ENZYMATIC-ENHANCED PRODUCTION OF GAHARU OIL: EFFECTS OF SHAKING SPEED AND WATER /GAHARU RATIO

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Bachelor in Chemical Engineering (Biotechnology)

Faculty of Chemical and Natural Resources Engineering
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MAY 2008

I declare that this thesis entitled "*Enzymatic-Enhanced Production of Gaharu Oil : Effects of Shaking Speed and Water/gaharu Ratio*" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree

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DEDICATION

Special dedication to my beloved father,mother, brothers,sister

&

Zaharatun Nadwa Binti Sha'rani

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ABSTRACT

Gaharu with scientific named Aquilaria is a very valuable plant where widely used for medicine, perfumery, and incense. Due to its rarity and the high growing demand for it, gaharu essential oil brings very high prices. One of the common methods to extract gaharu oil is by hydro distillation. However, the yield of gaharu oil using this method is still in small percentage. Previous researches proved that enzymatic hydrolysis during pre-treatment can give better result of gaharu oil yield extraction. Since enzymes are generally active over a specific range of reaction condition, research to improve the enzymatic hydrolysis has been conducted. Two parameters which are shaking speed (rpm) and water/gaharu ratio (v/w) that affect the enzymatic hydrolysis were studied. From the results, the yields for gaharu oil extraction were increased as the shaking speed increased from 50 rpm to 150 rpm. At the shaking speed of 200 rpm, the yield did not continue the same trend as the yield was decreased. The highest yield for varying rpm is 0.1092 at the 150 rpm. The yields for gaharu oil extraction were decreased due to the increasing water/gaharu ratio from 8:1 v/w to 20:1 v/w. The highest yield for varying water/gaharu ratio is 0.1092 at the ratio of 8: 1 v/w. Based on results obtained, the combination of 150 rpm and water/gaharu ratio of 8: 1 v/w during enzymatic hydrolysis pretreatment produced maximum gaharu oil yield extraction which is 0.1092%.

ABSTRAK

Gaharu dengan nama saintifik Aquilaria adalah tumbuhan yang sangat bernilai di mana sering digunakan untuk perubatan, minyak wangi dan colok. Disebabkan keistimewaannya dan pertumbuhan permintaan yang tinggi, harga minyak gaharu menjadi amat mahal. Salah satu kaedah yang paling biasa digunakan untuk mengekstrak minyak gaharu adalah penyulingan air. Namun, peratusan penghasilan minyak gaharu menggunakan kaedah ini masih di peratusan yang rendah. Kajian-kajian terdahulu telah membuktikan bahawa hidrolisis dengan enzim sebagai pra-rawatan boleh memberikan hasil pengekstrakan minyak gaharu yang lebih baik dengan peratus penghasilan lebih tinggi. Oleh kerana enzim aktif pada had keadaan tindak balas yang khusus, kajian untuk mengkaji hidrolisis dengan enzim telah dijalankan. Dua parameter iaitu kelajuan goncangan dan nisbah air gaharu yang memberikan kesan kepada proses hidrolisis menggunakan enzim telah dikaji. Parameter eksperimen yang optimum boleh digunakan untuk meningkatkan penghasilan minyak gaharu. Daripada eksperimen, penghasilan minyak gaharu meningkat apabile kelajuan goncangan meningkat dari 50 rpm kepada 150 rpm. Pada kelajuan mengoncang 200 rpm, penghasilan minyak tidak mengikut corak yang sama di mana penghasilan minyak adalah menurun. Peratus penghasilan yang tertinggi untuk kepelbagaian kelajuan mengoncang adalah 0.1092 % pada 150 rpm. Penghasilan minyak gaharu berkadar langsung berkurangan apabila pecahan air per gaharu meningkat dari 8:1 v/w kepada 20:1 v/w. Peratusan penghasilan minyak yang tertinggi untuk kepelbagaian nisbah air gaharu adalah 0.1092 % pada nisbah 8:1. Daripada keputusan, kelajuan goncangan 150 rpm dan pecahan air per gaharu 8:1 semasa hidrolisis dengan enzim menghasilkan peratus penghasilan ekstrak minyak gaharu yang maksimum iaitu sebanyak 0.1092%.

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LIST OF SYMBOLS

RPM	-	rotation per minute
v	-	volume
w	-	weight
h	-	hour
g	-	gram
mL	-	milliliter
L	-	liter
M	-	mole

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Gaharu with scientific named Aquilaria is classified under the family of *Thymelaeaceae*. Aquilaria is the only one tropical tree genus which currently has been focused by international attention (Chakrabarty *et al*., 1994; Ng *et al*., 1997). There are other's name for this incense wood given by local and the world like agaru, aloes wood, agarwood, oud, chen-xiang, eagle wood, jinkoh and others (Cheksum *et al*., 2002). The fragrance of agarwood can vary greatly depending on the country of origin, the density of resin and on the part of the tree from which it is harvested. Gaharu essential oil is very valuable which widely used for medicine properties, perfumery, and incense (Okugawa *et al*., 1993).

Aquilaria trees are now protected in most countries and the collection of agarwood is illegal from natural forests. International agreements, such as the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora), accepted by 169 countries, is designed to ensure trade in agarwood products from wild trees does not threaten the survival of Aquilaria. Despite these efforts agarwood products from illegally cut trees continues to be sold and unknowing consumers create a demand that helps to destroy the last old growth Aquilaria trees in existence (Blanchette, 2006).

