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Journal of Biobased Chemicals

Department of Chemical Engineering
Universitas Jember

PREFACE

We want to present our journal's 1th volume and edition, Journal of Biobased Chemicals, published by the Department of Chemical Engineering, University of Jember, Indonesia. This volume is expected to enhance the findings and research aboutnatural product and their derivatives, mainly in energy, chemicals, and materials. We present articles on biobased chemical products, processes, and management.

This new journal was envisioned and founded to represent the growing needs of biobased chemicals research as an emerging and increasingly vital field, now widely recognized as an ideal substitution for fossil-based chemicals. The journal aimsto deliver and provide notable and standardized research and findings through journal reporting. The journal is intended as a window or a library for practitioners and researchers to share their works, identify new issues, and organize further research. At the same time, industrial users could apply the invention for scale-up, problem-solving, and application.

Hopefully, this edition will contribute valuable thoughts for the readers and enhance future research on biobased chemical products. Finally, we thank all participants, including authors, reviewers, and editors, for contributing.

August 2020

Boy A. Fachri

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RESEARCH ARTICLE

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Microwave Assisted Extraction of Essential Oil from Fresh Basil (*Ocium Basilicium* L.*)* Leaves

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Abstract. In this research, the extraction of essential oil from fresh basil leaves is conducted using solvent-free microwave extraction (SFME) and microwave hydrodistillation (MHD) methods. Several parameters influence the extraction of basil oil using the SFME method: microwave power, the ratio of the mass of raw material to the volume of the distiller (F/D), material size, and the length of the extraction time. Additionally, the components contained in basil oil and changes in oil gland conditions in basil leaves before and after extraction were also evaluated. The optimum condition was obtained as follows: microwave power of 380 W, a ratio of 0.1 g/mL between the mass of raw material and the volume of the distiller (F/D), raw material size of intact (± 3 cm), and an extraction time of 60 min. Moreover, SFME has a shorter extraction time to produce yields than MHD methods. GC-MS analysis of basil oil identified 49 components. This study demonstrates that the SFME method is more effective than the MHD method for extracting basil oil from fresh leaves, based on both the time of extraction and yield.

Keywords: *basil oil, essential oil, microwave hydrodistillation, Ocimum basilicum* L.*, solvent-free microwave extraction*

1. Introduction

Essential oil is one of the potential agroindustry export commodities, serving as a mainstay for Indonesia's foreign exchange. The data on essential oil consumption and its derivation from the World Export-Import Statistics indicate an average annual growth of 5-10%. The increase was primarily driven by the growing demand for cosmetics, food flavorings, and fragrances. Essential oils that are mostly distilled in Indonesia include patchouli oil, cloves, nutmeg, citronella, fragrant root, eucalyptus oil, and others. Meanwhile, essential oils have the potential to be developed, including basil, gandapura, cardamom, cinnamon, and others [1].

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Basil (*Ocimum basilicum* L.) is one of the essential oil-producing plants in Indonesia that has not been fully utilized. Currently, the Indonesian people are more popular for consuming basil leaves as a vegetable (*Ocimum basilicum* L.). As a traditional medicine, basil leaves are used to treat fever, nausea, and increase milk production in breastfeeding mothers [2]. Additionally, basil offers various health benefits, including the treatment of diseases such as headaches, cough, diarrhea, constipation, skin diseases, worm infestations, and kidney failure. As a medicinal herb, basil leaves are also efficacious in various capacities, including anti-carcinogenic, anthelmintic, antiseptic, anti-rheumatic, anti-stress, and antibacterial properties [3]. Basil leaves contain various components, including saponins, flavonoids, tannins, and essential oils [4]. While the most important content is an essential oil, the essential oils in basil leaves can inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas fluorescen*ce, *Candida albicans*, *Streptococcus alfa,* and *Bacillus subtilis* [5].

In a previous study, essential oil from basil leaves was obtained from dry basil by using the Soxhlet method. Basil leaves were extracted using the Soxhlet method and ethyl acetate as a solvent. From this study, basil leaves were dried and sieved through a 40-mesh sieve. The optimal condition from this study was a 1:6 (w/v) ratio of dry basil leaves and solvent for 6 an extraction time [6]. Another method that was used is the microwave hydrodistillation (MHD) method. Based on a study conducted by Dalia et al. (2015), using 200 g of dried basil leaves and the MHD method for 6 h yielded 0.6% v/w. The MHD method yielded a small amount, and the extraction time was prolonged. It is necessary to consider using the green technique in the extraction of essential oils, which involves minimal solvent, energy, and time consumption. Nowadays, Microwave-assisted extraction is one of the new methods that have been developed for extracting essential oils. A study comparing the MHD and Solvent-Free Microwave Extraction (SFME) methods for extracting essential oils from basil leaves was conducted. From this study, dry basil leaves were extracted to obtain essential oils. According to this study, SFME has several significant advantages over MHD, including shorter extraction times, solvent savings, substantial energy savings, and environmental friendliness [8]. From the research, we can conclude that all studies using dry basil leaves to obtain essential oil have found SFME to be the optimal method for essential oil extraction.

This study aims to optimize the process of extracting the essential oil from fresh basil leaves. The selection of fresh basil leaves had not been used in previous studies and is expected to reduce costs due to the lack of a drying process required. This study will use two methods of microwave-assisted extraction. These methods are the MHD method and the SFME method. The selection of the SFME method for extracting essential oils does not require the addition of solvent, unlike other extraction methods, resulting in a higher yield and shorter extraction time [9].

2. Materials and Methods

2.1 Materials

Fresh basil (*Ocimum basilicum* L.) leaves were collected from Keputran market, Surabaya, East Java, Indonesia. The size of the fresh basil leaves used in this study was intact (± 3 cm), half-intact (± 1.5 cm), and chopped (± 0.5 cm). An analytical-grade anhydrous sodium sulfate was purchased from Sigma-Aldrich, Singapore.

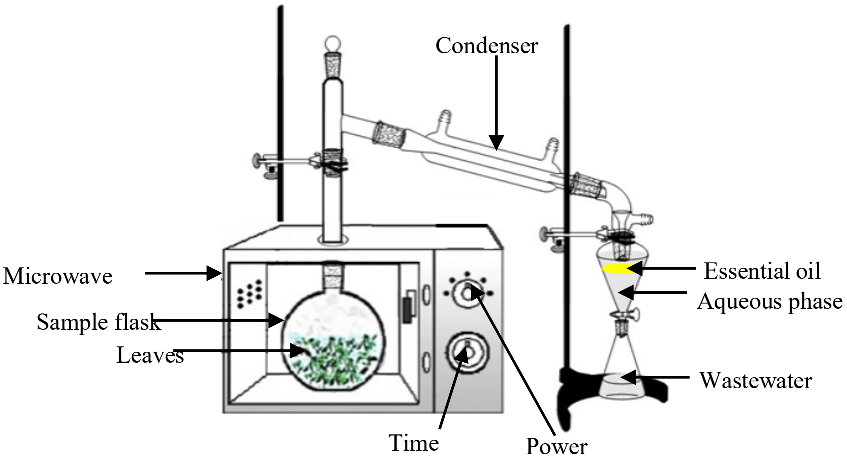


Figure 1. The experimental setup for the extraction of essential oil from fresh basil (*Ocimum basilicum* L.) leaves using a solvent-free microwave extraction method

In this SFME method (Figure 1), we used a microwave oven type EMM-2007X (20 L, 220 V, maximum power of 800 W) with a wave frequency of 2450 MHz. The dimensions of the PTFE-coated cavity of the microwave oven were 46.1 x 28 x 37.3 cm. The microwave oven was modified by drilling a hole at the top. A round-bottom flask with a capacity of 1000 mL was placed in the oven and connected to the Clevenger apparatus. Then, the hole was sealed with PTFE to prevent any heat loss inside.

The procedure for the SFME method was performed at 1 atm, the size variables of fresh basil leaves (chopped, intact, and half intact), and the ratio between the mass of raw material with a volume of distiller (F/D) (0.1; 0.175; and 0.25 g/mL)—the weighted material placed in a flask (1000 mL). Four power levels were operated using a microwave oven (100 W, 240 W, 380 W, and 540 W) for 60 min. Furthermore, to determine the effect of extraction time on the yield of basil oil, extractions using the SFME method were performed for up to 80 minutes. The extracted basil oil was dried over anhydrous sodium sulfate to remove water in the essential oil. Then, they were weighed and stored in vial bottles at 4 °C.

The yield of basil oil is calculated as follows:

$$\text{Yield (\%, w/w)} = \frac{Mass\ of\ extracted\ basil\ oil}{Mass\ of\ fresh\ basil\ leaves \times (1 - water\ content)} \times 100\% \qquad (1)$$

2.2 Microwave Hydrodistillation (MHD)

The extraction of basil oil using the MHD method (Figure 2) was done using the SFME method. 175 g intact fresh basil leaves (± 3 cm) and 400 mL of distilled water (ratio of raw material and a volume of solvent (F/S) of 0.4375 g/mL) were put into a 1000 mL distiller flask, and basil oil was extracted at a microwave power of 380 W for 180 min. Basil oil was collected in a vial, then dried over anhydrous sodium sulfate and stored at 4 ^{o}C.

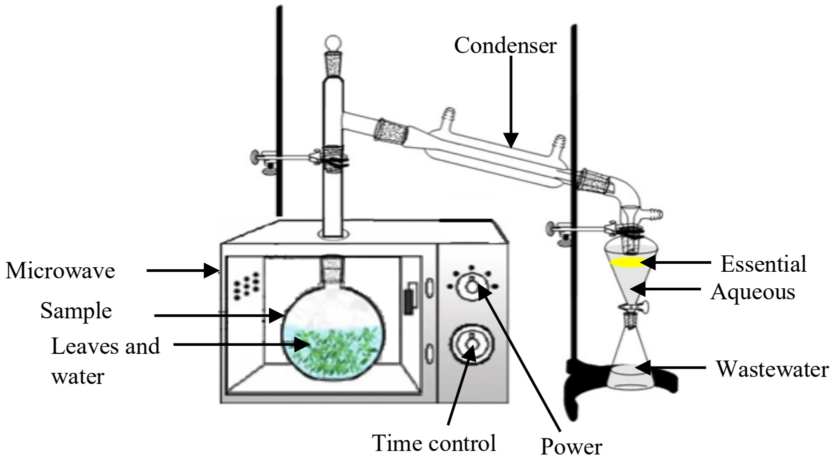


Figure 2. The experimental setup for the extraction of essential oil from fresh basil (*Ocimum basilicum* L.) leaves using the microwave hydrodistillation method

2.3 Chemical Analysis of Basil Oil Components by Gas Chromatography-Mass Spectrometry (GC-MS)

The components contained in basil oil were obtained by using gas chromatography-mass spectrometry (GC-MS) analysis. This analysis is not only used to know the components contained in essential oils, but also to know the level of each component. GC-MS was used in this study using an Agilent 6980N gas chromatograph with an Agilent 5973 mass spectrometric detector. Gas chromatography-mass spectrometry was incorporated with a chromatography column HP-5.5% phenyl methyl siloxane, 30 m length: 0.32 mm film thickness, 0.25 µm internal diameter.

2.4 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) analysis is a technique used to examine the surface structure of materials. The morphology of the fresh basil leaves was studied using SEM Evo MA 19. In this case, the material analyzed was the basil leaves before and after extraction.

3. Results and Discussions

3.1 Effect of Microwave Power on the Yield of Basil Oil: Comparison of Performance of Basil Extraction using SFME and MHD

The time of extraction is the main factor that affects the performance of MHD and SFME. In the extraction process, there are three important stages: the equilibrium phase, the transition phase, and the diffusion phase. The equilibrium phase occurs when the substrate is transferred to the outer layer of the matrix. Then, it is followed by the transition phase in which convection and diffusion occur in mass transfer. The last phase is diffusion. In the diffusion phase, the release of extracts through a diffusion mechanism is characterized, and in this phase, the extraction rate is slow. In the extraction process, mostly the extraction time is proportional to the yield. The longer the extraction time, the higher the yield will be [10].

In extracting basil oil using the MHD method and SFME method, the time of extraction is one of the factors that need to be considered. Table 1 shows the correlation between extraction time and basil oil yield. The MHD method requires more time to reach the equilibrium phase than the SFME method, which is 80 min. Whereas in the SFME method, within 80 min, the diffusion phase has reached. In general, the extraction of basil oil using the SFME method required a faster time (60 min) that could produce higher yields, which is 3.41 times greater than the MHD method. In SFME, extraction occurs in two kinds of heating: selective and volumetric. Selective heating is microwave radiation that can directly penetrate the distillation flask so that materials and solvents can absorb radiation effectively. Whereas volumetric heating is heating that occurs in the overall volume of material, so that heat is uniform and occurs more quickly. This method allows for obtaining high yield at lower extraction time when the extraction was done by SFME [11]. This statement proves that the SFME method is quite effective and efficient when compared to the MHD method.

Table 1. Comparison of the effect of time on yield between SFME and MAHD

Time (minutes)	**Yield (%)**	
	SFME	**MAHD**
0	0	0
20	1.662	0.249
40	2.120	0.268
60	2.281	0.388
80	2.373	0.400
100		0.503
120		0.573
140		0.603
160		0.666
180		0.668

3.2 Effect of operation condition on basil oil yield using SFME

Power in the microwave extraction process is an important operating condition that affects the extraction yield. In the extraction process, the amount of energy received by the raw material is converted into heat energy by microwave power, and the heat energy will help the process of removing essential oils from the raw material [10].

