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Original Article

OPTIMIZATION OF *LACTOBACILLUS PLANTARUM* FERMENTATION FOR ENHANCED PHENOLIC PRODUCTION FROM EXTRACT OF BAJAKAH BARK (U*NCARIA NERVOSA*) BY RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Objective: This research was purposed to optimize the fermentation medium and condition of *Lactobacillus plantarum* to enhance the production of phenolic compounds with the antibacterial activity tested against *Propionibacterium acnes* and the optimum conditions of bajakah bark fermentation using Response Surface Method (RSM) methods.

Methods: The antibacterial activity test ethanolic extract of bajakah bark was assayed using the agar diffusion perforator method and fermentation method using *L. plantarum*. Measurement of phenolic content fermentation and without fermentation was done using the standard methods with gallic acid as standard. The optimum conditions of bajakah bark fermentation using RSM methods using Software Design Expert 10.0.0.1.

Results: As the result, Response surface analysis revealed the optimum values of the tested significant variables for the production of phenolic were extracted in a concentration 40% (w/v) and 30% (w/v) sucrose for 3 d fermentation period. Under this optimal condition, the phenolic compounds were improved from 10.66 to 18.46 mg GAE/g extract. As well as the antibacterial activity of the fermented extract was increased by 1.35 times compared to the non-fermented extract.

Conclusion: In summary that, the optimized fermentation condition could be helpful for the production of antibacterial metabolites from bajakah bark by *L. plantarum*.

Keywords: Bajakah, *Propionibacterium acnes*, *Lactobacillus plantarum*, Phenolic, Response surface methodology

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INTRODUCTION

Kalimantan is an Indonesian island with a diverse biological wealth. Because it is difficult to obtain health services in remote areas, local people have relied on traditional medicinal plants for generations, which are believed to cure diseases [1]. The Bajakah is one of the plants empirically used as traditional medicine by the specific ethnic of Kalimantan [2]. This plant has traditionally been used to treat rheumatism, hyperpyrexia, hypertension, and headaches. Uncaria has yielded over 200 compounds, including indole alkaloids, triterpenes, flavonoids, phenols, and phenylpropanoids [3]. Aside from being the previously mentioned treatment, this compound can also act as an antibacterial. Antibiotics are commonly used in medicine to treat bacteria. Clinicians commonly use antibiotics to treat a variety of infectious diseases [4]. *Propionibacterium acnes* is a bacterium that is commonly found on human skin, particularly in the sebaceous region, and it dominates the skin's poly sebaceous follicles [5]. *P. acnes*, on the other hand, is known to be the pathogen responsible for acne vulgaris[6]. Natural anti-acne candidates can be obtained by utilizing microbes that use fermentation technology to increase levels of secondary metabolites as antibacterials in medicinal plants. In the uncaria genus, phenolic compounds act as antibacterials [7].

The bajakah plant is difficult to reach and must be preserved because it has medicinal properties, so a small amount of raw material with medicinal potential will be used by fermenting lactic acid bacteria because lactid acid bacteria can produces lactid acid to inhibition other bacteria [8]. Fermentation is a biocatalytic reaction that converts raw materials into products. Bacteria, yeast, and fungi are used as biocatalysts. The bioactive content, microbiological activity, and enzymes are all affected by fermentation [9]. The Response Surface Method (RSM) is an optimization method that can measure the relationship between the variables tested. Optimal conditions are measured by the RSM because it is a strategy to find the optimum response [10]. The purpose of this study was to determine which part of the bajakah plant has the best antibacterial activity, to determine the effect of sucrose concentration on increasing antibacterial activity, and to optimize the variables that increase the diameter of the inhibition zone using the Box-Behnken RSM experimental design, as well as to study the interaction between each variable. Until now there has been no research regarding the optimization of fermentation methods in Bajakah plants to increase their bioactivity.

MATERIALS AND METHODS

Materials

The materials used were ethanol extract of bajakah bark, *Lactobacillus plantarum* ATCC 8014 bacteria, *Propionibacterium acnes* ATCC 11828 bacteria, sucrose (Merck), De Man Rogosa and Sharpe Broth (MRSB) media (Merck), Mueller Hinton Agar (MHA) media (HIMEDIA), aquades (Smart-Lab), gallic acid (Sigma-Aldrich), Folin Ciocalteu reagent (Merck), $NaCO_3$(Merck). Ethanol absolute (Merck).

