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Biodiesel Production and Evaluation Using Chlorella Vulgaris That Has Been Grown in Industrial Dairy Wastewater

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Abstract: In this study, we used Chlorella vulgaris *(C.vulgaris)* grown in the dairy wastewater as biomass to produce a third-generation biodiesel. The lipids were extracted from the biomass and the biodiesel obtained by transesterification. Following ASTM and EN guidelines, the biodiesel was assessed along with its density, viscosity, heating value, and flash point. Gas chromatography was used to evaluate the concentration, composition, and type of fatty acid methyl esters. The results showed a biodiesel yield of 93% with a composition of 33% saturated, 28% monounsaturated, and 37% polyunsaturated. Engine testing was carried out to monitor the levels of CO, CO_2, NO_x, and smoke emissions as well as to assess the biodiesel performance. Results demonstrate the possibility of producing third-generation biodiesel from *C. Vulgaris* cultivated in dairy wastewater. The proposed process valorize the dairy wastewater and might represent an alternative approach for biodiesel production and the disposal of the wastewater.

Keywords: biodiesel; C. Vulgaris microalgae; transesterification; dairy wastewater

1. Introduction

In several countries, water pollution from dairy industries represents a serious environmental problem, which consists mainly of the pollution material released into the water effluents and the relative quantity of water used in the process [1,2]. The wastewater generated from the dairy industry is high in lipids, protein, and carbohydrates but also high in nitrogen and phosphorous-based components [3]. On average, the dairy industry generates from two to five liters of wastewater per liter of milk-based products produced [4]. In the last decades, attention has been paid to the use of microalgae as a resource for treating wastewater from various industrial activities, containing either organic or inorganic components [3,5]. Most of the studies focus on the use of microalgae, from various species, as a nature-based treatment technology or phytoremediation process to remove metals and other hazardous components from contaminated water. Through the last decades, more attention has been attributed to the use of wastewater as a medium for microalgae cultivation to produce secondary algae-based products, which have high interest in different industrial fields like pharmaceuticals, cosmetics, and food, to mention some [6]Gramegna et al., 2020). In such instances, microalgae represent an approach to reduce the issue related to the dairy industry wastewater, which has to be disposed of, but also to valorize the waste to produce indirectly valuable compounds [7]Silva et al., 2018).

Microalgae can assimilate and convert organic pollutants into cellular constituents such as lipids and carbohydrates. Conversely, other photosynthetic organisms, such as vascular plants, are used in conventional bioremediation models, which only use inorganic materials. Thus, achieving pollutant

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reduction in an environmentally friendly way has the potential for further exploitation of microalgal biomass. Moreover, many laboratory and industrial-scale studies report that microalgae could reach up to 70–80% of dry cell weight and be easily converted into highly valuable products among all biofuels [8–11].

Recent studies have demonstrated that microalgae are a promising feedstock for producing biofuels, in particular the so-called third-generation biodiesel [12,13]. In the last decade, there has been a pressing need to find alternative resources to replace fossil fuels. Initially, attention was focused on the production of biodiesel from crops such as soybean, rapeseed, Jatropha, Karanja, Mahua, Palm, and Castor oil, which are referred to as first- and second-generation biofuels. However, the production of these crops has several limitations, including the requirement for land for cultivation, irrigation, and climate dependence, as well as laborious and time-consuming processes. The entire production process, from cultivation to the final biofuels, is also more expensive than fossil fuels. Biofuels can be classified into four generations. First-generation biofuels are derived from biomass that is typically edible. Second-generation biofuels are produced from a variety of feedstocks, ranging from lignocellulosic to municipal solid waste. Third-generation biofuels are currently associated with algal biomass [14]. For the production of third-generation biofuels, several species of microalgae could be used for high lipid production, such as Nannocloropsis spp., Dunaliella salina, and Chlorella spp., with particular attention given to *C.vulgaris* [15,16]. The fourth generation is considered biodiesel, coming from the second and third generations of biomass. After genetic modification, *C. Reinhardtii* and *C. Vulgaris* are the most studied eukaryotic microalgae on which metabolic engineering has been focused.

C.vulgaris is a versatile microalga that can be found in various applications, for instance, as a food coloring, prescription nutrition ingredient, and detox agent [17–19]. It has been shown in clinical studies that supplements based on *C. Vulgaris* can have health benefits, such as reducing hyperlipidemia and hyperglycemia and protecting against oxidative stress, cancer, and chronic obstructive pulmonary disease [20]. Additionally, using *C. Vulgaris* biomass as a feedstock for biofuel production is a sustainable alternative to fossil fuels [21].

To reach high productivity and generate premium-quality biomass, it is important to control the cultivation conditions; including nutrient availability, temperature, and light as the most significant parameters. Choosing the appropriate nutrient medium is crucial for microalgal biomass production, and finding a cost-effective and nutrient-rich medium is important for large-scale operations. While chemical media are commonly used, partially replacing them with organic media like agricultural wastewater, vegetable compost waste, and palm oil mill effluent has been explored as a sustainable option. The present work provides an alternative approach for solving two important themes: dairy wastewater disposal and biofuel production. The microalga *C. Vulgaris* represents the linking point. Herein, we evaluate the growth, nutrient removal, and lipid accumulation of *C. Vulgaris* in dairy wastewater, followed by the extraction and characterization of the microalgae lipids and the production of third-generation biodiesel. The physical-chemical properties of the biodiesel have been evaluated in compliance with EN and ASTM standards.

The main goal of the work is to present a feasible approach to using wastewater to grow biomass, followed by biodiesel production (Figure 1). The objective is to reduce the cost and the environmental impact of both dairy wastewater treatment and biodiesel production and valorize waste, transforming it into valuable products.

