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New Proton Nuclear Magnetic Resonance-Based Derivation for Quantification of Alkyl Esters Generated Using Biocatalysis

Anirudh Sharma,[†] Atish Verma,[†] Vijay Luxami,[†] J. S. Melo,[‡] S. F. D'Souza,[‡] N. Tejo Prakash,[§] and Ranjana Prakash*[,†]

[†]School of Chemistry and Biochemistry, and [§]School of Energy and Environment, Thapar University, Patiala 147 004, India

[‡]Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay 400 085, India

Supporting Information

ABSTRACT: Monoalkyl esters of fatty acids commonly known as biodiesel are synthesized from triglycerides by the transesterification reaction with monohydric alcohol, usually methanol or ethanol. Biodiesel is an attractive alternative fuel for diesel engines because of its renewability, biodegradability, and nontoxicity. Several methods/approaches have been developed for analyzing the fuel quality of biodiesel. Mainly chromatographic techniques [e.g., gas chromatography (GC), high-performance liquid chromatography, etc.] are being used for the analysis of biodiesel. The equation for quantification of the transesterification reaction using proton nuclear magnetic resonance (^{1}H NMR) is available in the literature, wherein methanol/ethanol are being used as an acyl acceptor. In the present work, we report the equation based on ^{1}H NMR, which can be used for the quantification of the transesterification reaction, using other primary alcohols as an acyl acceptor. Simultaneously, we have also studied the effect of the chain length of alcohols on the extent of transesterification using whole cell catalysts. Transesterification was enhanced using butanol (67%) and pentanol (76%), followed by a decrease with hexanol (66%) and octanol (56%). The correlation coefficient (R^2) between GC and ^{1}H NMR methods was 0.97. The results obtained by the new ^{1}H NMR equation proposed in this work were well-correlated with GC analysis of the same samples.

1. INTRODUCTION

A high energy demand and increased environmental pollution-related problems because of the use of fossil fuels have necessitated the development and adaptation to renewable and ecofriendly fuels. Making use of biodiesel is one such initiative that has been projected as a renewable alternative to diesel fuel. Its reduced engine emission profiles and direct usability with existing diesel engines have attracted the world's attention to a large extent.[1]

Biodiesel consists of a long chain of fatty acid esters produced by the transesterification reaction of vegetable oils with alcohols using a suitable catalyst, viz., chemicals or enzymes. Chemical catalysts include various acids (e.g., H_2SO_4) and alkalies (e.g., NaOH). Transeterification by acid catalysis is much slower and more suitable for oils and fats with relatively high free fatty acid (FFA) and water contents. An acid-catalyzed reaction also commonly requires a high temperature. For alkali-catalyzed transesterification, the starting materials (oil or fats) must be devoid of moisture and FFA. The presence of a minor amount of FFA and moisture in the reaction mixture produces soap, which interferes in the process of transesterification and, thus, lowers the yield of esters.

In comparison to the chemical approach, the use of lipase (extracellular and intracellular) as the biocatalyst has promising potential because it eliminates obvious disadvantages of the chemical process. In addition, biocatalysis facilitates the yield of a high-purity product with less or no intensive downstream operation associated with the recovery of glycerol or catalyst.[2] Microbial lipase technology has shown enormous potential for making ester derivatives for various specific applications.[3,4] Despite numerous advantages, the enzymatic process has some drawbacks, such as a low reaction rate, low enzyme stability in the presence of excess methanol, and high cost of pure lipase. A higher level of oil supplementation can inhibit the activity of pure lipase during the transesterification reaction. As an effective alternative, whole cells that are capable of producing lipase in specific culture conditions have been shown to effectively catalyze the transesterification reaction, even in high oil supplementations.[5−7]

Methanol has been the most commonly used alcohol in the production of biodiesel. The degree of deactivation is estimated to be inversely proportional to the number of carbon atoms in the alcohol, which means that methanol is the most deactivating alcohol.[8,9] Other alcohols used include ethanol, propanol, isopropanol, butanol, and pentanol for the conversion of oil to alkyl esters.[10−17] It is also thought that the rate of the transesterification reaction using lipase increases with the length of the carbon chain of the alcohol, implying that the use of ethanol over the use of methanol increases the rate of the transesterification reaction.[18]

Besides, several studies have focused on the development and improvement of analytical methods for monitoring the yield of alkyl esters and determining their fuel quality.[19−24] Among the various approaches, the NMR method can be used for quantitative analysis based on the fact that the amplitude of a proton nuclear magnetic resonance (^{1}H NMR) signal is proportional to the number of hydrogen nuclei contained in the molecule.[20,23] Although gas chromatography (GC) and

