

Sustainable Biodiesel Production via Transesterification of *Bacillus sp.* (OQ135194) Lipids: Optimization Strategies

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Abstract: The contemporary necessity for an alternative to fossil fuels stems from the escalating levels of carbon dioxide, increasing fossil fuel consumption, and the accumulation of agricultural waste. Microorganisms capable of producing higher fatty acids offer a promising solution, diminishing our reliance on petroleum diesel. These lipid-accumulating microorganisms thrive on agro-waste-based media and can synthesize fatty acid methyl ester (FAME) from their lipid reserves. Bacterial growth requires minimal conditions and is not constrained by seasonal factors, making it a compelling alternative to fossil fuels. Although transesterification has been extensively investigated in lipids from plants, animals, and select microorganisms, the optimization of FAME production from bacterial oil remains a relatively unexplored territory. Recent research has focused on refining the transesterification process of bacterial lipids to maximize biodiesel yield. This pursuit of ideal conditions encompassed various base catalysts, revealing that the best results were achieved with a 1:9 oil-to-methanol ratio, a catalyst concentration of 0.6%, and a temperature of 75°C for 100 minutes. Spectroscopic techniques were employed to meticulously assess the quality and quantity of the resulting FAME, conclusively affirming the environmentally sustainable and economically viable nature of the produced biodiesel.

Keywords: biodiesel; transesterification; oleaginous microorganisms; FAME.

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1. Introduction

India's growing population puts the country in second place for crowding and seventh place for fatty acid output [1]. India ranked fifth in the world in petrol and diesel consumption, driven by increasing demand for public transport amid a growing population. India consumes close to 40% of all petroleum fuel produced, or 40 million tonnes, per year. Limited non-renewable fuel resources and rising demand for petroleum fuel generated a need for water, biological, and solar energy to overcome the paucity of energy sources [2–4]. The combustion of petroleum and coal releases greenhouse gases (GHGs), mostly CO₂, into the atmosphere [5].

Research on sustainable energy sources is currently driven by the extensive use of non-renewable fossil fuels [6]. Substitute sources of energy must be universally accessible, economical, recyclable, viable, and environmentally friendly [7]. Due to its low carbon content, lack of toxic substances, and improved fluidity, biodiesel is a fuel that shows promise and may eventually replace conventional energy sources [8]. Biodiesel production is the most promising substitute for petroleum products, attracting researchers' attention. Biodiesel can be produced from plant or animal fats via transesterification[9].

These days, third-generation technology and the most promising oil sources are being used to produce biofuel [10–12]. The most effective lipid producers in terms of cell dry weight are oleaginous bacteria [13,14]. When grown in a particular culture medium, algae and yeasts are said to contain higher lipid content, although bacteria are rarely reported to have a notable lipid content [15–17]. Several bacterial strains, including *Rhodococcus opacus* (PD630), *Bacillus subtilis* (RRL-8), and *Pseudomonas spp.* (RRL-28), have been observed to collect more oil when isolated from particular habitats [18,19].

Based on the total land area, there are 1.8 trillion tonnes of lignocellulosic biomass that can provide 33000 EJ of energy [20]. Using microbial bioengineering to convert sugars, starch, lignocellulose, or other C-containing biomass into lipids has several benefits over using traditional biodiesel feedstocks [21]. Various lignocellulosic waste products, along with varying carbon and nitrogen concentrations, are reportedly used by *Rhodococcus*, *Gordonia*, and other strains to enhance cellular oil production. The greatest accumulation is thought to occur during the stationary period of growth [22]. Simple culture conditions are adequate for growing bacteria, and their growth rate is higher [23,24]. Variations in lipid accumulation in cells are caused by a variety of carbon sources and bacterial strains [15].

The isolated oleaginous strain from dairy wastewater is the subject of the current research. Using processed agro-wastes, the chosen strain exhibits improved growth and lipid accumulation. The oil produced by the bacteria underwent transesterification, and the process was optimized to determine the optimal catalyst and concentration.

