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ORIGINAL ARTICLE

Pretreatment of lipid-extracted biomass of *Scenedesmus* sp. grown in wastewater for bioethanol production

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Abstract

Carbohydrates are more likely to be found in lipid-extracted microalgal biomass, which can be used to produce bioethanol with different pretreatment methods. The objective of this study was to prepare lipid-extracted biomass of *Scenedesmus* sp. grown in anaerobically digested brewery effluent for bioethanol production. Pretreatments such as autoclave, microwave, oven, and water bath heating with alkalis (NaOH and KOH), acids (HCL and H_2SO_4), and H_2O as hydrolytic agents, as well as optimization of an effective pretreatment method for carbohydrate and reducing sugar extractions, were used. Bioethanol was produced from lipid-extracted microalgal hydrolysate under optimum conditions. The findings showed that the highest contents of carbohydrates (222.59 ± 3.16 mg/g) and reducing sugar (150.52 ± 5.57 mg/g) were obtained using microwave pretreatment with HCl, whereas the lowest contents of carbohydrates (34.48 ± 1.36 mg/g) and reducing sugar (30.85 ± 3.22 mg/g) were obtained in water bath heating with H_2O. After optimizing the main parameters of microwave pretreatment, the highest carbohydrate and reducing sugar contents were increased by 24.72% and 27.92%, respectively, at the optimum conditions. The maximum bioethanol yield of 0.1 g/g lipid-extracted microalgal biomass with a fermentation efficiency of 94.84% was obtained at a fermentation time of 24 h. This study demonstrated that lipid-extracted biomass of microalgae obtained from wastewater has a high potential for bioethanol production and, consequently, the development of microalgae-based biorefineries.

Keywords Anaerobically digested brewery effluent · Bioethanol · Lipid-extracted microalgal biomass · Pretreatment · Reducing sugar

1 Introduction

The issues of energy security and greenhouse gas emission due to the utilization of fossil fuels have led to the development of alternative energy sources to satisfy the demands of energy in the world. Biofuels are considered an alternative to reduce dependence on fossil fuels, and they are derived from different types of biomass [1]. Among biomass, microalgae are recently perceived as a potentially renewable source for the production of biofuel due to their features such as higher photosynthetic efficiency, faster growth rate, and higher biomass production compared to other conventional biofuel sources [2]. Moreover, they are able to utilize available nutrients in wastewater and produce biomass, which can be converted into biofuels [3].

The growth of microalgae in wastewater has been suggested as a cost-effective method for biomass production with wastewater management and biofuel production [4]. Several previous studies have been undertaken to produce biomass for lipid and carbohydrate production from microalgae in wastewater. For instance, Mercado et al. [5] reported a maximum carbohydrates of 27% and lipids of 50% from *Scenedesmus* sp. grown in anaerobically digested (AD) dairy wastewater. Ansari et al. [6] found 35.1% carbohydrates and 30.8% lipids from *Scenedesmus obliquus* grown in raw aquaculture wastewater. Karpagam et al. [7] achieved a lipid content of 231.8 mg/L from *Scenedesmus* sp. grown in vegetable waste extract–treated growth media. These studies showed that microalgae store a substantial amount of lipids and carbohydrates when they are grown in wastewater.

Moreover, the use of lipid-extracted microalgal biomass (LEMB) for biofuel production or other applications can

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reduce the cost of biofuel production [8]. In previous studies, lipid-extracted biomass was called de-oiled or defatted biomass or residual biomass [9–11]. It was reported that LEMB has a higher carbohydrate yield than whole microalgal biomass (WMB) [11]. Therefore, the utilization of LEMB for carbohydrate and bioethanol production might promote the biorefinery concept. The production of bioethanol from microalgae can be done through either a step-by-step method or an integrated method. The step-by-step method involves first lipid extraction from microalgae and then bioethanol production from LEMB, whereas the integrated method involves simultaneous carbohydrate and lipid extraction from microalgal biomass [12].

Bioethanol production from microalgae involves steps such as microalgae cultivation, pretreatment, hydrolysis, fermentation, and distillation. Among these steps, pretreatment is an important one because it is used to enhance the release of fermentable sugars from microalgal biomass or affect the efficiency of bioconversion [13]. Pretreatment methods have commonly been employed for bioethanol production from various feedstocks, including microalgae. For instance, Manmai et al. [14] employed chemical and biological pretreatments on sunflower stalks and obtained higher reducing sugar, total sugar, and bioethanol with chemical pretreatment. Yirgu et al. [15] used microwave, autoclaved, oven, and water bath heating pretreatments of the whole microalgal biomass and obtained the highest reducing sugar using the microwave pretreatment. Like WMB, LEMB also requires pretreatment to modify the structure of internal carbohydrates and enhance the extraction of fermentable sugar. The pretreatment methods such as sonication, ultrasonic, chemical, and enzymatic and their combinations have been employed to extract reducing sugar from LEMB for the production of bioethanol [9, 11, 16].

The lipid-extracted biomass of *Scenedesmus* sp. after growing in synthetic medium and/or wastewater has been investigated for carbohydrate extraction with different pretreatments for bioethanol production. For example, Pancha et al. [9] achieved a maximum saccharification yield of 44% with 0.40 g bioethanol/g glucose from de-oiled *Scenedesmus* sp. biomass obtained from synthetic medium. Thangam et al. [17] obtained a maximum reducing sugar yield of 11.2% with a bioethanol yield of 10.48 g/L from the LEB of *Scenedesmus* sp. grown in domestic wastewater. *Scenedesmus* sp. can be used for lipid production from WMB, and carbohydrate and bioethanol production from lipid-extracted biomass after growing in a synthetic medium or wastewater. Most of the previous studies have used a synthetic medium for microalgae growth for bioethanol production from LEMB [9–11]. In addition, bioethanol production from the whole biomass of *Scenedesmus* has received significant attention. However, the utilization of LEB of *Scenedesmus* sp. after growing in wastewater for bioethanol production has rarely been reported.

