# PHYTOSTEROL ESTERS FORMATION FROM COCOA SHELL WASTE AND JATROPHA SEEDS OIL (CSW-TJO)

**SITI NURUL NAJIAH BINTI ABD RASID**

**BACHELOR OF CHEMICAL ENGINEERING**

**UNIVERSITI MALAYSIA PAHANG**

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**SITI NURUL NAJIAH BINTI ABD RASID**

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Signature :
Name of main supervisor : PM. DR. IR SAID NURDIN
Position : SENIOR LECTURER
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Date : 20th JUNE 2017

Dedicated to my dearest parents and siblings for their everlasting love and support.

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# ABSTRACT

Phytosterol ester from esterification phytosterol from plant source such as cocoa shell waste and jatropha oil methyl ester have attracted great interest as a new source since the conventional source which is from edible source was not relevant in present due to its shortage. Moreover, the urgent needs of phytosterols ester in various field industries especially in medical and food industries has forced large production of sterol ester. This research was aimed to produce phytosterols ester from cocoa shell waste and Jatropha il in the presence of solid catalyst (treated egg shell) and to investigate the effect of reaction time, reaction temperature and molar ratio of oil extracted from cocoa shell waste and jatropha oil. The practical study was carried out by using UAE-HD to extract phytosterol from cocoa shell waste and two-stage transesterfication method to extract methyl ester from the jatropha oil. Two-stage transesterfication is the suitable method used to reduce high free fatty acid content in jatropha oil and increase the methyl ester yield. Based on this research studies the highest yield of phytosterol was 0.001099274% and the optimum parameter for production of phytosterol ester was at reaction time 5 hours, temperature of 70$^{o}$C and ratio (cocoa shell waste oil to methyl ester) of 1:3.

# ABSTRAK

Phytosterol ester dari esterifikasi phytosterol dari sumber tanaman seperti sisa koko dan metil ester minyak jatropha telah menarik minat yang besar sebagai sumber baru sejak sumber konvensional yang berasal dari sumber yang tidak dapat dimakan sekarang tidak relevan kerana kekurangannya. Selain itu, keperluan mendesak phytosterols ester dalam pelbagai bidang industri terutamanya dalam industri perubatan dan makanan telah memaksa pengeluaran sterol ester yang besar. Kajian ini bertujuan untuk menghasilkan phytosterol ester dari sisa koko dan Jatropha oil dengan kehadiran pemangkin pepejal (kulit telur terawat) dan untuk mengkaji kesan masa reaksi, suhu tindak balas dan nisbah molar minyak yang diekstrak dari sisa kakao koko dan minyak jarak . Kajian praktikal dijalankan dengan menggunakan UAE-HD untuk mengeluarkan phytosterol dari sisa koko dan kaedah transesterfikasi dua peringkat untuk mengeluarkan metil ester dari minyak jatropha. Transesterfikasi dua peringkat adalah kaedah yang sesuai untuk mengurangkan kandungan asid lemak bebas yang tinggi dalam minyak jarak dan meningkatkan hasil metil ester. Berdasarkan kajian ini, kadar phytosterol ester tertinggi adalah 0.001099274% dan parameter optimum untuk pengeluaran phytosterol ester adalah pada masa tindak balas 5 jam, suhu 70ºC dan nisbah (minyak sisa koko ke metil ester) dari 1: 3.

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| | |
|---|---|
| °C | Degree Celcius |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| CSW | Cocoa Shell Waste |
| FFA | Free Fatty Acid |
| FKKSA | Fakulti Kejuruteraan Kimia & Sumber Asli |
| GC-MS | Gas Chromatography-Mass Spectomagraphy |
| $H_2SO_4$ | Sulphuric Acid |
| HPLC | High Performance Liquid Chromatography |
| JOME | Jatropha Oil Methyl Ester |
| Ps | Phytosterols |
| TAGs | Triacylglycerol |
| TJO | Treated Jatropha Oil |
| UAE-HD | Ultrasound Assisted Extraction-Hydrodistillation |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the Study

Plant sterols known as phytosterols is naturally occurring substances found in plants, which are similar to cholesterol. It is essential constituents of plant cell membranes because of their membrane stabilizing effect. In the past decades, phytosterols and their derivatives have been widely applied in different areas such as the pharmaceutical and food industries, and have recently received massive attention as nutraceutical additives (Moghadasian, 2000). Phytosterol are present in small quantities in many fruits, vegetable oils, canola, cereals and some food waste and large quantity in fat rich seeds like *theobroma cacao*.

Phytosterol esters can be produced by esterification of the natural sterol with fatty acids derived from food grade vegetable oils. The fatty acid ester chain may be saturated, mono- or polyunsaturated depending on the source of the vegetable oil (Roiaini et al., 2016). Phytosterol ester are added to food and supplement it has cholesterol lowering effect properties (Xiaoyu et al., n.d). As the added value to the production phytosterols from natural resource, the use of waste biomass which focussing on cocoa shell waste also one of valuable alternative and the research on it has received worldwide attention. The use of cocoa shell waste to generate energy decrease waste management problems and gives various advantages to the environment. There is a huge potential for bioenergy obtained from waste to decrease the speed of global warming. As per a recent report, by the year 2020, 19 million tons of oil equivalents could be derived from biomass. Out of this, 46% is obtained from bio-wastes like farm waste, agricultural waste, municipal solid waste and other biodegradable waste streams. The growing interest in the use of cocoa

resource in food and beverages processing industries had caused the abundant of waste from cocoa especially its shell since it is non-economic raw materials. Production of phytosterols from cocoa waste, shows many potentials advantages since the latter materials are also food for human and animals (Igbinadolor & Onilude, 2013).

Since there is urgent need of phytosterols ester in various field industries the usage of edible oil as the raw material for methyl ester production are not relevant in the present this is because vegetable oils that come from edible sources for example palm oil ,corn ,canola had caused food dilemma because it is disturbed the supply to food industries. Therefore, in recent years many researches have been studied to use non-edible oil such as Jatropha Curcas L as the source of phytosterols production, since it is not consume by human before processing because of its toxicity it will not interrupt the food supply (Syam et al., 2012). Plus, Jatropha plant contributing in rural development since it has many product that can be processed from it other than serves job vacancy to many rural areas (Bobade et al., 2013).

## 1.2 Motivation

Cocoa has played important roles in food sector since decades especially in beverages industries. The demand for this crop keep increasing by year. In 1989 to 1990 the production of cocoa in Malaysia at the top level which contributed to 414 thousand hectares and 247 thousand tonnes respectively (Hameed et al., 2009). Thus, alternatives routes are required in managing the shell waste into production of phytosterols. Since it does not compete with food crops in the same way as first-generation biofuels made from corn or palm oil. Cocoa waste offering low cost production which do not require an expensive extraction process which is UAE-HD non-chemical used. It is also in constant and readily available supply.

