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ORIGINAL PAPER

Biodiesel production potential of mixed microalgal culture grown in domestic wastewater

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Abstract In this study, a mixed microalgal culture grown in secondarily treated domestic wastewater effluent was investigated for biodiesel production using in situ transesterification method with conventional heating. The total lipid content of the mixed culture was found as 26.2 % ± 0.6 by weight of dry biomass, and 74 % of the lipids were contributed by total glycerides. In situ transesterification with conventional heating process under acidic conditions produced higher biodiesel yield with chloroform as the co-solvent (82.1 % ± 3.9) compared to hexane (55.3 % ± 3.9) under the same reaction conditions. The gas chromatography analysis showed that FAME composition was mainly composed of palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid methyl esters., and thus the mixed microalgal culture fed by domestic wastewaters has had comparable biodiesel conversion yields and FAME composition to mono-culture and pure cultures fed by synthetic culture media. Hence, this study showed that secondarily treated domestic wastewater could potentially be a suitable and sustainable medium for microalgae grown to be used as biodiesel feedstock.

Keywords Domestic wastewater · Secondary effluent · Mixed microalgae · Lipid · Biodiesel · In situ transesterification

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Introduction

Overwhelming amount of fossil fuel consumed every year to meet the global energy requirement risks the depletion of fossil fuel reserves, and through the emission of greenhouse gasses accelerates the rate of climate change process [1]. Therefore, partially replacing the fossil fuels by renewable energy resources is a promising solution to both climate change problem and global energy crisis. In this context, the use of biomass for energy production is regarded as a suitable alternative owing to its renewable and carbon neutral features [2].

Among the biomass alternatives, microalgae are believed to have great potential for the production of biodiesel. They have better photosynthetic efficiency, higher growth rate, greater biomass production, considerable lipid content and much less land requirement compared to other energy crops [3]. Since they also effectively provide biofixation (the mitigation) of CO_2 through photosynthesis, industrial-scale production of microalgal biomass will potentially reduce the effects of climate change. The average lipid content of microalgae species is commonly 20–50 % by weight of their dry biomass, although up to 80 % of lipid contents have been reported [4, 5]. Therefore, microalgal species are believed to be suitable stock material for biodiesel production.

Microalgae-based biodiesel production involves various processing steps, including biomass production, harvesting and drying of biomass, extraction of cellular lipids and conversion to biodiesel of the lipids [6, 7].

The first step of biomass production is costly, primarily because of low amount of biomass production and high cost of synthetic nutrients and growth medium used for growth of biomass [7–10]. Recently, the use of wastewater has been suggested to reduce the cost of biomass

production. The advantages of using wastewater for microalgae production include minimizing the fresh water use and meeting the nutrients requirements, e.g., nitrogen, phosphorous and trace elements directly from wastewater. In addition, reducing nutrient concentration in wastewater by this method benefits natural water environments [2, 11, 12]. The literature includes reports on microalgal production using various wastewater streams for biofuel production [2, 11–14]. For example, domestic wastewater was suggested as a more convenient nutrient source due to its non-toxic nature and wide-spread availability in large quantities. Earlier studies [2, 11, 15, 16] all reported promising biodiesel yields, however, most of these studies employed "pure cultures" in laboratory environment, yet in full-scale operation, maintaining pure cultures in domestic wastewater in ponds open to the atmosphere is hardly achievable. Different from the others, Wahlen et al. [17] and Sathish and Sims [18] used a mixed culture for investigation of its biodiesel production potential. The wild mixed culture used by Wahlen et al. [17] was obtained from a wastewater lagoon and used directly after concentrating by a centrifuge to analyze for its FAME content. In the second study [18], the mixed culture was obtained from a municipal wastewater lagoon and cultivated in a synthetic medium. Therefore, our study, to our knowledge, is the first that utilizes mixed culture and cultivates this culture in secondary effluent of municipal wastewater for biodiesel production.

Microalgal biomass, following its cultivation and harvesting, typically goes through first extraction of lipids and subsequently transesterification of lipids for biodiesel production. More recently, a direct (in situ) transesterification method, in which both extraction and transesterification processes occur in one step, has been applied to microalgal biomass [17, 19, 20]. This process allows a direct contact between lipid-containing biomass and the chemical solvent (methanol) in the presence of a catalyst. The advantage of direct transesterification over conventional biodiesel production method is that it minimizes the solvent separation step, decreases the processing time and thus reduces the process production cost, and may result in higher FAME yields [6, 19]. Various solvents including hexane, chloroform, ethanol, toluene or benzene have been used for in situ transesterification process [10, 20–22]. Johnson and Wen [20] investigated the effect of various co-solvents (chloroform, hexane or petroleum ether) on biodiesel production from *Schizochytrium limacinum* biomass by acid-catalyzed in situ transesterification process and reported higher FAME yield (72.8 %, based on the weight of algae oil) when chloroform was the co-solvent. Xu and Mi [21] investigated base-catalyzed in situ transesterification using toluene, diethyl ether and dichloromethane as co-solvents for biodiesel production from Spirulina sp. Ehimen et al. [10] evaluated in situ transesterification of Chlorella biomass by combining ultrasound and co-solvents.

