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ORIGINAL ARTICLE

Pretreatment and optimization of reducing sugar extraction from indigenous microalgae grown on brewery wastewater for bioethanol production

Zenebe Yirgu[1,2] · Seyoum Leta[2] · Ahmed Hussen[2] · Mohammed Mazharuddin Khan[2]

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Abstract
The aim of this study was to select a suitable pretreatment method and optimize total reducing sugar extraction from indigenous *Scenedesmus* sp. grown on brewery wastewater for bioethanol production. Microalgal biomass was pretreated using a microwave, autoclave, water bath, and oven with HCl, H_2SO_4, NaOH, and KOH, followed by the optimization of the best pretreatment method and hydrolytic agent using response surface methodology for reducing sugar extraction. Four independent variables (acid concentration, microwave power, temperature, and extraction time) were then considered in the optimization and model development process; results showed that the maximum reducing sugar content was achieved in a microwave with HCl. Analysis of variance (ANOVA) and regression coefficient (0.983) also showed that the developed model was significant ($P < 0.05$) and fitted to the experimental data, respectively. The optimum conditions of an acid concentration of 1.68 N, microwave power of 1200 W, the temperature of 145 °C, and extraction time of 19 min were predicted a maximum total reducing sugar production of 175.5 mg/g. The experimental result of total reducing sugar obtained at optimum conditions was 172.5 mg/g, which was well close to the predicted value, verifying the appropriateness of the model. The highest bioethanol yield of 0.08 g ethanol/g microalgal biomass was obtained at 24-h fermentation time with a fermentation efficiency of 88.15%. This study demonstrates the possibility of bioethanol production from indigenous microalgae grown on brewery wastewater through microwave-assisted acid extraction of reducing sugar.

Keywords Bioethanol · Reducing sugar · Microwave pretreatment · Response surface methodology · *Scenedesmus* sp

1 Introduction

The current energy sources are mainly fossil fuels such as coal, natural gas, and petroleum, which are intensively used to satisfy the energy demand of the world. However, these fuels are associated with issues such as the depletion of their sources and causing global warming due to the emission of greenhouse gas into the atmosphere [1]. Moreover, the utilization of traditional biomass fuels such as wood fuel, agricultural residues, and dung has an effect on human health and the environment in developing countries [2]. Besides these, the increase in population and industrialization also leads to increasing energy demand in the world [1]. Therefore, several efforts have been carried out to develop alternative energy sources that are renewable, sustainable, and environmentally friendly to meet the world's energy demands [3]. Among others, biomass is a commonly used alternative renewable energy source. It obtains from plant-based materials, which use for biofuel production such as bioethanol [4].

Bioethanol is a biofuel produced by the fermentation process from various biomass resources such as sugarcane and corn (first-generation), lignocellulosic biomass (second-generation), and algal biomass (third-generation) [5, 6]. The first-generation bioethanol feedstocks are used as food and feed for humans and animals, and thus, these feedstocks have the issues of food versus fuel conflict. However, the second and third-bioethanol feedstocks have received attention in different studies because they are considered non-food feedstocks, have no competition with food supplies, and are cheaper than the first-generation bioethanol feedstocks

✉ Zenebe Yirgu
zeneyw944@gmail.com; zenebe.yirgu@aau.edu.et

[1] Department of Environmental Science, Wolaita Sodo University, Sodo, Ethiopia

[2] Center for Environmental Science, Addis Ababa University, Addis Ababa, Ethiopia

[7]. Moreover, lignocellulosic materials are obtained from several sources, such as agricultural wastes, forest residues, aquatic plants, food/beverage wastes, and other industrial wastes [6]. Therefore, bioethanol production from the second- and third-generation feedstocks is perceived as more sustainable.

Lignocellulosic and algal biomasses need pretreatment steps to break down the structural features of the biomass and facilitate sugar release before the fermentation process. The pretreatment methods like physical, chemical, biological, and combination of those methods have been developed and employed for bioethanol production from lignocellulosic and algal biomasses [6]. For example, Nguyen et al. [8] utilized physical pretreatments such as boiling and autoclaving with algal enzymes for the hydrolysis of low-grade longan fruit wastes for bioethanol production and found a bioethanol yield of 0.066 g/g using separate hydrolysis and fermentation processes. Casabar et al. [9] employed alkaline pretreatment for pineapple fruit peel and obtained 5.98 g/L of bioethanol under separate hydrolysis and fermentation. However, the presence of a high content of lignin and hemicellulose in most lignocellulosic materials requires an efficient pretreatment method to produce lignin-free sugar. Hence, several studies have been conducted to produce bioethanol from seed weed (marine macroalgae) biomass [10, 11] and microalgae [12, 13].

Microalgae are lignin-free biomass with low hemicellulose content, which makes them much easier to convert into fermentable sugar compared to lignocellulosic materials [3]. Furthermore, the biochemical composition of microalgae is satisfactory for biofuel production coupled with the biorefinery approach, which refers to the extraction or production of all bioproducts from biomass with suitable processing methods [14]. Algal biomass consists of proteins, carbohydrates, lipids, and nucleic acids as biochemical components [15]. Microalgae are mainly composed of lipids (2%–40%), carbohydrates (4%–64%), and proteins (6%–61%). These biochemical compositions depend on the growth conditions such as nutrient stress and environmental conditions such as temperature, light, salinity, and pH [16]. Bioethanol production from microalgae has been conducted under different pretreatments such as acid with autoclave [12], alkali with oven [13], and acid and enzymatic [17]. For instance, Guo et al. [17] obtained a bioethanol yield of 0.103 g/g algal biomass from *Scenedesmus obliquus* under separate hydrolysis and fermentation processes. Therefore, microalgae have been perceived as one of the major feedstocks for bioethanol production.

The microalgae species such as *Scenedesmus* and *Chlorella* can accumulate high amounts of carbohydrates [18]. These microalgae species have also been proven to grow in various wastewater streams with biomass production and nutrient removal. Marchão et al. [19] cultivated *Scenedesmus obliquus* on brewery effluent under batch mode and found a maximum biomass production of 0.97 g/L with a removal efficiency of 99% of nitrogen and 43% phosphorus nutrient. Mercado et al. [20] used dairy wastewater for *Scenedesmus* sp. cultivation and achieved a maximum content of 51% lipids, 27% carbohydrates, and 20% proteins with a removal percentage of 88.4% total nitrogen and 97.1% phosphorus nutrients. Furthermore, Diniz et al. [21] found a maximum content of 19.5% carbohydrates, 12.5% lipids, and 31.1% proteins with maximum removal efficiency of 70% nitrogen and 90% phosphorus nutrients. Hence, wastewater-grown microalgae can produce bioethanol using various pretreatment methods with optimization strategies.

