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Biodiesel Production from *Oedogonium brevicingulatum* by in situ Transesterification

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Abstract: The production of biodiesel from the green alga *Oedogonium brevicingulatum* was studied phenotypically and genetically using a PCR reaction by the *18 S rDNA* gene. The production method used in situ transesterification, which involves direct production from the entire biomass without extraction in a single step. The biodiesel properties, including density, kinematic viscosity, cloud point, pour point, and acid value, were determined; their respective values are 0.920 g /cm^3, 5.51 mm^2 /s, 5 °C, 2 °C, and 0.54 mg KOH/g. The results also revealed that direct transesterification yielded about 75% biodiesel. By combining lipid extraction with direct transesterification, it is possible to produce biodiesel with fewer stages than would otherwise be required, eliminating the need for isolated and filtered algal oil.

Keywords: Biodiesel, catalyst, direct transesterification, molecular identification, phenotypic identification.

Introduction

The great development that has accompanied human life in all of its industrial, commercial, and economic aspects has relied primarily on fossil fuels, which pollute the air through greenhouse gas (GHG) emissions and contribute to global climate change (Abdullah & Hussein, 2020; Lamb et al., 2021; Kabeyi & Olanrewaju, 2022). Furthermore, global energy consumption is constantly increasing, resulting in diminishing fuel resources. Thus, production of alternative fuels and renewable energy sources is required (Kabeyi & Olanrewaju, 2022). Biodiesel is gaining popularity as a result of the massive increase in the depletion of fossil fuels, concerns about environmental pollution, and increased demand for transportation fuels (Bidir et al., 2021). Biodiesel can be used as a more sustainable alternative to fossil fuels because it is made from biomass (Mahlia et al., 2020; Alrubayae & Kadhim, 2020), there are many reasons to replace fossil fuels with biodiesel include the fact that bio diesel is biodegradable, renewable, and has low emissions of carbon monoxide, carbon dioxide, and hydrocarbons. However, the cost of raw materials remains a

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major constraint for biodiesel production (Akubude et al., 2019; Asl et al., 2020). As a result, new sources of fat must be sought (Ong et al., 2014; Silitonga et al., 2019). This has resulted in multiple generations of biodiesel production: first-generation biodiesel (made from edible vegetable oil, animal fats, and waste oil), second-generation biodiesel (made from non-food crop vegetable oils), and third-generation biodiesel (made from lipids extracted from algae) (Khan et al., 2017; Alwan & Al, 2021; Tsavatopoulou et al., 2021). Algae are a well-known group of photosynthetic organisms. The majority of them are autotrophic, while the remainder are heterotrophic. Microalgae can grow in various wastewaters and convert sunlight and atmospheric CO_2 into biomass. Their cells can convert and store energy instead of using it for growth and development (Barsanti et al., 2008; Khan et al., 2018). Therefore, microalgal biomass can be investigated as new systems for biofuels production that are a potential substitute for fossil fuels due to their renewability, sustainability, and short life cycle of algal growth, which does not require additional lands, improves air quality by absorbing atmospheric CO_2, and uses minimal water (Wang et al., 2008; Jaffer et al., 2022). Algae can be used to produce biodiesel through either a two-step transesterification process or a direct transesterification process. The oil is extracted from the algae and then converted into biodiesel in two steps by reacting the oil with alcohols in the presence of a catalyst. Whereas in direct transesterification, biodiesel is produced through direct contact between algae biomass and alcohol in the presence of a catalyst, and due to the removal of the algal oil extraction stage, this method is considered a more economical way to prepare algal biodiesel (Wahlen et al., 2011; Velasquez-Orta et al., 2013). So, the aim of this study is to estimate the biodiesel production from *Oedogonium brevicingulatum* green alga via direct transesterification.

Materials and methods

Collecting algal samples

Algal mass was collected directly from the aquatic environment in different water areas (Al-Qibla district and Saad Square) of the Basra Governorate in southern Iraq (Figure 1). Nets were used for collection and the algal samples were placed in sterile plastic containers after which they were brought to the laboratory. Algal samples were washed with tap water to remove impurities. Then they were then washed several times with distilled water to ensure cleanliness. The samples were as examined under a light microscope to determine the type of isolated algae, then morphological identified based on taxonomic sources, and genetically identified to determine its genetic sequences.

Figure 1. A. alga in the aquatic environment. B. Alga after washing.