2. Materials and Methods

2.1. Isolation, identification, and enrichment of lipid accumulators.

A sample of dairy waste was taken from the Dudhsagar dairy in Mehsana. 0.1 ml of the sample, which was taken in a sterile container, was used to activate the organisms. Luria Bertani broth was used for activation. After activation, the sample was spread on LB agar to provide the microbes with the nutrients needed for growth. The plates were then incubated at 37°C for 24 hours. The ability of the organisms to collect lipids was further examined after incubation. Based on the first observation, 26 isolates were selected and screened using Sudan staining to assess the presence of oil in the therganisms [25,26]. Selected 2 strains were further examined for the ability to produce more lipids from lignocellulosic waste. (wheat straw, peanut waste peel powder). Fermentation media contained (g/l), 100 g/l glucose, yeast extract - 0.9, NH₄Cl -1.0, KH₂PO₄-1.6, MgSO₄.7H₂O-0.45, CaCl₂ x 2H₂O-0.005, ZnSO₄ x 7H₂O - 0.001, FeCl₃ x 6H₂O-0.001, Na₂HPO₄-1.0. Glucose was used as a standard substrate. In production media, 8% waste samples were added to assess efficiency relative to the standard substrate. pH was neutralized. One isolate from the previously selected 2 was used for biochemical tests and 16S rRNA sequencing. Potential bacteria were cultivated in designed media using peanut waste peel powder as the substrate.

Bligh and Dyer's method, with slight modification, was used to extract fatty acids [27]. After performing the Bligh and Dyer method, organic solvents were evaporated using a rotary evaporator, and single-cell oil content was recorded. Extracted lipids were tested qualitatively using gas chromatography-mass spectrometry (GC-MS) [28,29].

2.2. *Transesterification.*

The rearrangement of alcohol from one ester to another is known as transesterification. The process was carried out using a capped Erlenmeyer flask. In contrast to acid-catalyzed transesterification, base-catalyzed transesterification offers several benefits, including a higher reaction rate, shorter reaction time, and a simpler setup [30]. Catalyst and methanol were allowed to react with each other by providing heat. The process was conducted using a magnetic stirrer. The mixture was introduced into the Erlenmeyer flask containing microbial oil after a period of time, once the base had dissolved in methanol. To determine the ideal conditions for the reaction, the reaction mixture was allowed to react. The solution was allowed to cool after the reaction finished, and its pH was adjusted to neutral. Washing was carried out for the liquid using warm water. Following the steps mentioned above, the fluids were separated using a separating funnel, in which the transesterified lipids, also known as fatty acid methyl esters, settled on the top layer [31]. For a better result, ethyl acetate was used [32]. The following formula was used to measure the formed biodiesel [32].

$$\text{Biodiesel yield}(\% \frac{v}{v}) = \frac{\text{Amount of biodiesel (ml)}}{\text{Amount of lipids (ml)}} \quad (1)$$

2.3. *Optimization of transesterification.*

Homogeneous alkaline catalysts such as KOH or NaOH are typically used to produce biofuel. These catalysts are frequently employed in factory-level biofuel production for several reasons, including the need for low reaction temperatures and low air pressure to catalyze the process, the ability to achieve rapid conversion rates, their accessibility, and their affordability. Compared to the acid-catalyzed transesterification reaction, the alkali-catalyzed transesterification reaction occurs 4000 times faster [33]. Two different catalysts, NaOH and KOH, were used to find the best. The rate of reaction for biodiesel production is influenced by temperature. It has a favorable impact on the procedure [34–38]. Temperature optimization was conducted to determine the optimal production temperature. A temperature range of 40°C to 80°C was used to determine the optimum temperature. The alcohol-to-oil ratio affects production, impedes glycerol dissociation, and influences the time required and yield [39]. While maintaining the same values for the other parameters, this criterion was optimized using several ratios, including 3:1, 6:1, 9:1, 12:1, and 15:1. Different catalyst concentrations can alter biodiesel yield[40]. By maintaining the other parameters constant, the catalyst% for this sample was tested between 0.1 and 1. Different time limits were set for different sets to determine the optimal time to complete the process for the highest yield. The time ranged from 40 to 120 minutes.

2.4. *Characterization of biodiesel.*

Fourier transform infrared spectroscopy was used to characterize the transesterified biofuel and determine the group composition. The fatty acid methyl ester was also characterized by gas chromatography and mass spectrometry to identify specific fatty acid

methyl esters. In addition to these techniques, nuclear magnetic resonance (NMR) spectra were recorded in deuterated chloroform with tetramethylsilane as an internal standard. The presence of various fatty acids in the chosen strain dominates the properties of biodiesel [41]. ¹³C and ¹H NMR spectra were recorded. Various physicochemical properties of biodiesel were examined using techniques from the American Oil Chemists' Society and the American Society for Testing and Materials [42] and are shown in Table 1.

Table 1. Compilation of established techniques for assessing Biodiesel quality.