In basil leaves oil extraction, determining the optimal power is important because it will affect the temperature during the extraction process. Figure 3 shows the effect of microwave power on temperature. The higher the microwave power, the faster the polar molecules of the material will rotate to produce heat energy, which is detected from an increase in temperature. The temperature-time profile for each power used in the extraction process is shown in Figure 3. The slope of the linear line determines the rate of temperature rise at the profile temperature. From Figure 3, the temperature increase for each power used in the SFME method is 3.35 °C/min, 14.48 °C/min, 18,7 °C/min, 23 °C/min for 100 W, 240 W, 380 W, 540 W microwave power, respectively.

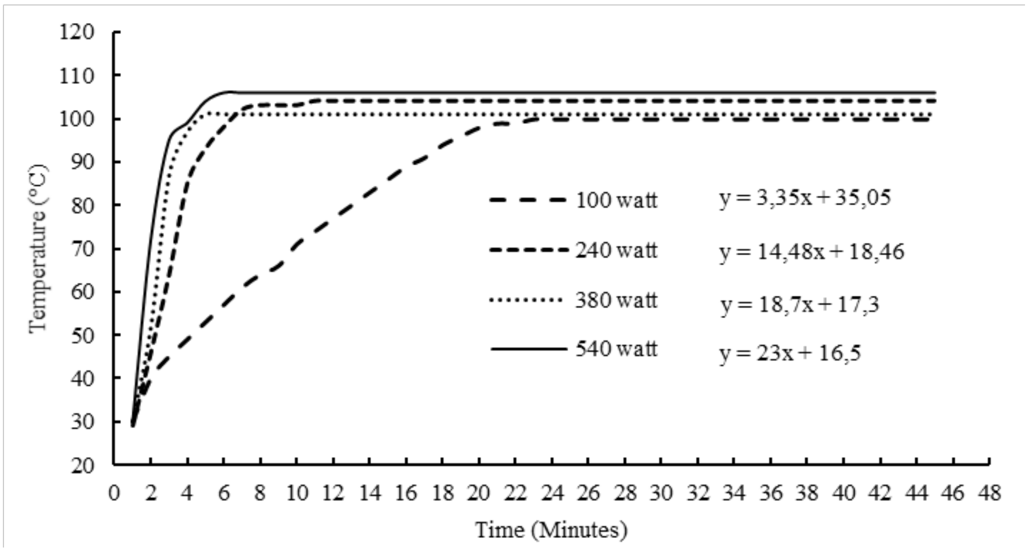
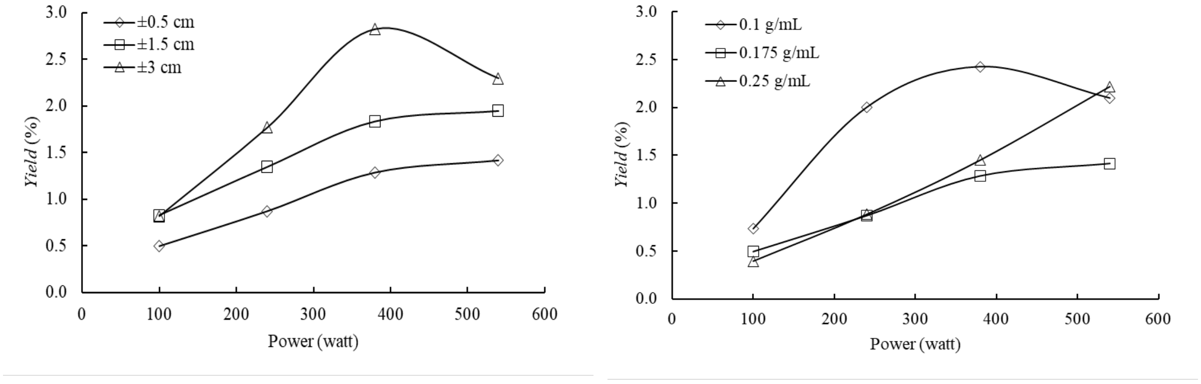


Figure 3. Temperature-time profile for each microwave power

Figure 3 shows the microwave power of 540 W, resulting in the fastest increase in temperature. However, in the extraction process using SFME methods, there is an essential factor of the material that affects the extraction process when using a power of 540 W, not necessarily the yield produced was the most exceptional yield. In this study, the microwave power that provided the highest yield was at 380 W.



(a) (b)

Figure 4. Effect of microwave power on basil oil yield at (a) ratio 0.175 g/ml and (b) size ± 0.5 cm

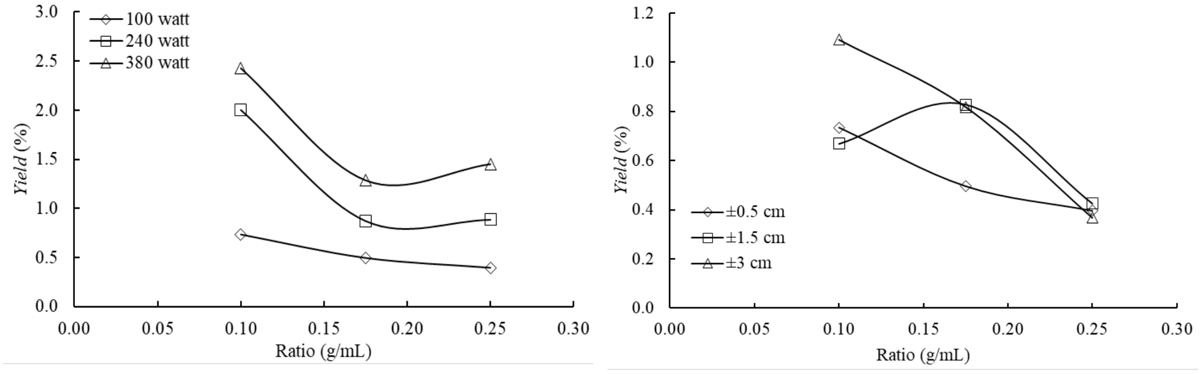
Based on Figure 4 (a), there was a tendency to increase the yield as the power increased. Thus, the amount of energy received by the material converted into heat would generate a higher yield of basil oil. Microwaves can accelerate the extraction process for the desorption of compounds targeted from the matrix with low and high power [12]. However, in the leaf intact (± 3 cm), the oil yield of basil was decreased when the power increased from 380

W to 540 W. This is because the ratio of the material space in the distillation flask to the ratio of material to the size of the whole leaf (± 3 cm) is more than the other flask. According to the previous study, the fuller the materials in the flask, the slower the oil extraction rate is, since the evaporation process of oil obstructs; thus, it decreases the yield of essential oils [13].

In Figure 4 (b), there is a tendency to increase yield as power increases. However, at the smallest ratio of 0.1 g / mL, the highest yield was obtained at 380 W. It was caused when the microwave power was 540 W, by degradation of materials and components of essential oils at the lowest material ratio, resulting in decreased yield. In this study, the highest yield of extracted basil oil from fresh ingredients by the SFME method was obtained when microwave power was 380 W. This microwave power was the most effective in this study.

3.3 Effect of Ratio Raw Material Mass and Distiller Volume (F/D) on Basil Oil Yield

The mass of material used was 100, 175, and 250 g for each variable size. The mass of this material affected the mass ratio of the material per volume distiller. Figure 5 shows the effect of the ratio of raw material mass and distiller volume (F/D) on basil oil yield, which was studied at the lowest microwave power of 100 W. Low microwave power is expected to minimize the significant effect of microwave heat so that the resulting data is more stable.



(a) (b)

Figure 5. The effect of the ratio on the yield basil oil size ±0.5 cm and (b) power 100 W

Based on Figures 5 (a) and (b), the higher the F/D ratio, the lower the yield. The optimum yield for the extraction of basil oil with the SFME method was found in the F/D ratio of 0.1 g/mL, which is caused by the smallest ratio of basil extracted with a medium density. The material density factor is the ratio between the mass of the material and the volume capacity of the distiller flask used. The ratio used relates to how much of the raw material enters the distiller flask. Thus, the oil extraction and evaporation process can proceed perfectly. The material density that is too high and uneven causes the formation of steam lines, "rat holes," which may reduce the yield and quality of essential oils [14]. From a previous study by Kusuma (2017) on patchouli oil extraction using the SFME method with several variations in the ratio of the mass of raw materials to volume of distillers (F/D) 0.06, 0.08, 0.1, and 0.12 g/mL. The highest yield of these variable ratios was obtained at the lowest ratio of 0.06 g/mL.

3.4 Effect of Material Size on Essential Oil Yield

In this study, the size of the materials used is intact (± 3 cm), half intact (± 1.5 cm), and chopped (± 0.5 cm). From Figure 5, we can see the effect of the material size of the basil leaf on the extraction yield using the SFME method. Based on Figure 5, the highest yield is in each power, and the ratio was the size of leaves ± 3cm. Whereas the leaf size ± 1.5cm and ± 0.5 cm yielded lower values, along with the smaller size of the material. This statement is contrary to the previous study. It is mentioned that the smaller material size would result in a higher yield because the small size of the material will accelerate the diffusion process [16]. In this study, the size of the material that produced the optimal yield was the largest. In this study, water content takes the effect of the extraction process. The largest leaf size has the most substantial moisture content. Thus, the extraction process cannot be easily scorched and can be extracted optimally. Meanwhile, the size of the materials with a smaller amount of water content quickly burns, or the ingredients cannot be extracted optimally.

The ratio of 0.1 g/mL resulted in the largest yield when the material size is ± 0.5 cm. This ratio had a difference with other ratios, which are 0.175 and 0.25 g/mL. With the effect of the water content in fresh basil ingredients, the extraction with the smallest ratio of 0.1 g/mL and the smallest material size of ± 0.5 cm resulted in a low yield. The small ratio and size of the material contained the water slightly. Thus, the material was scorched more quickly and burned, and that condition caused the material not to be extracted perfectly. However, in this condition, the extraction was carried out at 380 W. Thus, it was possible that the material had been extracted perfectly and produced a large yield.

3.5 Analysis of Gas Chromatography-Mass Spectrometry (GC-MS) Basil Oil

GC-MS analysis was not only used to find out the components contained in essential oils, but also used to determine the levels of each component. As shown in Table 2 on basil oil from fresh basil leaves, the number of components contained in it was 49 components, with the highest component content being E-Citral, 32.771% and Z-Citral, 27.618%. From these results, it was confirmed that the type of basil used in this study was the citral type, which is in line with research from Mondello et al. (2001) with components of basil essential oil in the form of geranial (E-Citral) 33.70% and mineral (Z-Citral) 27.90%.

Table 2. Components contained in essential oils of basil leaf (ratio of 0.175 g / ml, size ± 3 cm, and power 380 W) based on GC-MS analysis

No.	R.T. (min)	Compound	% Area
		Monoterpenes	
1	5.565	β-Ocimene	0.596
2	5.624	β-Myrcene	0.123
3	8.176	trans-chrysanthemal	0.651
4	10.449	Camphene	0.133
5	10.832	Bicyclo [3.1.0]hexane, 6-isopropylidene-1-methyl-	0.112
6	14.351	Naphtalene, decahydro-, cis-	0.732
		Oxygenated Monoterpenes	
7	7.201	D- (+) -Fenchone	0.153
8	7.453	Linalool L	2.607
9	8.002	cis/cis-Photocitral	0.184
10	9.761	Z-Citral	27.618
11	9.857	Piperiton	0.123
12	10.919	(+) -Carvotanacetone	0.269
13	11.398	D-Fenchyl alcohol	0.332
14	11.503	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate	0.649
15	11.973	E-ocimenone	0.141
		Sesquiterpenes	
16	10.275	E-Citral	32.771
17	11.102	(Z)-β-Farnesene	0.332
18	11.459	Copaene	0.24
19	11.66	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-, [1S-(1α,2β,4β)]-	0.426
20	11.903	α-Gurjunene	0.228
21	12.078	β-Caryophyllene	5.449
22	12.156	1H-Cyclopenta [1,3]cyclopropa [1,2]benzene, 2,3,3aα, 3bα., 4,5,6,7-octahydro-4α -isoprophyl-7β	0.113
23	12.217	α-Bergamotene	2.201
24	12.443	(E)-β-Farnesene	0.497