Identification of algae

Morphological identification of *Oedogonium brevicingulatum*

Oedogonium brevicingulatum was identified phenotypically by preparing temporary glass slides and examined under a light microscope to determine its morphological characteristics and identification based on taxonomic sources (Prescott, 1975).

Molecular Identification of algae

Extraction of DNA

Total genomic DNA of the *Oedogonium* specimen was extracted using the Geneaid Company's Genomic DNA mini kit (Table 1) and the material was isolated according to the plant tissue extraction protocol stated in (Motham et al., 2014). To verify the presence of DNA, electrophoresis was carried out using 0.8 % agarose gel and TBE buffer.

Table 1. Shows the components of the genomic DNA kit.

Component	GP100
GP1 Buffer	50 ml
GPX1 Buffer	50 ml
GP2 Buffer	15 ml
GP3 Buffer (Add Isopropanol)	30 ml (60 ml)
W1 Buffer	45 ml
Wash Buffer (Add Ethanol)	25 ml (100 ml)
Elution Buffer	30 ml
RNase A (10 mg/ml)	550 µl
Filter Columns	100
GD Columns	100
2 ml Collection Tubes	200

PCR polymerase chain reaction test method

The polymerase chain reaction test was performed using the *18 S r DNA* identified gene in accordance with the target DNA region of the presence gene. The test was carried out using the method of (Kepel et al., 2020), and the primers of the *18 S r DNA* gene were used as shown in Table 2.

Table 2. Showing the primers for the 18 S r DNA gene.

Primer	Sequence	Length bp	References
Forward 18 S r DNA	5'- TGATCCTTCYGCAGGTTCAC- 3'	1200	Moreno, 2012
Revers 18 S r DNA	5'-ACCTGGTTGATCCTGCCAG-3'	1200	

A 50 µL premix reaction was prepared by mixing 25 µL of Master Mix manufactured by Promega, 2 µL of Primer Forward, 2 µL of Primer Revers, 16 µL of Nuclease free water, and 5 µL of DNA template, then the mixture was centrifuged with a microfuge for (3-5) sec. to ensure the homogeneity of all materials in the tube and the samples were placed in a PCR sprint thermal cycler. The device was used in accordance with the programme shown in Table 3.

Table 3. Shows the program used in the PCR process.

No.	Stage	Temperature °C	Time	Cycle number
1	Initial denaturation	95	6 min.	1
2	Denaturation	95	45 sec.	35
3	Annealing	67	1 min.	
4	Extension	72	1 min.	
5	Final extension	72	5 min.	1

Electrophoresis process

To ensure that the DNA bands were found, the electrophoresis process was carried out according to the method of (Sambrook et al., 2012) by using 0.8% an agarose gel prepared by dissolving 0.2 g of Agarose in 25 ml of 1X TBE (Tris- HCl-Borate- EDTA) buffer. For PCR products, electrophoresis was performed by dissolving 0.5 g of Agarose in 25 ml of 1X TBE buffer.

Biodiesel production

Biodiesel was produced from *O. brevicingulatum* using the direct transesterification method, which was carried out concurrently with the extraction of oils from the alga (Benzidane et al., 2017). The alga was mixed with methanol at a 1:8 (w/v) ratio, and then concentrated sulfuric acid H_2SO_4 was added, equivalent to 60% of the alga's weight. The mixture was heated for 1.5 h at 90°C to maintain the atmospheric pressure inside the reaction and to avoid losing the solvent through evaporation and the system was equipped with a condenser (Figure 2).

After the experiment period expired, the mixture was centrifuged at 5000 rpm for 10 minutes to remove the algae residue, and the liquid layer was transferred to the separating funnel to obtain the biodiesel layer (by adding normal hexane solvent). The biodiesel (upper layer) was washed with 55 °C distilled water (30% v: v), the solvent was evaporated, and the biodiesel was heated for 15 min at 100 °C to remove the water and other solvent residues. The biodiesel yield was calculated relative to the content of algal oil % existing in the biomass, and it was determined as a percentage using the following equation (Cao et al., 2013):

$$\text{Biodiesel \%} = \frac{\text{Weigth of biodiesel products (g)}}{\text{Oil content (\%)X Weigth of alga (g)}} \text{ X } 100 \qquad (1)$$

Figure 2. The direct transesterification process.