According to Leo Sunari, head of the World Wildlife Fund (WWF) Gaharu project, gaharu is known throughout many Asian countries and commonly use from Asia to the Middle East. It is believed that only Aquilaria trees which are older than 25 years can produce high-grade gaharu, valuable, dark brown- or black-colored heartwood with a very strong smell. From India to Indonesia, market demand for this forest product is very strong and far greater than the supply.

As gaharu essential oil is in high demand today, the research to enhance the gaharu oil production is still dwindling. The small percentage of gaharu essential oil production is not enough to meet the market demand. Due to its rarity and the high growing demand for it, gaharu essential oil brings high prices.

Since gaharu is valuable, local entrepreneur has adopted water distillation technique that very much practice traditionally especially in rural areas of Cambodia and India (Chang *et al*., 2002). Nowadays, local entrepreneur prefer an effective technique that produce higher yield of gaharu oil which is using hydro distillation. It is reported that the proportion of mostly essential oil extracted by hydro distillation 93% and the remaining 7% is extracted by other method such as solvent extraction and CO_2 extraction. Hydro distillation method is believed to produces pure quality of gaharu essential oil because it only uses water rather than other method that use solvent such as solvent extraction. The partition between the water and oil phases of distillation make the separation of the oil is easy and more economical (Masango, 2001).

The gaharu oil production using hydro distillation can be improved if an enzymatic hydrolysis treatment is employed prior to extraction step (Fullbrook, 1983; Marek *et al*., 1990; Tano-Debrah *et al*., 1996). The cell wall degradation caused by the enzyme increases the permeability to the oil through membrane. The use of several enzymes as cellulases, hemicellulases, and amylases has been reported (Lanzani *et al*., 1975; Bhatnagar and Johari, 1987).

Like others plant, gaharu oil is found inside plant cells, linked with wide variety of carbohydrates. In order to facilitate its extraction from the plant cells, it is necessary to degrade the cells walls to increase the permeability for oil. Most carbohydrates in gaharu cell wall are in the form of lignocellulose in which is made up of mainly cellulose, hemicellulose, pectin, and lignin. Lignocellulose is generally found in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. One technique to hydrolyze this lignocellulose contents is by enzymatic hydrolysis. Degradation of lignocellulose, specifically cellulose by enzyme during enzymatic hydrolysis will increase the permeability to the gaharu oil through of cell wall.

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Beguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkali hydrolysis because enzyme hydrolysis usually operate at mild conditions, pH 4.8 and temperature 45 $^{\circ}$C – 50 $^{\circ}$C and does not have a corrosion problem (Duff and Murray , 1996).

1.2 Problem statement

Upon hydro distillation of Malaysian ‘gaharu’ (agarwood), an essential oil is obtained in 0.8% yield (Yaacob, 1999). This show that the yield of gaharu essential oil using hydro distillation still in small percentage compared with mostly types of essential oil extracted by the same method which is 93%. However the drawback of this method is that the still could get overheated, thus burning the aromatics and resulting in a burnt smell (Aromacures, 2006).

The results from previous research indicates that yield of extraction gaharu essential oil using enzyme as pre-treatment (enzymatic hydrolysis) give the highest result compare to extraction without enzyme pre-treatment. The oil production can

be improved if an enzymatic treatment is applied (Fullbrook, 1983; Marek *et al*., 1990; Tano-Debrah *et al*., 1996).

The advantages of this biological pretreatment (enzymatic pretreatment) include low energy requirement and mild environmental conditions. However, the rate of most hydrolysis in most biological pretreatment processes is very low (Sun and Cheng, 2002).

There are many factors that effects the enzymatic hydrolysis of cellulose in gaharu wood include substrates, cellulose activity, and reaction condition (pH, temperature, as well as other parameter). Enzymes are generally active over a specific range of reaction condition. Hence, to improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulose activity (Cantwell *et al*., 1988; Durand *et al*., 1988; Orpin, 1988).

This research investigated the effects of the specific parameters which are shaking speed (rpm) and water/gaharu (v/w) ratio in enzymatic hydrolysis using cellulase enzyme to enhance production of gaharu oil. As a result, the best parameter of reaction condition during enzymatic hydrolysis can be determined to produce maximum yield of the gaharu oil.

1.3 Scope

Use enzymatic hydrolysis during pre treatment in order to enhance the production of oil from gaharu wood. Further oil extraction process has been conducted via hydro distillation. The effects of shaking speed (rpm) and water/gaharu ratio (v/w) on gaharu oil extraction in enzymatic pre treatment has been investigated.

1.4 Objectives

The objectives of this research are:

- To study the effect of shaking speed (rpm) in enzymatic hydrolysis pre-treatment to get the maximum gaharu essential oil production.
- To study the effect of water/gaharu ratio (v/w) in enzymatic hydrolysis pre-treatment to get the maximum gaharu essential oil production.

CHAPTER 2

LITERATURE REVIEW

2.1 Gaharu

There are a few names for the resinous, fragrant and highly valuable heartwood produced by Aquilaria tree which are agarwood, eaglewood, gaharu and aloeswood. Most common name that use in scientific journal is agarwood. In Malaysia, this incense wood is familiar with name of gaharu (Cheksum *et al*, 2002).

