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ENZYME-ASSISTED EXTRACTION AND DETERMINATION OF PHYSICO-CHEMICAL PROPERTIES OF BIOFUEL FROM *CANARIUM OVATUM* (PILI) AND *ARTOCARPUS ALTILIS* (BREADFRUIT) AS POTENTIAL PLANT OIL FEEDSTOCK FOR BIODIESEL PRODUCTION IN THE PHILIPPINES

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Abstract: In this study, two oil extraction methods, enzyme assisted solvent extraction and multiple solvent extraction, were executed and evaluated to obtain the oil components of *Artocarpus altilis* (Breadfruit) and the non-edible parts (husk) of *Canarium ovatum* (Pili). Recovered oil percentage of normal solvent extractions for Breadfruit and Pili were 1.67% and 2.89%, respectively. Recovered oil percentages from the enzyme activity of Multifect® Pectinase FE were 2.80% for Breadfruit and 13.63% for Pili. Moreover, recovered oil percentages from the enzyme activity of Protex 6L has resulted to 1.60% for Breadfruit and 11.88% for Pili. Extracted oils were subjected to alkali-catalyzed and acid-catalyzed transesterification methods. Normal solvent extraction using n-hexane for Breadfruit and Pili samples and subsequent acid-catalyzed transesterification process had biodiesel yields of 70.33% and 88.59%, respectively. Biodiesel obtained from the activity of Multifect® Pectinase FE are 68.60% for Breadfruit and 83.85% for Pili. Biodiesel yields from the activity of Protex 6L (protease) followed by acid-catalyzed transesterification were 36.36% for Breadfruit and 85.51% for Pili. Fatty acid methyl esters derived from oils of the samples were characterized using Gas Chromatography – Mass Spectroscopy (GCMS) and Gas Chromatography – Flame Ionization Detector (GCFID). Kinematic viscosity and acid value of fatty acid methyl esters were concurred with the Philippine National Standard (PNS).

Key Words: Biofuel, Enzymatic, Canarium ovatum, Artocarpus altilis, Fatty Acid Methyl Ester

1. INTRODUCTION

Energy is a key factor in industrial development and in providing vital services that improve the quality of life, the engine of economic progress.(Amigun, et al., 2008) Currently, the Philippines is facing an increasing demand for energy in conjunction with limited supply and pollution problems. Quite a lot of studies and researches have been conducted in order to come up with viable alternative energy sources, which focuses on solar, geothermal, hydroelectric energies, as well as, wind power, wave power, tidal power, biofuels and biomass.(Conejos, 2008) Among several technologies available as solution to these problems is the use of Biodiesel which is becoming an important consideration as a promising source renewable energy source.

Biodiesel is derived from renewable local sources and thus, its utilization reduces the dependence on imported petroleum fuels.(Knothe)(Zhang. et al., 2003) It is biodegradable and non-toxic.(Zhang. et al., 2003) It encompasses exceptional lubricity.(Zhang. et al., 2003)(Von Wedel, 1999) It has less emissions, unburned hydrocarbons and particulate matter.(Knothe) The by-product, are being recycled thru photosynthesis, reducing the impact of biodiesel on global warming.(Zhang. et al., 2003)(Korbitz, 1999)(Agarwal, 2001)

Despite of the advantages, biodiesel cannot be commercially available in the market because of its higher price value compared to petrodiesel.(Bicol, 2007) Raw materials, used in synthesizing biodiesel are expensive. A report by Haas & Foglia, 2005, exhibited the cost of the least expensive raw materials accounts only for 70 to 85% of the total cost of biodiesel produced and the cost of most biodiesel feedstocks was assumed to be significantly higher than the cost of the marketed petrodiesel.(Foglia, et al. 2005)

The *Canarium ovatum* (Pili) is the most important nut-producing species in the Philippines.(Coronel, 1996) Economically, the Pili nut kernel is the most important part of the fruit because it has many uses.(West, et. al., 1923)(Garcia, 1941)(Brown, 1954)(Galang, 1955)(Oñate, 1967) Conversely, the *Artocarpus altilis* (Breadfruit) is a tropical fruit native to countries of the South Pacific and it is an important food in these areas.(Taylor, et. al., 2007) Its tree has a great productive ability with an average sized tree producing 400 to 600 fruits per year.(NTBG) According to Knothe, 2005, these crops can be good biodiesel feedstocks because oils derived from these have a closer kinematic viscosity than that of petroleum based diesels.(Knothe) Moreover, produced methyl ester from these crops demonstrates similarity to petrodiesel and best shows this suitability in cetane number or ignition quality.