Biodiesel properties

The biodiesel was chemically characterised using FTIR spectroscopy (Jasco - 4200 / Germany) and GC-MS (Agilent 5977 A MSD / USA) techniques. To confirm the conversion of fatty acid to fatty acid methyl esters, an FTIR spectrophotometer was utilised for FTIR analysis. Spectra were collected over the range of 400–4000 cm^{-1}. Main fatty acid methyl esters (FAMEs) were detected by GC-MS technique. Fuel properties Density (ASTM D1480), Kinematic viscosity (ASTM D445), cloud point (ASTM D2500), pour point (ASTM D97), acid values (ASTM D664) (Dolganyuk et al., 2020) of FAMEs were determined by performing standard ASTM tests.

Results and discussion

Morphological Identification of algae

After examining the algae samples with a light microscope, we determined, based on their morphology, that they belong to the green algae genus *Oedogonium* (Figure 3).

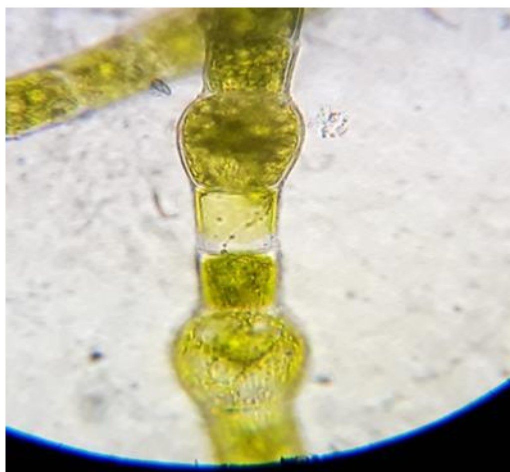

Figure 3. Light microscopy image of *O. brevicingulatum.*

Division: Chlorophyta
Class: Chlorophyceae
Order: Oedogoniales
Family: Oedogoniaceae
Genus: *Oedogonium*
Oedogonium brevicingulatum C.-C. Jao 1935

It is a green, filamentous, unbranched algae with three types of cells: the basal cell, which is responsible for fixing the algal thread and is known as the hold fast cell, the apical cell, and the cup cells, which are thought to be responsible for the reproduction process in the algae. Its cells are cylindrical in shape and range in length from (22-25) micrometres. Algae is common in fresh water, attaching itself to rocks and woody materials or attaching itself to algae and other plants. In addition to the presence of a large central vacuole, the algal cell has a parietal plastid dotted with starch-collecting centres and the lateral nucleus. Vegetative reproduction occurs through the fragmentation of the algal thread into small pieces, while asexual reproduction occurs through the formation of Zoospores, in addition to the sexual reproduction which is of the Oogamy type.

Molecular Identification of *O. brevicingulatum*

Gel electrophoresis of extracted DNA

Electrophoresis was performed, as well as the gel presentation of ultraviolet rays at a wavelength (nm) using a UV - device for the detection of DNA bundles, and the results revealed the appearance of a clear bundle of DNA of equal dimensions and large size, and the appearance of the bundles is evidence of the extraction process’s success (Figure 4).

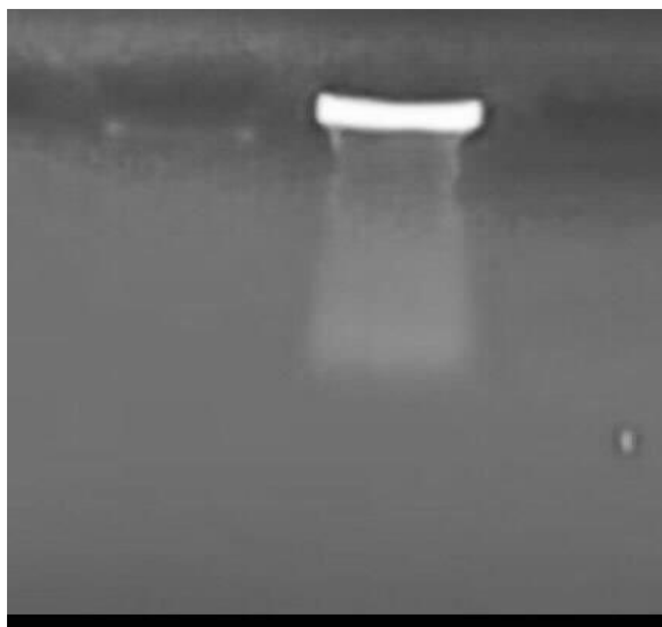

Figure 4. Agarose gel electrophoresis for genomic DNA extracted from *Oedogonium brevicingulatum*.