Properties	Test Method
Kinematic Viscosity @ 40°C	ASTM D 445 2018
Density @ 40°C	ASTM D 792
Specific gravity @ 40°C	ASTM D 792
Pour point	ASTM D 97 2017
Cloud point	ASTM D 2500
Flash point	ASTM D 92 2018
Acid number	AOCS Cd 3d 63 2009(RA2017)
Iodine number	AOCS Cd 1d 92 2009(RA2017)
Cetane number	ASTM D 613

3. Results and Discussion

3.1. Isolation, identification, and enrichment of lipid accumulators.

A recent study has focused on separating oil accumulators from sites rich in fat sources, such as dairy waste. Additionally, a suitable agro-waste was selected as a substrate: peanut waste peel powder. The sample was spread onto LB plates, where 26 isolates were discovered. One potent bacterium was found to increase oil production among the 26 isolates evaluated. The selected strain produced 5.10 g l⁻¹ dry weight and 3.4 g l⁻¹ oil using a standard fermentation medium, and it showed good oil productivity of 3.2 g l⁻¹ using peanut waste peel powder as a substrate (Figure 1). The selected organism's growth on LB media is depicted in Figure 2a. Using Sudan staining for initial screening, it was discovered that two isolates (RKC-1, RKC-2) have the potential to acquire fat. Figure 2b shows lipid granules in the selected strain by Sudan staining. Based on strong growth and fat buildup in fermentation media, one powerful organism (RKC-1) was identified among these two strains.

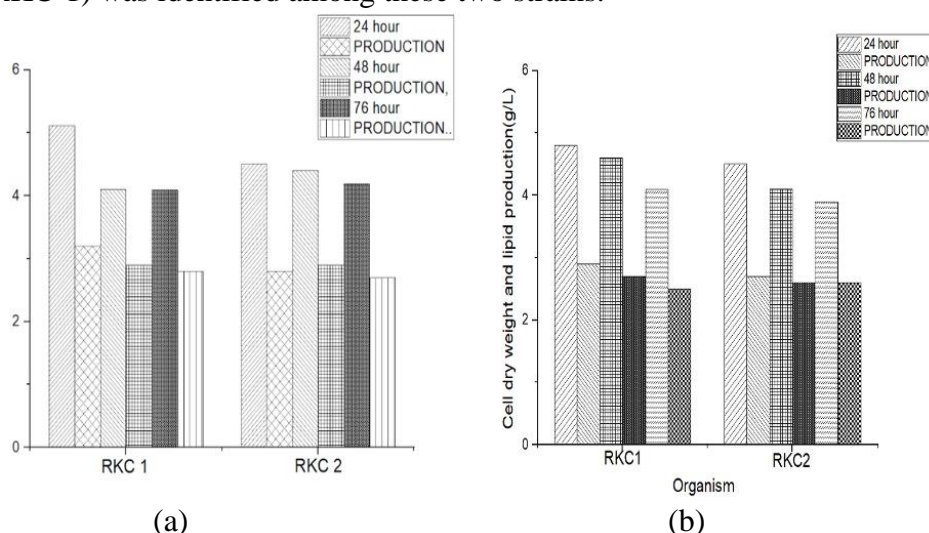


Figure 1. Effect of different substrates on several strains of chosen bacteria with regard to cell dry weight and oil accumulation (a) peanut waste peel powder; (b) wheat straw.

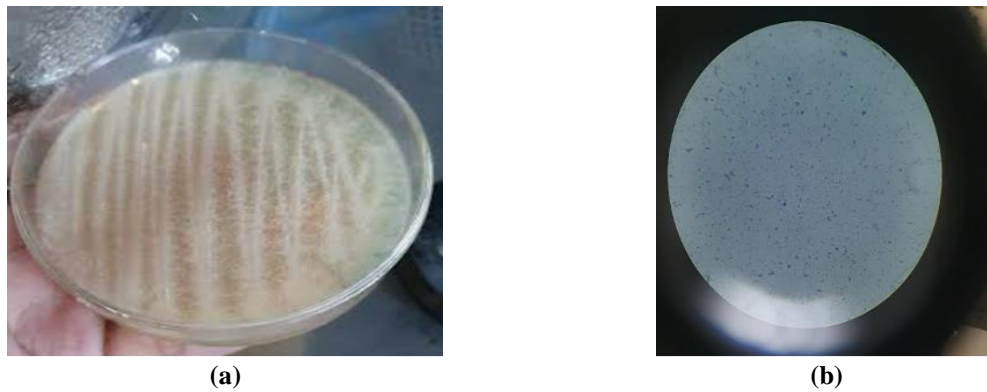


Figure 2. (a) Selected strain growth on LB agar plate; (b) microscopic image of selected strain with Sudan stain.

16s rRNA technique, biochemical characteristics, and morphology revealed the identity of the selected strain (Figure 3). The chosen strain was found to be *Bacillus sp.* The National Center for Biotechnology Information (NCBI) assigned the accession number OQ135194 to the strain after submitting the nucleotide sequence.

