Biodiesel production from Zygnema carinthiacum by direct transesterification

Sudad Asaad AlKinani[1], Athbi A. M[2], Afrodet A Saleh[3]
[1]Department of Ecology, College of Science, University of Basrah , Basra, Iraq
[2]Department of Biology, College of Education for Pure Sciences, University of Basrah, Basra, Iraq.
[2]Department of Pathological analyses College of Science, University of Basrah, Basra, Iraq
Email: sudad.mutashar@uobasrah.edu.iq

Abstract
Biodiesel production from *Zygnema carinthiacum* green alga which was studied identified phenotypically and genetically using PCR reaction by the Ribulose Bisphosphate Carboxylase Large subunit gene (*rbcL*). The production was investigated by conventional method involving directly producing from the whole biomass without extraction by one-step process. The biodiesel properties, including density, kinematic viscosity, cloud point, pour point, and acid value were determined , and their values are 0.867g /cm^3, 5.22 mm^2 / s, 3 °C, -5 °C, 0. 34 mg KOH / g., respectively. The results also showed that a yield of biodiesel about 79 % was obtained using direct transesterification. By combining lipid extraction with direct transesterification, it is possible to produce biodiesel with fewer stages than would otherwise be necessary, eliminating the requirement for isolated and filtered algal oil.
Keywords: Biodiesel ; Direct transesterification ; Catalyst ; Zygnema ; Phenotypic Identification ; Molecular Identification.

1. Introduction

Biodiesel is receiving more attentions, due to the huge increase in the depletion of fossil fuels, concerns about environmental pollution, and the increased demand for transportation fuels (Huang *et al.*, 2012 ; Bidir *et al.*, 2021) , in chemical aspects, biodiesel is a mixture of fatty acids methyl esters (FAMEs) produced from a reaction of triacylglycerols such as, vegetable oils, animal fats, algae lipids, or other fatty acids with alcohols in the occurrence of catalyst through the process of transesterification , the catalyst may be alkali, acid and enzymatic (Shi *et al.*, 2011). Indeed, algae are the best productive feedstock for biodiesel, it can produce 250 times as much oil per acre as soybeans, biodiesel from algae may be the only way to produce enough motor fuel to replace current gasoline use. Algae produce 7 to 31 times more oil than palm oil (Hossain *et al.*, 2008). Indeed, the biomass from algae is convertible by chemical or enzymatic transformations into biodiesel, almost all biomasses can be transformed into transport energy. The production of biofuels from algae has many advantages that go beyond replacing oil as transport energy (Chisti, 2007). Biodiesel production from algae is mostly obtained by direct transesterification (Eze *et al.*, 2014). In the transesterification reaction, triacylglycerols react with an alcohol to produce other esters of the same fatty acids and glycerol. This reaction can be expressed as three successive reversible reactions with the intermediate synthesis of glycerol, biodiesel, monoacylglycerols, and diacylglycerols (Dong *et al.*, 2013 ; Najeeb *et al.*, 2021). The raw biomass has also been directly trans- esterified when utilized with algae. Direct transesterification takes substantially less time than extraction and transesterification because it is a single-stage reaction. That, avoiding the processes of biomass oil extraction and cell disruption. Additionally, it stops lipids from being lost during the extraction process, which increases the output of crude biodiesel and FAMEs produced by direct transesterification (Johnson and Wen, 2009 ; Ehimen *et al.*, 2010). Biodiesel is better than petrodiesel because of several characteristics, including preserving the enviroment, reduced emission, higher combustion efficacy, renewability (Canakci and Sanli , 2008). So, the main aim of this study is to estimate the biodiesel production from *Zygnema carinthiacum* by direct transesterification.

2- Materials and methods

2-1 Collecting algal samples

Algal mass was collected from the aquatic environment directly from different water areas of Basra Governorate, South of Iraq. Algal samples were washed with tap water to get rid of the impurities stuck in them (Figure -1). Then they were washed with distilled water several times to ensure their cleanliness. The sample was examined under a light microscope to find out the types of isolated algae, then they were phenotypically identified based on the taxonomic sources, and they were also genetically identified

doi.org/10.31838/hiv23.02.218 Received: 23.10.22, Revised: 27.11.22, Accepted: 06.12.22

to knowing its genetic sequences.

Figure (1) : Primary washing process of algal sample.

