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Research article

Optimization of co-valorisation techniques for dairy and paper pulp wastewater in the cultivation of *Chlorococcum* sp. with a focus on mixture design, microwave-assisted pretreatment, and bioethanol production

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ABSTRACT

This study optimized biomass and lipid accumulation using mixed dairy and paper-pulp wastewater for the cultivation of *Chlorococcum* sp. The obtained microalgal biomass was thereafter subjected to microwave-assisted pretreatment for optimal fermentable sugar release. Microwave power (100–700 W), pretreatment time (1–7 min), and acid-liquid ratio (1–5 %) were the input parameters for the pretreatment optimization study. The wastewater mixture ratios (25:75, 50:50, 100:0) of dairy and paper-pulp wastewater (DWW and PWW respectively) were achieved using simplex lattice mixture design to obtain high biomass and lipid accumulation in *Chlorococcum* sp cultivation. The model recommended a mixture of 64.69 % DWW and 35.31 % PWW for optimal biomass concentration, and a ratio of 34.21 % DWW and 65.79 % PWW for maximum lipid accumulation, predicting biomass concentration of 1.17 g/L and lipid accumulation of 0.39 g/g. Experimental validation resulted in biomass concentration and lipid accumulation 0.94 g/L and 0.39 g/g, respectively. Moreover, the experimental confirmation of the predicted fermentable sugar (11.14 g/L) yielded 15.67 g/L with pretreatment set points of 2.52 % HCl for 4.06 min at 700 W. Additionally, the prospect of the optimized pretreated microalgal biomass for bioethanol production (7.85 g/L) was achieved. Findings from this study could facilitate the implementation of DWW and PWW wastewaters utilization that could significantly lower the use of scarce potable water in keeping with portable water, energy, and environmental sustainability nexus towards the realisation of a circular bioeconomy.

1. Introduction

The continuous depletion of the world's primary energy source, fossil fuels, necessitates the need for a sustainable and environmentally friendly alternative energy sources [1]. Major biofuel production as alternative to fossil fuel currently depends on first-generation biomass feedstock such as corn and soybean and as well as second-generation feedstock, including sugarcane bagasse

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and corncob [2,3]. A promising alternative to first- and second-generation feedstock for biofuel production is the third-generation microalgal biomass utilization [4–6]. Despite microalgae enormous potential as a feedstock for biofuel generation, their cultivation demands large amounts of freshwater, which challenges their viability for commercial-scale production [7]. Furthermore, diverting the freshwater supply for human consumption to energy production will have negative influence on freshwater supply and sustainability [6]. Consequently, studies on alternative water sources for microalgae cultivation is desirable. In addition, numerous industries like dairy, paper and pulp (P&P), petroleum, and mining utilize freshwater extensively for various processes, leading to the discharge of substantial volumes of wastewater into the environment without adequately treated, contaminating land, rivers, and lakes.

Wastewaters, particularly those from industrial sources, can threaten aquatic life, decrease the availability of clean drinking water for humans, and facilitate the spread of dangerous diseases [8]. The organic substance in wastewater, can affect oxygen levels in the discharge water environment, resulting in the death of aquatic creatures and causing air contamination through anaerobic decomposition [9]. Moreover, high nutrient content (nitrogen, potassium, and phosphorus), can lead to eutrophication and algal blooms, exposing humans to nitrate and nitrite toxicity [8]. Hence, a suitable utilization or remediation approach for these wastewaters such as dairy wastewater and P&P is being sought for. Presently, there is scarcity of report on co-utilization of dairy wastewater and P&P towards sustainable environment development. Such studies will provide data on wastewater utilization, valorisation, remediation towards low-cost wastewater treatment before their disposal.

Among different industries, the P&P sector has been identified as the predominant (42 %) producer of the overall industrial wastewater production [10]. Similarly, the dairy industry is another significant contributor to wastewater production. It utilizes freshwater in all stages of its operations, including cleaning, sanitization, heating, and cooling [11]. Consequently, it produces large quantities of wastewater with high organic load, nutrient content, and a wide pH range (4.7–11) [12]. It is noteworthy that these industrial wastewaters contain essential macronutrients such as phosphates and nitrates, making them a cost-effective medium for microalgal cultivation and aligning with the concept of microalgal wastewater biorefinery [12]. Microalgae biomass usually composes of lipids (20–80 %), carbohydrates (10–40 %), and proteins (10–50 %) [4,5,13]. The carbohydrate content of microalgal biomass is rich in fermentable sugars, which can be utilized as a feedstock for bioethanol production [2]. Several studies have investigated the cultivation of microalgae using wastewaters, with specific emphasis on wastewater treatment and the cost-effective production of microalgal biomass [14]. Nonetheless, these studies predominantly focus on the impact of a single type of effluent as a growth medium for microalgae. For example, Ummalyma and Sukumaran [15] investigated the utilization of dairy wastewater for the cultivation of microalgae *Chlorococcum* sp. RAP13. From their result, a maximum biomass yield of 1.94 g/L, lipid accumulation of 42 %, and a 93 % reduction in COD were obtained. Gurumoorthy and Saravanan [16] also investigated the production of biodiesel from *Nannochloropsis oculata* grown in PWW. The authors found that the maximum biomass accumulation was 7.7 g/L dry weight, with lipid accumulation of 42 %. On the other hand, single wastewater may not supply the necessary nutrients in the appropriate ratios to support microalgae growth [17]. This suggests that for efficient and sustainable production of microalgal biomass, it is essential to optimize the cultivation medium by combining different types of wastewaters in specific proportions. An effective multi-algae medium requires a well-structured protocol to ensure an optimal combination for microalgal cultivation [18]. There is a dearth of knowledge on multi-algae wastewater medium development. Knowledge on the appropriate wastewater mixture design for microalgal cultivation could facilitate its industrial biorefinery application an approach with high potential to mitigate some of the multidimensional human challenges (such as waste management, energy shortage, scarce portable water and sustainable environment development).

Despite the advantages of microalgae as a feedstock for bioethanol production, their potential is significantly constrained by the challenges such as release of sugars accompanying their implementation [19,20].

Therefore, efficient use of microalgal biomass for bioethanol production necessitates cell disruption (biomass pretreatment processes), making them susceptible to the subsequent process of hydrolysis [21]. Nevertheless, several of these pretreatment processes can be time consuming, energy intensives, leading to product degradation [19]. As a result, one method that has garnered substantial research interest as technique for breaking down microalgal cells is microwave-assisted (MW) pretreatment [22]. The MW approach involves the exposure of a solution to an electromagnetic field, causing the rotation of ions and dipolar molecules, resulting in the concurrent heating of the sample without direct contact [23]. Ultimately, uniform heat distribution throughout the biomass is achieved, resulting in efficient cell disruption [24]. Several research have studied the impact of the MW approach on the pretreatment of microalgal biomass to facilitate the extraction of fermentable sugars for the generation of bioethanol [25,26]. For instance, Hernández et al. [27] studied the effectiveness of MW pretreatment and reported a sugar yield of 21 mg/g dry weight from *Chlorella sorokiniana* at 150 W for 40s. Nevertheless, research on the utilization of *Chlorococcum* microalgal biomass for bioethanol production remains limited. Additionally, the interaction and optimization of irradiation time, microwave power, and liquid to solid operational parameters in *Chlorococcum* biomass pretreatment are not well understood. Enhanced comprehension of the interactive dynamics of the aforementioned pretreatment parameters in relation to *Chlorococcum* biomass could improve the efficiency of *Chlorococcum* biomass for extracting fermentable sugars, leading to increased bioethanol production. Therefore, this study aims to (i) identify the most suitable wastewater mixture for *Chlorococcum* growth and lipid accumulation, (ii) evaluate *Chlorococcum* sp. efficiency in wastewater remediation during cultivation, (iii) assess the impact of MW pretreatment on *Chlorococcum* biomass for fermentable sugar extraction, and (iv) explore bioethanol production from the MW-pretreated microalgal biomass.

2. Materials and methodologies

2.1. Microalgal species

The *Chlorococcum* sp. used in this study was identified in the brackish water ecosystem of Durban, KwaZulu-Natal province of South

Africa. The *Chlorococcum* sp, was thereafter cultivated and maintained using an enrichment medium consisting of 10 % BG-11 solution, 1 % trace metals solution and 89 % distilled water. The inoculum was agitated at 150 rpm, illuminated at an intensity of 54.36 μmol/m^2s and incubated for 14 days at room temperature.

2.2. Wastewater sample collection and screening for Chlorococcum sp cultivation

The dairy wastewater (DWW) and paper-and-pulp wastewater (PWW) utilized in this research was supplied by local dairy industry and paper-and-pulp industry located in KwaZulu-Natal Provine, South Africa. The DWW was collected from the storage tank containing both processing and cleaning wastewaters generated during dairy production. While the PWW sample was obtained from the secondary effluent treatment plant. Each wastewater sample (20 L) was filtered separately using Whatman filter paper to get rid of the solid particles. Subsequently, these filtered samples were combined in various proportions to make a wastewater mixture formulation ratio for the cultivation of *Chlorococcum* sp. A comprehensive analysis of the physicochemical characteristics of the DWW and PWW is presented in Table 1.

Thereafter the wastewater collection, a preliminary assessment was performed to evaluate the viability of DWW and PPW as a sole or combined medium, as well as treated or untreated for the cultivation of *Chlorococcum* sp. The pH of the treated wastewater was adjusted to pH 7.1 and autoclaved at 121 °C for 15 min before the wastewater was used for the cultivation. Then the cultivation experiments in an Erlenmeyer flask (100 mL working volume) were conducted under growth conditions of pH 7.1, ambient temperature, agitation speed (150 rpm) and cultivation duration of three weeks.

Furthermore, varied amount of DWW and PWW with or without BG11 medium supplementation was utilized to cultivate *Chlorococcum* sp with the most favourable condition (Table 2) then modelled and optimized.