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high-performance liquid chromatography (HPLC) are more sensitive techniques than NMR, the latter is a more rapid and easier method to use than the former. The area of the NMR peak mainly depends upon the number of protons and not the response factor, as obtained through chromatographic technique(s). Each component of alkyl esters in the sample has a different response factor, which needs to be predetermined and used in the quantitative determination by the chromatographic techniques. Such laborious processes can be eliminated in the case of NMR detection. However, equations based on ^{1}H NMR are reported for the quantification of methyl and ethyl esters[23,25] but not for the quantification of alkyl esters generated from other alcohols to the best of our knowledge.

In the present work, alkyl asters were generated using different alcohols (methanol to decanol and 2-methyl-propane-1-ol) as acyl acceptors using whole cell biocatalysts and an equation based on ^{1}H NMR was derived, which can be applied for the quantification of alkyl esters having primary alcohols other than methanol and ethanol as acyl acceptors. The yield of alkyl esters, as obtained from the derived equation, was further cross-validated with the yield quantified using GC. In addition, the study also demonstrates the use of dry biomass for biocatalysis and waste cooking oil (rice bran) as a substrate for whole-cell-catalyzed hydrolysis.

2. EXPERIMENTAL SECTION

2.1. Materials. The refined rice bran oil and cotton seed oil were procured from a retail market. Culture media, viz., Bushnall Hass Broth (BHB) and potato dextrose broth (PDB), and mycological peptone were purchased from Hi-Media, India. Other chemicals, such as ethanol (and other alcohols), hexane, ethyl acetate, silica gel (G) for thin-layer chromatography (TLC), biammonium hydrogen *ortho*-phosphate [$(NH_4)_2HPO_4$], potassium hydroxide (KOH), hydrochloric acid (HCl), sodium thiosulphate ($Na_2S_2O_3$), starch, potassium iodide (KI), and phenolphthalein, were purchased from SD Fine-Chem Limited, India. All of the reagents used were of analytical grade.

2.2. Preparation of Used Frying Oil. Used rice bran oil was generated following deep frying for 5 h. The FFA value of virgin and fried oil samples was determined using a standard method outlined by AOCS Ca5a-40.

2.3. Preparation of Biomass. The spores of *Aspergillus* sp. (MTCC 5436) isolated from biocontaminated clarified butter were inoculated aseptically in a 500 mL Erlenmeyer flask containing 200 mL of sterile PDB and incubated at 30 °C and 120 rpm for 3 days.[7] The active culture obtained from PDB was further used for experimentation. The minimal medium BHB containing $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.02 g/L), KH_2PO_4 (1.0 g/L), K_2HPO_4 (1.0 g/L), and $FeCl_3$ (0.05 g/L), supplemented with mycological peptone (0.5%, w/v), $(NH_4)_2HPO_4$ (0.5%, w/v), and virgin cotton seed oil (30%, v/v), was used as a growth medium. Mycological peptone and $(NH_4)_2HPO_4$ were used to supplement nitrogen, and cotton seed oil was used as a main carbon source for fungal growth. The culture flask was incubated at 30 °C and 120 rpm for 5 days. Fungal biomass was separated by filtering through Whatman filter paper, washed with hexane to remove the excess oil, and dried with blotting paper. The partially dried biomass was crushed in liquid nitrogen to make homogeneous powder using a pestle and mortar.

2.4. Transesterification Reaction. A total of 1.0 g of dried powdered biomass was taken in a round-bottomed flask containing 10 mL of used frying oil with FFA at 0.93 ± 0.05%. A total of 3.0 mL of alcohol (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, or 2-methyl-propane-1-ol) was added, and the mixture was stirred for 36 h on a magnetic stirrer at 30 °C. The reaction mixture along with biomass was washed 3 times with 10 mL of hexane to separate the product. The progress of the reaction was checked regularly by TLC.