Therefore, this study aimed to pretreat lipid-extracted biomass of *Scenedesmus* sp. obtained after growing in UASB (up-flow anaerobic sludge blanket) brewery effluent for carbohydrate and bioethanol production. The LEMB was pretreated via microwave, autoclave, oven, and water bath heating with the combination of acids and alkalis hydrolytic agents to select the effective pretreatment and hydrolytic agent. Moreover, optimization of the main variables using a one-variable-at-a-time approach was performed for carbohydrate and reducing sugar extractions from LEMB. Bioethanol was produced from reducing sugar obtained under optimum conditions.

2 Materials and methods

2.1 Microalgae cultivation in wastewater

The local microalga *Scenedesmus* sp. was cultivated in AD brewery effluent after being isolated from a water sample of Lake Ziway, Ethiopia. BBM (Basal Bold Medium) [18] was used for the isolation and inoculum preparation processes. The isolation of *Scenedesmus* sp. was carried out succeeding the techniques in Andersen and Kawachi [19] and identified on the basis of morphology features explained in Bellinger and Sigee [20] and Shubert and Gärtner [21]. AD brewery effluent (hereafter, brewery effluent) was kindly provided by St. Gorge Brewery Industry, found in Addis Ababa, Ethiopia. It was collected after the UASB reactor and first filtered through Whatman filter paper (No. 1) before the cultivation of microalgae. The COD (chemical oxygen demand), TN (total nitrogen), and TP (total phosphorus) concentrations of the brewery effluent samples were 399.58 ± 24.14, 53.42 ± 6.19, and 50.00 ± 2.64 mg/L, respectively. The cultivation was performed both in brewery effluent and BBM (as control) in a batch mode with 10% of the inoculum [22] and 1600 mL working volume in 2-L conical flasks as a photobioreactor. The cultivation conditions and biomass collection method were reported in our previous study [15].

2.2 Lipid extraction

The extraction of total lipids from the microalgal biomass was carried out according to the modified methods of Bligh and Dye [23]. The procedures used in this study were reported in Yirgu et al. [15]. The biomass leftover after lipid extraction was carefully collected and dried at 60 °C in an oven, and then stored until the analysis of carbohydrates, reducing sugars, and proteins, as well as bioethanol production.

2.3 Pretreatment of microalgal biomass

A combination of both chemical and thermal pretreatments was performed for the extraction of carbohydrates from LEMB and WMB. The LEMB and WMB were pretreated in an autoclave (Model, DIXONS and ST3028), oven heating (Model, GX65B), microwave (Milestone SK-10 and SK-12, Italy), and water bath heating (DK-98-II) with acids (HCl and H_2SO_4), alkalis (NaOH and KOH), and H_2O as hydrolytic agents, as reported in Yirgu et al. [15]. After pretreatment, the supernatant was then separated using centrifugation after cooling and neutralizing, and then taken for carbohydrate and reducing sugar content determination. After selecting an effective pretreatment method with a hydrolytic agent, optimization of main operational parameters was employed using a single parameter at a time approach.

2.4 Bioethanol production

Bioethanol was produced from the hydrolysates of LEMB obtained at optimum conditions. The fermentation process was performed with *Saccharomyces cerevisiae* (commercial baker's yeast), which was first activated and prepared using Luria Broth (LB) medium according to Harun et al. [24]. Fermentation was performed with a 50 mL working volume in 125-mL conical flasks containing the hydrolysate of LEMB with fermentation nutrients (0.1 g ammonium chloride (NH_4Cl), 0.2 g potassium dihydrogen phosphate (KH_2PO_4), and 0.5 g yeast extracted) [25]. The mixture was first adjusted to a pH of 5 [26] and then sterilized at 121 °C for 20 min in an autoclave. Ten percent of pre-cultured *Saccharomyces cerevisiae* yeast was added under aseptic conditions in the flasks to inoculate the sterilized hydrolysate solution [27]. Then, the flasks were placed on a shaker incubator (ZHWY-103B, China) at 150 rpm and 30 °C for 3 days. Bioethanol was determined after distillation within an interval of 24 h.

2.5 Analytical methods and calculations

2.5.1 Biomass production

The local microalgae growth was daily measured by optical density (OD) at 680 nm [28] using a UV/Vis spectrophotometer (Jenway, model 6705). The dry cell weight (DCW) for biomass yield estimation was determined according to the APHA method [29] for the total suspended solid. The linear relationship between OD_{680} and dry cell weight was obtained as follows: $DCW = 0.95*OD_{680} - 0.037$ ($R = 0.990$, $P < 0.05$). The biomass productivity of local *Scenedesmus* sp. was calculated using Eq. (1) [22].

$$P_B = (X_t - X_0)/(t_t - t_0) \quad (1)$$

where P_B is the biomass productivity, and X_t and X_0 are biomass concentrations at time t_t and at an initial time t_0, respectively.

2.5.2 Lipid and protein contents and productivities

The total lipid content after extraction and separation was determined using the gravimetric method. The total lipid content (LC) and lipid productivity (P_L) were determined according to Eqs. (2) and (3), respectively, [30].

$$\text{LC}\ (\%) = \frac{WLE}{WMB} \times 100 \quad (2)$$

where LC is the lipid content, WLE is the weight of lipid extracted, and WWMB is the weight of whole microalgal biomass

$$P_L\ (mg/L/d) = LC \times P_B\ (mg/L/d) \quad (3)$$

where P_L is the biomass productivity, LC is the lipid content, and P_b is the biomass productivity.

The total protein contents in lipid-extracted microalgal biomass were calculated based on the amount of TN, which was determined according to Kjeldahl's method as stated in the AOAC [31], and the procedures used in this method were reported by Yirgu et al. [15]. Protein productivity P_p over the cultivation time was determined according to Eq. (4) [6].

$$P_P\ (mg/L/d) = P_B\ (mg/L/d) \times PC \quad (4)$$

where P_P is the protein productivity, P_B is the biomass productivity, and PC is the protein content.

