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Determination of significant parameters and optimization production of virgin coconut oil using *Neurospora sitophila, Lactobacillus Plantarum,* and papain

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ABSTRACT

Virgin coconut oil (VCO) is a processed coconut product that contains high lauric acid which proved to have a health beneficial effect. Fermentation is one of the means to produce VCO. Factors that affect fermentation can be optimized to increase VCO recovery. The objective of the present study was to determine factors that significantly affecting VCO recovery using Plackett-Burman design (PBD), optimization of a fermentation process using Response Surface Methodology-Central Composite Design (RSM-CCD), and determine characteristics of a product. The experiment was performed by growing *Neurospora sitophila* and *Lactobacillus plantarum*, isolation, and activity determination of papain from papaya sap. Nine factors were screened using PBD and the three most significant factors were further optimized using RSM-CCD. The optimum condition was applied to produce VCO and the product was characterized for its physicochemical properties and fatty acid content. The results indicate that papain activity, incubation temperature, and pH were the most significant factors that affect VCO recovery. Further investigation using RSM-CCD indicates that optimum condition for VCO production was 1042.4 U, 46.4°C, and 5.07 for papain activity, incubation temperature, and pH, respectively. The recovery of VCO was 45.06 ± 0.42% and the physicochemical properties of VCO comply to APCC quality standard of coconut oil requirement with 56.7% lauryl acid content.

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INTRODUCTION

Coconut (*Cocos nucifera L.*) is a member of the *Arecaceae* family and it is native to Southeast Asia regions and the islands between India and the Pacific Ocean (Lima *et al.*, 2015). This makes Indonesia become one of biggest coconut-producing countries in the world (Krishna *et al.*, 2009).

At present, the area of coconut plantations in Indonesia is quite large. Directorate General of Estate Crops-Indonesia, 2018 Alouw and Wulandari (2020), stated that coconut production in 2018 was an estimated 2.9 million tonnes from 2.6 million ha of mature palms, with productivity, ca. 1.1 tonnes/ha/year. The present productivity is still far from the production potency of superior varieties, which can reach more than 2.8 tons/ha/year (Alouw and Wulandari, 2020). Directorate General of Estate Crops-Indonesia, 2019 Santika *et al.* (2020), stated, in 2018, Indonesia was the world's largest palm oil

producer, supplying more than 40 million tonnes of crude palm oil, or 56% of global production.

Coconut tree is known as the tree of life, because all parts of a coconut tree can be used for human life (Solangi and Iqbal, 2011). Coconut oil is the most popular coconut products that is used as a raw material in various industries like food, pharmaceuticals and cosmetics. However, coconut oil is usually extracted from copra through dry extraction and involves heating, purification, bleaching and scent removal. The process may lead to lower quality of oil, such as covers natural odor of the coconut, increases the content of free fatty acids, and damages the beneficial oil content such as vitamin E, phytosterol and polyphenols oil (Prapun *et al.*, 2016).

Virgin coconut oil (VCO) is produced from fresh and mature coconut meat without using heat but does not change the original nature of the oil. VCO can be an alternative to minimize the loss of beneficial contents in coconut oil (Prapun *et al.*, 2016). VCO has a high content of lauric acid (C-12) that belongs to a medium chain saturated fatty acid (MCFA) (Nurah *et al.*, 2017). MCFA in coconut oil is directly absorbed by the intestine and channeled to the liver as an energy source, not saved in the form of body fat (Boemeke *et al.*, 2015). According to APCC (2009), the percentage of lauric acid is around 45-56%. Lauric acid in VCO generally in the form of triglycerides, in the body can be converted to monolaurin, which has antiviral, antifungal and antibacterial activity that destroys various diseases caused by the organisms (Edem and Elijah, 2016). Consuming VCO can act as an anti-aging, prevent atherosclerosis, cancer, and diabetes mellitus (Akinnuga *et al.*, 2014).

A cheap and relatively safe method for VCO production is the enzymatic fermentation method using yeast, microbes, or other enzymes that are effective for breaking coconut emulsions (Rahayu *et al.*, 2008). Fresh coconut milk extract is a stable emulsion because it is stabilized by coconut proteins such as albumin and globulin and phospholipids (Patil and Benjakul, 2017; Raghavendra and Raghavarao, 2010). Activities possessed by microbes can destabilize coconut milk so that there is a separation between the phases of oil, coconut waste, and water (Raghavendra and Raghavarao, 2010).

Selection of the appropriate microbes and enzymes is very important in the VCO fermentation. There are various microbes commonly used in VCO fermentation. *Neurospora sitophila* known as mold which plays an important role in making "oncom", a traditional Indonesian food which has a distinctive orange color (Kanti and Sudiana, 2016). *N. sitophila* is able to produce protease enzymes that can break peptide bonds in proteins that trap oil in coconut milk emulsions.

The recovery of VCO can be further optimized by the addition of other microbes that can destabilize coconut milk emulsion, such as *Lactobacillus plantarum.* According to a recent study by Nurah *et al.* (2017), coconut milk fermentation using *L. plantarum* ATCC 14917 produced VCO with 48.94% lauric acid content. *L. plantarum* has the capacity to convert sugars into lactic acid which lowers the pH of coconut milk thereby encouraging protein denaturation and increasing the acquisition of VCO (Satheesh and Prasad, 2014).