The present study differs from the earlier studies using the acid-catalyzed in situ transesterification process on a mixed microalgal culture grown in domestic wastewater under controlled growth conditions, and thus is the first attempt to evaluate the real biodiesel production potential of mixed microalgal biomass fed by domestic wastewater. In this paper, we also present a comparison of the lipid and fatty acid methyl ester contents of our mixed culture to those reported in the literature for pure cultures grown in synthetic media as well as wastewater media.

Materials and methods

Mixed culture and acclimation to wastewater as the growth medium

The mixed microalgae culture used in these experiments was collected from various water bodies and acclimated to biologically treated domestic wastewater, which was obtained from the secondary effluent of Omerli Domestic Wastewater Treatment Plant (Istanbul, Turkey). The secondary effluent is a term used to indicate that the domestic wastewater has been subjected to the pre-treatment for removal of part of particulates and dissolved organic substances. Omerli plant uses Sequencing Batch Reactors to treat 500 m^3 day^{-1} of domestic wastewater biologically, without any additional advanced treatment for nitrogen (N) and phosphorus (P) removal. The characteristics of the domestic wastewater used as the growth medium for microalgae are given in Table 1. No additional macro- and micro-nutrients were added to the wastewater. No

Table 1 The characteristics of the secondary effluent used in this study

Parameters	Secondary effluent
pH	7.42
Dissolved oxygen (DO) (mg L^{-1})	8.39
Alkalinity (mg L^{-1} as $CaCO_3$)	110 ± 3
Chemical oxygen demand (COD) (mg L^{-1})	34.1 ± 4.7
Biological oxygen demand (BOD_5) (mg L^{-1})	6.0 ± 2.5
Ammonium nitrogen (NH_3–N) (mg L^{-1})	0.24 ± 0.03
Nitrate nitrogen (NO_3^-–N) (mg L^{-1})	15.0 ± 2.3
Phosphate phosphorus (PO_4^{-3}–P) (mg L^{-1})	1.9 ± 1.4
Total nitrogen (TN) (mg L^{-1})	17.0 ± 1.2
Total carbon (TC) (mg L^{-1})	50.5 ± 1.0
Inorganic carbon (IC) (mg L^{-1})	37.8 ± 1.2
Total organic carbon (TOC) (mg L^{-1})	12.7 ± 0.2

additional pre-treatment, such as dilution, autoclaving, UV radiation or filtration was applied to the wastewater.

The mixed microalgal biomass was acclimated to the wastewater medium in 20-L glass reactors operated as batch at a temperature of 24 ± 2 °C. The artificial light was provided continuously using day-light fluorescent lamps (85 W Philips Master TL-D-90 Graphica and 18 W Osram Lumiux de Luxe). Photosynthetically active radiation (PAR) was measured as 150 μ photon m^{-2} s^{-1} with LI-193 quantum sensors and recorded by an LI-1400 data logger. To stabilize the pH between 6.5 and 7.0, and to supply adequate inorganic carbon for algal growth, pure CO_2 was injected into the reactors through glass diffusers. No additional mixing was provided in the tank. The CO_2 flow was adjusted by an automatic control system (WTW pH 296) to continuously maintain pH in the range of 6.5–7.0 as the best pH conditions observed during our experiments. The mixed culture after 6 months of acclimation was observed by a light microscope (Scope 3025 Fluorescence Microscope) to contain primarily *Chlorella sp., Scenedesmus sp., Chlorococcale sp.* However, the dominant species was *Scenedesmus sp*.

Biomass production, concentration and harvesting

The acclimated cultures (100 mL volume) were transferred to 20-L reactors for biomass growth. The reactors were operated as batch, adding wastewater only to make-up the 0.5 L of loss of water per day due to evaporation. Under these conditions, the biomass concentration remained relatively steady at about 500 mg L^{-1}.