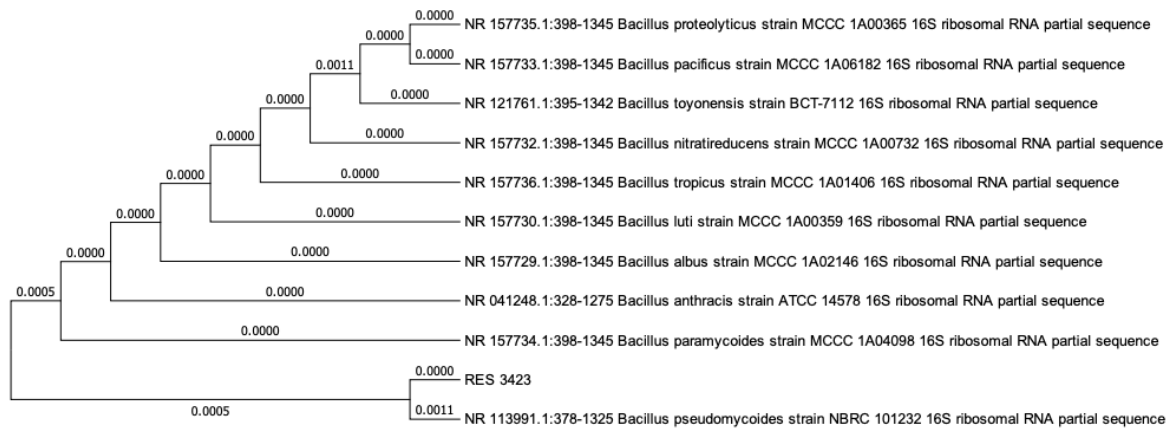


Figure 3. Phylogenetic relationships of *Bacillus sp.* (OQ135194).

According to Zhang *et al.* [43], *B. subtilis* HB1310 is an oleaginous bacterium that was isolated from walnuts with thin shells. The strain in question thrived on cotton stalk hydrolysate and could increase fat content by 39.8% after 48 hours [43]. Furthermore, Qadeer *et al.* [44] demonstrated that orange trash is the ideal substrate for *Bacillus cereus* KM15 growth to accumulate lipids. The procedure was run for a 72-hour incubation period at pH neutral [44].

3.2. Transesterification.

The production of fatty acid methyl ester is affected by both the test organism and the waste product, as well as by the addition of fermentation media [45,46]. Optimum criteria for maximum yield are reported, including a 9:1 molar ratio of alcohol to oil, a catalyst concentration of 0.6 g (%), a temperature of 75°C, and a reaction time of 100 minutes. Changes in the catalyst and its amount may affect diesel production [47]. In our investigation, sodium and potassium hydroxides were used to accelerate the reaction, with sodium hydroxide proving the most effective catalyst overall. While sodium hydroxide produced 86.1% FAME, potassium hydroxide produced 84.1%. The biodiesel yield was highest with NaOH, so it was selected as the catalyst for further testing (Figure 4a). NaOH was proven to be efficient, as a lower loss of ester was observed during the washing steps when the catalyst was NaOH. The molar ratio was optimized, and it was found that the yield was maximum at concentrations

above 0.5 and a molar ratio of 9:1. Saponification was observed at high catalyst concentrations [48,49]. According to reports, soap is produced from triacylglycerols when catalyst concentrations exceed 1% [50]. When the concentration was increased from 0.5, the yield decreased. However, it was also observed that a catalyst concentration below 0.5 failed to achieve a viscosity within the normal range. The best-suited result was achieved with a catalyst amount of 0.7% and a molar ratio of 9:1 to 12:1. Increasing the molar ratio did not affect yield. It remained constant, so it was convincing to maintain the NaOH concentration at 0.7% and the molar ratio at 9:1 (Figure 4b).

