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OPTIMIZATION OF BACTERIOCIN PRODUCTION FROM *Lactobacillus plantarum* IN05 BY USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Bacteriocins are ribosomally synthesized antimicrobial compound. Response surface methodology (RSM) is a combination of statistical and mathematical techniques used to create model and to analyze a response that is influenced by several factors. The objective of this study was to determine the optimum condition using RSM to obtain optimal bacteriocin production from *Lactobacillus plantarum*. Activity test was performed using well diffusion agar method. The four experimental factors, *i.e.* glucose concentration, pH, temperature and concentration of yeast extract with 30 treatment. The results indicated that the optimum condition of bacteriocin production from *L. plantarum* IN05 showed optimum inhibition activity against *Salmonella typhimurium* and *Listeria monocytogenes*, with the addition of 4 g/L glucose, 8.11 g/L yeast extract, pH of 5.3, and temperature of 30°C. The highest bacteriocin activities against *S. typhimurium* and *L. monocytogenes* were 3136 AU/mL and 2426 AU/mL, respectively.

Keywords: Bacteriocin production, *Lactobacillus plantarum* IN05, Optimization, Response surface methodology (RSM)

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial compound that is often used as a biopreservative agent because of its bactericidal effect against various types of pathogenic bacteria such as *Bacillus, Staphylococcus,* and *Listeria*. The use of bacteriocin in foods may prolong the shelf life without eliminating or reducing the nutritional value of the food. In addition, bacteriocin is a peptide compound that can be degraded by protease enzymes found in the intestine, making it safe for consumption because it leaves no residue (Gálvez et al. 2007).

Bacteriocins are generally produced by various types of lactic acid bacteria (LAB) safely consumed or more commonly known as Generally Recognized as Safe (GRAS) (Parada et al. 2007). Most LABs are isolated from fermentation products such as yogurt, bekasam (Desniar et al. 2011), and inasua (Mahulette et al. 2015). Inasua is a typical fish fermentation product of the people of Central Maluku (Teon, Nila, and Serua). Research conducted by Nara et al. (2013) isolated three genera of lactic acid bacteria from inasua are *Bacillus, Propionibacterium,* and *Lactobacillus*.

Research conducted by Mahulette et al., (2016) isolate some LAB's bacteria from inasuua are *L. rhamnosus* IN13, *L. plantarum* IN05 and *Leuconostoc mesenteroides* ITN17. Maulidayanti (2018) has characterized bacteriocin produced by *L. rhamnosus* IN13. The characteristics are resistant to cold temperatures up to 40 °C. After incubation for 4 weeks, remains stable in the range of pH 2.0 to 10, stable against the addition of salt with various concentrations, stable against several types of surfactants. However, there has been no research on the optimization of bacteriocin production produced by LAB's isolates from inasua product. This study is a follow up research from previous research to determine the optimum conditions of bacteriocin production from LAB's isolates from inasua product.

Bacteriocin produced from LAB is strongly influenced by environmental conditions such as incubation time, temperature, pH of media, carbon source, nitrogen source and NaCl concentration (Zamhir et al., 2016). Bacteriocin production increases along with increasing bacterial growth factors then decrease after reaching the optimum conditions (Subagiyo et al. 2015). Therefore, it is necessary to conduct research to find out the optimum condition of bacteriocin production in isolate from inasua. Therefore, Response surface methodology (RSM) is an appropriate method used in determining the optimum conditions required in a process to obtain optimal yields (Kaur et al., 2013).

Response surface methodology is a combined method of statistical and mathematical techniques used to create the model and to analyze a response influenced by several factors. This method has several advantages compared with conventio-

nal methods since it possible to determine the interaction between variables and the optimum point (Box et al. 1978) so that the optimization process is expected to be faster and more accurate. Many research by using RSM in various optimization process has been done such as optimization of protease enzyme produced by *Lactobacillus plantarum* (Nurtika et al., 2015) and medium optimization of β-glucanase production from *Bacillus subtilis* SAHA 32.6 (Dewi et al., 2016). Malheiros et al., (2015) reported that 4 most influential factors on bacteriocin production isolated from *Lactobacillus sakei* are glucose, tween 20, pH, and temperature. The optimization process was done by using RSM and the optimum condition of bacteriocin production was obtained at 25ºC, pH of 6.28, the glucose concentration of 5.5 g/L and Tween 20 concentration of 1.05%. Thirumurugan et al., (2005) reported that the optimum conditions for the growth of *Lactobacillus plantarum* ATMII bacteria using response surface methodology were 12.1 g/L yeast extract and 2.5 g/L Tween 80. Utilization of RSM in growth optimization has been frequently performed and successfully increased the production of bacteriocin (Venigalla et al., 2017). However, there has been no research on the optimization of bacteriocin production using RSM with bacteria isolated from inasua. The study aimed to determine the optimum condition for bacteriocin production from *L. plantarum* IN05 by using RSM.

