

## The Effect of Enzymatic Pre-Treatment in Agarwood Oil Extraction

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**Abstract**—Agarwood oil was extracted from a pre-treated agarwood chip soaked in a specific enzyme concentration of 1 %, 3 % and 5 % respectively. The effect of soaking time was also investigated by applying soaking period in the range of 3 to 14 days. Hydrodistillation was carried out using the Clevenger-type apparatus to obtain the essential oil of agarwood chips. Subsequently the quality of the oil and the identification of its oil compounds were analyzed using GC-MS. The morphology of agarwood chips prior to hydrodistillation was characterized using scanning electron microscope (SEM). The SEM results of enzymatic pre-treated sample showed an obvious change in the cell morphology as compared to the sample with standard soaking of 6 days. The highest yield of oil with the amount of 0.125 % is produced from the sample pre-treated with 3 % enzyme concentration as compared to 0.065 % of oil yield from the non-pre-treated sample employing similar soaking days. The GC-MS analysis revealed a total of 335 compounds from all samples of agarwood oil and similar compounds were found in the standard soaking and enzymatic pre-treated samples. Caryophyllene, Gurjunene, and Alloaromadendrene that contributes to the unique odour of agarwood oil are found mostly in all samples. Thus, the enzymatic pre-treatment indicates a comparable yield and oil quality and it is achievable even with a reduced soaking time of 6 days as compared to standard 14 days.

**Keywords**— agarwood; essential oil; enzymatic pre-treatment; GC-MS; hydrodistillation extraction.

### I. INTRODUCTION

The essential oil has been widely used for centuries primarily for medicine but has since expanded into fragrance, food, pharmaceutical [1] and also in the agricultural, hygienic, and cosmetic industries [2]. It is a complex mixture of volatile to semi-volatile components where they generally have strong odor [3] and according to Costa et al.[4], essential oils are an aromatic and natural complex of mixtures of volatiles extracted from various plants as secondary metabolites. Najafian et al. [1] stated that essential oil could be extracted from different parts of plants such as flowers, roots, barks, leaves, etc.

*Agarwood*, also known locally as *gaharu*, is the fragrant resin produced primarily by the *Aquilaria* species (family: *Thymelaeaceae*) and is highly valuable non-timber forest product and is high in demand globally [5]. It is also known by the name of *Oudh*, *Chen Xiang* and *Eagleswood* [6]. A few well-known agarwood-producing trees in the *Thymelaeaceae* family are *A. agallocha*, *A. crassna*, *A. bailloni*, and *A. Grandiflora* [7]. This resin is produced when the trees are unhealthy as a self-defense mechanism in which it can occur both naturally and/or through wounding or artificially treated with inoculation.

Some of the isolated major contributing constituents to the aromatic smell of *Agarwood* are the three fragrant

sesquiterpenes which are agarofuran, (-)-10-*epi-γ-eudesmol* and *agarospirol* [8]. The fragrant resin is usually used as perfumes, incense in religious ceremonies, and traditional medicines. Alam et al. [9] reported that *Aquilaria agallocha* leaves extract can be used as a laxative and some of its other beneficial properties are antimicrobial, antioxidant and antidiabetic.

*Hydrodistillation* is mostly applied by the commercial *agarwood* industries in Malaysia; even though this method is the simplest method, it is not effective in term of both time- and energy-consumption. The conventional method has major drawbacks in the operating process where the soaking may take 7 to 14 days [10] and another 4 to 10 days for the extraction completion. The long extraction times may influence the loss of some volatile fragrance constituents caused by the thermal degradation resulting in low oil yield [11], [12]. Thus, alternative methods such as introductions of pre-treatments steps to increase the yield and reduce soaking time have been extensively studied.

According to Puri et al. [13], enzyme-assisted extraction is gaining more attention as researchers seeks for eco-friendly extraction technologies and it was also mentioned that the application of enzymes increases the effect of solvent pre-treatment by either reducing the amount needed or increasing the extraction yield. A study by Dina et al. [14] further proved that enzyme-assisted extraction has the potential to improve the yield while maintaining bioactive

properties of the extracts. Baby and Ranganathan [15] reported that this method of extraction is a recent approach in plants' bio-ingredients extraction where it is applied in the extraction of essential oils from seeds such as corn, sunflower, soybean and etc. but there is less studies done for *agarwood* oil extraction.