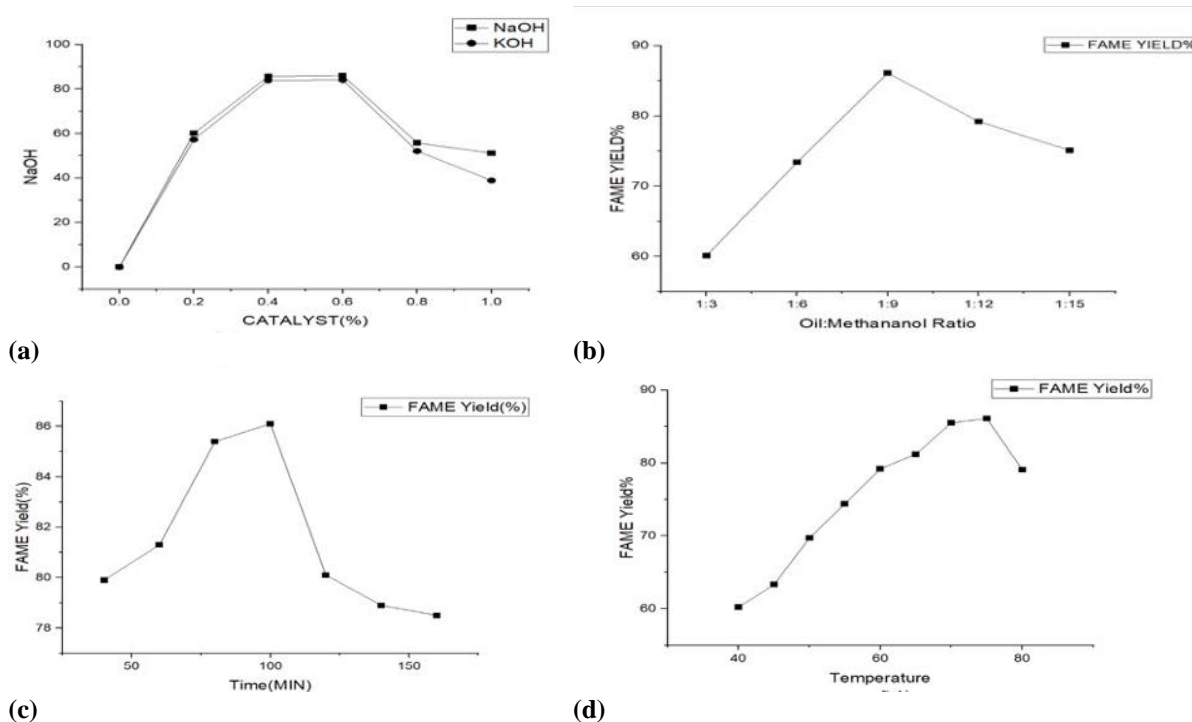


Figure 4. Optimization of fatty acid methyl ester (a) Catalyst; (b) Oil: methanol ratio; (c) Time (min); (d) Temperature (°C).

The reaction's duration and temperature were optimized. The time range was checked from 40 to 120 minutes with intervals of 20 minutes. 98% of ester productions finish in an hour, while 80% finish just five minutes, according to certain experiments [35–37,41]. An increase in biodiesel production was observed after 50 minutes. Similar outcomes were seen in the current investigation, with the highest lipid output occurring in the first 100 minutes (Figure 4c). The selected temperature range to find the optimum temperature was 40°C to 80°C with intervals of 5°C. Several researchers have detailed the desired effects of higher temperatures on biodiesel production [35–38,41]. Dorado *et al.* speculate that it may be due to a saponification-related side reaction that is negatively affected by temperature and catalyst concentration [51]. Methanol has a boiling point of 65°C, and at that temperature, FAME conversion improved because methanol boiling could enhance mass and heat transfer between the reactants and the catalyst. In our study, we found that 75°C was the optimal temperature for conversion (Figure 4d). Through optimization, an 86.1% transesterification yield was obtained. (Figure 5).



Figure 5. Fatty acid methyl ester formation.

3.3. Characterization of biodiesel.

The Bligh and Dyer method was performed and organic solvents were evaporated using a rotary evaporator, and single-cell oil content was recorded (Figure 6). Due to their appropriateness for the production of biofuel, free fatty acids (FFAs) are of great interest [23,52].



Figure 6. Extracted lipid sample collection after the Bligh and Dyer method by evaporation using Rota evaporator and FAME formation for gas chromatography and mass spectrometry.