2.1.1 Aquilaria species

Around the tropical region there has been reported that 15 species of Aquilaria exist in India, Burma, China, Myanmar and Malaysia region. In Malaysia there are 5 species of Aquilaria found which are *Aquilaria Hirta*, *Aquilaria Malaccensis, Aquilaria Rostrata, Aquilaria Microcorpa* and *Aquilaria Becanana*. A significant number of research studies have been conducted on *Aquilaria malaccensis* (Ng *et al*., 1997) which is well distributed throughout Peninsular Malysia except for Kedah and Perlis. *Aquilaria malaccensis* also considered threatened species due to its high value in today's market and has been included in 'The World List of Threatened Tress' (Oldfield *et al*., 1998).

2.1.2 Aquilaria tree and agarwood production

The Aquilaria tree is a large evergreen tree growing over 15-30 m tall and 1.5 – 2.5 m in diameter with white flowers (Chakrabarty *et al*., 1994). According to Professor Robert Blanchette from The University of Minnesota; agarwood is formed when aquilaria trees produce a resin as a defense mechanism against fungi infection or injury causing its normally soft, white wood to become hard and dark in color. This resin-soaked wood is called agarwood (Blanchette, 2006). Agarwood produced in grown Aquilaria tree is shown in Figure 2.0.

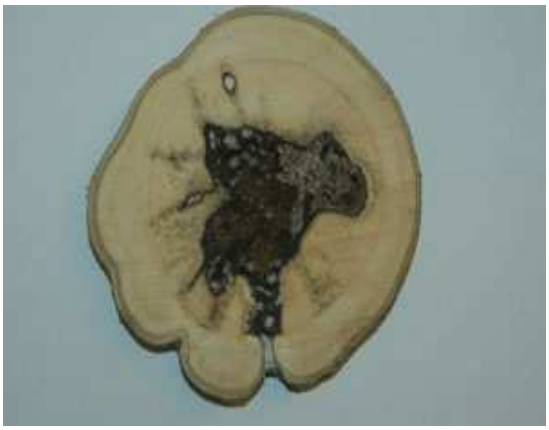

Figure 2.0: Agarwood produced in grown Aquilaria tree

The formation of agarwood occurs in the trunk and roots of trees are due to the infection by a parasitc ascomycetous mould, *Phialophora parasitica*, a dematiaceous (dark-walled) fungus. As a response, the tree produces a resin high in volatile organic compounds that aids in suppressing or retarding fungal growth. While the unaffected wood of the tree is relatively light in colour, the resin dramatically increases the mass and density of the affected wood, changing its colour from pale beige to dark brown or black.

High quality resin comes from a tree's natural immune response to a fungal attack. It is commonly known as agarwood #1 (first quality). An inferior resin is created using a forced method where aquilaria trees are deliberately wounded,

leaving them more susceptible to a fungal attack. This is commonly called as agarwood #2 which the second quality of agarwood (Ng *et al*., 1997).

2.1.3 Gaharu in Malaysia

Today, gaharu or agarwood is becoming more popular in Malaysia. This is due to an initiative taken by En Sulaiman Bin Doss Mohammed Khan, from Muar, Johor to create awareness of the precious sources of agarwood in Malaysia. This awareness is vital as Malaysia is rich in gaharu, mainly in Terengganu and Pahang. The Malaysia government recently financed some agencies to continue research and development of gaharu. The goal is also to increase the trade of agarwood in Malaysia (Ng *et al*., 1997).

Since price of gaharu is very high where the good quality gaharu can fetch around RM10, 000 per kg, gaharu collectors or buyers have to pay a royalty fee amounting to 10% of the raw material market price. Convention on International Trade in Endangered Species (CITES) have been issued an extraction permit and facilitate the traders in obtaining export of gaharu wood (Hilary, 2005).

2.1.4 Uses of gaharu

2.1.4.1 Medicine

Agarwood is one of the earliest recorded medicines found in early Chinese medical textbooks. The main function of agarwood is to remove the bad chi or energy from the body, which promotes circulation and blood flow. High grade agarwood powder is prescribed in Chinese medicine and used in the production of pharmaceutical tinctures (Yaacob, 1999).

Agarwood is used as a complex ointment for smallpox and various abdominal complaints. It also prescribed for dropsy, as a carminative, for heart palpitations and as a tonic taken particularly during pregnancy, after childbirth and disease of female genital organs (Chakrabarty *et al*., 1994).

2.1.4.2 Perfume

Perfume is a mixture of fragrant essential oils and aroma compounds, fixatives, and solvents used to give the human body, objects, and living spaces a pleasant smell. Agarwood is said to have been highly prized by European perfumes in the mid – 1990s (Chakrabarty *et al*., 1994).

In India, various grade of agarwood distilled separately before blending to produce final “minyak attar”. Minyak attar is water-based perfume containing agarwood oil which is traditionally used by Muslims to lace prayer clothes (Yaacob, 1999). Agarwood perfumes are seldom pure agarwood oil, but instead use an alcoholic or non alcoholic carrier. The cheapest agarwood perfumes are either synthetic or a blend of oils each with different qualities and fragrances. Agarwood essences have recently been used as fragrances in soaps and shampoo (Chakrabarty *et al*., 1994).

2.1.4.3 Incense

Agarwood powder and dust cannot be burned directly in incense holders, but can be used to make incense sticks or coils for indoor fragnance. Agarwood incense is burned to produce a pleasant aromatherapy. The aromatic smell of this incense is 100 % pure natural smell that is the specific smell of each different area of Agarwood. No chemicals or any artificial perfumes added. So they are very safe and no side effects to human health when burning. The most distinguishable advantage of Agarwood Incense is that it can be used in closed environments (Agarwood incense, 2007).

