bath at 30°C for 30 minutes. Absorbance was measured at 490 nm using a HACH DR 300 spectrophotometer. Glucose was used as the standard for the analysis, and the carbohydrate yield was calculated using the following equation:

$$Carbohydrate\ yield\ (L) = \frac{C}{V} \times M \quad (8)$$

Where C is the carbohydrate content (mg/mL) obtained from the calibration curve, V is the volume (mL) of the supernatant used for the analysis, and M is the total volume (mL) of the microalgal sample solution.

Elemental characterisation

Elemental characterization of the algal biomass involved analyzing its elemental composition to assess its suitability for biofuel production, especially biodiesel. Key elements investigated using a Perkin Elmer 2400 Series II CHNS/O Elemental Analyser (USA) included carbon (C), hydrogen (H), nitrogen (N), sulfur (S), and oxygen (O).

3.3. Design of Experiment (DOE) setup and analysis of variance

On completion of establishing the best suitable wastewater for sustaining the most attractive microalgae strain with the aim for highest growth rate (per day) and biomass productivity (cells/mL/day or mg/mL/day), the Design of Experiment was developed to consider, investigate and evaluate the effects of multiple basic parameters (pH, growth medium ratio and light illumination periods) affecting the response variables at bench scale level, in 1 L vessels. This design was based on

the Box-Behnken Design (BBD) and response surface methodology (RSM), commonly used for optimisation experiments. According to the DOE, 17 bench scale experimental runs for *Scendesmus sp.* and *Chlorella vulgaris* were compared. The faster growth rate and higher biomass yield of *Scenedesmus sp.* led to the elimination of *Chlorella vulgaris* at this point of study due to lower biomass production and slower growth rates. The response variables were analysed by Statistical Analysis of Variance (ANOVA) to compare the means of the different variable groups, understand main effects and interactions of single or combined independent variables and test hypotheses of factors in the experimental setup.

3.3.1. *Bench-scale setup and scale-up to 30 L pilot vessel*

Scenedesmus sp. and *Chlorella vulgaris* were first cultivated in 1 L reactor vessels bench-scale experimental setup under controlled operating parameters as per the Box-Behnken Design of Experiments (Fig. 1 a and b). Before inoculation, the sewage wastewater was sterlised for 24 hours using an 8 W UltraZap UV steriliser to prevent any contamination of the microalgal cells. A 1 L (working volume 500 mL) bioreactor setup was designed to evaluate the effect of pH (6.5 – 10), illumination light-dark cycles (24:0, 18:6, 12:12, 6:18, 0:24) at 60 μmol/m^2/s and varying ratios of Bold's Basal Medium (BBM) to sewage wastewater (4:0, 1:3, 2:2, 3:1, 4:0) on the overall growth rate and cell productivity of the selected microalgal strains. An air pump was used to continuously maintain optimal mixing and gas exchange within the reactors, while a blue light illumination source was used to enhance photosynthetic activity. The experiments were conducted at room temperature, which ranged between 23 – 29°C, with continuous monitoring of growth rates and cell productivities. Microalgae growth was measured daily at 10 am for the entire 18-day period to ensure consistent and accurate growth monitoring. Optical density using the spectrophotometer was based on direct readings expressed by the concentration of the microalgal cells in a properly homogenised solution. Optical density was expressed in μg/L units, representing the degree of light absorption by the solution at the 680 nm wavelength. This approach allowed for a precise investigation of the interactive effects of the experimental factors on the performance of the microalgal species under simulated wastewater treatment conditions. Most nutrients are absorbed during the time of cultivation until it reaches the stationary growth phase. Microalgae biomass was harvested during the stationary phase of the cultivation period, on days when the optical densities appeared highest, as large amounts of biomass were produced on these days. After harvesting is done and a new inoculation batch is input to grow. For small scale laboratory testing, biomass was harvested using centrifugation at 4000 rpm for 10 minutes. Once the optimised conditions and best performing conditions of the 1 L reaction vessels were established, the same conditions were transferred to a 30 L pilot vessel (30 cm diameter, 55 cm height) to analyse the effects of the reactor hydrodynamics (Fig. 1c). The overall methodological phenomenon is represented in Fig. 1d, and is the general operating procedure during all stages of cultivation setup.

Results

4.1. *Industrial wastewater characterisation*

Table 3 summarises the physical, chemical and biological characteristics of the investigated industrial effluent streams, with the aim of

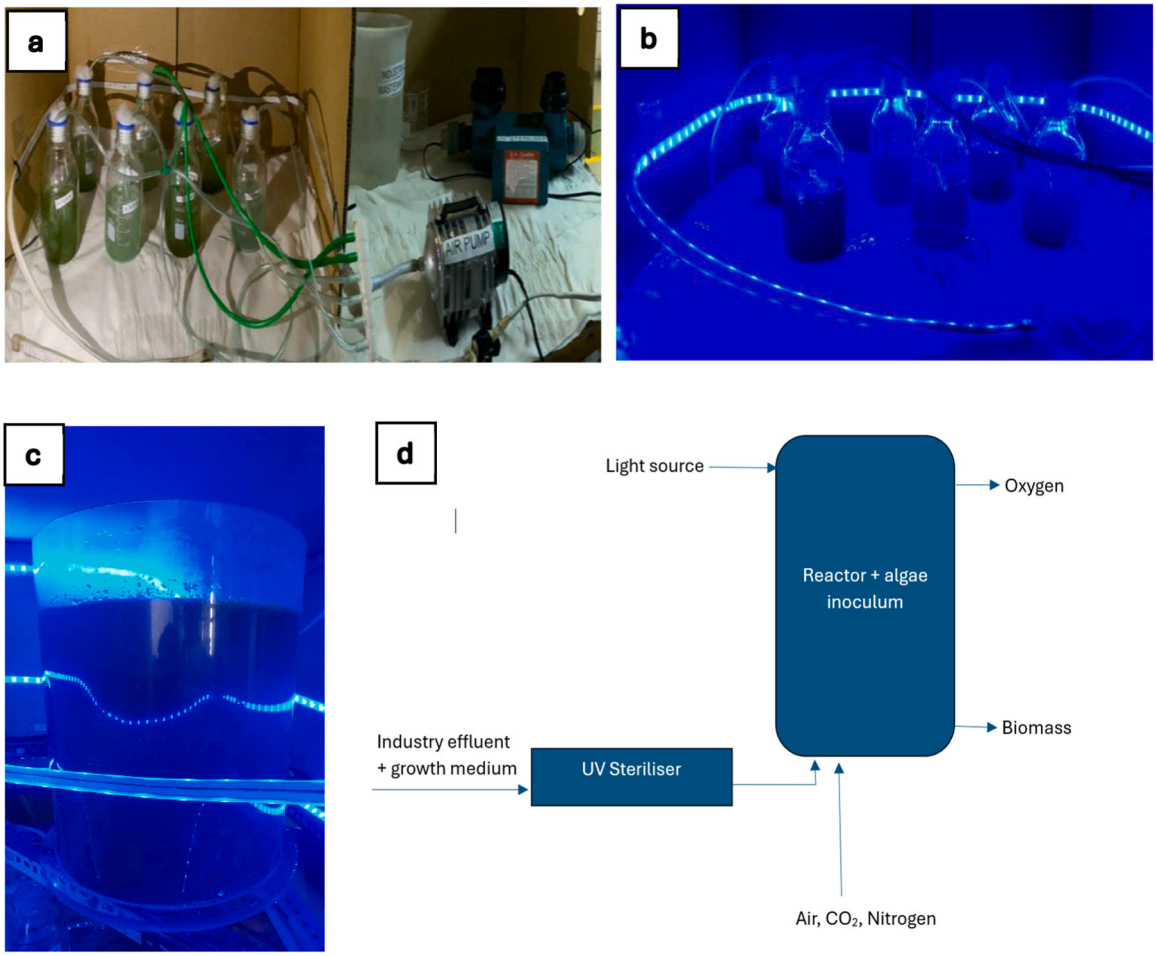

Fig. 1. (a) 1 L bench scale experimental set up, (b) illuminated 1 L bench scale setup, (c) 30 L reactor vessel setup and (d) overall schematic diagram.

Table 3
Full characterisation results of industrial wastewater sources.

Industrial source	Brewery	Sewage	Diary	Sugar
COD	6579 ± 226	1566 ± 145	10371 ± 289	1967 ± 130
BOD	3087 ± 162	1301 ± 97	1894 ± 217	1420 ± 94
TS	5863 ± 280	2743 ± 96	17654 ± 1874	2646 ± 109
TSS	3397 ±137	1756 ± 53	9979 ± 586	1754 ± 54
TDS	1711 ± 243	988 ± 70	6371 ± 487	969 ± 74
VS	2089 ± 120	2358 ± 158	3583 ± 173	2358 ± 158
pH	5.8 ± 0.2	3.52 ± 0.25	7.09 ± 1	4.9 ± 0.35
DO	0.19 ± 0.03 (2,5%)	0.38 ± 13 (3,9%)	5.13 ± 12 (50,3%)	0.39 ± 0.13 (3,9%)
Nitrates	0.05 ± 0.01	0.08 ± 0.01	0.06 ± 0.03	0.07 ± 0.015
Nitrites	1.49 ± 0.14	0.53 ± 0.01	0.53 ± 0.08	0.39 ± 0.07
Ammonia	0.85 ± 0.04	0.91 ± 0,01	0.91 ± 0.12	0.43 ± 0.05
Phosphates	14.97 ± 0.68	24.02 ± 0.66	0.69 ± 0.09	26.2 ± 0.64
Sulphates	26.30 ± 1.67	18.07 ± 1	0.59 ± 0.11	19.29 ± 1.12
Chlorine	0.5	2.3	4.7	0.9
Colour	Light brown	Deep yellow	Milky white/ cloudy	Brown

supporting microalgal cultivation. These results further emphasise the advantages of utilising large volumes of effluent for sustainable green processes, such as algal cultivation.

The characterisation of four wastewater streams established the physiochemical characteristics that influence their differences in organic load, nutrient availability and their capability for microalgal cultivation. Organic load, nutrient availability, solid content, pH and various other parameters play a significant role in microalgae growth, biomass productivity and biofuel yields. Brewery wastewater consisted of high COD (6579 ± 226 mg/L) and BOD (3087 ± 162 mg/L) concentrations, indicating the presence of a rich organic load, aiding in support of rapid microalgal growth (Fig. 2a), consistent with values reported by Yang et al. (2014) but higher than observations by Hossain et al. (2018). High TSS (3397 ± 137 mg/L) and TS (5863 ± 280 mg/L) indicate a higher concentration of solids compared to the other wastewater streams, which may pose challenges to light penetration and hinder photosynthetic activity. However, these issues can be mitigated through effective mixing and aeration. Additionally, brewery wastewater's volatile solids (VS) content (2089 ± 120 mg/L) provides ample biodegradable matter, facilitating algal proliferation. Furthermore, the high sulphate content (26.30 ± 1.67 mg/L) and relatively balanced nitrogen sources (nitrates 0.05 ± 0.01 mg/L, nitrites 1.49 ± 0.14 mg/L, and ammonia 0.85 ± 0.04 mg/L) support protein synthesis, making brewery wastewater an optimal candidate for high biomass productivity. Adjusting the slightly acidic pH (5.8 ± 0.2) and providing additional aeration to improve DO levels (0.19 ± 0.03 mg/L) are necessary steps to maximize algal growth potential in brewery wastewater.