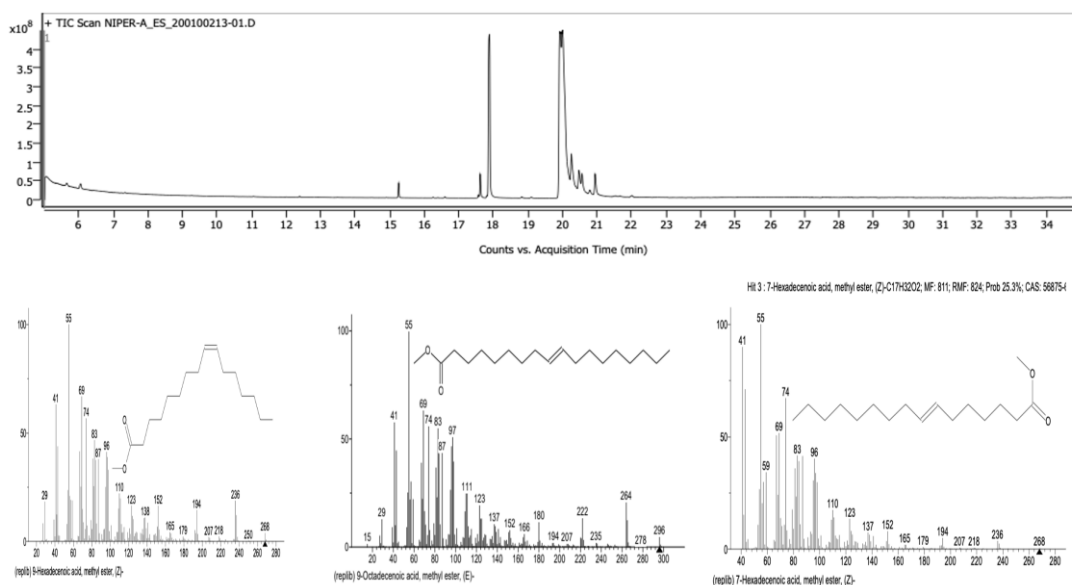


Figure 7. GC-MS spectrum.

The presence of FFAs in the isolated lipids confirmed their suitability for use in the transesterification procedure to produce biodiesel [53]. The GC-MS examination of a lipid

sample from crude oil isolates revealed that it contained a variety of FFAs, with oleic acid being the most often discovered free fatty acid, followed by n-hexadecanoic acid and octadecanoic acid [54]. Octadecanoic acid (43.81%), ethyl stearate, 9, 12-diepoxy (C19:0, 27.51%), hexadecanoic acid (16.16%), and octadecenoic acid (9.53%) were the lipids of *A. carneus* that were studied for biodiesel production. These lipids were comparable to the fatty acids of most oleaginous fungi [55]. In our work, GC-MS analysis revealed the presence of various fatty acids in the microbial cell, including 9-octadecenoic acid, 9-hexadecenoic acid, and 7-hexadecenoic acid (Figure 7). The presence of such monounsaturated fatty acids made the selected organism more potent for the production of FAME.

The presence of absorption bands between 3010 and 2854 cm^{-1} indicates the stretching of C-H [50]. Figure 8 shows the FTIR spectrum of our test sample. In our sample, we observed peaks at 2853.77, 2923.29, and 3006.63 cm^{-1} , indicative of C-H stretching. The characteristic absorption band at 1743 cm^{-1} identifies the carbonyl group of the RCOOR' type (C=O) [50]. Fatty acid methyl ester can be seen as a peak in the region of 1741–1750 cm^{-1} [56,57]. We validated the presence of the carbonyl group in RCOOR methyl esters by the observed absorption band at 1741.45 cm^{-1} . The 2850.41 cm^{-1} and 2924.32 cm^{-1} peaks, respectively, signify the vibrations of symmetric and asymmetric stretching of C-H alkane groups. The groups in the biodiesel ester chains could be methyl (CH_3) or methylene groups, and they require more energy to produce stretching vibrations within their bond than usual C-H bending vibrations of alkene groups, which are evident at low energy and frequency ranges [57,58]. The stretching vibrations of the C-H alkane groups are shown by the peaks we observed at 2853.77 and 2923.29, respectively. The band area between 1377.23 and 1465.03 cm^{-1} is caused by the fuel's bending vibration of C-H methyl groups [57]. From peaks at 1438.32 cm^{-1} and 1460.86 cm^{-1} , we identified alkane bending and primary alcohol stretching.

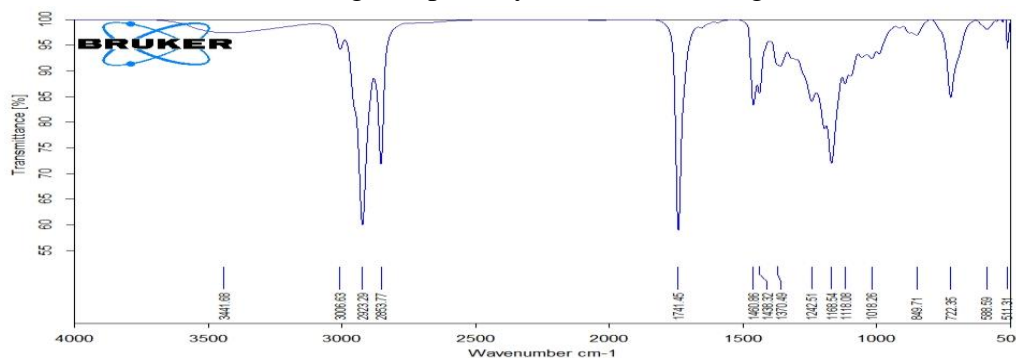


Figure 8. FTIR spectrum.