2. METHODOLOGY

2.1 Sample Collection and Preparation

Pili husks were gathered from the provinces of Sorsogon and Albay, and Breadfruits were freshly gathered from the *Artocarpus altilis* tree inside the De La Salle University campus located at the amphitheatre. Raw samples were chopped up into smaller pieces and then dried in an oven at 60°C for 2 days. Dried samples were milled using a grinder and stored in a sealed beaker at room temperature.

2.2 Oil Extraction

2.2.1 Enzyme Pre-treatment of Samples

Previously prepared Pili husk and Breadfruit were placed into two different separate one liter beakers and were each added with two hundred milliliters of prepared buffer sodium acetate–acetic acid, $Na^{+}CH_3COO^{-}/CH_3COOH$, with pH of 4.5. A 200mL of distilled water and Multifect® Pectinase FE equivalent to 0.1% of the weight of the sample were also added to each beaker. Beakers were sealed and placed into an oven set at 40°C. Reaction time of the enzymatic treatment was held up for approximately 120 hours. Integration and regulation of the enzymatic treatment were done every 24 hours. Procedure for pre-treatment using Protex 6L was done the same as pre-treatment using Multifect® Pectinase FE. However, buffer used was ammonium hydroxide–ammonium chloride, NH_4OH/NH_4Cl, with pH of 9.5.

2.2.2 Multiple Solvent Extraction using N-hexane

Solvent extraction was carried out using either 98% analytical grade N-hexane or petroleum ether. Solvent was incorporated separately to the enzyme pretreated and untreated samples. Mixtures were sealed and set aside for at least 24 hours. Resulting mixture was decanted and subjected for centrifugation for 3 minutes to exonerate out the included sediments and ensuing aqueous layer. Hexane extracts were collected and desiccated using Heidolph VV2000 Rotary Evaporator until the oil was free of solvent. All samples were re-extracted four more times.

2.3 Transesterification

2.3.1 Alkali-Catalyzed Transesterification

Extracted oil was placed in the reactor and preheated at 60°C–65°C to remove excess solvents. Upon heating, methanol and sodium hydroxide mixture, with an amount of methanol equivalent to 6:1 molar ratio of methanol to oil and an amount of sodium hydroxide equal to 1% of the oil by weight, was slowly injected into the reactor.(Bicol, 2007) Agitation and temperature was maintained at 60°C–65°C for an hour. Transesterified oil was transferred into a separatory funnel and allowed to settle for at least 24 hours. The upper fatty acid methyl ester layer was then collected.

2.3.2 Acid-Catalyzed Esterification

Procedure for esterification of the extracted oil was done with the same manner as the alkali-caatalyzed transesterification. But the methanol-sulfuric acid mixture injected was composed of an amount of methanol equivalent to 20:1 molar ratio of methanol to free fatty acid and an amount of sulfuric acid equal to 5% of the free fatty acid in the oil. Also, the bottom layer was collected since the methanol and water mixture rose on top. The free fatty acid content of oils was determined using AOCS Aa 6-38, Standard Test Method for Free Fatty Acid Determination.

2.4 Characterization of Fatty Acid Methyl Ester

2.4.1 Gas Chromatography Mass Spectrometry (GC-MS)

The transesterified oils were subjected to Perkin Elmer Clarus 500 Gas Chromatogram-Mass Spectrometer. The column used was 5% phenylmethylpolysiloxane with a length of 30 meters and a diameter of 0.25 millimeter. The parameters set were 80°C for initial temperature, an increase of 10°C per minute to a final temperature of 280°C.

2.4.2 Gas Chromatography Flame Ionization Detector (GC-FID)

The acid-catalyzed transesterified oils were subjected to 5890 Series II Gas Chromatograph-Flame Ionization Detector. The column used was HP-5 Cross-linked 5% phenylmethylsilicone with a length of 30 meters, a diameter of 0.32 millimeter and a film thickness of 0.25 micrometer. The parameters set were 80°C for initial temperature, an increase of 10°C per minute to a final temperature of 280°C.