Sewage wastewater appears with a more balanced composition for algal cultivation. Consisting of lower COD (1566 ± 145 mg/L) and BOD (1301 ± 97 mg/L) than brewery wastewater. Sewage effluent presents a moderate organic load that fosters steady, sustainable algal growth without the risk of organic overload. The phosphorus concentration (24.02 ± 0.66 mg/L) is significantly higher than in the other wastewaters, which is a key advantage since phosphorus is a critical nutrient for microalgal metabolism and growth. Sewage wastewater also has relatively low TSS (1756 ± 53 mg/L) and TS (2743 ± 96 mg/L), which is advantageous for light penetration and minimizes operational challenges in cultivation systems. While the acidic pH (3.52 ± 0.25) might inhibit growth, it can easily be adjusted to an optimal level for microalgal cultivation. The higher DO (0.38 ± 0.13 mg/L) compared to brewery wastewater further enhances the oxygen availability. The ammonia concentration (0.91 ± 0.01 mg/L) in sewage effluent offers a readily available nitrogen source, promoting protein synthesis. Although the dissolved oxygen levels (0.38 ± 13 mg/L) are still low, they are higher than in brewery wastewater, indicating slightly better natural oxygenation conditions.

Dairy wastewater was identified by the highest COD (10371 ± 289 mg/L) and BOD (1894 ± 217 mg/L) concentrations, representing a very high nutrient rich organic load. This, coupled with its elevated TSS (9979 ± 586 mg/L) and TS (17654 ± 1874 mg/L), suggests that dairy wastewater is nutrient-rich but may pose significant challenges due to the high solids content, which can interfere with light penetration, system fouling and reduce algal productivity (Fig. 2c). Furthermore, the low sulphate (0.59 ± 0.11 mg/L) and nitrate (0.06 ± 0.03 mg/L) levels, coupled with ammonia (0.91 ± 0.12 mg/L), indicate that additional supplementation of nitrogen and sulphur may be required to sustain optimal growth (Fig. 3). The pH (7.09 ± 1) is near neutral, which is ideal for algal cultivation, but the high total dissolved solids (TDS) (6371 ± 487 mg/L) could create osmotic stress on the algae, making dairy wastewater less suitable without significant dilution. The low phosphate (0.69 ± 0.09 mg/L) content is another limiting factor, as this nutrient is critical for sustained algal productivity.

Sugar wastewater has relatively low COD (1967 ± 130 mg/L) and BOD (1420 ± 94 mg/L), indicating a lower organic load compared to brewery and dairy effluents. However, the total solids (2646 ± 109 mg/L) and total suspended solids (1754 ± 54 mg/L) are on the lower side, which is favorable for light availability. The pH (4.9 ± 0.35) is slightly acidic, requiring adjustments for optimal algal growth. However, sugar wastewater also contains relatively low ammonia (0.43 ± 0.05 mg/L) and nitrate (0.07 ± 0.015 mg/L) concentrations, which may limit algal biomass productivity unless external nutrient supplementation is provided.

Although all four wastewater sources exhibit some potential for microalgal cultivation, sewage and brewery wastewater emerge as the most suitable options based on a balanced set of physiochemical properties. Sewage wastewater, although lower in organic content, offers a stable and nutrient-rich environment with lower suspended solids, making it more manageable in terms of light penetration and system operation. The high phosphate content in sewage wastewater further enhances its suitability for algal cultivation, contributing to biomass productivity and biofuel yield. Brewery wastewater, with its high organic load and sulphate content, supports algal cultivation conditions, provided that adequate mixing and aeration are maintained to counter the high TSS. This aligns with the findings of Gentili et al [16] who identified phosphorus as a limiting factor in wastewater-based algal growth. Unlike previous optimisation studies that relied on synthetic media such as BBM [13,16], this work provides a systematic comparison of real industrial effluents, offering a more realistic assessment of algal growth potential in practical wastewater management systems.

Dairy wastewater's high solid concentrations and organic load present operational challenges that would require significant system optimization. Similarly, sugar wastewater, while easier to manage in terms of suspended solids, lacks sufficient nitrogen content to sustain high microalgal growth without external supplementation. Therefore, sewage and brewery wastewaters provide the most balanced environments for cultivating microalgae, offering the best potential for scalable and efficient biofuel production. This was further confirmed by an elimination process (an intuitive way to filter the best candidates) used, whereby a scoring system for each of the industrial wastewater sources based on several key parameters (COD, BOD, DO, etc.) and their respective suitability for microalgal cultivation was adopted (Table 4), with score 1 being the best scored and 4 being worst scored, concluding that sewage and brewery wastewater were the lowest scored and suitable for algal cultivation needs.

4.2. *Morphological and molecular characterisation of microalgae biomass*

The morphological characterisation of the selected strains (*Chlorella vulgaris, Scenedesmus sp.* and *Spirulina platensis*) of the algal biomass provided physical traits of the species, including size, shape, color,

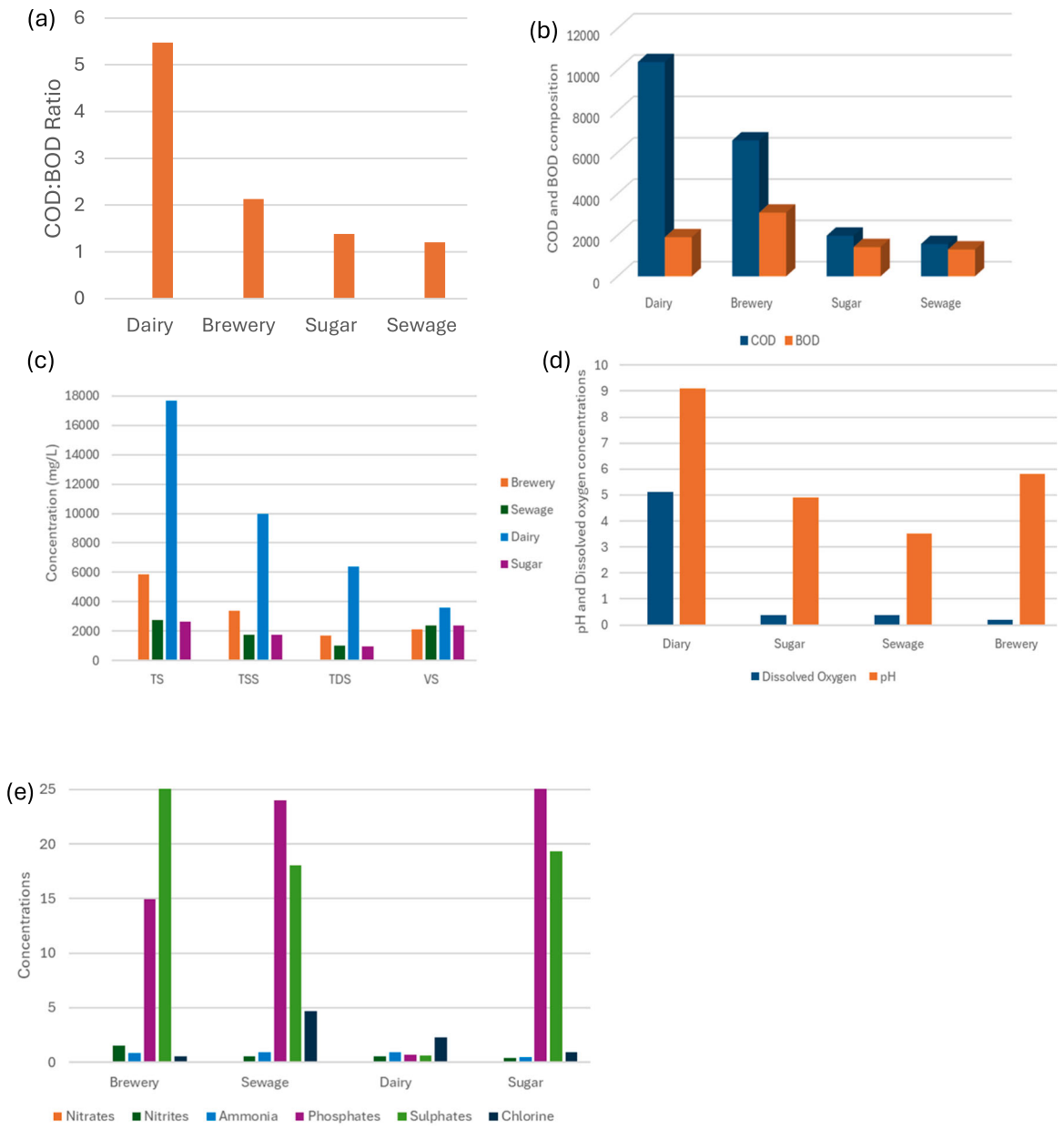

Fig. 2. (a) Relationship of COD and BOD ratio; (b) COD and BOD composition in wastewater, (c) Physical characterisation of wastewater, (d) Comparison of pH and DO between wastewaters, (e) Chemical characterisation of wastewater.

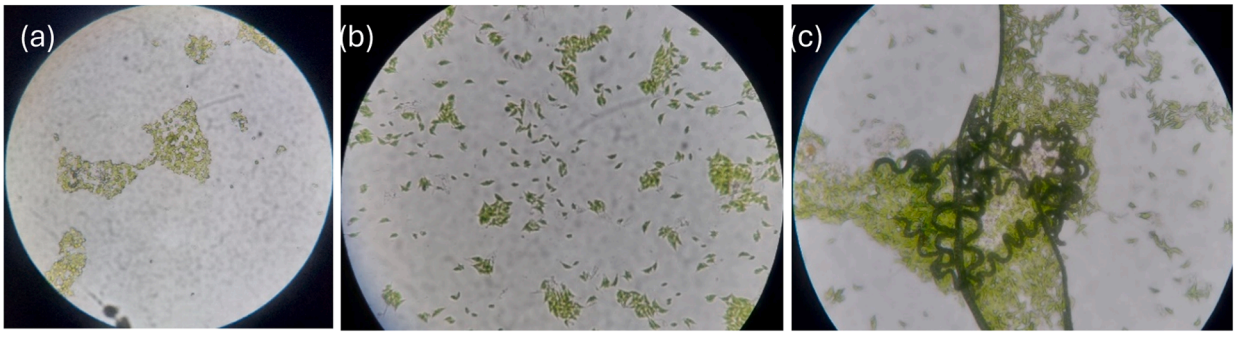

Fig. 3. (a) Chlorella vulgaris; (b) Scenedesmus sp.; and (c) Spirulina platensis.

Table 4
The elimination process used to establish the most suitable wastewater source.