Sample collection, processing, and extraction

The bark of the bajakah plant is cleaned using water flow and dried, then cut into small pieces. The bark that has been cut is then ground into powder. Bajakah stem powder was sieved using an aluminum sieve (1 mm) until particles of uniform size were obtained. The powder was weighed 500 g and macerated in 4L of 96% ethanol. Next, the macerate is stored in a maceration container for 3 x 24 h and after 6 h the macerate is stirred and closed tightly for 18 h. Then the macerate is stored for 24 h, then the solvent is added to replace the collected solvent, then closed again tightly. The all macerate is then concentrated using a rotary evaporator until a thick extract is obtained [11].

Preparation of bacterial suspension

1 ose *P. acnes* colony was taken from agar media containing MHA and saved in±2 ml of 0.95% sterile physiological NaCl. Next, homogenize the bacterial suspension using room temperature. The turbidity of the suspension that occurred was then compared with McFarland's 0.5 solution, reaching the equivalent of 1.5 x 10^8 cfu/ml [12].

Antibacterial activity test of the ethanol extract of bajakah bark

The 10% DMSO was mixed with the extract until it reached the desired concentration of 40% w/v. 20 µl of bacterial suspension was put into a sterile petri dish and 20 ml of MHA media was added to homogenize and solidify. After solidifying, the test media was perforated with a perforator with a diameter of 8 mm. Then add 50 µl of test extract. The test medium was incubated at 37 °C for 24 h and the diameter of the inhibition zone was measured using a caliper [13].

Antibacterial activity test of fermented ethanol extract of bajakah bark

The fermented extract was carried out by adding sucrose (20-40% w/v), bajakah extract (20-40% w/v), lactic acid bacteria suspension (6% v/v), and MRSB. Then incubated for 24 h at 42 °C, then supernatant was taken and centrifuged at 4000 rpm for 15 min, then pasteurized at 65 °C for 30 min. Then tested for antibacterial by well diffusion after that, it was incubated for 24 h at 37 °C.

Measurement of total phenolic content of the ethanol extract of bajakah bark

A. Preparation of gallic acid calibration curve with phenol Folin Ciocalteu reagent

Weight 50 mg of gallic acid, 1 ml of 96% ethanol was added, then distilled water was added to a final volume of 50 ml, so that a concentration of 1 mg/ml was obtained. 1.75 ml and 2 ml, respectively, then diluted with distilled water to a final volume of 10 ml to produce a concentration of 100 µg/ml gallic acid, respectively. Each concentration of solution 0.2 ml of gallic acid was pipetted and then add 15.8 ml of distilled water and 1 ml of Folin Ciocalteu reagent and shaken until homogeneous, kept for 8 min. 3 ml of 10% Na_2CO_3 solution was added, then shaken homogeneously, and then allowed to stand for 2 h at room temperature [14].

B. Determined total phenol content with the Folin Ciocalteu method

The extract 100 mg weighd of the extract then dissolved up to 10 ml with water flow to obtain a 10 mg/ml concentration. Concentration of 10 mg/ml, pipetted 1 ml and dilute it with distilled water up to 10 ml and obtain an extract concentration of 1 mg/ml. pipette 0.2 ml of extract, and 15.8 ml of distilled water and 1 ml of Folin Ciocalteu reagent were added and shaken. Allowed to stand for 8 min and then 3 ml of 10% Na_2CO_3 was added to the mixture. Leave the solution for 2 h at room temperature. Measured absorption with a UV-Vis at a maximum absorption wavelength of 765 nm [14].

C. Maximum wavelength setting

The absorption of the solution was measured with a UV-Vis spectrophotometer with 100 µg/ml gallic acid as a comparison at a wavelength of 500-700 nm, then a calibration curve for the relationship between gallic acid concentration (µg/ml) and absorption was made.

Optimization of variable fermentation of ethanol extract of bajakah bark with box-behnken RSM experiment design

The variables that increase the diameter of the inhibition zone are further investigated for the interaction between each factor, and the optimum value for extract fermentation with the largest inhibition zone diameter is determined. The Box-Behken RSM experimental design includes not only an upper limit (+1) and a lower limit (-), but also a middle limit (0). In addition, the ANOVA statistical test will be carried out to determine whether the results are significant. Furthermore, the optimum value of each variable is predicted in order to obtain the optimum diameter of the inhibition zone [15].