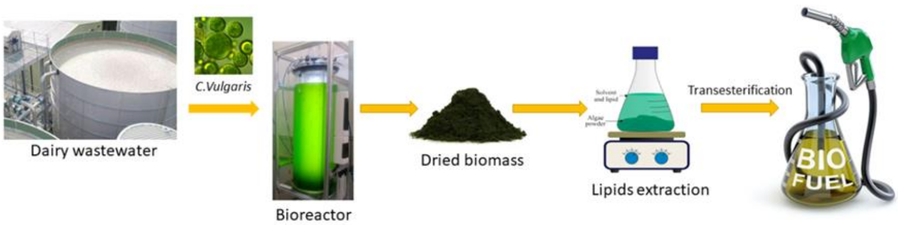

Figure 1. Schematic representation of the full process production of 3rd generation biodiesel.

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2. Materials and Methods

2.2. Physicochemical Characteristics of Wastewater

Wastewater was provided by a local dairy factory in glass bottles and did not undergo any purification process. For such reason, the solid particles were removed by filtration using filter paper and then the filtrate was sterilized in an autoclave for 30 minutes to avoid the development of bacterial contamination.

2.3. Microalgae Strain and Cultivation in Dairy Wastewater

The cultivation of Chlorella vulgaris strain was carried out for two weeks in Erlenmeyer flasks using BG11 medium and stored in an incubator at 25 °C and under a fluorescent lamp. The oxygen was provided by an air-compressed system. Four dilutions of the diary wastewater in distilled water (10, 24, 50, and 75 % v/v) were prepared and sterilized in an autoclave at 120°C for 30 minutes prior the use. Afterward, 25*106 cells/ml of chlorella vulgaris were added to each dilution and exposed to 3500 Lux at 25 °C. Cells counting was performed daily, by microscope (Olympus IX81 fluorescence microscope), until the stationary phase was reached.

2.4. Nutrient Content in the Wastewater

Dairy wastewater contains several organic and inorganic components coming from the processing of the milk. Ammonia, total nitrogen, and phosphate content were evaluated before and after the cultivation of *C. Vulgaris* as it directly affects not only the growth of the microalgae but also the quantitative and qualitative lipids content.

The nutrient removal in the media after algae cultivation was calculated using the following equation [11]:

$$W(\%) = \frac{C_I - C_N}{C_I} \times 100 \qquad [1]$$

Where C_I and C_N represent the average concentration of nutrients (mg/L) at the initial time and at time n, respectively.

2.5. Determination of Algal Biomass

A double beam UV–Vis spectrophotometer (UV-1800, Shimadzu) was used to identify the microalgae cell growth by measuring the absorbance at 680 nm [22]. The dry cell weight (DCW) was calculated as follows; 5 ml of aliquots of culture was filtered by a cellulose acetate membrane filter (0.45 µm pore size). Afterward, the filters were dried for 8 h at 90 ◦C and by the weight of the empty and full filter, the DCW was calculated. Then, by the absorbance values at 680 nm, the biomass concentration was obtained. A blank sample of each culture combination was used to reduce the interference of the medium color in the absorbance area. The filtrate was then centrifugated at 6000 rpm for 15 min; the supernatant was collected and the pellet was lyophilized and used for further qualitative and quantitative analysis.

The biomass growth productivity (PB), expressed as (mg/L/d) was calculated by the equation below [23]:

$$PB = \frac{W_2 - W_1}{t_2 - t_1} \qquad [2]$$

While the specific growth rate, µ (d^{-1}), as follows:

$$\mu = \frac{\ln(W_2 - W_1)}{t_2 - t_1} \qquad [3]$$

W_1 and W_2 are the dry cell weight (mg/L) at time starting (t_1) and ending (t_2) time, respectively.

2.6. Extraction of Algal Oil from C. Vulgaris

Algal oil was extracted from C. Vulgaris following the Folch method with minor modification [24]. 5 mL of methanol and 5 mL of chloroform were added to 100 mg of dried and ground algal biomass. The biomass-solvents mixture was vigorously shaken for 30 minutes to favor the extraction. These steps were repeated twice to maximize the extraction. Methanol: chloroform: distilled water

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(1:2:1) mixture was then added and the mixture again vigorously shacked and then let to settle in a separating funnel. The solvent layer was separated by using anhydrous Na_2SO_4. The final product was collected and dried in a ventilated oven at 50°C until a constant weight was achieved. The lipid content was quantified gravimetrically.

2.7. Biodiesel Production and FAME Profile

The biodiesel was obtained by transesterification following the procedure reported in our previous work [25]. In brief, 100 g of algae oil was firstly heated at 120 °C for 15 min under stirring to remove the moisture and then transferred to a 250 mL glass flask. Afterward, a 50 ml of methanol solution containing 2g of the catalyst (KOH) was prepared. A defined volume of the methoxide/ethoxide solution was poured into the oil to have the alcohol: oil molar ratio of 12:1, and the reaction was left to run at 65 °C for 30 minutes.

Afterward, the stirring and temperature were shut off, the solution transferred in a separating funnel and the two phases were left to separate by gravity. The biodiesel was separated from the glycerol and washed one time with an aqueous acetic acid solution (1% v/v) and three times with distilled water to remove side products, unreacted catalysts, and soaps. Then, the obtained biodiesel was heated at 150 °C for 30 min to remove the residual moisture coming from the washing process.

The biodiesel yield was calculated using the following equation [26]:

$$Y = \frac{M_b}{M_o} \quad [4]$$

where Y represents the yield (g), M_b is the mass of biodiesel (g), and M_o is the mass of algae oil (g).

The qualitative and quantitative analysis of the fatty-free methyl acid was performed by gas chromatography (FID detector) using the methodology reported in our previous work [25].

The sample was directly collected from the separation funnel and before being analyzed, the biodiesel was washed as reported above.

The following gas chromatography setup has been used: capillary column 30 × 0.31 × 0.25; helium as gas carrier, flow rate of 1 mL/min; temperature injection 280 °C; temperature detector 300°C; and temperature ramp 10 °C /min starting from 220 °C.