Other research has also shown that enzymatic pre-treatment can achieve a high yield of extraction of oils, flavors, and medicinal compounds [16]. A study done in a uniform concentration to compare the effect of different enzyme shows that cellulase afforded the highest yield of essential oil of *Fructus forsythia* [17] and is consistent with our findings. The enzyme-assisted extraction improved the efficiency of extraction attributing to the destruction of the cell wall components [18]. Boulila et al. [19] in their study stated that the enzyme pre-treatment did not induce transformation of the volatile components in bay leaves (*Laurus nobilis* L.) but contributed to the release of some glycosidically bound volatiles which enhanced the release of phenolic compounds.

In research by Yoswathana [20], which is followed closely, the agarwood is treated with a combination method of both chemical and biological pre-treatment method where it is first treated with 1 %  $H_2SO_4$  acid before the enzymatic treatment. Dimaki et al. [21] in their recent study showed the synergy between the combination of acidic and enzymatic pre-treatment which is a significant attribution to the high oil yield for *Sideritis spp.*. Thus, this research is done to investigate the effect of enzymatic pre-treatment with different enzyme concentration and incubation time on the yield of agarwood essential oil and to identify and compare the chemical compositions of oil extracted.

## II. THE MATERIAL AND METHOD

### A. Materials

Grounded *agarwood* chips were obtained from a Biobenua Lojistik Sdn. Bhd., a local *agarwood* plantation in Melaka. N-hexane and Sodium Hydroxide were bought from R&M Chemicals, Malaysia. *Sulfuric acid* and *Anhydrous sodium sulfate* were bought from JT Baker, USA and Merck, Germany respectively and *Cellulose* from *Trichoderma reesei* was bought from Sigma-Aldrich, USA.

### B. Materials Preparation

1) *Standard Preparation of Sample*: The process for standard preparation of sample is adopted from a local *agarwood* processing company, Biobenua Lojistik Sdn. Bhd. 200 g of the grounded *agarwood* chips is first weighed and mixed with deionized water by a 1:10 ratio volume of water to weight of sample (v/w). The sample is left to soak for 14 days in room temperature. Standard sample will be named as S6 and S14 for sample soaked for 6 days and 14 days respectively.

2) *Preparation of Sample with Acid*: 200 g of grounded *agarwood* is measured and transferred into an Erlenmeyer flask. The *agarwood* chip is first treated with 2000 mL of 1 % *Sulphuric Acid* ( $H_2SO_4$ ) and then autoclaved at 121 °C for 15 minutes as to breakdown the hemicellulose. The sample is

then incubated at 55 °C for 6 days. Sample from this pre-treatment is named as AO.

3) *Preparation of Sample with Enzymatic Pre-treatment*: *Agarwood* chip that was prepared according to section B (2) is allowed to cool down after the autoclave process completed. *Sodium Hydroxide* ( $NaOH$ ) is added to adjust the pH of the solution to 4.8 and then mixed with *cellulose enzyme* with different concentration of 1, 3 and 5 % (w/w) and followed by incubation for six days at 55 °C. The pretreated sample with 1, 3, and 5 % (w/w) is known as EP1, EP3, and EP5 respective of their concentration. The sample will be further investigated with three days incubation time with the same parameter once the best enzyme concentration has been found and will be named respective of the best concentration.

4) *Preparation of Sample with Enzyme*: Acetate buffer is prepared where the pH of the buffer is adjusted to 4.8 before the addition of 3 % cellulase enzyme. 200 g of grounded *agarwood* is soaked in the prepared solution and incubated in a water bath at 55 °C for six days. Sample for this pre-treatment is noted as EO.