PCR reaction

The PCR product electrophoresis results revealed that the bands appeared at 1200 bp (Figure 5). The genetic identification results were obtained by matching the *18 S r DNA* genome sequence with available GenBank sequencing data using the BLAST programme in the NCBI (National Centre for Biotechnology Information) database, and it was determined that the green macroalgae sampled are genetically identical to *Oedogonium brevicingulatum* at a rate of 99% (Table 4). Molecular Identification of alga species has become common, includes PCR and gene sequencing, in this identification can adequately distinguish between closely related species even at the level of the same species (Manoylov, 2014; Thomson et al., 2018). Molecular Identification is a universal tool in biological studies of living organisms in general and algae due to the increase in their biological diversity (Manoylov, 2014).

Biodiesel production

The methyl ester yield by direct transesterification was calculated in relation to the percentage of algal oil present in the biomass, and it was 75%.

Identification of biodiesel produced from *O. brevicingulatum*

Table 5 & Figure 6 shows the results of the GC-MS analysis of biodiesel produced from the alga *O. brevicingulatum* by direct transesterification. It was 18 types of fatty acids methyl esters, with the highest percentage being for α-Linolenic acid methyl ester 23.41%, and the percentage of total FAMEs was equal to 67.3%.

Table 4. Shows the results of matching samples with available GenBank sequencing data.

Type of gene	Closet species	Gene sequencing	Length bp	% Identical to GenBank	Accession no. of closet species
18 S r DNA	*Oedogonium brevicingulatum*	GCCATGCATGTCTAAGTATAA ACTGCTTATACTGTGAAACTG CGAATGGCTCATTAAATCAGT TATAGTTTATTTGATGGTACC TTACTACTCGGATAACCGTAG TAATTCTAGAGCTAATACGTG CGTAAATCCCGACTTCTGGAA GGGACGTATTTATTAGATAAA AGGCCGACCGGGTTTACCCGA CCTGCGGTGAATCATGATAAC TTCACGAATCGCATGGCCTTT GCGCCGGCGATGTTTCATTCA AATTTCTGCCCTATCAACTTTC GATGGTAGGATAGAGGCCTA CCATGGTGGTAACGGGTGACG GAGGATTAGGGTTCGATTCCG GAGAGGGAGCCTGAGAAACG GCTACCACATCCAAGGAAGG CAGCAGGCGCGCAAATTACCC AATCCTGACACAGGGAGGTA GTGACAATAAATAACAATACC GGGCCTTTCGGTCTGGTAATT GGAATGAGAACAATCTAAAT CCCTTATCGAGGATCCATTGG AGGGCAAGTCTGGTGCCAGC AGCCGCGGTAATTCCAGCTCC AATAGCGTATATTTAAGTTGT TGCAGTTAAAAAGCTCGTAGT TGGATTTCGGGTGAACCTCGC CGGTCCGCCATTGGTGAGCAC TGGCGGGGGTTACCTTCTTGC CGGGGACGGGCTCCTGGGCTT AATTGTCCGGGACTCGGAGTC GGCGTTGCTACTTTGAGTAAA ACGGAGTGTTCAAAGCAGGC CTACGCCTTGAACGATATAGC ATGGA	755	99	DQ413052.1

Fourier-Transform Infrared spectroscopy (FTIR) of biodiesel produced from *O. brevicingulatum*

The FTIR infrared spectroscopy technique is one of the most precise and rapid methods for determining the quality of the FAMEs after the transesterification reaction has been completed, as it was used to detect the formation of fatty acid methyl esters (Mahamuni & Adewuyi, 2009).

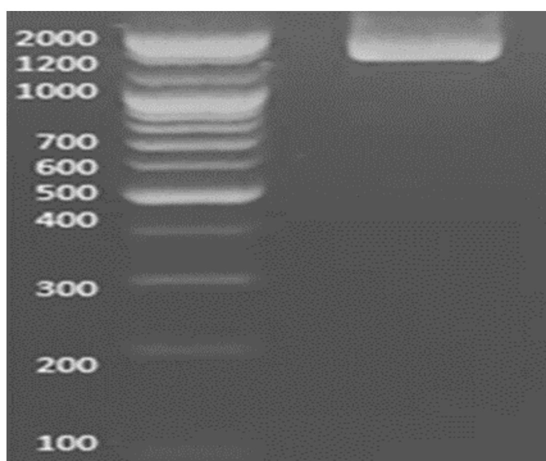

Figure 5. Shows the result of electrophoresis for PCR products of 18 S r DNA gene.