3. RESULTS AND DISCUSSION

3.1 Oil Extraction

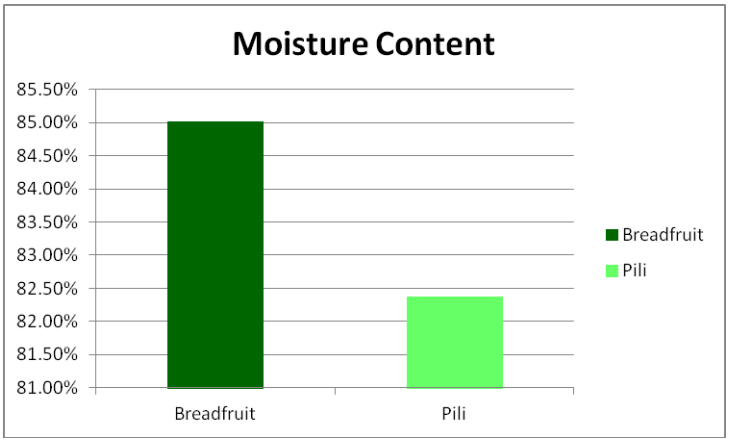

Figure 1. Moisture Content

As much as possible, water must be removed from the samples because it makes oil rancid and alters chemical reactions such as tranesterification reactions. Oven drying lessens the polar constituents, degrades macromolecules and preserves the sample. Moisture content of Breadfruit samples is 85.01% and the moisture content of Pili samples exhibit 82.38%.

Multifect® Pectinase FE and Protex 6L were introduced to degrade the cell walls, mainly composed of carbohydrate molecules and proteins of the plant samples. These enzyme preparations, promotes a higher oil extraction because it disassociates the matrix and facilitates the oil removal by an appropriate solvent.(Rosenthal, et. al., 1996) Pectinase degrades the cell wall of plants by decomposing pectin substances, which are chains of methylated or unmethylated polygalacturonic acids. On the other hand, protease hydrolyzes proteins present in the plant cell into smaller peptides or amino acids. After pre-treatment of enzymes, the solvent can further extract the lipids from the plant cell. This will optimize the extraction of oils.

Enzymatic reaction time was allowed for 120 hours to produce a higher yield of oil recovery percentage.(Passos, et. al., 2009) Multiple solvent extraction was done to the enzyme treated samples using either n-hexane or petroleum ether as a solvent. N-hexane and petroleum ether are highly non-polar solvents that are miscible with the non-polar lipids in the sample.

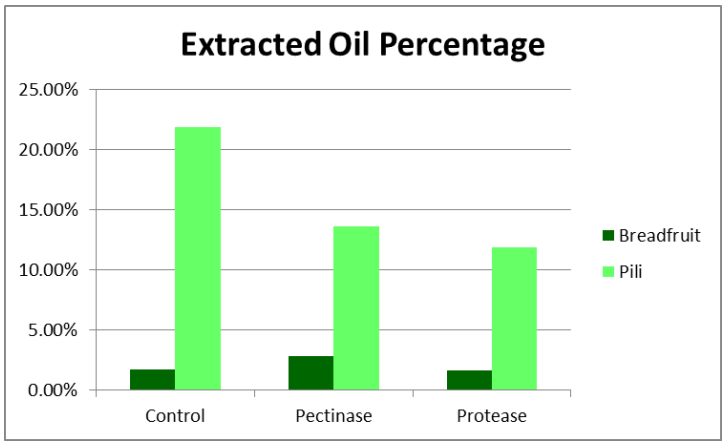

Figure 2. Percentage of Extracted Oil

Breadfruit subjected to the enzymatic activity of Multifect® Pectinase FE exhibited the highest percentage of oil recovery than the two extraction methods. This can be attributed to the high concentration of pectin components in Breadfruit. This is supported by the study of Rincón, A. M.; Padilla, F. C., 2004 that showed that 72.32g of amylopectin per 100g *Artocarpus altilis* native starch. This study conducted using UV-VIS spectroscopy at 600nm.(Rincón, et. al., 2004)

On the other hand, the normal solvent extraction of Pili presents the highest oil recovered percentage; this is probably due to the fact that Pili husk contains large amounts of cellulose instead of proteins and pectic substances. Study shows that *Canarium ovatum* contained 51.3% of cellulose, lignin 22.16%, pentosan 20.22%, ash 0.45%, SiO_2 0.04%.(Thiep, 1980)

3.2 Transesterification Reactions

3.2.1 Alkali-Catalyzed Transesterification

Pili husk and Breadfruit was subjected to alkali-catalyzed transesterification. Formation of soap was observed and this is probably due to the high percentage of free fatty acid in the extracted oil from Pili husk and Breadfruit samples. Therefore, the separation of glycerol and fatty acid methyl ester was prevented. Also, the said transesterification's by-product is water. The accumulation of water leads to the hydrolysis of the synthesized methyl esters into free fatty acid and methanol.