Jatropha Seed Oil or its scientific name Jatropha Curcas L. is one of the potential alternative to produce methyl ester that leads to the production of phytosterol ester. Jatropha oil is non-edible oil can be the edible oil replacement since edible oil is consume in food industries to reduce the competition in terms of nutritional purposes. Jatropha oil also renewable and easy to get because it is available in tropics country. For cultivation

in tropical and subtropical countries, commonly the oil yield is 33% (Peterson, 2008). Gonsalves (2006) claimed that the oil yield per hectare for Jatropha is among the highest for tree-borne oil seeds with seed production is between 0.4 tons per hectare per year to over 12 tons/hectare.

## 1.3 Problem Statement

Recent years have seen a significant increase of cocoa production and consumption, at the same time increasing its waste. For every year millions tonnes of cocoa will be consumed in food processing industries and keep increasing by year. The increased levels of production of product from cocoa resources has led to increase the abundant of waste of the cocoa since it is non-economic. .

The production phytosterols from vegetable oil such as palm, soy bean, rapeseed, corn, nuts and many more had disturbed its demand in food supply. These sources are also be used in food industries make them highly demand and the availability become limited. Since Jatropha seed plantation is growing in Asia and it that has potential oil that can be used for phytosterol production.

The usage shell is used in massive numbers by food manufacturers and restaurants and the shells are discarded as waste. Most of the eggshell waste is commonly disposed in disposal area without any pretreatment because it was conventionally useless (Tsai et al., 2008a).

## 1.4 Objectives

The following are the objective of this research:

1) To produce phytosterols ester from cocoa shell waste and Jatropha Seed Oil in the presence of solid catalyst (treated egg shell)

2) To examine the effect of reaction time, volume ratio of oil extracted from cocoa waste and jatropha oil and temperature on the phytosterols yield.

## 1.5 Scopes of Study

The following are the scope of this research:

1) Preparation of egg shell waste as solid catalyst.
2) Methyl Ester transesterification of jatropha seed oil in the presence of solid catalyst.
3) Phytosterols extraction from cocoa shell waste by Ultrasound Assisted extraction-Hydrodistillation (UAE-HD).
4) Evaluation the yield of phytosterol Ester formation by variation of parameter:
    i) Reaction time (1-5hours)
    ii) Volume ratio of oil extracted from cocoa waste and jatropha oil methyl ester (JOME) (1:1, 1:2, 1:3, 1:4 and 1:5)
    iii) Temperature (30-70 °C)
5) Analysis the formation of phytosterols ester by Gas Chromatography (GC-FID).

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

This chapter basically reviews on the advantages formation of Phytosterol Ester from cocoa shell waste (CSW) and Jatropha Oil methyl ester (JOME) in this research study.

## 2.2 Egg Shell Waste as Catalyst

Egg shells waste as catalyst in transesterification process of phytosterols may reduces the cost of raw material and equipment. Egg is consumed enourmously in food processing industries, restaurant and bakeries, as the shell is discarded as waste made it high in terms of availability. The waste management is not a desirable practice in view of the environmental odor from biodegradation (Tsai et al., 2008b). Many research found that eggshells may be used as a fertiliser and a feed additive for livestock and it appears to be able to effectively adsorb some heavy metals and organic compounds (Kuh & Kim, 2000; Koumanova et al., 2002; Chojnacka, 2005; Vijayaraghavan et al., 2005).

Having porous structure in the calci-fied eggshell, the abundance amount of high content of calcium carbonate, CaCO3 it is possible to prepare active heterogeneous catalyst from eggshell (Li et al., 2009). The conventional catalysts for this transesterification reaction are homogeneous strong bases (such as alkali metal hydroxides and alkoxides) and homogeneous acids such as H2SO4. Other than low cost and high availability, the development of solid catalysts also has recently gained

worldwide attention in terms of their ease of separation and lack of corrosion or toxicity problem (López et al., 2007; DaSilveira et al., 2007).

## 2.3 Cocoa (*Theobroma)* Shell Waste

Cocoa (*Theobroma*) tree is native to the Americas that originated in central America. Cocoa has been consumed since 5000 years ago until now. Nowadays, people around the world enjoy cocoa in thousands of different forms, consuming more than 3 million tons of cocoa beans annually since there are bulk food industries that produced product from cocoa such as confectioneries, beverages, bakeries and many more.

As reported by World Cocoa Foundation (2014), since 2008 to 2012 the world's production of cocoa has increased from 4.3 million metric tons to 4.8 million metric tons respectively. As the demand of cocoa is keep growing, its had caused abundant of waste from industries that really need an attention. Cocoa waste which referring to cocoa shell also known as (hull or husks) are the outer cocoa portions that encase nibs was seen as non-valuable and non-economic raw materials. The beans extracted from cocoa pods constitute only about 19% of the fresh weight of the pod and the remaining 81% in the form of fresh cocoa pod husk (CPH) and bean pulp juice are discarded as wastes. The sterols contained in roasted cocoa hull oul is approximately 10% bound sterol as comparison with other oil as shown in Table 2.1 below;

Sterol Levels in Saponified and Unsaponified Cocoa Oils

| | mg Sterols/100 gm Oil | | Percent "Free" Sterols |
|---|---|---|---|
| Sample | Unsaponified[1] | Saponified[2] | Present in Oil |
| Rice Bran Oil | 209 | 1,663 | 13 |
| Rice Germ Oil | 658 | 3,263 | 20 |
| Olive Oil | 117 | 113 | ~100 |
| Canola Oil | 172 | 556 | 31 |
| Corn Oil | 277 | 1,458 | 20 |
| Soybean Oil | 197 | 283 | 70 |
| Oat Bran Oil | 82 | 230 | 36 |
| Cocoa Butter | 86 | 205 | 42 |
| Roasted Cocoa Hull Oil | 4,396 | 4,674 | 94 |

[1]CSFC Analysis
[2]Method of Example 1

**Table 2.1** Comparison of percent "Free" sterols in different type of oil. (Source; Romanczyk et al., 2011)

Currently, most of cocoa shell waste is disposed at the plantation area use it as fertilizer after it is decompose for cocoa tree and a few farmers use it as animal feed (Syamsiro et al., 2012). The waste, which is the main residue generated during processing using the dry method, can be utilised as an organic fertiliser and when compressed will produce bio-energy that can be used as power generation. Major residues generated from cocoa processing in food industries which is its husk are also potential as biofuel feedstock (Duku et al., 2011).

## 2.4 Jatropha Curcas Linnaeus (J.Curcas L)

Jatropha is a genus of over 100 plants, woody shrub or tiny plant. It is native to central America which needs average in amounts of rainfall and soil fertility (Chandra et al., 2013). At one time, the seed crop that been produced can be used for over 50 years . The seeds contain about 25–35% oil that can be used as fuel directly or as a substitute to diesel in the transesterified form, the other by product can be used for producing soap, wax and cosmetics. It has properties in terms of chemical and physical that suitable to process into bio-energy moreover it can be directly use for cooking stoves and oil lamps (Chandra et al., 2013).