Pure agarwood is also burned as incense in Japan. The agarwood is breaks pieces off and burn. A revival in the ancient art of *Koh doh* (incense ceremony) in Japan has revitalized interest in agarwood (Katz, 1996).

2.2 Extraction

2.2.1 Definition of extraction

Extraction is a separation process to separate solute or removed undesirable solute component from the solid where the solid is contacted with a liquid phase. Fragrance extraction are processes which involve extracting aromatic compounds from the raw material using various methods such as distillation, solvent extraction and expression Currently, the most popular method that used many old times for essential oil extraction is distillation (Gilbert and Martin, 2002).

2.2.2 Distillation

Distillation accounts for the major share of essential oils being produced today. The choice of a particular process for the extraction of essential oil is generally dictated by the following: (1) sensitivity of the essential oils to the action of heat and water; (2) volatility of the essential oil; and (3) water solubility of the essential oil (Hand Book on Medicinal, 2007). After extraction, the properties of a good quality essential oil should be as close as possible to the essence of the original plant. The key to a good essential oil is through low pressure and low temperature processing. High temperatures, rapid processing and the use of solvents alter the molecular structure, will destroy the therapeutic value and alter the fragrance. This cause the usually method choose for oil extraction is hydro distillation.

2.2.3 Hydro distillation

Hydro distillation is one of the oldest and easiest methods being used for the extraction of essential oils using the water. It is not only the most ancient method of distillation but also the most versatile. In this method the plant material is fully dipped and boiled in the water with the resultant steam being captured and condensed. It involves the use of a common tub to boil water and plant material. Hydro distillation is the best method for tough materials like nuts, wood or root. However the disadvantage of this method is that the still could get overheated, thus burning the aromatics and resulting in a burnt smell (Aromacures, 2006).

2.3 Enzymatic hydrolysis

2.3.1 Cellulase

Enzymatic hydrolysis of cellulose is carried out by cellulase enzyme which is highly specified (Beguin and Aubert, 1994). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic material. These organism can be aerobic or anaerobic, mesophilic or thermophilic.

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase which attacks regions of low crystallinity in the cellulose fiber, creating free chain-end; (2) exoglucanase or cellobiohydrolase, which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) ß- glucosidase which hydrolyzes cellobiose to produce glucose. In addition to the three major groups of cellulose enzymes, there are also a number of ancillary enzymes that attack hemicellulose such as glucuronidase, xylanase, galactomannanase and glucomannanase (Duff and Murray, 1996).

2.3.2 Lignocellulose

Lignocellulose is the major carbohydrates component of cell wall that strengthens woody plant cells. Lignocellulose consists of about 35 to 50% cellulose, 20 to 35 % hemicelluloses and 10 to 25% lignin.

Cellulose is composed of linear chains of covalently linked glucose residues. It is very stable chemically and extremely insoluble. A hemicellulose can be any of several heteropolymers (matrix polysaccharides) present in cell walls along with cellulose. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random and amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base as well as myriad hemicellulase enzymes.

Lignin is further linked to both hemicelluloses and cellulose forming a physical seal around the latter two components that is an impenetrable barrier preventing penetration of solutions and enzymes. In general lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl and p-coumaryl) (Malherbe and Cloete, 2003).

2.3.3 Pretreatment of lignocellulosic material

Alkaline and acid (Nguyen, 1993; Grethlein and Converse, 1991) hydrolysis methods have been used to degrade lignocellulose. Weak acids tend to remove result in poor hydrolysis of cellulose whereas strong acid treatment occurs under relatively extreme corrosive conditions of high temperature and pH which necessitate the use of expensive equipment. Also, unspecific side reactions occur which yield non-specific by-products.

For many processes including degradation of lignocellulose, enzymes are preferred than acid or alkaline to hydrolyze since they are specific biocatalysts, can

operate under much milder reaction conditions, do not produce undesirable products and are environmentally friendly (Chahal, 1992).

2.3.4 Enzymatic hydrolysis pre treatment step

Enzyme is a protein (or protein-based molecule) that speeds up a chemical reaction in a living organism. An enzyme acts as catalyst for specific chemical reactions, converting a specific set of reactants (called substrates) into specific products. Enzymatic hydrolysis a process by which enzymes (biological catalysts) are used to break down cellulose in lignocelluloses (Biology Online, 2007).

The applications of the enzymatic hydrolysis treatment lead to a slightly higher fraction of easily extractable oil because the enzymatic attack of the cells causes additional breaking of the cell wall structure. Additional breaking will increase the permeability to the gaharu oil through of cell wall. Hence, the enzymatic hydrolysis pretreatment will enhance yield of extracted oil (Osburn, 1944).

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulose. Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulose on cellulose is partially responsible for this deactivation (Converse *et al*., 1988).

The cellulose hydrolysis by cellulase enzymes is influenced by many substrate and enzyme related factors (Esteghlalian *et al*., 1999) including heterogeneity of the reactants and a liquid enzyme acting upon a solid surface. Therefore adequate mixing is required to ensure sufficient contact between the substrate and enzyme, and to promote heat and mass balance. The soaking process purpose is to break down the parenchymatous cells and oil glands. In mixing and soaking process, water activity plays important role in swelling, expanding the

structure of fiber, increasing the surface area accessible to cellulolytic enzymes, and also facilitating the diffusion of enzymes (Fan *et al*., 1987).