In addition, addition of extracellular protease can improve degradation of peptide bonds. Protease are produced by plants, microorganisms or animals. However, animal originated protease are relatively expensive rarely encountered, while protease from microorganisms need more complicated steps to produce.

Therefore, proteases from plants are more preferred due to its simpler preparation procedure (Moodie, 2001). Papain is a plant protease isolated from unripe papaya latex (*Carica papaya* L.). Papain shows good proteolytic activity and can be applied in VCO production (Amri and Mamboya, 2012). Mansor *et al.* (2012), found that addition of 0.1% (w/w) papain produce VCO with 46.36% lauric acid.

Coconut milk fermentation is influenced by many factors. However, of all these factors could be only a few factors that affect the response significantly. Screening experiment is performed to determine significant factor(s) of a set of experimental factors that affecting the response.

One of the designs in a screening experiment is Plackett-Burman (Montgomery, 2017). In the present study, there are nine factors that were tested in the Plackett-Burman design, namely *L. plantarum* inoculum concentration, *N. sitophila* inoculum concentration, papain activity, time, temperature of incubation, pH, pasteurization temperature, centrifugation speed, and centrifugation time.

Important factors obtained from PBD were then further optimized using Response Surface Methodology-Central Composite Design (RSM-CCD) which has good accuracy in seeing the suitability of the model obtained (Edem and Elijah, 2016; Masyithah, 2017).

Therefore, in the present study factors that may affecting the recovery of VCO were screened using PBD and the most significant factors were further

optimized using RSM-CCD to improve the recovery of VCO in the fermentation process.

MATERIALS AND METHODS

Materials

Fresh and ripe coconuts were obtained from coconut plantation in Tasikmalaya, Indonesia. Culture of *N. sitophila* was obtained from Department of Microbiology, Institut Teknologi Bandung (ITB). *In contrast, L. Plantarum* InaCC B153 was obtained from Indonesian Culture Collection (InaCC) Indonesian Institute of Sciences, Bogor, Indonesia. Papain was isolated from the latex of raw papayas that were collected from a plantation in Sumedang, Indonesia. All chemicals and solvent used were of analytical grades.

Preparation Inoculum

N. sitophila was maintained in PDA agar whereas *L. Plantarum* was maintained in MRS agar. For inoculum preparation, each microorganism was inoculated into sterile coconut water and incubated at room temperature with 180 rpm shaking for 18 hours.

Preparation of Papain and Determination Its Protease Activity

Fresh unripe papaya was cut vertically with 1-2 mm deep, and the latex was collected in a container. Latex was dissolved in water (ratio 1:2 v/v) and centrifuged at 6000 rpm for 10 minutes. The supernatant was collected and used as the crude papain. Determination of protease activity of the crude papain extract using casein substrate was based on (Akpinar and Penner, 2001).

Plackett-Burman Experimental Design

The Plackett-Burman experimental design was used to evaluate the relative importance of several factors for VCO production through fermentation using *N. sitophila, L. Plantarum*, and papain. The nine selected independent factors that were assessed were *N. sitophila*inoculum concentration (%v/v), *L. Plantarum* inoculum concentration (%v/v), papain activity (U), time (hours), incubation temperature (°C), pH, pasteurization temperature (°C), centrifugation speed (rpm), and centrifugation time (minutes) as shown in Table 1. The number 1 (high level) and 0 (low level) represented the two different levels of the independent variables that were examined.

The experiment was carried out according to the Plackett-Burman design for conducting 12-experimental trials as shown in Table 2.

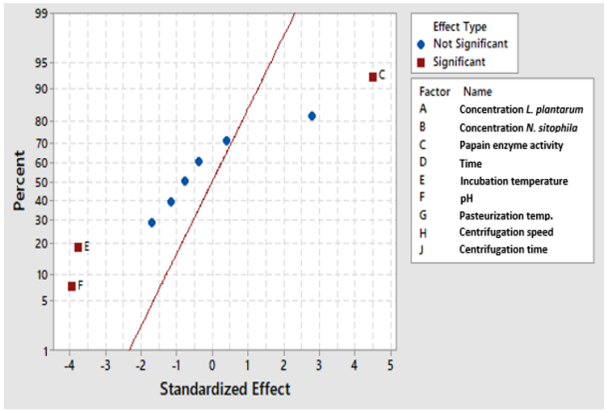

Figure 1: The normal plot effects standardized factors that influence VCO production using the Plackett-Burman design

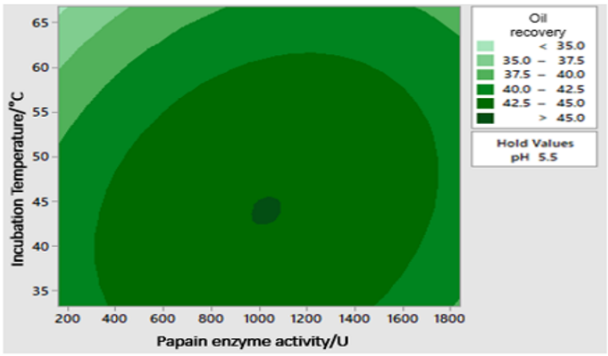

Figure 2: The contour plot between the addition of the papain enzyme to the incubation temperature