2-2 Identified of algal

2-2-1 Phenotypic identification of alga

The alga was phenotypically identified by preparing temporary glass slides and examined under a light microscope for the purpose of determining its phenotypic characteristics and Identification based on taxonomic sources (Desikachary, 1959; Prescott, 1975; Bourrely, 1980).

2-2-2 Molecular Identification of algae

2-2-2-1 Extraction of DNA

Total genomic DNA of the *Zygnema* specimen was obtained using Genomic DNA mini kit supplied by Geneaid Company, and the material was isolated according to the plant tissue extraction protocol as stated in (Motham *et al.*, 2014). To confirm the presence of DNA electrophoresis was performed.

2-2-2-2 PCR polymerase chain reaction test method

The polymerase chain reaction test was carried out by using the *rbcL* identified gene, according to the target DNA region of the presence of *rbcL* gene. The test was conducted according to the method of (Kepel *et al.*, 2020), the primers of *rbcL* gene were used as shown in the table (1) :

Table (1) showing the primers for the *rbcL* gene.

Primer	Sequence	Length bp	References
Forward rbcL	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	600-700	Kepel *et al.*, 2020
Revers rbcL	5'-GTAAAATCAAGTCCACCRCG-3'	600-700	

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) seconds to ensure the homogeneity of all materials in the tube and the samples were placed in a PCR sprint thermal cycler. The device was operated according to the program shown in Table (2):

Table (2) shows the program used in the PCR process.

No.	Stage	Temperature	Time	Cycle number
1	Initialdenaturation	95	3 min.	1
2	Denaturation	95	30 sec.	35
3	Annealing	50	30 sec.	
4	Extension	72	30 sec.	
5	Final extension	72	1 min.	1

2-2-2-3 Electrophoresis process

In order to make sure that the DNA bands were found, electrophoresis process was performed according to the method of (Sambrook *et al.*, 2012) by using an agarose gel at a concentration of 0.8 % by dissolving 0.2 g of agarose in 25 ml of TBE 1X solution. While for PCR products, electrophoresis was carried out by dissolving 0.5 g of agarose in 25 ml of TBE 1X solution.

2-3 Biodiesel production

Biodiesel was produced from alga *Z. carinthiacum* by direct transesterification method, this process was performed simultaneously with extracting oils from alga according to (Benzidane *et al.*, 2017). The alga was mixed with methanol at 1:8 (w /v), and then concentrated sulfuric acid H_2SO_4 was added to it, equivalent of 60% for the weight of alga. 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 the system was equipped with a condenser. After the expiry of the experiment period, the mixture was centrifuged at 5000 rpm for 10 minutes to get rid of the algae residue, then the liquid layer was transferred to the separating funnel to obtain biodiesel layer (upper layer). It 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 get rid of 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) :

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

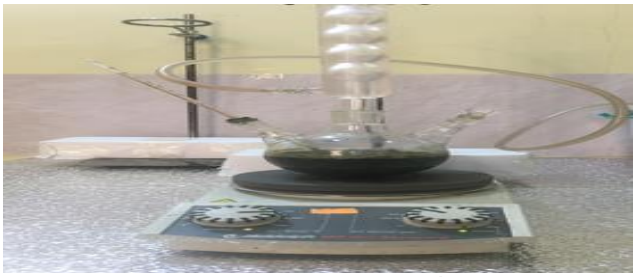


Figure (2): Show the direct transesterification process.

2-3-2 Biodiesel properties

Chemical characterization of the biodiesel produced was

performed by FTIR spectroscopy (Jasco - 4200 / Germany) and GC-MS (Agilent 5977 A MSD / USA) techniques. To confirm the conversion of fatty acid into fatty acid methyl esters FTIR analysis was done using an FTIR spectrophotometer. Spectra were taken for a scanning 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.

3- Results and discussion

3-1 Algae Identification

3-1-1 Phenotypic Identification

After examining the algae samples using a light microscope, based on the morphology we found that they are from the genus of green algae, which is the genus *Zygnema*. below are pictures showing the algae under the microscope

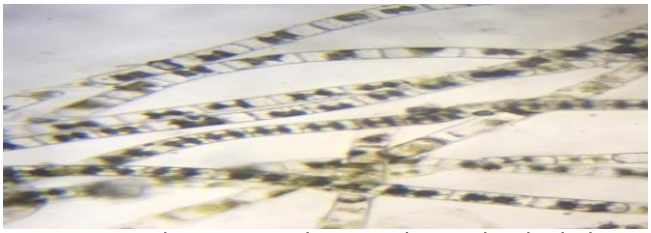


Figure (3) : Show Z. carinthiacum alga under the light microscope.