Optimization of pretreatment methods is a strategy for enhancing sugar extraction from biomass. Response surface methodology (RSM)-based optimization has currently been conducted for pretreating various lignocellulosic biomass. For instance, Ramaraj and Unpaprom [22] and Nguyen et al. [5] used RSM for pretreatment optimization for ethanol production from *Cyperus difformis* and low-grade longan fruit wastes, respectively. Sophanodorn et al. [23] also optimized reducing sugar extraction from tobacco stalk for bioethanol production. However, RSM has been widely used for the optimization of lipid and biodiesel production from microalgal biomass [24, 25]. Moreover, few studies have used the application of RSM on reducing sugar extraction optimization from microalgal biomass [26]. On the other hand, the lipid content with biodiesel production potential from *Scenedesmus* sp. grown on Basal Bold medium (BBM) was previously studied in Ethiopia [27].

Therefore, the aim of this study was to select a suitable pretreatment method and optimize total reducing sugar extraction from *Scenedesmus* sp. grown on brewery wastewater for bioethanol production. The optimization process was performed using response surface methodology (RSM). The biomass production and nutrient removal potential of *Scenedesmus* sp. were also examined. The microalgal biomass was first pretreated using acids (HCl and H_2SO_4) and alkalis (NaOH and KOH) in microwave, autoclave, water bath, and oven, and RSM was then employed to optimize the best pretreatment method and hydrolytic agent for reducing sugar extraction.

2 Materials and methods

2.1 Microalgae cultivation on brewery wastewater

The local microalga, *Scenedesmus* sp., was isolated following the procedure of Andersen and Kawachi [28] from water samples of Lake Ziway, Ethiopia, for brewery

wastewater treatment. The brewery wastewater was characterized for total nitrogen (TN) and total phosphorus (TP). The *Scenedesmus* sp. was cultivated on a brewery effluent using 2-L conical flasks with a maximum light intensity of 5500 lx and photoperiod of 12:12-h light–dark cycle at room temperature for 18 days. The flasks were aerated using an aquarium aerator (SB-648, China) to supply atmospheric CO_2 and maintain the mixing of the culture. At the end of cultivation, the biomass was harvested using a centrifuge and washed with distilled water. The biomass was then dried in an oven at 60 °C and prepared for reducing sugar extraction and bioethanol production.

2.2 Pretreatment of microalgal biomass

The pretreatment of microalgal biomass was performed in a microwave (Milestone SK-10 and SK-12, Italy), autoclave (Model, DIXONS and ST3028), water bath (DK-98-II), and an oven (Model, GX65B). The acids (HCl and H_2SO_4) and alkalis (NaOH and KOH) were used for the pretreatment to select the most effective hydrolytic agent. The acid and alkali concentrations used for hydrolysis were 3 N, which was chosen according to Miranda et al. [29]. A 5% (w/v) microalgal biomass was mixed separately with H_2O, HCl, H_2SO_4, KOH, and NaOH, and subjected to (i) microwave pretreatment at 1000 W and 120 °C for 15 min as modified from Boonmanumsin et al. [30], (ii) autoclave at 121 °C for 20 min as modified from Miranda et al. [29], (iii) oven heating at 120 °C for 20 min as adapted from Harun et al. [13], and (iv) water bath at 90 °C for 1 h. After pretreatment, the samples were cooled to room temperature, and neutralized and centrifuged to separate the supernatant. Then, the supernatant was analyzed for reducing sugar content to choose the effective pretreatment method and hydrolytic agent. Finally, RSM was used to optimize reducing sugar extraction after selecting effective pretreatment for bioethanol production.

2.3 Experimental design by response surface methodology

RSM was employed to optimize reducing sugar extraction after selecting microwave with HCl was effective pretreatment. The extraction experiments were performed according to the central composite design (CCD) with alpha ($\alpha = 1$), which is known as face-centered central composite design (FCCCD) with three-level for each factor [31]. Four independent variables such as HCl concentration, microwave power, temperature, and extraction time were investigated for reducing sugar extraction. The range and the coded levels of the independent variables are shown in Table 1. The number of experiments in the face-centered composite design calculated from $N = 2^n + 2n + Nc$, where N is the number of the experiments, n is the number of variables, and Nc is the number of run at the center [32]. Thus, in this study, 31 experiments were performed as design through MINITAB 18 software for the same blocks.

Table 1 Independent variables with their coded and actual values used in optimization process

Variables	Unit	Symbol	Coded value		
			Low (− 1)	Center (0)	High (1)
HCl Concentration	N	*A*	0.1	2.55	5
Microwave power	W	*B*	800	1000	1200
Temperature	°C	*C*	80	120	180
Extraction Time	min	*D*	5	17.5	30

2.4 Model validation

The validity of the model was confirmed by performing three replicate experiments under the optimum conditions of each variable. The average value of the experimental results at optimum conditions was compared with the predicted value to check the appropriateness of the model.

2.5 Bioethanol production

2.5.1 Yeast preparation

A commercial baker's yeast, *Saccharomyces cerevisiae*, was employed for the fermentation process. The activation and preparation of yeast were carried out according to Harun et al. [33].

2.5.2 Fermentation of microalgal hydrolysate

Fermentation was carried out in duplicate using 125-mL conical flasks with a working volume of 50 mL. Each flask containing microalgal hydrolysate was mixed with 0.5 g of yeast extract, 0.2 g of potassium dihydrogen phosphate (KH_2PO_4), and 0.1 g of ammonium chloride (NH_4Cl) as fermentation nutrients [1]. The solution was adjusted to a pH of 5 [34] and autoclaved at 121 °C for 20 min. The sterilized solution inoculated with pre-cultured *Saccharomyces cerevisiae* yeast under aseptic conditions [35]. The flasks were sealed with a rubber stopper and incubated on a shaker incubator (ZHWY-103B, China) at 30 °C and 150 rpm. [34] The samples were taken at an interval of 24 h and analyzed for bioethanol content after distillation.

2.6 Analytical methods and calculations

2.6.1 Biomass production and nutrient removal

Microalgal growth and biomass concentration were monitored by measuring optical density at 680 nm [36] using a JENWAY spectrophotometer (model 6705) during the experiment. The biomass concentration as dry weight was determined according to the standard method for the total suspended solids [37]. The correlation between the dry weight (DW, g/L) and the corresponding optical density (OD) was obtained as DW = 0.95 (OD)—0.037 with an R^2-value of 0.992. Total nitrogen (TN) and total phosphorus (TP) in a brewery effluent and microalgal culture were determined after filtration using a 0.45-µm syringe filter. TN was analyzed following the Hack DR 2400 spectrophotometer Manual [38]. TP was determined by the ascorbic acid method after persulfate digestion [37]. The removal efficiency of TN and TP was calculated from the following equation: $R_f = (C_o - C)/C_o$, where C_o and C are the initial and the final concentrations, respectively.