Biomass was then concentrated in the reactors by withdrawing 15 L of the spent wastewater every 3 days using a submerged hollow-fiber membrane filtration system, after which the same amount of wastewater was added to make-up the loss. This method allowed reaching a biomass concentration of about 3000 mg L^{-1}. This suspension was then centrifuged for 10 min at 6000 rpm to a microalgal paste and dried in an oven (Model ON-01E, Jeio Tech, Korea) for 24 h at 65 °C. The dried biomass was ground into a powder (mesh no: 40) and stored at −18 °C for further processing.

Extraction and measurement of total lipids

The total lipids were extracted from dry biomass powder using a modified version of Bligh and Dyer method [23]. The extraction procedure was carried out in triplicate using a mixture of chloroform–methanol–water at a ratio of 1:1:0.5 (v/v/v) [24]. Twenty-five mg of dry biomass powder was blended with 25 mL chloroform–methanol–water mixture in a glass test tube. The biomass–solvent mixture was vortexed for 1 min and the mixture was then allowed to stand for 5 min in a water bath at 75 °C. After the mixture was centrifuged in 6000 rpm for 10 min to provide phase separation, the lipids containing the chloroform phase were collected and transferred to a pre-weighed glass vial. The entire solvent was evaporated and the final weight was noted. The total lipid content was determined gravimetrically (based on w/w % of the dry biomass) by taking the difference in the pre- and the final weights of the vial.

In situ transesterification

In our study, in situ transesterification process was applied to microalgal biomass using the method of conventional heating with methanol as the solvent, and sulfuric acid (H_2SO_4) as the catalyst. The effects of co-solvents and the reaction time on biodiesel yield during in situ transesterification were investigated by conducting a set of experiments. The experiments involved the use of one hundred (100) mg of dry biomass powder mixed with 5 mL of methanol, 4 mL of co-solvent (chloroform or n-hexane) and 50 μL H_2SO_4 (1 % H_2SO_4 based on methanol, v/v). The reaction mixture was vortexed for 5 min and then heated in a water bath while being stirred at 500 rpm at 65 °C. Transesterification reaction was allowed to proceed for different reaction times of 2–8 h, and then the mixture was allowed to cool at room temperature. Ten (10) mL of distilled water was added into the mixture. The mixture was vortexed for 1 min and was then centrifuged at 6000 rpm for 15 min to achieve phase separation. The solvent layer containing the Fatty Acid Methyl Esters (FAME) was collected, filtered using a 0.22 μm syringe filter and transferred to a pre-weighed glass vial. The solvent was evaporated in a 40 °C water bath to a constant weight after which the mass of microalgal biodiesel product (FAME) was determined by taking the difference in the pre- and the final weights of the vial.

Neutral lipid and FAME analyses with GC-FID

The neutral lipid (mono–di–triglycerides) content of the lipids extracted from the biomass was analyzed by gas chromatography (GC). An Agilent 6890 N series gas chromatograph (GC) equipped with a flame ionization detector (FID), a DB-5HT (J&V Scientific) capillary column (30 m × 0.53 mm I.D. × 0.1 μm), a hydrogen generator (Shimadzu OPGU–2200S), and an auto-injector was used for this purpose. A modified version of the Standard EN 14105 method was used for the chromatographic analysis of the neutral lipids [25]. During sample preparation, for each 4 mg lipid sample, 40 μL Internal Standard#1 (IS-1), 50 μL Internal standard#2 (IS-2) and 100 μL N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) as silylating reagent were added, the mixture was vortexed for

2 min and then hold for 30 min for complete silylation, compared to 20 min holding time in the standard method. Furthermore, the 40 μL MSTFA addition recommended in the standard method was increased to 100 μL to ensure the volatilization of triglycerides [24].

For the FAME analysis, a modified version of standard DIN EN 14103 method was employed following in situ transesterification [26]. The GC system described above was used, except the column was a DB-WAX (polyethylene glycol, J&V Scientific) capillary column (30 m × 0.53 mm I.D. × 0.5 μm). The programming was as follows: The initial oven temperature at 100 °C was maintained for 2 min, then increased to the final temperature of 210 °C at a rate of 30 °C/min, and then maintained at 210 °C for 15 min. The temperature of the detector and the inlet was set at 250 °C, and the injection volume was set at 3 μL with the splitless mode. Helium was used as the carrier gas with a flow rate of 2 mL/min (constant flow), while nitrogen was used as make-up gas with a flow rate 30 mL/min. The total analysis time was 21 min.

Prior to the biodiesel analysis, FAME mix C8-C22 analytical standard (Supelco Co., USA) was dissolved in n-heptane for calibration. The retention time of the each FAME was determined using the GC/FID method described above. After the calibration for each FAME concentration, the FAME concentrations of the biodiesel products obtained from microalgae were dissolved in n-heptane and were analyzed under the same conditions.