### C. Hydrodistillation

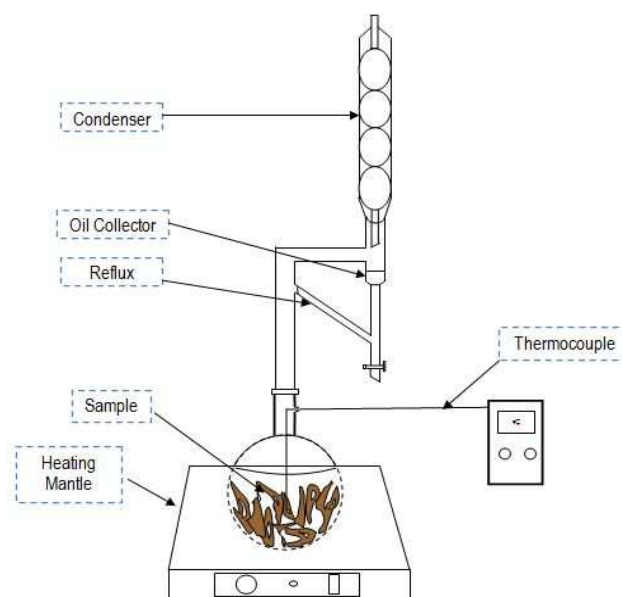


Fig. 1 Schematic Diagram

All samples prepared in sub-section B will undergo hydrodistillation using the Clevenger-type apparatus as shown in the schematic diagram in figure 1. The temperature during the process is kept at  $100 \pm 0.1$  °C. Oil formation is observed every 1 hour for a duration of 7 hours. The oil extracted will be collected and the moisture is removed by using anhydrous sodium sulfate and stored at 4 °C prior further analysis.

### D. Characterization of Agarwood Oil

1) *Gas Chromatography-Mass Spectrometer (GC-MS)*: GC-MS (QP 2010 series) was used to analyze chemical compounds from the extracted *agarwood* oil. The GC-MS is equipped with a VF-5ms fused silica capillary column and

ionization energy of 70 eV was used for the GC-MS detection. Helium gas (99.99 %) at a constant flow rate of 1 mL/min was used as the carrier gas. The temperature for the oven was set at 50 – 230 °C at an increasing rate of 5 °C/min

and the injector temperature was set at 230 °C. The peaks from GC-MS were identified using National Institute of Standards and Technology (NIST) mass spectral library.