Nuclear magnetic resonance (NMR) spectroscopy was used to confirm the biodiesel made from *Bacillus* transesterified lipids. Gelbard used the NMR method as the first spectroscopic method to observe the transesterification of triglycerides [59]. The presence of methyl esters in the biodiesel sample is confirmed by the ^1H NMR spectrum, which shows two distinctive peaks [60]. The aliphatic $-\text{CH}_2-$ signals are at 1.6 and 1.28 ppm [46]. Figure 9 shows the NMR spectrum of the test sample. In our experiment, we observed peaks in the 1.20-1.41 ppm range, corresponding to the protons of all the internal CH_2 groups in the fatty acid backbone. According to Doudin *et al.*, the end of chain aliphatic $-\text{CH}_3$ is at 0.86 ppm, and the methyl ester $-\text{CH}_3$ is at 3.63 ppm [61]. Satyarthi *et al.* reported a significant peak for $-\text{OCH}_3$ associated with FAME at delta 3.68 ppm [62]. We observed a peak at delta 1.00 ppm corresponding to the terminal methyl group, whereas the peaks at delta 3.74–3.76 ppm correspond to the protons of the COOCH_3 group. Doudin *et al.* reported the hydrogens of

unsaturated molecular species -CH=CH- from isolated and non-conjugated double bonds, as well as for the two outer hydrogens (-CH=CH-CH=CH-) of the conjugated double bonds at 5.31 ppm [61]. Additionally, for biodiesel derived from cottonseed oil, Moawia et al. reported an olefinic proton (-CH=CH) signal between 27 and 5.41 ppm [63]. We tracked the peaks of the olefinic (-CH=CH) moiety protons at delta 5.60–5.80 ppm. The signal for the -CH₂- group next to the carbonyl, which is used as an internal benchmark to roughly measure the other groups in the indole molecules, is at 2.27 ppm [61]. Our testing revealed that the peaks at delta 2.1–2.2 ppm correspond to CH₂ next to a carbonyl group.

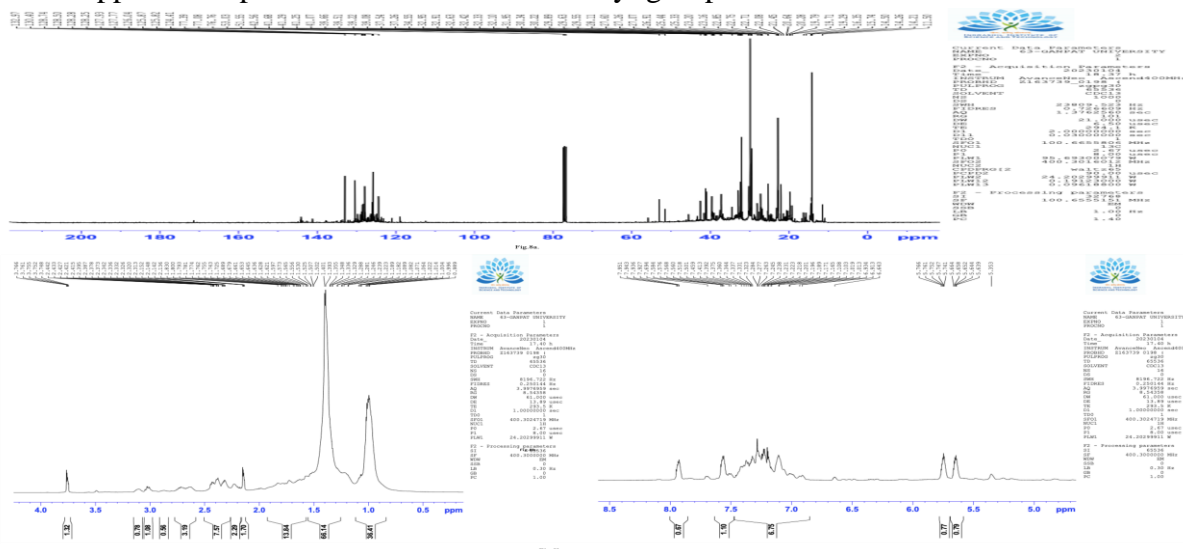


Figure 9. NMR spectrum.