2.6.2 Biochemical composition determination

Lipid extraction was done from dry microalgal biomass using the modified Bligh and Dyer's method [39]. Lipid was extracted by mixing 500 mg of microalgal biomass with chloroform–methanol (1:2 v/v) solvent in a test tube and vortexed for 5 min and kept at room temperature overnight. The next day, chloroform and water were added to make the final solvent ratio of chloroform/methanol/water of 1:2:1.8. Then, the mixture was filtered, and 1% of NaCl was added to wash the chloroform layer. The chloroform layer was separated in a pre-weight beaker and dried at 60 °C in an oven until a constant weight was obtained. Finally, the total lipid content was measured gravimetrically. Total lipid content calculated using Eq. (1) [40].

$$\text{Total lipid yield} = \frac{Lipidextractedweight}{Totalbiomass} \times 100\% \quad (1)$$

Total carbohydrate was determined on the basis of National Renewable Energy Laboratory (NREL) analytical methods used for biomass [41]. In this method, two-step acid hydrolysis was employed. The first hydrolyses were performed using 72% H_2SO_4 at 30 °C for 60 min in a water bath and the second was conducted in an autoclave at 121 °C for 60 min after reducing the concentration from 72% to 4% H_2SO_4 using distilled water. After neutralization, centrifugation, and dilution, total carbohydrate was determined by the phenol–sulfuric method [42] using glucose as the standard. A supernatant of 2 mL was mixed with 1 mL of 5% phenol solution and 5 mL of 98% H_2SO_4 in a test tube. The solution was mixed by a vortex and stand for 10 min. Then, absorbance was measured at 490 nm using a JENWAY spectrophotometer (model 6705).

Total protein was estimated on the basis of available total nitrogen in the microalgal biomass. Total nitrogen was analyzed by the Kjeldahl method following the AOAC [43]. A weight of 500 mg of microalgal biomass was hydrolyzed with 20 mL of concentrated sulfuric acid (H_2SO_4) containing 150:10 (mg/mg) of $KSO_4/CuSO_4$ powder catalyst in a heat block (Gerhardt digester) at 370 °C for 3 h and received the ammonia using boric acid. After that, the hydrolysates were cooled by adding distilled water and titrated with HCl. The crude protein content in microalgal biomass was calculated by multiplying the total nitrogen by the conventional conversion factor of 6.25.

2.6.3 Fourier transform infrared spectroscopy (FTIR) analysis

The structures of microalgal biomass before and after pretreatment were identified with FTIR spectrometer to detect the change in functional groups. The FTIR spectrum of raw and treated indigenous *Scenedesmus* sp. biomass was recorded in the spectral range of 4000–400 cm^{-1} which scanned at the resolution of 4 cm^{-1} using Perkin Elmer spectrum GX FTIR spectrometer to understand structural changes that occurred during various biomass pretreatments.

2.6.4 Determination of reducing sugar content

The reducing sugar content was estimated using the procedure of the DNS method [44]. The calibration curve was constructed using glucose as a standard with $R^2 = 0.9951$ with the test range. The measurement of reducing sugar was done by JENWAY spectrophotometer (model: 6705) at 540 nm using a blank as control. The content of reducing sugar was calculated using Eq. (2) [45].

$$\text{Reducing sugar content} = \frac{\text{Total reducing suagr (g/L)}}{\text{microalgal biomass (g/L)}} \quad (2)$$

2.6.5 Bioethanol determination

The fermentation broth was first distilled before the determination of bioethanol. Then, the bioethanol concentration was determined calorimetrically using the acidic potassium dichromate method according to the procedure described by Crowell and Ough [46]. A standard curve was constructed from absolute ethanol as described in Williams and Darwin Reese [47]. The experimental bioethanol

yield in percentage and the fermentation efficiency were calculated using Eqs. (3) and (4) [1]:

$$\text{Bioethanol yield} = \frac{\text{Bioethanol } (\frac{g}{L})}{\text{microalgal biomass } (\frac{g}{L})} \quad (3)$$

$$\text{Fermentation efficiency} = \frac{\text{Bioethanol obtained (g/L)}}{0.51 \times \text{ reducing sugar in the hydrolysate (g/L)}} \times 100 \quad (4)$$

where 0.51 was derived from the maximum theoretical ethanol yield per 1 g of glucose consumption.

2.7 Statistical analysis

The data obtained for reducing sugar extraction with different treatments are illustrated in figures that were generated using MS-Excel 2013. One-way ANOVA (using R-software) with the Tukey post hoc test was employed to compare total reducing sugar production with different hydrolytic agents and pretreatment methods. The differences were significant when $P < 0.05$.

The data obtained from the RSM design were analyzed using MINITAB (version 18) software to generate a mathematical model. A second-order polynomial equation was developed and used to relate the response to the independent variables and their interaction [48]. The following generalized second-order Eq. (5) was generated and employed in the response surface analysis.

$$Y = b_o + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i<j}^{k} b_{ij} X_i X_j + \cdots + e \quad (5)$$

where Y is the predicted reducing sugar. X_i and X_j are independent variables (coded values), β_o, β_i, β_{ii}, and β_{ij} stands for constant, linear, quadratic, and interaction coefficients, respectively, and ε is the error.

The significance of the model and individual model terms and their interaction was determined by analysis of variance (ANOVA). The statistical significance of the model was checked by the Fisher test (F-values) with P-values less than 0.05, considering as significant in response surface analysis. Regression coefficients (R^2-values) were used to evaluate the fitness of the developed model to the experimental data. The significance of the developed model was also checked by the lack-of-fit. A normal probability plot was used to examine the appropriateness of the model. The two-dimensional (2D) contour plots were generated for the model and used to visualize the relationship between the independent variables and the response variable (reducing sugar).

3 Results and discussion

3.1 Biomass production and nutrient removal

The brewery effluent used for *Scenedesmus* sp. cultivation had a TN of 53.42 ± 6.2 mg/L and TP of 50.00 ± 2.64 mg/L. Darpito et al. [49] used a brewery effluent whose concentration of 72.6 mg/L TN and 54.4 mg/L TP for microalgae cultivation. Therefore, the nutrient concentrations in the brewery effluent of this study were adequate to support microalgae cultivation. The biomass production and nutrient removal during the cultivation period of *Scenedesmus* sp. on brewery effluent are shown in Fig. 1. The biomass production increased with increasing cultivation time and reached a maximum biomass production of 1.05 ± 0.11 g/L at the end of cultivation. The TN and TP concentrations were steadily decreased and reached 2.93 ± 0.4 and 15.29 ± 1.3 mg/L with removal efficiencies of 94.52% and 69.42% at the end of the cultivation period, respectively. Darpito et al. [49] cultivate *Chlorella protothecoides* on brewery wastewater and found a maximum biomass production of 1.88 g/L with a removal efficiency of 96% TN and 90% TP. Ferreira et al. [50] also used brewery effluent for *Scenedesmus obliquus* cultivation and reported a maximum biomass production of 0.94 g/L with a removal efficiency of 88.5% TN and 40.8% TP. These studies showed there is a variation in TP removal efficiency from brewery effluent using different microalgae. This may be due to the removal of phosphorus is affected by algal physiology,