The total FAME (Fatty Acid Methyl Esters), expressed in percentage, was calculated referring to the total mass of the biodiesel using the following equation [26]:

$$FAME\ (\%) = \frac{\sum m}{b} \times 100 \quad [5]$$

where m is the mass of each fatty acid (g) and b is the total biodiesel mass (g).

The total content of FAME was compared to the EN 14214 standard. The measurements were performed in triplicate

2.8. Biodiesel Physical-Chemical Evaluation

2.8.1. Kinematic Viscosity

The kinematic viscosity represents one of the reasons why biodiesel is used as an alternative fuel instead of using directly vegetable oils or animal fats [27]. The kinematic viscosity of the biodiesel, the petrol-diesel, and their blends has been measured following the EN ISO 3104 and EN ISO 3105 procedures. According to EN ISO 3104, 05, the kinematic viscosity must be in the range of 1.9 and 6.0 mm2/s for the. The dynamic viscosity was determined using Anton-Paar MCR 502 at 40 °C as already described in the report in our previous work [25]. Each measurement has been performed in triplicate at three shear rates: 7.5 s^{-1}; 15 s^{-1} and 37 s^{-1}.

By knowing the dynamic viscosity, the kinematic viscosity was obtained by the following equation:

$$v = \frac{\eta}{\rho} \quad [6]$$

where v, η, ϱ, are the kinematic viscosity (m^2/s), the fluid density (Kg/m^3), and the dynamic viscosity (Pa·s), respectively.

2.8.2. Calorific Value

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The calorific value was measured by the oxygen bomb calorimeter following the ASTMD240-14 standard. The calorimeter was calibrated using benzoic acid at 20 °C [28].

2.8.3. Flash Point

The flash point of the biodiesel, fossil diesel, and their blends was measured with a Pensky–Martens closed-cup apparatus following the protocol reported by the EN 14214 standard. According to the EN standard, the minimum temperature is 101°C.

2.8.4. Density

The density is defined as the mass per unit volume. In biodiesel, there is a reverse correlation between the molecular weight of the fatty acid chains and the amount of unsaturation. In other words, the density increases when the molecular weight and the unsaturation decrease [29]. Generally, biodiesel fuels have a higher density than fossil diesel and produce more than three times the energy of the same amount of fossil fuel [30].

The biodiesel density was measured following the EN ISO (International Organization for Standardization) 12185 test method. The EN 14214 sets the density at 15 °C at between 860-900 kg/m^3.

2.8.5. Cetane Number

The cetane number (CN) represents the ignitability of the fuel. The CN is a fundamental property of biodiesel and is critical during engine start, especially in cold conditions [30]. CN is defined as the percentage by volume of the normal cetane in a mixture of normal cetane and α – methyl naphthalene which has the same ignition characteristics (ignition delay) as the test fuel when combustion is carried out in a standard engine under specified operating conditions [27].

The CN requirement for the engine depends on the composition of the fuel, which is related to the feedstock [31]Demirbas, 2007). The CN decreases as the unsaturation increases. Low cetane number leads to long ignition delay, conversely, biodiesel containing low unsaturated fatty acid has a higher CN meaning a fast and smooth engine operation [32,33]. Compared to fossil diesel, the CN in biodiesel is generally higher.

2.8.6. Elemental Analysis

The biodiesel and diesel content in carbon, hydrogen, nitrogen, sulfur, and oxygen has been measured by Thermo Scientific Flash 2000 CHNS/O.

2.9. Generator Performance Test

To evaluate the engine performance using the biodiesel-made oil extracted by *C.Vulgaris* cultivated in dairy wastewater, alone and blended with the fossil diesel, the electric generator tests were performed.

The generator was fueled with pure biodiesel (100%) and with biodiesel-diesel blends at 10, 25, 50, and 75% biodiesel.

The electric generator (Denyo DA-2805) connected to an electric dynamometer operated at 100%, 75%, 50%, 25%, and 0% of loading and 3,000 rpm.

At each load percentage, the following were measured; i) the engine speed; ii) the fuel flow rate; and iii) the brake torque. The concentration of CO, CO_2, and NO_x emitted were also monitored by an emission analyzer (Testo model 350XL), and the smoke concentration by a smoke meter (HORIBA model MEXA-130S).

The brake-specific fuel consumption (BSFC) was calculated as follows [34]:

$$BSFC = \frac{F_c}{P_b} \quad [7]$$

where Fc is the fuel consumption (g/h) while Pb represents the brake power (kW).

The brake mean effective pressure (BMEP) is an index of the engine load and was obtained by the equation [34]:

$$BMEP = \frac{\pi}{4VT} \quad [8]$$

where T is the engine torque (N·m) and V is the stroke volume of the engine piston (m³).

2.10. Statistical Analysis

Data for each parameter were analyzed statistically using the Analysis of variance (ANOVA)

3. Results and Discussion

3.1. Effect of Wastewater Dilution on C.vulgaris Biomass

In Table 1 the physicochemical characteristics of the wastewater used for growing algae are reported (mg/L).

Table 1. Chemical characteristics of the wastewater used for algae growing. Values are expressed in mg/L.

Parameters	NH_3	NO_3	PO_4	pH
	52	69	600	8.0

The results of the influence of the wastewater dilution at 10, 25 50, and 75% v/v in distilled water on the *C. Vulgaris* biomass are resumed in Figure 2A.

The algae biomass growth in the dairy wastewater at 10, 25, 50, and 75 % v/v dilutions was compared with the control growth medium. From the trend in Figure 2, the highest yield is observed at 50% dilution, and the highest biomass concentrations were found to be 3,59 (g/L) and 3.13 g/L at 50% and 25% dilution, respectively. Biomass in the control medium resulted in 2,05 g/L followed by 0,78 g/L and 0,57 g/L for 75% and 10% dilution.

Following the trend (Figure 2A) at the two extremes (10% and 75% dilution) the microalgae enter the stationary phase on the 10th day while at the same time, those cultivated at 50% and 25% v/v dilution are still growing and enter in the stationary phase only at the 12th day.