3.2.2 Acid-Catalyzed Esterification

AOCS Aa 6-38 or free fatty acids is a method that determines free fatty acids in the oil extracted from a seed sample by solvent extraction.(AOCS, 2009) Free fatty acid percentage obtained from Breadfruit oil has an average value of 5.94%. Pili oil free fatty acid content has an average value of 7.89%.

Table 1. Oil Characterization using AOCS Aa 6-38

Sample	Trial	Mass of oil sample	Volume of NaOH Solution Delivered	Mass of FFA reacted with NaOH	FFA Percentage	Average FFA Percentage
Breadfruit	1st	1.00 g	8.30 mL	0.053 g	5.38 %	5.94 %
	2nd	1.00 g	10.00 mL	0.065 g	6.49 %	
	3rd	1.00 g	9.16 mL	0.059 g	5.94 %	
Pili	1st	1.00 g	16.90 mL	0.120 g	10.96 %	7.89 %
	2nd	1.00 g	11.80 mL	0.077 g	7.65 %	
	3rd	1.00 g	7.80 mL	0.051 g	5.06 %	

Generally, for biodiesel producers to achieve maximum yield, free fatty acid content of the oil should be less than 0.5% by weight and the reaction should be free of moisture.(Freedman, et. al., 1984) High percentages of free fatty acid precede transesterifiaction reactions, especially alkali-catalyzed, to the formation of soap by base promoted hydrolysis or saponification. The accumulation of water or moisture hydrolyzes the synthesized methyl esters promoting the formation of fatty acid and methanol. Both oil samples are comprised of high percentages of free fatty acid and in order to produce a high yield of fatty acid methyl esters, procedures for acid-catalyzed esterification are essential. Acid-catalalyzed esterification serves as a pre-treatment to another transesterification reaction, because it decreases the free fatty acid content and reaction leads to the conversion of free fatty acid to methyl esters.

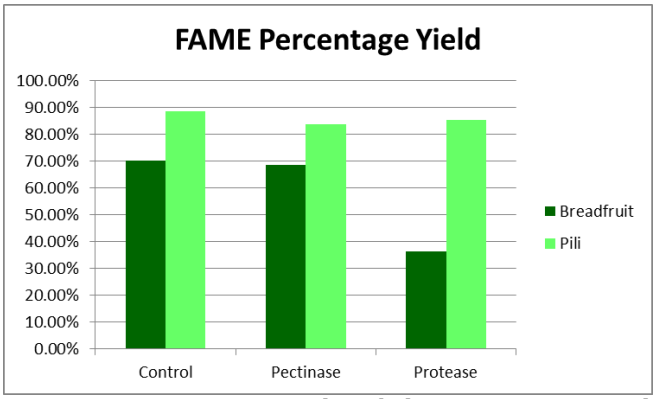

Figure 3. Fatty Acid Methyl Ester Percentage Yield

Acid-catalyzed esterification was performed and biodiesel yields were calculated. Normal N-hexane extractions of Breadfruit and Pili samples had biodiesel yields of 70.33% and 88.59%, respectively. Biodiesel yields from the activity of Multifect® Pectinase FE are 68.60% for Breadfruit and

83.85% for Pili. Biodiesel yields from the activity of Protex 6L are 36.36% for Breadfruit and 85.51% for Pili. Conventional solvent extraction has higher oil percentage yield than enzyme assisted extraction. However, extracted oil from Breadfruit and Pili husk samples treated using enzyme-assisted extraction has better Fatty acid methyl ester yields than that using the conventional solvent extraction.