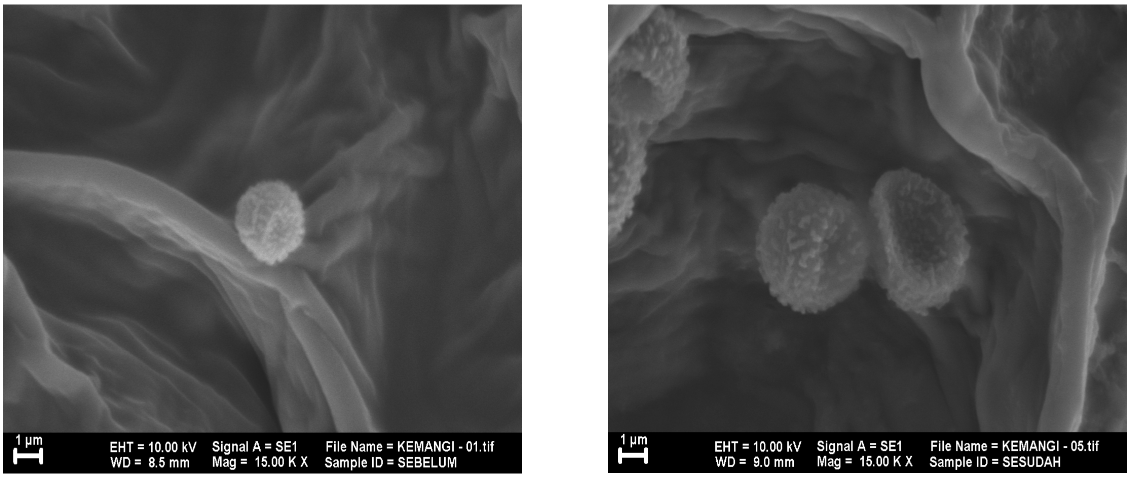
No.	R.T. (min)	Compound	% Area
25	12.487	α-Humulene	1.576
26	12.557	Bicyclo [7.2.0] undec-4-ene, 4,11,11-trimethyl-8-methylene-	0.123
27	12.739	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1α,4aα.,8aα)-	0.124
28	12.826	Germacrene-D	2.66
29	13.096	(+,-)-β-Bisabolene	0.113
30	13.192	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1α,4aβ,8aα)-	0.116
31	13.297	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	0.69
32	13.532	cis-α-Bisabolene	4.471
33	13.671	2-Pentadecen-4-yne, (Z)-	0.233
		Oxygenated Sesquiterpenes	
34	13.68	Caryophyllene oxide	3.079
35	14.673	d-Nerolidol	0.261
		Other Compounds	
36	8.672	Ethenylcyclohexane	2.074
37	19.393	1,3,5-Cycloheptatriene, 2,4-dihexyl-7, 7-dimethyl-	0.383
		OtherOxygenated Compounds	
38	5.546	Methyl heptenone	0.689
39	8.089	Dicyclopropyl Ketone	0.18
40	8.402	(S,E)-3-Methyl-2-methylene-4 -hexenal	1.601
41	8.551	Rosefuran epoxide	0.12
42	8.908	Methyl chavicol	0.21
43	10.728	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	0.17
44	11.242	Neryl acetate	0.712
45	11.538	cis-3-Hexenyl Lactate	0.286
46	16.031	Benzyl benzoate	2.878
47	17.094	Benzyl salicylate	0.268
48	17.999	Geranyl benzoate	0.15
49	19.645	2-Buten-1-one, 1-(2,2,5a-trimethylperhydro-1-benzoxiren-1-yl)	0.154
		Monoterpenes	2.347
		Oxygenated Monoterpenes	32.076
		Sesquiterpenes	52.363
		Oxygenated Sesquiterpenes	3.34
		Other Compounds	2.457

No.	R.T. (min)	Compound	% Area
		Other Oxygenated Compounds	7.418

The components contained in essential oils can be classified into several compounds, namely monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, other compounds, and other oxygenated compounds. The oxygenated compound has more influence on the aroma of essential oils compared to other compounds [18]. In this study, based on the GC-MS test, it was found that the number of oxygenated compounds in this basil oil was 42.834%.

3.6 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a test used to analyze the surface structure of materials. In this study, the material analyzed was basil leaves before and after extraction. Based on Figure 6, there are still intact oil glands (perfect shape) in the cross-section of basil leaves before being extracted. However, the oil gland extraction process was not complete because the oil inside had been taken. In the sample of basil leaves after extracting in Figure 6 (b), the shape of the oil gland was only slightly concave and not damaged. This was due to the ratio of the material used when the extraction process was a large ratio of 0.25 g / mL, so there was a possibility that the leaves, which were used for SEM samples, were not perfectly extracted because of the density factor of the material.



(a) (b)

Figure 6. SEM results of fresh basil leaves with a magnification of 15,000 times (a) before extraction and (b) after extraction

4. Conclusion

In this study, the optimal operating conditions using the SFME method were 380 W microwave power, the ratio between the mass of the raw material and the volume of the distiller (F / D) 0.1 g / mL, the size of whole basil leaves (± 3 cm), and an extraction time of 60 min. Based on GC-MS analysis, there are 49 components in basil oil, and the most significant component is E-Citral. Thus, the basil ingredients used are classified as Citral type. SEM analysis was used to observe changes in the shape of the oil glands found in basil leaves before and after extraction, which shows that the oil has been extracted. The SFME method significantly reduced extraction time and increased extraction yield compared to the MHD method. SFME was 60 min faster than MHD, yielding a 3.41-fold higher result. Thus, the SFME method can be applied to extract basil oil as an effective and efficient method to obtain higher yields.

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Natural Sources Screening for Antimicrobial Agents of Herbs, Spices, and Extracts: A Semi-Qualitative Study

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Abstract. Indonesia has been recognized for its rich natural ingredients such as spices, herbs, and extracts for decades. Furthermore, these components have also been used as an herbal medicine for a long time. Meanwhile, the apparent capability comparison of several components on antimicrobial activity has not been updated yet. This study was conducted to compare the comparison of antimicrobial activity of several materials, such as noni (*Morinda citrifolia*), garlic (*Allium sativum*), celery (*Apium graveolens*), galangal (*Alpinia galangal*), ginger (*Zingiber officinale*), yellow turmeric (*Curcuma longa*), lime (*Citrus aurantifolia*), papaya (*Carica papaya*) leaf, betel (*Piper betel*) leaf, and cutcherry (*Kaempferia galangal*), using agar dilution method. Two types of bacteria are used for the test, namely gram-negative bacteria and gram-positive bacteria, with a total of seven bacteria. The media used were TSA (Trypticase Soy Agar) media for gram-negative bacteria and MRSA (Methicillin-Resistant Staphylococcus Aureus) media for gram-positive bacteria. This study was conducted using a fast-screening method, which referred to a semi-qualitative method. Several components, such as noni, lime, and betel leaf, showed a significant result of antimicrobial activity. Otherwise, other compounds, surprisingly, could not suppress bacterial growth.

Keywords: *herbs, spices, extracts, antimicrobial activity, and agar dilution method*

1. Introduction

The abundance of natural resources in Indonesia has not been mapped accordingly, particularly in terms of antimicrobial agent capability. As a tropical country, Indonesia has several beneficial plants, bushes, shrubs, and even animals, and the derivative components such as herbs, spices, and extracts from leaves, bulbs, rhizomes, and flowers. For decades, researchers were interested in studying natural herbs from Indonesia as famous as “Jamu” [1].

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With the recent advances in technology, the mapping of antimicrobial capability from several resources should be started to valorize the product value. Wherein, after the initial study of the antimicrobial screening process, would be a basis for conducting further research to find suitable substances in nature as antimicrobial agents.

Many researchers have studied antimicrobial activity from many natural resources [2–4]. Antimicrobial activity is the capability of substances to suppress or kill single or a combination of microorganisms, such as fungi, mold, microbes, and algae. The antimicrobial attributes are useful properties that have broad application in many industries, such as textile, food, beverage, medicine, and so forth. Mostly, screening of substances from nature was initially conducted on the macroscopic scale and followed by microscopic identification of the responsible chemical as antimicrobial agents. There is a limitation for continuous study from the macroscopic to the microscopic scale due to the abundance of chemicals in an originally natural source. Mostly, the screening of natural herbs is presented by Minimum Inhibitory Concentration (MIC) [5]. As a rapid detection, MIC would be beneficial for comparing the strength of several prospective antibacterial agents. Usually, the comparison is made with the antibiotic concentration [5].

This study mainly discusses the screening process of several ingredients, such as noni (*Morinda citrifolia*), garlic (*Allium sativum*), celery (*Apium graveolens*), galangal (*Alpinia galangal*), ginger (*Zingiber officinale*), yellow turmeric (*Curcuma longa*), lime (*Citrus aurantifolia*), papaya (*Carica papaya*) leaf, betel (*Piper betel*) leaf, and cutcherry (*Kaempferia galangal*), for prospective antimicrobial agents. The selection of ingredients is mostly caused by the medicinal story behind the ingredients. Their availability is also a factor to be considered because of mass production, as the ingredients are capable of being antimicrobial agents. The study also used a positive control using antibiotics and a negative control without any addition to give a comparison of the antimicrobial agent capability. So, the results were analyzed semi-qualitatively with a comparison with the positive control. This study was conducted due to the limited data available in Indonesia, mostly the nature components screening process conducted in another country.

2. Materials and Methods

2.1 Materials

Materials consisted of three types of substances, namely antimicrobial agents, bacterial colonies, and growth medium. Antimicrobial agents consisted of antibiotics as the positive control, and natural ingredients for the screening process of antimicrobial activity were studied, as shown in Table 1. Meanwhile, the bacteria used in this study can be seen in Table 2. The growth medium, such as TSA (Trypticase Soy Agar) and MRSA (Methicillin-Resistant Staphylococcus Aureus), was obtained from Nugen Bioscience Indonesia laboratory. All works were conducted in Nugen Bioscience Indonesia Laboratory, Jakarta.

Table 1. Natural ingredients as a prospective antimicrobial agent

Plant species	Description	Origin
Morinda citrifolia	Juice	Jakarta, Indonesia
Allium sativum	Bulb	Traditional market, Jakarta, Indonesia
Apium graveolens	Leaves	Traditional market, Jakarta, Indonesia
Alpinia galangal	Rhizome	Traditional market, Jakarta, Indonesia
Zingiber officinale	Rhizome	Traditional market, Jakarta, Indonesia
Curcuma longa	Rhizome	Traditional market, Jakarta, Indonesia
Citrus aurantifolia	Juice	Traditional market, Jakarta, Indonesia
Carica papaya	Leaves	Traditional market, Jakarta, Indonesia
Piper betle	Leaves	Traditional market, Jakarta, Indonesia
Kaempferia galangal	Rhizome	Jakarta, Indonesia
Doxysol (2 g/L)	Antibiotic	Sumber Hidup Satwa -International
Comoxy 500 (2 g/L)	Antibiotic	Sumber Hidup Satwa-International
Amcol (2 g/L)	Antibiotic	Sumber Hidup Satwa-International

2.2 Natural ingredients extraction

Besides *Morinda citrifolia* and *Citrus aurantifolia*, which were only heated to sterilization, the extraction of natural ingredients was conducted with a Soxhlet extractor using distilled water as the solvent. The extraction was conducted at 70 °C with a Feed-Solvent Ratio (1:2) for 6 hours. The solution of the extraction would be used as the solution sample in the antimicrobial screening test.

2.3 Antimicrobial screening agent

The method was the agar dilution method using Trypticase Soy Agar (TSA) growth media for gram-negative bacteria and Methicillin-Resistant Staphylococcus Aureus (MRSA) growth media for gram-positive bacteria, which was adopted from Baker et al. (1991). The selection of bacteria shown in Table 2 was the occurrence in the hatchery. The study carried out the screening process of several ingredients from nature. The screening process was conducted in two steps. The first step was to evaluate the inhibitory activity of each ingredient.

The last step was a deepened comparison of the promising antimicrobial agent. Positive control with the addition of antibiotics, namely Doxysol, Comoxy, and Amcol, was also conducted in this study. Meanwhile, a negative control was conducted as a bacterial growth basis comparison with another treatment. Bacterial growth score was used on a scale of 0 – 4. This scale was used to determine the sufficient growth of bacteria despite the inhibition zone diameter. Thus, this study was referred to as a semi-qualitative study despite being a qualitative study.

Table 2. Bacteria used in this study

Bacteria species	Code	Source
Salmonella spp.	4054-36P306*	Chicken yolk
	3912-36P297*	Chicken air sac
Escherichia coli	4014-36P304*	Chicken bone Chicken heart
	3958-36P301*	Chicken liver
	4098-36P308*	
Pseudomonas aeruginosa	4032-36P306*	Chicken lung
	3909-36P296*	Chicken navel
Lactobacillus plantarum	N1A1	Microbiology Department
Lactobacillus rhamonsus	MD4^{b}	Microbiology Department
Enterococcus faecium	-	Microbiology Department
Pediococcus pentosaceus	-	Microbiology Department

3. Results and Discussions

The first screening process resulted that only noni, lime, and betel leaf were adequate to suppress the bacteria growth, as seen in Table 3. It is shown by less amount of number in Table 3 with a green column. Other substances are not strong enough to suppress bacterial growth, as shown with numbers 3 and 4 in the screening test. As hypothesized, the negative control had a prolific bacterial colony. *Allium sativum*, *Apium graveolens*, and *Kaempferia galangal* had a higher growth score than the negative control. The occurrence of total sugar and carbohydrates in those natural substances was believed to support bacterial growth. *Allium sativum*, *Apium graveolens*, and *Kaempferia galangal* have total sugar content as much as 14.8 mg/g [7], 338.8 mg/g [8], and 19.0 mg/g [9], respectively.

Meanwhile, positive control is mostly very effective as expected, but Comoxy and Amcol seem to lose against *Salmonella spp.*, which was isolated from the chicken yolk. It is believed to be caused by bacterial resistance against the antibiotic, which has become a recent issue in the hatchery.

The successive components, as antimicrobial agents at the first screening, were subsequently evaluated to find the optimum condition. As seen in Table 4, lime was seemly the most potent antimicrobial agent compared to others with a lower concentration, which could be achieved to suppress microbial growth at 10%. This condition would be the optimum condition for this study. This concentration could be optimized further to find a concentration economically in the application. Meanwhile, noni was sufficient to be used at 50% concentration with the growth media, and piper betel was effective at 40% concentration with the growth media.

The mapping of this study could be seen in Table 5. Another study was also presented to give additional information on tropical natural resources, which could be used as an antimicrobial agent. Microscopic-scale research should be conducted in the future to give a perspective on responsible substances in lime. Meanwhile, another abundance of natural sources in Indonesia should be conceivably evaluated macroscopically to give a large map of Indonesia's natural source potential as microbial agents.

The initial study is strongly believed to be very useful for further implementation and application in both industry and society. The dense map of Indonesia's natural resources could enhance the possibility of antimicrobial products. Otherwise, natural sources that do not have potential could not be studied further to minimize the research sustainability. The application of each natural resource should be matched with the nature of its.

Further study needs to be conducted to find the chemical substances that act as an antimicrobial agent through the microscopic study. Meanwhile, the optimum condition was obtained with the addition of 1:10 lime to the growth media. This research is beneficial in a broad application, such as food ingredients, natural medicine for both human beings and animal husbandry, and antimicrobial applications in natural dyes.