2.5.3 Carbohydrate determination

The carbohydrate contents in WMB and LEMB were analyzed using a phenol–sulfuric acid method [32]. In brief, 1.0 mL of 5% phenol solution and 5 mL concentrated H_2SO_4 were added to a test tube containing 2 mL of supernatant. A test tube was vortexed for 1 min and then maintained in a 30 °C water bath. The color of the mixture was then turned to orange, which is the result of the reaction between phenol and carbohydrates. A calibration curve was constructed to determine the amount of carbohydrates using glucose as a standard, ranging from 10 to 80 µg/mL. The carbohydrate content was determined on the basis of absorbance read at 490 nm using a UV/Vis spectrophotometer (Jenway).

2.5.4 Reducing sugar determination

The procedures of the DNS method with minor modifications were used to determine reducing sugar content [33]. Briefly,

an equal amount of hydrolysate of microalgal biomass and DNS reagent (1 mL each) was mixed in capped test tubes and heated in boiling water (95 °C). Eight milliliters of distilled water was added to the test tubes after cooling using running tap water to room temperature. The calibration curve was constructed using D-glucose as a standard, ranging from 0.1 to 0.3 mg/mL with $R^2 = 0.9951$ in the test range. The amounts of reducing sugar were determined according to the absorbance read at 540 nm using a UV/Vis spectrophotometer (Jenway).

2.5.5 Bioethanol determination

The bioethanol concentration was estimated according to the procedures provided by Crowell and Ough [34] using the potassium dichromate method. Briefly, the distilled samples of bioethanol (2 mL) and the acidic potassium dichromate reagent (10 mL) were mixed in a test tube and then heated in a water bath for 2 h at 60 °C. The bioethanol concentration was determined by measuring absorbance at 600 nm using a UV/Vis spectrophotometer. A calibration curve was prepared using absolute ethanol solution as standard [35], ranging from 1 to 3 mg/mL which provided R^2 of 0.997. The bioethanol yield kinetics were determined according to Manmai et al. [14] using the following Eqs. (5), (6), (7), and (8).

$$\text{Bioethanol yield}\,(\%) = \frac{\textit{Bioethanol obtained from fermentation}\,(g)}{\textit{Microalgal biomass}\,(g)} \times 100 \tag{5}$$

$$\text{Bioethanol yield}(\%) = \frac{\textit{Bioethanol obtained from fermentation}\,(\frac{g}{L})}{\textit{Reducing sugar in LEMB}\,(\frac{g}{L})} \tag{6}$$

$$\textit{Bioethanol productivity}\,(g/L/h) = \frac{\textit{Bioethanol obtained from fermentation}\,(\frac{g}{L})}{\textit{Fermentation time}\,(h)} \tag{7}$$

$$\textit{Fermentation efficiency}\,(\%) = \frac{\textit{bioethanol obtained from fermentation}\,(\frac{g}{L})}{0.51 \times \textit{reducing sugar in hydrolysate}\,(\frac{g}{L})} \times 100 \tag{8}$$

2.6 Data analysis

All experiments in this study were performed in triplicate, except for the fermentation process (duplicate), and findings were presented as the mean ± standard deviation. Paired sample *t*-tests were used for the comparison of biomass production, total lipids, and protein production using Microsoft Excel 2013. One-way ANOVA with the Tukey post hoc test using R-software was performed to compare carbohydrate and reducing sugar yields with different pretreatments. The results are significant at a significant level of 95% ($P < 0.05$).

3 Results and discussion

3.1 Biomass production

The local microalga *Scenedesmus* sp. used in the present study was selected on the basis of its proven capability to grow in different wastewaters and to accumulate relatively high amounts of lipids and carbohydrates in its biomass. *Scenedesmus* sp. was cultivated in BBM (as a control) and brewery effluent until maximum biomass was obtained, which was on the18th day. Figure 1 depicts the biomass production of *Scenedesmus* sp. in BBM and brewery effluent over the cultivation period. The maximum biomass production and productivity obtained in BBM were 1.26 ± 1.05 and 93.30 mg/L/d, and in AD brewery effluent, they were 1.05 ± 0.10 g/L and 64.33 mg/L/d, respectively. It is clearly observed that the biomass production and productivity obtained in BBM were higher than those attained in AD brewery effluent. This attribution might be due to the balanced nutrient and mineral composition in BBM rather than AD brewery effluent for microalgae growth. The biomass production obtained in AD brewery effluent was comparable with the maximum result achieved by Ferreira et al. [36] and Marchão et al. [37] using *Scenedesmus obliquus* in brewery effluent. Diniz et al. [38] and Ansari et al. [22] obtained a maximum biomass production of 0.445 and 0.258 g/L using *Scenedesmus* sp. in institutional and municipal wastewater, respectively, which are lower than this study. Likewise, both of these studies reported lower biomass productivity using *Scenedesmus* sp. than in this study. However, Ferreira et al. [36] reported higher biomass productivity than this study. As a result, this study demonstrated that the brewery effluent has great potential for the production of microalgal biomass for biofuel feedstock.