3.3 Chromatographic Analysis

The transesterified Pili husk and Breadfruit oil samples were analyzed using Gas Chromatography – Mass Spectrometry. The peaks in the chromatograms were identified using the instrument's library. The acid-catalyzed transesterified Pili husk and Breadfruit oil samples were analyzed using GCFID. The transesterified Pili husk oil samples were diluted with Petroleum ether, into ratio of 1:37 and those transesterified Breadfruit oil samples into ratio of 1:75, for the chromatograms to show better resolution of the peaks. All the chromatograms obtained from GCFID were then correlated with their corresponding chromatograms acquired from GCMS by observing the shapes and sizes of the peaks.

Table 2. Chromatographic Results of Pili FAME

GC-MS Results			GC-FID Results		
Retention Time (min)	% Concentration	Identity	Retention Time (min)	% Peak Area	Inferred Identity
16.41	3.60%	Methyl hexadec-9-enoate	10.730	3.6758	Methyl hexadec-9-enoate
17.15	34.17%	Hexadecanoic acid, methyl ester	12.390	18.570	Hexadecanoic acid, methyl ester
19.11	81.01%	9-Octadecenoic acid (Z) – methyl ester	14.263	62.021	9-Octadecenoic acid (Z) – methyl ester

Table 3. Chromatographic Results of Breadfruit FAME

GC-MS Results			GC-FID Results		
Retention Time (min)	% Concentration	Identity	Retention Time (min)	% Peak Area	Inferred Identity
16.68	23.86%	Hexadecanoic acid, methyl ester	12.887	14.654	Hexadecanoic acid, methyl ester
18.42	61.48%	9-Octadecenoic acid (Z) – methyl ester	14.583	28.503	9-Octadecenoic acid (Z) – methyl ester
22.11	8.56%	Docosanoic acid, methyl ester	16.403	12.103	Docosanoic acid, methyl ester

3.4 Physico-Chemical Properties of Biofuel

ASTM D445-96 is a test method that determines kinematic viscosity of liquid petroleum products by measuring the time for a volume of liquid to flow under gravity through a calibrated viscometer.(ASTM, 1972) Kinematic viscosities of Biodiesel from Breadfruit and Pili were 43.92mm^2/s and 16.77mm^2/s, respectively. Kinematic viscosities of Breadfruit and Pili biodiesels failed to meet the limit of kinematic viscosity set by Philippine National Standard.

The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in 1 gram of test sample.(AOCS, 2009) Using the AOCS Cd 3d-63, Standard Test Method for Acid Value**,** the acid values for Breadfruit and Pili biodiesel were 4.28mg KOH/g and 12.90mg KOH/g, respectively. Determined acid values for Breadfruit and Pili biodiesels had failed to meet the acid value limits set by the Philippine National Standard.

Table 4. Identified Physico-Chemical Properties of Tested Biofuel Samples

Sample	Kinematic Viscosity (mm^2/s)	Acid Value	PNS Kinematic Viscosity Standard	PNS Acid Value Standard
Breadfruit Biodiesel	43.92 mm^2/s	4.28 mg KOH/g	2.0 – 4.5 mm^2/s	0.50 mg KOH/g
Pili Biodiesel	16.77 mm^2/s	12.90 mg KOH/g		

4. CONCLUSIONS

The study showed that higher oil percentage yield can be acquired from Pili husk samples than Breadfruit samples. Untreated Pili husk samples and Breadfruit samples pre-treated with Multifect® Pectinase FE gave higher oil percentages compared to other extraction methods. Alkali-catalyzed transesterification undergoes base promoted hydrolysis compared to those undergone alkali-catalyzed transesterification. Standard test methods done for determining the acid value and kinematic viscosity of the produced biofuel did not meet the Philippine National Standard. For further studies, Cellulase can be used to pre-treat the pili husk samples. In determining the FFA content of extracted oils, proper titration should be observed. To produce better biofuels, transesterification methods can be compared and developed. Lastly GCMS would be better than GCFID in determining the identity of the produced fatty acid methyl ester.

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6. REFERENCES

Agarwal, A.K. and Das, L.M. (2001). Biodiesel development and characterization for use as a fuel in compression ignition engines. *J. Eng. Gas Turbines Power*. 123. pp.440–7.

American Oil Chemists' Society. (2009). Acid Value. *Sampling and Analysis Of Commercial Fats And Oils*. p.1.

American Oil Chemists' Society. (2009). Free Fatty Acids. *Sampling and Analysis Of Vegetable Oil Source Materials*. pp. 1-2.