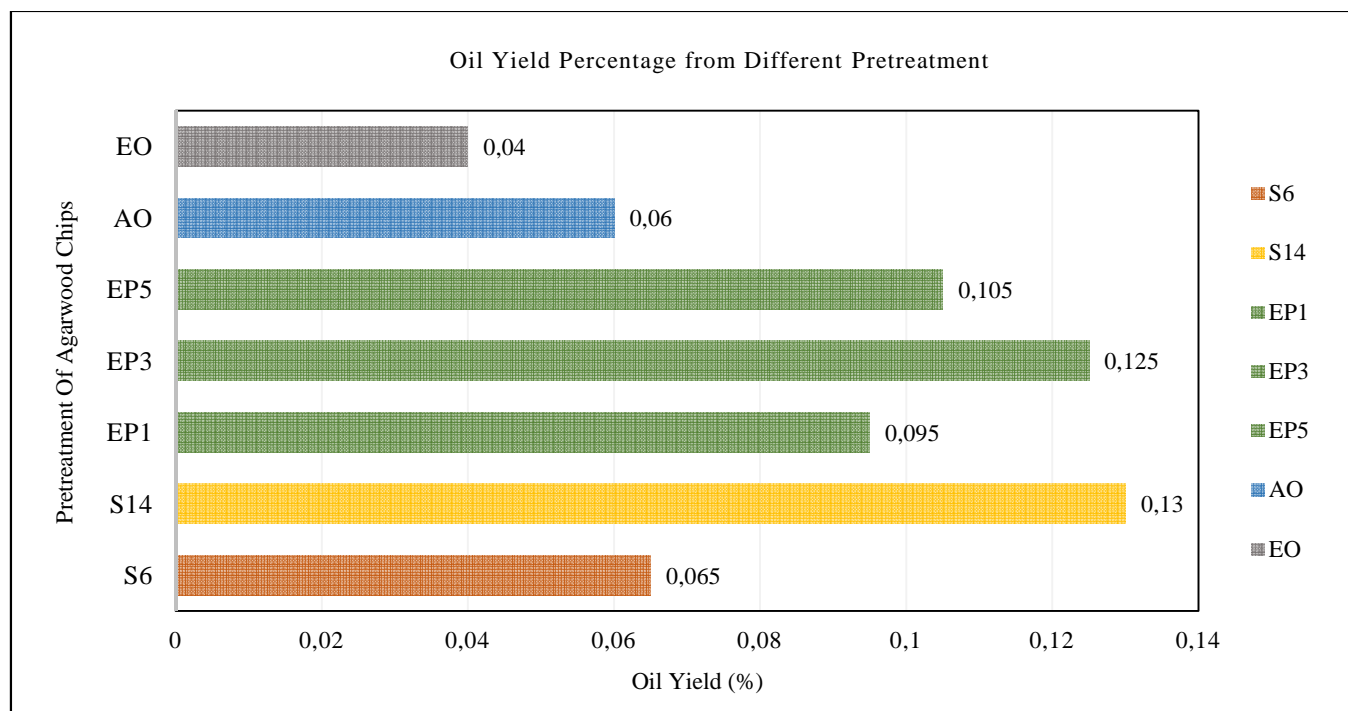


Fig. 2 Comparison on The Percentage of Agarwood Oil Yield with Different Pre-treatment.

2) *Scanning Electron Microscope (SEM)*: The evaluation of the agarwood cell structure characteristics for all samples were done using a Scanning Electron Microscope (SEM). The cell structures were observed at 500 times magnification. The samples were coated with a layer of gold (Au) to enhance the captured image using the mini Sputter Coater (Quorum Technologies, SC7620) and followed by SEM imaging using Hitachi Tabletop Microscope (TM-3000).

### III. RESULTS AND DISCUSSION

#### A. Effect of Pre-Treatment on Agarwood Oil Yield

The effect of different pre-treatment on the yield of agarwood oil is summarized in figure 2. The percentage of oil yield for S14 is higher compare to S6, which is 0.13 % and 0.065 % respectively, where this supports the data provided by the local company where longer soaking time produced higher oil yield. A study done by Jok et al. [22] showed that agarwood chips that were soaked for 14 days have the highest yield however, further soaking time will only reduce the oil yield. As the soaking time increased, the acidity of the medium increases too as to corrode the cell wall of the agarwood chips, but over time, it may cause excessive breakage and releasing the chemical compounds to the soaking medium [23]. Further research by Jok et al. [24] confirmed that longer soaking time might increase the yield but over soaking will somehow give a lower yield which can

result in the wastage of the essential compounds of the agarwood chips into the soaking water.

Figure 2 also shows that EP3 has the highest yield of 0.125 %, which is close to oil yield obtained from sample S14, which is 0.13 %. AO has a yield of 0.06 %, and EO sample has the lowest yield of 0.04 % as shown in figure 2. This proved that acid pre-treatment is necessary to increase enzyme-to-cellulose accessibility to increase the oil yield of agarwood through the degradation of the resin's cell wall material. This is supported by Chen et al. [25], where during the dilute acid pre-treatment, hemicellulose bond is removed thus the increase of the sample porosity which subsequently improves the enzymatic digestibility of cellulose and Van Dyk and Pletschke [26] also reported that by the degradation of hemicellulose which acts as physical barriers that hamper the access of cellulose to cellulose may increase the release of essential oil. The increase in the extraction yield was observed with enzyme concentration up to 3 % but no substantial increase observed for enzyme concentration of 5 % which is supported by Baby and Ranganathan [15] in their study where the increase of yield of essential oil was observed up to 1 % of enzyme concentration and no corresponding significant increase for higher concentration.