2.3.5 Shaking speed (rpm) effects

Mukataka *et al*., (1983) have shown that excessively high mixing speeds (>200 rpm) could lower the extend of cellulose conversion while moderate mixing speed (100-200 rpm) provide a good combination of fast hydrolysis rate and high conversion yield.

From the study, increasing the shaking speed from 25 to 150 rpm enhanced the interaction between the substrate and had no appreciable adverse impact on the activity of enzymes, as reflected by the slightly higher conversion yields of the 150 rpm runs. As a result, the continuous and high speed shaking produced the highest conversion yield whereas the intermittent and low speed shaking regimes resulted in lower conversion (Hanna *et al*., 2001).

2.3.6 Water/gaharu ratio effects

The lowest value of the range of the water/seeds ratio has chosen to allow a good mixing in the shaking equipment. Best conditions for oil extraction are found to be low water/seed ratio where the maximum yield conversion is obtained (Sineiro *et al*., 1997).

CHAPTER 3

METHODOLOGY

3.1 Substrate preparation

3.1.1 Raw material preparation

Gaharu woods were bought from local entrepreneur in Gua Musang, Kelantan. First step is chopping process where gaharu woods were chopped into small pieces for easier grinding process. Second step is grinding process. The pieces of gaharu woods were ground into powders used grinder type of Disk Mill FFC23.

3.1.2 Buffer solution preparation

Buffer solution used for substrate preparation is 0.1 M NaoH solution and 0.1 M KHP (Potassium hydrogen phthalate) solution. For 0.1 M NaoH solution, 8g of NaOH powder were mixed with 2L distilled water in the beaker. Then, the solution was stirred using magnetic stir bar. For 0.1 M KHP solution, 40.846 g Potassium hydrogen phthalate powder were mixed with 2L distilled water in the beaker. Then the solution was stirred using magnetic stir bar. In buffer solution preparation, 363 mL of 0.1 M NaoH and 1100 mL of 0.1 M KHP were mixed with 2537 mL of distilled water in the 5L flask. The mixture was shaking with hand until the mixture being homogeneous. The pH of buffer solution was checked using pH meter. The

buffer solution should be in range of 4.5 into 5.0. The optimum pH for cellulase enzyme activity is 4.8.

3.1.3 Sample preparation

Sample preparation was dependent on the water/gaharu ratio values which are 8:1 v/w, 12:1 v/w, 16:1 v/w and 20:1 v/w. Gaharu powder was weighed in the correct amount and put into the glassware. The buffer solution was poured into the glassware and mix using the ladle. When the gaharu powder and buffer solution really mix, they were poured into 5L flask. When the half of mixture was poured, the enzyme Celluclast 1.5 L which is Cellulase from *Trichoderma reesei* was put following enzyme/substrate ratio value which is 1: 100 w/w. The position of syringe must be in straight to avoid the enzyme attach the wall of flask. Then, the balance of mixture was poured into the flask.

3.2 Enzymatic hydrolysis pretreatment

The enzymatic hydrolysis pretreatment to enhance gaharu oil extractability was performed during the mixing stages, which carry out in the double stack shaking incubator (brand: INFFORS model: MULTITRON 11). The sample was shaking at temperature 50 $^{\circ}$C for 3 hours. The experimental parameter of shaking speed were varies in the range of 50 rpm, 100 rpm, 150 rpm, and 200 rpm respectively.

3.3 Extraction process

A hydro distillation unit for the extraction process was set up as shown in Figure 3.0. The sample was poured into the flask of hydro distillation unit. The water flow was continuously turned on. Hexane was putted using the pipette until the layer between the hexane and water has been seeing. Tissue and aluminum foil needs to be

wrapped all over the apparatus to make sure there was no heat loss occurs. The extraction of gaharu oil was run for 3 days using hydro distillation unit. The temperature used is 100 $^{\circ}$C (boiling point of water).

The gaharu oil in present in the flask was vaporizes and passes through a condenser. After the extraction process ended, the hydro distillation unit was cooled in one day. The layer of gaharu oil and water were collected in a receiving flask. Sodium Sulphate (Na_2SO_4) was putted into a mixture of with a function to absorb the water content in the mixture. Then the mixture was transferred into the sample bottle using the pipette. The sample bottle used have been weighted and labelled first. The sample bottle was put into the fume wood for hexane evaporation. The gaharu oil was weighted until get the constant value. The same steps were repeated for all experimental parameters.

Figure 3.0: Hydro distillation unit

3.4 Data collecting and analysis

The weight of gaharu oil extracted was calculated as below:

weight of bottle and gaharu oil (g) – weight of bottle (g)

The percentages of gaharu oil yield for every parameter were calculated as below:

$$\text{Oil yield (\%)} = \frac{\text{weight of gaharu oil extracted (g)}}{\text{dry weight sample (g)}} \times 100$$