2.3. Mixture design modelling and optimization

Based on the literature and preliminary study results (section 2.2), eight experimental runs with different input compositions were generated using the simplex lattice mixture design (Design-Expert software) [18]. This experimental design aimed to identify the optimal combined wastewater while assessing the impact of single wastewater on the hybrid wastewater. Table 3 shows the experimental design utilizing the simplex mixed design network. The components tested were PWW and DWW, with each component's proportion ranging from 0 to 100 %.

2.4. Bench-top pilot Chlorococcum cultivation scale up

In view of large-scale potential of using the formulated and optimized wastewater mixture, a preliminary scale up trail was carried out. *Chlorococcum* sp. was cultivated for 21 days in a lab-scale photobioreactor illuminated with four fluorescent bulbs for optimal illumination using the optimized condition in section 2.3. The photobioreactor consists of 15 wells, each with a maximum capacity of 1 L, arranged into three rows of five wells each. Each well has dimensions of 27 cm in length, 10 cm in width, 7.5 cm in depth, and a working volume of 800 mL. The culture was mixed using a submerged paddle operating at 43 rpm, with mixing speed regulated by automated sensors and actuators. *Chlorococcum* sp biomass was harvested by centrifuging at 4500 g for 10 min. The supernatant was removed, and the resulting pellet was dried and kept at ambient temperature [28]. The obtained dried biomass was afterwards pretreated for the release of fermentable sugar.

2.5. Chlorococcum sp biomass pretreatment modelling and optimization

In Section 2.5, the Response Surface Methodology (RSM) was employed to design fifteen (15) pretreatment experimental runs for

Table 1
Physico-chemical parameters of DWW and PWW.

Parameter	Unit	Dairy wastewater	Paper and pulp wastewater
pH	–	2.87	6.94
Colour	–	White	Brown
COD	mg/L	876	955
TN	mg/L	736.25	562.25
TP	mg/L	27.07	1.20
Na	mg/L	237.73	1153.73
K	mg/L	27.73	68.40
Ca	mg/L	50.80	48.00
Mg	mg/L	5.47	18.13
Fe	mg/L	0.24	0.04
Cu	mg/L	0.01	0.004
Zn	mg/L	0.13	0.04
Mn	mg/L	0.04	0.76
Al	mg/L	0.15	0.52

TN-Total nitrogen, TP- Total phosphorous.

Table 2
Selected mixtures for the optimization model.

Mixture	BG11 (%)	PWW (%)	DWW (%)	BG11+PWW + DWW (%)
DWBG25 (A)	25	0	75	100
DWBG50 (B)	50	0	50	100
DWPWBG25 (C)	25	25	50	100
DWPWBG50 (D)	50	25	25	100
A + B + C + D	150	50	200	400

DWBG25: Dairy wastewater (75 %) and blue-green algae 11 (25 %); DWBG50: Dairy wastewater (50 %) and blue-green algae 11 (50 %); DWPWBG25: Dairy wastewater (50 %), paper and pulp wastewater (25 %) and blue-green algae 11 (25 %); DWPWBG50: Dairy wastewater (25 %), paper and pulp wastewater (25 %) and blue-green algae 11 (50 %).

Table 3
Simplex lattice design for the DWW and PWW mixture design.

Run	A:DWW	B:PWW	Biomass (g/L)	Lipid yield (g/g)
1	50.00	50.00	1.07	0.40
2	50.00	50.00	1.05	0.40
3	100.00	0.00	0.88	0.50
4	25.00	75.00	0.68	0.38
5	100.00	0.00	0.90	0.50
6	75.00	25.00	1.16	0.40
7	0.00	100.00	0.91	0.36
8	0.00	100.00	0.91	0.36

Chlorococcum sp. cultivation. The RSM used in this study was three-level Box-Behnken design (Design Expert 7.0 software, Stat Ease Inc, USA). The input parameter ranges were chosen based on literature [29]. The chosen parameters included microwave power (400–800 W), pretreatment time (1–5 min), and liquid ratio (1–10 % v/v). The resulting empirical data were then fitted into the polynomial model equations. These equations correlate the input parameters with the response variables, specifically the release of fermentable sugars. The general form of the model is shown in Eq. (1).

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{11} X_1^2 + \alpha_{22} X_2^2 + \alpha_{33} X_3^2 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{23} X_2 X_3 \quad (1)$$

Where Y represents the response output, α_0 is the intercept, $\alpha_1 X_1$ to $\alpha_3 X_3$ are the linear coefficients, $\alpha_{11} X_1^2$ to $\alpha_{33} X_3^2$ are the quadratic coefficients and $\alpha_{12} X_1 X_2$ to $\alpha_{23} X_2 X_3$ shows the interaction of coefficients. The model was afterwards assessed using analysis of variance (ANOVA). The optimal pretreatment conditions for maximizing fermentable sugar yield were determined by solving the equation, and these conditions were subsequently validated.

2.6. Enzymatic saccharification of the pretreated microalgae hydrolysate

The enzymes employed in this research included cellulase, α-amylase, and amyloglucosidase. The enzymatic hydrolysis of the pretreated microalgae hydrolysate was conducted in a 100 mL Erlenmeyer flask at 100 rpm. First, cellulose was introduced into flask and incubated at 55 °C, pH of 5.5 for 2 h. This was followed by liquefaction with α-amylase at 90 °C, pH 7 for 1 h, with enzyme denaturation achieved via incubation of the mixture at 95 °C for 10 min. Subsequently, the saccharification stage was carried out with amyloglucosidase at 60 °C, pH 4.5 for 24 h. Thereafter, saccharification enzyme denaturation at 96 °C for 10 min. The resulting sugar-rich hydrolysate was then centrifuged at 5000 rpm for 5 min to obtain a supernatant, which was used for fermentable sugar evaluation. The saccharification efficiency was determined using Eq. (2).

$$Saccharification\ (\%) = \frac{Sugar\ recovered\ (g/g) \times 0.9}{Carbohydrate\ unit\ (hemicellulose, cellulose)\ in\ pretreated\ sample} \times 100 \quad (2)$$

2.7. Analytical methods

The physicochemical properties of the two wastewater samples were assessed following the protocols set by the American Public Health Association [30]. *Chlorococcum* sp. biomass concentration was determined by measuring the optical density at 680 nm and the dry weight of the biomass was then calculated using a pre-established calibration curve that relates optical density to dry biomass weight.

The nutrient removal efficiency from the mixed wastewater was calculated using Eq. (3):

$$P_R = \frac{P_0 - P_1}{P_0} \times 100\% \quad (3)$$

where P_R is the efficiency of parameter removed, P_0 and P_1 are initial and final concentrations of the parameter, respectively.

Lipid content was determined using the Bligh and Dyer [31] solvent extraction method. Briefly, 1 g of dry microalgal biomass was homogenized with 80 mL of distilled water (H_2O) and heated for 5 min at 2450 MHz using a Samsung microwave oven (Model: ME9114S1, South Korea). Then, 100 mL of chloroform and 200 mL of methanol were added to the disrupted cells and the mixture was vortexed for 30 s. An additional 100 mL of chloroform was then added, and the mixture was homogenized for another 30 s. Thereafter, extra 100 mL of H_2O was introduced with vortexing for a further 30 s. The resultant mixture was filtered via a pre-weighed Whatman filter paper. Subsequent to filtration, the chloroform present in the filtrate (containing chloroform and lipid) was allowed to be evaporated. Then, the lipid content was quantified gravimetrically.

Fermentable sugar content was quantified in the pretreated hydrolysate using 3,5-dinitrosalicylic acid method [32].

Moreover, the structural components of pretreated *Chlorococcum* sp. biomass including cellulose, hemicellulose, and lignin, were examined following established protocols. While the morphological features of the dried microalgal biomass samples (untreated and optimally pretreated) were examined under Scanning Electron Microscopy (SEM) to obtain inherent structure and potential structural changes. Additionally, the samples were evaluated for the presence of functional group using Fourier Transform Infrared Spectroscopy (FTIR) and the spectra were recorded between 450 and 4000 cm^{-1}.

2.8. Bioethanol concentration determination

Bioethanol concentration in the fermented broth was obtained with Vernier ethanol sensor (United States of America), and bioethanol productivity was estimated using Eq. (4).

$$\textit{Bioethanol productivity}\ (g/L/h) = \frac{\textit{Highest biothanol concentration}\ (g/L)}{\textit{Bioethanol production period}\ (h)} \tag{4}$$

3. Results and discussion

3.1. Physicochemical properties of DWW and PPW

The physicochemical characteristics of the DWW and the PWW are depicted in Table 1. The result indicated that both DWW and PWW were turbid with DWW appearing whitish and PWW brownish. The turbidity and the brownish colouration of PWW are most likely due to the presence of lignin and its derivatives which were its major constituents [33]. The brownish colouration of PWW in comparison to the white colouration of DWW may have influenced light penetration during *Chlorococcum* sp cultivation as microalgae require light for photosynthesis and growth. Given that microalgae rely on light for photosynthesis and growth, limited light penetration could have adversely impacted their growth performance. This factor likely contributed to the lower accumulation of biomass and lipid yield obtained in our study with PWW or a high proportion of PWW mixture component.

Additionally, both DWW (876 mg/L) and PWW (955 mg/L) exhibited significant COD levels, which align with the findings of Harrison et al. [34], who reported COD values ranging from 700 to 1200 mg/L for PWW. The author attributed the significant COD values to the presence of lignin and its derivatives which are recalcitrant and consequently raises the organic loading in the wastewater [33]. Furthermore, DWW had higher nutritional contents such as total nitrogen (TN) and total phosphorus (TP) in comparison to PWW (Table 1). The DWW's high nutrient concentration can be attributed to its high organic load and high concentrations of nitrogen, phosphorus, protein, and dissolved sugar [15]. The growth of microalgae relies on these crucial nutrients. In addition to these nutrients, the wastewaters employed in this study comprised of trace amounts of metals such as Fe, Ca, Mn, Mg, and Zn (Table 1). The presence of these nutrients in microalgae cultivation had an impact on culture performance which in turn improved microalgae biomass concentration and lipid yield. Decreasing the COD, N and P in wastewater such as dairy wastewater is an energy demanding process using either conventional or highly established protocols [35]. In addition, the costs of producing nitrogen and phosphorus for agricultural fertilizers are expensive due to the high energy requirements (Nitrogen-11.1 kWh/kg and Phosphorus-10 kWh/kg) [35]. Thus, nutrient recycling, reutilizing through wastewater remediation is crucial and desirable for environmental sustainability [36]. For instance, an efficient, safe, and less expensive method of treating wastewater from excess nutrients is by using microalgae to consume the excess N, P organic carbon, COD, and ammonia such as was implemented in the present study. In this study, *Chlorococcum* sp. cultivation was efficient in DWW and PWW remediation with simultaneous high biomass production that was subsequently utilized for bioethanol production. Similarly study on microalgal wastewater remediation has been reported, De Francisci et al. (2017), used wastewater for the cultivation of *Chlorella sorokiniana* and wastewater remediation. The authors reported a 50 % COD reduction, 94.2 % and 82.7 % drop in N and P contents respectively.