Industrial source	COD	BOD	COD: BOD	DO	TS, TSS, TDS, VS	Nx	Sx/P	Cl	Total
Brewery	2	4	3	1	2	1	2	1	16
Sewage	1	1	1	3	1	1	1	2	12
Dairy	4	3	4	4	3	1	1	4	24
Sugar	3	2	2	2	1	1	3	3	17

structure and development. This was used for confirmation purposes in this study to accurately identify species based on appearance. The molecular composition was done to understand the species diversity and potential towards the direct objectives of this study, i.e. lipid composition, highest growth rates, wastewater treatment and biofuel production, intended for industrial or large-scale applications.

Chlorella vulgaris (Fig. 3a) displayed a 2 to 10 μm diameter unicellular, spherical morphology, with a high chlorophyll content depicted by its bright green color. Chlorella has thick, smooth cell walls made of polysaccharides, providing structural integrity and protection, thus allowing it to thrive in various environments, from freshwater to wastewater, making it suitable for high biomass productivity, lipid accumulation, nutrient removal and biodiesel production. *Scenedesmus sp.* (Fig. 3b) appears in small, green, non-motile but robust colonies known as Coenobia, which consist of four or more elongated, cylindrical or slightly oval cells with a protective spine. Each cell measures approximately 5 – 10 μm in length and 2 – 4 μm in width. The cells have smooth surfaces and rigid cell walls, often with spines or projections that help protect them from grazing and more adaptable to survival in open systems. *Spirulina platensis* (Fig. 3c) with a filamentous cyanobacterium (commonly called blue-green algae) with a distinct spiral, helical structure, making harvesting easier. Spirulina appears blue green due to the presence of the pigment phycocyanin, in addition to chlorophyll. The filaments are unbranched and approximately 3 – 5 μm in diameter, while varying in length. Its flexible, unbranched filaments form long strands, and its thin cell wall, composed of peptidoglycan, makes it easier for biomass extraction, although its lower lipid content leans more potential toward bioethanol and biogas production.

Composition of microalgal strains

In order to start the preliminary bench scale experimentation in the 1 L reactor vessels and to select the most suitable microalgal strain for biofuel production and wastewater recovery, a full detailed characterisation was done (Table 5). These results were evaluated and scored values 1 (best suitable) and 3 (least desirable) according to their suitability for the dual objective of this study, i.e. biofuel production and wastewater production (Table 6).

Table 5
Microalgal biomass characterisation (compositions).

Compound (%)	Chlorella vulgaris	Scenedesmus sp.	Spirulina platensis
Chemical composition			
Carbohydrates	23.26	24.5	12.2
Lipids	15.9	28.55	9.05
Proteins	48.2	52.22	62.3
Proximate composition			
Fixed carbon	11.56	24.76	11.95
Volatile solids	67.37	68.21	65.3
Moisture content	80.23	85.23	72.57
Ash content	7.28	3.45	8.23
Elemental composition			
Carbon (C)	39.28	34.36	33.11
Hydrogen (H)	5.47	5.74	5.91
Oxygen (O)	30	35	33.89
Nitrogen (N)	4.88	4.22	3.01
Sulphur (S)	3.07	2.99	2.67

Table 6
Elimination process of microalgae for suitability.

Microalgae strain	Chlorella vulgaris	Scenedesmus sp.	Spirulina platensis
Chemical composition			
Lipids	2	1	3
Carbohydrates	1	1	2
Proteins	1	2	3
Proximate composition			
Fixed carbon	1	2	1
Volatile solids	2	1	3
Moisture content	2	2	1
Ash content	2	1	3
Elemental composition			
C	2	1	3
H	2	2	1
O	1	3	2
N	1	2	3
S	3	2	1
Total	20	20	26

Lipid content was one of the significant factors considered during this study, as it is crucial for biodiesel conversions, especially having maximum TAG's present and available for transesterification. *Scenedesmus sp.* demonstrated higher lipid content (28.55 %) compared to *Chlorella vulgaris* (15.9 %), while *Spirulina platensis* had the lowest performance in terms of lipids (9.05 %). The carbohydrate content found and in *Scenedesmus sp* (24.5 %) and *Chlorella vulgaris* (23.26 %) was almost equal, which is highly beneficial in biofuel production, showing potential for bioethanol, biogas production and its ability to integrate into other biorefinery processes (Fig. 4b).

Proximate analysis indicated the fixed carbon portion responsible for biochar and syngas production was highly contributed by *Scenedesmus sp.* Although the fixed carbon, volatile solids and moisture content of *Scenedesmus sp.* displays some challenges in terms of energy recovery, thermal recovery or limitations due to high moisture, the lipid content of *Scenedesmus sp.* was the highest and most attractive compared to the most attractive proximate composition (*Spirulina platensis*). The proximate composition of *Chlorella vulgaris* exhibited lower scores for fixed carbon and ash content compared to Spirulina, highlighting its efficiency in nutrient utilization and biomass yield, both of which are critical factors for biodiesel production (Fig. 4a).

The elemental composition of the microalgae is crucial for fuel quality and being in compliance with the regulations of the environment (Fig. 4c). The elemental composition (C) depicted that *Chlorella vulgaris* appeared as the main energy contribution, consisting of the highest carbon content (39.28 %) assisting in better calorific value and energy rich biofuels. Furthermore, having a good C: H ratio is essential for complete combustion and a better quality of biodiesel. Too high O_2 lowers the heating value, although it aids in biodegradability. Low nitrogen and sulphur levels are preferred for biofuel production as too high nitrogen levels lead to higher NOx emissions. Similarly, high sulphur causes SOx emissions, acid rains and corrosion. Favorable nitrogen-to-carbon ratios were present in *Chlorella vulgaris* (0.1243) and *Scenedesmus sp* (0.1228), enhancing the growth rates and lipid accumulation during cultivation, thereby improving biodiesel yield as compared to *Spirulina platensis* (0.0909).

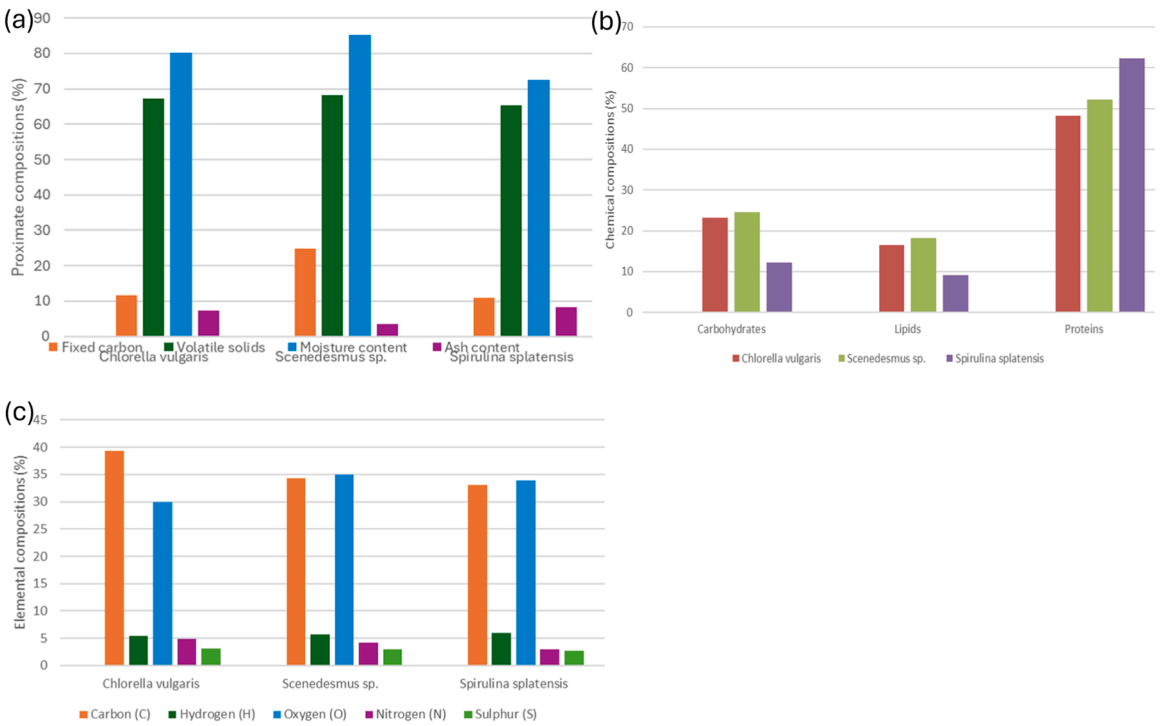

Fig. 4. (a) Proximate composition of microalgae; (b) Chemical composition of microalgae, (c) Elemental composition of microalgae.

Volatile solids portions indicate materials that can be converted into other liquids (bio-oil) and gases; *Scenedesmus sp*. contains 68.21 % but also has the lowest ash composition (3.45 %). Too high ash content leads to poor fuel quality, clogging of engines and lower calorific values; hence, *Scenedesmus sp*. applicability gains attention. An unattractive characteristic of *Scenedesmus sp* was its moisture content (85,23 %), as it is responsible for combustion efficiency and overall process efficiency, as more energy is required for drying. A lower moisture content would be advantageous for better thermal conversion and biodiesel extraction. Ultimately, the tie in total scores between *Chlorella vulgaris* and *Scenedesmus sp.* suggests that while both microalgae can potentially serve as valuable sources for biodiesel production, the higher lipid content and superior nutrient profile of *Scenedesmus sp.* make it a more favourable candidate for further research and application in biodiesel synthesis, paving the way for a greener future. While Chlorella is often reported as the standard strain for wastewater-based cultivation (Ahorsu et al., 2018), our results demonstrate that Scenedesmus sp. offers superior lipid productivity and robustness in real wastewater environments, highlighting its novelty for decentralised wastewater-to-biofuel applications.

Analysis of the key factors affecting the growth rate of Scenedesmus sp

Each process parameter investigated was analysed with a scatter plot, as it is most significant and crucial in statistical analysis, indicating the effect of each parameter on the output response. Tables 7-16 show the model's coded value versus the obtained output, i.e., growth rate. Another crucial test utilised in statistical analysis observes the effect of each variable and the model's coefficients, as displayed in Table 7 with the ANOVA outputs. The f-value, R^2 (coefficient of determination), adjusted R^2, predicted R^2 and the lack of fit indicate how accurately the selected regression model relates to/fits the obtained experimental data (reference). The coefficients suggest how much of allowable variance can be achieved in the output of the regression model, as numbers closer to 1 signify high accuracy, reliability and effectiveness, i.e. $0.9910 < R^2 < 1$. The F-values further show how significant the difference between the mean and operating conditions is. Additionally, the p-values coincide with the f-value, as the p-value shows the probability of the regression model being valid. The p-values for light illumination and growth medium ratio are <0.0001. This model CI (confidence interval) was 95 %, leaving the $\alpha = 5$ %. Hence, a p-value lower than 0,05 can be considered significant, while a p-value > 0,1 is considered insignificant. The p-values for the operating parameters appeared <0.05, hence having significant effects when included in the model. Unlike those studies, our results were obtained using real sewage wastewater, showing that statistical optimisation methods remain predictive even under complex and variable nutrient conditions. The model fit ($R^2 = 0.9910$; adjusted $R^2 = 0.9820$) was higher than that reported in similar optimisation studies (e.g., Abazari et al [15], $R^2 = 0.9864$), confirming the reliability and robustness of the regression model in wastewater-based cultivation systems.