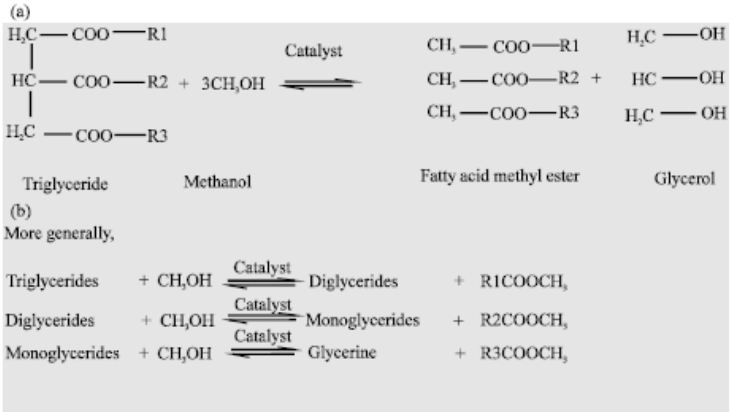
Jatropha is easy to establish, grows relatively quickly and is hardy, being drought tolerant, low in nutrient requirement, not susceptible to pests nor diseases made it the most profitable and possible bioenergy-plant to cultivate (Wahl et al., 2009). While focussing in asia especially in Malaysia the cultivation has about of 1.5 million hectares of marginal land and the take-up rate for growing Jatropha is rising gradually (Hon & Joseph., 2010). Use of edible oil from palm oil, soybean, nuts and many more to produce phytosterols is not feasible in view of a big gap in demand and supply of such oils as nutritional purposes and they are far too expensive to be used at this current. Since Jatropha oil is non-edible it is thus will not affect the supply of edible oil in food industries (Syam, 2012). Besides of energy and nutritional purposes security, Jatropha oil also guarantees potential opportunity to rural community in reducing poverty and underemployment problem (Brittaine, 2010).

According to (Yaakob Z, 2013) the only way to reduce the phytosterols production costs is to use the less expensive feedstock containing fatty acids such as non-edible oils, animal fats, biomass waste and by products of the refining vegetables oils. The lipid fraction of Jatropha oil seed will be extracted and analyzed for their chemical and physical properties. The fatty acid and triacylglycerol (TAGs) composition of the extracted lipid will be revealed using the gas chromatography (GC) and high pressure liquid chromatography (HPLC) method.

## 2.5 Transesterification

Transesterification reaction is commonly used in producing phytosterols which refers to catalyse chemical reaction of vegetable (fats and oil) triglycerides and alcohol to give ethyl esters of fatty acids and glycerol (Syam et al., 2012; Zhang et al., 2003). The reaction is carried out under normal conditions and as can be seen below the reaction only involves triglyceride and the alcohol. The increase the usage of alcohol will lead to the increase of efficiency in this transesterification process (Tapanes et al., 2008; Meher et al., 2006). In the production of phytosterols process, Jatropha Curcas L seed will be extracting to form crude oil and the crude oil (triglyceride) then will mix together with methanol in the presence of catalyst to produce methyl esters as shown in figure below;

**FIGURE 2.1** Transesterification reactions of glycerides with methanol, (a) Overall reaction and (b) Stepwise reactions (Source Islam et al., 2013). The R1, R2 and R3 are alkyl group.



This reaction will proceed either exceedingly slowly or not at all so catalysts are used to speed the reaction rate and increase yield. It is important to note that the acid or base are not consumed by the transesterification reaction, thus they are not reactants, but catalysts. Common catalysts being use for transesterification include sodium hydroxide, potassium hydroxide, and sodium methoxide. Catalyst that comes from alkali metal alkoxides are the most effective catalyst as compared to acidic catalyst in transesterification (Barnwal & Sharma, 2005). As mentioned, in this research catalyst would be use is solid catalyst from treated egg shells which is more economic in terms of cost and availability.

Other than that, the parameter such as temperature, purity of the reactant (mainly water content) and free fatty acid (FFA) content should be taken into the account. According to study by Ma et al., (1998) the yield of methyl ester in every 0.9% fatty acid pesent in the mixture achieved the lowest value which less than 5% then they claimed that the fatty acid contents must be kept under 0.5% so it is necessary to conduct a pretreatment process before proceed to transesterification process. Fatty acid and water contents always gives bad effects, since the present of fatty acids and water causes soap formation, consumes and cause low activation of the catalyst, all which will result a low conversion and more complicated production process, commonly in the purification step.

## 2.6 Ultrasound-assisted Extraction Hydrodistillation (UAE-HD)

Among the newer techniques used in extraction technology which are microwave, batch extraction, ultrasound irradiation and many more, ultrasound assisted extraction-Hydrodistillation has shown to be an effective method to assist the extraction of oil and and other components from vegetables and wastes.

Recently, there is an increased interest in new technologies related to mass transfer enhancement. Maeda et al. and Thanh et al. produced biodiesel from vegetable oils assisted by ultrasound which is a useful tool for strengthening the mass transfer of immiscible liquids. According to earlier researched by (Li, 2004) it is proven that the use of ultrasound in extraction produce large yield and safer produces.

Ultrasound method is considered as an efficient method for extracting natural compounds from herbs because of allowing the penetration of solvents into cellular materials and increasing mass transfer that results in the enhancement of extraction. Not only produces greater yield it is also has advantages in terms of time consuming and can be run at low temperature. The most widely used solvent to test extracted edible oils from plant sources is n-hexane since it is inexpensive (Gholivanda, 2014). Due to reduce negative impact to environment and safety alcohol for example ethanol recently use as the alternative solvent (Li, 2004). Unfortunately, most likely alternatives solvent as the replacer less efficient and at the same time increase costs for solvent and equipment since it will corrode the equipment.

# CHAPTER 3

# METHODOLOGY

## 3.1 Introduction

This chapter presents on the materials used and the precise experimental procedure to achieve the objective of this research.

## 3.2 Materials and Chemical Used

Cocoa Shell waste that will be collected from cocoa industry Barry Callebaut Manufacturing in Pasir Gudang, Johor. Meanwhile, egg shell waste was obtained from Executive Café, Universiti Malaysia Pahang.

Chemical such as Jatropha oil was purchased from Semangat Angkasa Enterprise. Sulphuric acid (95% purity), methanol (99% purity), Potassium hydroxide, ammonia, n-hexane (99% purity) GC grade, methanol (99% purity) HPLC grade, acetonitrile HPLC grade were obtained from FKKSA Laboratory.