Table 3. Bacterial growth in every treatment

Treatment	**Extract in Growth Media**	***[a]Salmonella spp.***		***[a]Escherichia coli***			***[a]Pseudomonas aeruginosa***		***Lactobacillus Plantarum***	***Lactobacillus rhamonsus***	***Enterococcus faecium***	***Pediococcus pentosaceus***
		(-)		(-)			(-)		(+)	(+)	(+)	(+)
		TSA							MRSA			
NC	-	3	3	3	3	3	3	3	3	3	3	3
NO	50%	0	0	0	0	0	2	2	0	0	0	0
GL	50%	4	3	3	3	3	3	3	2	1	0	0
CE	50%	4	3	3	3	3	4	4	3	3	3	3
GA	50%	2	1	3	3	3	3	3	0	2	2	2
GI	50%	3	3	3	3	3	3	3	3	3	3	3
YT	50%	3	3	3	3	3	3	3	3	3	3	3
LI	50%	0	0	0	0	0	0	0	0	0	0	0
PL	50%	3	3	3	3	3	3	3	3	3	3	3
BL	50%	0	0	0	0	0	0	0	0	1	0	1
CU	50%	4	3	3	3	3	4	4	2	2	2	2
DO	50%	0	0	0	0	0	0	0	0	0.5	0	0
CO	50%	3	0	0	0	0	0	0	0	0.5	0	0
AM	50%	3	0	0	0	0	0	0	0	0.5	0	0

4	extensive growth
3	good growth
2	moderate growth
1	weak growth
0	no growth

[a] bacterial code followed Table 2; **NC**: Negative Control; **NO**: Noni (Morinda citrifolia); **GL**: garlic (Allium sativum); **CE**: celery (Apium graveolens); **GA**: galangal (Alpinia galangal); **GI**: ginger (Zingiber officinale); **YT**: yellow turmeric (Curcuma longa); **LI**: lime (Citrus aurantifolia); **PA**: papaya (Carica papaya) leaf; **BL**: betel (Piper betle) leaf, **CU**: cutcherry (Kaempferia galangal); **DO**: Doxysol; **CO**: Comoxy; and **AM**: Amcol.

Table 4. Bacterial growth for prospectus antimicrobial agent comparison and optimization

Treatment	**Extract in Growth Media**	[a]***Salmonella spp.***		[a]***Escherichia coli***			[a]***Pseudomonas aeruginosa***		***Lactobacillus Plantarum***	***Lactobacillus rhamonsus***	***Enterococcus faecium***	***Pediococcus pentosaceus***
		(-)		(-)			(-)		(+)	(+)	(+)	(+)
		TSA							MRSA			
NO	50%	1	0	0	0	0	0	0	1	1	1	1
	40%	1	1	0	0	0	1	1	1	1	1	1
	30%	2	1	2	2	2	4	4	2	2	2	2
	20%	3	2	3	3	3	4	4	3	3	3	3
	10%	3	2	3	3	3	4	4	4	4	4	4
LI	50%	0	0	0	0	0	0	0	0	0	0	0
	40%	0	0	0	0	0	0	0	0	0	0	0
	30%	0	0	0	0	0	0	0	0	0	0	0
	20%	0	0	0	0	0	0	0	0	1	0	0
	10%	0	0	0	0	0	0	0	2	2	1	0
BL	50%	0	0	0	0	0	0	0	2	2	2	2
	40%	0	0	0	0	0	0	0	3	3	3	3
	30%	2	2	3	3	2	2	2	3	3	3	3
	20%	3	3	3	3	3	3	3	4	4	4	4
	10%	4	4	4	4	4	4	4	4	4	4	4

4	extensive growth
3	good growth
2	moderate growth
1	weak growth
0	no growth

[a] bacterial code followed Table 2; **NO**: Noni (Morinda citrifolia); **LI**: lime (Citrus aurantifolia); and **BL**: betel (Piper betle) leaf.

Table 5. Recent work and references for tropical antimicrobial agents

Common name	**Scientific name**	**Compound**	**Class**	**Activity**	**Relative toxicity** [a]	**Bacteria growth** [b]	**Reference(s)** [c]
Aloe	*Aloe vera*	Latex	Complex mixture	Corynebacterium, Salmonella, Streptococcus, S. aureus	2.7		[10]
Betel pepper	*Piper betel*	Catechols, eugenol	Essential oils	General	1.0	0.0 & 0.5	[3], TS
Chili, peppers, paprika	*Capsicum annuum*	Capsaicin	Terpenoid	Bacteria	2.0		[11–13]
Clove	*Syzygium aromaticum*		Terpenoid	General	1.7		[3]
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide, Sulfated terpenoids	General	-		[14–17],TS
Ginseng	*Panax notoginseng*		Saponins	E. coli, Sporothrix schenckii, Staphylococcus, Trichophyton	2.7	3.1 & 0.8	[3]
Mountain tobacco	*Arnica montana*	Helanins	Lactones	General	2		[3]
Papaya	*Carica papaya*	Latex	Mix of terpenoids, organic acids, alkaloids	General	3		[18–20], TS
Noni	*Morinda citrifolia*	-	-	General	-	3.0 & 3.0	TS
celery	*Apium graveolens*	-	-	Bacteria	-		TS
galangal	*Alpinia galangal*	-	-	Bacteria	-	0.6 & 0.0	TS
ginger	*Zingiber officinale*	-	-	Bacteria	-	3.4 & 3.0	TS
yellow turmeric	*Curcuma longa*	-	-	Bacteria	-	2.6 & 1.5	TS
lime	*Citrus aurantifolia*	-	-	Bacteria	-	3.0 & 3.0	TS
Cutcherry	*Kaempferia galangal*	-	-	Bacteria	-	3.0 & 3.0	TS

[a]Data from reference Duke (2002); [b]gram-negative & gram-positive (scale 0 – 4); [c]Table is based on data compiled from references [21–22]; TS: This Study

4. Conclusion

The screening process of several ingredients as an antimicrobial agent has been successfully conducted. Most of the ingredients did not give a glance at the antimicrobial activity. Only noni, lime, and betel extract showed inhibitory properties against bacteria, both gram-negative and positive; in other words, these three materials could be antimicrobial agents derived from nature. Lime, betel leaves extract, and noni are the strongest components with the order of higher levels, respectively.

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Biobutanol Production Using FED-Batch High Cell Density Extractive Fermentation

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Abstract. Butanol, as a product with specific toxicity for its producer, is necessary to be maintained in low concentration during fermentation. In-situ integrated recovery using extensive volume extraction was used in high cell density fermentation to prevent the lag phase in the prime condition. *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 was employed to ferment glucose in extractive fermentation with oleyl alcohol as extractant. As the results of fed-batch cultures using high cell density with different extractant to broth volume ratios (Ve/Vb), 0.8 g/l butanol concentration in the broth was maintained with a ratio of 10, which was much lower than 4.4 g/l with the ratio of 0.5. Besides, the Ve/Vb ratio of 10 demonstrated 2.7-fold higher total butanol concentration (28 g/l) than that 11 g/l obtained with a Ve/Vb ratio of 0.5. These results indicated that larger Ve/Vb improved total butanol concentration by reducing butanol toxicity in broth.

Keywords: *butanol fermentation, extraction, oleyl alcohol, tributyrin, high cell density*

1. Introduction

Butanol had the desirable properties as an alternative for premixed combustion engine fuel. It fulfilled the requirement for the spark timing and octane number as well as minimized particles emission and low carbon monoxide [1–3]. As an intermediate chemical and solvent, butanol had been used for the commercial production of various materials [4–6]. Today, butanol is majorly produced from petrochemicals [7], biobutanol is also producible via acetone-butanol-ethanol (ABE) fermentation. The global butanol demand as a chemical feedstock has been growing increasingly [8].

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Integrated separation and fermentation enhanced butanol production by maintaining low concentration of butanol in the media [9]. Liquid-liquid extraction provided remarkable advantages for butanol fermentation, compared with other separation methods. The equipment for extraction unit is simple and easy to be installed, the recovery of the extractant is easy, butanol is extracted selectively and requires low consumption of energy [9–13]. To select the suitable extractant, butanol distribution coefficient, biocompatibility with butanol producer, and the ease of separation from butanol and the fermentation broth were consider [13].

The total butanol concentration may be increased if the amount of carbon source is increased. However, excessively high substrate concentration would inhibit the metabolism of *Clostridia* [9]. It is shown, by the long lag phase, before the strain can produce organic acid and solvent. As in the batch mode, fermentation substrate concentration would decrease after being consumed by the cells; it is difficult to obtain higher butanol production since the substrate is limited. Glucose concentration above 50 g/l inhibited the substrate consumption [14].

During fed-batch culture, medium or substrate is fed temporarily or continuously into the reactor, but the product is only harvested on the final of the fermentation. Feeding solution is fed up with the maximum growth of the cell or more simply, until the maximum capacity of the reactor. To maximize the cell formation rate in a constant cell mass yield, the substrate concentration should be maintained at the value that maximizes the specific growth rate [15].

The objective of this study was to develop extractive acetone-butanol-ethanol fermentation with high cell density to enhance substrate consumption in fed-batch fermentation. The work to observe large Ve/Vb with high cell density has not been reported. To confirm what problem prevented the total butanol from increasing, the kinetics of fermentation was studied by taking a sample in several points. Then, to increase the amount of carbon source but maintaining the substrate concentration at the normal level, fed-batch fermentation was studied.

2. Materials and Methods

2.1 Materials

C. saccharoperbutylacetonicum N1-4 ATCC 13564 was stored in the form of sand stock. Five spoons of this sand stock were inoculated in 9 ml of PG (Potato Glucose) medium in the test tube containing (g/l) grated fresh potato 150, glucose 10, $(NH_4)_2SO_4$ 0.5, and $CaCO_3$ 3. *C. saccharoperbutylacetonicum* N1-4 were heat-shocked in 100°C water for 1 minute then incubated at 30°C for 24 h. All suspension was incubated in the anaerobic environment using anaeropack. This suspension was kept at 4°C as the working stock.

2.2 Inoculation

One ml of the spore suspension is refreshed in 9 ml PG medium (10% inoculation) in the test tube. *C. saccharoperbutylacetonicum* N1-4 were heat shocked in 100°C water for 1 minutes then incubated at 30°C for 24 h. All suspension were incubated in the anaerobic environment using anaeropack.

This refreshed culture was then inoculated in the preculture using 90 ml of TYA (Tryptone – Yeast – Acetate) (10% inoculation) containing glucose 20 g/l in 200 ml flask. The medium was sparged with nitrogen gas for 10 minutes, then incubated for 15 h.

The first preculture was then continued with the second preculture to obtain high cell mass. Second preculture used 1000 ml of TYA and incubated in the optimum temperature for 15 h. Cells from the second preculture was concentrated using centrifugation with 6000 rpm for 25 minutes.

2.3 Fermentation Kinetics of High Cell Density Batch Extractive Fermentation

The concentrated cell was then inoculated in the main culture with 10% inoculation volume to set the initial cell density to 10 g/l dry cell weight. High cell density batch fermentation was conducted using TYA containing 50 g/l glucose with 40 ml working volume in serum bottle. Oleyl alcohol was used as extractant with Ve/Vb was 0.1 (4 ml), 0.5 (20 ml), 1 (40 ml), and 10 (400 ml). Main fermentation was conducted until 96 h and was sampled for the initial several sampling time for composition in the broth and extractant phase.

2.4 Fed – Batch Fermentation with High Cell Density Extractive Fermentation

The concentrated cell was then inoculated in the main culture with 10% inoculation volume to set the initial cell density to 10 g/l dry cell weight. High cell density fed batch fermentation was conducted using TYA containing 50 g/l glucose with 60 ml working volume in jar fermentor. Oleyl alcohol was used as extractant with Ve/Vb was 0.5 (30 ml) and 10 (600 ml). The mixture was agitated at 120 – 130 rpm. Main fermentation was conducted until 96 h and was sampled for several sampling time composition in the broth and extractant phase. When the glucose concentration in the broth was less than 20 g/l, 240 g/l glucose solution was fed into the fermentor.

2.5 Analysis

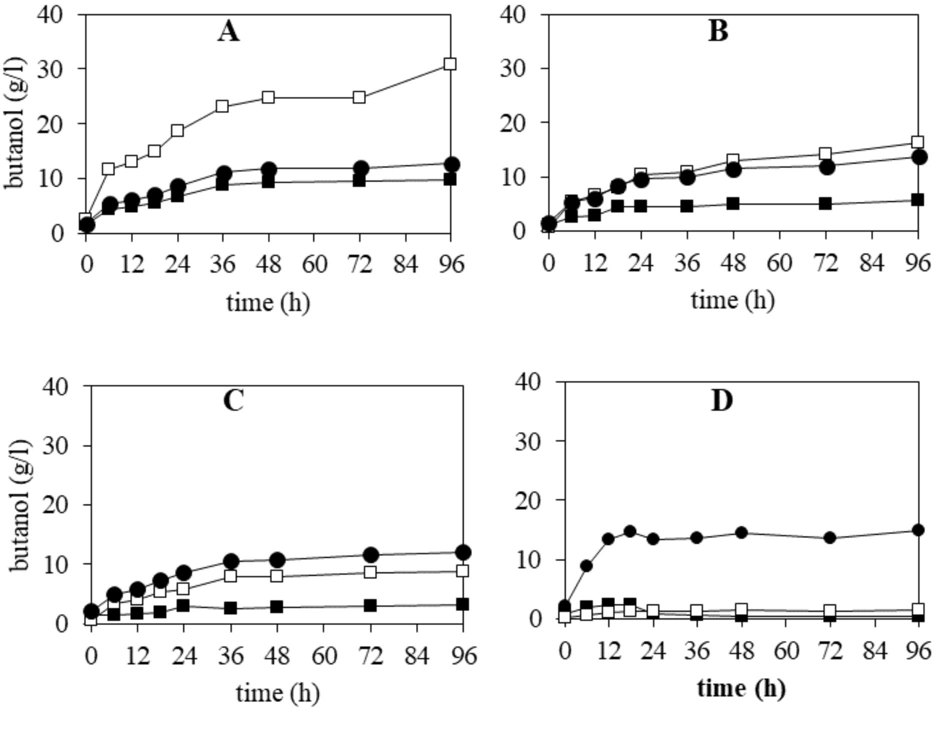
Glucose concentration as substrate in the aqueous phase was measured using High Pressure Liquid Chromatography with SH1011 column and a refractive index detector. Sulfuric acid, 0.05 M, was used as the mobile phase at flow rate 1.0 mL/min. The HPLC analysis was performed at a column temperature of 50 °C.