Table 7
ANOVA for reduced quadratic model of growth rate response.

Source	Sum of Squares	df	Mean square	F-value	p-value	
Model	1.03	8	0.1282	110.17	<0.0001	significant
A-pH	0.0001	1	0.0001	0.0966	0.7638	
B-Light illumination	0.1445	1	0.1445	124.09	<0.0001	
C-Growth medium: WW ratio	0.2064	1	0.2064	177.31	<0.0001	
AB	0.0116	1	0.0116	9.93	0.0136	
BC	0.0900	1	0.0900	77.32	<0.0001	
A^2	0.2502	1	0.2505	214.91	<0.0001	
B^2	0.1992	1	0.1992	171.11	<0.0001	
C^2	0.0684	1	0.0684	58.80	<0.0001	
Residual	0.0093	8	0.0012			
Lack of Fit	0.0077	4	0.0019	4.82	0.0784	Not significant
Pure Error	0.0016	4	0.0004			
Core Total	1.04	16				

Table 8
Coefficients of coded factors in model.

Factor	Coefficient Estimate	df	Standard Error	95% Cl Low	95% Cl High	VIF
Intercept	0.6600	1	0.0153	0.6248	0.6952	
A: pH	0.0038	1	0.0121	-0.0241	0.0316	1.0000
B: Light Illumination	0.1344	1	0.0121	0.1066	0.1622	1.0000
C: Growth medium: WW ratio	0.1606	1	0.0121	0.1328	0.1884	1.0000
AB	-0.0538	1	0.0171	-0.0931	-0.0144	1.0000
BC	0.1500	1	0.0171	0.1107	0.1893	1.0000
A^2	-0.2438	1	0.0166	-0.2821	-0.2054	1.01
B^2	-0.2175	1	0.0166	-0,2558	-0.1792	1.01
C^2	-0.1275	1	0.0166	-0.1658	-0.0892	1.01

Table 9
Model fit statistics.

Std. Dev.	0.0341	R^2	0.9910
Mean	0.3829	**Adjusted R^2**	0.9820
C.V. %	8.91	**Predicted R^2**	0.8805
		Adeq Precision	30.3377

Table 10
Fit summary of the model's response on growth rate.

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.1342	<0.0001	o.1865	-0.0246	
2Fl	0.6402	<0.0001	0.0996	-0.3989	
Quadratic	**<0.0001**	**0.0538**	**0.9798**	**0.8808**	**Suggested**
Cubic	0.0538		0.9938		Aliased

Table 11
Sequential Model Sum of Squares depicting growth rate.

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Mean vs Total	2.49	1	2.49			
Linear vs Mean	0.3510	3	0.1170	2.22	0.1342	
2Fl vs Linear	0.1017	3	0,0339	0.5819	0.6402	
Quadratic vs 2Fl	**0.5735**	**3**	**0.1912**	**146.14**	**>0.0001**	**Suggested**
Cubic vs Quadratic	0.0076	3	0.0025	6.30	0.0538	Aliased
Residual	0.0016	4	0.0004			
Total	3.53	17	0.2075			

Table 12
Analysis of results obtained from initial DOE experimental runs.

	Run 8	Run 7	Run 9	Run 13
pH	8.25	8.25	8.25	6.5
Light illumination	24	12	24	24
BBM: ww	4:0	2:2	0:4	2:2
Growth rate (per day)	0,79	0,69	0,21	0,355

4.3.1. Evaluation of model significance and fit using ANOVA

The analysis of variance (Table 7) investigates the significance of the developed model and each of the investigated variables. The significant factors represented from the ANOVA table are B (light illumination) and C (growth medium ratio), with p-values of <0.0001. Their respective positive coefficient data B: + 0,1344 and C: + 0,1606 represent the positive effect they have on algae growth. Evaluating the coefficient of light illumination (+ 0,1344) indicates that sufficient light illumination is vital to enhance photosynthetic activity and improve overall growth rates. However, the quadratic term B^2 suggests diminishing returns, i.e., decreased or hindered growth rates at ranges exceeding the optimum. The large positive coefficients and significant p-values in the regression equation for growth medium ratio and light illumination strongly suggest a pronounced effect on algal growth rates, confirming that the nutrient availability provided by the sewage wastewater medium and the light source are crucial for promoting photosynthetic and cellular activity. The growth medium to wastewater ratio with p-value <0.0001 remains the most significant factor, further represented by the sum of squares equalling 0,2064. Additionally, a large coefficient indicates how the ratio of the growth medium positively contributes to the growth rate of microalgae. Interaction term BC (light illumination and growth medium ratio) with p-value <0.0001 also appears as significant with a positive sum of squares value of + 0,0900 and a coefficient (0,1500), suggesting that the synergistic effect between light and nutrient availability works together to boost photosynthetic activity. Although factor C remains the primary significant factor (Table 8).

Factor A, pH (+0.0038) has a much smaller coefficient but a non-significant p-value, confirming that maintaining the correct pH balance during cultivation is essential, but small fluctuations will be less influential on the effect of the response variable. Therefore, from the ANOVA, it can be concluded that the effects of each main contributor influence the growth rate of *Scenedesmus sp.*, relative to the magnitudes of its coefficients representing their importance. In the hierarchy order of factor growth medium ratio being the strongest contributor, followed by light illumination and lastly pH. Factor A (pH) and interaction factor AC (pH x growth medium ratio) or AB (pH x light illumination) both have p-values exceeding 0.0001, indicating that no significant interaction between pH and factor B or factor C exist. Hence this suggests that algal growth is tolerant with minor pH variations during the studied experimental range, emphasised by a negligible experimental range (+ 0,0038). Evaluating the non-linear terms, all these factors (A, B and C) exhibit diminishing returns due to their negative quadratic terms; hence emphasising the importance of the optimisation of each individual factor, within a specific range. Analysing B^2 and C^2 both appear significant

Table 13
Model constraints and selected ranges during optimisation.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: pH	Is in range	8	8.5	1	1	3
B: Light illumination	Is in range	12	24	1	1	3
C: Growth medium: WW ratio	Is in range	2	3.5	1	1	3
Growth rate	Maximise	0.04	0.79	1	1	3
Cell productivity	Maximise	19500	543000	1	1	3

Table 14
Best DOE optimisation runs with highest potential for 1 L bench scale experiments.

	pH	Light illumination (hours)	BBM: WW	Desirability	Growth rate per day	Cell productivity (cells/mL/day)
Run 1	8	18	3:1	0.944	0.757	475916.46
Run 8	8.3	18	3:1	0.929	0.790	449100.73
Run 14	8	18	2.5: 1.5	0.917	0.724	453076.65
Run 19	8	18	2:2	0.857	0.676	410730.429

Table 15
Final results obtained from 1 L optimised conditions and the optimised 30 L conditions, with varying compositions of air supply streams.

	1 L optimised	30 L at 1 L optimised conditions	30 L + physical adjustments	30 L + operational adjustments	Optimised 30 L	Optimised 30 L	Optimised 30 L
pH	8.3	8.3	8.3	8.3	8.3	8.3	8.3
Light illumination (60 μmol/m^2/s for 18 hour duration)	18	18	18	18	18	18	18
Growth medium ratio	2:2	2:2	2:2	2:2	2:2	2:2	2:2
Aeration unit	1 × 3 cm air stone @ 5 L/hr	1 × 10 cm shower head @ 5 L/hr	2 × 10 cm shower head @ 7.5 L/hr	2 × 10 cm shower head @ 7.5 L/hr	2 × 10 cm shower head @ 7.5 L/hr	2 × 10 cm shower head @ 7.5 L/hr	2 × 10 cm shower head @ 7.5 L/hr
Air supply	100 % air	100 % air	100 % air	50 % air, 50 % nitrogen	100 % nitrogen	100 % nitrogen 5 % CO_2 injections	100 % air 5 % CO_2 injections
Light distance (cm)	20	20	20	20	20	20	20
Diameter of the reactor (cm)	10	30	30	30	30	30	30
Height of the reactor (cm)	15	55	55	55	55	55	55
Growth rate per day	0.79	0.577	0.641	0.69	0.72	0.752	0.70
Cell productivity	64×10^4	46.89×10^4	52.04×10^4	55.89×10^4	58.59×10^4	60.92×10^4	56.89×10^4

Table 16
Final cumulative growth rates and compositions on completion of cultivation cycle.

	1 L Normal conditions	1 L optimised conditions	30 L optimised conditions
Growth rate (per day)	0.69	0.79	0.752
Biomass Produced (mg/L/day)	56,71	64,925	61,805
Cumulative biomass after 18 days (mg/L)	1020,71	1168	1112,5

with p-values <0.0001, having coefficient data of – 0,2438 and – 0,1275 respectively. This suggests that diminishing returns with potential light inhibition are possible at high light intensities. Additionally, high/extreme nutrient concentrations become inhibitory, due to possible toxicity or sub-optimal levels.

The statistical fit analysis of the study (Table 9) model shows a good overall significance (F= 101,17 and p-value <0.0001). The F-value indicates the model significance and the interactions of variables with the response to the growth rate. The predicted R^2 indicated the estimated coefficient of determination of the model, whereas the R^2 value was the actual overall mean, a better predictor of the expected output. This model's R^2 was 0,9901, while the predicted R^2 was 0,8805. The predicted R^2 of 0,8805 is reasonably close to the adjusted R^2 (difference less than 0,2), which is acceptable and statistically valid, confirming the model's predicted power. The adjusted R^2 of 0,9820 value indicates that 98,2 % of the variability in growth is explained by the model. The residual lack of fit model statistical analysis F-value of 4,82 with a p-value of 0,0784 which indicates the model is not statistically significant, which is good as it confirms the model adequately fits the experimental data.

The model fits also analysed the adequate precision statistical term, which investigated the response to noise ratio with general values over 4, accounting for model accuracy. The regression model precision values were above 4, suggesting the model was satisfactory. The model was confirmed best fit due to the adequate precision and signal noise ratio of 30,3377, which was desirable to navigate the design space towards the optimisation of maximum algal growth rate. The predicted residual error sum of squares (PRESS) calculates the error of variation that cannot be measured by the model (Raychaudhuri and Behera, 2020). Low PRESS values indicate the model has a good fit, while high PRESS values indicate the model is not the best fitting one. The PRESS value for this model was 0,1237, suggesting the model is one of the best fit.