The results of the biodiesel analysis produced from *O. brevicingulatum* shown in the Table 6 & Figure 7. The peaks were at 2926 and 2855 cm^{-1}, indicating the presence of asymmetric and symmetric CH_2 stretch, which indicated the presence of alkanes and the methyl group in the methyl esters of fatty acids (Maity et al., 2014), and that the appearance of the peak at 2361cm^{-1} it indicated the C=O stretch which indicated the presence of ester (Kaur, 2018). It was also observed that the peak appeared at 1459 cm^{-1}, which indicated the presence of the C-H stretch and thus the presence of the asymmetric and symmetric CH_3 group (Lawer-Yolar et al., 2021). as for the appearance of peaks at (1742 and 1171) cm^{-1}, indicating the presence of the two stretches (C = O and C- O), respectively, which indicated the presence of the ester group in the methyl esters of fatty acids (Lawer- Yolar et al., 2021).

Biodiesel properties:

The results showed that the characteristics of the biodiesel produced from algae by direct transesterification fall within the values of the standard characteristics of diesel specified by the American Society for Testing and Materials (ASTM), as shown in Table 7. The density of the biodiesel produced was 0.920 g/cm^3 which is similar to what was stated in the study (Pandit & Fulekar, 2019) where the density of biodiesel produced from *Chlorella vulgaris* was 0.9 g / cm^3., the Kinetic viscosity was 5.51 mm^2/s which was identical to the value of the kinematic viscosity of biodiesel produced from cooking oils in a study of (Abdalla & Oshaik, 2013), the cloud point of the biodiesel was 5 °C, it was identical to what was obtained by (Ahmed et al., 2015), where the cloud point of biodiesel produced from the oil of *Spirogyra sp.* in their studies also equal to 5 ° C , the pour point was 2 °C, these results were identical to the results obtained by (Foroutan et al., 2019) , the acid value of the biodiesel was (0.54) mg KOH/ g. this result was similar to the result obtained by (Ahmad et al., 2013), where the acid value of the biodiesel produced from *Rhizoclonium hieroglyphicum* oil was 0.5 mg KOH/g , the yield of biodiesel was 75 %.

Table 5. The GC-MS analysis of biodiesel produced from *O. brevicingulatum*

NO.	Name of fatty acid methyl esters	Chemical formula	Molar mass (g/mol)	FAMEs %
1	α - Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292.46	23.41
2	Palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	12.68
3	Linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294.5	11.99
4	Roughanic acid, methyl ester	$C_{17}H_{28}O_2$	264.4	9.74
5	Eicosapentaenoic acid, methyl ester	$C_{21}H_{32}O_2$	316.5	1.98
6	Stearic acid, methyl ester	$C_{19}H_{38}O_2$	298.5	1.61
7	7- 10, Hexadecadienoic acid methyl ester	$C_{17}H_{30}O_2$	266.4	1.36
8	Myristic acid , methyl ester	$C_{15}H_{30}O_2$	242.4	1.31
9	Cis- 11,14- eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322.5	0.47
10	Stearidonic acid, methyl ester	$C_{19}H_{30}O_2$	290.4	0.47
11	Arachidonic acid, methyl ester	$C_{21}H_{34}O_2$	318.5	0.49
12	Lauric acid methyl ester	$C_{13}H_{26}O_2$	214.34	0.41
13	γ- Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292.5	0.35
14	Capric acid, methyl ester	$C_{11}H_{22}O_2$	186.29	0.34
15	Oleic acid, methyl ester	$C_{19}H_{36}O_2$	296.5	0.20
16	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284.5	0.20
17	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256.4	0.16
18	Lignoceric acid, methyl ester	$C_{25}H_{50}O_2$	382.7	0.13
				67.3