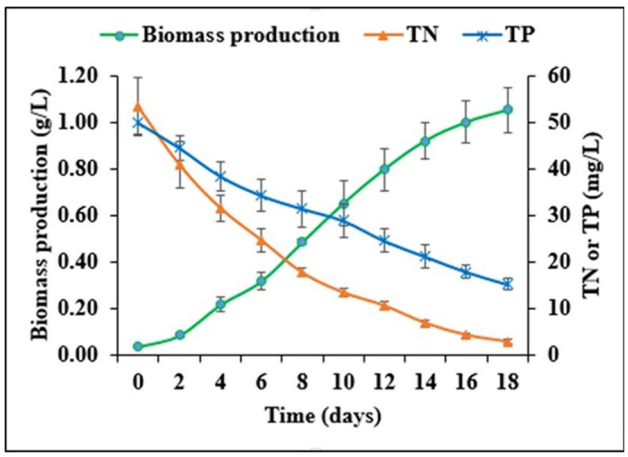

Fig. 1 Biomass production and nutrient removal potential of *Scenedesmus* sp. overcultivation period in brewery effluent

phosphate concentration, and chemical form of available phosphate, light intensity, pH, and temperature [51]

3.2 Biochemical composition of Scenedesmus sp. biomass

The biochemical composition of the *Scenedesmus* sp. was found to be 13.67% of lipids, 26.65% of carbohydrates, and 49.44% of proteins. These results showed that the total protein was the dominant biochemical compound, whereas lipid was the least in the indigenous *Scenedesmus* sp. The carbohydrate content in the microalgal biomass of this study shows a promising result for bioethanol production. Table 2 provides the comparison of lipid, carbohydrate, and protein contents of *Scenedesmus* sp. reported in this study with other studies. Gupta et al. [52] and Sivaramakrishnan and Incharoensakdi [53] have obtained higher lipid contents (18.3% and 40%) but lower carbohydrate contents (12.6% and 22.2%) and protein contents (30.4% and 19%) from *Scenedesmus* sp. grown on domestic and municipal wastewaters, respectively, than this study. Diniz et al. [21] found lower carbohydrate and lipid contents from *Scenedesmus* sp. grown on municipal wastewater than in this study. This shows that *Scenedesmus* sp. grown on brewery effluent accumulates more carbohydrates than grown on domestic and municipal wastewater. However, the result of carbohydrate content obtained in this study was comparable to those reported by Mercado et al. [20], who found a carbohydrate content of 27% from *Scenedesmus* sp. grown on dairy wastewater. The differences in biochemical compositions of *Scenedesmus* sp. with various studies could be due to cultivation conditions such as temperature, light, pH, and nutrients in wastewater [54].

3.3 Effect of pretreatment on reducing sugar production

Pretreatments of the microalgae biomass with different hydrolytic agents (H_2O, HCl, H_2SO_4, NaOH, and KOH) in microwave, autoclave, oven, and water bath were investigated to identify the most effective pretreatment method and hydrolytic agent. Figure 2 shows the total reducing sugar content obtained from *Scenedesmus* sp. biomass after microwave, autoclave, water bath, and oven pretreatments. Results showed acid-assisted hydrolysis produced a higher reducing sugar than alkaline-assisted hydrolysis in all pretreatment methods. The maximum reducing sugar contents obtained were 146.56 ± 0.93 and 115.98 ± 2.05 mg/g in the microwave and water bath using HCl, whereas 122.49 ± 0.85 and 112.63 ± 0.38 mg/g in autoclave and oven using H_2SO_4, respectively. Regarding alkaline pretreatment, both KOH and NaOH provided a maximum reducing sugar content in an autoclave. This indicates that the hydrolytic agent types and pretreatment methods have an effect on reducing sugar production from microalgal biomass. The highest reducing sugar content obtained in this study was 146.03 ± 0.93 mg/g in the microwave using HCl. This result was significantly different ($P < 0.05$) from the results obtained in the other pretreatment methods. Thus, the acid HCl and microwave pretreatment method were selected for the reducing sugar extraction process optimization.

The production of reducing sugar using different acids and alkalis pretreatment on microalgae biomass does not provide reproducible results [29]. For example, Shokrkar et al. [56] used the hydrolytic agents H_2SO_4, H_2PO_4, and NaOH, and found a maximum sugar extraction with HCl from mixed microalgal biomass. Moreover, Park et al. [57] reported that HCl was more effective than H_2SO_4 and HNO_3 for reducing sugar extraction from *Chlorella vulgaris*. These two studies are following the present study regarding the effectiveness of HCl. However, Miranda et al. [29] reported H_2SO_4 was more effective when compared to HCl and NaOH for sugar extraction from *Scenedesmus obliquus*. On the other hand, Hernández et al. [58] and Shokrkar et al. [56] employed acid and alkaline-assisted hydrolysis for microalgal biomass and they found that alkaline-assisted hydrolysis using NaOH released less sugar than acid-assisted hydrolysis using HCl and H_2SO_4, which agreed with this study. Therefore, it can be concluded that the selection of a hydrolytic agent for sugar extraction depends on the nature of microalgae species.

Table 2 Comparison of biomass composition profile of *Scenedesmus* sp. in other studies with the present study

Growth medium	Lipids (%)	Carbohydrates (%)	Proteins (%)	Reference
Urban wastewater	8.1	11.7	8.1	[55]
Municipal wastewater	13.4	18.1	26.4	[21]
BG11	40	22.2	19	[53]
Domestic wastewater	18.3	12.6	30.4	[52]
Dairy wastewater	51	27	20	[20]
Brewery effluent	13.67	26.65	49.44	The present study