Solvent concentration was analyzed using Flame Ignition Detector Gas Chromatography. Acetone, ethanol, butanol, acetate, and butyrate compound can be measured. To measure concentration in aqueous phase, water was used as the solvent for the samples. To measure concentration in extractant phase, methanol was used as the solvent for the samples. Helium was used as the mobile phase and the analysis was performed at a column temperature of 50 – 170 °C.

3. Results and Discussions

3.1 Fermentation Kinetics of High Cell Density Extractive Fermentation

C. saccharoperbutylacetonicum N1-4 strain was further studied due to its operability near ambient temperature [15]. This strain was cultured in high cell density to increase the biomass population from the initial condition [16]. A large extractant to the broth volume ratio was used to study its effect on cell growth and biobutanol fermentation.



■ broth; □ extractant; ● total butanol concentration

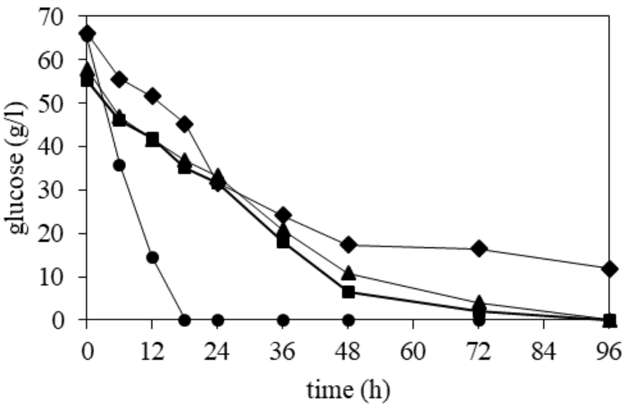
Figure 1. Time course of butanol production by *C. Saccharoperbutylacetonicum* N1-4 with high cell density in Ve/Vb ratio (A) 0.1; (B) 0.5; (C) 1.0; (D) 10.0

Figure 1 shows the fermentation kinetics of the batch extractive fermentation with high cell density. By using the high cell density fermentation, productivity was high in the early time, between 0 – 24 hours (as shown by the steeper gradient). Compared with previous studies, batch extractive fermentation was conducted using biodiesel [15], oleyl alcohol, and dodecanol [17]; they resulted in a maximum dry cell weight of 1.9 g/l, 2.1 g/l and 1.43 g/l respectively. In this study, the cell density was set at 10 g/l at the initial time and kept stable during fermentation, which provided faster substrate consumption and butanol production. However, the final total butanol produced was not significantly different by using various extractant/broth ratio. Especially for the Ve/Vb value of 10, the butanol concentration was kept stable from the 18 h up to 96 h.

Table 1. Fermentation performance of *C. saccharoperbutylacetonicum* N1-4 with high cell density and various Ve/Vb ratio

Ve/Vb	total butanol concentration (g/l)	yield (C-mol/C-mol)		productivity (g/l/h)
		butanol	solvent	max at 0-6 h
0.1	12.7	0.442	0.598	0.621
0.5	13.7	0.742	0.992	0.643
1.0	12.0	0.699	0.921	0.471
10.0	14.8	0.459	0.560	1.138

In Table 1, the highest broth-based total butanol production was obtained using a Ve/Vb ratio of 10 of 14.8 g/l. Although it did not affect the yield, the productivity of butanol using high cell density fermentation was high, and the highest one was obtained by Ve/Vb ratio of 10 of 1.138 g/l/h. It was almost twice of butanol productivity using other ratios. Extraction with a high volume of extractant was sufficient to enhance the productivity of the strain by maintaining low toxicity of butanol in the media [9].



using Ve/Vb ♦ 0.1; ■ 0.5; ▲ 1.0; ● 10.0

Figure 2. Glucose consumption along high cell density batch extractive fermentation using Ve/Vb

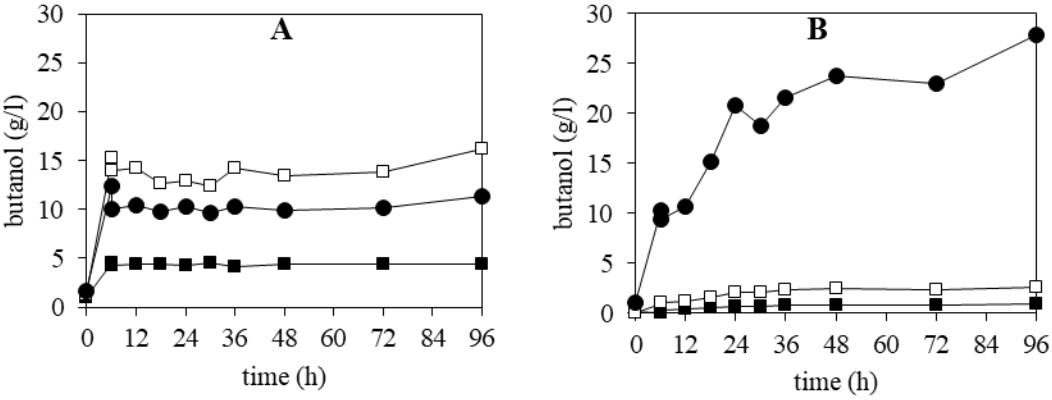
The cause of the unenhanced butanol production was related to Figure 2. This figure shows that by using the Ve/Vb ratio of 0.1, the glucose substrate was not consumed totally and remained at the end of fermentation. The toxic concentration 15 g/l has been approached and caused the strain to enter the death phase [18]. By using the ratio of 0.5 and 1.0, the glucose consumption was similar and consumed after 96 h. By using the Ve/Vb ratio of 10.0, the glucose was consumed totally, and no glucose remained after 18 h. High cell density was a well-known method to improve the glucose consumption [16, 19], but in this study, large Ve/Vb successfully fasten the glucose consumption time from 96 h by using Ve/Vb of 1.0 to only 18 h by Ve/Vb of 10.

The shortage of glucose indicated that butanol production could be increased if only the substrate is more fed to the broth. Higher Ve/Vb ratio is essential to optimize the yield of butanol based on the volume of broth. To produce the same amount of butanol, a more significant amount of recyclable extractant is cost saving rather than using the more expendable nutrient medium.

3.2 Fed – Batch Fermentation with High Cell Density Extractive Fermentation

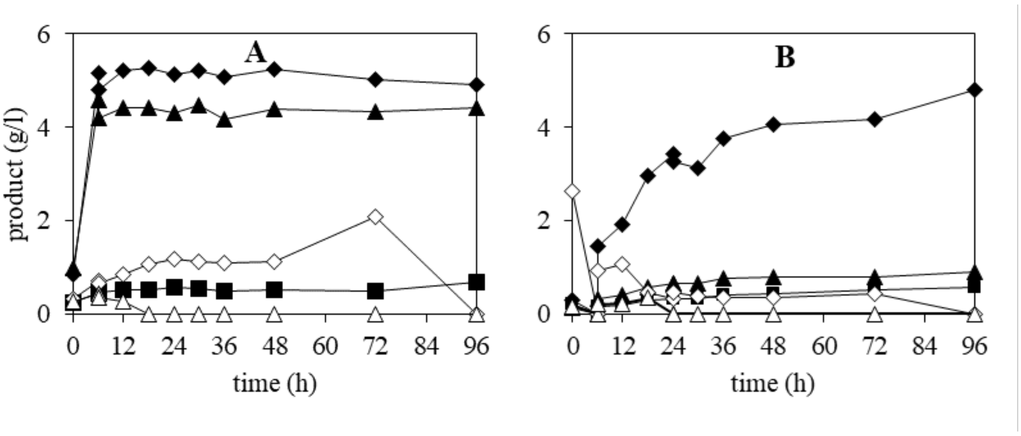
The result of the previous experiment, which showed rapid consumption of the glucose, was attempted to be solved by conducting fed-batch fermentation. Two values of Ve/Vb of 0.5 and 10 were selected to investigate its effect. Glucose was fed twice at 6 h and 24 h.

Figure 3 shows that broth-based total butanol concentration using Ve/Vb of 10 was around twice fold of 27.85 g/l compared with Ve/Vb 0.5 of 11.40 g/l. Compared with the similar extractive fed-batch fermentation with the Ve/Vb of 1.0 using oleyl alcohol as extractant, the total broth-based butanol production 19.5 g/l [20] and using Ve/Vb of 4; the total butanol production was 12.3 g/l [21]. The butanol concentration in the broth was still low at 0.9 g/l, which was still far below the inhibition limit. As the concentration in the broth was low, so was the butanol concentration in extractant, only 2.7 g/l. As expected from ABE fermentation with high cell density, beside the substrate, nutrient was also consumed rapidly and was necessary to be fed in order to keep the strain activity to produce butanol. A high cell density method was commonly implemented for continuous fermentation [16, 19, 22]. Figure 4 shows that the product concentration in the broth using Ve/Vb of 0.5 was still high, around 5 g/l for butanol and acetone, which may lead to the reducing effect on the strain activity. On the other hand, using the Ve/Vb ratio of 10.0, the product concentration in the broth was lower than 1 g/l. Unexpectedly, the acetone was accumulated in the broth in around 5 g/l. Acetone was also observed to cause toxicity effect to the *Clostridial* strain [23]. Further extraction of acetone during fermentation using extractant with a high distribution coefficient for acetone such as tributyrin was sufficient to enhance butanol production [9, 21].



■ broth; □ extractant; ● broth based total production

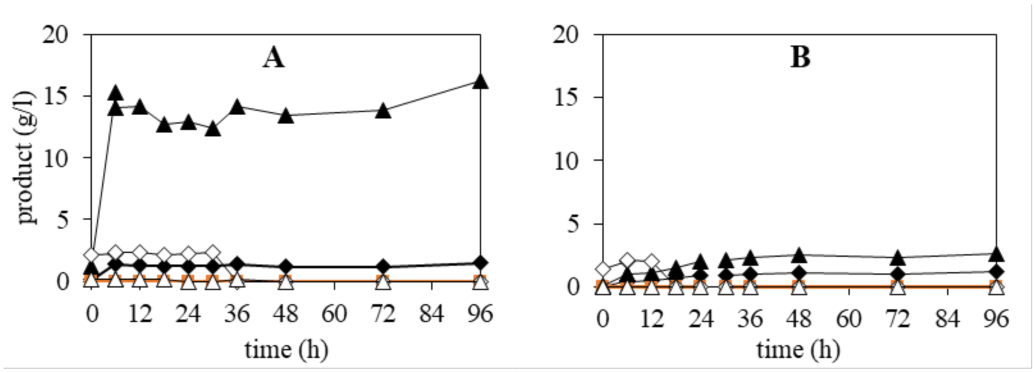
Figure 3. Butanol production using Ve/Vb ratio A) 0.5 and B) 10.0 in fed batch fermentation using high cell density



♦ acetone; ■ ethanol; ▲ butanol; ◊ acetate; Δ butyrate

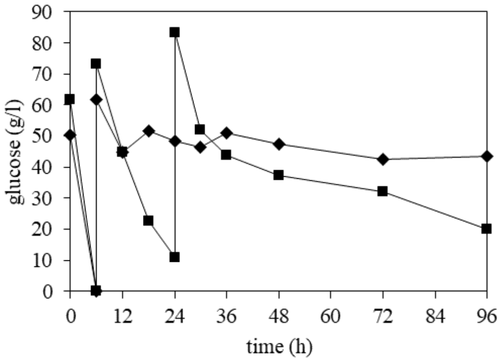
Figure 4. Product concentration in broth using Ve/Vb ratio of A) 0.5 and B) 10.0

Product concentration in the extractant was shown in Figure 5, both for the Ve/Vb ratio of 0.5 and 10.0. The concentration of the product was still low. As the amount of product was similar, using a smaller Ve/Vb ratio, butanol concentration in the extractant was higher. However, using a larger Ve/Vb ratio, the extraction capacity is expected to be more optimized.



♦ acetone; ■ ethanol; ▲ butanol; ◊ acetate; Δ butyrate

Figure 5. Product concentration in extractant using Ve/Vb ratio of A) 0.5 and B) 10.0



♦ 0.5; ■ 10

Figure 6. Glucose concentration in broth with high cell density fermentation in Ve/Vb

Even though in Figure 4B, the butanol concentration was still deficient in the broth and extractant, sugar consumption (Figure 6) and butanol production could not increase. Figure 6 showed that initial glucose was consumed very quickly in the first 6 hours. After glucose feeding at 6 h, glucose consumption was stopped at 12 h using Ve/Vb of 0.5 and was continued up to 96 h using Ve/Vb of 10. Second, glucose feeding was added only to Ve/Vb of 10. The consumed glucose was 4.22 g and 12.18 g, respectively, for Ve/Vb of 0.5 and 10 (Table 2). Applying fed-batch fermentation, large Ve/Vb effectively enhanced the glucose consumption compared to the batch fermentation. The yield of butanol and solvent was increased by 34% from 0.309 to 0.419 C-mol/C-mol and from 0.421 to 0.614 C-mol/C-mol, respectively.