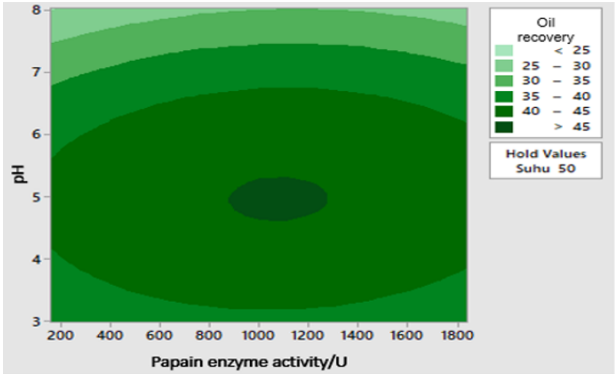

Figure 3: The contour plot between the addition of the papain enzyme to the pH of coconut milk cream

Table 1: Variables and their levels employed in Plackett-Burman design for screening of significant variables affecting on VCO production yield by fermentation method

Variable code	Variables	Value	
		0	1
X_1	*L. plantarum* inoculum concentration (% v/v)	-	10
X_2	*N. sitophila* inoculum concentration (% v/v)	-	10
X_3	Papain activity (U)	-	1,250
X_4	Fermentation time (h)	12	36
X_5	Incubation temperature (°C)	37	55
X_6	pH	4	7
X_7	Pasteurization temperature (°C)	-	75
X_8	Centrifugation speed (rpm)	1,000	4,000
X_9	Centrifugation time (minutes)	10	30

(-) means the variable is not included in experiment

Table 2: Plackett-Burman experimental design to determine significant variables by analyzing nine variables

Experimental Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
2	1	1	0	1	0	0	0	1	1
3	0	1	1	0	1	0	0	0	1
4	1	0	1	1	0	1	0	0	0
5	1	1	0	1	1	0	1	0	0
6	1	1	1	0	1	1	0	1	0
7	0	1	1	1	0	1	1	0	1
8	0	0	1	1	1	0	1	1	0
9	0	0	0	1	1	1	0	1	1
10	1	0	0	0	1	1	1	0	1
11	0	1	0	0	0	1	1	1	0
12	0	0	0	0	0	0	0	0	0

Table 3: Coded level and number level for X_1, X_2, and X_3

Independent Variable		Coded level				
		-1.682	-1	0	1	1.682
Papain activity (U)	X1	159.10	500	1,000	1,500	1,840.90
Incubation temperature (°C)	X2	33.18	40	50	60	66.82
pH	X3	2.98	4	5.5	7	8.02

Table 4: Design matrix of central composite design obtained from RSM in term of coded and actual variables in coded level

Experimental Run	Coded (Actual) Variables		
	Papain activity, X_1 (U)	Incubation temperature, X_2 (°C)	pH, X_3
1	-1 (500)	-1 (40)	-1 (4)
2	1 (1,500)	-1 (40)	-1 (4)
3	-1 (500)	1 (60)	-1 (4)
4	1 (1,500)	1 (60)	-1 (4)
5	-1 (500)	-1 (40)	1 (7)
6	1 (1,500)	-1 (40)	1 (7)
7	-1 (500)	1 (60)	1 (7)
8	1 (1,500)	1 (60)	1 (7)
9	-1.682 (159.10)	0 (50)	0 (5.5)
10	1.682 (1,840.90)	0 (50)	0 (5.5)
11	0 (1,000)	-1.682 (33.18)	0 (5.5)
12	0 (1,000)	1.682 (66.82)	0 (5.5)
13	0 (1,000)	0 (50)	-1.682 (2.98)
14	0 (1,000)	0 (50)	1.682 (8.02)
15	0 (1,000)	0 (50)	0 (5.5)
16	0 (1,000)	0 (50)	0 (5.5)
17	0 (1,000)	0 (50)	0 (5.5)
18	0 (1,000)	0 (50)	0 (5.5)
19	0 (1,000)	0 (50)	0 (5.5)
20	0 (1,000)	0 (50)	0 (5.5)

Table 5: Result of oil recovery by using Plackett-Burman design

Experimental Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	Oil Recovery (%), Y
1	1	0	1	0	0	0	1	1	1	31.7
2	1	1	0	1	0	0	0	1	1	33.3
3	0	1	1	0	1	0	0	0	1	35.0
4	1	0	1	1	0	1	0	0	0	26.7
5	1	1	0	1	1	0	1	0	0	18.3
6	1	1	1	0	1	1	0	1	0	28.3
7	0	1	1	1	0	1	1	0	1	33.3
8	0	0	1	1	1	0	1	1	0	33.3
9	0	0	0	1	1	1	0	1	1	0.3
10	1	0	0	0	1	1	1	0	1	0.0
11	0	1	0	0	0	1	1	1	0	25.0
12	0	0	0	0	0	0	0	0	0	31.7