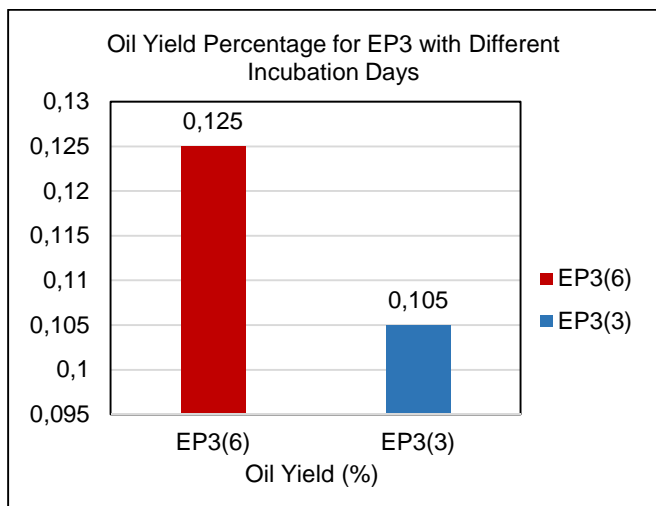


Fig. 3 Comparison on The Percentage of *Agarwood* Oil Yield for EP3 Samples with Incubation Days of 6 and three days.

TABLE I  
SOME OF THE COMPOUNDS IDENTIFIED IN *AGARWOOD* ESSENTIAL OIL VIA GC-MS.

Chemical Compound	% Of Total Area						
	S6	S14	EP1	EP3	EP5	AO	EO
<i>Caryophyllene</i>	0.318	0.225	2.035	2.496	-	1.346	-
<i>Valencene</i>	0.637	1.603	0.632	4.073	2.028	2.995	4.456
<i>Gurjunene</i>	-	8.081	1.724	2.939	-	-	-
<i>Aristolene</i>	1.256	1.884	1.198	2.218	2.009	3.202	2.152
<i>Alloaromadendrene</i>	-	2.531	0.859	2.202	2.458	-	-

#### B. GC-MS on Chemical Compounds in *Agarwood* Oil Extracted with Different Pre-treatment Method

The GC-MS analysis revealed a total of 335 compounds from all seven samples of *agarwood* oils. Some of the identified chemical compounds are tabulated in table I in the percentage of whole Essential Oil. The compositions of the compounds obtained differ with different pre-treatment method, which is supported by a study by Yoswathana [20]. Among the compounds found that contributes to the unique odor of *agarwood* oil are *sesquiterpenes* such as *Caryophyllene*, *Gurjunene*, *Alloaromadendrene*, and *Gualene*. However, some of the main compounds, such as *jinkoh-eremol*, *epoxybulnesene*, and  $\beta$ -*agarofuran* are missing from the results [27]. According to Chen et al. [28], different artificial methods used in stimulation the formation of the resin may produce different *agarwood* qualities which supported also by the study done Hashim et al. [29] which concluded that various factors may affect the different compositions of *agarwood* essential oil such as species/origin of the tree and methods of stimulation of *agarwood* resin.

From the table I, similar compounds can be seen increasing and decreasing depending on the different methods applied. Such as the percent total of *Caryophyllene* were notably increased in EP3 from 0.3 % to around 2.0 % but was missing in EP5 sample. Based on data obtained from the GC-MS analysis, *Caryophyllene* was identified in EP5, but the total area is too small, making it negligible. As previously mentioned, based on Terry and Heinrich [30] and

Once the best enzyme concentration between 1, 3, and 5 % have been met, the experiment was continued with reduced incubation time from 6 to 3 days for further investigation. As discussed previously, the best result obtained was from EP3, with the highest yield of 0.125 % with an enzyme concentration of 3 %. EP3 (6) refers to sample incubated for six-day, and EP3 (3) is for sample incubated for three days. Figure 3 shows a comparison between EP3 (6) and EP3 (3) with an oil yield of 0.125 % and 0.105 %, respectively. EP3 (3) with lower incubation time gives a lower percentage of *agarwood* oil yield as compared to that of EP3 (6). This shows that three days of incubation time cannot produce a better yield than six days. As mentioned previously, Jok et al. [22] stated in their study, where 14 days of soaking time produced the highest yield of oil as compared to 7 days for the sample with no pre-treatment. Thus, by further reducing the soaking day, the amount of oil yield becomes less significant.