Additionally, DWW had a pH of 2.87, while PWW had a pH of 6.94 (Table 1). These pH values were unsuitable for supporting *Chlorococcum* growth. Typically, microalgae growth, similar to other cellular processes, is pH dependent. *Chlorococcum* growth was significantly improved after the pH of the wastewater was adjusted to 7.1. The pH as a growth requirement is highly influential in regulating microalgal growth and controls different nutrient availability as well as their uptake. Additionally, pH affects photosynthetic activities, decrease ammonia toxicity, and controls the availability of inorganic carbon to cells. Ultimately, the pH influences cellular activities and growth performance.

3.2. Effects of wastewater sterilization and augmentation on microalgae growth

The proliferation of *Chlorococcum* sp. was evaluated in unsterilized and non-supplemented DWW and PWW. The results revealed

that *Chlorococcum* sp. growth was not stimulated by the unsterilized wastewaters (DWW and PWW), whether separately or mixed. However, the growth of other microorganisms such bacteria and fungi were detected. Previous studies have shown that most unsterilized wastewaters contain bacteria, fungi, and zooplankton, which cause biotic pollution. Biotic pollution has been known to inhibit the growth of microalgae [37,38]. In contrast, no biotic pollution was observed with sterilized DWW and PWW for the cultivation of *Chlorococcum* sp. Nevertheless, neither the separated nor mixed sterilized wastewaters were able to adequately support *Chlorococcum* sp. growth. Therefore, these findings underscore the necessity of supplementing the sterilized wastewaters with a minimal amount of BG-11 microalgae formulated growth media. The inclusion of the BG-11 formulated microalgae growth nutrient is crucial as a growth initiator. After the inclusion of minimal growth initiator (BG-11 formulated growth nutrient) desired *Chlorococcum* sp. growth were observed.

Table 2 shows the DWW and PWW supplemented with BG11 at low concentration for *Chlorococcum* sp. growth initiator and cultivation. The results revealed that the wastewater mixture comprising (75 and 25 %), (50 and 50 %), and (25 and 75 %) of DWW and BG11, respectively, stimulated *Chlorococcum* sp growth. On the other hand, for the growth of *Chlorococcum* sp requires at least 50 % formulated BG11 when using a mixture containing PWW, In literature, PWW has been reported to be deficient in nutrients such as phosphorous and nitrogen that are needed for microalgae cultivation [34]. This nutritional limitation in PWW suggests that nutrient augmentation is imperative to substantiate the use of PWW as a growth medium for *Chlorococcum* sp. cultivation. These findings

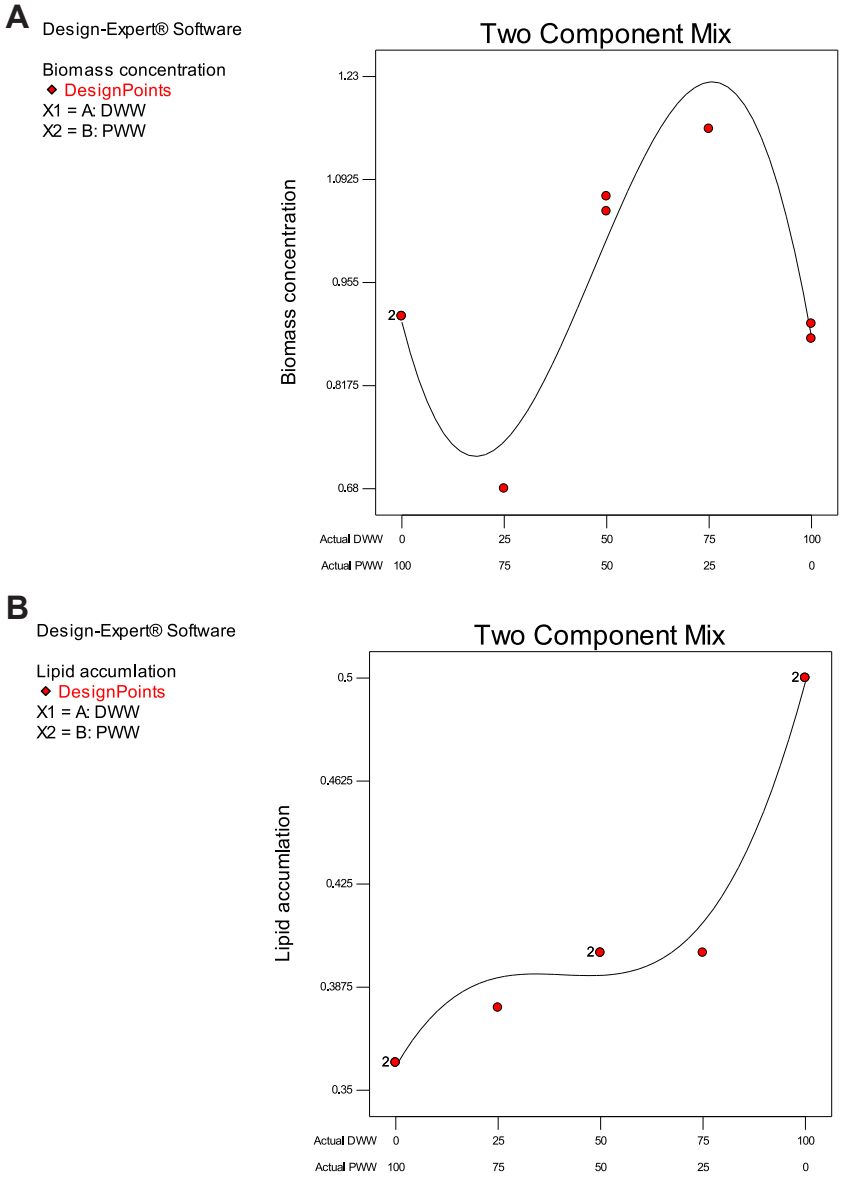

Fig. 1. 2-Dimensional plot showing the interactive effect of mixed DWW and PWW on algal biomass concentration (A), and the interactive effect of blended DWW and PWW on lipid yield (N).

indicate that the DWW is a better nutrient source for stimulating microalgae growth compared to PWW. The combinations of DWW, PWW, and BG11 in ratios of (50, 25, and 25 %), (25, 50, and 25 %), and (25, 25, and 50 %) respectively, prove to be suitable growth media for *Chlorococcum* sp. (see supplementary document). The growth pattern of *Chlorococcum* sp. in blended wastewater media was comparable to that of the standard BG11 growth media and no significant differences were observed (see supplementary document). This indicates that the mixed wastewater contains abundant of nutrients for *Chlorococcum* sp. cultivation. Furthermore, these results showed that the hybridity promotes *Chlorococcum* sp. growth in comparison to the potential of individual wastewater to stimulate and support microalgae growth. Therefore, it is crucial to ensure their optimal complementary blend to achieve maximum *Chlorococcum* sp. growth as individual wastewaters might lack certain required nutrients. Additionally, the obtained growth media formulation indicates that the DWW and PWW can be effectively used to cultivate *Chlorococcum* sp. This is desirable and economically advantageous as it has the potential to eliminate the cost associated with wastewater treatment while utilizing the wastewater for microalgae cultivation. The biomass produced from this cultivation can serve as a feedstock for biofuel production. To further determine the optimal blend of the growth media formulation, a simplex lattice mixture design model was employed aiming to achieve the best complementary mixture. (Table 3).

3.3. Wastewater mixture design optimization

The data obtained from the cultivation of DWW and PWW mixture was employed to develop an optimization model for biomass concentration and lipid accumulation. Thereafter, analysis of variance (ANOVA) was used to evaluate the model's fitness (see supplementary document). The responses for biomass concentration and lipid accumulation yielded high F-values of 14.30 and 76.21, and low p-values of 0.0132 and 0.0006, respectively. These high F-values and low p-values indicate the model's significance. Additionally, the regression coefficient (R^2) for biomass concentration and lipid accumulation models was 0.91 and 0.98, respectively, suggesting that these models can elucidate 91 % and 98 % of the experimental data (see supplementary document). The model's equations are represented in Eq, (5 and 6).

$$\text{Biomass concentration} = 0.88 * A + 0.9 * B + 0.48 *A * B + 2.61 *A *B * (A - B) \quad (5)$$

$$\text{Lipid accumulation} = 0.5 * A + 0.36 * B - 0.15 * A * B - 0.27 * A *B * (A - B) \quad (6)$$

where A and B are the various components of the mixture.

The biomass concentration and lipid yield obtained for each experimental run is shown in Table 3. The biomass concentration and the lipid yield ranged from 0.68 g/L to 1.16 g/L and from 0.36 g/g to 0.50 g/g, respectively. The process gave higher responses for biomass concentration and lipid yield when DWW and PWW were blended at their median values compared to using the wastewater individually and at high concentration of PWW. When the mixtures were blended at 50 % each of DWW and PWW (Runs 1 and 2), biomass concentrations 1.07 and 1.05 g/L were obtained respectively. Similarly, with Runs 1 and 2 lipid accumulation of 40 g/g was obtained for both experimental runs. Furthermore, the mixed wastewater containing 75 % of DWW and 25 % of PWW resulted in 1.16 g/L of biomass concentration. While, at a higher percentage of PWW (75 %), the lowest biomass concentration of 0.68 g/L was obtained. The observed impact of DWW on achieving increased biomass concentration for *Chlorococcum* cultivation might likely be attributed to the increased nitrogen (736.25 mg/L) and phosphorous (27.07 mg/L) present in DWW (Table 1). These nutrients are also readily available for *Chlorococcum* growth. Nitrogen and phosphorus are vital nutrients necessary for the growth of microalgae during cultivation [39,40]. Thus, the increase in biomass concentration in mixed wastewater with median (50 %) DWW or high DWW (75 %) was anticipated.