MATERIALS AND METHODS

Lactic acid bacteria isolate: Lactic acid bacteria isolate used is *Lactobacillus plantarum* IN05 which was isolated from inasua, fish fermentation product from Teon, Nila and Serua area in Seram Island, Central Maluku, Indonesia (Mahulette et al., 2016). The isolate was collected in the laboratory of Microbiology, Department of Biology, FMIPA-IPB. Bacterial isolate was subcultured on medium de Mann Rogosa Sharpe Agar (MRSA). Isolates used as indicator were *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644.

Production of bacteriocin: Recultured bacterial isolate were inoculated into MRSB media then incubated for 24 hours at 37°C. A total of 1 ml of bacterial culture was then inoculated into 50 mL MRSB and incubated at 37° C for 24 h. A total of 5 mL of bacterial isolates were then centrifuged at 6000 g for 10 min. The supernatant was adjusted to pH of 6.5 and filtered using 0.22 micrometer milipore. The resultant filter was a cell-free neutral supernatant (Choi et al., 2011).

Enzyme sensitivity test: The bacteriocin sensitivity test against protease enzyme was performed using well diffusion agar method. Test on protease enzyme sensitivity was done by adding 10 µL proteinase K (1 mg/mL) into 100 µL cell-free supernatant then incubated for 2 hours at 37°C, subsequently bacteriocin activity was measured (Patil et al., 2011). The control treatment is supernatant without enzyme treatment.

Optimization of bacteriocin production: Factors used in this study were glucose concentration (4-7 g/L), pH (4-8), temperature (20-40ºC) and concentration of yeast extract (4-16g/L). The design used to determine the factors that affect bacterio-cin activity was the central composite design (CCD). The relationship between the response and the free factor is illustrated by the equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2$$

Y is the response factor of bacteriocin activity, X_i and X_j are the free factors tested, β_0 is the intercept coefficient, β_i coefficient for linear effect, β_{ij} coefficient for interaction effect, β_{ii} is coefficient for quadratic effect. After processing the data based on the research design using RSM, the next stage was model validation to obtain the optimum conditions based on the best solution recommended by software; the activity of the rough extract of bacteriocin was then re-tested on the indicator bacteria.

Activity test: The bacteriocin activity test was performed using well diffusion agar method (Venigalla et al., 2017). A total of 50µL of bacteriocin was put into a well on the mueller hinton agar (MHA) medium that had been inoculated with test bacteria with a cell density of 106CFU/ mL, then incubated at 37°C for 24 hours. After 24 hours, the halo zone formed was observed. The bacteriocin activity unit was expressed in Unit Activity (AU/mL). AU/mL is defined as the area of resistance per unit volume of bacteriocin samples tested (Engelhardt et al., 2017). Bacteriocin activity was calculated based on the equation (Usmiati and Marwati 2007):

$$\text{Bacteriocin activity} = \frac{Lz - Ls}{V}$$

Lz: Area of halo zone (mm^2), Ls: area of well (mm^2), V: Volume of sample (mL).

Data analysis: The data obtained was in the form of bacteriocin activity against bacteria *S. typhimurium* and *L. monocytogenes* after process optimization. The data were then analyzed using Design-Expert version 7.0.0. Trial software.

RESULT AND DISCUSSION

Enzyme sensitivity test: This test aimed to see the sensitivity of the bacteriocin compound from the isolates. The results of enzyme sensitivity test on *L. plantarum* IN05 shows the absence of bacteriocin activity when supernatant is treated with Proteinase K (Figure 3). Bacteriocin is a peptide or protein that will be easily degraded by protease enzymes such as proteinase K (Jack et al., 1995), causing no activity. Therefore, it may be assumed that the activity produced by *L. plantarum* IN 05 was a bacteriocin activity.