Generally, *C. Vulgaris* takes up to 14 – 16 days to reach its stationary growth phase with the BG-11 culture medium (control) [35].

The primary role of the culture medium is to initiate the exponential growth process until the microalgae have adapted to their new medium and to keep the stationary phase going. Thus, it is important to identify the optimal dilution of the dairy wastewater. The light source also plays a significant role in biomass growth; herein, 24 h of LED fluorescence light is on both sides of the photobioreactor to avoid biomass loss during the night.

The light availability strongly influences the algae growth and the media dilution plays an important role. The control media is colorless while the dairy wastewater is opaque. The different opacity in the wastewater-based media might obstruct light from reaching the microalgae and limit their growth. It can be the reason explanation of the lowest biomass growth at higher concentrations of wastewater [3].

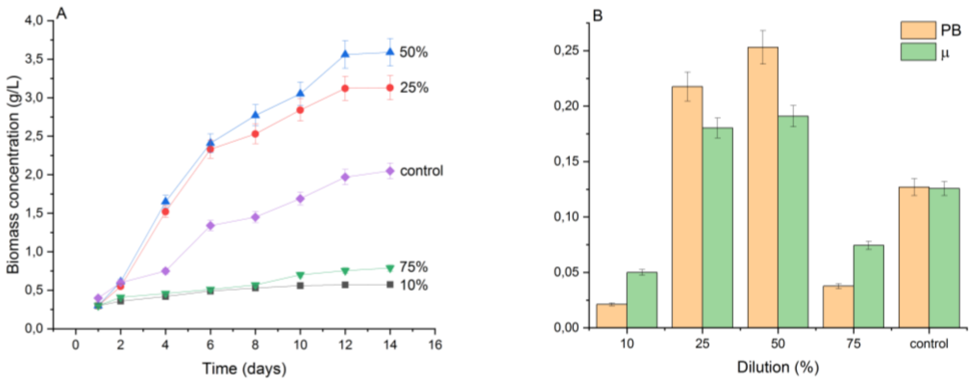

Figure 2. A) Biomass concentration expressed in g/L versus time (days) at different wastewater dilutions. Control represents water as a growing medium. The data are expressed as the average with the respective SD (n=3); B) biomass growth productivity (PB) and biomass growth rate (μ) at different wastewater dilutions (v/v in %). The results refer to the 14th day of cultivation. The average values are reported with the SD (n=3).

In Table 2, the calculated parameters including daily biomass productivity and basic growth rate of *C. Vulgaris* on the 14th day are resumed. Although the medium with lower dilution was the lightest in color intensity after the control, the biomass productivity measured was almost 6 times lower than in the control.

Table 2. Dry cell weight content (DCW), biomass productivity (PB), and specific growth rate (μ) of *C. Vulgaris* grown in control media and dairy wastewater and various dilutions. Support of Figure 2 B.

Growing media	DCW (g/L)	Pb (mg/L/d)	⚜(d^{-1})
Control	2.05±0.08	0.12±0.01	0.12± 0.02
10%	0.57±0.01	0.02±0.01	0.05±0.01
25%	3.13±0.02	0.21±0.03	0.18±0.03
50%	3.59±0.06	0.25±0.01	0.19±0.03
75%	0.78±0.02	0.03±0.01	0.07±0.01

As reported in a similar work [22] the reduction in biomass growth could be ascribed to the lack of nutrient supply in the culture medium. For an optimal cultivation process, a proper mix of nutrient sources with adequate light penetration must be ensured. Thus, it is not desirable to entirely substitute the inorganic medium [36]. Other influencing factors such as temperature, and CO_2 supply were kept constant during the cultivation process to avoid any additional influences on biomass development [37].

3.2. Effect of Different Dilutions of Wastewater on Nutrient Removal

The data in Table 3 shows a difference between nitrates, phosphate, and ammonia biological removal at different wastewater dilutions. The higher values of nitrate and ammonia removal are observed at 75% dilution, while phosphate is at 50%. The lowest values for all the parameters are at 10% dilution.

Table 3. Removal of nitrate, phosphate, and ammonia from wastewater at different dilutions by the microalgae after the 14th day of growing.

	Wastewater Dilution (v/v)			
Parameters	**10%**	**25%**	**50%**	**75%**
NO_3	17.14 ± 0.03	19.23 ± 0.01	29.16 ± 0.05	38.34 ± 0.01
PO_4	19.87 ± 0.01	23.44 ± 0.01	39.72 ± 0.01	39.07 ± 0.01
NH_3	19.53 ± 0.01	36.18 ± 0.01	41.94 ± 0.01	43.61 ± 0.01

The reason for efficient performance in wastewater and effluent of the dairy industry may be the presence of enough nutrients, including nitrogen and phosphorus, essential microalgae growth. Previous works [3,38] have shown that algae need NH3-N and phosphorus to produce amino acids and phospholipids respectively [39]. In another study dealing with the isolation of Chlorella vulgaris from pig wastewater and its ability to remove nutrients from untreated wastewater, it has been reported that total nitrogen removal after 12 days is 90% which concerns the ability of *C.vulgaris* to absorb nitrogen; this study is consistent with the present study.

3.3. Lipid Extraction from Chlorella vulgaris Biomass

The green microalga, *C. Vulgaris* was found to be dominant in the environment producing a greater quantity of biomass and high lipids yield [3,11,39]. The total content of lipids for the cultivated *C. Vulgaris* was found to be 24 ± 2% depending upon the different cell disruption methods adopted (data not shown). It was comparatively higher than a recent study with a lipid content of 18.7 wt. % was reported [40]. Microalgae lipids extraction is known to increase with the degree of cell disruption. During the extraction, cells are disintegrated and the intracellular lipids and cellular bodies are released into the surrounding microenvironment [41,42]. Herein, the grinding method was used to disrupt the cell components and maximize the lipid release. The lipid content in *C. Vulgaris* was found to be 3 mg/L after grinding. These results agree with those reported in previous studies [43]. This method has been demonstrated to be valid for scaling up.