Fig. 1 depicts the interactive impacts of the process variables on biomass concentration and lipid yield via two-dimensional contour plots for the generated process models. It was found that the different percentage of DWW content (0, 25, 50, 75 and 100 %) exhibited a linear relationship with the biomass concentration. As the DWW content increased from 0 to 75 % while the PWW content decreased from 100 to 0 %, the microalgal biomass concentration increased from 0.90 to 1.19 g/L (Fig. 1). However, further increasing the DWW content from 75 to 100 % led to a decline in biomass concentration from 1.19 to 0.95 g/L. Similar results were observed for DWW and PWW mixture in terms of lipid accumulation. It was noted that the percentage of DWW content had a direct correlation with the lipid accumulation observed (Fig. 1). As the percentage of PWW content decreased while the DWW content increased from 0 % to 100 %, the *Chlorococcum* lipid content increased from 0.36 to 0.50 g/g (Fig. 1). Based on these observations, wastewater blend with a high concentration of DWW resulted in higher biomass concentration. Similarly, a greater percentage of DWW in the mixed wastewater significantly enhanced lipid yield. The high productivities can be linked to the nutritional components of DWW, which were in adequate proportions (Table 1) and available for *Chlorococcum* growth, as previously elucidated [39,40].

Table 4
Experimental validation of the wastewater mixture design.

Response	Components (%)		Response values	
	DWW	PWW	Predicted value	Observed value
Biomass concentration	64.69	35.31	1.17 g/L	0.94 g/L
Lipid accumulation	34.21	65.79	0.39 g/g	0.39 g/g

3.4. Validation of mixture design optimization

In this study, the biomass model estimated a biomass concentration of 1.17 g/L for a mixture comprising 64.69 % DWW and 35.31 % PWW, whilst the lipid model predicted a lipid accumulation of 0.39 g/g for a blend comprising 34.21 % DWW and 65.79 % PWW (Table 4). The experimental validation yielded biomass concentrations of 0.94 g/L and lipid accumulation of 0.39 g/g (Table 4). Biomass concentration (g/L) showed rapid increase over the first 5–15 days of cultivation, followed by gradual progression until day 20. *Chlorococcum* sp. thrive well in the DWW and PWW mixture. Therefore, combining DWW and PWW can serve as a viable alternative to commercial microalgae media. This highlights the promising prospect of optimized wastewater blends or combinations in the growth of microalgae for mass production of microalgal biomass and lipids. Also, the findings present a complementary mixture of DWW and PWW as excellent media for *Chlorococcum* cultivation and can be considered as a suitable cost-effective alternative microalgal growth medium towards a biorefinery biofuel production.

The increment in the lipid yield might be ascribed to the higher percentage of PWW in comparison to the DWW [41,42]. The ratio of DWW to PWW used in the *Chlorococcum* cultivation to obtain the high lipid yield is approximately 1:2, which corresponds to a percentage composition of 35 % DWW and 65 % PWW, respectively. According to Harrison et al. [34], paper and pulp wastewater lacks an adequate amount of nitrogen [34]. Similarly, Vitova et al. [43] indicated that lack of nitrogen stimulated increased lipid accumulation in microalgae. Moreover, Gentili [35] conducted a study utilizing a unique combination of PWW with DWW and municipal wastewater. The author obtained reduced lipid content (32 %) when using a mixture of PWW and DWW (2:1) in contrast to the lipid content observed in this study. Gentili [35] also noted that the increased lipid accumulation is possibly associated with nitrogen depletion, as all the available nitrogen was consumed within a short period of time. Various studies have shown that microalgae tend to accumulate higher amounts of lipids when using ammonium nitrogen source, rather than nitrate, nitrite, yeast and urea [44]. This is because these alternative nitrogen sources typically need to be converted to ammonium nitrogen type through different metabolic pathways before they can be transformed into amino acids within the microalgal cells.

On the other hand, the high total nitrogen (TN) content detected in DWW utilized in this study, potentially led to the increased lipid accumulation observed. The TN primarily consists of NH4-N, and the conversion of ammonia into amino acids demands less energy compared to other nitrogen sources, making it a preferred nitrogen source by microalgae [45]. Additionally, Sharma et al. [42] conducted research to examine the efficacy of different culture media, such as Blue green-11 (BG-11), Fog's medium, Bold basal medium, and Basal medium, in promoting the growth and lipid productivity of microalgae. The authors revealed the highest lipid accumulation (38 % dry biomass weight) with microalgal was achieved in the BG-11 medium. The result obtained was 1.03 times less than the outcome observed in this present study involving the combination of DWW and PWW. This provides additional evidence that developing the optimal wastewater composition is a viable strategy for enhancing the combination of remediation and bioenergy generation through the utilization of different microalgae species. Furthermore, the blend of DWW and PWW in a 1:2 ratio may serve

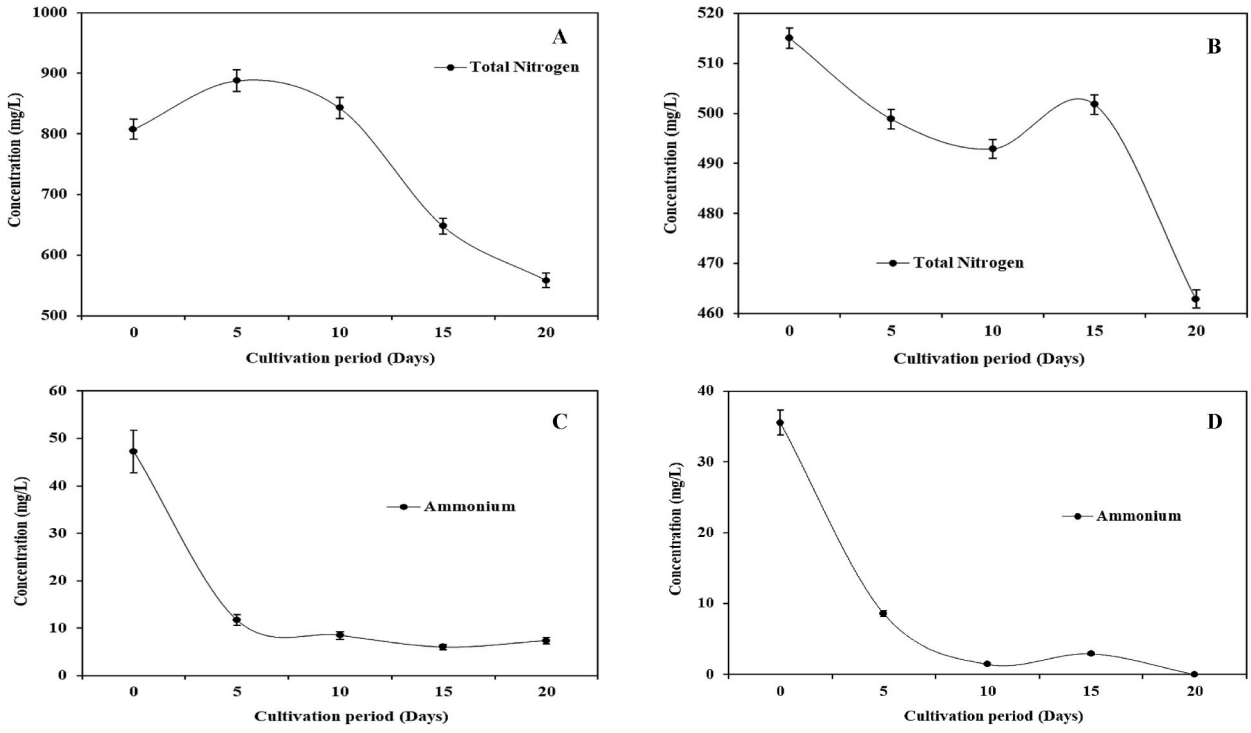

Fig. 2. Variations of TN concentration in (a) biomass accumulation mixture model and (b) lipid accumulation mixture model, and the variation of NH_4-N concentration in (a) biomass accumulation mixture model and (b) lipid accumulation mixture model.

as a viable substitute for the BG11 growth medium in the *Chlorococcum* cultivation, leading to a cost-efficient enhancement of lipid yield.

3.5. Nutrient removal efficiencies using Chlorococcum

In this present study, a notable decrease in the nutrients such as TN, NH_4-H, phosphorus (P), as well as metals (magnesium and calcium) and trace elements (zinc, manganese, copper, and iron) was observed during the mixed wastewater cultivation of *Chlorococcum* sp. Figs. 2–4, illustrate the nutrient compositions of the experimental mixtures of the 20-days cultivation period. The total nitrogen (TN) removal efficiencies, as depicted in Fig. 2A and B, were 30.86 % for the biomass concentration model and 10.13 % for the lipid accumulation model. The substantial utilization of nitrogen suggests that the nitrogen compounds detected in the mixed wastewater were readily accessible by *Chlorococcum* sp for growth. The study's findings showed a higher result compared to the findings of Ding et al. [36], who did not observe any variation in TN concentration while cultivating microalgae in DWW. The authors proposed that the microalgae's incapability to eliminate nitrogen might be due to the presence of substantial amounts of intricate organic nitrogen sources in the DWW.