3.5 Process flow of the experiment

Process flow for the research experiment is shown in Figure 3.1

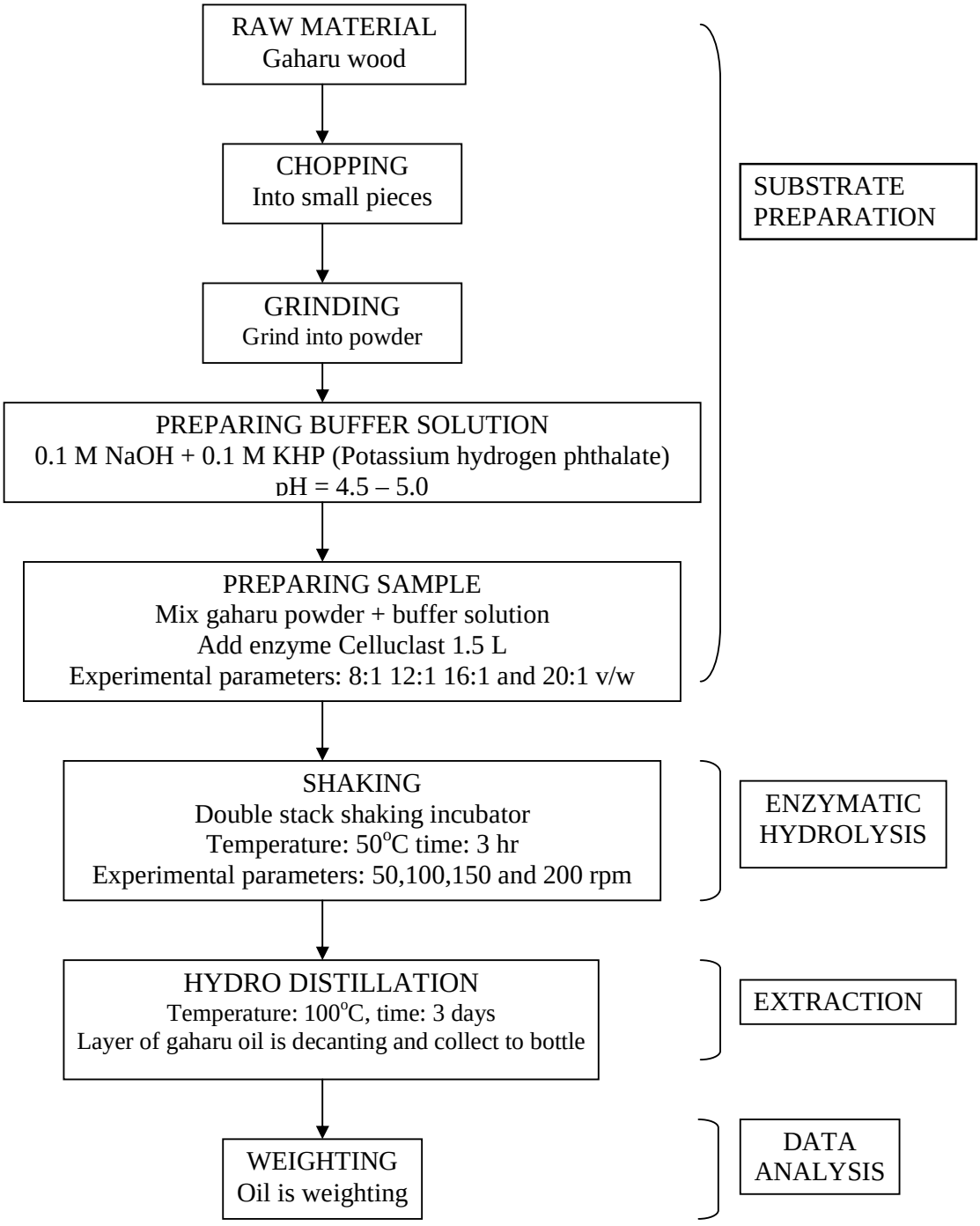

Figure 3.1: Process flow for the research experiment

CHAPTER 4

RESULTS AND DISCUSSION

The effects of two specific parameters which are shaking speed and water/ gaharu ratio in enzymatic hydrolysis using cellulose enzyme have been investigated. The yields of gaharu oil for each parametes are compared and the optimum parameters for enzymatic hydrolysis pretreatment based on maximum gaharu oil yield extraction. The yields of gaharu oil are shown in Table 4.0.

Table 4.0: Yield of gaharu oil extraction

Variable	rpm	Ratio water /gaharu	Ratio enzyme/ gaharu	Treatment time (hr)	Extraction time (day)	Yield %
Shaking speed	50	8:1	1:100	3	3	0.0995
	100	8:1	1:100	3	3	0.1068
	150	8:1	1:100	3	3	0.1092
	200	8:1	1:100	3	3	0.0923
Ratio water/ gaharu	150	8:1	1:100	3	3	0.1092
	150	12:1	1:100	3	3	0.0859
	150	16:1	1:100	3	3	0.0707
	150	20:1	1:100	3	3	0.0543