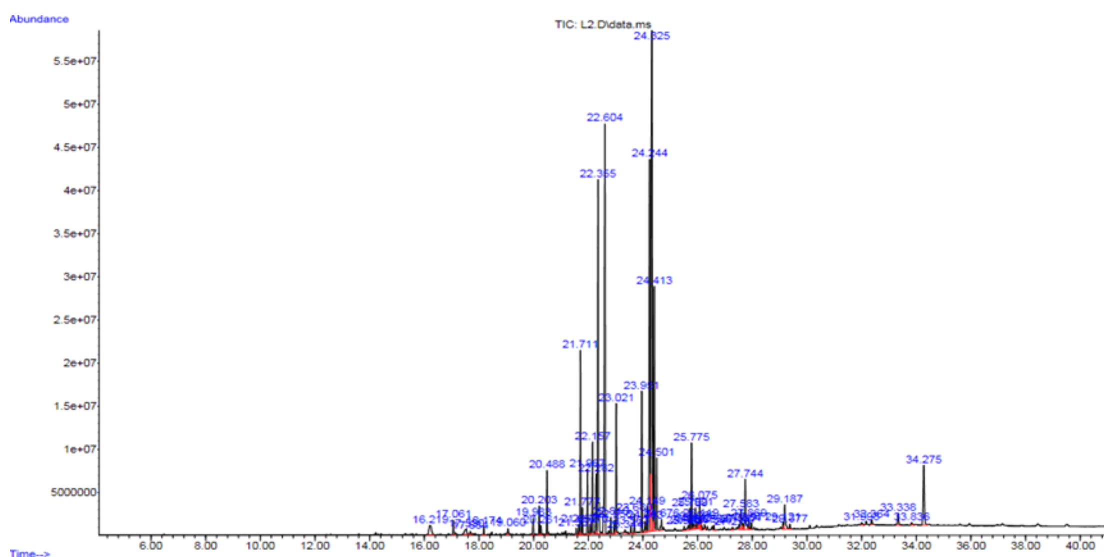

Figure 6. The GC-MS spectrum for biodiesel produced from *O. brevicingulatum.*

Table 6. The FTIR analysis of biodiesel produced from *O. brevicingulatum.*

FAMEs	Frequency cm^{-1}	Bonds	Functional groups
FAMEs from *O.brevicingulatum*	2926 (m)	CH_2 asymmetric stretching	Alkenes
	2855 (m)	CH_2 symmetric stretching	Alkenes
	2361 (m)	C=O Stretch	Esters
	1742 (m)	C=O Stretch	Esters
	1459 (m)	- C=C- stretch	Alkanes
	1171 (s)	C- O stretch	Esters

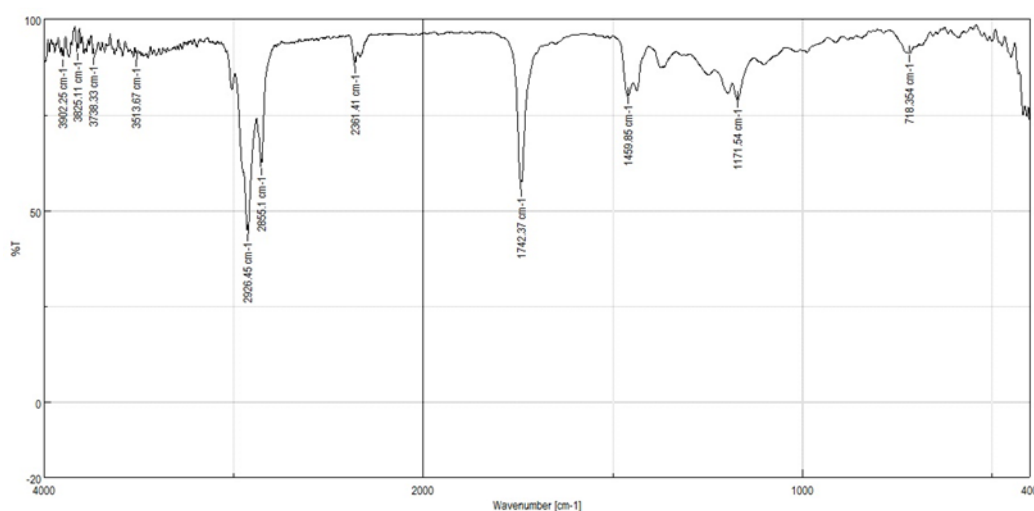

Figure 7: FTIR spectrum of biodiesel produced from *O. brevicingulatum* by direct transesterification.

Table 7. The properties of biodiesel produced from *O. brevicingulatum.*

Properties	biodiesel produced	ASTM standards
Density (g/cm^3)	0.92	0.86-0.9
Kinematic viscosity mm^2/s (40 °C)	5.51	1.9-6
Cloud point (°C)	5	-3 to 12
Pour point (°C)	2	-15 to +10
Acid value (mg KOH / g)	0.54	< 0.8
yield of biodiesel %	75	

Conclusion

O. brevicingulatum was used in this study to produce biodiesel via direct transesterification. The results demonstrated that these algae were an important source of biodiesel, that all properties of the biodiesel produced were within the

limits of ASTM standards, and that direct transesterification produced a high conversion rate, and that it could be an alternate, effective, and cost-effective process for producing biodiesel from algae.

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