RESULTS AND DISCUSSION

Antibacterial activity testing ethanol extract of bajakah bark

The antibacterial activities ethanol extracts of bajakah bark are demonstrated in table 1. Zone of inhibition antibacterial activity test ethanol extracts of bajakah bark with 3 replications.

Table 1: Antibacterial activity ethanol extract of bajakah bark

Part	Replication 1 (mm)	Replication 2 (mm)	Replication 3 (mm)	Average inhibition zone (mm)
Bark	14.2	15	14.5	14.56±0.4

Based on the result of the antibacterial activity test, the ethanol extract of bajakah bark produced an inhibition zone diameter of 14.56±0.4 mm at an extract concentration of 40% (w/v). Antibacterial activity on the bark of bajakah plant is in a strong category. Based on Strout and Davis (1971) state that the level of inhibition of bacterial growth if the inhibition zone is 5 mm or less then the level of inhibition is categorized as weak, 5-10 mm is categorized as moderate, 10-19 mm is categorized as strong, and 20 mm or more is categorized as very strong [16]. According to [17], various plants of the genus Uncaria have cytotoxic, anti-inflammatory, antiviral, immunostimulant, antioxidant, and antibacterial properties. No previous studies have investigated the antibacterial activity of the bajakah bark.

Antibacterial activity of fermented ethanol extract bajakah bark

Zone of inhibition antibacterial activity test of fermented extracts using optimum RSM results. Based on method using variable sucrose concentration (20-40% w/v), extract concentration (20-40% w/v) and fermentation time (1-3 d). The fermented extract inhibition zone based on the optimum conditions of RSM was 19.66 mm, with optimum conditions were 40% (w/v) extract concentration, 30% (w/v) sucrose concentration, 3 d fermentation time.

Table 2: Antibacterial activity of fermented ethanol extract bajakah bark

Condition	Fermentation time (days)	Extract concentration (20-40% w/v)	Sucrose concentration (20-40% w/v)	Inhibition zone (mm)*
1	2	20	20	8.13±0.13
2	2	20	40	12.5±0.5
3	3	30	20	11.0±0.00
4	1	40	30	9.46±0.06
5	1	30	20	8.90±0.00
6	3	40	30	19.66±0.05
7	2	30	30	9.03±0.04
8	2	30	30	9.20±0.1
9	2	40	20	8.33±0.05
10	2	40	40	9.03±0.03
11	2	30	30	8.80±0.15
12	1	30	40	9.80±0.00

Condition	Fermentation time (days)	Extract concentration (20-40% w/v)	Sucrose concentration (20-40% w/v)	Inhibition zone (mm)*
13	1	20	20	8.80±0.11
14	3	30	40	12.66±0.27
15	2	30	30	9.40±0.1
16	2	30	30	9.50±0.2
17	3	20	30	12.03±0.01

*Notes: All assays were done in three replications (n = 3)

Fermentation affects the bioactive content, microbiological activity, and enzyme [18]. Based on the results obtained, the antibacterial activity of the fermented extract increased by 1.35 times compared to the nonfermented extract. Increase in antibacterial activity from 14.56±0.4 mm to 19.66±0.11 mm. This is also in line with the opinion [19] which states that fermentation can increase biological activity. Fermentation in this study used *L. plantarum* bacteria which is thought to be one of the factors for successful fermentation. In line with the results of research [20], which stated that fermentation using *L. plantarum* could increase the antibacterial activity of black garlic.

Measurement total phenolic content of the ethanol extract bajakah bark

Table 3: Result of determination phenolic content ethanol extract of bajakah bark

Part	Replication 1 (mg GAE/g Extract)	Replication 2 (mg GAE/g Extract)	Replication 3 (mg GAE/g Extract)	Phenolic content (mg GAE/g Extract)
Bark	10.57	10.66	10.75	10.66±0.09

Absorbance with 3 replications ethanol extract of the bajakah bark was 0.118±0.001. Determination of phenol content is based on from the calibration curve, the regression equation y = 0.0011x+0.0007 was obtained and the coefficient of determination (R^2) was 0.9907 which means that 99.0% absorption was affected by concentration. as shown in (fig. 1).