In contrast, the TN removal efficiency observed in our study was lower when compared to previous research findings. For example, Yao et al. [46] assessed the TN removal efficiency during the cultivation of *Chlorella sorokiniana* and *Desmodesmus communis* using a 1:3 ratio of swine wastewater to secondary treated municipal wastewater with 5 % CO_2. Their study reported high TN removal efficiencies of 88.05 % for *C. sorokiniana* and 83.18 % for *D. communis*. The removal of total nitrogen from wastewater by nutrient assimilation is significantly dependent on the nitrogen source or type. Research indicates that microalgae show a preference for ammonia and simpler organic nitrogen sources like yeast extract and urea, which require less energy for conversion to ammonia [39,40]. Unlike the simpler organic nitrogen, complex organic nitrogen compounds necessitate significant amount of energy for their conversion to ammonia, making it challenging for microalgae to uptake nitrogen from these sources. Hence, it can be inferred that the wastewater blends used in this present study contained complex nitrogen sources that *Chlorococcum* sp. found difficult to readily assimilate.

Fig. 2C and D shows the variations in NH4-N concentration during the growth of *Chlorococcum* sp. for biomass and lipid accumulation. Fig. 2C indicates a significant decrease in NH4-N within the initial five days of cultivation for biomass concentration. This suggests that *Chlorococcum* sp. efficiently assimilate the nitrogen source with minimal energy requirement to adapt and thrive in the wastewater mixture. This finding corroborates with the results reported by Ruangsomboon [45]. The author indicated that majority of microalgae exhibit a preference for ammonium compounds as their nitrogen source because it requires less energy for assimilation into amino acids compared to other nitrogen types. After the initial five days, the NH4-N removal efficiency decreased until reaching removal efficiency of 87.21 % at day 15 of cultivation. The decrease in NH4-N removal may possibly be attributed to *Chlorococcum* sp. reaching its optimal NH4-N uptake. By the end of the cultivation period, a slight rise in NH4-N concentration was observed, potentially indicating a reduction in alternative nitrogen sources available. This observation is in accordance with the results by Cai et al. [39], which reported that microalgae typically convert nitrogen sources into ammonia before incorporating them into amino acids. Additionally, similar trends were observed in our study with *Chlorococcum* sp., where significant NH4-N reduction occurred after five days of cultivation, followed by sustained removal throughout the 15-days period, achieving complete NH3-N (100 %) removal efficiency as indicated by the lipid accumulation model (Fig. 2D). Likewise, the NH4-N removal obtained in this study was reported in other studies [35,47]. For instance, Wang et al. [47] reported 100 % NH4-N removal efficiency after cultivation of *Chlorella* sp. in a medium supplemented with digested dairy manure. Similarly, Gentili [35] reported 99 % NH4-N removal efficiency via microalgal cultivation in a mixture of municipal, dairy, as well as pulp and paper wastewater for biomass and lipid production. Fig. 3 illustrates the decline in phosphorous (P) nutrient during *Chlorococcum* sp. cultivation using the wastewater mixture. The variations in P concentration within both the biomass concentration and lipid accumulation cultivation mixtures exhibited comparable removal trends, demonstrating efficient P removal. Notably, the lipid accumulation model achieved a higher removal efficiency of 84.62 %, compared to 59.34 % in

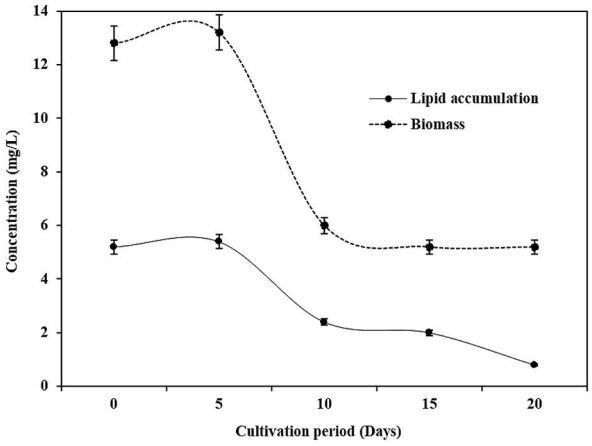

Fig. 3. Changes in phosphorus concentration in biomass concentration and lipid accumulation mixture models.

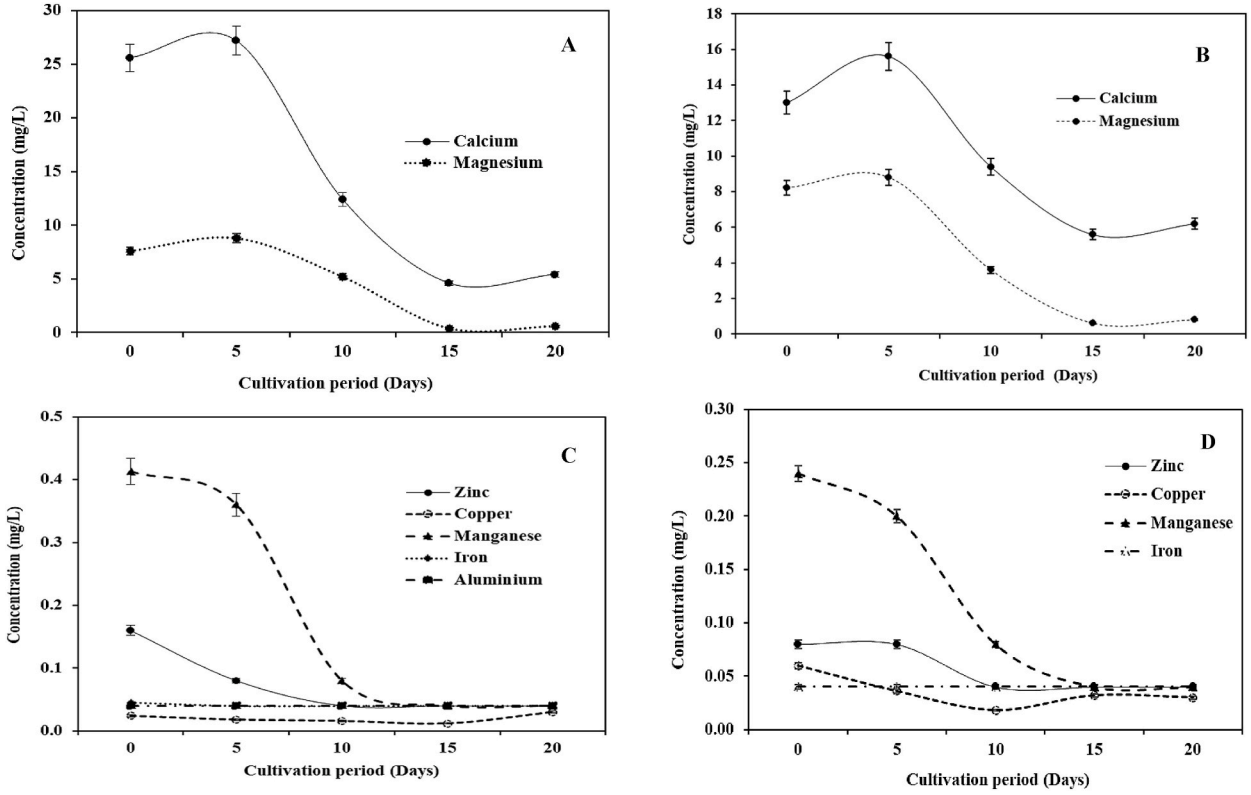

Fig. 4. Changes in Ca and Mg concentrations in (a) biomass concentration mixture model and (b) lipid accumulation mixture model. The variation of trace element concentrations in (c) biomass concentration mixture model and (d) lipid accumulation mixture design model.

the biomass concentration medium. These results revealed that the P removal efficiencies observed in this study can be attributed to the substantial utilization of P by *Chlorococcum* sp. for growth and lipid production. According to Luo et al. [48] and Ren et al. [49], P is a crucial component of adenosine triphosphate (ATP), deoxyribonucleic acid (DNA), and ribonucleic acid (RNA), hence it plays a vital role in several cell metabolisms including chlorophyll production and fatty acid metabolism.

The reduction in other essential nutrient such as Ca and Mg are depicted in Fig. 4A and B. The biomass concentration model recorded removal efficiencies of 78.91 % for Ca and 92.11 % for Mg. In contrast, the lipid accumulation model showed reductions of 52.31 % for Ca and 90.24 % for Mg. Both Ca and Mg are crucial for microalgal chlorophyll formation and growth [48]. Therefore, the notable removal efficiencies of these nutrients by *Chlorococcum* sp. might have contributed to the significant biomass and lipid accumulation reported in this study. In accordance with the findings of McGinn et al. [50], it was stated that Mg ions have the ability to increase enzyme (such as acetyl-coenzyme A carboxylase) activities responsible for fatty acid production and supports the synthesis of neutral lipid in microalgal cells.

Table 5
Box-Behnken design for microwave-assisted pretreatment of *Chlorococcum* biomass.

Run	A: MW Power (Watt)	B: Acid ratio (v/v)	C: Pretreatment time (min)	Response 1: Reducing sugar (g/L)
1	700.00	5.00	4.00	9.006
2	400.00	5.00	1.00	7.728
3	700.00	1.00	4.00	10.626
4	700.00	3.00	1.00	10.104
5	400.00	3.00	4.00	9.287
6	100.00	5.00	4.00	8.532
7	400.00	1.00	1.00	6.852
8	100.00	3.00	7.00	7.404
9	400.00	3.00	4.00	9.287
10	400.00	3.00	4.00	9.287
11	400.00	5.00	7.00	8.180
12	700.00	3.00	7.00	10.734
13	400.00	1.00	7.00	6.510
14	100.00	3.00	1.00	6.798
15	100.00	1.00	4.00	7.338

Additionally, Fig. 4C and D depict the profiles of trace elements in the mixed wastewater media. Both media showed 100 % removal efficiency for Zn and Mn. This high removal efficiency is ascribed to *Chlorococcum* sp. ability to readily absorb these elements. Zinc (Zn^{2+}) plays a crucial role in aiding photosynthetic efficiency within microalgal cells, while manganese (Mn^{2+}) acts as a co-enzyme, essential for activating enzyme activities involved in glycolysis and tricarboxylic acid cycle. The significant removal efficiencies of these trace elements in our study indicate their efficient assimilation by *Chlorococcum* sp. In addition, efficient removal of zinc and manganese in this study is desirable and attractive for wastewater remediation and sustainable green environment [51].