Division: Chlorophyta
Class: Chlorophyceae
Order: Zygnematale
Family: Zygnemataceae
Genus: *Zygnema*
Species: *Zygnema carinthiacum*
Genus of *Zygnema* green alga was distributed into freshwaters (rivers, streams, and ponds). It is one of the unbranched filamentous algae, young filaments are attached, while the mature filaments are floating. The cells are cylindrical, characterized by the presence of double star-shaped chloroplast, in each of them a starchy center, the nucleus occupies the center of the space between the two chloroplast and is attached to protoplasmic filaments, there is a large central gap (Guiry, 2013).

3-2 Molecular Identification

3-2-1 Extraction of DNA

DNA genetic material of the green algae under study was obtained using Genomic DNA mini kit supplied by Geneaid Company, the isolated DNA samples were kept in sterilized tubes at -20 °C until used.

3-2-2 Electrophoresis process on agarose gel

Electrophoresis was carried out on agarose gel at a concentration of 0.8 % of the isolated DNA samples of green alga and the gel presentation of ultraviolet rays at a wavelength (nm) using a U.V device Transilluminator for the detection of DNA bundles and the results showed the appearance of a clear bundle of DNA of equal dimensions and of large size, and the appearance of the bundles is evidence of the success of the extraction process used with these samples.

3-2-3 PCR reaction

The electrophoresis results for PCR products showed that the bands appeared at 600 bp (Figure-4). The genetic identification results were obtained by matching the *rbcL* genome sequence with available Gene Bank sequencing data by the BLAST program in the NCBI (National Center for Biotechnology Information) database, it was indicated that green macroalgae sampled are genetically match with *Zygnema carinthiacum* strain RS011 in the rate of 100 %. The results of matching samples with Gen Bank presented in Table (3).

Table (3) : Shows the results of matching samples with available Gene Bank sequencing data.

Algae	Type of gene	Closet species and strain and accession no.	%Identical to GenBank	Accession no. of closet species
Zygnema	rbcL	*Zygnema carinthiacum* strain RS011	100	JF965535.1

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).

Figure (4) : Show the result of electrophoresis for PCR products.

3-3 Biodiesel production

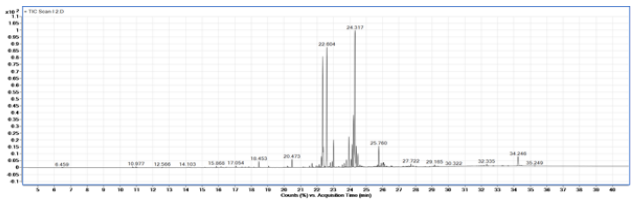
The methyl ester yield by direct transesterification was intended relative to the percentage of algal oil present in the biomass and it was equal to 79 % .

3-3-2 Identification of biodiesel produced from Z. carinthiacum

Table (4) show the results of the GC-MS analysis of biodiesel produced from alga *Z. carinthiacum* by direct transesterification, it was appeared 14 types of fatty acids methyl esters, with the highest percentage was for α -Linolenic acid methyl ester 30.62 %, The percentage of total FAMEs was equal to 82.92 %

Table (4): Shows the results of GC-MS analysis of biodiesel produced from alga *Z. carinthiacum*

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	30.62
2	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	270.45	18.94
3	Roughanic acid methyl ester	$C_{17}H_{28}O_2$	264.4	15.95
4	Linoleic acid methyl ester	$C_{19}H_{34}O_2$	294.5	7.15
5	Stearidonic acid methyl ester	$C_{19}H_{30}O_2$	290.4	2.46
6	Arachidonic acid methyl ester	$C_{21}H_{34}O_2$	318.5	2.85
7	Stearic acid methyl ester	$C_{19}H_{38}O_2$	298.5	1.31
8	7-10, Hexadecadienoic acid methyl ester	$C_{17}H_{30}O_2$	266.4	1.22
9	Myristic acid methyl ester	$C_{15}H_{30}O_2$	242.4	0.80
10	Cis-11,14-eicosadienoic acid methyl ester	$C_{21}H_{38}O_2$	322.5	0.44
11	Cis-11-eicosenoic acid methyl ester	$C_{21}H_{40}O_2$	324.5	0.50
12	Heptadecanoic acid methyl ester	$C_{18}H_{36}O_2$	284.5	0.31
13	Caprylic acid methyl ester	$C_9H_{18}O_2$	158.24	0.20
14	Pentadecanoic acid methyl ester	$C_{16}H_{32}O_2$	256.4	0.17
				82.92