### 3.3 Overall Workflow on the Production of Phytosterol Ester

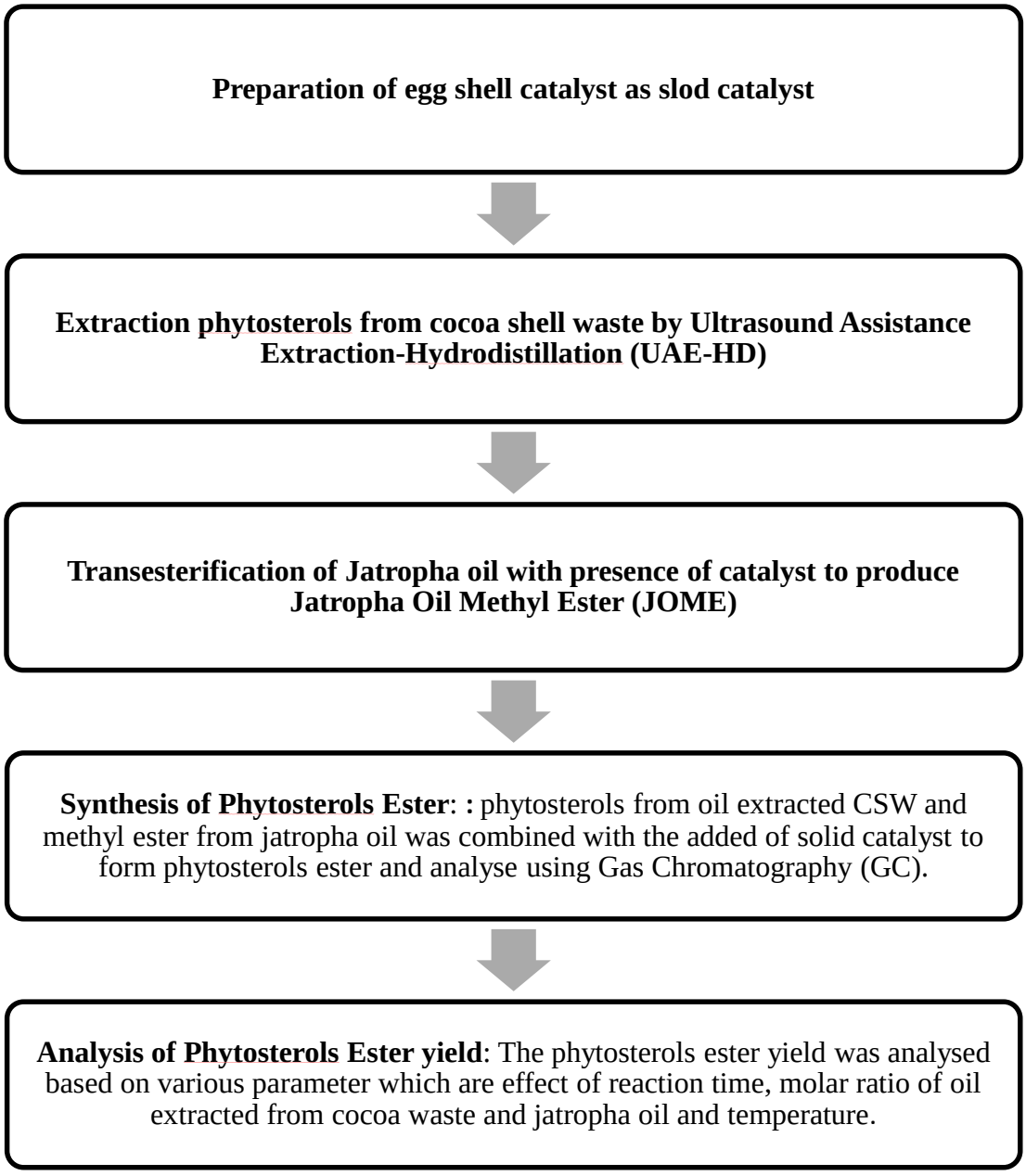



**Figure 3.1** Flowchart showing the overall workflow of Phytosterol Ester formation.

## 3.4 Process Flowchart of Phytosterol Ester Formation

The synthesis route of phytosterol ester is simplified as shown in diagram below;

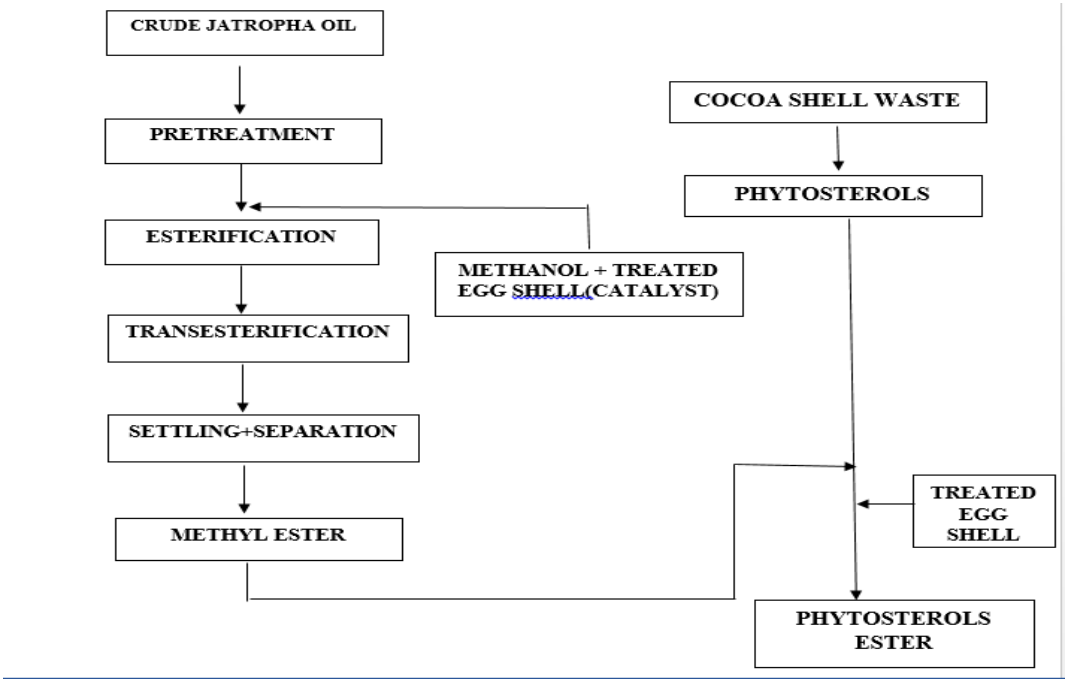



**Figure 3.4** Synthesis route of Phytosterol Ester Formation

## 3.5 Experimental work

### 3.5.1 Preparation, Activation and Impregnation of Waste Egg Shells as Solid Catalysts

The waste egg shells that had been collected was washed cleanly with water. The washed shells were dried at 110◦C for overnight. Then, the well-dried shells were crushed into course particles using pestle and mortar before grinding them into fine particles using a grinder machine, model Retcsh Laboratory Knife Mills GRINDOMIX GM 200 with blade size of 0.08 mm. The powder of waste shells was sieved using 65µm sieve. The eggshell powder was calcined in Carbolite muffle furnace at 1000◦C for 2 hours with heating rate of 10◦C/min. This was the completion of non-impregnated egg solid catalysts preparation. The potassium- impregnated solid catalysts were produced by subjecting the

prepared non-impregnated egg solid catalysts through wet impregnation method (Wei., et al 2009). 50ml of 2M potassium hydroxide solution was added to 10g of non-impregnated egg shell catalyst suspended in 100ml of 0.5wt% ammonia solution. The suspension of non-impregnated egg shell catalyst, ammonia solution and potassium hydroxide solution was heated at 80◦C and stirred vigorously for 2 hours. Then, the suspension was filtered using filter paper and the filtrate was washed few times with water. The filtrate was calcined in the Carbolite muffle furnace at 400◦C with heating rate of 10◦C/min overnight. The catalysts were cooled down to room temperature and stored in air tight container. This was the completion of potassium impregnated egg solid catalysts synthesis.