Results and discussion

Lipid content

Using the modified Bligh and Dyer method noted above, the mean total lipid content of the mixed microalgae culture was measured as 26.2 % ± 0.6 of dry biomass. A comparison of the lipid contents of microalgae species grown in different culture media is given in Table 2. Accordingly, the lipid content of the microalgal culture grown in wastewater in this study was found similar or higher than the recent values reported in the literature [3, 6, 15, 17, 27, 28]. The lipid content is known to vary not only by the species of microalgal cells, but also the growing conditions and the growth phase of the cells. These types of information are not usually available in the literature for the studies listed in Table 2. However, we report that in our study, the mixed culture was subjected to very low concentrations of nutrients at the time of the harvesting, the cells were at the stationary growth phase and N and P concentrations in water phase were about 3 mg L^{-1} and 0.1 mg L^{-1}, respectively. Under such nutrient limiting conditions, the cells are expected to accumulate lipids.

Total glycerides (TG) are the parts of the lipids that are converted to biodiesel. For analysis of TGs, the preferred method involves esterification to form fatty acid methyl esters (FAME), which then are analyzed by the GC method. Using this approach in our studies, the TG content of the microalgal lipid samples was found as approximately 74 % of the lipids, which means that 74 % of the lipids of the mixed microalgal cells produced in this study can potentially be converted into biodiesel.

Biodiesel yield

Separation of lipids and FAME conversion with the help of various solvents, including hexane, chloroform, ethanol, toluene or benzene has been reported in the literature [10, 20–22]. In this study, chloroform and hexane were used as co-solvents with methanol during the conversion of microalgal biomass into biodiesel via in situ transesterification with conventional heating. During this process, the use of a co-solvent with methanol is expected to increase the formation of FAMEs by helping with cell rupture and extraction of the lipids from the cells into the reaction medium. In our study, higher conversion efficiency was observed with chloroform than with n-hexane (82.1 % ± 3.9 vs. 55.3 % ± 3.9, respectively, based on weight of lipid). Since, n-hexane showed in our studies lower lipid extraction efficiency compared to the

Table 2 Lipid contents of various microalgae cultures grown in wastewater vs. synthetic media

Microalgae culture	Growth medium	Lipid content (wt %)[a]	References
Mixed culture	Wastewater	26.2 ± 0.6	This study
Mixed culture	Wastewater	14.4 ± 0.4	[17]
Chlorella saccharophila	BG11 medium	12.9 ± 1.2	[27]
Chlorella saccharophila	Wastewater	18.1 ± 1.3	[27]
Chlorella sp.	BG11 medium	27.6	[6]
Chlorella pyrenoidosa	Brostol's medium	19.0 ± 0.6	[28]
Scenedesmus sp. LX1	Wastewater	33	[29]
Scenedesmus obliquus	BG11 medium	20	[3]

[a] 100 × (mg of lipid/mg of biomass)

chloroform, low lipid recoveries and, as a result, low FAME yields were observed when n-hexane used as the co-solvent, as was in previous studies [20, 30].

According to the European Union (EU) biodiesel standard (EN 14214), the FAME conversion should be higher than 96.5 wt % as a good practice to eliminate further purification of the product. Although the highest FAME conversion yield achieved in this study is lower than that value, it is quite possible to increase this amount of yield above the limit value using various process modifications such as ultrasound and microwave applications. Previous studies have already shown that the FAME yields obtained from conventional applications of in situ transesterification of algal biomass could be enhanced about 25 % by ultrasonication [10] and about 34 % by microwave irradiation [31].

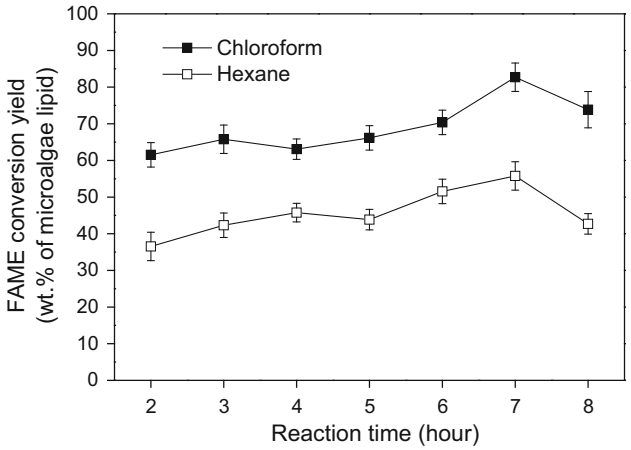

Fig. 1 Effect of chloroform and *n*-hexane on biodiesel yields [reaction conditions: 5 mL of methanol, 4 mL of co-solvent (hexane or chloroform) and 50 µL H_2SO_4 (1 % H_2SO_4 based on methanol, v/v]