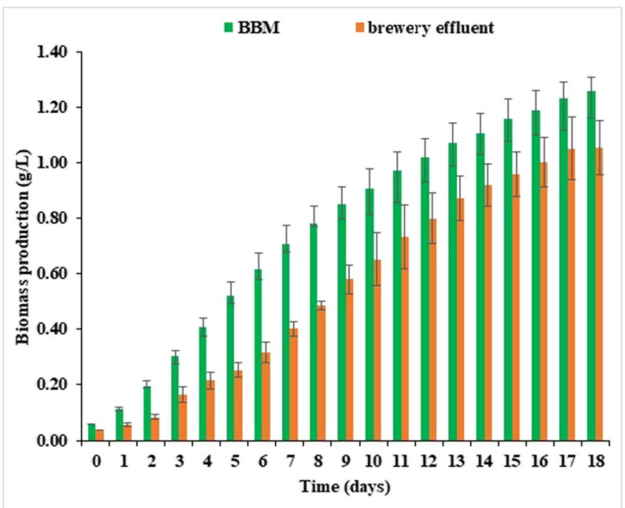

Fig. 1 Biomass production by local *Scenedesmus* sp. in BBM and brewery effluent

3.2 Lipid and protein extraction from wastewater-grown *Scenedesmus* sp.

The microalgal biomass of *Scenedesmus* sp. obtained from AD brewery effluent had a lipid content of 13.67 ± 0.31% and a lipid productivity of 8.79 ± 0.20 mg/L/d. The lipid contents of local *Scenedesmus* sp. were categorized under the moderate lipid content range of microalgae, which is about 10 to18% [39]. The lipid and protein contents and productivities of *Scenedesmus* sp. in different wastewaters are presented in Table 1. *Scenedesmus* sp. achieved a similar lipid content when it grew in institutional wastewater [22], municipal wastewater [38], and brewery effluent (this study). Nayak et al. [40], Gupta et al. [41], and Thangam et al. [17] reported different values of lipid contents from *Scenedesmus* sp. grown in domestic wastewater. Dairy wastewater [5] offered a better lipid accumulation in *Scenedesmus* sp. compared to the other wastewaters except the domestic wastewater used by Thangam et al. [17]. The lipid productivity found in this study was similar to that reported by Ansari et al. [22] using institutional wastewater but greater than that reported by Diniz et al. [38] using municipal wastewater. However, it was lower than those achieved in domestic wastewater [17, 40] and dairy wastewater [5]. The differences in lipid content and productivity of *Scenedesmus* sp. grown in different wastewaters may be due to the availability of nutrients in the wastewater and cultivation conditions (temperature, pH, light intensity, photoperiod, etc.).

The total protein content found in LEMB was 53.98 ± 0.08%, with a productivity of 34.72 ± 0.05 mg/L/day. This showed that the total protein obtained in LEMB was 8.41% higher than that obtained in WMB, as reported in our previous study [15]. Likewise, protein productivity achieved on the basis of proteins from LEMB was 9.18% higher than that obtained on the basis of WMB. As a result, the use of lipid-extracted microalgal biomass is a sustainable approach to extracting more protein than the whole microalgal biomass. The accumulation of proteins on the biomass of *Scenedesmus* sp. varied with wastewater streams (Table 1). The protein content obtained in this study was higher than those achieved by Diniz et al. [38], Ansari et al. [6], and Gupta et al. [41] in domestic, aquaculture, and municipal wastewater, respectively. However, protein productivity calculated from the data reported by Mercado et al. [5] was higher than in this study. Ansari et al. [8] found that the lipid-extracted biomass of *Scenedesmus* sp. grown in BG11 has a higher protein content than WMB, which is similar to that attained in this study. As a result, brewery effluent seemed to be suitable for the accumulation of more total proteins in the cells of *Scenedesmus* sp.

3.3 Carbohydrate extraction

3.3.1 Effect of pretreatment on carbohydrate extraction

Carbohydrates were extracted from WMB and LEMB using microwave, autoclave, oven, and water bath heating pretreatments with acid and alkali hydrolytic agents to identify the effective pretreatment (Fig. 2a–d). Results showed that microwave pretreatment provided higher carbohydrates from WMB and LEMB with all hydrolytic agents compared to autoclave, oven, and water bath heating pretreatments. The highest carbohydrate yields in LEMB and WMB were obtained using HCl, followed by H_2SO_4, NaOH, KOH, and H_2O. Moreover, the LEMB provides higher carbohydrate contents than those obtained from the WMB in all pretreatment methods. The highest carbohydrate content obtained from LEMB was 222.59 ± 0.89 mg/g using microwave with HCl, whereas the lowest was 81.90 ± 2.21 mg/g using water bath heating with H_2O. The highest and lowest carbohydrate contents obtained from LEMB are 7.16% and 33.12% higher, respectively, than those achieved from WMB. Furthermore, the carbohydrate yield obtained in a microwave using HCl differed significantly ($P < 0.05$) from that obtained using the

Table 1 Lipid and protein contents and productivities of *Scenedesmus* sp. grown in different wastewater streams

Growth medium	Biomass productivity (mg/L/day)	Lipid content (%)	Lipid productivity (mg/L/day)	Protein content (%)	Protein productivity (mg/L/day)	Reference
Domestic wastewater	196	33.30	65.17	-	-	[40]
Institution wastewater	58.70	13.00	7.63*	-	-	[22]
Municipal wastewater	54.20	12.50	6.77*	31.10	16.86*	[38]
Aquaculture wastewater	89.61	30.85	27.65	19.52	17.50	[6]
Domestic wastewater	-	18.30	-	30.40	-	[41]
Dairy wastewater	1750	51.00	892.5*	20.00	350.0*	[5]
Domestic wastewater	-	50.50	19.00	-	-	[17]
Brewery effluent (WMB)	-	-	-	49.44	31.80*	[15]
Brewery effluent (LEMB)	64.33	13.67	8.72	53.98	34.72	The present study