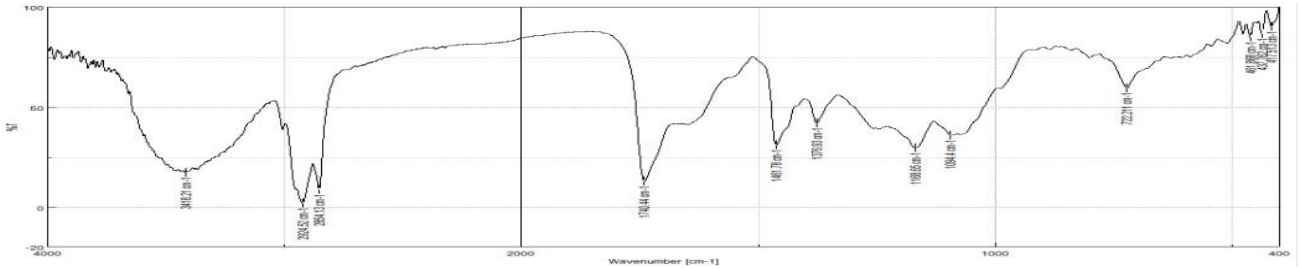
Shape (1): The GC-MS spectrum for biodiesel produced from alga Z. carinthiacum.

3-3-3 Infrared spectroscopy (FTIR) of biodiesel produced from Z. carinthiacum

FTIR analysis of biodiesel were showed the presence of peaks at 3418 cm $^{-1}$ an indication of the presence of hydrogen bond O-H stretch indicating the presence of phenolic hydroxyl (Vieira *et al.*, 2021) , and at (2924 and 2854) cm $^{-1}$ an indication the presence of the symmetrical and asymmetrical CH_2 bond , which indicates the presence of alkanes, also refers to the methyl group in the methyl esters of fatty acids (Maity *et al.*, 2014) . The presence of a peak at 1461 cm^{-1} indicates the presence of the C-H bond indicating that the methyl, methylene group were found , and the peak at 1376 cm^{-1} indicates the presence of a methyl CH_3 group (Samuel *et al.*, 2020) . The symmetry in the ester methylation, while the peaks at (1740, 1168 and 1094) cm^{-1} are an indication of the presence of the (C=O and C-O) bond, respectively, which is due to the presence of the ester groups (Arik *et al.*, 2022 ; Martínez Gil *et al.*, 2022).

Table (5): Shows the FTIR analysis of biodiesel produced from *Z. carinthiacum*

FAMEs	Frequency cm^{-1}	Bonds	Functional groups
FAMEs from *Z. carinthiacum*	3418(s,b)	O-H stretch	Alcohols , phenols
	2924(m)	CH_2 asymmetric stretching	Alkenes
	2854(m)	CH_2 symmetric stretching	Alkenes
	1740(m)	C=O Stretch	Esters
	1461(m)	C-H bend	Alkanes
	1376(m)	CH_3 deformation symmetric	Alkanes
	1168 (s)	C-O stretch	Esters
	1094(s)	C-O stretch	Esters



Shape (2): The FTIR spectrum of biodiesel produced from Z. carinthiacum by direct transesterification.

3-3 Biodiesel properties

All properties of biodiesel produced from green alga *Z. carinthiacum* were approximately equal or better than fossil diesel. Furthermore, all properties is within the limits of ASTM standards as shown in table (6) (Mccurdy *et al.* 2014). The density of the biodiesel produced was 0.867 g/cm^3 which similar to 0,869 g/cm^3 as reported by (Emilia *et al.*, 2014), the Kinetic viscosity was 5.22 mm^2/s which was comparable to 5.156 mm^2/s. from beef tallow as reported by (Sutapa *et al.*, 2017), the cloud point of the biodiesel was 3 °C , it was identical to what was stated by (Fernando and Kapilan , 2020) as the cloud point of biodiesel produced from *Sargassum sp.* in their study was 3 °C , the pour point was -5 °C, these results were identical to the results obtained by (Ong *et al.*, 2020) of biodiesel produced from Grape seed, the acid value of the biodiesel was (0.34) mg KOH/ g. this result was similar to the result obtained by (Kaewdaeng *et al.*, 2017), the yield of biodiesel was 79 %.