Tajuddin et al. [31], some of the compounds identified in the table, I provide the woody aroma to *agarwood* oil. *Caryophyllene* contributes to the spicy-wood aroma, while *Gurjunene* contributes to the woody balsamic aroma and *Alloaromadendrene* to the woody aroma. According to Hosni et al. [32], enzymatic pre-treatment led to an increase in the amounts of some of the compounds which may affect the aroma produced and eventually increase the yield of the essential oil. Compounds in Table I for EP3 shows an increase in the amount as compared to other samples but produced less *Gurjunene* from S14 which is also further supported by Hosni et al. [32] that enzymatic pre-treatment may induce a decrease of the compounds.

TABLE II  
COMPARISON OF THE COMPOUNDS IDENTIFIED IN *AGARWOOD* ESSENTIAL OIL VIA GC-MS FOR EP3 (6) AND EP3 (3).

Chemical Compound	% Of Total Area	
	EP3(6)	EP3(3)
<i>Caryophyllene</i>	2.496	0.075
<i>Valencene</i>	4.073	-
<i>Gurjunene</i>	2.939	0.531
<i>Aristolene</i>	2.218	-
<i>Alloaromadendrene</i>	2.202	-

As for the EP3 (3) sample, only 17 compounds were detected from the GC-MS analysis. The percentage of the total area for the same compounds as the table I was tabulated in table II with comparison to EP3 sample that was

incubated for six days. From table II, *Caryophyllene* and *Gurjunene* were among the main compounds identified for EP3 (3) with 0.075 % and 0.531 % as compared to EP3 (6) with 2.496 % and 2.939 % respectively. This showed that with reduced incubation time, fewer compounds could be extracted despite the enzymatic pre-treatment. This finding is in contrast with the research by Nor et al. [33] where there were no significant differences in the compounds found by GC-MS analysis with shaking time of 1, 3 and 7 days but Boulila et al. [19] did mention in their study that enzymatic pre-treatment doesn't induce transformation to their samples' volatile compounds. This proved that longer soaking or incubation time than three days is necessary to extract all the compounds required even though the yield obtained is a considerable amount. There were no reported effects for *agarwood* compounds composition on soaking time less than five days with enzymatic pre-treatment [20], and Jok et al. [22] did research comparing the effect of different soaking time of 0, 7, 14, 21 and 28 days with no pre-treatment.

### C. Scanning Electron Microscope (SEM)

The SEM micrographs were taken for all *agarwood* chips before hydrodistillation process. The images were captured at 500x magnification [22] as shown in figure 4, 5, 6, and 7. Figure 4 (a, b) shows SEM captured images for untreated *Agarwood* chips clearly show that it has a smooth surface structure.

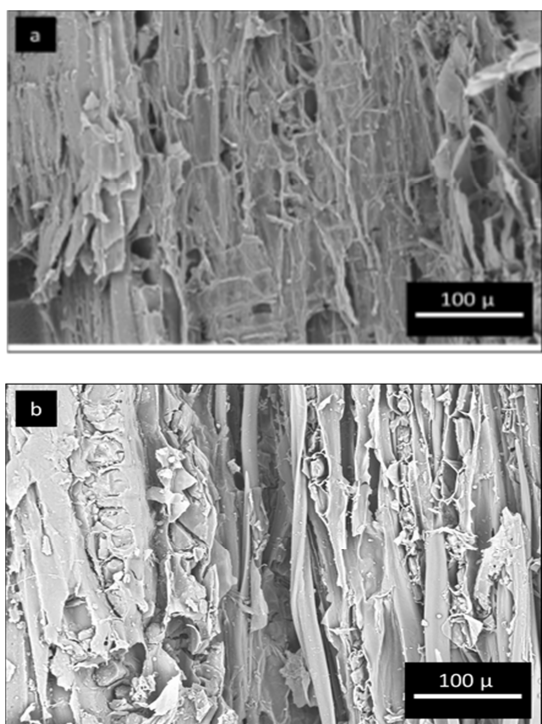


Fig. 4 Scanning Electron Microscopy (SEM) of *Agarwood* Chips before Hydrodistillation for Sample without Pre-treatment; a) S14; b) S6