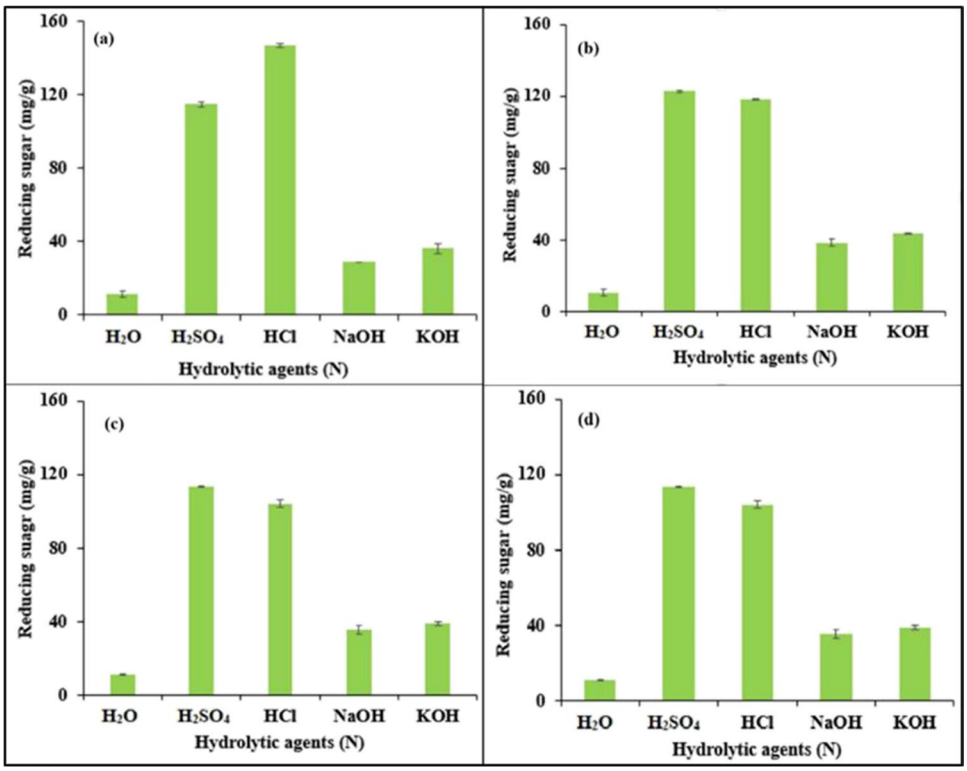

Fig. 2 Effect of hydrolytic agents on reducing sugar production using (**a**) microwave, (**b**) autoclave, (**c**) oven, and (**d**) water bath pretreatments

3.4 Optimization of microwave-assisted hydrolysis using RSM

3.4.1 Regression equation development

The microwave-assisted HCl hydrolysis produced the highest reducing sugar compared with the other pretreatment methods. Therefore, microwave-assisted HCl hydrolysis was chosen and used for reducing sugar extraction optimization from *Scenedesmus* sp. in this study. The complete experimental design with actual and predicted values of the RSM experiments is provided in Table 3. The experimental data were analyzed using the Minitab software, and a regression equation (Eq. (5)) for the quadratic model was obtained.

$$\begin{aligned} Y = &-264 + 41.82A + 0.026B + 4.528C \\ &+ 0.09D - 2.258A^2 - 0.000006B^2 \\ &- 0.02005C^2 - 0.0357D^2 - 0.03033\ \mathrm{AB} \\ &+ 0.0184\mathrm{AC} - 0.0230\mathrm{AD} + 0.000933\mathrm{BC} \\ &+ 0.000295\mathrm{BD} + 0.00664\mathrm{CD} \end{aligned} \quad (5)$$

where Y is the predicted reducing sugar and A, B, C, and D are linear terms that stand for HCl concentration, microwave power, temperature, and extraction time, respectively. AB, AC, AC, BC, BD, and CD are the interaction terms, and A^2, B^2, C^2, and D^2 are the quadratic terms. A positive or a negative sign before a coefficient in the regression equation represents either a synergistic effect or an antagonistic effect [59]. All quadratic terms (A^2, B^2, C^2, and D^2) had negative coefficients, implying antagonistic effects on reducing sugar. Except for the interaction of A and D, the other interactions had positive coefficients, indicating synergistic effects on reducing sugar. The parameters with synergic effect show that increasing these parameters will increase the reducing sugar production. Nevertheless, parameters with antagonistic effects mean that increasing these parameters will lead to a decrease in reducing sugar production.

3.4.2 Statistical analysis of regression model

The ANOVA for the fitted quadratic model of reducing sugar extraction is depicted in Table 4. The model would be significant if F-value becomes greater and the p-value turns smaller [60]. As shown in the ANOVA table (Table 4), the model F-value of 78.36 and P-value of 0.000 indicate that the model was significant. The model terms of A, B, C, A^2, C^2, AB, BC, and CD were significant in reducing sugar extraction. Moreover, the model's lack-of-fit was insignificant ($P > 0.05$) relative to the pure error. The coefficient of variation (CV) measures the reliability of the experiments and the lower CV indicates the higher reliability of the results [61]. In this study, a relatively low CV was obtained (2.37%), indicating that a better reliability of the experimental values.

Table 3 Face-centered central composite design matrix and the response of reducing sugar production

Std order	Run	Coded value				Actual value				Reducing sugar (mg/g)	
		A	*B*	*C*	*D*	*A*	*B*	*C*	*D*	Actual value	Predicted value
29	1	0	0	0	0	2.55	1000	130	17.5	158.62	157.23
23	2	0	0	0	−1	2.55	1000	130	5.00	152.85	152.43
4	3	1	1	−1	−1	5.00	1200	80	5.00	65.920	63.060
22	4	0	0	1	0	2.55	1000	180	17.5	125.67	127.56
31	5	0	0	0	0	2.55	1000	130	17.5	167.12	157.22
2	6	1	−1	−1	−1	5.00	800	80	5.00	78.640	87.810
24	7	0	0	0	1	2.55	1000	130	30.0	145.22	150.83
10	8	1	−1	−1	1	5.00	800	80	30.0	76.486	75.030
12	9	1	1	−1	1	5.00	1200	80	30.0	60.280	53.230
27	10	0	0	0	0	2.55	1000	130	17.5	157.24	157.22
1	11	−1	−1	−1	−1	0.10	800	80	5.00	59.250	51.560
3	12	−1	1	−1	−1	0.10	1200	80	5.00	85.020	86.250
14	13	1	−1	1	1	5.00	800	180	30.0	112.39	110.14
6	14	1	−1	1	−1	5.00	800	180	5.0	111.26	106.33
18	15	1	0	0	0	5.00	1000	130	17.5	144.81	148.48
20	16	0	1	0	0	2.55	1200	130	17.5	162.18	169.52
5	17	−1	−1	1	−1	0.10	800	180	5.00	55.028	61.060
19	18	0	−1	0	0	2.55	800	130	17.5	146.57	144.42
30	19	0	0	0	0	2.55	1000	130	17.5	167.12	157.22
17	20	−1	0	0	0	0.10	1000	130	17.5	137.32	138.85
8	21	1	1	1	−1	5.00	1200	180	5.00	120.60	118.89
11	22	−1	1	−1	1	0.10	1200	80	30.0	74.595	79.240
15	23	−1	1	1	1	0.10	1200	180	30.0	152.83	142.64
13	24	−1	−1	1	1	0.10	800	180	30.0	65.110	67.690
26	25	0	0	0	0	2.55	1000	130	17.5	150.23	157.22
21	26	0	0	−1	0	2.55	1000	80	17.5	83.305	86.600
16	27	1	1	1	1	5.00	1200	180	30.0	118.24	125.65
7	28	−1	1	1	−1	0.10	1200	180	5.00	131.89	133.06
9	29	−1	−1	−1	1	0.10	800	80	30.0	40.900	41.590
28	30	0	0	0	0	2.55	1000	130	17.5	157.13	157.22
25	31	0	0	0	0	2.55	1000	130	17.5	158.62	157.22

The fitness of the model was checked by examining the determination coefficients R^2 (correlation coefficient), adjusted determination coefficient R^2, and predicted determination coefficient R^2, which are 0.983, 0.968 and 0.904, respectively. The closeness of the determination coefficient (R^2) to 1, indicating the fitness of the model to actual data [62]. The R^2 value was 0.983, indicating that only 1.70% of the total variation cannot be explained by the model. The adjusted R^2 value of the model was 0.968 which did not differ notably from the R^2-value, indicating a high degree of correlation between actual and predicted values [63]. Furthermore, the predicted R^2 value (0.904) closes to the adjusted R^2 value (0.968) and the difference less than 0.2 was in a reasonable agreement. The correlation between the actual and the predicted reducing sugar values is shown in Fig. 3(a). As shown in Fig. 3(a), all points were relatively close to the straight line, which suggests that the model was adequate in predicting the response (reducing sugar). Figure 3(b) shows a normal probability of plot of residuals, which indicated that all residuals closely fall on a straight line. This showed that the errors are normally distributed.