3.6. Optimization of reducing sugar release from Chlorococcum feedstock

The data obtained from the acid-microwave assisted pretreatment regimes are shown in Table 5. These data were utilized to develop a polynomial equation (Eq. (7)) that correlated reducing sugar concentrations with HCl concentration, microwave intensity, and microwave time. The assessment of the model's accuracy was also conducted via analysis of variance (ANOVA) (see supplementary document). Additionally, the significance of these polynomial models was evident from the low p-value of 0.0410 and the high F value of 6.02 (see supplementary document). In addition, the model achieved 0.92 coefficient of determination (R^2), suggesting its accuracy to explain at least 92 % of the variations in the obtained data.

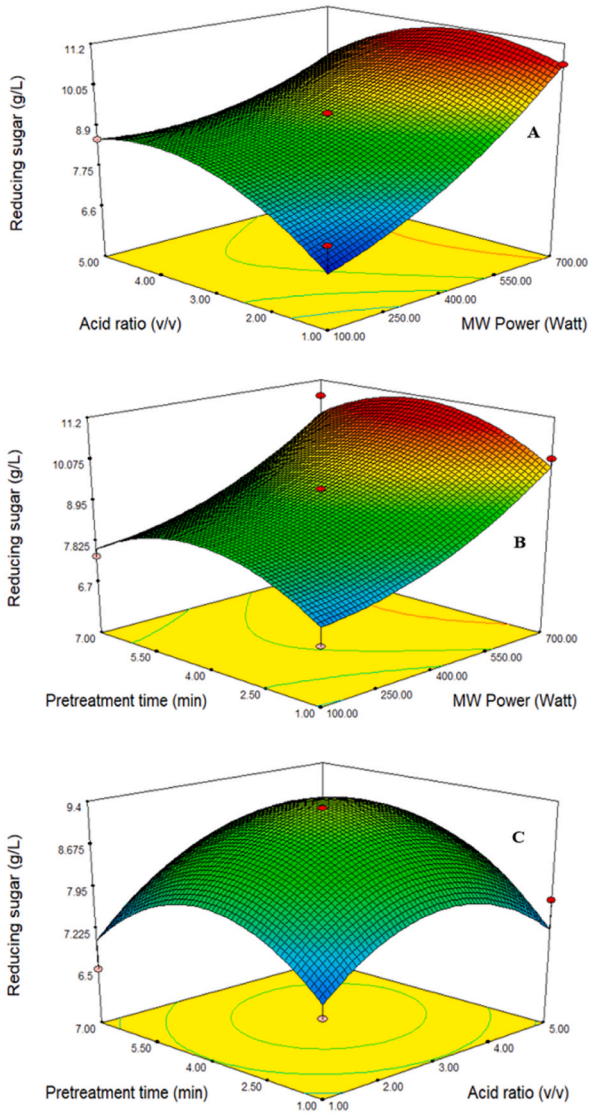

Fig. 5. 3-D response surface plot showing the interaction of acid ratio and microwave power on reducing sugar yield (A), the interaction of pretreatment time and microwave power on reducing sugar yield (B), and the interaction of pretreatment time and microwave power on reducing sugar yield (C).

$$\text{Reducing sugar (g/L)} = 9.29 + 1.30A + 0.27B + 0.17C – 0.70AB + 6.000E\text{-}0.003AC + 0.20BC + 0.52A^2 – 0.93B^2 – 1.04C^2 \quad (7)$$

The range of reducing sugar concentration varied between 6.51 g/L and 10.73 g/L, demonstrating how the input variables (microwave power, acid-liquid ratio, and pretreatment time) significantly influenced the release of reducing sugar. As presented in Table 5, the microwave pretreatment using 5 % HCl concentration resulted in reducing sugar of 7.72 g/L while reducing sugar concentration of 10.73 g/L was obtained at 3 % acid concentration. Moreover, pretreatment process using acid concentration as low as 1 % resulted in low reducing sugar concentration (6.51 g/L). Previous study by Sindhu et al. [52], also reported similar effects of pretreatment acid concentration on the release of fermentable sugars (glucose, xylose, and galactose) from cellulosic plant biomass like wheat straw. Furthermore, in this study, the acid-microwave pretreatment regime at low microwave power (100 W) produced lower reducing sugar concentration of 6.80 g/L compared to pretreatment regime at higher microwave power (700 W) which yielded 10.73 g/L. Similarly, low pretreatment time of 1 min led to a reduced reducing sugar concentration of 6.80 g/L, whereas a longer pretreatment time of 7 min resulted in a higher reducing sugar release of 10.73 g/L. These could be ascribed to the efficient fractionation of *Chlorococcum* biomass aided by the interaction of acid and microwave power at longer pretreatment time. Furthermore, the data indicates that the acid-microwave-assisted acid pretreatment efficiently degrade the hemicellulose and the lignin units of the microalgae biomass, thereby improving enzymatic digestibility [52].

Furthermore, the interactive effect of the acid-microwave pretreatment parameters was evaluated using three-dimensional response surface graphs (Fig. 5). As shown in Fig. 5A, when the acid concentration remained at 4 (v/v) and the microwave power was raised from 100 to 700 W, the concentration of reducing sugar rose from 8.50 to 10.99 g/L. Similarly, in Fig. 5B, maintaining the pretreatment time at 5.50 min with an increase in microwave power from 100 to 700 W led to an increase in reducing sugar from 7.55 to 10.85 g/L. While the combined effects of pretreatment time and acid concentration on reducing sugar release are illustrated Fig. 5C. By using lower acid concentrations (<5 v/v), higher reducing sugar concentrations (>7.00 g/L) can be achieved while still allowing for a longer pretreatment time (7.00 min). Also, increasing the pretreatment time from 5.5 to 7 min led to lower concentrations of reducing sugar. On the other hand, when the acid concentration was kept at 4 % v/v and the pretreatment time at 5.50 min, there was peak reducing sugar concentration of 8.80 g/L (Fig. 5C). These findings indicate the different pretreatment parameters affected the breakdown of the cell wall structure to varying degrees, resulting in the release of different concentration of fermentable sugars, as shown in Fig. 5A–C. Taking into account the factors influencing fermentable sugar released in the pretreatment process, it is imperative to optimize the pretreatment conditions.

3.7. Reducing sugar model validation

The optimal setpoints of microwave power (700 W), acid concentration (2.52 %), pretreatment time (4.06 min) and a reducing sugar concentration response of 11.14 g/L (Table 6) were predicted by the model. The experimental validation resulted in a reducing sugar yield of 15.67 g/L (Table 6). This demonstrated that high microwave intensity couple with low acid concentration within a short period favours the degradation of the microalgae biomass for optimal sugar release [53]. Moreover, the pretreatment regime effectiveness was revealed by the extent of hemicellulose (9.01 %), cellulose (0.86 %), and lignin (0.33 %) components obtained after the pretreatment regime (see supplementary document). The lignin content increased by 98.52 % after the pretreatment process. This could be due to the impact of the treatment and the pseudo-lignin formation. Additionally, the compositional analysis of the pretreated microalgae showed a rise in hemicellulose with 47.49 % and cellulose 12.24 %. Similar increases in hemicellulose, cellulose, and lignin content have been reported following the chemical hydrolysis of sugarcane bagasse and sorghum straw [54]. The rise in cellulose content may be due to the increased cellulose accessibility following the hemicellulose solubilization. Similarly, Ruangmee and Sangwichien [55] observed an increase in cellulose during the alkali pretreatment of cattail leaves.

Furthermore, the electron micrograph showed the microalgal biomass subjected to optimal microwave-assisted pretreatment experienced degradation of both surface and architectural structure, leading to exposure of inner materials (Fig. 6A). The microwave-assisted pretreatment caused substantial structural damage and induced alterations in cellulose crystallinity, ultimately enhancing the solubilization of the internal components of the microalgal biomass. In contrast, the untreated microalgal biomass retained a relatively intact architectural structure (Fig. 6B).

Also, the microalgae biomass obtained after pretreatment was analyzed using the FTIR (see supplementary document). Significant changes in the peaks within the 800–3800 cm^{-1} band were detected in the microalgae sample that underwent microwave-assisted pretreatment. The bands at 900 cm^{-1} represent the polysaccharide adsorption, while those at 1200 cm^{-1} indicate the C-O-C adsorption. These findings suggest that the microwave-assisted pretreatment led to the cleavage of the cell wall structure. Conversely, there was a noticeable decrease in the protein peaks, indicated by the band between 1040 cm^{-1} and 1760 cm^{-1}. This suggests that protein components (displayed by the N-H stretching for amine I and II protein) in the microalgae biomass underwent degradation during the pretreatment process. The reduction in the bands indicates a decline in protein content in the pretreated microalgae

Table 6
Optimum levels of variables during microwave-assisted pretreatment.

Independent variables		Predicated optimum levels
Microwave power		700 W
Acid ratio		2.52 % (v/v)
Pretreatment time		4.06 min
Response	**Predicted value**	**Observed value**
Reducing sugar	11.14 g/L	15.67 g/L

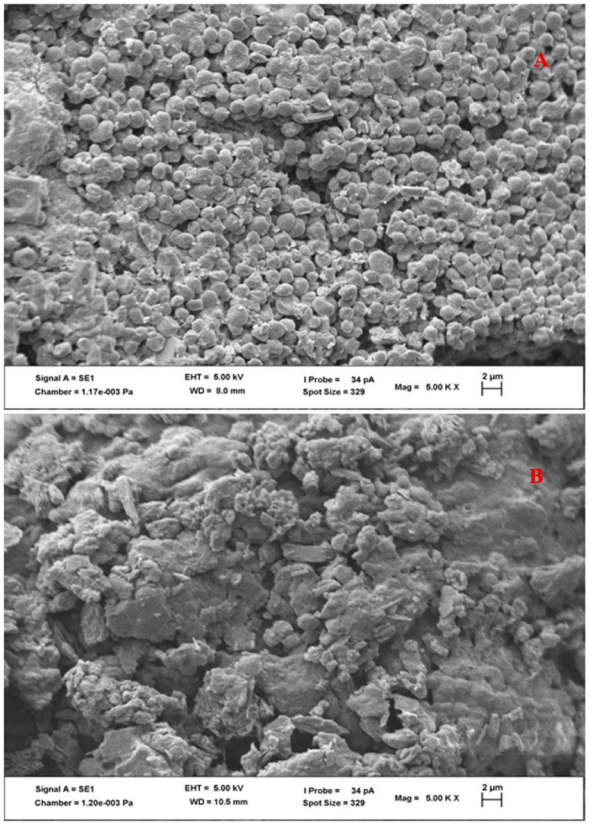

Fig. 6. Scanning electron micrograph of the optimally pretreated microalgae (A), the untreated microalgae (B).

biomass. The decrease in protein content can be attributed to the elimination of protein units from the microalgal biomass during microwave-assisted pretreatment, caused by degradation and deterioration during the pretreatment process [20]. Protein biomolecules have been known to be heat sensitive; consequently, they are denatured at high or extreme temperature. The FTIR spectra analysis further confirmed the impact of the pretreatment regime on *Chlorococcum* biomass inherent structure for the release of the unit components and functional groups, leading to improved enzymatic hydrolysis efficiency. Also, this showed the pretreatment strategy implemented can be considered as a promising approach for the release of fermentable sugar from *Chlorococcum* biomass as a promising feedstock for biofuel production.