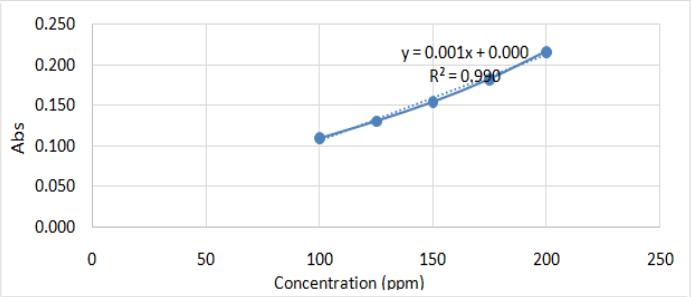

Fig. 1: Gallic acid callibration curve on phenol test (n = 5)

Table 4: Result of determination TPC fermented ethanol extract of bajakah bark

Part	Replication 1 (mg GAE/g extract)	Replication 2 (mg GAE/g extract)	Replication 3 (mg GAE/g extract)	Total phenolic content (mg GAE/g extract)
Bark	18.46	18.29	18.63	18.46±0.17

Based on fig. 1, the linear regression equation y = 0.0011x+0.0007, R^2= 0.9907 curve creation. This calibration is useful to help determine the phenol content in the sample through the regression equation of the calibration curve, y = 0.0011x+0.0007, and the price of the coefficient of determination (R^2) = 0.9907. The value of R^2 which is close to 1 proves that the regression equation is linear [21]. The concentration of the sample solution can be determined using a calibration curve by measuring the absorbance of the sample, and then the total phenolic content of the extract of bajakah bark is calculated by using the linear regressions equation. Based on the linear equation above, the equivalent levels of gallic acid with ethanol solvent are obtained. Based on the results of this study, the total phenolic content of the ethanol extract of bajakah bark was 10.66 mg GAE/g extract, meaning that in every gram extract ethanol of bajakah bark, there is phenolic equivalent to 10.66 mg of gallic acid. Phenolic compounds contained in ethanol extract bajakah bark are secondary metabolites. Total phenolic content of fermented ethanol extract bajakah bark was 18.46 mg GAE/g extract, meaning that in every gram ethanol extract of bajakah bark there is phenolic equivalent to 18.46 mg of gallic acid. Phenolic compounds contained in ethanol extract of bajakah bark are secondary metabolites.

Optimization of variable fermentation of ethanol extract of bajakah bark with box-behnken RSM experiment design

The Box-Behnken RSM experimental design was used to determine the optimum values for the three variables affecting the diameter of the inhibition zone in extract fermentation. Table 5 illustrates three variables that increase the diameter of the inhibition zone.

Table 5: Variables in RSM box-behnken experimental design

No	Variable	Code	Unit	Limit		
				Lower (-1)	Middle (0)	On (+1)
1	Fermentation Time	A	Day	1	2	3
2	Extract Concentration	B	%	20	30	40
3	Sucrose Concentration	C	%	20	30	40

Table 6 shows how the resulting increase in the diameter of the actual inhibition zone (R1) was entered into the experimental design table for the three variables A (fermentation time), B (extract concentration), and C (sucrose concentration).

Table 6: Obtaining the diameter of the inhibition zone (mm) (R1) from the experimental results and predicting the diameter of the inhibition zone for optimization of fermentation with the box-behnken RSM experimental design (R2)

run	A	B	C	R1 *Actual*	R1 *Predicted*
1	0	-1	-1	8.13	8.44
2	0	-1	+1	12.50	12.19
3	+1	0	-1	11.00	10.88
4	-1	+1	0	9.46	9.46
5	-1	0	-1	8.90	8.40
6	+1	+1	0	19.66	19.76
7	0	0	0	9.03	9.19
8	0	0	0	9.20	9.19
9	0	+1	-1	8.33	8.64
10	0	+1	+1	9.03	8.72
11	0	0	0	8.80	9.19
12	-1	0	+1	9.80	10.30
13	-1	-1	0	8.80	8.80
14	+1	0	+1	12.66	12.78
15	0	0	0	9.40	9.19
16	0	0	0	9.50	9.19
17	+1	-1	0	12.03	12.03

From the results of the diameter of the inhibition zone for each experiment, the data acquisition was run in Software Design Expert 10.0.0.1. The results showed that the three variables, including fermentation time (A), extract concentration (B), and sucrose concentration (C) had a significant increase in the diameter of the inhibition zone in extract fermentation. The AB interaction shows a fairly high significant interaction on the acquisition of inhibition zone diameter compared to the BC interaction which is shown in (fig. 2).