4.3.2. *Interaction amongst main terms and synergistic effects*

The regression model further represents how the combination of the paired factors influences *Scenedesmus sp*. growth rate beyond their independent effects. A significant p-value for the interaction term BC (light illumination x BBM:WW ratio) indicates that the combined effect of these two factors is important, where the effect of one factor depends on a specific level of the other. The positive coefficient of BC (+0.1500), together with a significant p-value suggests a synergistic effect, where the increase of light illumination amplifies the benefit of the wastewater growth medium. This relationship explains how sufficient nutrients present in the growth medium support the more efficient utilisation of light energy for photosynthesis, leading to an improvement in growth rate of *Scenedesmus sp*. This synergistic effect is observed in experimental runs where the light illumination and wastewater ratio are varied, compared to the other runs with either factor at lower levels. When both factor B and factor C were high, the growth rate of *Scenedesmus sp*. was exhilarated/amplified beyond the results of the individual effect of each factor. Mid to high levels of this BC relationship also demonstrate this effect clearly. Run 8 (B:18, C:3, μ = 0,790) illustrates how both these high levels synergize, producing the peak in the growth rate. BC combination allowed the microalgae to have sufficient nutrients and the opportunity to utilise light for an extended period. The same synergistic relationship is seen in run 7 (B:18,25, C: 3, μ = 0,690), confirming the benefit of combining these two factors. Run 4 (B: 18, C:1, μ = 0,180) attained significantly lower growth rates compared to run 7 and 8, indicating that although factor B is high, the low factor C ratio limited the growth nutrients available, hence restricting the growth rate; further emphasizing the importance of interactions between the investigating parameters.

Interchanging the investigated factors as seen in run 3, having low light illumination period (B:12) and high wastewater growth medium ratio (C:3), achieved moderate growth rates of μ = 0,650. This further supports that the growth medium to wastewater ratio alone is a factor of importance that boosts growth, but the low light availability hinders photosynthetic activity, leading to lower growth rates. Concluding that nutrient availability alone cannot drive maximum growth, but limited nutrients limit growth, indicating that synergistic interaction and balance are pivotal. From the bench scale experiments carried out for *Scenedesmus sp*., Run 7 and 8 demonstrated the critical importance of synergistic effects between the variables under investigation, specifically light illumination and the BBM-to-wastewater growth medium ratio. The growth rates demonstrated how the nutrient availability (Factor C) was complemented by higher light periods (Factor B) to attain maximum photosynthetic activity and growth rates. Whereas in runs 4 and 3, a clear example of how limitations/sub-optimal conditions of either factor hindered overall growth rates.

4.3.3. Model quadratic returns and diminishing returns

The quadratic returns A^2, B^2 and C^2 are indicators of the non-linear relationships between the investigated factors (ABC) and the output response (μ = growth rate). However, these positive effects of factors eventually start producing badly/decrease and may turn negative, hence indicating a "diminishing return". Interpreting the ANOVA coefficient in terms of the coded factors, a significant p-value indicates that there is a curved relationship present between the factor and the response. For example, factor A^2 (pH) negative coefficient (– 0,2438) indicated that although pH fluctuations during the cultivation period did not cause a significant change in the growth rate, the initial pH is vital to improve growth and set the basis for cultivation. Moreover, excessively high pH or too low pH reduced growth significantly. Factor B^2 (light illumination period) coefficient (– 0,2175) suggests that excessive lighting can cause photoinhibition and damage photosynthetic processes, resulting in reduced growth rates. Factor C^2 (BBM to sewage wastewater ratio) negative coefficient (– 0,1275) shows that optimal nutrient concentrations are vital, but beyond the optimal ratio can be toxic to microalgal strains, leading to resource wastage and reduced growth rates. Therefore, the interpretation of the significant quadratic terms confirms that each factor performs best within its optimal range, being able to perform its best by achieving the maximum growth rates. Whereas, exceeding the optimal range results in diminishing returns or adverse effects on the growth rates (Table 10).

4.3.4. Holistic view of the significance of the model

At the bench scale level, it was critical to evaluate and analyse the combined significance of the main effects, interactions, synergize and the quadratic terms. By doing so, the combined effects illustrate the complexity of microalgal growth dynamics and provide actionable insights for process optimisation and experimental design when upscaling to larger reactors. Holistically, pH, growth medium ratio and light illumination serve as the main effects during cultivation, with the growth medium ratio as the dominant factor driving growth, with light illumination serving as a critical supporting factor. Whereas pH concentrations are vital but less impactful compared to factor B and factor C. From the synergistic analysis, it is logical to state that light availability and growth medium ratio should be optimised together for the best growth rates. Moreover, the presence of the quadratic terms further emphasises the need of finding and maintaining the optimal range for each factor of investigation.

4.3.5. Interpretation of the regression model equation

The regression model and fit summary were essential techniques in statistical analysis, utilising multiple mathematical functions on the DOE software on the expected response, such as number of transformations, natural log, inverse square root, square root, base 10, logarithm, logit, inverse, arcsine square root and power transformation. The fit summary table of statistics assisted in selecting the best suitable model for in-depth analysis. According to Raychaudhuri and Behera [49], the quadratic model was aliased as there was sufficient data to predict all the parameter coefficients of the model. The equation below represents an equation of growth rate in coded factors. Utilising coded factors provided the ability to predict the response for specific levels of each factor by comparing their coefficients. Each coefficient represents the expected output change per unit change, provided all others remain constant. It further explains how each factor's interactions contribute to the overall growth rate. Positive coefficients indicate factors that enhance the growth of microalgae cultivation, whereas negative terms represent diminishing returns. The basic interpretation of this regression equation is that the intercept (+ 0,6600) is the average achievable growth rate when all operational factors are at their mid-points / central values (0). A pH value (A: 0,0038) indicates pH has a minimal positive effect, expecting that for every one unit increase in the pH, the growth rate will increase by 0,0038 units. Whereas light illumination (B: +0, 1344) has a more substantial positive effect, where an increase in light illumination improves growth rate. The growth medium to wastewater ratio (C: +0,1606) possesses the strongest positive linear main effect, boosting microalgal growth. Interaction factors pH x light illumination (AB: – 0,0538) has a relatively weak relation and statistically insignificant p-value (0,0136) between each, compared to the strong light illumination x growth medium ratio (BC: +0,1500) and p-value <0.0001, suggesting an intense synergistic effect between both factors leading to enhanced growth. AC (0,0109) also appears weak and insignificant (p-value 0,7797), indicating that pH and growth medium ratio do not interact significantly. The quadratic factors A^2, B^2 and C^2 in the regression equation all appear negative, hence meaning that optimal ranges are present and diminishing returns are highly possible, as drastic values will reduce overall growth. From this, it is ideal to state that further optimisation should focus on the significant regions (e.g., BC) where maximization of growth is possible and large amounts of biomass were achieved.

$$\begin{aligned} \textit{Growth rate}\ (\mu) = {} & 0,6600 + 0,0038\,A + 0,1344\,B + 0,1606C \\ & + 0,0538\,AB + 0,1500\,BC + 0,0109\,AC \\ & - 0,2438\,A^2 - 0,2175\,B^2 - 0,1275\,C^2 \end{aligned} \tag{9}$$

4.3.6. Diagnostic analysis, validation of model accuracy, RSM and normality of residuals

The diagnostic analysis provides critical insights and patterns associated with the quality and validation of the regression model. Below combines the comprehensive evaluation of all findings from the normal probability plot, residual patterns, actual vs predicted growth rates and the contour surface plots. The validation of the model was done to distinguish specific areas of predicted accuracy and improvement.

Most experimental data points align closely along the 45 ° diagonal reference line, indicating the residuals are approximately normally distributed. This alignment validates the assumption of normality, a key prerequisite for the reliability of the ANOVA results. Minor deviations are visible at the extremes, as seen in run 8 (Residual + 0,0300) and run 9 (Residual + 0,0312). Since these points deviate from the expected diagonal trends, these are the possible outliers of the data distribution. Furthermore, this can be seen from the color representative of green zone falling in the normality range, with the extremes falling into the yellow and red zones.

4.3.6.1. Residuals vs predicted values. In figures 5 a and b, the homoscedasticity is evaluated. Judging from this graph, the majority of the residuals appear scattered randomly around 0.00. However, a slight clustering of data around 0,65 and 0,75 growth rates. This model further accounts for the variability in growth rates (e.g. 0,65 – 0,75). Outliers (Run 13 = – 0,0388 and Run 9 = +0,0469) indicate deviations that contribute to the clustering, whereas Run 16 (Residual = – 0,0027) and

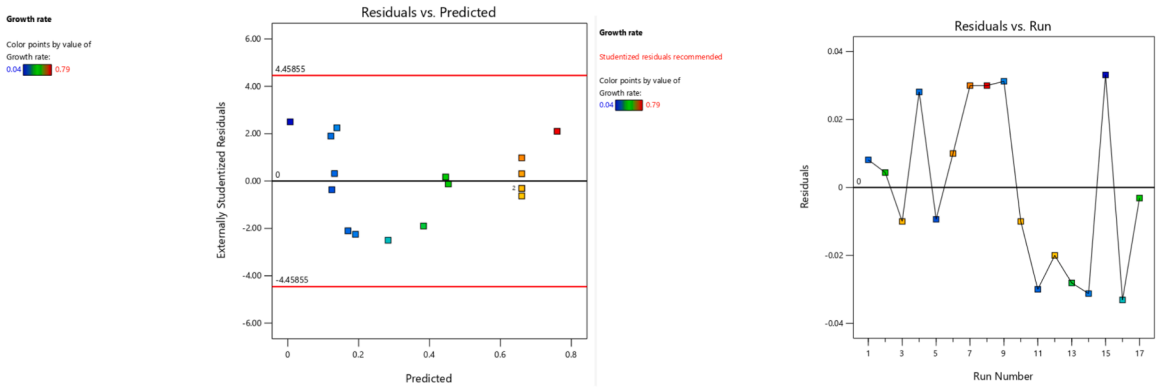

Fig. 5. (a) Residual vs Predicted; (b) Residual vs run.

other residuals closer to 0, appear ideal and align with the model assumptions.

4.3.6.2. Residuals vs factors. The residual vs factor plot examines whether the residuals are affected or influenced by a specific factor pH (A), light (B) or growth medium (C). Looking at the figures 6 a – c, there are no significant clusters observed, but the presence of extreme deviations (red zones) at extreme levels of light (B) and growth medium (C) is highly likely due to the effects of the quadratic factors. This is further supported by runs 1 and 4, where a pH of 10, residuals are minimal, hence a well-developed model. The deviation at run 9 (+ 0,0312) indicates that there is likely due to a significant interaction between the BC factors.

4.3.6.3. Predicted vs actual growth rates. To validate the regression model, diagnostic tests are vital to confirm the ANOVA assumptions. The predicted versus actual plot is useful to visually compare and validate the experimental data against the model's predictions, illustrating that the majority of the points align closely to the 45 ° diagonal line, confirming the model's accuracy (Fig. 8). However, there are minor deviations and higher growth rates observed. This can be seen in run 8 with a predicted growth rate of 0,7581 where the actual growth rate was 0,79; and in run 9 with an underprediction of growth rate 0,1631 and actual growth rate of 0,21. However, these deviations are potentially a reflection of biological variability of the strain and appear minor, hence not undermining the accuracy of the model's reliability. This layout simply shows the model was capable of predicting the experimental data.