2.5. Identification and Quantification of Alkyl Esters. The product (ester) obtained was analyzed using TLC with silica gel G as the stationary phase and hexane/ethyl acetate (9:1) as the mobile phase. The chromatogram was developed in the iodine chamber. Further, the product was quantified by GC using methyl heptadecanoate as a standard. The percentage of alkyl ester of fatty acid present in the sample was determined according to EN ISO 5508 with internal calibration (10 mg/mL methyl heptadecanoate). The sample was prepared by weighing 250 mg of alkyl ester in a 10 mL vial, followed by the addition of 5 mL of methyl heptadecanoate (10 mg/mL). A total of 1.0 μL of sample was injected into GC-5765 (Nucon, India) equipped with a flame ionization detector. A fused silica capillary column (0.25 mm internal diameter, 30 m length, and 0.25 μm film thickness, wall coated with EC wax/polythene glycol) was used to separate alkyl ester. The flow rates of nitrogen as a carrier gas and hydrogen were 30 mL/min, while that of zero air was 300 mL/min. The injector and detector temperatures were maintained at 230 and 240 °C, respectively. The oven initial temperature (160 °C) hold time was 1 min, and the final oven temperature was 240 °C. The rate of increase in the temperature was 4 °C/min, and complete program duration was 45 min. A split injection ratio of 1:30 and a split flow rate of 30 mL/min were maintained. The ester content C, expressed as a mass fraction in percent, was calculated using the following formula:

$$C = \frac{(\sum A) - A_{EI}}{A_{EI}} \frac{C_{EI} V_{EI}}{m} \times 100$$

where $\sum A$ is the total peak area from the alkyl esters of oil, A_{EI} is the peak area corresponding to methyl heptadecanoate, C_{EI} is the concentration in milligrams per milliliter of the methyl heptadecanoate solution, V_{EI} is the volume in milliliters of methyl heptadecanoate solution being used, and m is the mass in milligrams of the sample.

The alkyl esters were analyzed further, using ^{1}H NMR (Bruker-Advance II-400 with 5 mm BBO probes) with $CDCl_3$ as a solvent, and chemical shifts were expressed in parts per million with tetramethylsilane (TMS) as an internal standard. A duplicate analysis was carried out using JEOL (400 MHz, ^{1}H NMR). Table 1 presents the mean and standard deviation (SD) between the two analytical values. Methyl and ethyl ester contents in the reaction mixture were quantified using the equation proposed by Gelbard et al.[23] and Ghesti et al.,[25] respectively.

Table 1. Effect of Different Alcohols on the Extent of Transesterification[a]

	percent conversion	
acyl acceptor	GC	^{1}H NMR
methanol	8.13 ± 0.06	9.47 ± 0.49
ethanol	7.90 ± 0.58	12.76 ± 0.00
propanol	3.84 ± 0.26	4.88 ± 0.38
butanol	73.79 ± 4.27	67.15 ± 2.89
pentanol	75.55 ± 2.30	76.35 ± 1.24
hexanol	59.96 ± 1.40	66.25 ± 1.87
heptanol	75.41 ± 2.49	73.40 ± 0.96
octanol	54.91 ± 4.79	55.69 ± 2.53
nonanol	50.62 ± 1.08	46.40 ± 2.06
decanol	40.27 ± 1.63	47.92 ± 1.39
2-methyl-propane-1-ol	67.26 ± 2.72	65.94

[a]Data are the mean ± SD (n = 3) in the case of GC.

The alkyl esters produced by the reaction with various other primary alcohols, viz., propanol to decanol and 2-methyl-propane-1-ol, were quantified by deriving a modified equation proposed by Gelbard et al.[23] for methyl esters given below, wherein the triplet at 4.00−4.10 ppm of methylenic protons indicates alkyl ester ($-CO_2\mathbf{CH_2}(CH_2)_xCH_3$) formation. The signals at 2.30 ppm result from the protons on the CH_2 groups adjacent to the alkyl or glyceryl ester moieties ($-\mathbf{CH_2}CO_2CH_2(CH_2)_xCH_3$ for alkyl esters)