3.4. Physical-Chemical Characterization

3.4.1. Biodiesel Yield, Kinematic Viscosity, Density, and Calorific Value

In our previous work [25], we found the optimal condition for the transesterification reaction using methanol and KOH to maximize the yield. Previously we used a different feedstock, including sunflower and rapeseed oil as a source of lipids, however here we propose the same condition but a different oil source. The reaction conditions were as follows; i) methanol to oil molar ratio 12; ii) KOH as catalyst (40 mg/ml methanol); iii) reaction time and temperature 30°C and 65°C, respectively. Herein, the biodiesel yield was 93 % ± 4, which is slightly less than the value obtained using rapeseed and sunflower oil. However, comparing the obtained yield, with those reported in similar works in which *C. Vulgaris* has been used as biomass for biodiesel production [44–46] the yield can be considered satisfactory as it exceeds the 90g per 100g of vegetable oil (Table 4).

Table 4. Physical-chemical properties of biodiesel obtained by *C.Vulgaris* compared to diesel and the EN and ASTM values. *cetane number of oil extracted from *C.Vulgaris.*

	Biodiesel from *C.Vulgaris*	**Diesel**	**EN14214**	**ASTM D61**
Yield (g/100g oil)	93 ± 4			
K.Viscosity (40 °C mm^2/s^2)				
7.5 s^{-1}	4.9 ±0.3			
15 s^{-1}	4.3 ±0.1	2.6	3.5-5.0	1.9-6.0
37 s^{-1}	4.1 ±0.4			
Calorific value (MJ/Kg)	37.5 ±1.4	42.2		

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Density (15 °C, g/cm3)	0.875 ±0.019	0.850	0.860-0.900	0.880
Flashpoint (°C)	131 ±2	73	101 min	130 min
Cetane number	50.21 ±0.13 (36.19 ±0.13)*	56.66 ±0.19		
Carbon	81.1	87.1		
Hydrogen	10.6	13.6		
Oxygen	7.5			
Nitrogen	0.8			
C/H	7.6	6.4		

In the automotive, the biodiesel viscosity represents a key parameter to determine as it directly influences the starting and the performance of the diesel engine. The kinematic viscosity at 40 °C is the parameter required by biodiesel and petroleum diesel standard.

As reported in published works dealing with biodiesel production by transesterification using KOH as a catalyst [47]; the viscosity is a result of the transesterification conditions and in particular the type of alcohol and the alcohol to oil molar ratio. The kinematic viscosity measured at 40°C, following the methodology described by the EN standards, falls in the range between 3.6 and 5.2 mm^2/s^2 in a reverse relation with the shear rate. In fact, at a higher shear rate, the lowest kinematic viscosity has been observed. However, as can be seen, the viscosity values at each shear rate are comparable and no statistical differences are present.

Together with the kinematic viscosity, the biodiesel density provides information to predict the engine power performance and consequently the fuel consumption [48]. The biodiesel's density is related to the purity of the feedstock, the fatty acids composition, and the total content. The density at 15°C was 0.875 ± 0.019 and it represents an acceptable value as it falls in the required range of 0.860 – 0.900 gm/cm3

The calorific value is the thermal energy released by the combustion of a unit of fuel, giving the energy content of the fuel [49]. As for the viscosity, the calorific value depends on the nature of the feedstock and only in minor by the transesterification conditions. Using *C. Vulgaris* as fat source, the calorific value of the produced biodiesel was 37.5 ± 1.4 MJ/Kg, which is a positive value in line with the EN 14214 and ASTM D6751 requirements.

3.4.2. Flash Point

The flash point is the lowest temperature at which a sufficient concentration of vapors is released from the fuel, which upon mixing with air ignite [50]. The presence of fatty acid methyl esters is responsible for the higher biodiesel flashpoint compared to the fossil diesel making it safer to handle at high temperatures. As biodiesel, in the automotive, is used in a mixture with fossil diesel, the flashpoint of pure biodiesel and blends of biodiesel-diesel has been evaluated and the results are reported in Figure 3.

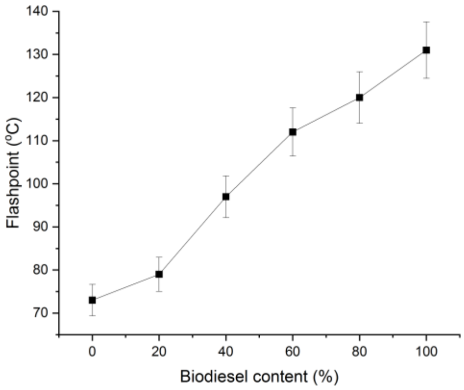

Figure 3. Correlation between flash point (°C) and biodiesel content in the biodiesel-diesel mixture. 0% diesel, 100% pure biodiesel. The average value and the S.D. are reported (n=3).

The flash point in pure biodiesel varies according to the source and the quality of the oil; herein the flash point temperature, obtained, was 131°C, almost double that of the fossil diesel 73°C. As already reported [25], in the biodiesel-diesel blend by increasing the content of diesel, the flash point decreases I in a quasi-linear correlation. The clear correlation between the biodiesel quantity in a diesel-biodiesel blend and its flash point. This means that the experimental determination of a flash point temperature also provides information regarding the biodiesel content of diesel-biodiesel blends. Conversely, if the percentage of biodiesel in a diesel-biodiesel blend is known, the flashpoint can be estimated if the flash points of diesel and pure biodiesel. From the obtained results, it is also clear that, for diesel-biodiesel blends with lower contents of biodiesel, the flash point is mainly determined by that of the mineral diesel.