4.3.6.4. Leverage plots. Analysis of the leverage plots (Fig. 9 a and b) is another form of validation of the model. Average leverage is defined by the following equation, where N_{mc} is the number of model coefficients and N_{er} is the total number of experimental runs.

$$L_{average} = \frac{N_{mc}}{N_{er}} \tag{10}$$

For this model, the N_{mc} was 9 and the actual experimental runs N_{er} were 17. Hence, the leverage was 0,529. Any experimental runs having a

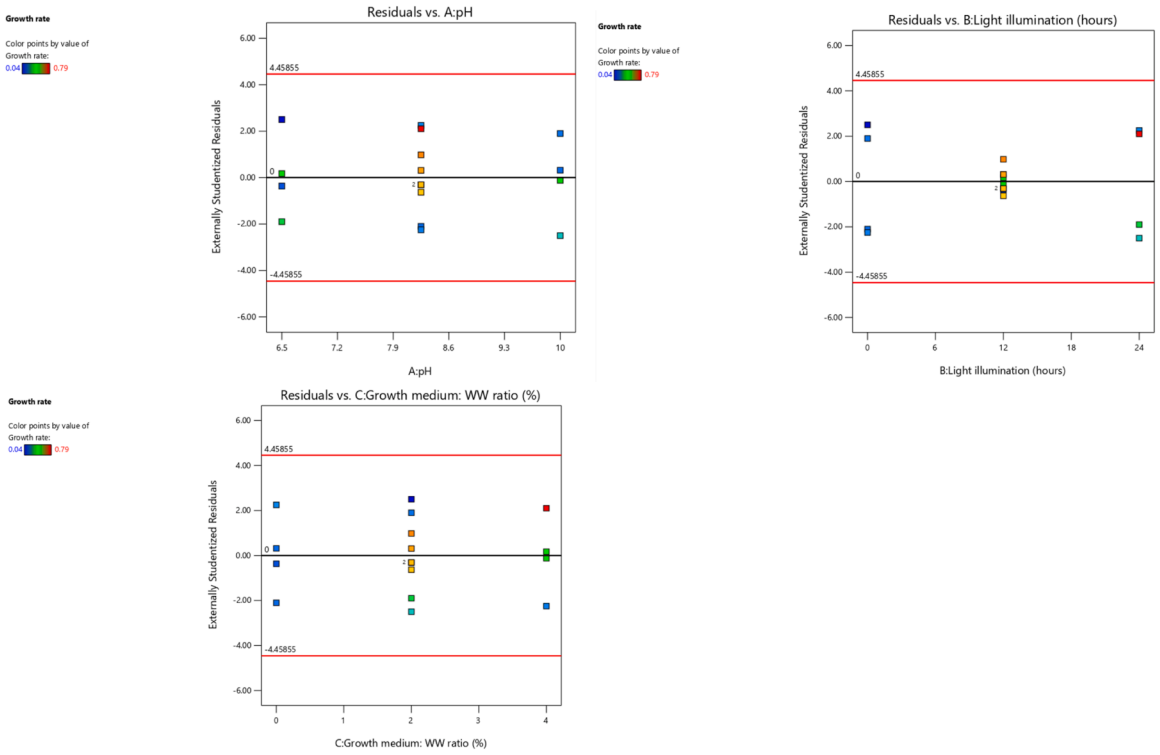

Fig. 6. Residual vs investigated parameter (a)pH, (b)light illumination and (c) growth medium ratio.

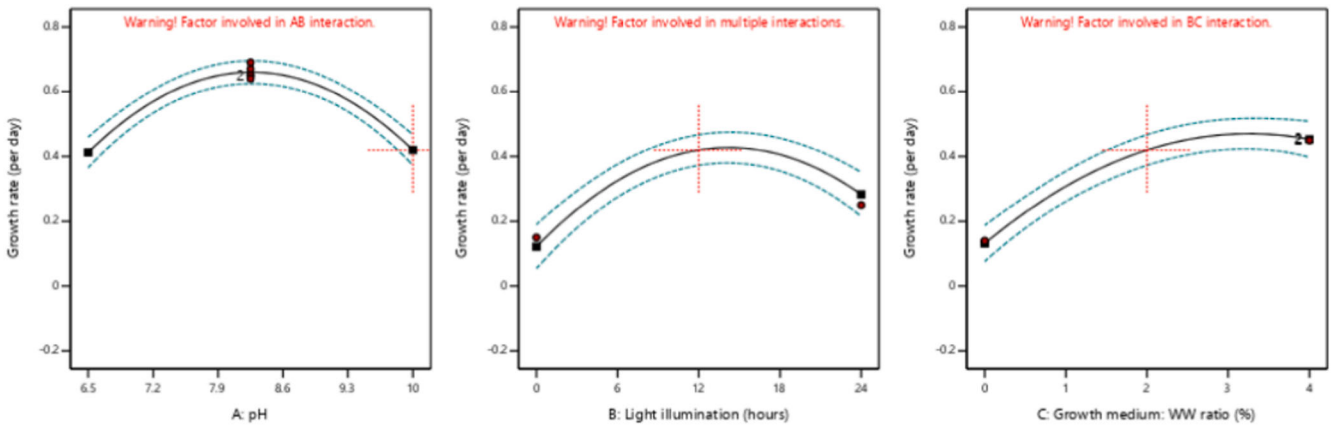

Fig. 7. Multiple factor interactions on growth rate.

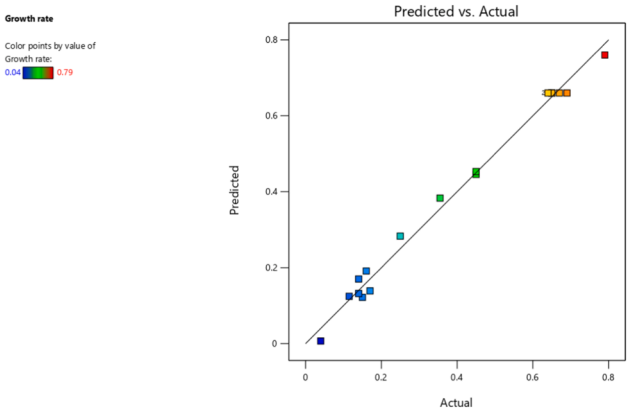

Fig. 8. Predicted vs Actual.

leverage above $L_{average}$ or > 1 has a too high leverage [60]. Evidently, from all the experimental runs, no run leverage was greater than $2 \times 0{,}529$ or > 1, proving the leverage values were not too high.

The last statistical diagnostic plot analysed for the validation of the model was the plot of external residuals vs the run number (Fig. 11b), illustrating the t-values of each experimental run. This represents the degree of deviation of all runs, also referred to as the outliers. Fig. 11b also demarcates the boundary lines obtained via the degree of freedom and the tail value. The tail value is calculated from the equation below

$$tail\ value = \frac{\propto}{2N_{er}} = \frac{0,05}{17 \times 2} \quad (11)$$

The model's alpha value was set at 5% with $2N_{er}$ being 34. Using Eq. 11, the tail value was calculated to be 0,00147. From this, the degree of freedom of the residuals (DOFs) can be calculated as DOF = Ner – n – 1; where n is the number of process variables present in the model. DOF = 17 – 3 – 1 = 13. Using this DOF value of 13 and tail value of 0,00147, the residual limit (Lr) from the one tailed/two tailed t-table, the residual limit is $Lr = (\pm\, t_{(tail\ value,\ DOF)}) = (\pm\, t_{(0,00147)}) = \pm\, 3{,}570$. This suggests that any t-value $> +\,3{,}570$ or $< -3{,}570$ is acceptable (Fig. 9 a and b). All residuals are falling within the residual limits (Lr), confirming all experimental data points are accepted and fitted by the model. This further confirms that no problems were evident or present with the regression model or the points of data.

4.3.6.4. Contour and surface plots. From the surface plots in Fig. 11 a and b, a clear visual representation of how each factor interactions influence the overall growth rate. From the experimental data, the light illumination (B) and growth medium ratio (C) interactions (BC) highlight a strong peak, confirming that the interactions are significant. According to the contour plot in Fig. 10 a and b, it can be seen that the highest growth rates are achieved in regions with moderate-to-high levels of light and growth medium. These surface contour plots further validate the regression model and emphasize the importance of optimising the inter-relation factors, light and growth medium ratios to achieve the maximum growth rate (BC: $p < 0.0001$). Run 8 ($\mu = 0{,}79$/ day) indicates the highest growth rate near the optimal light (24 hours) and growth medium ratio (4:0), whereas at lower light levels (12 hours) and moderate growth medium ratio (2:0), conversely in run 7, the growth rate achieved was lower ($\mu = 0{,}69$/ day) but still significant, hence emphasising the importance of synergistic optimisation. The

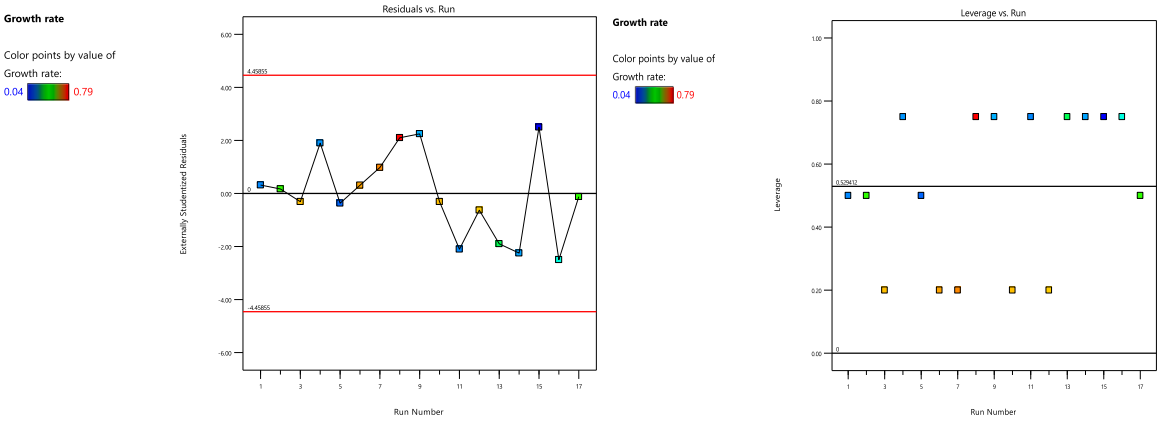

Fig. 9. (a) Residual vs Run; (b) Leverage vs Run.