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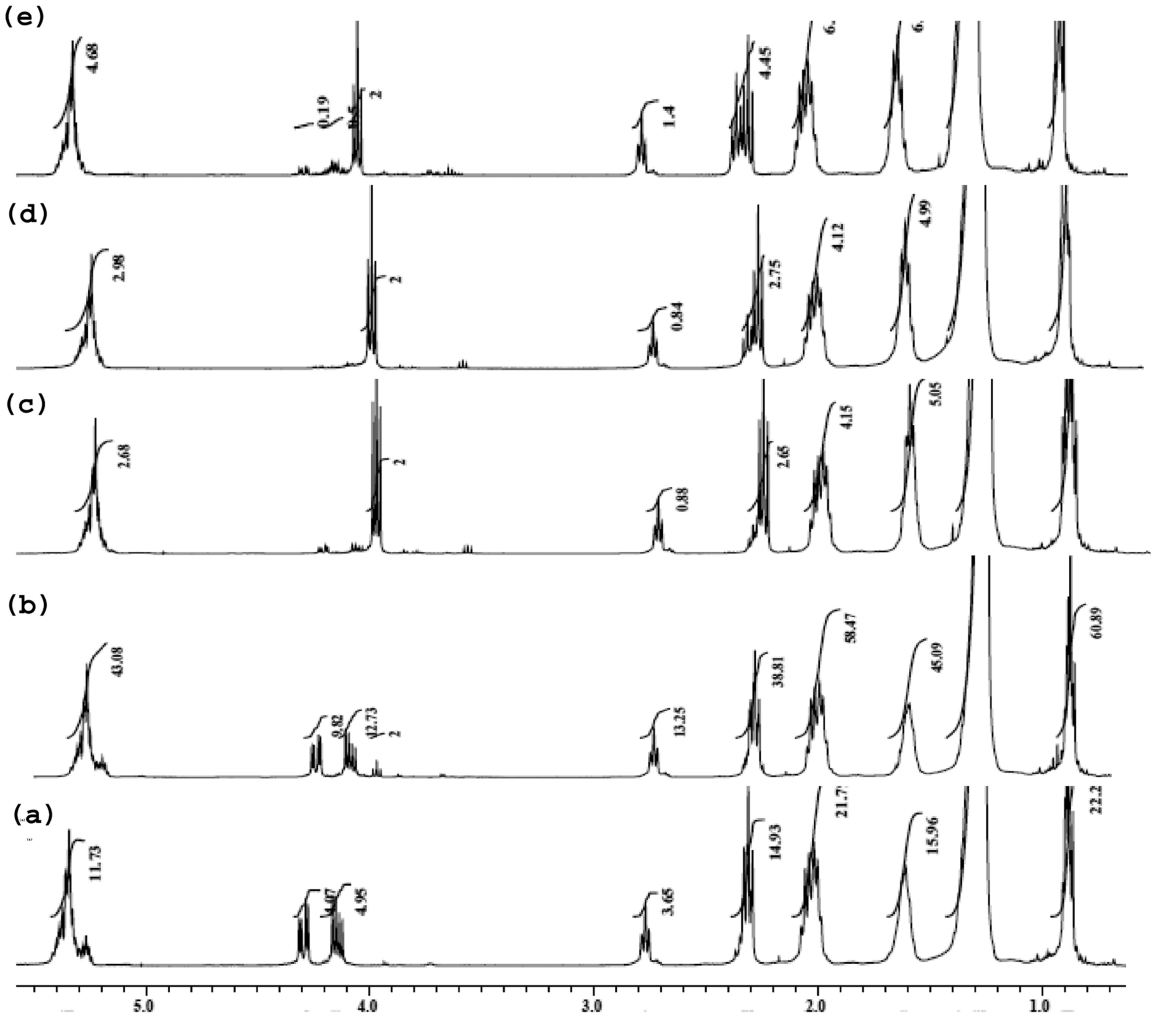

Figure 1. ^{1}H NMR spectra for (a) pure oil, (b) alkyl ester of propanol, (c) pentanol, (d) heptanol, and (e) nonanol.

$$C = 100\left(\frac{AE_{\alpha\text{-}CH_2}}{A_{\alpha\text{-}CH_2}}\right)$$

where C is the conversion of triacylglycerol of the feedstock (vegetable oil) to the corresponding alkyl ester, $AE_{\alpha\text{-}CH_2}$ is the integration value of the methylene protons of the alkyl esters (the triplet peak), and $A_{\alpha\text{-}CH_2}$ is the integration value of the methylene protons.

3. RESULTS AND DISCUSSION

3.1. GC and ^{1}H NMR Analyses. The present work is based on the whole-cell-catalyzed transesterification reaction of used rice bran oil in the presence of alcohols of different chain lengths. Preliminary examination by TLC indicates the formation of ester in each case. Table 1 presents the results obtained from GC analysis, showing a noticeable influence of the chain length of alcohol on the extent of transesterification.