Table 6: Result of actual and predicted oil recovery by using RSM-CCD

Experimental Run	Coded (Actual) Variables				
	Papain activity, X_1 (U)	Incubation temperature, X_2 (°C)	pH, X_3	Actual oil recovery (%) [Y]	Predicted oil recovery (%)
1	-1 (500)	-1 (40)	-1 (4)	40.3	41.34
2	1 (1500)	-1 (40)	-1 (4)	41.0	41.22
3	-1 (500)	1 (60)	-1 (4)	39.5	41.26
4	1 (1500)	1 (60)	-1 (4)	39.8	43.20
5	-1 (500)	-1 (40)	1 (7)	38.7	38.65
6	1 (1500)	-1 (40)	1 (7)	37.7	39.25
7	-1 (500)	1 (60)	1 (7)	29.2	32.34
8	1 (1500)	1 (60)	1 (7)	32.7	35.00
9	-1.682 (159.10)	0 (50)	0 (5.5)	42.2	40.22
10	1.682 (1840.90)	0 (50)	0 (5.5)	44.0	42.36
11	0 (1000)	-1.682 (33.18)	0 (5.5)	43.8	44.22
12	0 (1000)	1.682 (66.82)	0 (5.5)	44.8	40.57
13	0 (1000)	0 (50)	-1.682 (2.98)	40.7	38.97
14	0 (1000)	0 (50)	1.682 (8.02)	32.0	29.82
15	0 (1000)	0 (50)	0 (5.5)	44.7	44.83
16	0 (1000)	0 (50)	0 (5.5)	45.0	44.83
17	0 (1000)	0 (50)	0 (5.5)	44.7	44.83
18	0 (1000)	0 (50)	0 (5.5)	43.7	44.83
19	0 (1000)	0 (50)	0 (5.5)	44.0	44.83
20	0 (1000)	0 (50)	0 (5.5)	45.3	44.83

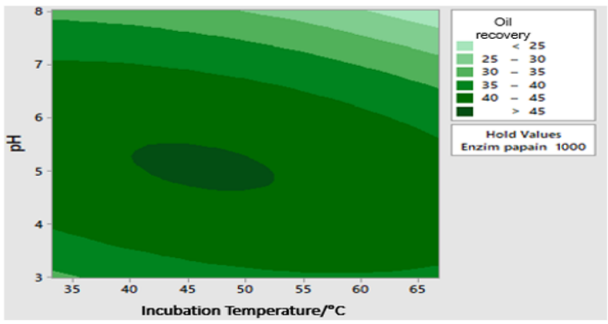

Figure 4: The contour plot between the incubation temperature to the pH of coconut milk cream

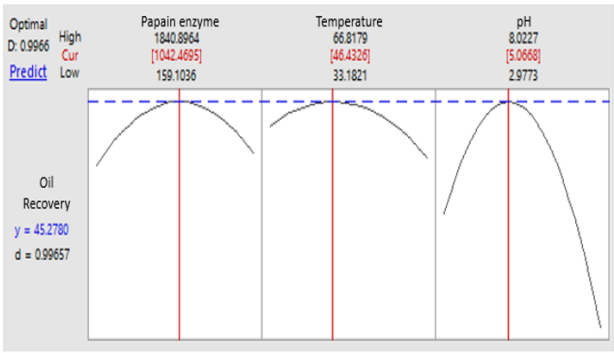

Figure 5: Estimation optimum value of the acquisition of VCO using the enzyme variables papain enzyme activity, temperature, and pH, which produce oil recovery of 45.28%

Response Surface Methodology Experimental Design

RSM with central composite design (CCD) was used to analyze the interaction effect between the variable of fermentation of coconut milk and optimize VCO production. The Minitab 17® software was used for the design and analysis of the experimental data.

The independent variables which were used in RSM-CCD experimental design were obtained from Plackett-Burman's data analysis result. The independent variables were papain activity (X_1), incu-

Table 7: Analysis of variance in the acquisition of VCO by the enzyme factors papain, temperature and pH using the RSM-CCD. P value <0.05 indicates a significant variable

Source	DF	Adj SS	Adj MS	F value	P value
Model	9	352.325	39.147	5.84	0.005
Linear	3	119.565	39.885	5.94	0.014
X_1	1	3.088	3.088	0.46	0.513
X_2	1	16.006	16.006	2.39	0.153
X_3	1	100.472	100.472	14.98	0.003
Quadratic	3	210.928	70.309	10.48	0.002
X_1	1	24.160	24.160	3.60	0.087
X_2X_2	1	10.627	10.627	1.58	0.237
X_3X_3	1	195.924	195.924	29.22	0.000
Two-way interaction	3	21.832	7.277	1.09	0.399
X_1X_2	1	2.136	2.136	0.32	0.585
X_1X_3	1	0.269	0.269	0.04	0.845
X_2X_3	1	19.427	19.427	2.90	0.120
Error	10	67.058	6.706		
Lack of fit	5	65.132	13.026	33.82	0.001
Pure error	5	1.926	0.385		
Total	19	419.383			