*Calculated value

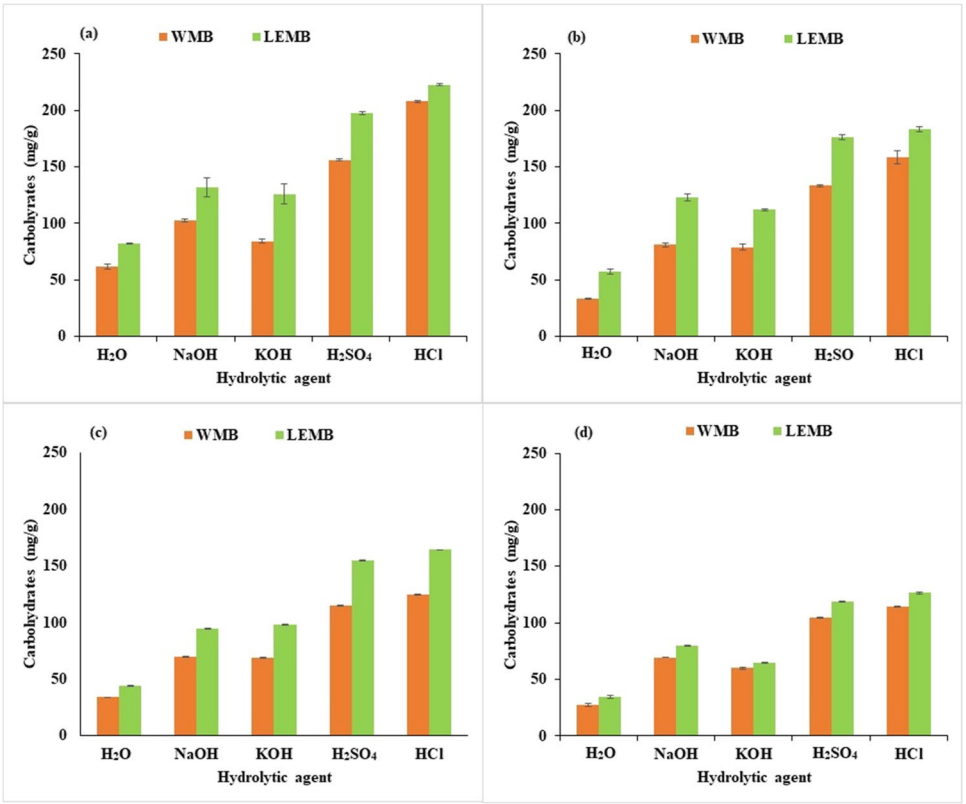

Fig. 2 Carbohydrate production from the WMB and LEMB in **a** microwave, **b** autoclave, **c** oven, and **d** water bath

other hydrolytic agents (H_2SO_4, NaOH, KOH, and H_2O).The results obtained in this study also showed that alkaline pretreatment was less effective for carbohydrate extraction compared to acid pretreatment. The higher carbohydrate yield obtained in LEMB could be explained due to the organic solvent used for lipid extraction weakening the structural integrity of the cell wall by dissolving the cellulose [42]; therefore, this enhances the digestibility of the LEMB. Lee et al. [43] and Lee et al. [11] conducted a study to extract carbohydrates from LEMB and WMB for bioethanol production and reported that LEMB has a higher carbohydrate yield than WMB of *Dunaliella tertiolecta* and *Chlorella* sp., respectively. Moreover, these two studies found that HCl is more effective for releasing carbohydrates and reducing sugar than H_2SO_4 and the results are higher than those obtained in this study. However, Ansari et al. [44] and Vardon et al. [45] reported a lower carbohydrate yield in LEMB than WMB of *Scenedesmus obliquus* and *Scenedesmus* sp., respectively. Furthermore, they achieved a lower carbohydrate content from LEMB when compared to those obtained in this study.

3.3.2 Effect of pretreatment on reducing sugar extraction

Like carbohydrates, the LEMB released a higher amount of reducing sugar in all pretreatment methods than the WMB (Fig. 3a–d). Pretreatment with HCl released a higher reducing sugar in microwave pretreatment while H_2SO_4 released a higher reducing sugar in autoclave, oven, and water bath heating pretreatments. The highest reducing sugar content obtained in LEMB was 159.19 ± 1.05 mg/g in microwave pretreatment using HCl, while the lowest reducing sugar content achieved was 30 ± 3.22 mg/g in water bath heating with H_2O. The highest reducing sugar content obtained in LEMB is 7.77% higher than that obtained from WMB. Microwave pretreatment was more effective with HCl, whereas autoclave, oven, and water bath heating were more effective with H_2SO_4 for reducing sugar extraction. Alkaline pretreatment in this study released a higher reducing sugar from LEMB than WMB; however, the results were lower than acid pretreatment. Ansari et al. [8] reported a reducing yield of 12.37–19.51% from the LEB of *Scenedesmus obliquus* using autoclave pretreatment with H_2SO_4. Additionally, Thangam et al. [17] found a maximum reducing sugar yield of 112 mg/g from the LEB of *Scenedesmus* sp. grown in domestic wastewater using heating at 120 °C with H_2SO_4. However, Pancha et al. [9] obtained the highest reducing sugar yield (29.35%) from LEB of *Scenedesmus* sp. with HCl compared to H_2SO4, HNO_3, H_3PO_4, NaOH, and KOH. Furthermore, they obtained a lower reducing sugar yield with alkaline (NaOH or KOH) pretreatment, which concurred with this study. A lower sugar yield with alkaline pretreatment was also reported by Hernández et al. [46] compared to acid pretreatment. The lower sugar yield using

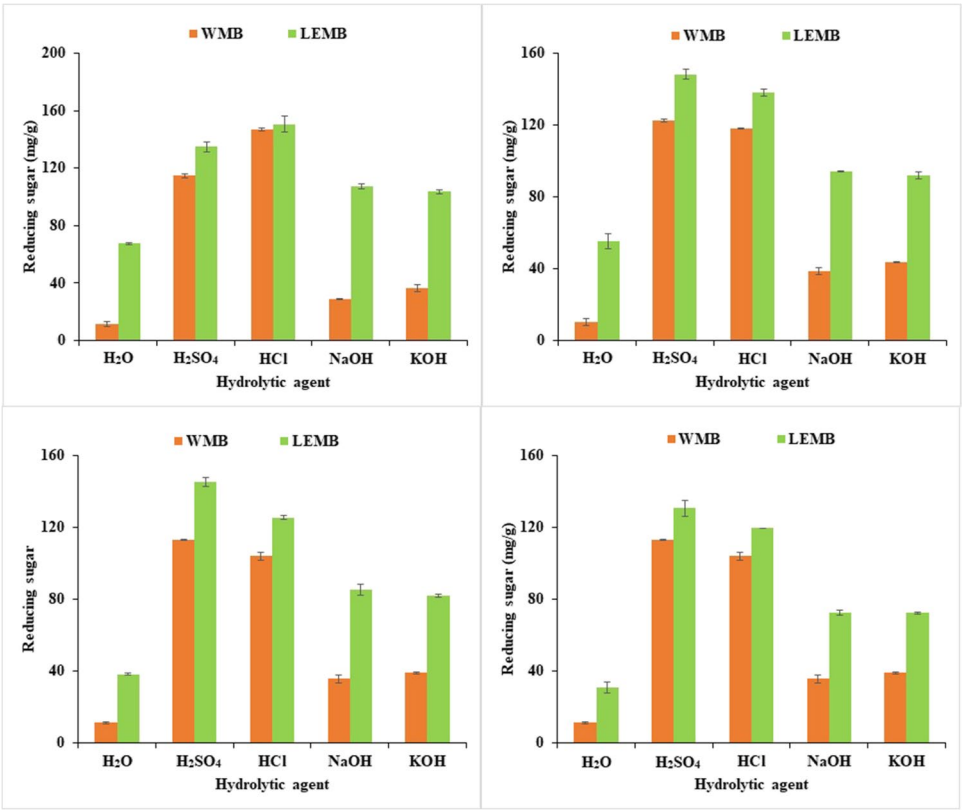

Fig. 3 Reducing sugar content obtained from the WMB and LEMB using the pretreatment of **a** microwave, **b** autoclave, **c** oven heating, and **d** water bath heating

alkaline agents for microalgal biomass hydrolysis is predominantly because of the degradation of sugar at high pH and alkaline agents which mostly hydrolyze fiber polymer compared to complex sugar [9, 46].