Table 2. Fermentation result

Ve/Vb	total butanol concentration (g/l)	consumed glucose (g)	yield (C-mol/C-mol)	
			butanol	solvent
0.5	11.40	4.22	0.309	0.431
10.0	27.85	12.18	0.419	0.614

For the cost-saving purification process, it is necessary to obtain the butanol concentration in the extractant as high as possible. Probably, the Ve/Vb ratio of 10 was too large for the fed-batch process. The Ve/Vb ratio needs to be optimized more precisely. High acetone concentration, which was not selectively extracted, may also affect the butanol production.

4. Conclusion

ABE extractive fermentation using high cell density in large extractant volume has been successfully developed. Larger Ve/Vb improved total butanol concentration and provide higher butanol yield by reducing butanol toxicity. Using high initial cell density, lag phase during early fermentation was avoided and the higher glucose concentration was consumed. These benefits are potential for butanol production with high productivity. This study requires further investigation to improve the feasibility for massive production.

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RESEARCH ARTICLE

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Quantitavie Structure-Activity Relationship Study of Ester-Based Ferulic Acid Derivatives Againts Cervical Cancer Cell (HELA)

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Abstract. Quantitative structure-activity relationship (QSAR) has been studied for ferulic acid derivatives to determine the QSAR model able to predict anticancer. As the subject of this research was a set of experimentally calculated IC_{50} value data of 6 ferulic acid derivatives against cervical cancer cells (HELA). QSAR analysis was based on multilinear regression calculation on fitting subset using log $(1/IC_{50})$ as the dependent variable, and dipole moment, partition coefficient in the n-octanol/water, and atomic net charges of the aromatic carbons as independent variables. The values of the descriptors were obtained from semiempirical PM3 quantum mechanics calculation. The relationship between log $(1/IC_{50})$ and the descriptors was described by the result in the QSAR model. The QSAR model for ferulic acid derivatives against HELA cell lines was developed with the statistical parameters of $R=0.998$; $R^2=0.999$; $SE=0.00857$; and $F=394$. The calculated log $(1/IC_{50})$ using QSAR Hansch Model for ferulic acid derivatives have excellent agreement with experimental data of Log $(1/IC_{50})$.

Keywords: *QSAR study, ferulic acid derivatives, descriptors*

1. Introduction

Indonesia is the world's fourth-largest coffee producer, in which the production reached up to 668.70 thousand tons in 2017 [1]. The processing of coffee cherry into coffee beans generates by-products such as pulp coffee, which is the primary residue of this process (40%-45%). Recently, several studies focused on the composition of pulp coffee and their secondary application. The main constituents of coffee pulp are fiber with 80% composition, followed by lignin 12%, crude protein 9.9%, ash 1.5%, and moisture 7.6% [2]. Besides, highly valuable

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*Coressponding author : heldawikaamini@unej.ac.id bioactive compounds are existing in the coffee pulp such as caffeic acid, ferulic acid, and chlorogenic acid [2–3].

Phenolic acids have been reported widely of the activity as antioxidants [4–6]. Other application of phenolic acids includes anticancer [7], antiviral [8], antitumor [9], antidiabetic [10], antihypertensive [11]. Hydroxycinnamic acid-like ferulic acid, caffeic acid, and chlorogenic acid are natural compounds in coffee pulp [12]. Approximately 10% weight of the dry coffee pulp contain these bioactive compound [2]. These available contents are promising for the reuse of coffee pulp for various purposes.

Ferulic acid has shown to regulate the key enzymes which were responsible for free radical-induced cell damage [13]. Ferulic acid is biosynthesized from amino acid phenylalanine through a shikimic acid pathway. Ferulic acid has an anticancer activity that possesses free carboxylic acid with gastric irritation as an inevitable adverse effect when consumed orally. Thus, ester derivatives of ferulic acid are favorable in used as drugs, as reported by Serafim et. al (2011) caffeic and ferulic acid ester derivatives exerted cytotoxicity to the breast cancer cells compared with the parent compound as well as the study for HELA cells [13]. Modification of the carboxylic group with a different alkyl group of ferulic acid including methyl, ethyl, n-propyl, n-butyl, bromo ethyl, and chloroethyl affected the anticancer activity [13, 15].

The value of an anticancer activity is an important parameter in drug design. Anticancer activity might measure both experimental and theoretical. The properties of the predicted derivative compounds can be predicted using the QSAR technique using the built molecular descriptors. This consists of the similarities between molecules in a large database of existing molecules with known properties [16]. This technique has been used in various procedures for various applications such as anticancer [16–17], antibacterial [18], antifungal [19], and hormone controlling [20]. The result of the QSAR prediction model shows a high correlation with the experimental data [16, 18].

Cervical cancer is the second most happening cancer in women in the world [21]. HELA is one type of cervical cancer cell that has been widely studied for the elimination using natural anticancer agents including flavonoids and polyphenols [22]. As far as our knowledge, the QSAR study has not been reported specifically for the active compound in HELA cells treatment. This study is expected to build the model to design the compound with optimized anticancer activity using QSAR.

In this research, we study about QSAR of six ferulic acid derivatives to establish the relationship between structural characteristics of the ferulic acid derivatives molecules and their activities using the semi-empirical method of PM3 (Parametric Method 3).

2. Methods

2.1 Studied Compounds

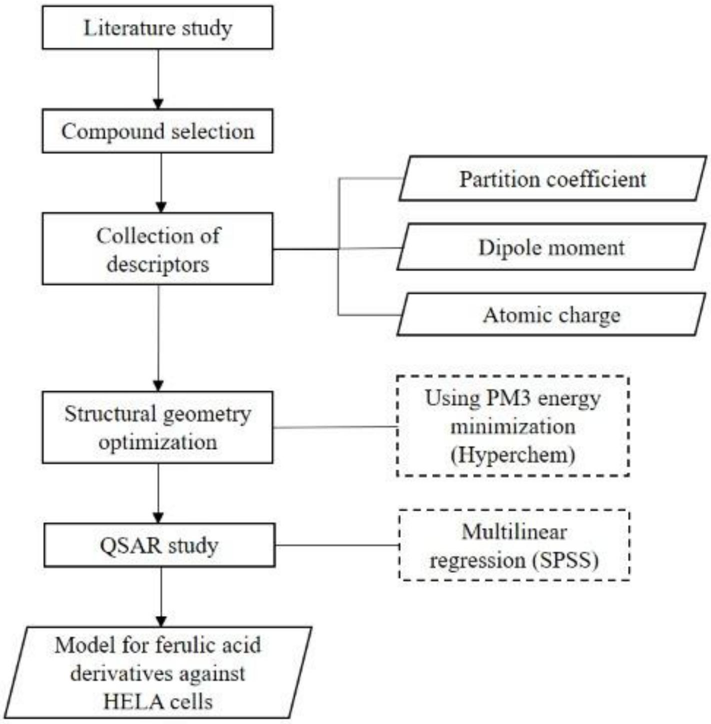


Figure 1. Experimental methods

The methods are summarized in Figure 1. In this study, we calculated properties of 6 known ferulic acid derivatives compounds, as shown in Table 1. The structure of ferulic acid derivatives including the ones with methyl, ethyl, n-propyl, n-butyl, bromo-ethyl, and chloro ethyl, is shown in Figure 2. These compounds were observed for their activities against HELA cancer lines as being studied by Kiran et al (2015).

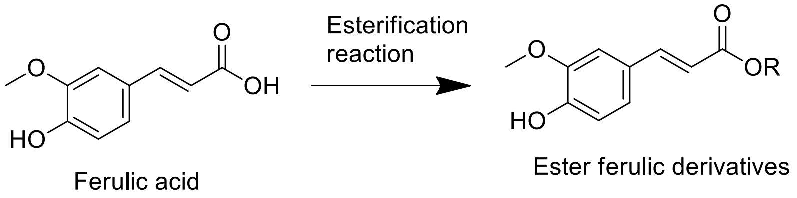
Table 1. The experimental anticancer activity of ferulic acid derivatives against HELA cell lines [13]

Compound	R’	Anticancer Activity	
		IC_{50} (µg/mL)	Log ($1/IC_{50}$)
FE1	Methyl	92	-1.96
FE2	Ethyl	70	-1.85
FE3	n-propyl	64	-1.81

Compound	R’	Anticancer Activity	
		IC_{50} (µg/mL)	Log ($1/IC_{50}$)
FE4	n-butyl	61	-1.79
FE5	chloroethyl	32	-1.51
FE6	bromoethyl	55	-1.74

2.2 Equipment

Intel (R) Celeron (C) processor was used with 204 MB RAM as computer hardware. The computational chemical calculation was conducted using Hyperchem 7.02 computational chemistry software. The statistical analysis was performed with SPSS 16.020. Two-dimensional molecular structure was created using ChemDraw Ultra 8.0.



FE1 : R=CH_3 (methyl)
FE2 : R=CH_2-CH_3 (ethyl)
FE3 : R=CH_2-CH_2-CH_3 (n-propyl)
FE4 : R=CH_2-CH_2-CH_2-CH_3 (n-butyl)
FE5 : R=CH_2-CH_2-Cl (chloro ethyl)
FE6 : R=CH_2-CH_2-Br (bromo ethyl)

Figure 2. Ferulic derivatives [13]

2.3 Procedures

2.3.1 Collection of descriptors

QSAR equation was built by the relationship between activity and descriptors. The logarithm of partition coefficient in the n-octanol/water (log P), the atomic charge, and the dipole moment (μ) were selected as the descriptors. Table 2 shows the descriptors and the calculation methods. The calculations for electronic descriptors are performed using computational chemistry modeling with the geometry optimization procedure of each compound structure. E as described previously [23]. Each compound was created into a 2D structure model using the ChemDraw Ultra 8.0 and equipped with hydrogen atoms to form a 3D structure.

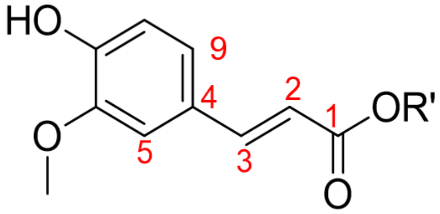


Figure 3. The label of selected carbon charge in ferulic acid derivatives

We analyze the net atomic charge of carbon number 1, 2, 3, 4, 5, 9, which have a high probability affect in the different attachment of alkyl group for ferulic acid derivatives. Figure 3 shows the position of the carbon. The net atomic charge, the dipole moment, and the partition coefficient were determined as described in the previous report [23]. Then the most stable molecular geometry was optimized by minimizing the molecular energy using PM3 method, the convergence limit was 0.001 kcal / Å.mol for ferulic acid derivatives, according to Polak-Ribiere algorithm.

Table 2. List of descriptors and how to optimize them

No	Symbol	Descriptor	Unit	Calculation Method
1	qC_1, qC_2, qC_3, qC_4, qC_5, qC_{12}	The atomic charge of C_1, C_2, C_3, C_4, and C_{12}	Coulomb	Semiempirical method of PM3, Hyperchem, compound optimization
2	μ	dipole moment	Debye	Semiempirical method of PM3, Hyperchem, compound optimization
3	Log P	The partition coefficient of n-octanol/water	-	QSAR Properties, Semiempirical method of PM3, Hyperchem, compound optimization

2.3.2 QSAR Study

The most representative QSAR equation to predict IC_{50} was determined by multilinear regression statistical analysis with backward method using SPSS. The QSAR equation was optimized by fitting six ferulic acid derivatives and the influence of the dependent and independent variables to the QSAR equation. The variables were determined as described previously in Rahmawati et al. (2020). The following regression equation was expressed as the resul of QSAR approach:

$$\text{Log}\,(1/IC_{50}) = k_1 \log P + k_2 qC_1 + k_3 qC_2 + k_4 qC_3 + k_5 qC_4 + k_6 qC_5 + k_7 qC_9 + k_8 \mu + k_9 \quad (1)$$

Statistical analysis was performed as explained in the previous report [23].

3. Results and Discussions

3.1 The Result of Descriptors Calculation on Ferulic Acid Derivatives

This research was performed by PM3 semi-empirical method for optimizing the structure of six ferulic acid derivatives. Semi-empirical methods are more reliable than *ab-initio* methods for QSAR study and produce the best model for the QSAR model [24–25]. The six compounds are FE1 which attached methyl group on carboxylic moiety in ferulic structure. FE2 has an ethyl group attached in carboxylic moiety and FE3 has n-propyl group bind to a carboxylic moiety in ferulic structure. Besides, incorporating an n-butyl group on carboxylic moiety in ferulic structure named as FE4. FE5 and FE6 have chloroethyl and bromoethyl substituent, respectively, which are shown in Figure 2. These compounds resulted in the relatively higher anticancer activity against HELA cell lines compared with other ferulic acid derivatives with longer alkyl chain studied [8, 13].

In this research, we study about QSAR between dependent variable anticancer activity (see Table 1) and eight independent descriptors. The anticancer activity of ferulic acid derivatives was expressed as IC_{50} (μg/ml) which is the concentration of the compound that inhibited proliferation rate of HELA cell lines by 50% as compared to the control untreated cells which were reported on the previous research [13]. Table 1 shows that all the synthesized of ferulic acid derivatives are having higher anticancer activity than the parent compound. Longing the alkyl chain from methyl to n-butyl (FE1-FE4) was observed, generating higher anticancer activity. Furthermore, FE5 having chloroethyl is most favorable, which produces the lowest IC_{50} value against HELA cell lines.