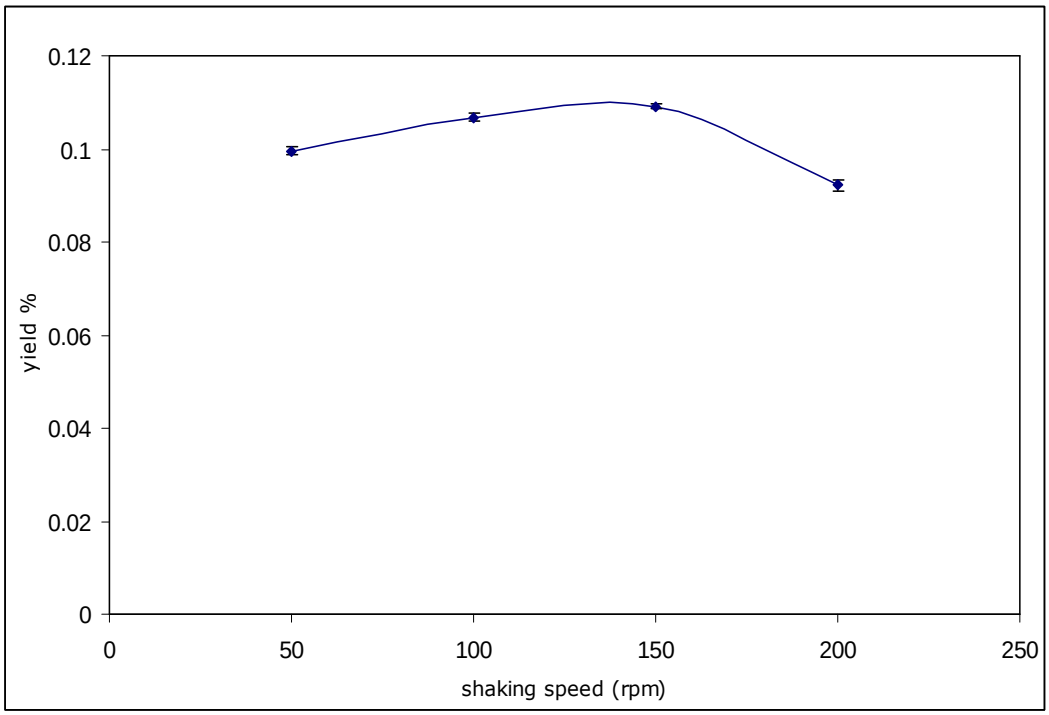

Figure 4.1: Shaking speed versus yield

From Figure 4.1, it is observed that a considerable increase in gaharu oil extraction yield occured when the speed shaking (rpm) increased from 50 rpm to 150 rpm. Same results have obtained by Hanna *et al*, (2001). The speed shaking will enhance the interaction between gaharu and cellulose enzyme. This cause more cellulase enzyme attack to the cell wall of gaharu, and degradation being increase. The cell wall degradation considered as a reduction in fiber content. The enzymatic attack of the cell walls causes an enhanced pressing efficiency as well as reduction in fiber content (Sineiro *et al*, 1996). That cause gaharu oil easily passes through the cell wall and caused the increament of the yield of gaharu oil being extracted (Osburn, 1944). At 200 rpm, the yield of gaharu oil didn't continue the same trend where it was decreased. Excessively high speeds lower the conversion yield as reported by Hanna *et al*, (2001). The enzyme is believed to be exposed to high shear stresses. These stresses are responsible for the surface denaturation of enzyme and reduced the activity of enzyme to degrade the cell wall of gaharu (Meenal *et al*, 2006). The highest yield for these parameters is 0.1092% at 150 rpm.

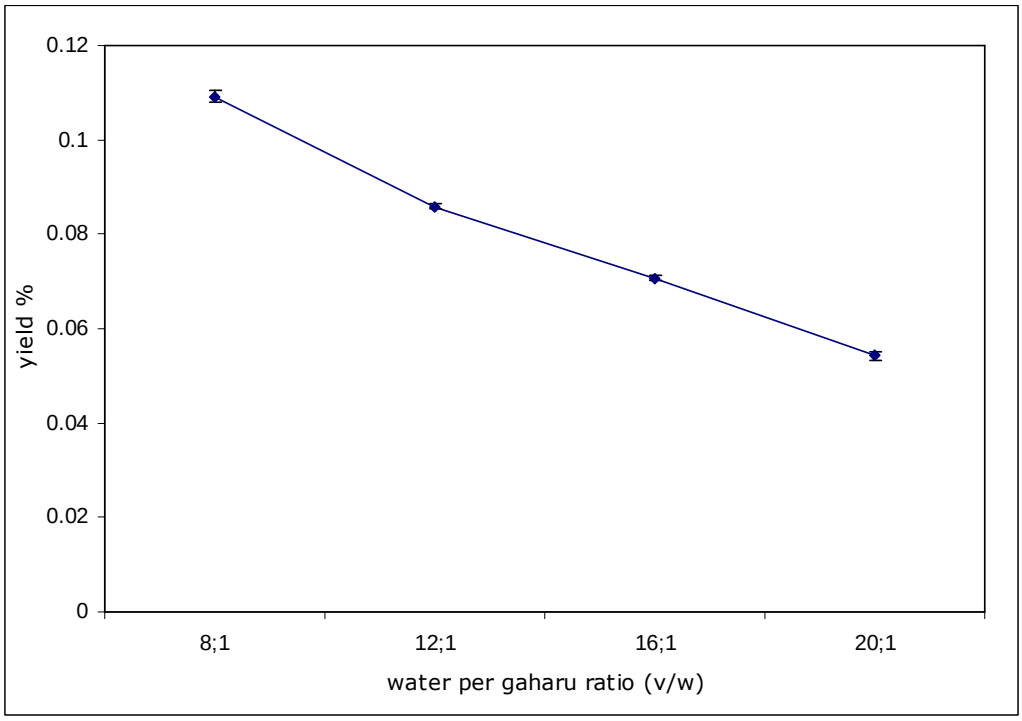

Figure 4.2: Water/gaharu ratio versus yield

As shown in Figure 4.2, a decrease in gaharu oil extraction yield occur when the water/gaharu ratio increase from 8:1 v/w to 20:1 v/w. Same results were reported by (Sineiro *et al*., 1997). Water in the sample plays an important role in hydrolytic reaction. From Dominguez *et al* (1996) report, water favouring the diffusion and mobility of both the enzyme and the products. In this condition water may inhibiting the enzymatic reaction. This proved that high water content can inhibit the enzyme activity during enzymatic hydrolysis pretreatment if the water content is too high. By increasing the ratio from 8:1 v/w into 20:1 v/w, the volume of water is become larger and causes the inhibition activity being increase. At the same time the enzyme activity being reduced and less degradation of gaharu cell wall by cellulose enzyme occur. The permeability of gaharu oil through cell wall decrease hence less of gaharu oil can be extracted. Therefore it is observed that increasing the water per gaharu ratio reduced the yield of gaharu oil extracted. The highest yield for this varying water/gaharu ratio is 0.1092% at 8:1 v/w.