American Society for Testing and Materials (1972). *ASTM viscosity tables for kinematic viscosity conversions and viscosity index calculations*. Philadelphia,

Amigun, B., Rovani S. and Blottnitz H. V. (2008). Commercialisation of biofuel industry in Africa: A review. *Environmental, Renewable and Sustainable Energy Reviews*. 12. pp. 53-68.

Bicol, J.P.G. (2007). *Initial evaluation of fatty acid methyl esters (biodiesel) from Pili pulp (Canarium ovatum) and sigarilyas seed (psophocarpus tetragonolobus) as diesel engine fuel.* (Bachelor's dissertation). De La Salle University, Manila.

Brown W.H. (1954). *Useful plants in the Philippines*. Republic of the Philippines. Department of Agriculture. National Research and Technology. Bureau of Printing. Manila. Bull. 10(2), pp. 241-256.

Conejos, J. R. (2011) *Optimization Of Microwave-Enhanced Transesterification For Coconut Biodiesel Production*. (Master's thesis). De La Salle University Manila.

Coronel, R. E. (1996). Pili nut: Canarium ovatum Engl. Engels, J., Hammer, K. and Heller, J. (Ed). *Promoting the conservation and use of underutilized and neglected crops*. International Plant Genetic Resources Institute: Rome, Italy, pp 7-17.

Foglia, T.A. and Haas, M.J. (2005). Alternate Feedstocks and Technologies for Biodiesel Production. Knothe, G.. Krahl, J.. Van Gerpen, J., (Ed). *The Biodiesel Handbook*. AOCS Press: Champaign, Illinois. pp 42-62.

Freedman, B., Pryde, E.H. & Mounts, T.L. (1984). Variables affecting the yields of fatty esters from transeterified vegetable oils. *Journal of American Oil Chemists' Societ*. 61, pp. 1638 – 43.

Galang, F.G. (1955). *Fruit and nut growing in the Philippines. Ch. 18 (Burseraceae Family)*. AIA Printing press. Malabon, Rizal. pp. 246-247.

Garcia, G.M. (1941) *A comparative study of nutrients of nuts of Pili (Canarium ovatum Engl.)*. Philipp.. Agric. 30(2). 96-106.

Knothe, G. Introduction. Knothe, G, Krahl, J. Van Gerpen, J. (Ed). *The Biodiesel Handbook*. AOCS Press: Champaign, Illinois. pp 1-16

Korbitz, W. (1999). Biodiesel production in Europe and North American, an encouraging prospect. *Renewable Energy*. 16, pp. 1078–83.

National Tropical Botanical Garden (NTBG) Hunger initiative. Breadfruit Institute. National Tropical Botanical Garden. Retrieved August 05, 2012 from http://www.ntbg.org/Breadfruit/hunger.phb.

Oñate, L.U. (1967). *Preserving fruits. Farm Bull, 23, Coll. Agric.* Univ. Philippines, Los Baños, Laguna, the Philippines.

Passos, C. P., Yilmaz, S. Silva, C. M.. and Coimbra, M. A.. Enhancement of grape seed oil extraction using cell wall degrading enzyme cocktail. Food Chemistry. 115. 2009. 48–53

Rincón, A. M. . Padilla, F. C., (2004). Physicochemical properties of breadfruit (Artocarpus Altilis) starch from Margarita island, Venezuela. *Archivos Latinoamericanos de Nutrición. ALAN* v.54 n.4 Caracas dic.

Rosenthal, A., Pyle, D. L. and Niranjan, K. (1996). Aqueous and enzymatic processes for edible oil extraction. *Enzyme and Microbial Technology*. 19(6). 402–420.

Taylor M. B. and Tuia V. S. (2007). *Breadfruit in the pacific region. Acta Horticulturae*. (ISHS) 757. pp. 43-50.

Thiep, T. X. (1980).The results of research on Canarium album. *Tap chi Lam nghiep [Journal of Forestry]* (12): 23-24

Von Wedel, R. (1999). *Technical handbook for marine biodiesel in recreational boats. Prepared for National Renewable Energy Laboratory*. U.S. Department of Energy. p. 32

West, V.N. and Balce S. (1923).The composition of Pili-nut oil. *Philipp. J. Sci*. 23(3): pp. 269-276.

Zhang. et al. (January 2003). Biodiesel production from waste cooking oil: process design and technological assessment. *Bioresource Technology*. 89. pp. 1-16.