3.3.3 Effects of operational parameters on carbohydrate extraction

The optimizations of carbohydrate and reducing sugar extractions were carried out in microwave for four main operational parameters: acid concentrations, temperature, microwave power, and extraction time. The weight of LEMB (5% (w/v)) was constant throughout the optimization of the above variables with a single parameter at a time approach. The results obtained from the optimization process are illustrated in Fig. 4a–d. Figure 4a shows the effect of acid concentrations ranging from 0.1 to 6 N on carbohydrate and reducing sugar extractions from LEMB at 1000 W and 120 °C for 15 min. The carbohydrate and reducing sugar yields increased with acid concentration and reached maximum at the concentrations of 1 N and 2 N, respectively. After these acid concentrations, both carbohydrate and reducing sugar contents decreased and reached the lowest at 6 N. Therefore, the acid concentrations of 2 N for carbohydrates and 1 N for reducing sugar were considered optimal acid concentrations. The contents of 247.86 ± 1.30 and 165.075 ± 2.19 mg/g were found at the optimum acid concentration of 2 and 1 N for carbohydrates and reducing sugar, respectively. The carbohydrate contents achieved at 1 N and 2 N differed significantly ($P < 0.05$) from those attained at concentrations of 0.1, 0.5, 3, 4, 5, and 6 N. However, the reducing sugar contents found at 1 N and 2 N did not differ significantly. The decrease in sugar content with increasing acid concentration may be attributed to monosaccharide degradation into sugar degradation products such as furfural [47].

Figure 4b shows the effect of microwave power ranging from 600 to 1400 W on carbohydrate and reducing sugar extractions with a fixing temperature of 120 °C, an acid concentration of 1 N for reducing sugar and 2 N for carbohydrates, and an extraction time of 15 min. The maximum carbohydrate content obtained was 247.96 ± 1.54 mg/g at a microwave power of 1200 °C, which differed significantly ($P < 0.05$) from those achieved at 600, 800, 1000, and 1400 W. The maximum reducing sugar content was also determined to be 167.92 ± 2.79 at 1200 W, which did not differ significantly ($P > 0.05$) from the value obtained at 1400 W but did differ significantly ($P < 0.05$) from the reducing sugar contents obtained at 600, 800, and 1000 W. Therefore, the optimal value of microwave power for carbohydrate and reducing sugar extraction was 1200 W.

Figure 4c displays the effect of temperatures ranging from 80 to 180 °C on carbohydrate and reducing sugar extraction. The other parameters were fixed at 1200 W, 2 N for carbohydrates/1 N for reducing sugar, and a 15 min extraction time. The maximum carbohydrate content of 276.96 ± 2.13 mg/g and the reducing sugar content of 181.27 ± 2.49 mg/g were obtained at 140 °C. These carbohydrate and reducing sugar

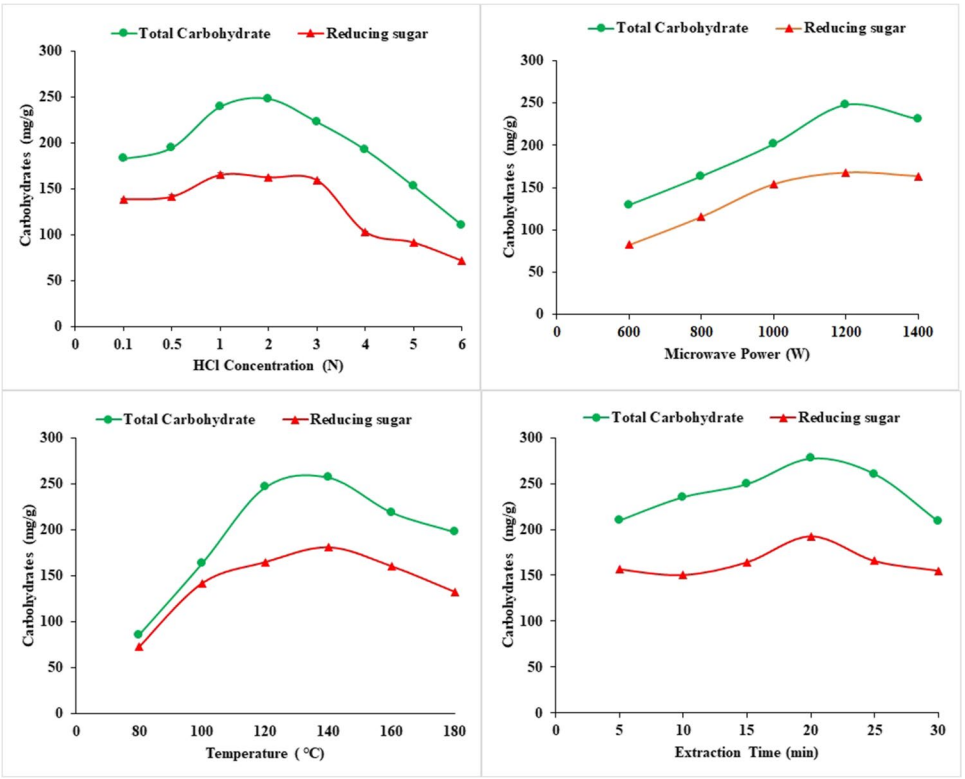

Fig. 4 Optimum carbohydrate and reducing sugar yields obtained using **a** acid concentrations, **b** microwave power, **c** temperature, and **d** extraction time

values were significantly different from those found at temperatures of 80 °C, 100 °C, 120 °C, 160 °C, and 180 °C. Hence, the optimum temperature for carbohydrate and reducing sugar extraction was 140 °C.