Furthermore, two enzymatic probing efforts were employed for the saccharification of pretreated microalgae hydrolysate. In the initial investigation, three enzymes namely cellulase, amylase, and amyloglucosidase were used for the hydrolytic saccharification, resulting in reducing sugar concentration of 15.67 g/L. The subsequent investigation aimed to determine the effects of excluding the cellulase enzyme step. This resulted in reducing sugar concentration of 15.60 g/L. There was no significant variance from the results of the first experimental investigation. Therefore, due to economic considerations, the exclusion of the cellulase enzyme step is recommended for industrial scale up. High saccharification efficiency (78 %) was obtained with the pretreated microalgae hydrolysate showing the high processibility of the *Chlorococcum* hydrolysate [56].

3.8. Preliminary assessment of pretreated biomass for bioethanol production

The harvested and optimally pretreated biomass was subsequently utilized for bioethanol production in a simultaneous saccharification and fermentation (SSF) process. The SSF fermentability of the pretreated and enzymatically saccharified *Chlorococcum* hydrolysate using *Saccharomyces cerevisiae* resulted in maximum bioethanol production of 7.85 g/L after 12 h (see supplementary document) and bioethanol productivity of 0.98 g/L/h. The productivity in this study is comparable to other studies [57,58]. Lower bioethanol productivities of 0.12 g/L/h and 0.35 g/L/h were reported by Srimachai et al. [57] as well as Rork and Gueguim-Kana [58] using oil palm frond juice and waste sorghum leaves as feedstock separately. This was 8.2-fold and 2.8-fold lower respectively in comparison to the productivity obtained in this study. These data strongly suggest the high processibility of the pretreated *Chlorococcum* sp, hydrolysate for bioethanol production. The result from this study could facilitates the implementation of waste-based bioenergy generation that might lower the cost of bioenergy generation in keeping with the waste management, energy and sustainable environment nexus [59–61].

4. Conclusion

In this study a complementary wastewater mixture of DWW and PWW was formulated and optimized for *Chlorococcum* sp. cultivation. The best mixture ratio for biomass accumulation was 64.69 % DWW and 35.31 % PWW while the ratio of 34.21 % DWW and 65.79 % PWW was achieved for high lipid accumulation. Afterwards, the feasibility of cultivating *Chlorococcum* sp. in the optimized formulated growth medium for improved biomass and lipid accumulation was demonstrated. Also, the cultivation of *Chlorococcum* sp. was effective in excess nutrient removal from the wastewater mixture, a potential wastewater remediation approach. Moreover, a highly efficient microwave-assisted acid pretreatment technique for the pretreatment of cultivated *Chlorococcum* sp, biomass was developed. This resulted in the release of maximum fermentable sugar of 15.67 g/L and the obtained sugar was successfully exploited for bioethanol production (7.85 g/L). These findings have demonstrated the potentials of wastewater-based microalgae cultivation as a viable alternative feedstock for a low-cost biofuel production and sustainable environmental management.

CRediT authorship contribution statement

Emmanuel C. Ngerem: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Isaac A. Sanusi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Data curation, Conceptualization. **Gueguim E.B. Kana:** Writing – review & editing, Supervision, Software, Resources, Methodology, Conceptualization. **Ademola O. Olaniran:** Writing – review & editing, Supervision, Resources, Conceptualization.

Availability of data

Data will be provided on reasonable request.

Ethical statements

The article has not been submitted for publication consideration elsewhere.

This work has not been published previously anywhere.

The submitted article was approved by all the contributing authors and the university for publication.

The article after acceptance will not be published somewhere else in the present form or electronically and in any language without proper consent of the copyright-holder.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42531.

References

[1] S. Dutta, F. Neto, M.C. Coelho, Microalgae biofuels: a comparative study on techno-economic analysis & life-cycle assessment, Algal Res. 20 (2016) 44–52.
[2] J.T.T. Chye, L.Y. Jun, L.S. Yon, S. Pan, M.K. Danquah, Biofuel production from algal biomass, Bioenergy Biofuels 621 (2018).
[3] B.D.C. Bishop, A.I. Sanusi, E.B.G. Kana, Enhanced substrate suitability of autoclave-assisted acid pre-treated waste sugarcane molasses: pre-treatment optimization, sequential nano-based detoxification strategies, and bioproduct production, Biomass Convers. Biorefin. (2024), https://doi.org/10.1007/s13399-024-06127-7.
[4] S.B. Eregie, I.A. Sanusi, G.E. Kana, A.O. Olaniran, Effect of ultra-violet light radiation on Scenedesmus vacuolatus growth kinetics, metabolic performance, and preliminary biodegradation study, Biodegradation 35 (1) (2023) 71–86.
[5] Z. Yaakob, E. Ali, A. Zainal, M. Mohamad, M.S. Takriff, An overview: biomolecules from microalgae for animal feed and aquaculture, J. Biol. Res.-Thessaloniki 21 (2014) 6.
[6] E.C. Odjadjare, T. Mutanda, A.O. Olaniran, Potential biotechnological application of microalgae: a critical review, Crit. Rev. Biotechnol. 37 (2017) 37–52.
[7] W.Y. Cheah, T.C. Ling, P.L. Show, J.C. Juan, J.S. Chang, D.J. Lee, Cultivation in wastewaters for energy: a microalgae platform, Appl. Energy 179 (2017) 609–625.
[8] A. Conradie, G.O. Sigge, T.E. Cloete, Influence of winemaking practices on the characteristics of winery wastewater and water usage of wineries, S. Afr. J. Enol. Vitic. 35 (2014) 10–19.