According to Doudin *et al.*, the signals for the end-chain methyl (-CH₃) carbon are at 13.9 ppm, and the signals for the aliphatic methylene (-CH₂-carbons are in the range of 34 to 27 ppm [61]. In our analysis, long-chain hydrocarbons with carbons in the methylene group were found in the range of delta 20.00–35.00 ppm (-CH₂), and the terminal methyl carbon (-CH₃) peaks are at delta 14.00–15.00 ppm. The signals for unsaturated (-CH=CH-) carbons and the outside carbons of the non-conjugated (-CH=CH-CH₂-CH=CH-) are both at 129.8 ppm, whereas the inner carbons of the non-conjugated (-CH=CH-CH₂-CH=CH-) are at 127.9 ppm [61]. The peaks found in our investigation are associated with olefin carbon (-CH=CH-) at delta 125.00–140.00 ppm. At 174.0 ppm, the carbonyl carbon signal (-COO-) is detectable [61]. In our study, the peak detected at delta 170.00 ppm corresponds to carbonyl carbon (C=O). The peaks at delta 76.00–78.00 ppm correspond to solvent (CDCl₃).

The American Society for Testing and Materials (ASTM) investigated the characteristics of biodiesel, and the recommended values for biodiesel under ASTM D6751 are shown in Table 2.

Table 2. Characteristics of Biodiesel synthesized through *Bacillus sp* OQ135194 (Taken from open access sources) [47].

Properties	Petrodiesel	Standard biodiesel	Biodiesel from <i>Bacillus sp.</i> (OQ135194)	Units
Kinematic viscosity @ 40°C	0.834	1.9 - 6.0	4.6	mm ² s ⁻¹
Density @ 40°C		0.86 - 0.90	0.8821	g cm ⁻³
Specific gravity @ 40°C	0.85	0.88	0.8891	g cm ⁻³
Pour point	-35 to 15	-15 to 16	-13.0	°C
Cloud point	-15 to 5	-3 to 12	3.0	°C
Flash point	60–80	100–170	105.5	°C
Acid number	0.5	0.5	0.67	mg KOH g ⁻¹
Iodine number	80–135	-	114.54	%(m/m)
Cetane number	15–20	47 minimum	60	-

The viscosity of the fuel is one of its most crucial characteristics, as it affects temperature and how the fuel injector operates. According to ASTM guidelines, the viscosity of the *Bacillus* fuel was $4.6 \text{ Mm}^2\text{s}^{-1}$, which is within permitted bounds. Higher cloud and pour points indicate higher polyunsaturated fat content and lower fuel viscosity. Additionally, these features are better suited to a range of weather conditions. At 40°C , biofuel from the test organism had a density of 0.8821 g cm^{-3} , which is likewise within the acceptable range of ASTM criteria. The product's specific gravity was slightly higher than the ASTM standard (0.8891 g cm^{-3} at 40°C). The pour point is the temperature at which fuel continues to exist in its fluid state, as opposed to the cloud point, which is the temperature at which biodiesel starts to change into wax. The FAME's cloud point and pour point, which are both within the normal range for biodiesel, are 3°C and -13°C , respectively. The lowest temperature at which vaporization is feasible is called the flash point. Our test sample's flash point, 105.5°C , falls within the range for conventional biofuels. In our research, we were able to find biodiesel with a 60 cetane rating. The cetane number is used to evaluate the efficiency or caliber of diesel fuel. The engine of an automobile burns fuel more efficiently the higher the number.

4. Conclusions

Bacillus sp. (OQ135194) was shown to be particularly efficient at using peanut waste peel powder as agricultural waste and accumulating monounsaturated fatty acids. It was identified from a dairy waste sample. The organism accumulated oil using powdered peanut waste peel, and these fatty acids have been shown to be useful in the synthesis of FAME. When the oil: methanol ratio was set at 1:9, and the temperature was raised to 75°C for 100 minutes, 0.6% sodium hydroxide enhanced the yield of fatty acid methyl ester. The biodiesel produced by the test organism from agricultural waste met the requirements set by the ASTM and ASAM standards. The biodiesel's cetane value was within the permitted range for fuel quality. We can infer from this that the chosen strain has the potential to be used in the production of biodiesel. This bacterium converts environmentally hazardous agricultural waste into biodiesel, reducing reliance on non-renewable energy sources. As a result, it may be exploited as a source for a possible fuel factory.

Author Contributions

Conceptualization, R.C. and P.P.; methodology, R.C. and P.P.; investigation, R.C. and P.P.; resources, R.C. and P.P.; data curation, R.C. and P.P.; writing—original draft preparation, R.C.; writing—review and editing, R.C. and P.P.; visualization, R.C.; supervision, P.P.; project administration, R.C. and P.P. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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