Table 3. Descriptor data as independent variables for ferulic acid derivatives

No	Compound	Net Atomic Charge (Coulomb)						Dipol μ (deybe)	LogP
		C_1	C_2	C_3	C_4	C_5	C_{12}		
1	FE1	0.4105	-0.2007	0.004779	-0.08484	-0.09780	-0.07153	4.334	-0.60
2	FE2	0.4149	-0.2003	0.003293	-0.08413	-0.09809	-0.07191	4.354	-0.25
3	FE3	0.4150	-0.2004	0.003415	-0.08422	-0.09804	-0.07189	4.353	0.21
4	FE4	0.4149	-0.2005	0.003572	-0.08425	-0.09805	-0.07185	4.356	0.61
5	FE5	0.05136	0.08998	-0.1772	-0.07007	-0.09100	0.4191	1.836	0.12
6	FE6	0.05136	0.08983	-0.1771	-0.07018	-0.09058	0.4118	1.479	0.42
7	Ferulic acid	0.4199	-0.1986	0.01114	-0.08772	-0.09657	-0.06980	4.491	-0.63

The result of the calculation of atomic net charge, dipole, and log P of six ester ferulic derivatives are shown in Table 3. The substitution of a different chain of alkyl groups influenced the carbon net charge. The c of C_1 in ferulic acid derivatives slightly decreased relative to that of the atomic charge of C_1 in ferulic acid. This tendency is like the net atomic charge of C_2, C_3, C_5, and C_{12}. This indicates incorporating different alkyl chain can induce atoms in the adjacent position. The longer carbon chain establishes more non-polar compounds, so the solubility of the compound in lipid is greater and the anticancer activity enhances, respectively (See Table 1). The solubility in water and lipid of the compound enhanced its bioavailability in the cells, so the anticancer activity was performed more [26–27]. Ferulic acid has lower log P of -0.63. Furthermore, the dipole of ferulic ester declined to compare to the dipole of ferulic acid. Substitution of alkyl enhanced the value of log P. Higher hydrophobicity of a compound resulted in a better interaction with the binding site of the protein during the metastasis phase of cancer cell development. This was reported in research using MMP-9 as a target protein using molecular docking [28]. On the other hand, the substituent chloroethyl in FE5 and bromoethyl in FE6 bear significantly lower dipole than other studied compounds which increase anticancer activity. The molecule with smaller dipole value demonstrated higher polarity, affecting the binding interaction with oxidative species. The addition of the ferulic compound provided the ability to reduce reactive oxygen species (ROS) which stimulated cancer cell proliferation [29]. High polarity molecule bound more comfortably with ROS leading to the prevention of cell proliferation by activating caspases, as well as associated protein which changes the chemical and morphological properties of the cells causing cell elimination by apoptosis [30].

3.2 Analysis of QSAR on Ferulic Acid Derivatives

The relationship between chemical structure and biological activity (anticancer activity) was conducted by statistical calculation using the SPSS program. The best correlation between descriptors and anticancer activity for ferulic acid derivatives is shown in Table 4. It is seen that the descriptors which have a strong correlation with anticancer activity are log P, dipole (μ), and the net charge in C_4 and $C_{5.}$ The prolongation of the alkyl group might increase nonpolar properties followed by enhancing of log P-value. Also, it shows that the importance of the halogen atom for enhancing anticancer activity. SE is the standard error of the estimated which explains error value about the calculation. It is seen that the SE value of this calculation is small (0.00857). Besides, the R^2 for the calculation is excellent at 0.998. This value represents that the calculation result can significantly explain the descriptors of the response data around its mean. It is seen that the equation is excluded from several descriptors, which are qC_1, qC_2, qC_3, and qC_9. This result indicated that those descriptors didn't influence the anticancer activity significantly. The activities of the ferulic acid derivatives were determined by log P, dipole, qC_4, qC_5. Atomic charges of C_4 and C_5 were the most affecting descriptors followed by dipole and log P. The prolongation of the alkyl chain of ferulic acid derivatives and incorporation halogen atom in ethyl moiety showed higher log P followed by a higher anticancer activity. This work suggests esterification with long alkyl chain and incorporation halogen atom in ferulic acid is favorable to give the higher anticancer activity of ferulic acid derivatives.

Table 4. The result of the best correlation between descriptors and anticancer activity for ferulic acid derivatives against HELA cell lines

Equation	$11.0 + 129C_4 + 53.8C_5 + 0.085\log P + 0.751\mu$
R	1
R^2	0.999
SE	0.00857
Sig	0.038
F	394

We were employed PRESS (predicted residual error sum of square) as cross-validation of this calculation. PRESS statistic is calculated as the sum of the squares of all the resulting prediction errors. The calculated Log ($1/IC_{50}$) has a low PRESS value of 7.50E-05 (see Table 5) which indicates the calculation of calculated log ($1/IC_{50}$) using the QSAR Hansch Model for ferulic acid derivatives has excellent agreement with experimental data of Log ($1/IC_{50}$).

Table 5. Experimental log ($1/IC_{50}$), calculated log ($1/IC_{50}$), and PRESS value for ferulic acid derivatives against HELA cell lines

Compound	Experimental Log ($1/IC_{50}$)	Calculated Log ($1/IC_{50}$)	Residual error	[Residual error]^2
FE1	-1.964	-1.963	-1.17E-03	1.38E-06
FE2	-1.845	-1.842	-3.26E-03	1.06E-05
FE3	-1.806	-1.813	6.49E-03	4.22E-05
FE4	-1.785	-1.781	-4.55E-03	2.07E-05
FE5	-1.505	-1.505	-2.54E-04	6.43E-08
FE6	-1.740	-1.740	-1.02E-05	1.03E-10
		PRESS		7.50E-05

4. Conclusion

Based on that best QSAR model, the chemical descriptors that strongly influence anticancer activity are partition coefficient of n-octanol/water (Log P), dipole moment (μ), and atom charge in C_4 and C_5 on ferulic acid derivatives. In the future study, this research suggests to design the new compound with higher Log P and lower dipole moment for giving excellent bio-activity. The result of this study could be used to develop the structure of new ferulic acid derivatives to be produced in the lab-scale to confirm their actual performance.

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Optimization Of Subcritical Water Assisted by Nitrogen Prior to Enzymatic Hydrolysis for Reducing Sugar Production

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Abstract. The objective of this study is to optimize the three significant parameters of the subcritical water (SCW) process for sugar production from coconut husk by using response surface methodology (RSM). In this study, the effect of temperature, reaction time and solvent-feed (S/F) ratio was evaluated and discussed comprehensively. The results showed that the S/F ratio had the most significant effect on sugar yield. The optimal sugar yield was obtained at the highest S/F ratio of 23.4, the shortest time of 4.8 min, and the highest temperature of 183.6 ºC of the SCW process. The characterization results confirmed that the lignocellulose structure was changed remarkably then contribute to the efficient processes of enzymatic hydrolysis. The parameters evaluation using RSM in this study suggests that SCW hydrolysis could be subjected to commercial purposes.

Keywords: *response surface methodology, subcritical water, enzymatic hydrolysis, sugar production, coconut husk*

1. Introduction

Biofuel from lignocellulosic biomass is considered as a promising new solution for energy problems because of cheapness, abundance, and energy security [1]. Before converting holocellulose into biofuels through biological processes, a pretreatment process is needed to

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*Corresponding author : maktum@unej.ac.id destroy the complex and recalcitrant biomass structures [2]. Conventional methods using chemical solutions pose several advantages and disadvantages that affect the feasibility of industrial-scale production [3]. Although it has been proven capable of devastating the lignin wall to provide bacteria access to the holocellulose compound, alternative methods are still demanded due to environmental issues from the resulting waste [4]. Therefore, the attention of researchers now is focused on developing sophisticated green technologies that are more sustainable to improve biomass processing.

Among the leading pretreatments, subcritical water technology, which can simultaneously function for pretreatment and hydrolysis, has received considerable attention in recent years [5]. Its rapid reaction characteristics and environmentally friendly nature make this technology the most attractive process for converting biomass. Moreover, the tunable solvent properties at different temperature ranges show that the selective optimization of hydrolysis conditions can be performed [6]. On the other hand, this technology still has weaknesses in terms of the formation of derivative products such as furfural compounds and phenolic compounds which act as inhibitors for further biological processing [7], [8]. It is considered that economically viable processes are only achieved by minimizing pretreatment costs while maximizing the yield of sugar.

Recently, we reported that the integration of subcritical water and enzymatic hydrolysis processes has successfully generated the fermentable sugar from coconut husk by the severity factor approach [9], [11]. The addition of surfactants to the subcritical water process could also increase the production of sugars [12], [13]. Kinetic studies of the subcritical water hydrolysis have also been reported previously [14]. Although the work has prospered in producing biohydrogen, the yield is still relatively low due to the obtained sugar was low [11]. Therefore, optimization of the subcritical water process needs to be conducted and developed. To best our knowledge, the optimization of the conversion of coconut husks using subcritical water and enzymatic hydrolysis has not been reported.

To fill these research gaps, in this work, the other significant variables (solvent/feed (S/F) ratio, time and temperature of reaction) in the SCW process have been examined and optimized by using response surface methodology to produce maximum sugar yield. Furthermore, solid characterization has been comprehensively discussed to confirm the optimization values. Subsequently, the SCW treated solids were subjected to enzymatic hydrolysis to get the remaining sugar. The optimization of the SCW technique before enzymatic and fermentation processes is projected making the processes more commercially viable.

2. Materials and Methods

2.1 Materials

Coconut husk was collected from Manado City, North Sulawesi, Indonesia. The preparation procedure of the sample was adapted from the previous work [9]. Some chemicals such as Dinitrosalycilic acid, commercial c*ellulase,* and *xylanase* were purchased from Sigma Aldrich, Japan.

2.2 SCW Process

The SCW equipment used in this study was modified from the preceding work [10]. The process was run by supplying the UHP Nitrogen gas (PT. Aneka Gas, Sidoarjo, Indonesia) to the reactor with a constant pressure of 80 bar. Process parameters were varied at temperatures of 130 to 170 °C for 15 to 45 min since the condition reached. The sample was washed and dried in the oven at a constant temperature of 60 °C and stored at 4 °C before being analyzed.

Central Composite Design (CCD) was applied to optimize the critical factor of the SCW process. The effect of three independent variables (Solvent/Feed (S/F) ratio, time and temperature of reaction) at three levels toward sugar yield as the response was investigated by using Minitab 16 (Minitab Inc, ITS Surabaya, Indonesia).

2.3 Enzymatic Hydrolysis

The experimental and analytical procedures for enzymatic hydrolysis was conducted based on previous work [12]. All the coconut husk solid was hydrolyzed using a combination of commercial *cellulase* and *xylanase*. The hydrolysis process was carried out in a shaker incubator and operated at 60 °C and 125 rpm. Sugar obtained during the process was analyzed every 8 h for 48 h.

2.4 Analytical Method

The reducing sugar was obtained following SCW and enzymatic hydrolysis process. It was measured by using the Dinitrosalicylic acid (DNS) method [15]. Hydrolysis data were analyzed by applying the two-way analysis of variance (ANOVA). Data are presented as mean standard deviation based on triplicate analyses. Pretreated and unpretreated samples were examined by using Scanning Electron Microscopy (SEM) Evo MA 19 (Carl Zeiss, England), X-Ray Diffraction (XRD) X'Pert PRO (PANalytical B.V, Holland) and Fourier Transform Infrared (FTIR) Nicolet iS10 spectrophotometer.

3. Results and Discussions

3.1 Response Surface of SCW process

At this work, three critical parameters of the SCW process were evaluated to determine variable which has a significant effect in reducing the concentration of sugar using response surface methodology (RSM). Table 1 shows the design and result of the three parameters in the SCW process using a central composite design (CCD). As shown in Table 1, the yields obtained from the SCW pretreatment process varied from 3.37% to 9.89%. The highest reducing sugar yield of 9.89% was attained under a reaction condition of 170 °C for 15 min and the S/F ratio of 20.

Table 1. Central composite design matrix along with predicted and experimental values for reducing sugar yield in SCW process

Runs	Reaction time (min)	S/F ratio	Reaction temperature (°C)	Sugar concentration (g/L)	Reducing sugar yield (%)	
					Observed	Predicted
1	30	15	150	4.795	4.295	4.255
2	30	23.4	150	4.026	4.026	4.426
3	30	15	150	6.499	4.299	4.255
4	15	20	130	3.589	3.589	3.219
5	30	15	150	4.841	4.241	4.255
6	15	10	130	3.423	3.423	3.280
7	15	20	170	4.946	4.946	4.802
8	30	15	150	3.363	4.263	4.255
9	45	10	170	7.585	7.585	7.474
10	30	6.6	150	6.379	7.379	7.697
11	45	10	130	6.711	6.982	6.642
12	45	20	170	3.725	3.981	3.641
13	45	20	130	3.061	3.016	2.834
14	55.2	15	150	3.649	4.078	4.451
15	30	15	150	2.609	4.258	4.255
16	4.8	15	150	2.247	2.247	2.602
17	30	15	116.4	2.564	2.729	3.129
18	30	15	183.6	4.177	4.830	5.158
19	15	10	170	5.188	5.188	4.888
20	30	15	150	3.544	4.244	4.255

The experiment data of sugar yield was used to determine the coefficient of the regression equation (Eq. 1) where X_1, X_2, and X_3 are reaction time, S/F ratio, and temperature. The second-order polynomial equation can be used to predict the yield of reducing sugar.

$$\begin{aligned} Yield = {} & -20.4 + 0.513X_1 - 0.07X_2 + 0.192X_3 - 0.00189X_1X_1 + 0.0168X_2X_2 \\ & - 0.0005X_3X_3 - 0.01531X_1X_2 - 0.00095X_1X_3 + 0.00175X_2X_3 \end{aligned} \tag{1}$$

In the general SCW Process, the temperature is one of the significant parameters toward cracking and hydrolysis performance of lignocellulose. It due to the properties of SCW which help in the hydrolysis process (i.e., dielectric constant, density, viscosity, etc.) is a function of the temperature [16]. Figure 1 depicts a 3-D plot of the response surface for the reducing sugar yield. As shown in Figure 1, by increasing temperature, reducing sugar production was increased. The degradation of hemicellulose and cellulose occurred at high temperatures because of water more reactive to break down the complex carbohydrate molecules. Hydrolysis biomass run slowly, on the temperature below 100 °C. Prado et al. (2016) revealed that free sugars and most hemicelluloses reacted above 170 °C. Muharja et al. (2017) showed the same result that hemicellulose extract from coconut husk increase from 67.8% to 73.94% with increasing temperature from 150 to 160 °C.