[9] M.P. Zacharof, Grape winery waste as feedstock for bioconversions: applying the biorefinery concept, Waste Biomass Valoriz. 8 (4) (2017) 1011–1025.
[10] O. Ashrafi, L. Yerushalmi, F. Haghighat, Wastewater treatment in the pulp-and-paper industry: a review of treatment processes and the associated greenhouse gas emission, J. Environ. Manag. 158 (2015) 146–157.
[11] L. Esterhuizen, A. Fossey, E. Potgieter, Groundwater quality on dairy farms in central South Africa, WaterSA 41 (2015) 194–198.
[12] E. Daneshvar, M.J. Zarrinmehr, E. Koutra, M. Kornaros, O. Farhadian, A. Bhatnagar, Sequential cultivation of microalgae in raw and recycled dairy wastewater: microalgal growth, wastewater treatment and biochemical composition, Bioresour. Technol. 273 (2019) 556–564.
[13] S.B. Eregie, I.A. Sanusi, G.E. Kana, O.O. Ademola, Synergistic effect of process parameters and nanoparticles on spent lubricant oil waste biodegradation by UV-exposed *Scenedesmus vacuolatus*: process modelling, kinetics and degradation pathways, Bioresour. Technol. Rep. 24 (2024) 101627.
[14] N. Arora, A. Patel, K. Sartaj, P.A. Pruthi, V. Pruthi, Bioremediation of domestic and industrial wastewaters integrated with enhanced biodiesel production using novel oleaginous microalgae, Environ. Sci. Pollut. Control Ser. 23 (20) (2016) 20997–21007.
[15] S.B. Ummalyma, R.K. Sukumaran, Cultivation of microalgae in dairy effluent for oil production and removal of organic pollution load, Bioresour. Technol. 165 (2014) 295–301.
[16] P. Gurumoorthy, A. Saravanan, Biofuel production from marine microalgae using paper and pulp industry wastewater, Int. J. Chem. Sci. 14 (2016) 3249–3255.
[17] L. Moreno-Garcia, Y. Gariépy, N. Bourdeau, S. Barnabé, G.S.V. Raghavan, Optimization of the proportions of four wastewaters in a blend for the cultivation of microalgae using a mixture design, Bioresour. Technol. 283 (2019) 168–173.
[18] N.S. Nouadjep, E. Nso, E.G. Kana, C. Kapseu, Simplex lattice mixture design application for biodiesel production: formulation and characterization of hybrid oil as feedstock, Fuel 252 (2019) 135–142.
[19] J. Martín-Juárez, G. Markou, K. Muylaert, A. Lorenzo-Hernando, S. Bolado, Breakthroughs in bioalcohol production from microalgae: solving the hurdles, in: Microalgae-Based Biofuels and Bioproducts, Woodhead Publishing, 2017, pp. 183–207.
[20] R.V. Kapoore, T.O. Butler, J. Pandhal, S. Vaidyanathan, Microwave-assisted extraction for microalgae: from biofuels to biorefinery, Biology 7 (1) (2018) 18.
[21] A. Karemore, R. Sen, Downstream processing of microalgal feedstock for lipid and carbohydrate in a biorefinery concept: a holistic approach for biofuel applications, RSC Adv. 6 (35) (2016) 29486–29496.
[22] D.A. Esquivel-Hernández, I.P. Ibarra-Garza, J. Rodríguez-Rodríguez, S.P. Cuéllar-Bermúdez, M.D.J. Rostro-Alanis, G.S. Alemán-Nava, J.S. García-Pérez, R. Parra-Saldívar, Green extraction technologies for high-value metabolites from algae: a review, Biofuels, Bioprod. Biorefin. 11 (1) (2017) 215–231.
[23] P. Biller, C. Friedman, A.B. Ross, Hydrothermal microwave processing of microalgae as a pre-treatment and extraction technique for biofuels and bio-products, Bioresour. Technol. 136 (2013) 188–195.
[24] J. Iqbal, C. Theegala, Microwave assisted lipid extraction from microalgae using biodiesel as co-solvent, Algal Res. 2 (1) (2013) 34–42.
[25] H.C. Theofany, M. Yheni, H.B. Aditiya, N.S. Sepwin, Microwave irradiation pre-treatment in third generation bioethanol production from tropical green algae, J. Phys. Conf. 1402 (4) (2019) 044046. IOP Publishing.
[26] J.C. Naidoo, P. Moodley, I.A. Sanusi, Y. Sewsynker-Sukai, E.L. Meyer, E.G. Kana, Microwave-assisted sequential green liquor-inorganic salt pretreatment for enhanced sugar recovery from sorghum leaves towards bioethanol and biohydrogen production, Renew. Energy 225 (2024) 120225.
[27] D. Hernández, B. Riaño, M. Coca, M.C. García-González, Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production, Chem. Eng. J. 262 (2015) 939–945.
[28] G. Singh, S.K. Patidar, Microalgae harvesting techniques: a review, J. Environ. Manag. 217 (2018) 499–508.
[29] R. Feng, A.A. Zaidi, K. Zhang, Y. Shi, Optimisation of microwave pretreatment for biogas enhancement through anaerobic digestion of microalgal biomass, Periodica Polytech., Chem. Eng. 63 (1) (2019) 65–72.
[30] American Public Health Association [APHA], American Water Works Association [AWWA], and Water Environment Federation [WEF], Standard Methods for the Examination of Water and Wastewater, nineteenth ed., American Public Health Association, New York, NY, 1998.
[31] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (8) (1959) 911–917.
[32] G. Miller, Modified 3,5–Dinitrosalicylic acid (DNS) method for reducing sugars, Anal. Chem. 31 (3) (1959) 426–428, https://doi.org/10.1021/ac60147a030.
[33] V. Sharma, U.K. Garg, D. Arora, Impact of pulp and paper mill effluent on physico-chemical properties of soil, Arch. Appl. Sci. Res. 6 (2) (2014) 12–17.
[34] S. Harrison, M. Johnstone-Robertson, R. Pott, B. Verster, S. Rumjeet, L. Nkadimeng, Towards wastewater biorefineries: integrated bioreactor and process design for combined water treatment and resource productivity, Water Res. Commission Rep. 2580 (1) (2016) 2016.
[35] F.G. Gentili, Microalgal biomass and lipid production in mixed municipal, dairy, pulp and paper wastewater together with added flue gases, Bioresour. Technol. 169 (2014) 27–32.
[36] J. Ding, F. Zhao, Y. Cao, L. Xing, W. Liu, S. Mei, S. Li, Cultivation of microalgae in dairy farm wastewater without sterilization, Int. J. Phytoremediation 17 (3) (2015) 222–227.
[37] H. Wang, W. Zhang, L. Chen, J. Wang, T. Liu, The contamination and control of biological pollutants in mass cultivation of microalgae, Bioresour. Technol. 128 (2013) 745–750.
[38] S.Y. Chiu, C.Y. Kao, T.Y. Chen, Y.B. Chang, C.M. Kuo, C.S. Lin, Cultivation of microalgal *Chlorella* for biomass and lipid production using wastewater as nutrient resource, Bioresour. Technol. 184 (2015) 179–189.
[39] T. Cai, S.Y. Park, Y. Li, Nutrient recovery from wastewater streams by microalgae: status and prospects, Renew. Sustain. Energy Rev. 19 (2013) 360–369.
[40] S.A. Razzak, M.M. Hossain, R.A. Lucky, A.S. Bassi, H. de Lasa, Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing—a review, Renew. Sustain. Energy Rev. 27 (2013) 622–653.
[41] W. Wang, F. Han, Y. Li, Y. Wu, J. Wang, R. Pan, G. Shen, Medium screening and optimization for photoautotrophic culture of *Chlorella pyrenoidosa* with high lipid productivity indoors and outdoors, Bioresour. Technol. 170 (2014) 395–403.
[42] A.K. Sharma, P.K. Sahoo, S. Singhal, A. Patel, Impact of various media and organic carbon sources on biofuel production potential from *Chlorella* spp, 3 Biotech 6 (2) (2016) 116.
[43] M. Vitova, K. Bisova, S. Kawano, V. Zachleder, Accumulation of energy reserves in algae: from cell cycles to biotechnological applications, Biotechnol. Adv. 33 (6) (2015) 1204–1218.
[44] G. Kim, G. Mujtaba, K. Lee, Effects of nitrogen sources on cell growth and biochemical composition of marine chlorophyte *Tetraselmis* sp. for lipid production, ALGAE 31 (3) (2016) 257–266.
[45] S. Ruangsomboon, Effects of different media and nitrogen sources and levels on growth and lipid of green microalga *Botryococcus braunii* KMITL and its biodiesel properties based on fatty acid composition, Bioresour. Technol. 191 (2015) 377–384.
[46] L. Yao, J. Shi, X. Miao, Mixed wastewater coupled with CO_2 for microalgae culturing and nutrient removal, PLoS One 10 (9) (2015) e0139117.
[47] L. Wang, M. Min, Y. Li, P. Chen, Y. Chen, Y. Liu, Y. Wang, R. Ruan, Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant, Appl. Biochem. Biotechnol. 162 (4) (2010) 1174–1186.
[48] L. Luo, H. Ren, X. Pei, G. Xie, D. Xing, Y. Dai, N. Ren, B. Liu, Simultaneous nutrition removal and high-efficiency biomass and lipid accumulation by microalgae using anaerobically digested effluent from cattle manure combined with municipal wastewater, Biotechnol. Biofuels 12 (1) (2019) 1–15.
[49] H.Y. Ren, J.N. Zhu, F. Kong, D. Xing, L. Zhao, J. Ma, N.Q. Ren, B.F. Liu, Ultrasonic enhanced simultaneous algal lipid production and nutrients removal from non-sterile domestic wastewater, Energy Convers. Manag. 180 (2019) 680–688.
[50] P.J. McGinn, K.E. Dickinson, K.C. Park, C.G. Whitney, S.P. MacQuarrie, F.J. Black, J.C. Frigon, S.R. Guiot, S.J. O'Leary, Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus* sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode, Algal Res. 1 (2) (2012) 155–165.
[51] F. Hamidi, A.N. Baghani, M. Kasraee, M. Salari, M.H. Mehdinejad, Modeling, optimization and efficient use of MMT K10 nanoclay for Pb (II) removal using RSM, ANN and GA, Sci. Rep. 13 (2023) 8434, https://doi.org/10.1038/s41598-023-35709-0, 2023.
[52] R. Sindhu, M. Kuttiraja, P. Binod, R.K. Sukumaran, A. Pandey, Bioethanol production from dilute acid pre-treated Indian bamboo variety (*Dendrocalamus* sp.) by separate hydrolysis and fermentation, Ind. Crop. Prod. 52 (2014) 169–176.
[53] H. Shokrkar, S. Ebrahimi, M. Zamani, Bioethanol production from acidic and enzymatic hydrolysates of mixed microalgae culture, Fuel 200 (2017) 380–386.

[54] D.C. Rorke, T.N. Suinyuy, E.G. Kana, Microwave-assisted chemical pre-treatment of waste sorghum leaves: process optimization and development of an intelligent model for determination of volatile compound fractions, Bioresour. Technol. 224 (2016) 590–600.
[55] A. Ruangmee, C. Sangwichien, Response surface optimization of enzymatic hydrolysis of narrow-leaf cattail for bioethanol production, Energy Convers. Manag. 73 (2013) 381–388.
[56] G. Gupta, M. Baranwal, S. Saxena, M.S. Reddy, Utilization of banana waste as a resource material for biofuels and other value-added products, Biomass Convers. Biorefin. (2022) 1–20.
[57] T. Srimachai, K. Nuithitikul, S. O-thong, P. Kongjan, K. Panpong, Optimization and kinetic modeling of ethanol production from oil palm frond juice in batch fermentation, Energy Proc. 79 (2015) 111–118, https://doi.org/10.1016/j.egypro.2015.11.490.
[58] D.C.S. Rorke, E.B. Gueguim-Kana, Kinetics of bioethanol production from waste sorghum leaves using *Saccharomyces cerevisiae* BY4743, Fermentation 3 (2017) 19, https://doi.org/10.3390/fermentation3020019.
[59] A.N. Baghani, S. Sadjadi, K. Yaghmaeian, A.H. Mahvi, M. Yunesian, R. Nabizadeh, Solid alcohol biofuel based on waste cooking oil: preparation, properties, micromorphology, heating value optimization and its application as candle wax, Renew. Energy 192 (2022) 617–630, https://doi.org/10.1016/j.renene.2022.04.100.
[60] R. Nabizadeh, I.L. García, S. Sadjadi, K. Yaghmaeian, A.H. Mahvi, M. Yunesian, A.N. Baghani, Biodiesel production from supernatant waste cooking oil by a simple one-step technique: calorific value optimization using response surface methodology (RSM) based on D-optimal design, J. Mater. Cycles Waste Manag. 25 (2023) 3567–3583, https://doi.org/10.1007/s10163-023-01779-5, 2023.
[61] F. Eslami, K. Yaghmaeian, R. Shokoohi, R. Sajjadipoya, A. Rahmani, H. Askarpur, A.N. Baghani, H.J. Mansoorian, F.J. Ansari, Nano-pumice derived from pumice mine waste as a low-cost electrode catalyst for microbial fuel cell treating edible vegetable oil refinery wastewater for bioenergy generation and reuse, Heliyon 10 (2024) e40495, https://doi.org/10.1016/j.heliyon.2024.e40495, 2024.