FAME yields investigated as a function of reaction times ranging from 2 to 8 h are presented in Fig. 1. The maximum FAME yields under the reaction conditions (5 mL of methanol, 4 mL of co-solvent and 50 µL H_2SO_4, and heating at 65 °C) were achieved for both co-solvents at the reaction time of 7th hour; while increasing the reaction time for another hour resulted in reduced FAME yield. The similar results were also observed by Cheng et al. [32] and Carvalho and Malcata [33]. This was concluded that the FAME yields were decreased with the further reaction time due to the degradation of free fatty acids by the sulfuric acid [32, 33].

FAME composition

The characterization of the FAME composition was made by GC analysis of the microalgal biodiesel sample with the highest FAME conversion yield (82.1 % ± 3.9 wt %) obtained from in situ transesterification with chloroform. As presented in Table 3, palmitic (C16:0), palmitoleic

Table 3 Composition of FAME obtained from the mixed culture in this study, and comparison with literature values

Fatty acid	FAME composition (wt % of total FAME)			
	Mixed culture[a]	*Scenedesmus obliquus*[b]	*Chlorococcum sp.*[c]	*Chlorella pyrenoidosa*[d]
Lauric (C12:0)	1.27	–	–	–
Myristic (14:0)	4.95	1.48	0.5	–
Pentadecanoic (C15:0)	0.62	–	–	–
Palmitic (C16:0)	26.72	21.78	18.8	19.46
Palmitoleic (C16:1)	8.94	5.95	5.7	4.66
Hexadecadienoic (C16:2)	–	3.96	2.3	12.75
Hexadecatrienoic (C16:3)	–	0.68	–	19.22
Hexadecatetraenoic (C16:4)	–	0.43	–	–
Heptadecanoic (C17:0)	1.98	–	0.5	–
Stearic (C18:0)	5.82	0.45	2.8	0.21
Oleic (C18:1)	23.50	17.93	63.3	2.68
Linoleic (C18:2)	14.02	21.74	4.7	19.76
Linolenic (C18:3)	9.83	3.76	–	21.25
Stearidonic (C18:4)	–	0.21	–	–
Arachidic (C20:0)	2.35	–	1.1	–

[a] This study

[b] [35]

[c] [1]

[d] [28]

(C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid methyl esters were found to be as the predominant FAME constituents. Earlier studies also reported palmitic, palmitoleic, stearic, oleic and linoleic acid methyl esters as the major constituents of the transesterified microalgal lipids [1, 34, 35, 36]. Hence, our results showed that the mixed culture fed by secondary effluent produced lipids with contents and composition comparable to those of pure microalgal species reported in the literature.

Out of the FAME constituents, the saturated fatty acids (C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0) were 43.7 %, the monounsaturated fatty acids (C16:1, C18:1) were 32.4 %, and the polyunsaturated fatty acids (C18:2, C18:3) were 23.9 % of the total. The degree of saturation and the chain length of the fatty acids are known to affect the biodiesel properties such as oxidation stability, cetane number, the iodine value [3, 37]. According to the American Society for Testing and Materials (ASTM D6751) biodiesel standard and the European Union (EU) biodiesel standard (EN 14214), good quality biodiesel should have a high oxidation stability, high cetane number and low iodine value [38]. It is known that in biodiesel, both low amounts of polyunsaturated fatty acids and high oleic acid content improve oxidation stability, whereas high amounts of fully saturated fatty acids (e.g., palmitic and stearic) result in high cetane number and low iodine value [36, 37]. Although the oxidation stability, the cetane number and the iodine value of the biodiesel obtained in this work were not directly measured, the low polyunsaturated FAME content and high oleic and palmitic acid compositions indicate that the FAME obtained from the mixed culture may satisfy the fuel specifications in these three parameters. Our results are in agreement with the literature, which report that most of the physicochemical properties of biodiesel derived from pure microalgal species already meet the most of the limit values established by the ASTM D6751 and EN 14214 biodiesel standards [5, 35, 36, 39].

Conclusion

The mixed culture of microalgae grown in secondary effluent wastewater was investigated for the production potential of biodiesel via in situ transesterification. With high total lipid and TG contents, this mixed culture demonstrated to have a fatty acid composition that could make it a suitable and sustainable feedstock for production of good quality biodiesel. Furthermore, for biodiesel production, the mixed culture that is fed by nutrients in wastewater, by producing comparable quality and quantity of lipids, proved to be a less costly alternative to pure cultures fed by synthetic nutrients and fresh water.

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