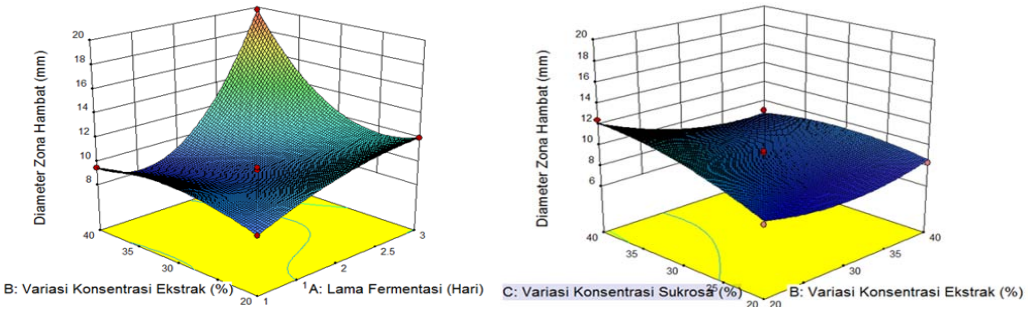

Fig. 2: 3D Surface interaction between AB (left) and BC (right) variables in the Box-behnken RSM experimental design

Factor	Name	Levels	Low Level	High Level	SDT. Dev	Coding
A	Fermentation Time	3.00	1.00	3.00	0	Actual
B	Extract Concentration	40.00	20.00	40.00	0	Actual
C	Sucrose Concentration	30.00	20.00	40.00	0	Actual

	Predictions	Predictions				CI for Means		99% of Population	
Response	Means	Median	Observation	SDT. Dev	SE Means	95%CI Low	95%CI High	95%CI Low	95%CI High
Inhibition Zone Diameter	19.76	19.76	19.66	0.456405	0.456405	18.6432			

Fig. 3: Prediction of optimum fermentation for inhibition zone diameter (19.76 mm) with variable A (time fermentation), B (extract concentration), and C (sucrose concentration)

The relationships between inhibition zona diameter (*Y*) and the tested variables were obtained by application of RSM. By employing multiple regression analysis on the experimental data, the response variable (*Y*) and the tested variables can be related by the following second-order polynomial equation, e. i. inhibition zone diameter = 9.19+1.24*A–0.82*B+0.95*C+1.77*AB–0.92*BC+2.21*A2+1.12*B2–0.81*C2+2.92*A2B+2.14*AB2. Where Y was the predicted inhibitor zona diameter, A was fermentation time, B was extract concentration, and C was sucrose concentration. ANOVA testing with desain expert 10.0.1 showed that coefficient determination (R^2) of extract fermentation was 0.9900, which means that 99%. Coefficient determination (R^2) value must be in the range 0-1. Coefficient determination (R^2) value is to 1, the better model will be and the respon prediction better.

RSM using Box-Behnken design (BBD) was employed to determine the optimal levels of the three selected variables. The respective levels with the coded levels for the factors are listed in table 5.

The experimental design and result are shown in table 6. Based on result analysis of variance (ANOVA), the high model *F*-value (59.50) and low *p*-value (<0.05) implied the model was highly significant. The fitness of the model could be examined by the coefficient of determination R^2 [22], which was calculated to be 0.9900, indicating that 99.00% of the sample variation was attributed to the variables and only less than 1% of the total variance could not be explained by the model. A regression model, having an R^2 value higher than 0.9 was considered as having a very high correlation. Therefore, the present R^2-value reflected a very good fit between the observed and predicted responses and implied that the model is reliable for zona inhibitor diameter in the present study. The extract fermentation coefficient (R^2) is 0.9900, indicating that 99.00% of the model's variance can be explained. The value of R^2 must be in the range 0-1. The closer R^2 is to 1, the better the model and the better the response prediction.

CONCLUSION

Bajakah bark has antibacterial activity against *Propionibacterium acnes* was 14.56±0.4 mm. Fermentation methods can increase the content of phenolic compounds thereby increasing antibacterial activity. The optimum conditions obtained by optimizing the Behnken RSM Box using Software Design Expert 10.0.0.1 for extract fermentation are 3 d of fermentation, 40% extract concentration and 30% sucrose concentration with a predicted inhibition zone diameter of 19.76 mm.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The Authors declare that there are no conflicts of interest in this article.

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