For the enzymatic pre-treatment which used both acid and enzyme, figure 5 (c, d, e) showed that the outer layers were more disrupted and flakier surface structure caused by the removal of hemicellulose which contributes to the increase of oil yield for EP1, EP3 and EP5 as compared to S6 despite the similar soaking time. Figure 5 (d) showing the most disruption as compared to (c) and (e)

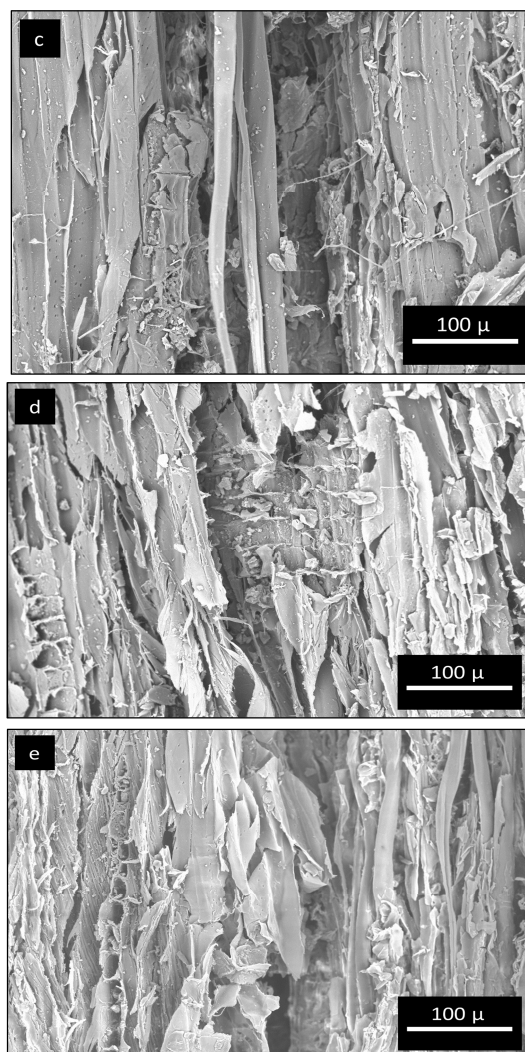


Fig. 5 Scanning Electron Microscopy (SEM) of *Agarwood* Chips before Hydrodistillation for Sample with Enzymatic Pre-treatment where c) EP1 d) EP3 and e) EP5.

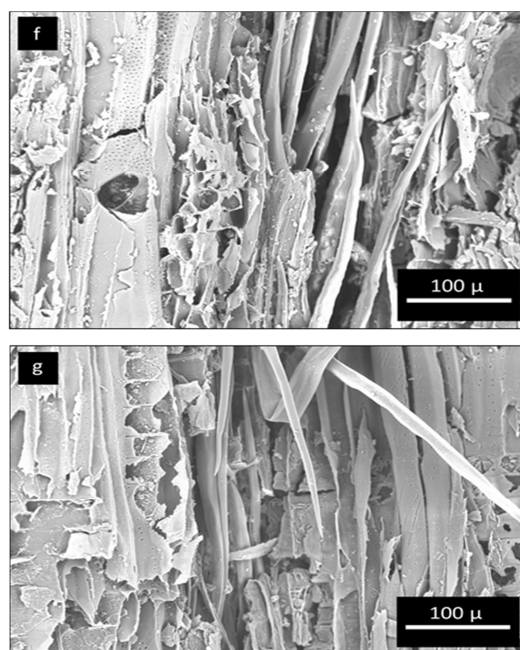


Fig. 6 Scanning Electron Microscopy (SEM) of *Agarwood* Chips before Hydrodistillation for f) AO and, g) EO