### 3.5.2 Ultrasound-Assisted Extraction-Hydrodistillation (UAE-HD)

Ultrasonic-Assisted extraction hydrodistillation were performed with an ultrasound cleaning bath-Delta/ DC150H and Clevenger apparatus set-up. The cocoa shells were dried in the oven at temperature 110ºC for 24 hours. After dried, the cocoa shells were grinded using a grinder machine, model Retcsh Laboratory Knife Mills GRINDOMIX GM 200 with blade size of 1.0 mm and sieved using siever size 71µm. Approximately 100 g of the grinded cocoa shells were extracted with 600 ml of deionised water (ratio 1:6) in an ultrasonic apparatus. The temperature of the bath was controlled at 30ºC (Rohaini et al), the frequency was set at 6kHz. The grinded cocoa shells and water were mixed, stirred and homogenized a few minutes before ultrasonicated at suggested pre-treatment time for 60 min. After the pre-treatment step, the sonicated mixture were removed and subjected to hydrodistillation process. The hydrodistillation was carried out by using Clevenger equipment at 100ºC until the extracted oil appeared. The collected is then separated using n-hexane and weighed. The samples of oil collected was analysed using HPLC. The same procedure was repeated by manipulating the extraction operating condition which is at pre-treatment time (30, 40, 50, 70 and 90 min) while the ratio 1:6 (raw material to water ratio) and temperature 30ºC was remained constant.

### 3.5.3 Trans-Esterification of Jatropha Oil to Methyl Ester (JOME)

**Esterification:** Jatropha oil contains 6%-20% (wt) free fatty acids (Khrishna et al, 2009). The methyl ester is produced by chemically reacting jatropha oil with an methanol, in the presence of catalyst. A two-stage process were used for the transesterification of jatropha oil ( Naik et al, 2008). The first stage (acid catalysed) of the process is to reduce the free fatty acids (FFA) content in jatropha oil by esterification with methanol (99 % purity)and acid catalyst sulphuric acid (98% purity). Required amount of methanol (1:5 of oil to methanol molar ratio) was mixed with 37wt% of Suphuric acid amounted 3wt% of the jatropha oil used and poured into the 250ml two necked flask. This mixture of solution was heated until the temperature reaches 50◦C with condenser system attached and also continuous stirring to produce the homogeneous solution and activation of the catalysts itself. Once the solution reaches 50◦C, 50ml of jatropha oil was poured into the reaction medium. Then, the process temperature was increased to 60◦C and continued until the free fatty acid of jatropha oil reduces below 2% which was an hour. After the reaction was completed, the reaction contents were poured into a separation funnel to allow the solution to cool down. Then, the reaction product was washed with hot water of 70◦C and two separate phases were created. The washing was continued until clear water was found. The organic phase was collected and dried under the vacuum at 110◦C for 30min to remove the excess methanol and moisture content. This step was done in order to remove completely the moisture in the esterified oil to avoid soap formation during alkali transesterification.

**Transesterification Reaction:** Tarnsesterification of was done by mixing the esterified jatropha oil with methanol (1:5 of oil to methanol molar ratio) in the presence of impregnated egg shell shell catalyst which amounted of 3wt% of oil used. The solution was properly stirred at temperature up to 60◦C and the esterified oil was added into the flask and stirred vigorously for one hour. The methyl ester dewatering was done as well as during esterification process.

### 3.5.4 Esterification of CSW oil and JOME for the formation of Phytosterol Ester

The cocoa shell waste oil collected from extraction using UAE-HD was then mixed with methyl esters from trans-esterification of jatropha oil and stirred at 650rpm

for 5 hours reaction time. The temperature was set at 70ºC and at ratio 1:3(CSW oil to methyl ester ratio) the experiment was repeated with different parameters which are the reaction time (1, 2, 3 and 4 hours), temperature (30, 40, 50, 60 and 70◦C) and CSW oil to methyl ester ratio (1:1, 1:2, 1:3, 1:4, 1:5). The sample was then analysed using GC-FID analysis.

## 3.6 Analysis of Phytosterol Yield from CSW Oil by HPLC

### 3.6.1 Standard Preparation

10 mg of standard β-Sitosterol were accurately weighted and dissolved in 10 ml of methanol to prepare 1000µg/ml stock solution. 0.2ml, 4ml, 6ml, 8ml and 1ml solution withdrawn from stock solution and added 1 ml methanol of each solution, the prepared dilution were 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, and 1000µg/ml. The standard stock solutions was prepared in calibrated flask.

### 3.6.2 HPLC System and Condition

A Prominence series HPLC system equipped with an LC20AT solvent delivery unit, autosampler, CTO-20AC Column oven and SPD-M20 photodiode detector was used in this analysis. The chromatographic separation was performed using a Cosmosil C18 column (250 x 4.6 mm i.d) at 25ºC. The mobile phase consist of acetonitrile and water (85:15 v/v), were filtered through a 0.2 µm membrane filter and degassed in an ultrasonic bath prior to use. The mobile phase was delivered at flow rate of 2.0 ml/min in the isocratic mode. Quantitation of stigmasterol and β-sitosterol was performed by injecting 202 µl volume with the UV detector set at 202 nm (Shah et al., 2010).

## 3.7 Analysis of Phytosterol Ester Yield Formation from Esterification of CSW Oil and JOME by GC-FID

The 5 mg of reference compound, β-Sitosterol were accurately weighted and dissolved in 5 ml of methanol to prepare 1000µg/ml stock solution. 0.2ml, 4ml, 6ml, 8ml and 1ml solution withdrawn from stock solution and n- hexane was added until each solution reached 1 ml, the prepared dilution were 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, and 1000µg/ml. The standard stock solutions was prepared in calibrated flask. The samples of phytosterol ester was diluted by using n-hexane

### 3.7.1 Standard Preparation

The 5 mg of reference compound, β-Sitosterol were accurately weighted and dissolved in 5 ml of n-hexane (99% purity GC grade) to prepare 1000µg/ml stock solution. 0.2ml, 4ml, 6ml, 8ml and 1ml solution withdrawn from stock solution and n-hexane was added until each solution reached 1 ml, the prepared dilution were 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, and 1000µg/ml. The standard stock solutions was prepared in calibrated flask. The samples of phytosterol ester obtained from different parameters was diluted by using n-hexane which is 100uL sampel + 1000uL Hexane (0.1ml sampel + 1ml Hexane).