3.4.3 Effect of variables on reducing sugar production

The two-dimension contour plot was drawn to visualize the mutual interaction between independent variables and to determine the optimum levels of each variable for the maximum response (reducing sugar content) [64]. The plots were made on the basis of the regression equation. Figure 4(a-f) shows the two-dimension contour plot as a function of two independent variables, while the other two independent variables have been kept as constant (at

Table 4 ANOVA for the fitted quadratic regression model for optimization of reducing sugar extraction

Source	DF	Adj SS	Adj MS	*F*-value	*P*-value
Model	14	48,381.1	3455.80	66.46	0.000
Linear	4	10,814.1	2703.53	51.99	0.000
A	1	417.5	417.47	8.03	0.012
B	1	2835.0	2835.02	54.52	0.000
C	1	7550.1	7550.08	145.19	0.000
D	1	11.6	11.56	0.22	0.644
Square	4	32,267.9	8066.97	155.14	0.000
A^2	1	476.8	476.82	9.17	0.008
B^2	1	0.2	0.15	0.00	0.958
C^2	1	6522.7	6522.72	125.44	0.000
D^2	1	80.9	80.91	1.56	0.230
2-way interaction	6	5299.1	883.19	16.98	0.000
*A***B*	1	3534.0	3533.96	67.96	0.000
*A***C*	1	81.2	81.23	1.56	0.229
*A***D*	1	7.9	7.94	0.15	0.701
*B***C*	1	1392.0	1392.01	26.77	0.000
*B***D*	1	8.7	8.72	0.17	0.688
*C***D*	1	275.3	275.29	5.29	0.035
Error	16	832.0	52.00		
Lack-of-fit	10	617.7	61.77	1.73	0.260
Pure error	6	214.3	35.72		
Total	30	49,213.1			

fixed values). As shown in Fig. 4a, the maximum reducing sugar (171.2 mg/g) was obtained at an acid concentration of 1.64 N and microwave power of around 1200 W. This shows that lower acid concentration with higher microwave power is more favorable to get a maximum reducing sugar. As shown in Fig. 4b, a similar reducing sugar content was observed when the temperature was between 134 and 150 °C at any given acid concentration. However, the maximum reducing sugar content (156.2 mg/g) was achieved at the acid concentration of 3.0 N and temperature of 140.6 °C, which were taken as optimum values. As shown in Fig. 4c, reducing sugar content was increased with an increase in acid concentration from 0.1 to 3.0 N and extraction time from 5 to 16.5 min and then decreased as both kept increasing. Therefore, the maximum reducing sugar of 157.7 mg/g was attained at an acid concentration of 3.0 N and extraction time of 16.4 min.

As can be seen in Fig. 4d, reducing sugar content was first increased and then decreased with increasing temperature. However, at a temperature greater than 100 °C, reducing sugar content was increased with an increase in microwave power. Maximum reducing sugar content (173.9 mg/g) was obtained at a microwave power and temperature of around 1200 W and 145 °C, respectively. From Fig. 4e, it was observed that the reducing sugar content was increased with an increase in microwave power throughout the extraction time. Nevertheless, the reducing sugar content was first increased as the extraction time was changed from 5 to 17.20 min and then decreased as the extraction time increased. The microwave power of around 1200 W and extraction time of 17.4 min was found to be optimum for a maximum reducing sugar content (169.5 mg/g). As shown in Fig. 4f, reducing sugar content was increased as both temperature and extraction time increased and decreased with both variables kept increasing. The maximum reducing sugar (159.3 mg/g) was obtained at a temperature of 140.2 °C and extraction time of 17.6 min. Among the above six interactions, the interaction between the microwave power and temperature provided the highest reducing sugar, showing that this interaction had the greatest effect on reducing sugar extraction. However, the interaction of the acid concentration and extraction time produced the smallest reducing sugar so that it had the least effect on reducing sugar extraction.

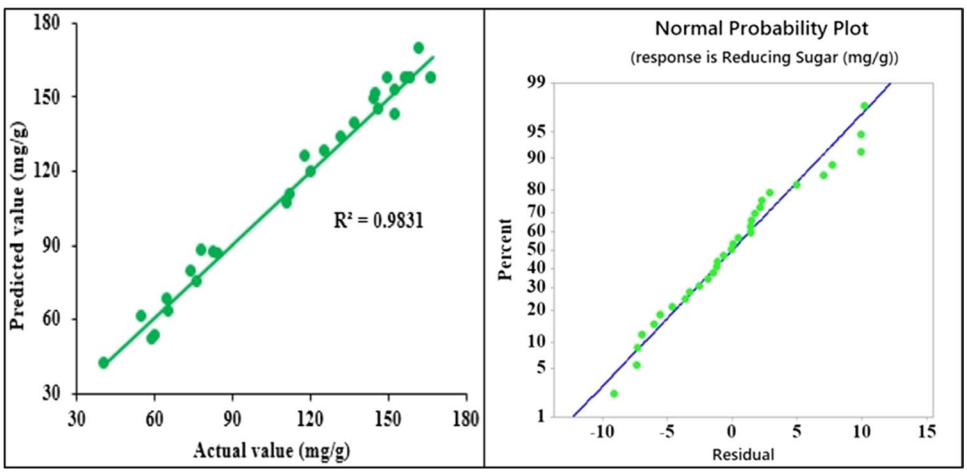

Fig. 3 Correlation between experimental and predicted values of (**a**) reducing sugar and (**b**) normal distribution plot

