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CHARACTERISTICS OF METHYL ESTERS DERIVED FROM ENZYME ASSISTED EXTRACTS OF *BARRINGTONIA ASIATICA* AND *RICINUS COMMUNIS*

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Barringtonia asiatica, also known as Botong nut, and *Ricinus communis*, also known as Castor bean, were both studied as possible sources of biodiesel. The samples were subjected to enzymatic pre-treatment with the use of protease and pectinase enzymes followed by solvent extraction with n-hexane and petroleum ether as solvents. The results showed that the Castor beans have a higher yield of oil than Botong nut samples. The samples which underwent multiple n-hexane extractions and were not subjected to enzymatic pre-treatment showed higher average oil percentage yield. Enzymatic treatment of the samples followed by solvent extraction exhibited lower amount of oil recovered. However, results also showed that the enzymatic pre-treatment rendered ease into the recovery of the sample oil inasmuch as two consecutive solvent extraction procedures are enough to collect the oil from the sample. The oil samples were transesterified using both acid-catalyzed and base-catalyzed transesterification methods. The fatty acid methyl esters were analyzed using Gas Chromatography-Mass Spectrometry and Gas Chromatography-Flame Ionization Detector to determine its constituents. The kinematic viscosity of the castor bean sample was determined to be 2.813 mm^2/s, which passed the Philippine National Standard specifications. However, the acid number of castor bean FAME which measured 21.3 mg KOH/g sample did not meet the said specifications.

Key Words: *Barringtonia asiatica; Ricinus communis;* Fatty Acid Methyl Esters; Enzyme-assisted extraction; Biofuel

1. INTRODUCTION

Biodiesel production is a growing market due to the need for clean and renewable sources of energy. To help extend the supply of petroleum products, vegetable oils and used cooking oils are commonly made into biodiesel. Biodiesel is said to give better performance, energy efficiency and lesser greenhouse gas emissions as compared to the regular diesel available in the market[1]. According to the Biofuels Act of 2006[2], the law mandates a minimum of 2% blend of biodiesel in all diesel products to decrease the amount of oil imported and to have a cheaper source of diesel. The total consumption of petro diesel in the Philippines is estimated at about seven billion liters per year. However, according to the International Energy Statistics[3] as of 2010, the Philippines is only producing around 400 thousand liters of biodiesel a year. This annual production is insufficient to satisfy the mandate of the Biofuels Act. Thus, this calls for a need for new sources and feedstock for biodiesel production.

Ricinus communis (Castor beans) and *Barringtonia asiatica* (Botong nut) are said to be possible sources of biodiesel. These two plant sources are grown locally and in large scale without competing with food demands. The castor plant is included in the family of Euphorbiaceae, flowering plants. Its oil is commonly used as lubricants, dye, drying agent in paints and varnishes, and in the production of waxes. In folk medicine it is used commonly as antidote and laxative. In other countries such as Brazil, the oil extracted from castor beans is now used to produce biodiesel[4]. It consists principally of ricinoleic acid with only small amounts of dihydroxystearic, linoleic, oleic, and stearic acids as well as the unsaponifiable matter that contains sitosterol[5]. On the other hand, seeds of *Barringtonia asiatica* contain about 2.9% of fixed oil including olein, palmitin, and stearin, 0.54% of galic acid, and a glucoside, barringtonin of 3.271%[6] which can also be considered as an alternative fuel.

This study aims to determine if *Barringtonia asiatica* and *Ricinus communis* are effective sources of biodiesel through extraction of their oils and transesterification to produce a fatty acid methyl ester. The properties of the

biodiesel produced would be assessed using the Philippine National Standards (PNS) specifications with the use of standard test methods from the American Society for Testing and Materials (ASTM) and the American Oil Chemists' Society (AOCS).