Table (6): Show the biodiesel properties

Properties	biodiesel produced	ASTM standards
Density (g/cm^3)	0.867	0.86-0.9
Kinematic viscosity mm^2/s (40 °C)	5.22	1.9-6
Cloud point (°C)	3	-3 to 12
Pour point (°C)	-5	-15 to +10
Acid value (mg KOH / g)	0.34	< 0.8
yield of biodiesel %	79	

4- Conclusion

In this study, the green alga *Z. carinthiacum* was studied to produce biodiesel by direct transesterification. The results showed that these algae were an important source to produce biodiesel, all properties of the biodiesel produced were within the limits of ASTM standards, and the result indicated that the direct transesterification produced a high conversion rate, and could be an alternate, effective and economical process for the production of biodiesel from algae.

Acknowledgment

We are thankful for the authorities of Basrah University (Iraq) for supporting this research work as a part of Ph.D.

References

Arik, N., Horzum, N., and Truong, Y. B. (2022). Development and Characterizations of Engineered Electrospun Bio-Based Polyurethane Containing Essential Oils. Membranes, 12(2), 209.

Benzidane, D., Baba, H. M. B., and Abi-Ayad, S. M. E. A. (2017). Biodiesel production from marine microalgae Nannochloropsis gaditana by in situ transesterification process. African Journal of Biotechnology, 16(22), 1270-1277.

Bidir, M. G., Millerjothi, N. K., Adaramola, M. S., & Hagos, F. Y. (2021). The role of nanoparticles on biofuel production and as an additive in ternary blend fuelled diesel engine: A review. Energy Reports, 7, 3614-3627.

Bourrely , P. (1980). Les Algues deuu douce , initiation a la systematigue . soc. Nouv.Edit .Boubee, Paris : pp 517 . Cited by Venkatarman ,L.V. and Becker ,E.W,(1985).

Canakci, M., and Sanli, H. (2008). Biodiesel production from various feedstocks and their effects on the fuel properties. Journal of Industrial Microbiology and Biotechnology, 35(5), 431-441.

Cao, H., Zhang, Z., Wu, X., and Miao, X. (2013). Direct biodiesel production from wet microalgae biomass of Chlorella pyrenoidosa through in situ transesterification. BioMed research international.

Chisti, Y. (2007). Biodiesel from microalgae. Biotechnology advances, 25(3), 294-306.

Desikachary, T. U. (1959). Cyanophyta. Indian council of Agricultural research , New Delhi , 517 pp.

Dolganyuk, V., Andreeva, A., Budenkova, E., Sukhikh, S., Babich, O., Ivanova, S., and Ulrikh, E. (2020). Study of morphological features and determination of the fatty acid composition of the microalgae lipid complex. Biomolecules, 10(11), 1571.

Dong, T., Wang, J., Miao, C., Zheng, Y., and Chen, S. (2013). Two-step in situ biodiesel production from microalgae with high free fatty acid content. Bioresource Technology, 136, 8-15.

Ehimen, E. A., Sun, Z. F., and Carrington, C. G. (2010). Variables affecting the in situ transesterification of microalgae lipids. Fuel, 89(3), 677-684.

Emilia, P., Carmen, G., and Tamara, R. (2014) BIODIESEL PRODUCTION FROM WASTE COOKING OIL. Analele Universității din Oradea, Fascicula Protecția Mediului Vol. XXIII,

Eze, V. C., Phan, A. N., and Harvey, A. P. (2014). A more robust model of the biodiesel reaction, allowing identification of process conditions for significantly enhanced rate and water tolerance. Bioresource technology, 156, 222-231.

Fernando, M. N., and Kapilan, R. (2020). Small scale biodiesel production from Sargassum sp and optimization of conditions for yield enhancement. Journal of Science, 11(1).

Guiry, M. D. (2013). Taxonomy and nomenclature of the Conjugatophyceae (= Zygnematophyceae). Algae, 28(1), 1-29.