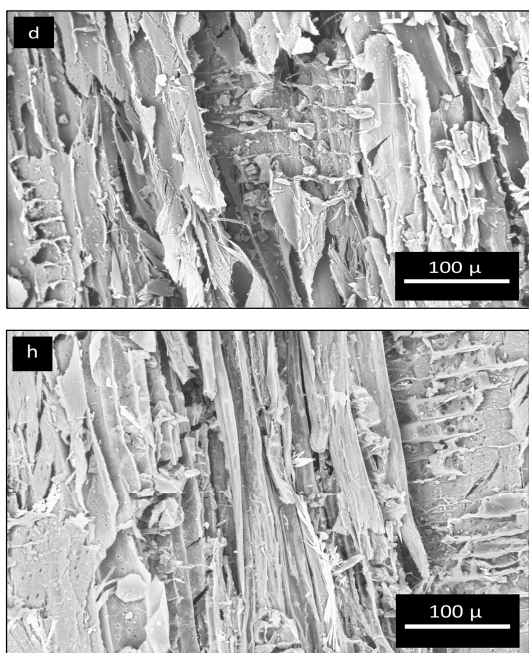


Fig. 7 Scanning Electron Microscopy (SEM) of Agarwood Chips before Hydrodistillation for EP3 with Different Incubation Days where d) EP3 (6) and h) EP3 (3)

The oil yield obtained from the sample in figure 5 (d) is 0.125 %, whereas the yield for sample in figure 5 (c, e) are 0.095% and 0.105 % respectively. This showed that enzyme concentration of 3 % is the optimal amount in extracting the essential oil of *agarwood* where the *cellulose enzyme* can digest more cellulose in the cell wall composition when the hemicellulose bonds were removed which was mentioned previously in A for the effect of pre-treatment on *agarwood* oil yield [15], [25], [26]. Figure 6 (f, g) showed a much smoother and undisrupted surface compared to number 5 (d) where the oil yield obtained from these samples were 0.06 % and 0.04 % respectively. Samples with a combination of pre-treatment from EP1, EP3, and EP5 has more yield of essential oil compared to AO and EO with EP3 having the highest yield. The alteration of the structure should be one of the reasons for the increase of enzyme accessibility towards cellulose during the hydrolysis, and this is by the findings reported by Chen et al. [28].

Figure 7 shows the comparison between *agarwood* chips that have undergone enzymatic pre-treatment with 3 % enzyme concentration but with different incubation days of 6 and three days. Figure 7 (d) as previously discussed in figure 5 (d), has more disrupted and flakier surface structure but as for figure 7 (h), it has a rough surface structure but doesn't look as disrupted or flakier, and the oil yields obtained were 0.125 % and 0.105 % respectively. Fazila et al. [34] found that unsoaked sample produced 1.72 % of oil yield as compared to sample soaked for seven days with sulfuric acid, which gave 6.78 %. They also mentioned that in previous studies, it showed that sufficient soaking could rupture the parenchyma cells thus enables the diffusion from the oil glands and this further clarifies the need of adequate soaking or incubation time for a more ruptured cell wall.

#### IV. CONCLUSION

The results of enzymatic pre-treatment on *agarwood* chips indicate that comparable yield is achieved even with a reduced soaking time of 6 days compared to standard 14 days soaking time. Optimum oil recovery was observed in the sample with 3 % enzymatic pre-treatment with 0.125 % oil yield. Common compounds identified using GC-MS were *Caryophyllene*, *Gurjunene*, *Alloaromadendrene*, and *Gualene* that make the unique *agarwood*'s wood odor where *Caryophyllene* contributes to the spicy-wood aroma while *Gurjunene* contributes to the woody balsamic aroma and *Alloaromadendrene* to the woody fragrance. The aroma profile of pretreated *agarwood* oil has improved as compared to the standard. SEM results showed that the surface morphology of samples with enzymatic pre-treatment was more disrupted and have flakier surface structure caused by the removal of hemicellulose which contributes to the increase of oil yield for enzymatic pre-treated samples as compared to the standard sample despite the similar soaking time. Thus, enzymatic pre-treatment can likely be used in *agarwood* oil extraction with improved oil yield and less soaking time and with comparable oil quality.

#### ACKNOWLEDGMENT

The authors are grateful to Universiti Teknologi MARA, Malaysia for the research grant, 600-RMI/FRGS5/ (15/2015) and would like to express their gratitude to Biobenua Logistik Sdn. Bhd. for the supply of *agarwood* chips.

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