2. METHODOLOGY

2.1. MATERIALS

The chemicals used for solvent extraction were n-hexane and petroleum ether. For the preparation of the buffer solution for pectinase pre-treatment ofthe samples, acetic acid (CH_3COOH) and sodium acetate ($NaCH_3COO$) were used while for protease pre-treatment of the samples, ammonium hydroxide (NH_4OH) and ammonium chloride (NH_4Cl). In the base-catalyzed transesterification process, toluene (C_7H_8), methanol (CH_3OH), and sodium metal were reacted to produce methoxide solution while Sulfuric acid (H_2SO_4) and methanol (CH_3OH) were reacted for the acid-catalyzed transesterification. For the determination of acid number, Potassium hydroxide (KOH) was standardized against Potassium Hydrogen Phthalate ($C_8H_5KO_4$). A solution of toluene and isopropyl as the solvent and phenolphthalein indicator in isopropyl alcohol were prepared. All chemicals and reagents were bought from Scharlau and J.T. Baker and were provided by De La Salle University – Manila.

The enzymes used for enzymatic pre-treatment of the samples were Protex 6L (protease, alkaline serine endopeptidase) and Multifect Pectinase FE (pectinase, complex type of enzyme derived from *Aspergillus niger* var.) which were obtained from Di-catalyst International Corporation.

2.2. SAMPLE PREPARATION

Botong nuts were obtained from De La Salle University, Manila and University of the Philippines, Los Baños, Laguna. They were deshelled and the "meat" inside were freeze-dried for one week using Labconco Freeze-dry System Freesome 4.5 to remove the moisture in the sample. Then, the freeze-dried Botong nut samples were ground using an Osterizer blender and weighed using a triple-beam balance. The sun-dried castor beans were bought from Richfund Import & Export, Co. Ltd. Makati City. The sun-dried castor beans were ground using an Osterizer blender and weighed using a triple-beam balance.

2.3. EXTRACTION METHODS

2.3.1. Enzymatic Pre-treatment

2.3.1.1. Protease

Both castor beans and Botong nut samples were weighed and placed into separate one (1) L beakers. An ammonium hydroxide-ammonium chloride (NH_4OH/NH_4Cl) buffer solution with a pH of 9.5 and an amount of 0.1% (w/w) of Protex 6L enzyme were added. The samples were then left to stand for twenty-four (24) hours.

2.3.1.2. Pectinase

Both fruit samples were weighed and placed into separate one (1) L beakers. A sodium acetate-acetic acid ($NaCH_3COO/CH_3COOH$) buffer solution with a pH of 4.5 was added to the samples. Amounts of 0.1% (w/w) of Multifect Pectinase FE were then added to the mixtures and were left to stand for twenty-four (24) hours.

2.3.2. Solvent Extraction

2.3.2.1. n-Hexane

One hundred fifty (150) mL of n-hexane was added to every one hundred (100) grams of fruit sample. The mixture was stirred for one (1) minute and was left to stand for twenty-four (24) hours. The solvent was separated from the extracted oil through filtration and was subjected to rotary evaporation using Heidolph w2000 Rotary Evaporator set to 40ºC and 200 rpm. The solvent was collected after rotary evaporation to be reused for successive filtrations. For successive extractions, this procedure was done 10 times for both pre-treated and control samples.

2.3.2.2. Petroleum Ether

One hundred fifty (150) mL of petroleum ether was added to every one hundred (100) grams of fruit sample. The mixture was stirred for one (1) minute and was left to stand for three (3) hours. The solvent was separated from the extracted oil through filtration and was subjected to rotary evaporation using Heidolph w2000 Rotary Evaporator set to 40ºC and 200 rpm. The solvent was collected after rotary evaporation to be reused for successive filtrations.

2.4. TRANSESTERIFICATION PROCESS

The oil was first placed into another round bottom flask. A measured amount of toluene was then added into it and was pre-heated to around 60 – 70°C. The prepared sodium methoxide or methanol-sulfuric acid solution was added slowly and after two (2) hours, the solution was cooled down and was transferred into a separatory funnel to separate the glycerol layer and the fatty acid methyl esters.