Table 8: Verification result of VCO yield by optimization fermentation process with three replications

Condition	Variables			
	Papain activity/U	Incubation temperature/°C	pH	Oil recovery/ (%)
Center point	1000	50	5.5	44.83
Optimum	1042.47	46.43	5.07	45.28
Experiment 1	1,042.47	46.43	5.07	44.67
Experiment 2	1,042.47	46.43	5.07	45.50
Experiment 3	1,042.47	46.43	5.07	45.00
Average Exp.				45.06 ± 0.42

Table 9: Physicochemical properties of VCO comply APCC standard for comparison

Parameters	VCO sample	APCC standard
Saponification value/(mg KOH/g oil)	255.82 ± 3.67	250 – 260
Density/(g/mL)	$0.9169 \pm 0{,}00$	0.915 – 0.920
Moisture content/(%)	0.13 ± 0.03	Max 0.1
Free fatty acid (FFA)/(%)	0.11 ± 0.02	Max 0.2
Acid number/(mg KOH/g oil)	0.30 ± 0.06	0.2 – 0.5
Peroxide value/(mg eq/kg oil)	0.9059 ± 0.23	Max 3
Color	Colorless	Colorless

Table 10: Data concentration of VCO fatty acids were compared to the FAME standard of 20 mg / mL

Peak	Type of fatty acid	Retention time	Area	Concentration/ (mg/mL)	Concentration/ (%)
1	C8 (Caprylic)	4.434	8,903,093	1.7039	13.56
2	C10 (Capric)	7.084	9,597,499	1.2396	9.87
3	C12 (Lauric)	10.910	75,884,331	7.1278	56.73
4	C14 (Myristic)	15.673	24,369,230	1.5780	12.56
5	C16 (Palmitic)	18.581	9,049,552	0.5012	3.99
6	C18:2 (Linoleic)	20.482	1,110,235	0.0598	0.48
7	C18:1 (Oleic)	20.542	5,707,548	0.2612	2.08
8	C18 (Stearic)	20.795	2,198,836	0.0920	0.73
Total				12.5635	100

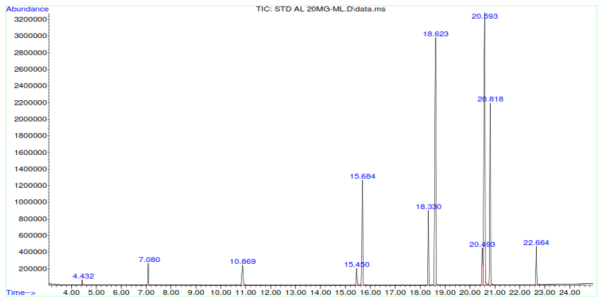

Figure 6: Gas chromatogram of FAME of Standard (Larodan 20 mg/mL), eleven peaks appeared with different retention times

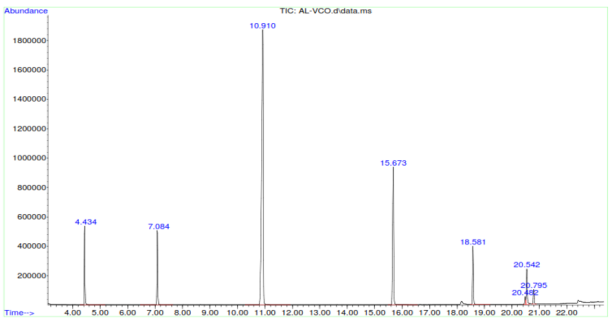

Figure 7: The optimized VCO sample, only eight peaks appeared compared to retention time of standard

bation temperature (X_2), and pH (X_3). Each variable has five different levels of code from low values (-1), medium (0), high level (+1) and axial point ($\pm\ \alpha$) to determine the curvature and quadratic model. CCD design with three independent variables has a rotatability number (α)=(32)1/4 = 1.6818 $\approx$ 1.682. Therefore, $\pm$1.682 is included in the coding. The response (dependent variable) was oil recovery (%). RSM analyzed experimental data by using second-order polynomial equations (Masyithah, 2017). The coded level and number level for X_1, X_2, and X_3 variables were shown in Table 3.

In addition to the three variables varied, the other variables were made fixed, namely as follows: inoculum concentration of *N. sitophila* (10% v/v), inoculum concentration of *L. plantarum* (10% v/v), fermentation time (48 hours), pasteurization temperature (75°C), centrifugation speed (4,000 rpm), and centrifugation time (10 minutes). A central composite design and six replications at the center point (total number were 20 experiments) were conducted for optimization of fermentation condition. After coded, the CCD experimental design is shown as coded level in Table 4.

Fermentation of Coconut Milk

Fresh and ripe coconuts were dehusked and grated, then hot distilled water was added to reach 1:1 ratio, and it was left for 2 hours in a container until two layers appeared.