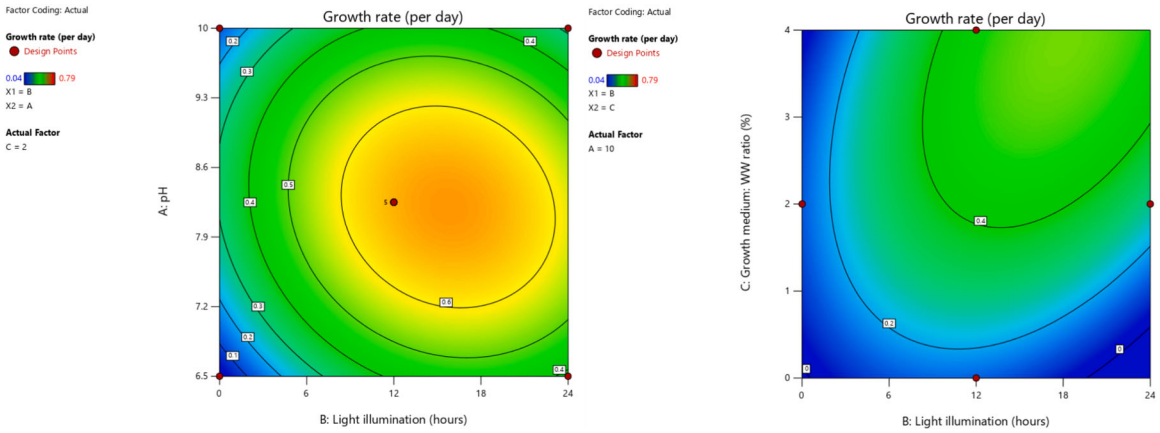

Fig. 10. Contour plot of signification factors (a) light illumination with pH and (b) light illumination with growth medium ratio.

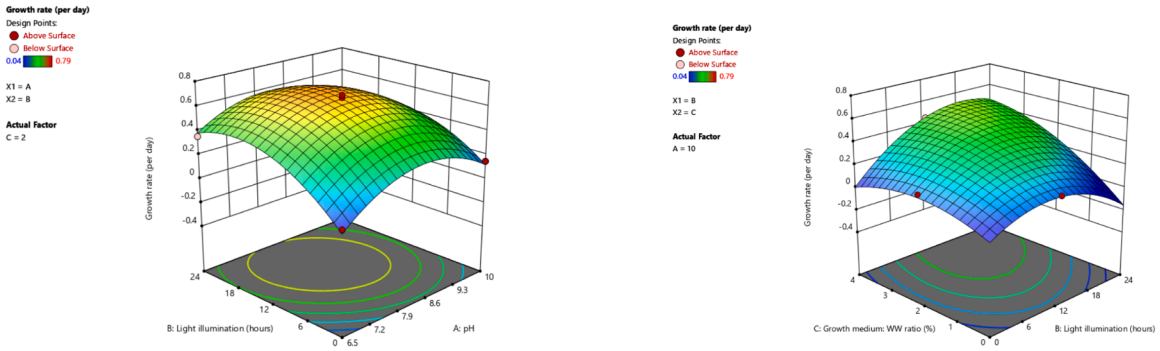

Fig. 11. Surface plot of signification factors (a) light illumination with pH and (b) light illumination with growth medium ratio.

contour surface plots highlight regions of optimal conditions with the maximum growth rates achieved at moderate-to-high (18 – 24 hours light illumination) and high growth medium ratios (3 – 4 parts BBM). Overall, the diagnostic analysis provides evidence that the regression model is robust and performs well. Small deviations are present and expected, suggesting areas for further improvement or refinement. Overall, this model provides reliable predictions for optimising the growth of *Scenedesmus sp* (Fig. 7).

Analysis of results obtained from initial DOE experimental runs

The highest performing runs from the Design of Experiments were Run 7 and Run 8 with 0,79/day and 0,69/day. Reducing light exposure from 24 hours to 12 hours, and the BBM: sewage wastewater growth medium ratio from (4:0) to (2:2) while maintaining a pH (8.25), the growth rate decreased from 0,79/day to 0,69/day. Whereas run 8 studied full BBM growth medium as compared to run 9, with full sewage wastewater, which drastically reduced the growth rate, even under sufficient lighting conditions. This motivates the fact that light can be evaluated at lower levels of 24 hours, such as an 18 hour cycle with a 2:2 ratio growth medium (Run 13), being able to still achieve a decent growth rate outside the optimum levels of pH. The model's exploration of the optimal range of light exposure and growth medium could provide a more efficient alternative without compromising growth rates.

Considering the fact that this study's dual objective is biofuel production with simultaneous wastewater treatment, this was taken into account for the optimisation process to reduce the standard BBM growth medium and utilise the maximum wastewater as possible. Hence, run 1 in the optimisation DOE solution is highlighted as the optimal combination (pH: 8,0, light: 20:4 and growth medium 3:1) due to its high growth rate, strong cell productivity and the highest desirability (0,956) amongst all runs. Optimisation run 1 balances the maximum growth rate, while maintaining the cell productivity under experimental conditions (Table 12).

4.5. Optimisation of bench scale experimental data

On completion of the ANOVA analysis and model interpretation, it is vital to determine the best combination of growth factors (A, B and C) to achieve the maximum growth rate of *Scenedesmus sp*. According to the model prediction, the highest growth rate achievable was of 0,772/day under the conditions pH: 8, light illumination: 20.8 hours and growth medium ratio 3 BBM to 1 wastewater. The optimal pH of 8 indicated limited individual influence (not significant linear A term). However, the A^2 quadratic term is significant, indicating that pH has an optimal range for supporting algal growth. At a pH of 8, enzymatic activity and nutrient absorption are likely at their peak for efficient cellular metabolism. The strong positive B term (< 0.0001) confirms light importance to the model, enabling maximum growth rate. However, B^2 also suggests diminishing returns or potential photoinhibition at excessively high levels. Likewise, factor C requires a balance to avoid nutrient saturation or toxicity (Fig. 14).

4.5.1. Numerical and graphical optimisation for maximum growth rates of *Scenedesmus sp*

On confirmation of model accuracy and suitability, the last step was to optimise the solutions via numerical and graphical methods. From Fig. 13, numerical ramp charts were utilised to find the optimal pH, light illumination time and growth medium ratio to obtain the highest algal growth rate possible, hence setting the target goal to maximum for growth. Operational parameters were all considered vitally important, with (+++) plus signs method selected. According to the best optimum

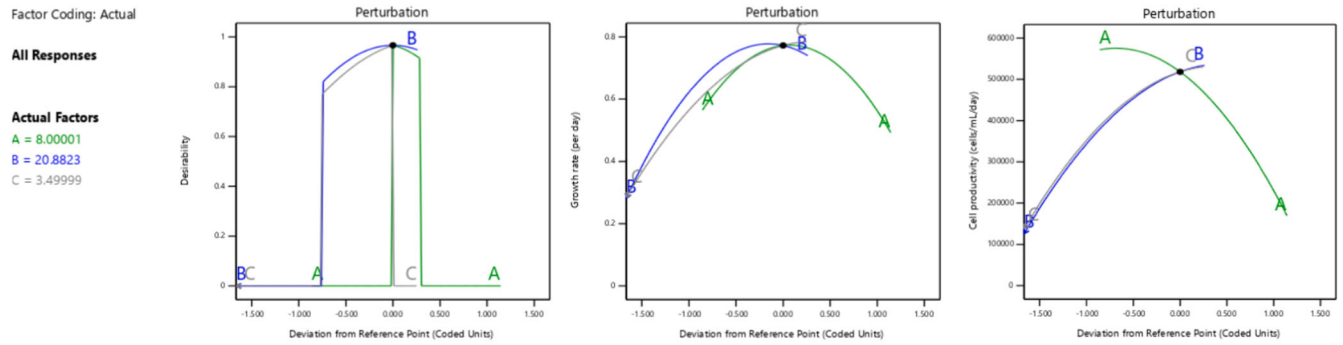

Fig. 12. Optimisation based on deviation from reference points.

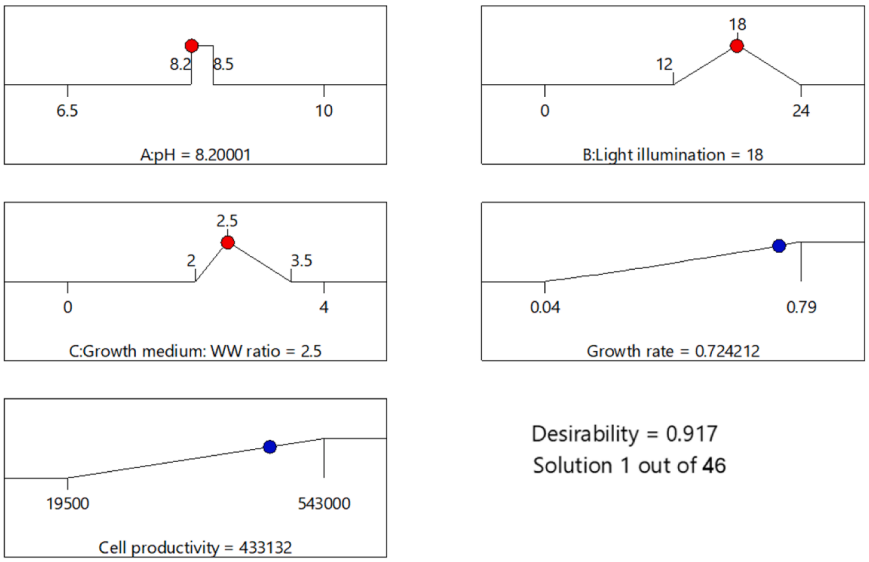

Fig. 13. Numerical optimisation ramps plots with solutions for output responses required for scaling up.

process conditions, pH of 8, light illumination of 18 hours, and growth medium 2:2 was selected with a desirability of 0.917 % out of 46 iterations (Fig. 12).

Table 1 in the literature review indicates other studies that utilised BBD for the optimisation of growth of algae, showing how the various studies select and optimise various parameters, enhancing the applicability and efficiency of various BBD optimisation studies, as each study is unique in cultivating the algae under different environmental conditions or experimental design set ups.

Detailed factor analysis for optimisation

Taking into consideration all experimental runs, the best growth rates were achieved at pH ranges 8.2 – 8.5. This aligns with the quadratic term A^2 in the regression model, suggesting the optimal pH range for maximising growth. According to the DOE optimisation results and the model's regression equation, it was suggested that run 1 was the desired run to produce the highest growth rate. Optimisation run 1 (pH: 8, light: 20 and growth ratio 3:1) achieves the highest growth rate of 0.772/day with desirability of 0.956. However, a slight increase of pH in optimisation run 8 (pH: 8.3, light: 19 and growth ratio 3:1) achieves the highest predicted growth rate of 0.767/day and a decreased desirability 0.950. Hence, both pH concentrations were tested at the same light illumination periods to establish which was the ideal starting pH for cultivation. This was aligned to the model and a minute difference between the experimental and predicted growth was achieved (Table 14).