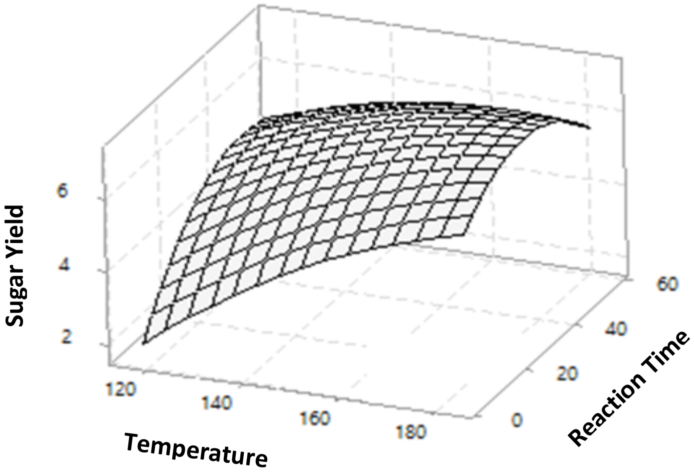

Figure 1. Surface response from RSM design

Another critical parameter on hydrolysis using SCW is reaction time. As shown in Figure 1, the yield of reducing sugar increased with increasing time of reaction and then decreased. This can be explained by the fact that the contact between the solvent and the substrate causes the carbohydrate complex molecules to be increasingly hydrolyzed. This result is like the study of cellulose recovery from water hyacinth by Thi et al. (2017). They revealed that reducing sugar increased at 15 to 35 min and then decreased.

Tabel 2. Analysis of variance (ANOVA) of model parameters

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Model	9	48.6301	5.4033	1.86	0.173
Linear	3	30.8721	10.2907	3.55	0.056
Temperature	1	8.4775	8.4775	2.92	0.118
Reaction Time	1	2.3972	2.3972	0.83	0.385
S/F Ratio	1	19.9974	19.9974	6.89	**0.025**
Square	3	6.3153	2.1051	0.73	0.560
Temperature*Temperature	1	0.5708	0.5708	0.20	0.667
Time*Time	1	2.6065	2.6065	0.90	0.366
S/F Ratio*S/F Ratio	1	2.5600	2.5600	0.88	0.370
2-Way Interaction	3	11.4426	3.8142	1.31	0.323

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Temperature* Time	1	0.6441	0.6441	0.22	0.648
Temperature*S/F Ratio	1	0.2415	0.2415	0.08	0.779
Time*S/F Ratio	1	10.5570	10.5570	3.64	0.086
Error	10	29.0098	2.9010		
Lack-of-Fit	5	7.2083	1.4417	0.33	**0.875**
Pure Error	5	21.8015	4.3603		
Total	19	77.6399			

Note. The bold letter denoted the significant value (at a confidence level of 95%).
DF, Adj. SS, and Adj. MS means Degree of Freedom, Adjusted Sum of Squares, and Adjusted Mean of Squares, respectively.

The amount of water affected sugar yield. In this study, S/F ratio is the most significant parameter in the hydrolysis process (see Table 2). As shown in Figure 2, hydrolysis of reducing sugar increased with increasing S/F ratio. Based on the result of this study, more solvents used in the process cause complex carbohydrate molecules to be more easily hydrolyzed. Higher S/F ratio caused the solution in the reactor is not easily saturated. In the batch process, the saturation of the solution becomes very important because it affects the solubility of the hydrolyzed sugar. The same result was reviewed by Prado et al., (2016) where sugar yield increased with increasing S/F for batch SCW system.

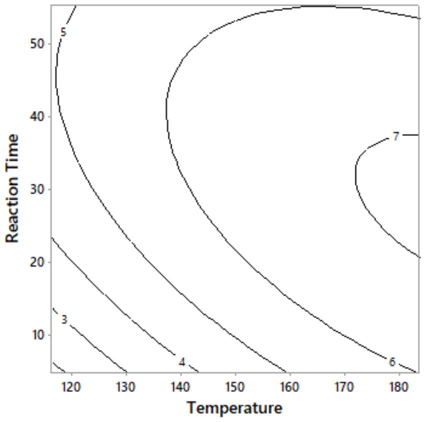

Figure 2. Contour response from RSM Design

3.2 Statistical Analysis and Model Fitting

P-value is a parameter to determine the significant factor of the experiment. A p-value of less than 0.05 indicates factors that are significant at the probability level of 95%. Table 2 is an analysis of variance (ANOVA) to fitness the models. From Table 2, the linear coefficient (reaction time, temperature), square coefficient, and cross coefficient were not significant. Surprisingly, the S/F ratio is a highly influential parameter in reducing sugar yield through SCW. The lack-of-fit test is showing that the CCD model is sufficiently precise for predicting reducing sugar yield by SCW hydrolysis. Based on the model, the operating conditions for the optimum reducing sugar yield were on the temperature reaction 183.6 ° C with the S/F ratio of 23.4 for 4.8 min.

3.3 Enzymatic Hydrolysis

Figure 3 shows the time courses of the hydrolysis process following SCW employed. The sugar concentration increased significantly from 0 to 8 h of hydrolysis time. This phenomenon occurred may be due to enzymes have hydrolyzed cellulose and hemicellulose entirely during that condition for an initial 8 h. A study by Sánchez-Ramírez et al., (2017) revealed that after incubation at 60 ° C, enzyme activity diminished continuously and reached 20% of its initial activity after 4 h of hydrolysis. As shown in Figure 3, the highest yield of reducing sugar obtained after enzymatic hydrolysis for 48 h was 2.58 g/L. Using two-way ANOVA, it is known that the SCW process has a significant effect on the concentration of sugar compared to the hydrolysis of untreated solid.

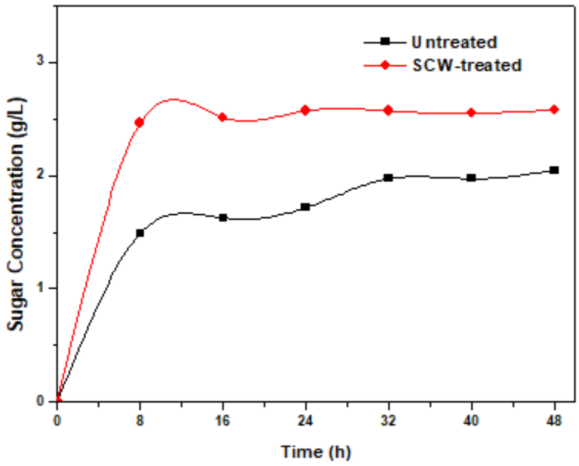

Figure 3. Time courses of the sugar concentration during enzymatic hydrolysis of (a) untreated and (b) SCW treated solid at optimum conditions

3.4 Characteristic of Solid Residue

Figure 4 shows the comparison of diffraction patterns between untreated and SCW treated coconut husk at optimum conditions. XRD was used to detect CrI values from coconut husk. The value CrI of coconut husk before and after pretreatment was 14.69% and 8.33%, respectively. The SCW treated coconut husk showed a broad diffraction pattern with higher intensity compared to the untreated coconut husk. Ciftci and Saldana (2015) reported that the crystalline index of sweet blue lupin increased from 11.5% to 58.6% after SCW pretreatment. Mohan et al. (2015) also reported the crystalline index of the sample increased from 50.55% to 65.83% after pretreatment. This can be explained by the degradation of amorphous lignin and hemicellulose after SCW pretreatment, leading to an increase in the amount of crystalline part from cellulose enriched residue.

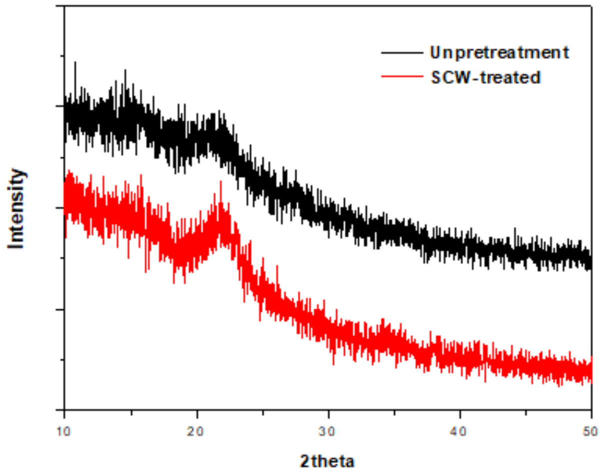

Figure 4. XRD pattern of (a) untreated and (b) SCW treated solid at optimum conditions

Fourier transform infrared spectroscopy (FTIR) was used to identify and characterize the treated coconut husk solid after SCW pretreatment. The FTIR spectra of untreated and SCW treated samples are shown in Figure 5. The emergence of the peak at the wavelength of 1300 indicates the attenuation of the OH bond between the cellulose. A vibration of aromatic rings indicates a peak of 1600. The range is 1030 indicate the stretching of cellulose and hemicellulose in C-O. The emergence of vibration peaks that exist in pretreatment samples indicates the process of lignification, dissolution of hemicellulose and cellulose.

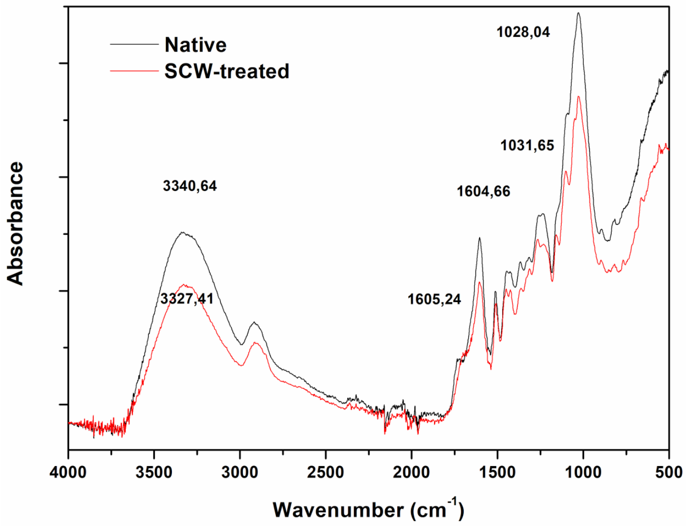

Figure 5. FTIR spectra of (a) untreated and (b) SCW treated solid at optimum conditions

The surface morphology of untreated and SCW-treated solid was examined using SEM. Figure 6 shows the SEM images of (a) untreated and (b) SCW-treated solid at optimum conditions. The surface of coconut husk before SCW pretreatment is marked by some boundary edges and does not show the presence of any pores, trenches, or surface cracks, while cracks and trenches can be seen on the surface of the SCW-treated solid. These images of microscopic structures difference demonstrate that the SCW pretreatment could efficiently disintegrate the lignocellulose cell wall, resulting in exposure of internal structure [20]. These crystal structural changes have been managed to expose cellulose to enzyme access and increase the digestibility of enzymatic hydrolysis [22].

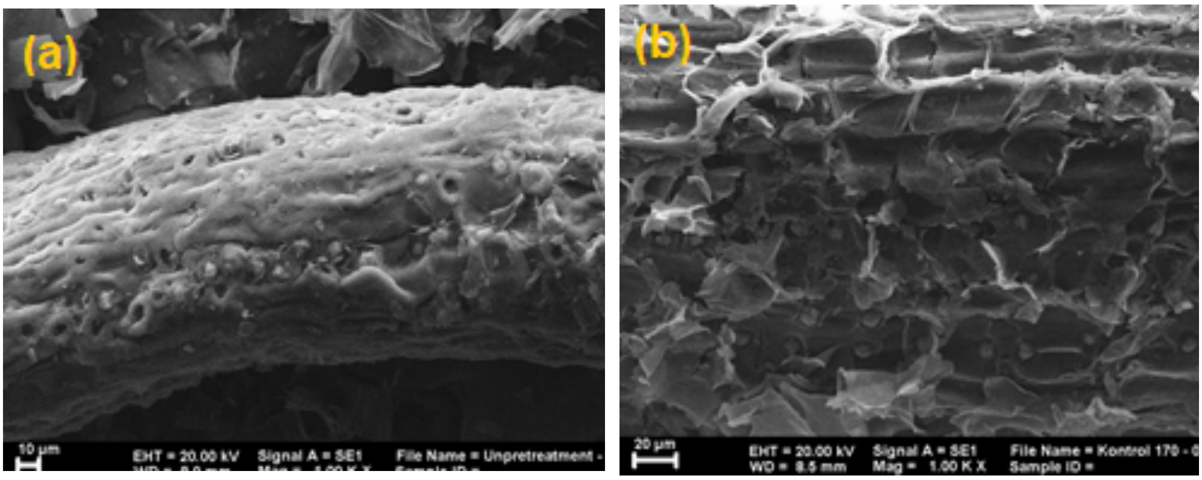

Figure 6. The morphological structure of (a) untreated and (b) SCW treated solid at optimum conditions

4. Conclusion

Reducing sugar has been successfully gained from coconut husk using a combination of SCW and enzymatic hydrolysis. The highest yield of sugar was 9.89% with a sugar concentration of 4.946 g/L. The variable water-solid ratio has a significant effect, where the high ratio will produce a high yield. From the optimization, the concentration of reducing sugar from the RSM model was 183.6 °C for 4.8 min and 23.4 S/F ratio

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