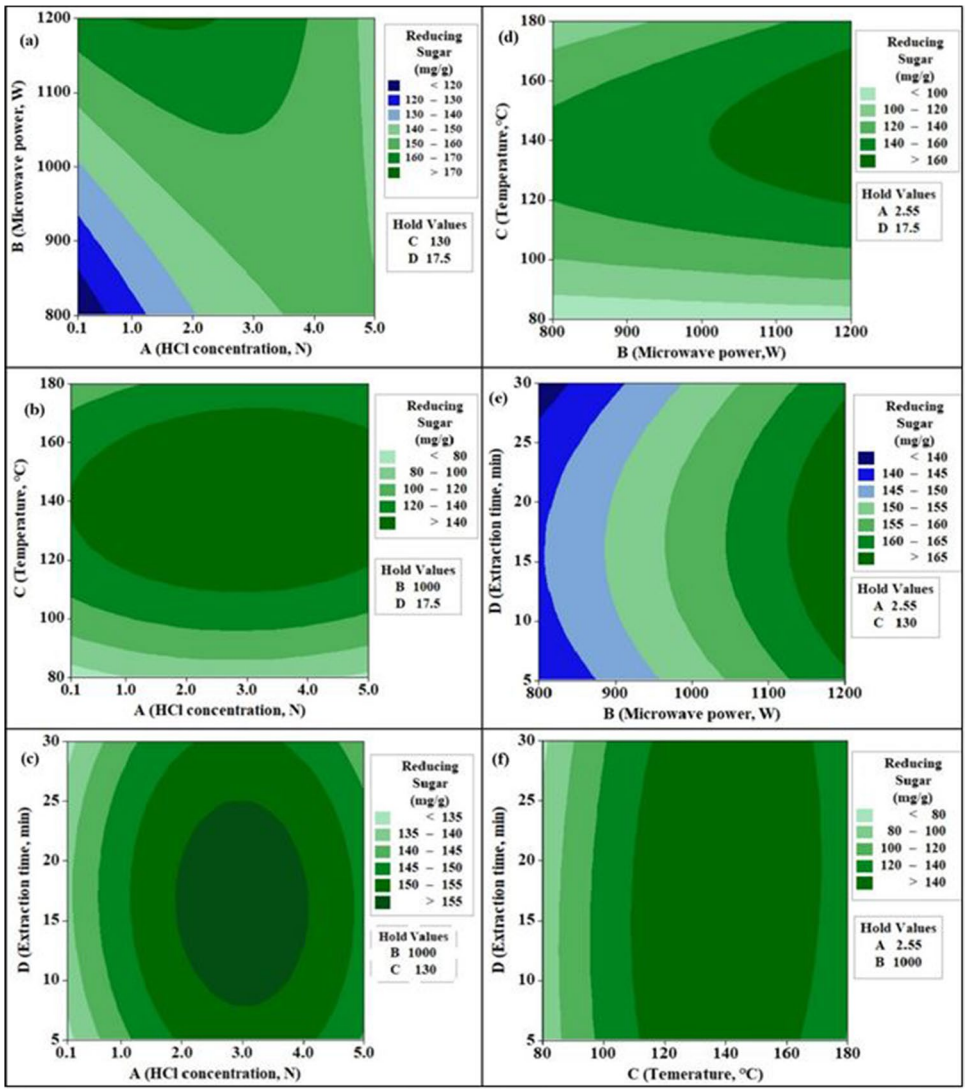

Fig. 4 Counter plot showing effect of variables on total reducing sugar (**a**) acid concentration versus microwave power, (**b**) acid concentration versus temperature, (**c**) acid concentration versus extraction time, (**d**) microwave power versus temperature, (**e**) microwave power versus extraction time, and (**f**) temperature versus extraction

3.4.4 Optimization and validation experiment

Optimization was carried out using RSM to obtain the maximum reducing sugar production that jointly fulfills all process conditions. An acid concentration of 1.68 N, microwave power of 1200 W, the temperature of 144.65 °C, and extraction time of 18.89 min were found to be optimal for reducing sugar extraction from microalga biomass. Under these optimum conditions of independent variables, the theoretical (predicted) reducing sugar yield was estimated to be 175.5 mg/g. The capability of the model equation for predicting the optimum reducing sugar content was validated by the above optimum conditions with a small modification. The temperature and extraction time were modified as 145 °C and 19 min, respectively, but acid concentration and microwave power were taken as they were. Three replicate experiments were performed using the modified optimum conditions. The average reducing sugar obtained was 172.5 mg/g, which was close to the predicted value. The hydrolysate obtained at optimal condition was then employed for bioethanol fermentation.

The reducing sugar content obtained in this study was higher than those obtained by Hernández et al. [58], who reported a maximum sugar of 88 mg/g from *Scenedesmus almeriensis* using acid hydrolysis with sulfuric acid for 60 min at 121 °C. Kassim and Bhattacharya [26] also reported a lower reducing sugar content (88 mg/g) from *Chlorella* sp. using alkaline pretreatment compared to this study. Miranda et al. [29] reported a higher sugar content of around 286 mg/g under the condition of 2 N H_2SO_4 at 120 °C for 30 min in an autoclave from *Scenedesmus obliquus*. This showed that pretreatment methods can be affected the release of sugar from microalgal biomass. Most previous studies on sugar extraction from microalgal biomass have been using synthetic mediums for microalgae growth [29, 58]. Therefore, the use of wastewater such as

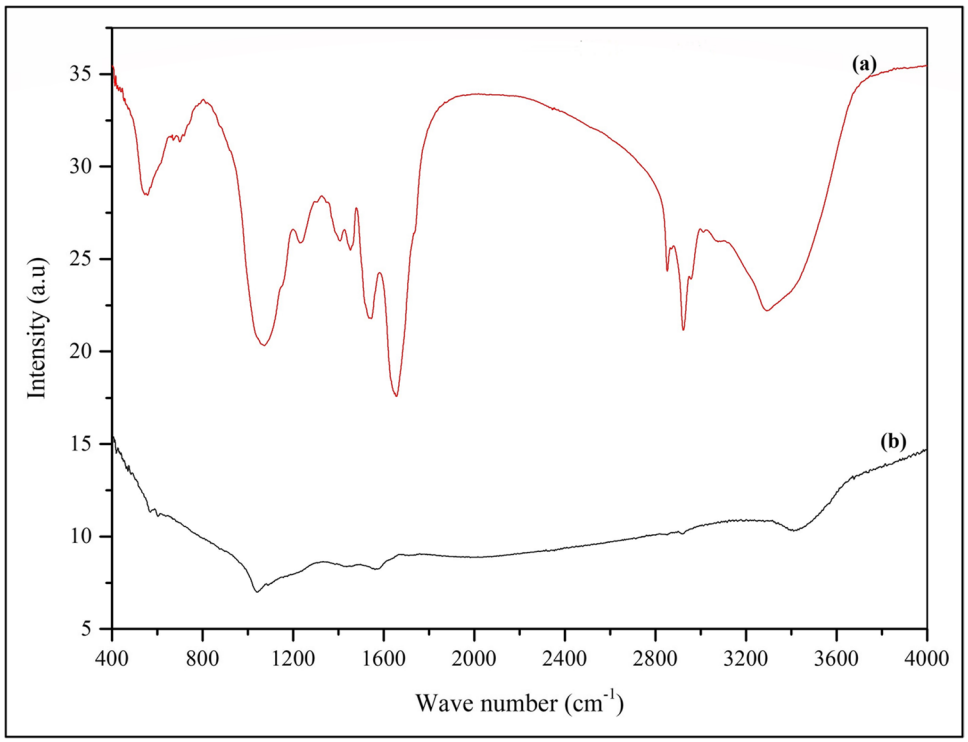

Fig. 5 FTIR spectra of (**a**) untreated and (**b**) pretreated biomass of *Scenedesmus* sp. under optimum conditions

brewery effluent for microalgae growth was a more sustainable approach to extract reducing sugar and for subsequent bioethanol production.