3.4.3. FAME Composition

The lipids extracted from *C. Vulgaris* were in the range of 9-11% of the dried weight (30 g of the algal dried biomass). The content, obtained by gravimetric analysis, makes the selected biomass and its growing approach a suitable strategy for the 3rd generation type biodiesel production.

It is well established that the quality of the biodiesel is determined by the fatty acid content, in particular their molecular weight and the saturation and unsaturation degree. Biodiesels containing saturated and mono-unsaturated fatty acids result in superior quality as reported by international standards. The FAME (Fatty Acid Methyl Ester) profile of the obtained biodiesel from *C.Vulgaris* biomass is resumed in Table 5. The data shows a major content of saturated and mono-unsaturated FAME. The mains are Tridecanoic acid (C13:0), Palmitic acid (C16:0), Erucic acid (22:1) Myristoleic acid (C14:1), Linolelaidic acid (C18:2), G Linolenic acid (C18:3). The obtained results indicate that the biodiesel obtained from *C. vulgaris* cultivated in dairy wastewater has 33.53% saturated, 28.68% monounsaturated and 37.12% polyunsaturated fatty acids content [24].

Table 5. Fatty Acid Methyl Ester (FAME) present in biodiesel from *C.Vulgaris* cultivated in dairy wastewater. The content is expressed in percentages referring to the total amount of FAME.

FAME (Fatty Acid Methyl Ester)	(%)
Tridecanoic acid (13:0)	4.02
Myristoleic acid (14:1)	7.95

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Palmitic acid (16:0)	52.4
Stearic acid	17.3
Linolelaidic acid (18:2)	6.83
Linoleic acid (18:3)	7.90
Oleic acid	5.21
cis, 11, 14-Eicosadienoic acid (20:2)	4.70
Arachidonic acid (20:4)	3.09
Erucic acid (22:1)	4.46
Tricosanoid acid (23:0)	3.33

3.5. Generator Performance Test

In Figure 4 A and B, the generator performance test results are reported. As can be seen, no significant differences are observable in the BMEP among the 3 formulations; fossil diesel, biodiesel, and blends fossil diesel-biodiesel. Conversely, differences are observable in the BSFC, for pure biodiesel was almost 20% higher than in the mineral diesel. Following reported studies, such difference could be ascribed to the difference in the calorific value of the two fuels. The blends, as expected have values of BSFC displaced between the 2 extremes; 100% biodiesel and 100% fossil diesel.

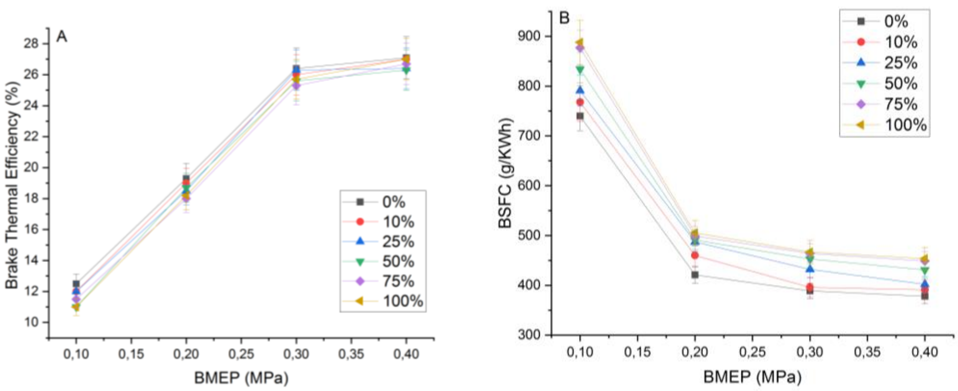

Figure 4. A) brake thermal efficiency (%) and B) brake specific fuel consumption VS BMEP in various biodiesel-diesel blends. 0%= pure diesel; 100% = pure biodiesel. Average values are reported with SD (n=3).

Figure 5 presents correlations between biodiesel concentration and the variation in emissions. As increasing biodiesel concentration, CO and smoke emissions decreased, while NO_x and CO_2 increased. The percent change in emissions of CO_2, NO_x, CO, and smoke, using pure-form biodiesel, was +18%, +15%, -60%, and -19%, respectively. These results were attributed to the improvement of combustibility and the increase in combustion temperature due to oxygen-containing biodiesel. Biodiesel has carbon neutrality, which prevents the increase of CO_2 emission in the atmosphere. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

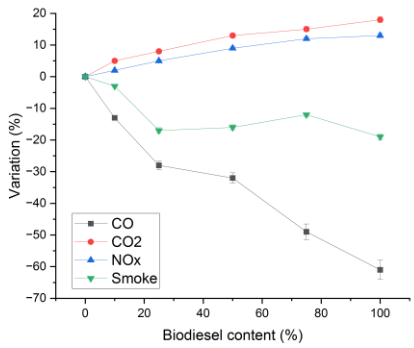

Figure 5. Variation of CO, CO_2, NO_x, and smoke emissions in different blends of diesel-biodiesel. The variation is expressed as a percentage in comparison to the emission from diesel. (0%= pure diesel considered as zero point; 100% = pure biodiesel). Average values are reported with SD (n=3).

4. Conclusions

The application of dairy wastewater as a growth medium for *C.Vulgaris* may result in a promising production of lipids. The resulting lipids can then be used for producing biodiesel, an environmentally friendly substitute for conventional petroleum-based fuels. The outcomes show that dairy wastewater, which is high in nutrients like phosphorus and nitrogen which are eutrophic agents, can support the growth of *C. Vulgaris* algae. When cultivated on dairy effluent instead of traditional growth media, the algae's lipid content was found to be much higher, indicating that utilizing this wastewater could substantially boost the synthesis of lipids. By providing a method of wastewater treatment, the use of dairy effluent for the cultivation of algae additionally offers the added benefit of reducing the environmental effect of dairy farming. Growing *C. Vulgaris* algae in dairy wastewater has the potential to be a profitable and environmentally friendly approach to produce biodiesel. A new sector for the generation of renewable energy from dairy effluent could be established as a result of more research and development in this field.