The ^{1}H NMR results further confirmed the formation of alkyl esters. The ^{1}H NMR of methyl ester indicated a singlet in the region of 3.60 ppm because of the proton of methyl ester (see Supplementary Figure 1 of the Supporting Information). In the case of ethyl ester, the appearance of a quartet of $-OCH_2$ at 4.10−4.20 ppm confirmed the formation of the ester (see Supplementary Figure 2 of the Supporting Information). With reference to other alkyl esters, ^{1}H NMR of alkyl ester obtained after transesterification indicated the appearance of a triplet at an integration value around 4.10 ppm. The integration value of this triplet was used in the modified equation for the quantification of different alkyl esters. The small triplet at 3.60 ppm (panels c and d of Figure 1) is due to methylene protons of unreacted alcohols.

To the best of our knowledge, there is no equation known in the literature to quantify alkyl ester based on ^{1}H NMR for acyl acceptors other than methanol and ethanol. The new derivation can be applied for the quantification of any alkyl ester generated from primary alcohols other than ethanol and methanol, whereas the derivation given by Gelbard et al.[23] can only be applied for quantification of methyl esters. The objective behind proposing this equation was to exploit the efficacy of the NMR technique because of obvious advantages, such as faster and easier adaptable analysis, non-destructive measurements, and ease with smaller amounts of samples.[24,25] In the present study, the percent conversion obtained by GC significantly correlated (R^2 = 0.98) with the percent conversion obtained by ^{1}H NMR (Figure 2).

The extent of transesterification enhanced from butanol (≅70%) to pentanol (≅76%) and decreased in hexanol (≅63%) and octanol (≅55%). These observations differ from those reported by Romero et al.,[26] wherein there was no noticeable influence on the esterification effect when propanol, butanol, hexanol, and octanol were used as acyl acceptors.

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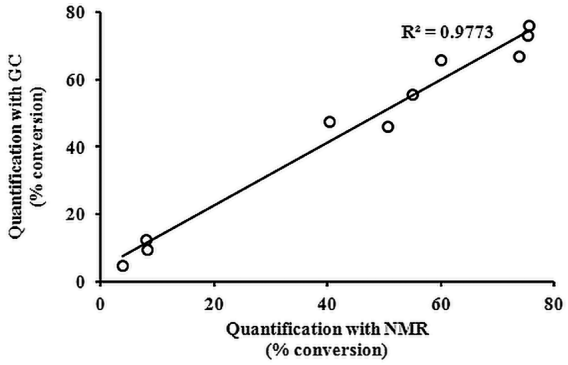

Figure 2. Correlation between the alkyl ester yields quantified using GC and NMR.

In the case of the transesterification reaction employing methanol, methanolysis takes place between two immiscible liquids.[27,28] Upon formation of diacyl and monoacyl glyceridic intermediates in sufficient quantities, they serve as surfactants that improve mass transfer of triacylglycerides into the methanol phase.[29] Ethanol is of particular interest primarily because it is less expensive than methanol in some regions of the world (such as Brazil). However, the ethanolysis reaction proceeds at a slower rate than methanolysis because of the higher reactivity of the methoxide anion in comparison to ethoxide. As the length of the carbon chain of the alkoxide anion increases, a corresponding decrease in nucleophilicity occurs, resulting in reduced reactivity of ethoxide in comparison to methoxide.[30] The observations obtained in the case of chemical catalysis by other researchers also support our results.[31] However, there are limited reports on the use of other alcohols as acyl acceptors in whole-cell-catalyzed transesterification. Long-chain alcohols, 2-propanol and *n*-butanol, have a less negative effect on lipase stability, and they also improve low-temperature properties of the fuel.[32] However, excess alcohol leads to inactivation of the enzyme, and glycerol, a major byproduct, can block the immobilized enzyme, resulting in low enzymatic activity.[33] Butanol may also be obtained from biological materials, thus yielding completely bio-based biodiesel as well.[34,35] Butanol is completely miscible with vegetable oils and animal fats because it is significantly less polar than methanol and ethanol.[36] Consequently, transesterification reactions employing butanol are monophasic throughout.[37,38] The monophasic nature of the butanolysis reaction further enhances the rate and extent of the reaction.

4. CONCLUSION

The present study thus proposes an easier method for quantification of alkyl esters with data obtained through ^{1}H NMR spectroscopic analysis with primary alcohols as acyl acceptors. The study also outlines the application of whole cell catalysis for the transesterification of waste edible oils, which can be an alternative approach for the generation of alkyl-ester-based biodiesel in place of chemical catalysis.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra used in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +91-175-2393119. Fax: +91-175-2364498. E-mail: rprakash@thapar.edu.

Notes

The authors declare no competing financial interest.

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