2.5. CHARACTERIZATION METHODS

2.5.1.*Gas Chromatography – Mass Spectroscopy orGas Chromatography - Flame Ionization Detector Analysis of Transesterified Products*

Perkin Elmer Clarus 500 Gas Chromatogram-Mass Spectrometer (GC-MS) with 5% phenylmethylpolysiloxane column having a length of 30 meters and a diameter of 0.25 millimeter was used to characterize the transesterified oil samples extracted using n-Hexane solvent. 5890 Series II Gas Chromatograph - Flame Ionization Detector (GC-FID) with a column of HP-5 Cross-linked 5% phenylmethylsilicone, having a length of 30 meters, a diameter of 0.32 millimeters and a film thickness of 0.25 micrometer was used to characterize transesterified oil samples extracted using Petroleum ether solvent. The parameters for both analyses were set to 100°C for initial temperature for two (2) minutes, a rate of 10°C per minute to a final temperature of 250°C.

2.5.2. Determination of Kinematic Viscosity Using ASTM Method D445

The calibration constant of the Ostwald Glass-Capillary Viscometer apparatus was first determined using distilled water at a temperature of 40°C. The biofuel produced was added to the viscometer and heated to 40°C in a water bath. The time it took for the liquid sample to reach point B from point A was measured. The Kinematic Viscosity of the biofuel produced was then calculated.

2.5.3 Acid Number Determination Using AOCS Method Cd 3d-63

The acid number of the biofuel produced that was dissolved in a toluene-isopropyl alcohol solution was obtained by titration of the standardized 0.1M KOH solution with the sample using 1.0% phenolphthalein in isopropyl alcohol as indicator.

3. RESULTS AND DISCUSSIONS

3.1. Optimization of Extracted Triglyceride Oils

3.1.1. Single n-Hexane Extraction of Botong Nut and Castor Bean Triglyceride Oil

The first portions for both Botong nut and Castor bean were for the control sample where single extraction with the solvent n-hexane was done. The second portion was treated with pectinase and was subjected to solvent extraction with n-hexane afterwards. The third portion was mixed with protease and was subsequently subjected to solvent extraction.

The extraction results show that the castor beans had a higher percentage yield than that of the Botong nut samples because of the composition of the samples having different percentages of extractable oil present. However, comparing the extraction methods, both fruit samples showed similar results. The enzyme pre-treatment for both plant samples showed lower yield than that of the control caused by the short period of time of exposure of the enzyme to the sample, only breaking down a small amount of pectin or protein of the samples. Protease enzymes showed higher yield than that of pectinase enzymes because of the higher amounts of protein present in both samples.

Table 1. Percentage Triglyceride Yield of Single Extraction on Castor Beans

Trial	Control (% yield)	Pectinase (% yield)	Protease (% yield)
1	4.28	4.52	4.79
2	5.95	0.10	3.04
3	2.90	8.33	7.03
4		0.83	1.95
5		2.25	2.75
Average	4.38	3.21	3.91

Table 2. Percentage Triglyceride Yield of Single Extraction on Botong Nut

Trial	Control (% yield)	Pectinase (% yield)	Protease (% yield)
1	0.20	0.32	0.26
2	0.80	0.12	0.12
3	0.93	0	1.20
Average	0.64	0.15	0.52

3.1.2. Successive Extraction of Triglyceride Oil using n-Hexane and Petroleum Ether as Solvents

Castor bean samples were subjected to exhaustive and successive solvent extraction. These samples were divided into two, having one portion subjected to hexane extraction, while the other for petroleum ether extraction.