Hossain, A. S., Salleh, A., Boyce, A. N., Chowdhury, P., and Naqiuddin, M. (2008). Biodiesel fuel

production from algae as renewable energy. American journal of biochemistry and biotechnology, 4(3), 250-254.
Huang, D., Zhou, H., and Lin, L. (2012). Biodiesel: an alternative to conventional fuel. Energy procedia, 16, 1874-1885.
Johnson, M. B., and Wen, Z. (2009). Production of biodiesel fuel from the microalga Schizochytrium limacinum by direct transesterification of algal biomass. Energy & Fuels, 23(10), 5179-5183.
Kaewdaeng, S., Sintuya, P., and Nirunsin, R. (2017). Biodiesel production using calcium oxide from river snail shell ash as catalyst. Energy Procedia, 138, 937-942.
Kepel, R. C., Mantiri, D. M., and Sahami, F. M. (2020). Phylogeny and molecular identification of green macroalgae, Ulva prolifera (OF Müller, 1778) in Totok Bay, Maluku Sea, and Blongko waters, Sulawesi Sea, North Sulawesi, Indonesia. Aquaculture, Aquarium, Conservation and Legislation, 13(4), 2196-2203.
McCurdy, A. T., Higham, A. J., Morgan, M. R., Quinn, J. C., and Seefeldt, L. C. (2014). Two-step process for production of biodiesel blends from oleaginous yeast and microalgae. Fuel, 137, 269-276.
Maity, J. P., Bundschuh, J., Chen, C. Y., and Bhattacharya, P. (2014). Microalgae for third generation biofuel production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future perspectives–A mini review. Energy, 78, 104-113.
Manoylov, K. M. (2014). Taxonomic identification of algae (morphological and molecular): species concepts, methodologies, and their implications for ecological bioassessment. Journal of phycology, 50(3), 409-424.
Martínez Gil, J. M., Vivas Reyes, R., Bastidas Barranco, M. J., Giraldo, L., and Moreno-Piraján, J. C. (2022). Biocatalysts Synthesized with Lipase from Pseudomonas cepacia on Glycol-Modified ZIF-8: Characterization and Utilization in the Synthesis of Green Biodiesel. Molecules, 27(17), 5396.
Motham, M., Pumas, C., and Peerapornpisal, Y. (2014). Improvement of DNA extraction protocols for Nostochopsis spp. Chiang Mai J Sci, 41, 557-567.
Najeeb, J., Akram, S., Mumtaz, M. W., Danish, M., Irfan, A., Touqeer, T., and Choong, T. S. Y. (2021). Nanobiocatalysts for biodiesel synthesis through transesterification—a review. Catalysts, 11(2), 171.
Ong, H. C., Mofijur, M., Silitonga, A. S., Gumilang, D., Kusumo, F., and Mahlia, T. M. I. (2020). Physicochemical properties of biodiesel synthesised from grape seed, philippine tung, kesambi, and palm oils. Energies, 13(6), 1319.
Prescott, G.W. (1975) . Alage of the western great lake area . 6th ed. , William, C. Brown Co. Publishers. Dubugue , Towa , PP 977.
Sambrook , J.; Fritsch, E. F. and Maniatis, A. (2012). Molecular cloning, 2nd ed. New York: Cold Spring Harbor Laboratory Press. 228 pp.
Samuel, E. O., Sunny, E. I., Anselm, I. I., and Michael, O. D. (2020). Spectroscopic and chemical analysis of a Nigerian hybrid Hevea brasiliensis (Rubber) seed oil for product quality assessment in technical applications. African Journal of Biotechnology, 19(9), 636-643.
Shi, S., Valle-Rodríguez, J. O., Siewers, V., and Nielsen, J. (2011). Prospects for microbial biodiesel production. Biotechnology journal, 6(3), 277-285.
Sutapa, I. W., Wati, S. N. I., and Sekewael, S. J. (2017). Biodiesel Production From Beef Tallow, Catalyzed by Potasium Hidroxide in Ethanol. International Journal of Engineering and Science Applications, 3(2), 169-177.
Thomson, S. A., Pyle, R. L., Ahyong, S. T., Alonso-Zarazaga, M., Ammirati, J., Araya, J. F., and Segers, H. (2018). Taxonomy based on science is necessary for global conservation. PLoS biology, 16(3), e2005075.
Vieira, M. G., Batista, L. R., Muniz, A. S., Lichston, J. E., Oliveira, A. R., César-Oliveira, M. A., and Antoniosi Filho, N. R. (2021). Synthesis of antioxidant additive from safflower seed oil. Journal of the Brazilian Chemical Society, 32, 40-46.