Light illumination optimal ranges occur between 16 – 18 hours as illustrated in the contour surface plots, with the best combination clustering around 18 hours, achieving a high desirability close to the maximum predicted growth rate of 0.757/day. The growth medium ratio typically has the top performing ratio of 3:1, however the slight reduction in BBM to 2.5 decreased the growth rate slightly to 0.724/day with a 0.917 desirability (optimisation run 14). This can be optimised further as it predicts it is still highly possible to achieve significant growth when cultivating in a 2:2 medium ratio. Comparing the optimal optimization results of run 1, the growth rate decreased from 0.757/day to 0.676/day as the growth medium ratio shifted from 3:1 to 2:2, reflecting a slight difference of 0.081/day. Since the dual objective of this study was to find alternative solutions to wastewater treatment, this is acceptable and given priority for further upscaling processes during this study. Instead of using a cultivation medium ratio of 3:1, a 2:2 growth medium was investigated as suggested by optimisation run 19, but with a higher pH of 8 – 8.3. Therefore, optimisation runs will focus on Run 8 and Run 19 to establish an achievable comparison between all outcomes.

On completion of repeatedly cultivating *Scenedesmus sp*. under the optimised conditions as suggested by run 19 (pH: 8, light:18 and growth medium 2:2) and a slight increase in pH to (pH: 8.2 – 8.5, light:18 and growth medium 2:2), a growth rate of 0.79/day was achieved at bench scale 1 L cultivation levels. This output is within the acceptable range and supported by previous studies [50,51].

To upscale to a 30 L reactor, the established optimised conditions of the 1 L vessels (pH: 8.3, light: 18 and growth medium 2:2) were kept as the starting point of ideal cultivation conditions. However, when these

same baseline conditions were maintained while upscaling, variations and a sudden difference in the growth rate of *Scenedesmus sp*. were observed (Schulz & Wucherpfennig, 2024). Maintaining the same operating conditions during the upscaling to the 30 L reactor led to hydrodynamic changes as the column had a larger diameter than the 1 L, aeration changes, difference in shear forces, variation of light penetration, presence of dead zones and several other contributing factors [50].

Table 15 above provides a comparative analysis of the growth rate of *Scenedesmus sp*. and various outcomes when cultivated across the two reactor setups, namely the 1 L versus 30 L; highlighting the impact that optimisation has on algal biomass productivity and growth rate. Across all setups, maintaining the three basic optimal parameters of pH (8.3), light illumination (18 hours) and growth medium ratio (2:2), it was possible to isolate the effects of other contributing hydrodynamic variables such as aeration rate, supply and reactor design to accommodate some of the challenges of upscaling. Scaling up from the optimised 1 L conditions to the 30 L reactor without any additional changes resulted in a notable decline in both the growth rate (0,79/day to 0,577/day) and cell productivity (64×10^4 to $46{,}89 \times 10^4$). This general pattern shows the challenges associated with upscaling, such as reduced mixing, insufficient agitation, insufficient light penetration, insufficient nutrient supply and the presence of large dead zones.

To address these challenges, additional variables were incorporated into the 30 L after visual interpretation of barely any agitation and stagnated zones, where no movement was seen. An additional air supply shower head operating at an increased flow rate (7,5 L/hr) to enhance mixing was added to the 30 L reactor. Operating at these conditions, a maximum growth rate of 0,641/day was achieved.

At this point, it was possible to incorporate an additional air supply line as the apparatus was able to accommodate this, unlike the 1 L reactor vessels. Now operating at conditions of pH 8.3, 18 hour light illumination, growth medium ratio 2:2, 2 shower head air supply at 7,5 L/hr, 50 % air and 50 % nitrogen, the growth rate had increased to 0,69/day. This showed that utilizing a shared air supply stream with nitrogen increased the growth rate by 7,6 %. As this increase in growth rate was evident, a 100 % nitrogen stream was tested at the optimum conditions, leading to a higher growth rate of 0.72/day. As per previous studies by Sun et al, (2025) and Rodas-Zuluaga et al. (2021) proved that the CO_2 levels become strained or depleted during cultivation, and hinders growth, an additional 3 % CO_2 injection to increase carbon availability was investigated. Operating the column at 100 % nitrogen supply, with 5 % CO_2 sparging as Yi et al. (2022) the highest growth rate of 0.752/day was obtained. This demonstrated the effect of additional CO_2 sparged into the medium, as a 100 % full air supply with 5% CO_2 sparging produced 0,70/day. Our findings suggest that whether cultivated with 100 % nitrogen supply and 5 % CO_2 or 100 % air and 5 % CO_2 sparging, the difference in growth rate is significantly small (±0.052/day), as seen in a similar study by Yi et al. (2022). Fig. 14 demonstrates the respective growth patterns together with the effects and challenges experienced during upscaling processes.

However, improving aeration within the column improved the light intensity, photosynthetic efficiency and efficient mixing of nutrients in the larger column. This table also clearly demonstrates that thoughtful optimisation, and factual hypothesis could mitigate or improve scale up challenges in algal cultivation. The slight adjustments in the 30 L reactor proved effective in enhancing productivity while making the system more scalable while achieving growth rates closer to the 1 L benchmark. This step of methodology in this study highlights the importance of addressing *specific factors* and *optimal ranges* when transitioning from lab scale to larger bioreactors, bubble columns or airlift reactors.

Discussion

With the exponential rise of our global population and the upkeeping of its demands, large volumes of wastewater will continuously be generated as a byproduct of necessary processing, which is harmful and a threat to humans, animals, ecosystems and the environment. Large volumes of wastewater are released from sewage systems, endangering health and causing an ecological imbalance due to the strong and irregular organic pollutants. Utilizing this wastewater for microalgal cultivation will not only absorb harmful substances from the wastewater and promote a safer environment but also aid in wastewater treatment and the removal of high-toxic compounds. The model developed during ANOVA can predict growth rates based on the 1 L reactor vessels and further optimisation showed significant factors (<0.0005) influencing the overall effect within the reactor. Optimisation improved growth rates and biomass productivities, supporting the fact that nutrient and CO_2 availability, sufficient lighting and aeration need to be carefully maintained. However, the model needs to be developed to suit various reactor designs and configurations, as the effect of hydrodynamics significantly challenges the output responses. The quadratic regression models produced from the BBD validate the experimental data obtained by RSM, as it was selected to evaluate 3 levels of each factor while reducing the number of experimental runs compared to CCD. This tailored methodology at each stage produced attainable and practical results as the model demonstrated high R^2 (0,9910) and adjusted R^2 (0,9820) values. Furthermore, the model was validated by the predicted vs actual plots, residuals versus runs and residuals vs run number.

The results achieved from this study are consistent with other previous studies that utilised the BBD and statistical optimisation techniques for cultivating microalgae and added extended knowledge to the field. Previous study achieved a growth rate of 0,80/day and 315mg/L

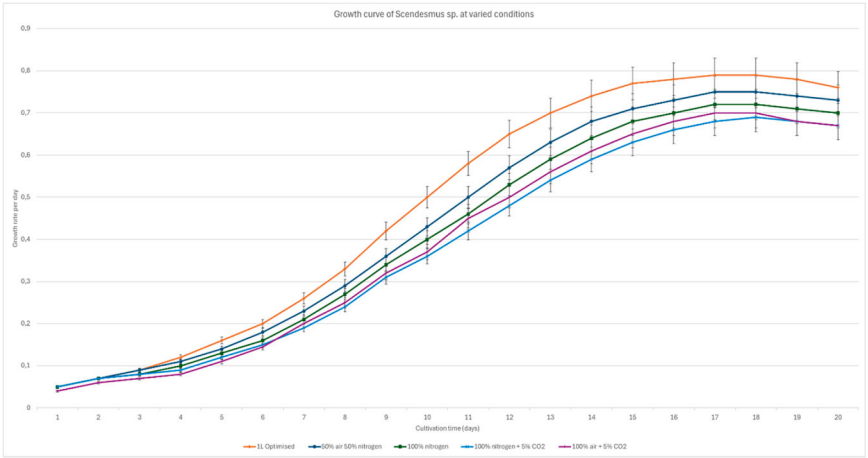

Fig. 14. Growth curve of Scenedesmus sp. at the various optimised equipment setups using various input air conditions.

of lipids using synthetic growth medium [12], whereas this study achieved 0,752/day and 28.55 % lipids using wastewater, further enforcing its applicability and sustainable practices. Similarly, a 0,84/day growth rate of Chlorella vulgaris was achieved but using synthetic growth medium [14]. Although this study's growth rate was lower, utilising real wastewater improved the feasibility and ecological relevance of the overall cultivation process. Furthermore, this study targeted a major limitation of previous studies that focused on ideal laboratory conditions. Understanding the strong synergistic relationship between the light illumination period and the growth medium ratio. The use of BBD in conjunction with ANOVA allowed the precise identification of dominant factors influencing algal growth and biomass productivity. Upscaling from a 1 L to 30 L photobioreactor shows that successful bench scale optimisation can be achieved on larger systems.

Conclusion

This is the first study to optimise Scenedesmus sp. growth using BBD in real sewage wastewater and to demonstrate a viable pathway for integrating biofuel production with wastewater remediation. This work identified light illumination and nutrient interactions as key drivers of biomass productivity, achieving at 1 L bench-scale a growth rate of 0.79/day and validating scalability to 30 L with a reduction in performance. The potential of this feedstock will play a significant role in providing energy security and mitigating the global water challenges faced. Furthermore, this study demonstrated the potential of integrating microalgae cultivation with industrial wastewater treatment to produce biofuels while achieving significant wastewater remediation. The selection of high-lipid microalgae strains such as *Chlorella vulgaris, Scenedesmus sp.*, and *Spirulina platensis* has proven effective in both lipid accumulation and pollutant removal. Upscaling and large-scale production challenges show great potential of being mitigated since microalgae have a rich and versatile composition of lipids, proteins and carbohydrates, attractive to produce clean energy and a wide range of products beneficial to mankind. These findings advance current knowledge by showing that optimisation models remain predictive under realistic wastewater conditions and can be successfully scaled up. In conclusion, cultivating microalgae in sewage wastewater is a sustainable solution concerning the current global energy and water crisis. The findings highlight the importance of optimizing these processes; the economic feasibility of microalgae-based biodiesel production can be significantly improved, contributing to the broader adoption of renewable energy solutions. Overall, this study contributes to the existing literature by novel contributions and practical implications in three key ways (i) It is the first to apply BBD optimisation of algal cultivation in real sewage wastewater rather than synthetic media, (ii) It demonstrates that statistical optimisation models remain predictive during scale-up, validating their use for pilot systems and (iii) it provides practical insights into synergistic factor interactions that can guide design of low-cost, decentralised wastewater-based algal biorefineries, highlighting the potential for scalable, circular bioeconomy solutions in industrial and municipal contexts.

Future research should investigate more controlled cultivation conditions, as this study incorporated semi-controlled conditions where room temperature and humidity could influence variance in the full potential of the system. Additionally, although promising results were seen during this upscaling, exploring continuous cultivation of co-cultivation strategies in bigger photobioreactors like airlift reactors, comparable to industry, should be investigated, as other additional factors may arise. Future work focusing on refining downstream separation technologies will be essential to enhance resource recovery efficiency and biofuel yield, thereby contributing critical insights to this evolving field of sustainable energy research. Future research should also expand on co-cultivation techno-economic analysis to further strengthen the industrial feasibility of wastewater-based algal biorefineries.

CRediT authorship contribution statement

Nikita Singh: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Manimagalay Chetty:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Sudesh Rathilal:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

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