Table 3. Percentage Triglyceride Yield of Successive Solvent Extraction using Hexane on Castor Beans

Extraction	Control (% Yield)	Pectinase (% Yield)	Protease (% Yield)
1	5.95	2.25	2.75
2	4.05	3.90	6.15
3	1.00	0.00	0.00
4	2.00	0.00	0.00
5	3.80	0.00	0.00
6	1.20		
7	0.00		
Total	18.00	6.15	8.90
Average (of first 2 extractions)	5.00	3.08	4.45

Table 4. Percentage Triglyceride Yield of Successive Solvent Extraction using Petroleum Ether as Solvent on Castor Bean

Extraction	Control (% Yield)	Pectinase (%Yield)	Protease (% Yield)
1	2.65	2.92	2.00
2	2.25	1.74	2.54
3	1.00	1.84	2.88
4	2.60	2.90	3.28
5	2.00	1.96	2.18
Total	10.05	11.36	12.88
Average	2.10	2.27	2.58

In comparison with all the successive extraction methods, n-hexane without enzymatic pre-treatment had the highest average percentage yield of oil extracted. However, it needed an optimum of six successive extractions to maximize the amounts of triglyceride oil that can be collected. On the other hand, the extraction methods that utilized petroleum ether as solvent needed more repetitions of extractions to get the maximum amount of triglyceride oil that can be collected. This would be disadvantageous due to the amount of solvent that is needed to be used to collect a large amount of oil from the plant. The enzyme pre-treated samples using n-hexane as solvent showed fewer extractions but smaller amounts of triglyceride oil extracted. This may entail a lower amount of solvent to be used and a shorter period of time to collect triglyceride oil from the sample. With the enzymatic pre-treated samples for both solvents, the protease enzyme yielded higher than that of the pectinase enzyme, which may be due to the higher amounts of peptide bonds present in the plant sample. Another difference between the two extraction methods was the color of the oil. The n-hexane extracts pre-treated with the enzymes showed darker shades of brown than that of the petroleum extracts, having the sample pre-treated with protease as the darkest. This may be attributed to the composition of the oil extracts and/or to the color of the enzyme used.

3.2. Components of Transesterified Products Analyzed Using Gas Chromatography – Mass Spectrometry (GC-MS) and Gas Chromatography – Flame Ionization Detector (GC-FID)

The castor bean transesterified products Botong nut transesterified products pre-treated with pectinase and protease were analyzed using the Gas Chromatography-Mass Spectrometry (GC-MS) or Gas Chromatography-Flame Ionization Detector (GC-FID). The figures and tables below show the different retention times and percentage relative peak area of the different compounds found in the transesterified products.

Table 5. Castor Pre-treated with Pectinase GCMS Results

Retention Time	% Relative Peak Area	Compound Name
2.37	1.81	o-Xylene
6.43	1.00	Dodecane
15.97	3.80	Palmitic Acid Methyl Ester
17.94	0.89	Elaidic acid, methyl ester
20.33	90.68	9-Octadecenoic acid, 12-hydroxy-, methyl ester, (Z)-

Table 6. Castor Pre-treated with Protease GCMS Results

Retention Time	% Relative Peak Area	Compound Name
2.38	2.95	p-Xylene
6.44	1.18	Dodecane
15.96	1.44×10^{-6}	Palmitic Acid Methyl Ester
20.07	89.30	9-Octadecenoic acid, 12-hydroxy-, methyl ester, (Z)-
28.99	6.57	Squalene

Table 7. Botong Pre-treated with Pectinase GCMS Results

Retention Time	% Relative Peak Area	Compound Name
13.54	32.06	2-Propenoic acid, 2-methyl-, hexyl ester
18.21	5.45	Oleic Acid Methyl Ester
21.40	59.57	Ricinoleic Acid Methyl Ester

The GC-FID peaks were co-related with the results from the GC-MS since both showed the same pattern of peaks. Correlation was possible given that the columns of both instruments are the same. The difference on the diameter and the detector would only have an effect on the retention time and peak area.