3.5 FTIR analysis

FTIR spectroscopy is an instrumental method that is frequently used to investigate the structural constituents and changes that occurred during the pretreatment of algae and lignocellulosic biomasses [65]. The FTIR spectra of raw and pretreated microalgal biomass after microwave-assisted acid pretreatment are shown in Fig. 4. The spectra show peaks near 3400–3200 cm^{-1}, 1705–1460 cm^{-1}, 2920–2860 cm^{-1}, and 1200–900 cm^{-1}. The peaks between 3400 and 3200 cm^{-1} could link to symmetric O–H of water and N–H stretching of protein [66]. The noticeable peaks observed between 2920 and 2926 cm^{-1} and 2850 and 2860 cm^{-1} could be linked to asymmetric CH_2 of methylene groups and symmetric CH_2 of methyl groups, respectively, which were also the major characteristic for lipids. The prominent peaks between 1705 and 1575 cm^{-1}, and 1575 and 1460 cm^{-1} could be linked to C = O of amide I and N–H bending vibration of amide II, respectively, which were mainly for protein [24].

The spectra peaks between 1200 and 900 cm^{-1} were dominated by functional groups associated with carbohydrate (C–O, C–C, C–O–C, P = O stretching vibration) [67]. The spectra bands in 1500–1300 regions provide information on the deformation vibrations of C–H bonds in lipids and proteins and also to C–O symmetric stretching vibration of carboxylic groups [68]. Analysis of the FTIR spectrum of pretreated microalgal biomass showed an obvious effect on the peaks. A significant reduction in intensity was observed in all regions after pretreatment. The major peaks between 3200 and 3400 cm^{-1} (Fig. 5) are reduced in pretreated biomass, which might be attributed to rupture the hydrogen bonding of cellulose. The peaks between 1500 and 1300 cm^{-1} are also reduced in pretreated biomass that might be due to the destruction of the cell walls that contain carbohydrates. From the spectrum of pretreated biomass, it was possible to conclude that the most main macromolecules were removed during pretreatment.

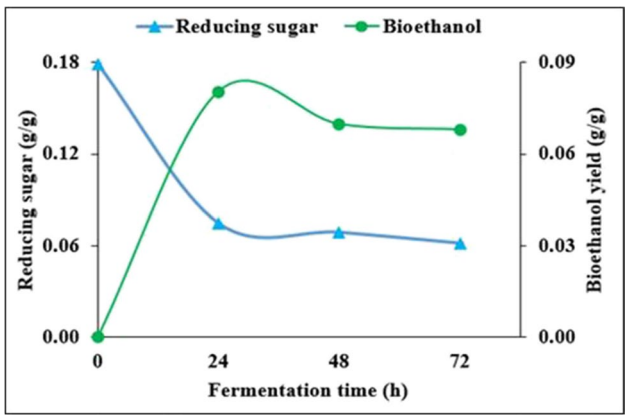

Fig. 6 Changes in bioethanol and reducing sugar concentration during the fermentation period

Table 5 Comparison of bioethanol yield obtained in this study with the previous studies

Biomass types	Pretreatment method	Ethanol yield (g/g biomass)	Reference
Scenedesmus obliquus	H_2SO_4, autoclave at 110 °C	0.103	[17]
Scenedesmus sp.	H_2SO_4, autoclave at 121 °C	0.076	[53]
Scenedesmus acuminatus	H_2SO_4, autoclave at 121 °C	0.120	[54]
Sunflower stalk	NaOH, at room temperature for 3 days and enzymatic hydrolysis	0.188	[45]
Longan waste fruit	Physical pretreatment and enzymatic hydrolysis	0.066	[8]
Scenedesmus sp.	HCl, microwave-assisted pretreatment	0.080	The present study

3.6 Bioethanol production

The hydrolysate derived from microalgal biomass at optimum conditions was investigated for bioethanol production using a commercial baker's yeast, *Saccharomyces cerevisiae*. This yeast has commonly been employed for bioethanol production from different feedstocks such as seed weed (marine macroalgae) [11, 69], *Scenedesmus acuminatus* [54], and Sunflower stalk [45]. Figure 6 shows the bioethanol production profiles from *Scenedesmus* sp. within 72-h fermentation time in this study. The results showed that the maximum bioethanol yield was obtained at 24-h fermentation time, but beyond this time, it was observed a slight decrease of bioethanol yield. The maximum fermentation yield of bioethanol obtained was 0.08 g/g microalgal biomass. The theoretical yield of conversion of fermentable sugar of microalgal biomass to bioethanol was estimated and found to be 88.15% of hydrolysis (based on total reducing sugar content in microalgal biomass).

Table 5 shows a comparison of bioethanol yield between the biomass of microalgae and lignocellulosic materials. The result for bioethanol yield in this study was similar to the study of Sivaramakrishnan and Incharoensakdi [53], who obtained from *Scenedesmus* sp. However, it was slightly lower than the results achieved by Guo et al. [17] and Chandra et al. [54], who produced bioethanol from *Scenedesmus obliquus* and *Scenedesmus acuminatus*, respectively. Unlike this study, most of the previous studies used a synthetic medium for microalgae growth, which added an extra cost toward bioethanol production. Concerning bioethanol from lignocellulosic materials, Manmai et al. [45] optimized reducing sugar extraction from sunflower stack using RSM for bioethanol production and found a maximum of 0.188 g bioethanol/g biomass, which is higher than that obtained in this study. However, Nguyen [8] reported a lower bioethanol yield from low-grade waste longan fruits using physical pretreatment and enzymatically hydrolysis. Therefore, the bioethanol obtained from *Scenedesmus* sp. grown on brewery effluent was more attractive compared to the bioethanol obtained in previous studies.

4 Conclusion

The data in this study showed that the feasibility of bioethanol production from indigenous microalgae cultivated on brewery wastewater. Results showed that the types of pretreatment and hydrolytic agent used for microalgal biomass can significantly affect reducing sugar production. The microwave pretreatment with HCl produces a higher reducing sugar from microalgal biomass than autoclave, water bath, and oven pretreatments. The RSM optimization of microwave-assisted extraction produced a maximum of 172.75 mg/g reducing sugar with the optimum conditions of 1.68 N, 1200 W, 145 °C, and 19 min. The hydrolysate obtained at the optimal condition was then employed for the fermentation process. The maximum bioethanol yield obtained was 0.08 g/g microalgal biomass with 88.15% fermentation efficiency. Based on the findings of this study, it can be suggested that the utilization of brewery wastewater as a growth medium for indigenous *Scenedesmus* sp. provides a promising approach for biomass and bioethanol production from microalgae grown on wastewater.

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Author contributions M.M.K. and Z.Y. isolated and identified *Scenedesmus sp.*, designed the study, conducted the experiments, collected and analyzed the data, and wrote the manuscript. S.L. and A.H. designed the experiments, supervised the research, analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the manuscript.

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

Ethical approval and consent to participate.
Not applicable.

Declarations

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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