The upper layer was coconut milk, and the bottom layer was skim. The layers were separated into a different container. The coconut milk was pasteurized at 75°C for 10 minutes. Then 10% (v/v) of each *N. sitophila* and *L. Plantarum* inoculum was added. Papain (range in Table 2) was also added into 300 mL of coconut milk. Acetic acid and sodium carbonate were added to adjust pH (range was shown in Table 2). The mixture was stirred until homogeneous and then incubated for 48 hours at a designated temperature (range in Table 2). As the layers of oil and water became separated, the oil layer was decanted. And then it was centrifuged at 4000 rpm for 10 minutes to separate the waste and recover clear VCO. This procedure was modified from Masyithah (2017).

Oil Recovery

The determination of oil recovery was calculated according to the volume of coconut milk which was

used for the fermentation process to the quantity of oil extracted by fermentation based on Mansor *et al.* (2012).

Physicochemical Properties of VCO

The saponification value, density, moisture content, free fatty acid, acid number, peroxide value, and colour were determined by AOAC method (Ghani *et al.*, 2018; Srivastava *et al.*, 2016).

Fatty Acid Composition

The fatty acid composition was determined by AOAC method using boron trifluoride (Nielsen, 2017). The extracted fatty acid methyl ester (FAME) composition was identified using a gas chromatography-mass spectrometer GC 7890A-MS 5975C Agilent Technologies by injecting one μL sample. The separation of the compounds was performed on Agilent HP-5 MS column (29.81 m$\times$0.25 mm$\times$0.25 μm). Helium used as carrier gas with a flow rate of 1.00 mL/min. The injector temperature was 250°C. Programmed temperatures started from 100°C for 2 minutes, went up to 150°C with a rate increased of 10°C/min for 5 minutes and a final temperature of 315°C with an increase of 10°C/min for 10 minutes. The mass-selective detector was set with a mass range of 40-550 amu. The FAME in VCO was identified by matching the unknown retention index of compounds with standard compounds. The concentration of the fatty acid was determined by comparing the height of sample peak to an external standard, and mass percentage of the fatty acids were then calculated as the ratio of the concentration of each fatty acid to total fatty acid.

RESULTS AND DISCUSSIONS

The production of VCO from coconut milk was performed using a mixture of *N. sitophila*, *L. Plantarum,* and papain. This mixture was expected to give a better separation of the layers of the fermented coconut milk that can lead to higher oil recovery in a short time. During the process of enzymatic fermentation, the microbes produce proteases which can degrade protein molecules that surround oil in coconut milk so that the oil phase could be separated (Montgomery, 2017).

In this research, at least three types of proteases were present in the fermentation process. The first is pepsin-like aspartic proteases produced by *N. sitophila*, which tend to cleave dipeptide bonds that have hydrophobic residues (Mahajan and Badgujar, 2010). Next, *L. Plantarum* produces trypsin-like serine protease, which cleaves peptides on the carboxyl side of the amino acids lysine or arginine, which is not followed by a proline (Margono *et al.*, 2014). Lastly, papain which belongs to cysteine proteases, could cleave peptides after basic amino acids such as arginine or lysine (Amri and Mamboya, 2012). The analysis result of crude *papain* proteolytic *activity* was 117.87 U/mL. Because of the three types of proteases cleave different amino acids in proteins that trap oil, which is expected to optimize the destabilization of coconut milk emulsions in VCO fermentation process.

High-temperature treatment can be applied during pasteurization, which besides removing undesirable microorganisms, it can also help destabilize coconut milk emulsions. High temperature can eliminate hydrophobic interactions of protein and oil so that the desired oil can be completely free of protein and can be separated from its emulsions (Singh and Ye, 2014). Besides, after the fermentation process is complete, centrifugation of the VCO produced needs to be conducted to help terminate the oil-water emulsion that has not been wholly destabilized, and to separate oil, water, and its waste into three layers. Nour *et al.* (2009), reported that centrifugal force used in VCO production was directly proportional to its yield.

Selection of Significant Factors Affecting Oil Recovery by Plackett-Burman Design

VCO production was processed through fermentation using mature coconut meat ($\pm$12 month) because its fat content was higher than young coconut so that the oil recovery obtained would be higher (Appaiah *et al.*, 2015). Coconut milk cream is the upper layer of coconut milk contain fats and proteins which used to produce VCO.

Plackett-Burman design was used to determine the significant parameters of nine parameters (presented in Table 2) that influence the production of VCO through enzymatic fermentation using *N. sitophila, L. Plantarum* and papain. The result of oil recovery through Plackett-Burman design shown in Table 5.

Total 12 experiments was conducted, the treatment number 10 provides the lowest yield (no oil obtained), it may because some factors made the destabilization of coconut milk did not work, such as the pH fermentation was not suitable and time of fermentation was too short. Whereas the treatment number 3 provides the highest yield (35.0%). The percentage of oil recovery results were processed using Minitab 17® and the following equation is obtained:

$$Y = 71.4 - 3.39X_1 + 8.27X_2 + 13.28X_3 - 0.044X_4 - 0.614X_5 - 3.870X_6 - 2.29X_7 + 0.000393X_8 - 0.247X_9 \quad (1)$$

where X_1 = *L. plantarum* inoculum concentration (%v/v); X_2 = *N. sitophila* inoculum concentration (%v/v); X_3 = papain activity (U); X_4 = time (h); X_5 = incubation temperature (°C); X_6 = pH; X_7 = pasteurization temperature (°C); X_8 = centrifugation speed (rpm); X_9 = centrifugation time (minutes); Y = oil recovery (%).