### 3.7.2 Gas Chromatography System and Condition

A Hewlett-Packard 5890-series II gas chromatograph equipped with a flame ionisation detector was used A sample volume of 1 ml of the reaction solution was injected using a HP-5. The injector was heated at 260 ºC and was used in the splitless mode. The FID-detector was heated at 290 ºC. The column temperature was programmed from 220 ºC (held for 1 min) to 290 ºC at 5ºC/min and held at 290 ºC for 10 min. Helium 5.0 was used as carrier gas at 17 p.s.i. Column head pressure in the constant pressure mode (P. Breinholder et al., 2002)

### 3.8 Phytosterol Yield Determination

The percentage of phytosterol recovery was calculated by using the following equation:

$$\text{Phytosterol Yield (\%)} = \frac{\left[concentration\left(\frac{mg}{L}\right) x\ volume\ of\ sample(L)\ x\ Dilution\ Factor(DF)\right]}{weight\ of\ sample\ collected\ (mg)}\ x\ 100 \quad (1)$$

To get the concentration of phytosterol calibration curve of internal standard β-sitosterol at different concentration must be plotted first and straight line equation will be obtained as below:

$$Y = mX + C \quad (2)$$

The concentration of phytosterol from each sample obtained from X by inserting the peak area at Y.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Introduction

This chapter presents and discusses the result obtained after carrying out research on the performance of UAE-HD method on the extraction process of oil from cocoa shell waste at different pre-treatment time and the effect of phytosterol ester yield after the esterification of oil from CSW and JOME at different reaction time, molar ratio and temperature.

## 4.2 Phytosterol Yield of Extracted oil from Cocoa Shell Waste (CSW) by HPLC

Figure 4.1 shows the yield of phytosterol obtained from the oil of CSW at different pre-treatment time (30 min, 40 min, 60 min, 70 min and 90 min) in a fix fix raw material to water ratio of 1:6 and ultrasonic frequency of 6kHz was analysed by HPLC and the result obtained as below;

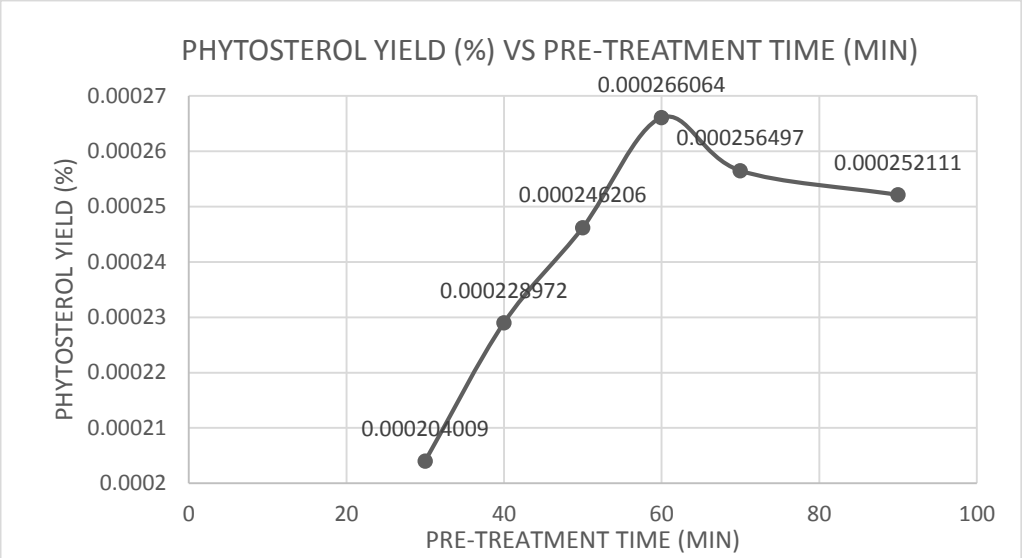



**Figure 4.1** Phytosterol Yield at different ultranic pre-treatment time using 6 kHz and 1:6 ratio.

From the result it is clearly indicates that the yield increased with the increasing ultrasonic pre-treatment time. However, increasing pre-treatment time more than 60 minutes tended to decrease the yield. The efficiency of the ultrasonic pre-treatment time on the plant material was exhibited at the 60$^{th}$ minute. The result that was obtained which is the highest phytosterol yield is 0.000266064% at 60 minutes pre-treatment time. The yield obtained at min 30, 40, 50, 70, and 90 were 0.000204009%, 0.000228972%, 0.000246206%, 0.000256497% and 0.000252111% respectively. After the-pre-treatment time was prolonged to 70 to minutes it showed the decrease in the phytosterol yield. This could be due to decomposition of the extracts by prolonged sonication or due to the initial rinsing effect of sonication, which facilitated the release of most of the active constituents inside the cells to the water (Annegowda et. Al., 2012). Therefore it can be conclude that ultrasonic pre-treatment may affect the phytosterol yield.

## 4.1 Phytosterol Ester Yield by GC-FID

After the trans-esterification of CSW oil and Jome at different reaction time, molar ratio and temperature the samples was analysed by GC-FID to obtain the yield of phytosterol ester.

### 4.1.1 Efeect of ratio (CSW oil – JOME)

Figure 4.4 presents the yield of phytosterol ester obtained by manipulating the extracted oil from CSW and JOME (1:1, 1:2, 1:3, 1:4, and 1:5) by keeping the temperature at 70ºC and reaction time 5 hours. For every 4ml of oil used, 8ml of JOME was mixed together.