Figure 4d illustrates the effect of extraction time ranging from 5 to 30 min on carbohydrate and reducing sugar extraction from LEMB. The variables acid concentration, microwave power, and temperature were held constant at 2 N (for carbohydrates)/1 N (for reducing sugar), 1200 W, and 140 °C, respectively. As shown in Fig. 4d, the carbohydrate and reducing sugar yields from LEMB increased with extraction time, reaching a maximum at 20 min but decreasing after this time. At 20 min, the carbohydrates of 277.24 ± 0.98 mg/g and the reducing sugar of 192.54 ± 1.37 mg/g were obtained from LEMB. The total carbohydrate content as well as reducing sugar content obtained at 20 min significantly differed from those obtained at 5, 10, 15, 25, and 30 min. Hence, the extraction time of 20 min was taken as the optimal value for carbohydrate and reducing sugar extraction.

Finally, the optimum conditions obtained for carbohydrate and reducing sugar extractions from LEMB were found to be the combination of 2 N, 1200 W, 140 °C, and 20 min and 1 N, 1200 W, 140 °C, and 20 min, respectively. At optimum conditions, the carbohydrate content of 277.24 ± 0.98 mg/g (27.72%) and reducing sugar of 192.54 ± 1.37 mg/g (19.25%) were obtained from the LEB of *Scenedesmus* sp. According to Demirbas [48], Scenedesmus sp. contains between 21 to 52% of carbohydrates, which includes the results of this study. Therefore, this study found that microwave-assisted acid hydrolysis has the potential to improve carbohydrates and reducing sugar released from lipid-extracted microalgal biomass.

3.4 Bioethanol production from LEMB

Microalgal carbohydrates are not readily fermentable due to the fact that they are mostly found as starch in chloroplasts and as cellulose in the cell walls. The process of solvent extraction with the microwave pretreatment method used in this study enhanced the breakdown of the complex structure of polysaccharides in microalgae. In the present study, bioethanol was produced from the hydrolysate of LEMB obtained at optimum conditions using the yeast *Saccharomyces cerevisiae*. The bioethanol yield kinetic parameters and reducing sugar reduction during the fermentation period are provided in Table 2. As shown in Table 2, the concentration of bioethanol was increased and reached a maximum at a 24 h fermentation time, whereas the concentration of reducing sugar was decreased. The decrease in reducing sugar over the 24 h fermentation period demonstrated that the *Saccharomyces cerevisiae* yeast utilized the LEMB hydrolysate as a substrate. The highest bioethanol concentration, bioethanol yield, bioethanol productivity, and fermentation efficiency were found to be 6.04 g/L, 0.1 g/g LEMB, 0.084 g/L/d, and 94.84% at the fermentation time of 24 h, respectively. The bioethanol yield and fermentation efficiency obtained from LEMB were increased by 25% and 7.5%,

Table 2 Kinetic parameters of bioethanol production and reducing sugar reduction during the fermentation period

Fermentation time (h)	Reducing sugar (g/L)	Bioethanol concentration (g/L)	Bioethanol yield (g/g LEMB)	Bioethanol yield (g/g reducing sugar)	Bioethanol productivity (g/L/h)	Fermentation efficiency (%)
0	13.05 ± 1.13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	0.000 ± 0.00	0.000 ± 0.00
24	4.24 ± 0.08	6.04 ± 0.04	0.10 ± 0.01	0.48 ± 0.00	0.084 ± 0.01	94.84 ± 0.00
48	3.71 ± 0.35	4.86 ± 1.19	0.08 ± 0.00	0.39 ± 0.07	0.067 ± 0.02	75.95 ± 0.14
72	3.45 ± 0.03	3.97 ± 0.04	0.08 ± 0.00	0.32 ± 0.02	0.055 ± 0.00	62.55 ± 0.05

respectively, compared to the results obtained from the whole microalgal biomass which were reported in our previous study [15]. The high fermentation efficiency obtained in this study probably indicated that the hydrolysis of LEMB does not form fermentation inhibitors, which are common in lignocellulosic biomass [11]. This could be one of the advantages of using LEMB for bioethanol production compared to lignocellulosic biomass. Moreover, the production of bioethanol from the biomass left after lipid extraction may enhance the biorefinery concept through coupling with biodiesel production.

Bioethanol production from LEB of different microalgae in other studies is provided in Table 3. Dhandayuthapani et al. [16] reported a maximum yield of bioethanol (0.087 g/g LEMB) from *Chlorella sorokiniana* grown in sterilized municipal wastewater using ultrasonic pretreatment. Lee et al. [43] studied chemo-enzymatic saccharification for bioethanol production from LEB of *Dunaliella tertiolecta* grown in a synthetic medium, and they found a maximum reducing sugar of 42% and bioethanol of 0.14 g/g from LEMB. Lee et al. [11] attained a maximum yield of bioethanol (0.16 g/g LEMB) from *Chlorella* sp. grown in a synthetic medium. Chng et al. [49] obtained a maximum of 0.26 g/g bioethanol yield from the LEB of *Scenedesmus dimorphus* grown in a synthetic medium without any pretreatment. The bioethanol yields obtained from LEMB in most previous studies were higher than that found in this study, except that reported by Dhandayuthapani et al. [16]. On the other hand, the bioethanol yield obtained in this study was higher than that achieved from whole microalgal biomass. Yu et al. [25] and Sivaramakrishnan and Incharoensakdi [50] found a maximum bioethanol yield of 0.076 g/g biomass from *Scenedesmus* sp. and *Chlorella* sp., respectively. Reyimu and Ozçimen [51] reported a maximum bioethanol yield of 0.04 g/g bioethanol yield from the whole biomass of *Nannochloropsis oculata* grown in municipal wastewater. However, most of the previous studies reported utilizing a synthetic medium for microalgae growth and subsequently producing bioethanol. This may add an extra cost to the production of bioethanol and other biochemical compounds from microalgae. Therefore, the application of wastewater as a growth medium for microalgae like in the present study is more attractive in order to reduce bioethanol production costs.