Based on the ANOVA stepwise Plackett-Burman design with 90% confidence level, Figure 1 shows that only three factors are significant which has *p* value<0.1, namely the papain (p = 0.046), incubation temperature (p = 0.064), and pH (p =0.059). The three factors could be significant for fermentation process because extracellular protease may work faster to break the peptide bonds than protease from a microbe, whereas incubation temperature and pH must be significant because it is important to be regulated as microbes and enzyme has their optimum temperature and pH. Therefore, these three factors were then used to proceed to the fermentation stage using RSM to optimize the response of the VCO recovery.

Optimization of VCO Production through Response Surface Methodology

Figure 1 shows the independent parameters obtained from Plackett-Burman analysis (papain activity, incubation temperature, and pH fermentation) that will use for RSM design. In addition to the three varied variables, other factors were made fixed, namely inoculum concentration of *N. sitophila* (10% v/v), inoculum concentration of *L. Plantarum* (10% v/v), fermentation time (48 hours), pasteurization temperature (75°C), centrifugation speed (4000 rpm), and centrifugation time (10 min)

Result of actual and predicted oil recovery by using RSM-CCD shown in Table 6. Total of 20 experiments was conducted, the treatment number 20 (papain enzyme activity 1000 U, incubation temperature 50°C, and pH fermentation 5.5) provides the highest yield (45.3%). Whereas the treatment number 14 (papain enzyme activity 500 U, incubation temperature 60°, and pH fermentation 7) provides the lowest yield (29.2%). In comparison with the results of Soxhlet (51.22%), the recovery of VCO was more moderate. This indicates that the process of preparation of coconut milk was not optimum, which cause the oil not extracted entirely from grated coconut. The regression analysis showed that the second-order polynomial model represented the relationship between significant parameters and response with *p*-value <0,05. The smaller *p*-value the notable is the appropriate variables (Masyithah, 2017).

The Anova results show the most significant variables are a linear term of pH (X_3) with *p*-value 0.003 and the quadratic term of pH (X_3 X_3) with *p*-value < 0.001. Both linear or quadratic term of variables that have *p*-value $\leq$ 0.05 is significant for the fermentation process. In contrast, the other variables such as a linear term of papain activity (X_1), linear term temperature (X_2), a quadratic term of papain enzyme (X_1 X_1), a quadratic term of temperature (X_2X_2), the interaction between papain enzyme and temperature (X_1 X_2), the interaction between papain enzyme and pH (X_1 X_3), and interaction between temperature and pH (X_2 X_3) have a *p*-value $\geq$ 0.05 which means these variables have no significant effect to the acquisition of VCO as shown in Table 7.

The best fitting of response function is represented by the following equation 2 processed using Minitab 17® software:

$$Y = -39.2 + 0.0048\,X_1 + 1.219\,X_2 + 21.17\,X_3 - 0.000005\,X_1^2 - 0.00859\,X_2^2 - 1.639\,X_3^2 + 0.000103\,X_1X_2 + 0.00024\,X_1\,X_3 - 0.1039\,X_2\,X_3\ \ldots\ldots\ldots \quad (2)$$

where Y is the percentage of oil recovery, and X_1, X_2, X_3 are papain enzyme activity, incubation temperature, and pH fermentation, respectively.

Analysis of Response Surface

The two-dimensional contour plot, as shown in Figures 2, 3 and 4 was obtained using Minitab 17® software which describes the interactive effect between independent parameters in fermentation at respective zero levels. Figure 2 shows the interactive effects of papain enzyme activity and incubation temperature. The surface plot in Figure 2 indicates that the oil recovery could reach up to 45% at the middle papain enzyme activity and moderate incubation temperature. Figure 2 describes that the increase of incubation temperature and the decrease of enzyme activity would reduce the amount of oil recovery. The green colour in the figure illustrates when the colour becomes darker, the higher oil recovery can be obtained. The optimal oil recovery from Figure 2 is >45% at papain enzyme activity 1000-1100 U and incubation temperature 42-45°C.

Papain is a protease that can hydrolyze peptide bonds in protein molecules which surround oil in

coconut milk, the more proteolytic enzymes, the more oil can be extracted, but in this research an optimum enzyme which is used around 1000 U/300 mL. For the incubation temperature, the increase in temperature will decrease oil recovery because the microbes were used in fermentation could be dead.

The relationship between papain activity and pH with oil recovery is presented in Figure 3. The surface plot describes that moderate pH fermentation around 5 (not too acid or basic) and papain enzyme activity around 1000-1200 U/300 mL significantly increased the oil recovery up to 45%.