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References

1. Raghunath, B. V., Punnagaiarasi, A., Rajarajan, G., Irshad, A., Elango, A., & Mahesh Kumar, G. (2016). Impact of dairy effluent on environment—a review. Integrated Waste Management in India: Status and Future Prospects for Environmental Sustainability, 239-249)

2. Choudhury, P.; Ray, R.N.; Bandyopadhyay, T.K.; Basak, B.; Muthuraj, M.; Bhunia, B. Process Engineering for Stable Power Recovery from Dairy Wastewater Using Microbial Fuel Cell. Int. J. Hydrogen Energy 2021, 46, 3171–3182)
3. Khalaji, M., Hosseini, S. A., Ghorbani, R., Agh, N., Rezaei, H., Kornaros, M., & Koutra, E. (2021). Treatment of dairy wastewater by microalgae Chlorella vulgaris for biofuels production. *Biomass Conversion and Biorefinery*, 1-7.
4. Dongre, A.; Sogani, M.; Sonu, K.; Syed, Z.; Sharma, G. Treatment of Dairy Wastewaters: Evaluating Microbial Fuel Cell Tools and Mechanism. In Environmental Issues and Sustainable Development; IntechOpen: Rijeka, Croatia, 2020
5. You, X., Yang, L., Zhou, X., & Zhang, Y. (2022). Sustainability and carbon neutrality trends for microalgae-based wastewater treatment: A review. Environmental Research, 209, 112860)
6. Gramegna, G., Scortica, A., Scafati, V., Ferella, F., Gurrieri, L., Giovannoni, M., & Benedetti, M. (2020). Exploring the potential of microalgae in the recycling of dairy wastes. *Bioresource Technology Reports*, 12, 100604
7. Silva CKD, Almeida ACAD, Costa JAV, Morais MGD (2018) Cyanobacterial biomass by reuse of wastewater-containing hypochlorite. Ind Biotechnol 14:265–269
8. Brennan L, Owende P (2010) Biofuels from microalgae—areview of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14:557–577
9. Wang L, Min M, Li Y, Chen P, Chen Y, Liu Y, Wang Y, Ruan R (2010) Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnol 162:1174–1186)
10. Chisti Y. Biodiesel from microalgae. *Biotechnol Adv*. 2007;25:294–306.
11. Ratomski P, Hawrot-Paw M. Influence of nutrient-stress conditions on Chlorella vulgaris biomass production and lipid content. Catalysts. 2021;11:573.
12. Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews, 14(1), 217-232
13. Chen, J., Li, J., Dong, W., Zhang, X., Tyagi, R. D., Drogui, P., & Su-rampalli, R. Y. (2018). The potential of microalgae in biodiesel production. Renewable and Sustainable Energy Reviews, 90, 336-346.
14. Roland Arthur Lee, Jean-Michel Lavoie, From first- to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity, Animal Frontiers, Volume 3, Issue 2, April 2013, Pages 6–11.
15. Hosseini Tafreshi A, Shariati M (2009) Dunaliella biotechnology: methods and applications. *J Appl Microbiol* 107:14–35
16. Tao R, Kinnunen V, Praveenkumar R, Lakaniemi A-M, Rintala JA (2017) Comparison of Scenedesmus acuminatus and Chlorella vulgaris cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge. J Appl Phycol 29:2845–2856
17. Gouveia, L., Batista, A. P., Miranda, A., Empis, J., & Raymundo, A. (2007). Chlorella vulgaris biomass used as colouring source in traditional butter cookies. Innovative food science & emerging technologies, 8(3), 433-436.
18. Martins, C. F., Pestana, J. M., Alfaia, C. M., Costa, M., Ribeiro, D. M., Coelho, D., & Prates, J. A. (2021). Effects of Chlorella vul-garis as a feed ingredient on the quality and nutritional value of weaned piglets' meat. Foods, 10(6), 1155.)
19. Kim, Y. J., Kwon, S., & Kim, M. K. (2009). Effect of Chlorella vul-garis intake on cadmium detoxification in rats fed cadmium. Nutrition Research and Practice, 3(2), 89-94.