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusions

This study on effect of shaking speed and water/gaharu ratio during enzymatic hydrolysis pretreatment had been successfully carried out. Thus, by completing this research, several conclusions can be made which are:

1. The yields for gaharu oil extraction were increased as the shaking speed increased from 50 rpm to 150 rpm. At the shaking speed of 200 rpm, the yield did not follow the same pattern where the yield being decrease. The highest yield for varying shaking speed is 0.1092 % at 150 rpm.

2. The yields for gaharu oil extraction were decrease proportional to increasing water/gaharu ratio from 8:1 v/w to 20:1 v/w. The highest yield for varying water/gaharu ratio is 0.1092% at 8:1 v/w.

3. Shaking speed at 150 rpm and water/gaharu ratio of 8:1 v/w in enzymatic hydrolysis pretreatment are optimum parameters that given maximum gaharu oil yield extraction which is 0.1092%.

5.2 Recommendations

Enzymatic hydrolysis pretreatment before extraction process is an important research that must be continued due to the high value and demand of gaharu nowadays. From this research, there are several recommendations that should be done in future study to improve the yield and quality of gaharu oil extracted which are:

- More effective hydro distillation units should be designed. During this research, there was gaharu oil stuck at the wall of equipment apparatus. The new technology should be used to improve the recovery of gaharu oil extracted.

- Further decrease in water per gaharu ratio v/w should be done to find the trend effects of these parameters clearly. Due to apparatus problem, water/gaharu ratio below than 8:1 v/w cannot be done. Preparing suitable apparatus and equipment was needed to make sure these parameters can be done successfully. Parameters recommend are 6:1 v/w and 2:1 v/w.

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APPENDICES A

Preparation of buffer solutions

1. **Preparing 0.1 M NaOH solution**
   Volume = 2 L

   Weight = MV x JMR
   = 0.1(2) (40)
   = 8 g

   8 g NaOH powder + 2L distilled water

2. **Preparing 0.1 M KHP solution**
   Volume = 2 L

   Weight = MV x JMR
   =0.1 (2) (204.23)
   = 40.846 g

   40.846 g KHP powder + 2L distilled water

3. **Preparing buffer solution**
   Volume = 4 L

   33 mL NaOH x 11 = 363 mL
   100 mL KHP x 11 = 1100 mL
   Distilled water = 2537 mL
   4000 mL of Buffer Solution

APPENDICES B

Preparation of samples

1. **Preparing sample for ratio 8:1 v/w**

8 : 1
3200 mL : 400 g
buffer solution gaharu powder

1 : 100
4 mL : 400 g
cellulase enzyme gaharu powder

2. **Preparing sample for ratio 12:1 v/w**

12 : 1
3600 mL : 300 g
buffer solution gaharu powder

1 : 100
3 mL : 300 g
cellulase enzyme gaharu powder

3. Preparing sample for ratio 16:1 v/w

16 : 1
4000 mL : 250 g
buffer solution gaharu powder

1 : 100
2.5 mL : 250 g
cellulase enzyme gaharu powder

4. Preparing sample for ratio 20:1 v/w

20 : 1
4000 mL : 200 g
buffer solution gaharu powder

1 : 100
2 mL : 200 g
cellulase enzyme gaharu powder

APPENDICES C

Yield calculations

1. 8:1 v/w and 150 rpm

Weight of bottle = 13.2553 g
Weight of bottle + oil = 13.6921 g
Weight of oil = 0.4368 g

Yield = (0.4368 g / 400 g) x 100
= 0.1092 %

2. 12:1 v/w and 150 rpm

Weight of bottle = 13.2262 g
Weight of bottle + oil = 13.4840 g
Weight of oil = 0.2578 g

Yield = (0.2578 g / 300 g) x 100
= 0.0859 %

3. 16:1 v/w and 150 rpm

Weight of bottle = 4.7463 g
Weight of bottle + oil = 4.9230 g
Weight of oil = 0.1767 g

Yield = (0.1767 g / 250 g) x 100
= 0.0707 %

4. 20:1 v/w and 150 rpm

Weight of bottle = 4.7404 g
Weight of bottle + oil = 4.8490 g
Weight of oil = 0.1086 g

Yield = (0.1086 g / 200 g) x 100
= 0.0543 %

5. 50 rpm and 8:1 v/w

Weight of bottle = 4.6432 g
Weight of bottle + oil = 5.0411 g
Weight of oil = 0.3979 g

Yield = (0.3979 g / 400 g) x 100
= 0.0995 %

6. 100 rpm and 8:1 v/w

Weight of bottle = 4.6330 g
Weight of bottle + oil = 5.0600 g
Weight of oil = 0.4270 g

Yield = (0.4270 g / 400 g) x 100
= 0.1068 %

7. 200 rpm and 8:1 v/w

Weight of bottle = 13.3720 g
Weight of bottle + oil = 13.7410 g
Weight of oil = 0.3690 g

Yield = (0.3690 g / 400 g) x 100
= 0.0923 %