Microbes and enzyme have an optimum pH that influences its ability to work. If the pH fermentation is not suitable, the fermentation would not be optimal. Figure 4 shows the relationship between incubation temperature and pH with oil recovery. The pH fermentation 4.6-5.5 and incubation temperature 40-52°C could be achieved of oil recovery > 45%.

The estimated optimum value of VCO production was obtained by analyzing the surface and contour plots. The optimization was carried out to get the VCO with the most acquisition and was conducted three replications to obtain satisfactory results. The optimum value of the papain enzyme activity, incubation temperature, and pH were 1042.47 U, 46.43°C and 5.07, respectively, which would produce an oil recovery of 45.28% as shown in Figure 5.

The amount of papain used in this experiment based on the optimization was 1042.47 U for 300 mL of coconut milk cream. The optimum incubation temperature used in the present experiment was 46.43°C. This value is around the mid-value between the optimum temperature for *L. Plantarum* (±40°C) and the optimum temperature of the papain (50-60°C) (Noori *et al.*, 2016; Winarti *et al.*, 2007). While the optimum pH is 5.07, which is in agreement to the optimum pH of papain reported by Kusumadjaja and Dewi (2005), who reported that the activity of the papain enzyme increases when the pH ranges were 5-6 but decreased the activity when the pH is in alkaline condition, and also is per Noori *et al.* (2016), who reported that the optimum pH of *N. sitophila* and *L. Plantarum* which ranges from 5-7.

Research on optimum conditions carried out with three repetitions resulted in the acquisition of VCO of 44.67%, 45.50%, and 45.00%, as shown in Table 8. The average optimization result of oil recovery was 45.06 ± 0.42%, which has approached the target value of 45.28%. It means that the Equation 2 model can be quite good because the estimated value is per the response of practical VCO acquisition.

Physicochemical Properties of VCO

The physicochemical of VCO comply the APCC (2009) requirement, as shown in Table 9. All the measurement of physicochemical properties of VCO fulfilled the criteria established by APCC standard, except the moisture content (0.13%) which is a little bit higher than the recommended value by APCC (max 0.1%). It may because of the separation between VCO and its wastewater did not complete.

Moisture content is vital in determining the quality of the oil produced. It must be kept as low as possible to increase oil resistance to prevent hydrolysis and the process of rancidity (Nurah *et al.*, 2017). Water in the oil can cause a hydrolysis reaction that can cause the oil to smell rancid because the oil turns into ketone compounds. High levels of water in oil can also give the higher FFA in oil because the oil becomes hydrolyzed (Anwar and Salima, 2016). Fatty acids are produced through triglyceride hydrolysis reaction caused by water, enzymes and microbe activity and play a role in the taste and aroma of oil (Anwar and Salima, 2016). The other characteristic that can increase the process of rancidity is a peroxide number. The peroxide number in coconut oil shows the oxidative levels, so it must be kept lower to give better oil quality (Marina *et al.*, 2013).

In this experiment, VCO obtained was colourless and also had aroma and taste like fresh coconut. Because of all the measurement of physicochemical properties of VCO fulfilled the requirements established by APCC standard, it means that VCO obtained has a good quality and may have a long storage time because of its low moisture content, FFA, and peroxide value.

Fatty Acid Analysis

The chromatograms of VCO sample and standard FAME is presented in Figures 6 and 7. The standard FAME has fatty acid components with known concentration, so to get the type of fatty acids from a peak that appears on the chromatogram was conducted by comparing it to standard retention time and comparing its area to standard to determine the concentration of each fatty acid.

In Figure 6, eleven peaks of standard Larodan were read on the chromatograms with different retention times, namely caprylic acid (4.432), capric acid (7.080), lauric acid (10.869), myristoleic acid (15.450), myristic acid (15.684), palmitoleic acid (18.330), palmitic acid (18.623), linoleic acid (20.493), oleic acid (20.593), stearic acid (20.818), and eicosanoic acid (22.664), respectively.

Whereas in the VCO sample Figure 7, only eight

peaks were detected as presented in Table 10. Three fatty acids were not found in the VCO sample, are myristoleic acid, palmitoleic acid, and eicosanoic acid.

The content of lauric acid in this study was higher than lauric acid produced by Nurah *et al.* (2017), who used *L. Plantarum* (48.94%), *N. sitophila* (48.06%), and Mansor *et al.* (2012), who used 0.1% (w/w) papain (46.36%). Therefore it is clear that lauric acid content of VCO produced by a fermentation process using a mixture of *N. sitophila*, *L. Plantarum* and papain produced higher lauric acid compared to the application of single microorganisms or protease alone.

CONCLUSIONS

Papain activity, incubation temperature, and fermentation pH were found as significant factors affecting VCO recovery according to Plackett-Burman design in coconut milk fermentation process. The optimum conditions for the VCO production process which was determined using RSM-CCD found that optimum condition was achieved when papain activity, incubation temperature, and pH were adjusted to 1042.47 U, 46.43°C, and pH 5.07, respectively. The recovery of VCO at this condition was 45.06% $\pm$ 0.42%, with 56.7% of lauric acid content.

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Competing Interest Statement

The authors declare that they have no conflict of interest for this study.

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