20. Galasso, C., Gentile, A., Orefice, I., Ianora, A., Bruno, A., Noonan, D.M., Sansone, C., Albini, A., Brunet, C., 2019. Microalgal derivatives as potential nutraceutical and food supplements for human health: A focus on cancer prevention
21. Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae : current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb. Cell Fact*. 17 (36), 1–21.
22. Peter, A. P., Chew, K. W., Koyande, A. K., Yuk-Heng, S., Ting, H. Y., Rajendran, S., ... & Show, P. L. (2021). Cultivation of Chlorella vulgaris on dairy waste using vision imaging for biomass growth monitoring. *Bioresource technology*, *341*, 125892.
23. Goh, B. H. H., Ong, H. C., Cheah, M. Y., Chen, W. H., Yu, K. L., & Mahlia, T. M. I. (2019). Sustainability of direct biodiesel synthesis from microalgae biomass: A critical review. *Renewable and Sustainable Energy Reviews*, *107*, 59-74.
24. Davoodbasha, M., Pugazhendhi, A., Kim, J. W., Lee, S. Y., & Nooruddin, T. (2021). Biodiesel production through transesterification of Chlorella vulgaris: Synthesis and characterization of CaO nanocatalyst. *Fuel*, *300*, 121018.
25. Khan, E., Ozaltin, K., Spagnuolo, D., Bernal-Ballen, A., Piskunov, M. V., & Di Martino, A. (2023). Biodiesel from Rapeseed and Sunflower Oil: Effect of the Transesterification Conditions and Oxidation Stability. *Energies*, *16*(2), 657.
26. Roosta, A., & Bardool, R. (2019). A predictive correlation for dynamic viscosity of fatty acid methyl esters and biodiesel. *Journal of the American Oil Chemists′ Society*, *96*(7), 741-750.
27. Hossain, A. K., & Davies, P. A. (2010). Plant oils as fuels for compression ignition engines: A technical review and life-cycle analysis. *Renewable energy*, *35*(1), 1-13.
28. Verevkin, S. P., Pimerzin, A. A., Glotov, A. P., & Vutolkina, A. V. (2022). Biofuels energetics: Measurements and evaluation of calorific values of triglycerides. *Fuel*, *326*, 125101.
29. Giakoumis, E. G. (2013). A statistical investigation of biodiesel physical and chemical properties, and their correlation with the degree of unsaturation. *Renewable Energy*, *50*, 858-878.
30. Tesfa, B., Mishra, R., Gu, F., & Powles, N. (2010). Prediction models for density and viscosity of biodiesel and their effects on fuel supply system in CI engines. *Renewable energy*, *35*(12), 2752-2760.
31. Demirbas, A. (2007). Progress and recent trends in biofuels. *Progress in energy and combustion science*, *33*(1), 1-18.
32. Nabi, M. N., Rahman, M. M., & Akhter, M. S. (2009). Biodiesel from cotton seed oil and its effect on engine performance and exhaust emissions. *Applied thermal engineering*, *29*(11-12), 2265-2270.
33. Chalatlon, V., Roy, M. M., Dutta, A., & Kumar, S. (2011). Jatropha oil production and an experimental investigation of its use as an alternative fuel in a DI diesel engine. *Journal of Petroleum Technology and Alternative Fuels*, *2*(5), 76-85.
34. Emaish, H., Abualnaja, K. M., Kandil, E. E., & Abdelsalam, N. R. (2021). Evaluation of the performance and gas emissions of a tractor diesel engine using blended fuel diesel and biodiesel to determine the best loading stages. *Scientific Reports*, *11*(1), 9811.
35. Chew, K. W., Chia, S. R., Show, P. L., Ling, T. C., Arya, S. S., & Chang, J. S. (2018). Food waste compost as an organic nutrient source for the cultivation of Chlorella vulgaris. *Bioresource technology*, *267*, 356-362.
36. Machado, A., Pereira, H., Costa, M., Santos, T., Carvalho, B., Soares, M., ... & Silva, J. (2020). Development of an organic culture medium for autotrophic production of Chlorella vulgaris biomass. *Applied Sciences*, *10*(6), 2156.
37. Eloka-Eboka, A. C., & Inambao, F. L. (2017). Effects of CO2 sequestration on lipid and biomass productivity in microalgal biomass production. *Applied Energy*, *195*, 1100-1111.
38. Kothari R, Prasad R, Kumar V, Singh D (2013) Production of biodiesel from microalgae Chlamydomonas polypyrenoideum grown on dairy industry wastewater. *Bioresour Technol* 144:499– 503.
39. Barsanti L, Gualtieri P (2014) Algae: anatomy, biochemistry, and biotechnology. CRC press, p 295.

 doi:10.20944/preprints202403.1061.v1

40. dos Santos, R. R., Moreira, D. M., Kunigami, C. N., Aranda, D. A. G., & Teixeira, C. M. L. L. (2015). Comparison between several methods of total lipid extraction from Chlorella vulgaris biomass. *Ultrasonics sonochemistry*, *22*, 95-99.
41. Kumar, R. R., Rao, P. H., & Arumugam, M. (2014). Lipid extraction methods from microalgae: a comprehensive review. Front Energy Res 2: 61–69.
42. Couto, D., Melo, T., Conde, T. A., Moreira, A. S., Ferreira, P., Costa, M., & Domingues, P. (2022). Food grade extraction of Chlorella vulgaris polar lipids: A comparative lipidomic study. *Food Chemistry*, *375*, 131685.
43. Nguyen, T. D. P., Nguyen, D. H., Lim, J. W., Chang, C. K., Leong, H. Y., Tran, T. N. T., ... & Show, P. L. (2019). Investigation of the relationship between bacteria growth and lipid production cultivating of microalgae Chlorella vulgaris in seafood wastewater. *Energies*, *12*(12), 2282.
44. Asadi, P., Rad, H. A., & Qaderi, F. (2020). Lipid and biodiesel production by cultivation isolated strain Chlorella sorokiniana pa. 91 and Chlorella vulgaris in dairy wastewater treatment plant effluents. *Journal of Environmental Health Science and Engineering*, *18*, 573-585.
45. Scarponi, P., Izzo, F. C., Bravi, M., & Cavinato, C. (2021). C. vulgaris growth batch tests using winery waste digestate as promising raw material for biodiesel and stearin production. *Waste Management*, *136*, 266-272.
46. Katırcıoğlu Sınmaz, G., Erden, B., & Şengil, I. A. (2023). Cultivation of Chlorella vulgaris in alkaline condition for biodiesel feedstock after biological treatment of poultry slaughterhouse wastewater. *International Journal of Environmental Science and Technology*, 20(3), 3237-3246.
47. Sharma, A., Kodgire, P., & Kachhwaha, S. S. (2020). Investigation of ultrasound-assisted KOH and CaO catalyzed transesterification for biodiesel production from waste cotton-seed cooking oil: Process optimization and conversion rate evaluation. *Journal of Cleaner Production*, *259*, 120982.
48. Aydın, S. (2020). Comprehensive analysis of combustion, performance and emissions of power generator diesel engine fueled with different source of biodiesel blends. Energy, 205, 118074.
49. Dey, P., Ray, S., & Newar, A. (2021). Defining a waste vegetable oil-biodiesel based diesel substitute blend fuel by response surface optimization of density and calorific value. Fuel, 283, 118978.
50. Santos, S. M., Nascimento, D. C., Costa, M. C., Neto, A. M., & Fregolente, L. V. (2020). Flash point prediction: Reviewing em-pirical models for hydrocarbons, petroleum fraction, biodiesel, and blends. Fuel, 263, 116375.