3.5 Mass balance in bioethanol production from microalgal biomass

The overall mass balance of lipid and bioethanol production was analyzed from 1 kg of whole microalgal biomass. Figure 5 demonstrates the overall mass balance diagram of bioethanol production from lipid-extracted biomass. From 1 kg of WMB,

Table 3 Comparison of bioethanol production from LEB of different microalgae with their growth medium

Species	Growth medium	Pretreatment method	Bioethanol yield (g/g LEMB)	Fermentation efficiency (%)	Reference
D. tertiolecta	Synthetic	Enzymatic saccharification	0.14	82.00	[43]
Chlorella sp.	Synthetic	Enzymatic saccharification	0.16	79.30	[11]
Scenedesmus bij	Synthetic	Oven heating and H_2SO_4 at 130 °C	0.16	-	[52]
Scenedesmus sp.	Synthetic (BG-11)	Enzymatic saccharification	0.40	78.00	[9]
Scenedesmus dim	Synthetic (BBM)	Without any pretreatment	0.26	95.60	[49]
Chlamydomonas sp.	Synthetic (BBM)	Sonication and HCl at 121 °C	0.18	-	[53]
C. sorokiniana	Municipal WW	Ultrasonic pretreatment	0.087	-	[16]
Scenedesmus sp.	Domestic WW	Heating and H_2SO_4 at 120 °C	10.6*	-	[17]
N. oculata	Synthetic (F_2)	Chemo-enzymatic saccharification	0.26$^+$	65.50	[10]
Scenedesmus sp.	Brewery effluent	Microwave and HCl at 120 °C	0.10	94.84	This study

*The unit is in mg/L; $^+$the unit is in g/g sugar consumed

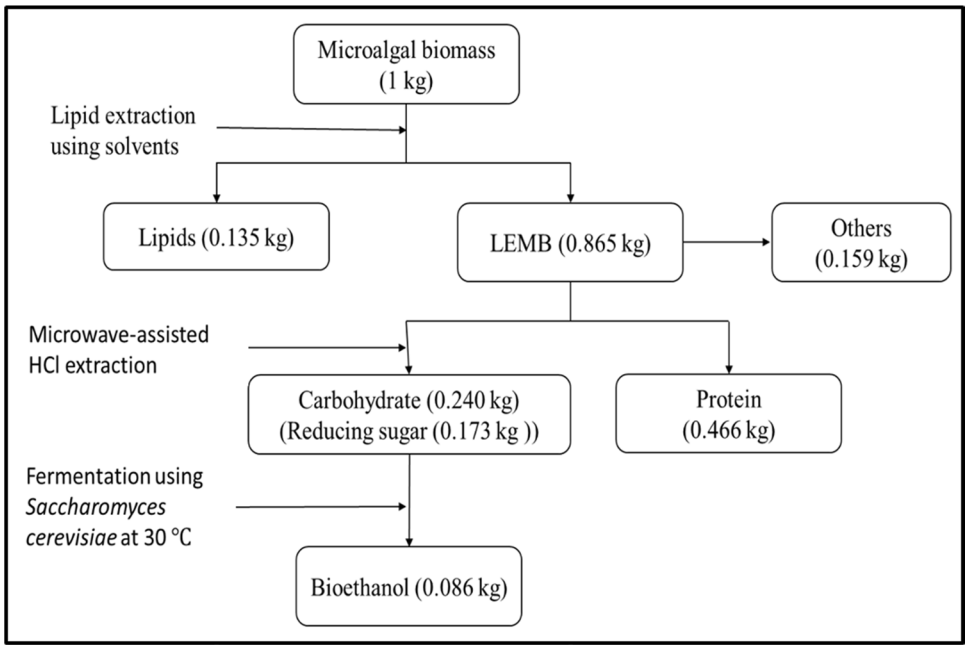

Fig. 5 The overall mass balance of bioethanol production from microalgal biomass

0.135 kg of lipids and 0.865 g of LEMB can be obtained. The LEMB contains 0.466 kg of proteins, 0.240 kg of carbohydrates (0.173 kg of reducing sugar and 0.067 kg of non-reducing sugar), and 0.159 kg of others. The hydrolysate containing reducing sugar (0.173 kg) was fermented using *Saccharomyces cerevisiae* at 30 °C for 72 h. The fermentation process can convert the reducing sugar with 94.84% fermentation efficiency and produce 0.086 kg of bioethanol from 0.865 kg of LEMB, indicating a 0.1 g bioethanol yield/g LEMB. Moreover, the lipid content may be enhanced in the combination of solvent extraction with the pretreatment methods and used for biodiesel production. Therefore, local *Scenedesmus* sp. has great potential for a sustainable approach to developing microalgae-based biorefineries.

4 Conclusion

The results of the present study demonstrated the feasibility of the production of bioethanol from LEB of local microalga grown on AD brewery effluent. Results showed that microwave pretreatment with HCl was the most effective pretreatment method for carbohydrate and reducing sugar extraction. The maximum yields of reducing sugar and carbohydrates obtained from LEMB were 159.19 and 222.5 mg/g, which were increased by 20.94% and 19.74% after optimization, respectively. The maximum bioethanol yield achieved was 0.1 g/g LEMB with a fermentation efficiency of 94.84%. The results found in this study suggested that LEMB is a promising biomass for bioethanol production with the appropriate pretreatment method and that it has great potential for protein production. Moreover, the utilization of wastewater as a growth medium is a cost-effective and eco-friendly approach for carbohydrate and bioethanol production. Furthermore, the lipid production from local microalgae can be enhanced through pretreatment methods and then, it can be used for biodiesel production.

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Author contribution ZY, MMK, and TA isolated and identified *Scenedesmus* sp., designed the study, conducted the experiments, collected and analyzed the data, and wrote the manuscript. SL and AH designed the experiments, supervised the research, analyzed and interpreted the data, and edited the manuscript. All authors read and approved the manuscript.

Data availability The data sets used in this study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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