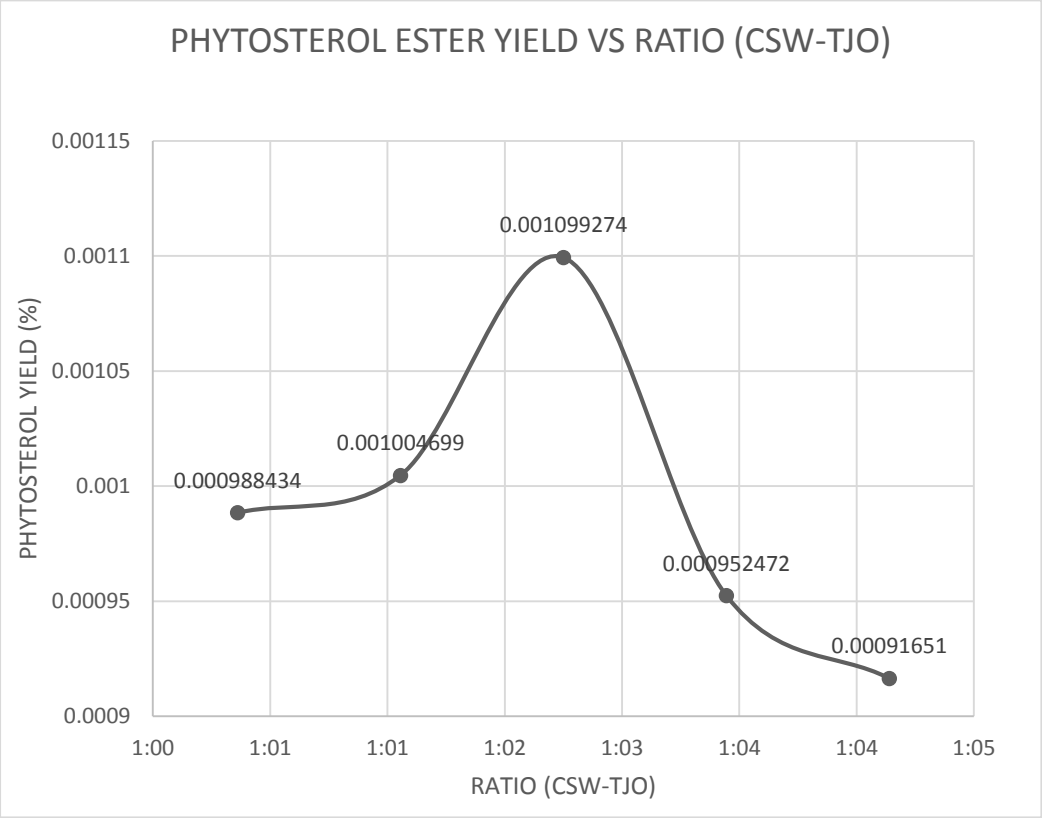



**Figure 4.2** Phytosterol Ester yield at different ratio

The effect of ratio upon the yield of extracted phytosterol ester as displayed in figure 4.2. At the first ratio which is 1:2 of the sample preparation, the phytosterol ester yield increased from 0.000988434% up to 0.001004699%, the maximum yield of phytosterol was 0.001099274% at ratio 1:3 before the drop in yield starting from ratio 1:4 to 1:5 at 0.00052472% and 0.00091651% respectively. This could be concluded, the yield increase when the amount of CSW oil and JOME is at optimum level and beyond its ideal state yield of phytosterol ester will decrease because the reduction would minimize the degradation, transesterification or oxidation process in plant materials (Ranitha, 2013).

### 4.1.2 Effect of Temperature

The effect of temperature (30, 40, 50, 60 ,70 ºC) on the phytosterol ester yield was studied by maintaining the ratio at 1:3 (CSW oil to JOME) and reaction time 5 hours as shown in figure 4.3

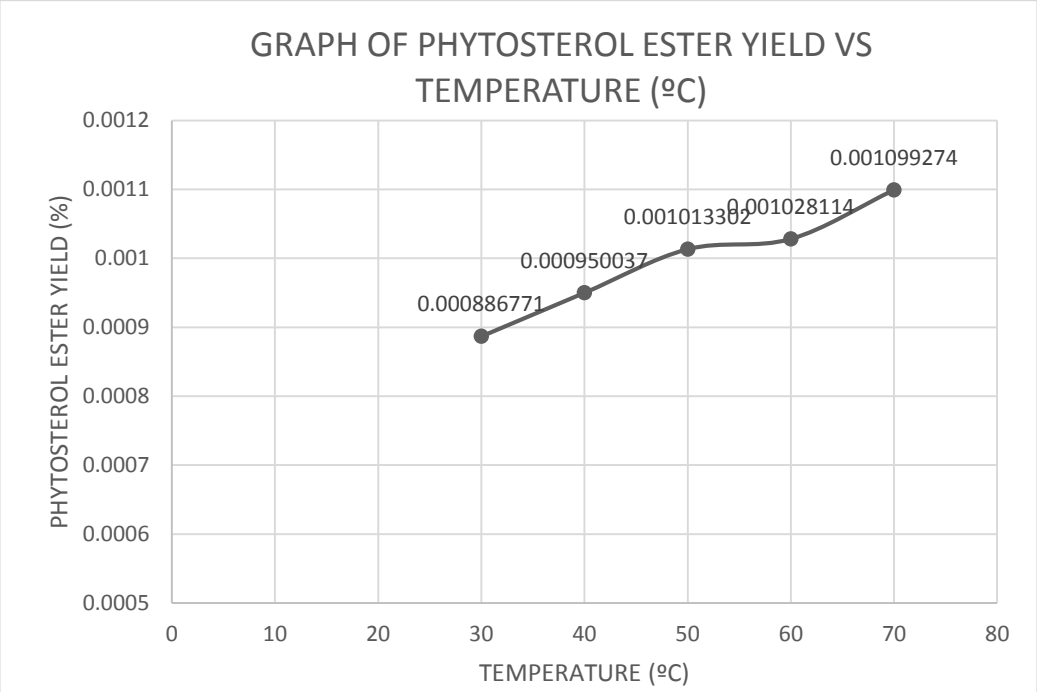



**Figure 4.3** Phytosterol ester yield at different temperature

Figure 4.3 clarifies the effect of temperature on the yield of phytosterol ester obtained from the process increase significantly from 30 ºC to 70 ºC. The maximum amount of phytosterol ester obtained was achieved at the temperature of 70 ºC. The maximum yield of extracted phytosterol achieved was 0.001099274%. Phytosterols ester and their fatty acid esters are quite stable compounds and undergo only limited degradation during only under harsh conditions, such as high temperatures (>100°C) in the presence of oxygen, oxidation of the phytosterol ester moiety may occur.

### 4.1.3 Effect of Reaction Time

Figure 4.4 shows the yield of phytosterol ester at different reaction time ( 1 hour, 2 hours, 3 hours, 4 hours and 5 hours) by fixing the ratio at 1:3 (CSW oil to JOME) and temperature of 70 ºC

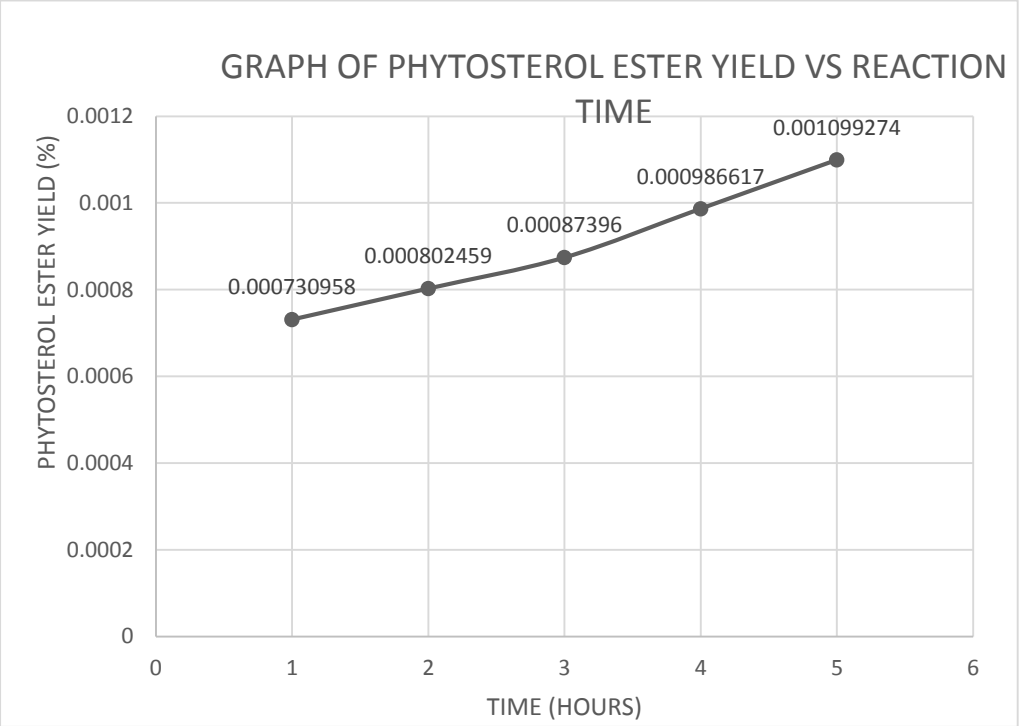



**Figure 4.4** Phytosterol ester yield at different reaction time

Figure 4.4 clarifies the effect of reaction timeon the yield of phytosterol ester obtained from the process increase significantly from 1 hour reaction time to 5 hours. The maximum amount of phytosterol ester obtained was achieved at 5 hours reaction time. The maximum yield of extracted phytosterol achieved was 0.001099274%. This is because the solution Tran-Esterification has reached the optimum homogenity.