Table 8. Castor Pre-treated with Pectinase GC-FID Results

Retention Time	% Relative Peak Area	GCMS Retention Time	Inferred Compound Identity
10.65	2.00	15.97	Palmitic Acid Methyl Ester
12.36	14.70	17.94	Elaidic acid, methyl ester
12.55	1.90	18.12	Stearic Acid Methyl Ester
14.43	76.20	20.33	9-Octadecenoic acid, 12-hydroxy-, methyl ester, (Z)-

Table 9. Castor Pre-treated with Protease GC-FID Results

Retention Time	% Relative Peak Area	GCMS Retention Time	Compound Name
10.67	1.60	15.96	Palmitic Acid Methyl Ester
12.35	12.20	17.82	Linoleic Acid Methyl Ester
12.56	1.90	18.11	Stearic Acid Methyl Ester
14.36	67.20	20.08	9-Octadecenoic acid, 12-hydroxy-, methyl ester, (Z)-

3.3. Analysis of the Biodiesel Product Using Standard Test Methods

3.3.1 Kinematic Viscosity

The kinematic viscosity measurement of the remaining transesterified products were done using ASTM Method D445 using an Ostwald viscometer. The results show that the biodiesel made fits the specifications of the Philippine National Standard for kinematic viscosity. The average measured viscosity was found to be 2.813 mm^2/s included between the ranges of 2.0 to 4.5 mm^2/s which is the standard. This is a sign of good fuel injection for the engine system since the viscosity of the sample would give a fast transfer of fuel to the combustion chamber.

3.3.1 Acid Number

The acid number of the transesterified products was measured using the AOCS Method Cd 3d-63. The sample was dissolved with equal parts of toluene and isopropyl alcohol, and titrated with standardized potassium hydroxide solution. The results show that it did not meet the Philippine National Standard of 0.5 mg of KOH per gram of sample. The sample had an average acid number of 21.3 mg of KOH per gram of sample, a higher value compared to the standard specification. This may cause problems to the diesel engine due to the particles it may leave in the combustion chamber as it ignites

Table 10. Kinematic Viscosity of the Transesterified Castor Oil

Trial 1	Time (seconds)	Kinematic Viscosity (mm^2/s)
1	26.43	2.817
2	26.18	2.791
3	26.32	2.805
4	26.34	2.808
5	26.68	2.844
Average	26.39	2.813

Table 11. Acid Number Results of the Transesterified Castor Oil

Trial	Weight of Sample	mL of KOH	Acid Number
1	0.8738	3.65	21.4
2	0.6843	2.90	21.5
3	0.7760	3.20	21.0
		Average	21.3

4. CONCLUSION

Barringtonia asiatica (Botong nut) and *Ricinus communis* (Castor bean) were studied as possible sources of biodiesel through enzymatic pre-treatment with the use of the protease and pectinase enzyme. Different methods of solvent extraction with n-hexane and petroleum ether solvents were also done for optimization of percentage triglyceride oil yield. The castor bean samples showed higher percentage yield of oil than the Botong nut samples due to the higher amounts of triglyceride oil in the sample. The method found to have higher average percentage yield was the successive solvent extraction with n-hexane solvent without enzymatic pre-treatment, having five repetitions of extraction as the optimum number of successive extractions. However, enzymatic pre-treatment with protease enzyme using n-hexane as solvent can decrease the number of extractions, yielding relatively a good amount of triglyceride oil.

The components of the transesterified triglyceride oil from the castor bean and botong nut were identified using Gas Chromatography-Mass Spectrometry (GC-FID) and Gas Chromatography-Flame Ionization Detector (GC-FID). The results show that the transesterification process was indeed successful for both the acid-catalyzed and base-catalyzed reactions.

The kinematic viscosity and acid number of the transesterified castor bean oil were determined. The kinematic viscosity of the sample passed the Philippine National Standard (PNS) which is a good sign of fuel injection to the combustion chamber. However, the acid number failed to go below the limit given by the PNS, possibly causing the formation of engine deposits that can damage the engine.

For further studies, optimization on the recovery of the solvents can be done which may give a cheaper alternative method for oil extraction. Comparisons between the controlled and enzyme pre-treated samples on the engine performance and properties can be done. Enzymatic pre-treatment with other types of enzymes such as alcalase may also be done to further optimize the breakdown of certain parts of the plant. Extraction of oils from the botong nut may be studied with the use of various solvents or extraction methods for better yield. Comparisons between the two types or modifications on the transeseterification methods can be done to make sure that the biodiesel made meets the Philippine National Standards.

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