## 4.2 Optimization by Design Expert

According to Design Expert, there are a few optimum conditions are predicted to maximize the phytosterol yield. The predicted optimum conditions from the design expert result with their confirmed values respectively such as listed in table below. The percentage of phytosterol from the design expert result is compared with the experimental

value. The calculation of error is calculated based on the formula presented below. The acceptable percentage error is below 10%.

$$\%\ error = \frac{M(exp) - M\ (DE)}{M(exp)} x\ 100$$

M (exp) = Percentage of phytosterol from the experimental value

M (DE) = Percentage of phytosterol from the Design Expert

**Table 1.1** : Optimum Condition Results from Design Expert

| Time (hour) | Ratio (CSW oil-JOME) | Temperature (ºC) | Yield of phytosterol (%) | Error (%) |
|---|---|---|---|---|
| 5.00 | 1:3 | 70 | 0.00103378 | 9.6984 |
| 4.68 | 1:3 | 70 | 0.00104635 | 8.4231 |

The table above shows that, the maximum yield of phytosterol could be achieved was 0.00104635% and has the least value of error which is 8.4231% if compared with the experimental result. The optimum condition to produce phytosterol ester at the highest yield based on the design expert result is at the parameter is 4.68 hours and exposed with the temperature 70 ºC and ratio 1:3.

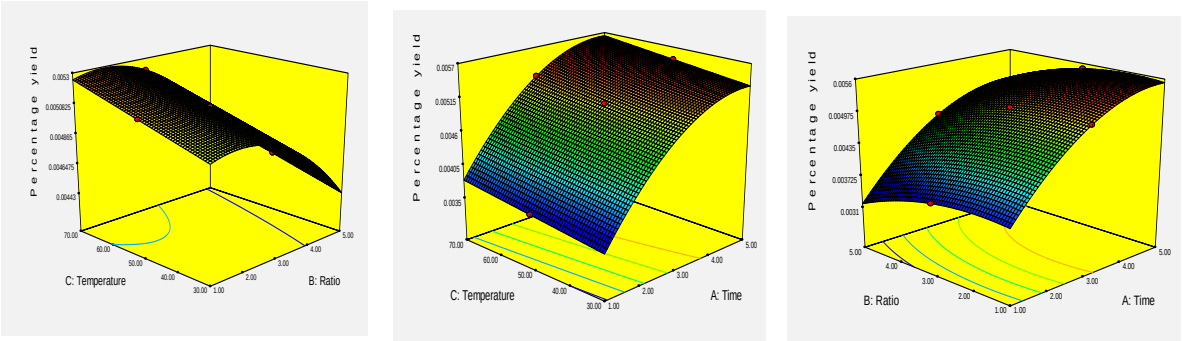



**Figure 4.5: 3D** Response Plot by Design Expert

# CHAPTER 5

# CONCLUSION AND RECOMMENDATION

## 5.1 Conclusion

This research was carried out to evaluate the phytosterol ester formation from Trans-Esterification of cocoa shell waste oil cointaining phytosterol with Jatropha Seeds oil methyl ester (JOME) with the added of solid catalyst. Three vital factor which may influence the yield of phytosterol ester were studied and optimized. Hence, the optimum operating condition which were attained to achieve maximum yield of phytosterol ester which is 0.001099274% were at 5 hours reaction time, temperature at 70 ºC and ratio 1:3 (CSW oil- JOME). This proved that the presences of phyosterol in cocoa shell waste and phytosterol ester could be derived by tran-esterification of natural sterol from Cocoa shell waste oil and Jatropha oil methyl ester (JOME), it can be conclude that by utilizing waste material such as cocoa shell waste and extraction using UAE-HD can be implied for phytosterol ester production.

## 5.2 Recommendation

While the research work was in progress, some issues were raised regarding other factors which is might influence the yield. Those factors were the interaction of amount catalyst on the phytosterol ester formation and the kinetics study on the effect of reaction time, temperature and ratio (CSW oil to JOME) on the yield of phytosterol ester. Due to shortage of time those factors could not be studied. By investigating those effects thoroughly; a clearer view on the optimal operating conditions for the phytosterol ester formation could be gained. Therefore, it is recommended that further study on the factors stated above to be done.

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# APPENDIX

A1: Phytosterol Yield from CSW oil extraction

**Table A1.1 :** Phytosterol yield CSW oil extraction at different pre-treatment time

| Time (min) | CSW to water ratio | Ultrasonic frequency | Yield (%) |
|---|---|---|---|
| 30 | 1:6 r | 6 | 0.000204009 |
| 40 | 1:6 r | 6 | 0.000228972 |
| 50 | 1:6 r | 6 | 0.000246206 |
| 60 | 1:6 r | 6 | 0.000266064 |
| 70 | 1:6 r | 6 | 0.000256497 |
| 90 | 1:6 r | 6 | 0.000252111 |

A2 : Phytosterol Ester Yield from Esterification of CSW oil and JOME

**Table A2.1:** Phytosterol Ester Yield from Esterification of CSW oil and JOME at different ratio

| Ratio (CSW oil-JOME) | Temperature (ºC) | Reaction Time (hours) | Yield (%) |
|---|---|---|---|
| 1:1r | 70 | 5 | 0.000988434 |
| 1:2r | 70 | 5 | 0.001004699 |
| 1:3r | 70 | 5 | 0.001099274 |
| 1:4r | 70 | 5 | 0.000952472 |
| 1:5r | 70 | 5 | 0.000916610 |

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**Table A2.2:** Phytosterol Ester Yield from Esterification of CSW oil and JOME at different temperature.

| Temperature (ºC) | Ratio (CSW oil-JOME) | Reaction Time (hours) | Yield (%) |
|---|---|---|---|
| 30 | 1:3r | 5 | 0.000886771 |
| 40 | 1:3r | 5 | 0.000950037 |
| 50 | 1:3r | 5 | 0.001013302 |
| 60 | 1:3r | 5 | 0.001028114 |
| 70 | 1:3r | 5 | 0.001099274 |

**Table A2.3:** Phytosterol Ester Yield from Esterification of CSW oil and JOME at different reaction time.

| Reaction Time (hours) | Ratio (CSW oil-JOME) | Temperature (ºC) | Yield (%) |
|---|---|---|---|
| 1 | 1:3r | 70 | 0.0007309580 |
| 2 | 1:3r | 70 | 0.0008022459 |
| 3 | 1:3r | 70 | 0.000873980 |
| 4 | 1:3r | 70 | 0.0009866170 |
| 5 | 1:3r | 70 | 0.0010992740 |

**Figure A.1** Step by step procedure of Esterification CSW oil and JOME.