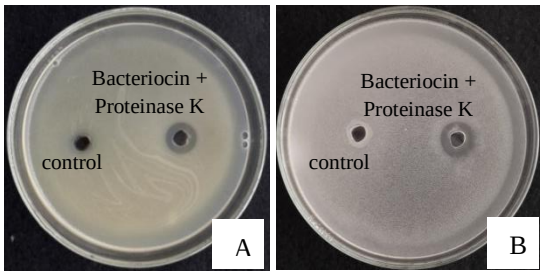


Figure 1. The results of enzyme sensitivity test against pathogenic bacteria (A) *S. typhimurium* and (B) *L. monocytogenes.*

Optimization of bacteriocin production: The results of bacteriocin activity against *S. Typhimurium* and *L. monocytogenes* before optimization were 1476AU/mL and 1291AU/mL, respectively. Optimization of bacteriocin production consisted of 30 experimental units, each activity of the unit was tested against *S. typhimurium* and *L. monocytogenes*. The response obtained was the activity of bacteriocin (Table 1). Data from observation results were computed using Design-Expert 7.0.0. Trial software. The model suggested by the program to both responses is the quadratic model and the accuracy of the quadratic model was tested by variance analysis.

Table 1 Experimental units and result of bacteriocin activity test after optimization.

Run	Factor				Response	
	Glucose Conc. (g/L)	pH	Temperature (°C)	Yeast Extract Concentration (g/L)	Bacteriocin Activity (AU/mL)	
					Salmonella typhimurium	*Listeria monocytogenes*
1	7.0	4.0	20.0	16.0	2081	1988
2	5.5	6.0	30.0	12.0	2624	2557
3	5.5	2.0	30.0	12.0	0	0
4	7.0	8.0	40.0	16.0	1749	1585
5	7.0	4.0	20.0	8.0	2203	2760
6	5.5	6.0	30.0	12.0	2746	2089
7	5.5	6.0	30.0	12.0	2669	2426
8	4.0	8.0	40.0	16.0	2404	1928
9	7.0	8.0	40.0	8.0	1233	1696
10	7.0	8.0	20.0	8.0	2016	2028
11	7.0	4.0	40.0	8.0	1088	889
12	4.0	4.0	20.0	16.0	2377	2171
13	8.5	6.0	30.0	12.0	2760	3160
14	7.0	4.0	40.0	16.0	336	331
15	4.0	8.0	40.0	8.0	2203	1440
16	5.5	6.0	30.0	12.0	2673	2383
17	5.5	6.0	10.0	12.0	952	540
18	4.0	4.0	40.0	16.0	1668	2383
19	7.0	8.0	20.0	16.0	2130	2213
20	5.5	10.0	30.0	12.0	0	0
21	5.5	6.0	30.0	20.0	2340	2255
22	5.5	6.0	30.0	12.0	2783	3306
23	4.0	4.0	40.0	8.0	2436	2130
24	5.5	6.0	50.0	12.0	0	0
25	4.0	8.0	20.0	16.0	1811	1335
26	5.5	6.0	30.0	4.0	2683	2340
27	2.5	6.0	30.0	12.0	3406	2969
28	5.5	6.0	30.0	12.0	3304	3257
29	4.0	8.0	20.0	8.0	1830	1180
30	4.0	4.0	20.0	8.0	3128	2426

The result of the variance analysis for the quadratic model shows that the model is significant ($p < 0.0001$) could explain the data obtained. Test on lack of fit was done to see the suitability between the model and the quadratic design. The result shows that the lack of fit is not significant with the p-value of more than α 0.05, suggesting that the obtained model has conformity with the quadratic design. Thus the quadratic model is a model that represents the response which could be used for the optimization of the bacteriocin production process of the *Lactobacillus plantarum* IN 05 isolate.

Low coefficient variation (CV) values show high experimental accuracy, CV value in this exp-

eriment was 15.79% in first response and 21.92% in second response. The good value of adequate precisions is those more than four; the value of adequate precision which more than 4 of the research indicates that this model can be used to navigate the design space of the surface response. The difference between values between adjusted (Adj) R^2 and predicted R^2 should not exceed 0.2, it indicates that the model is able to determine the optimum conditions of the factors affecting the productivity of bacteriocin.

Optimization of bacteriocin production against *Salmonella typhimurium:* Based on the analy-sis, coefficient of regression R-Squared (R^2) = 0.9440. The value explains that the four factors affect the diversity of responses of 94.40 % the rest is influenced by other factors that are not examined. The concentration of glucose as a carbon source, temperature, and pH source in a linear fashion shows a significant effect. Based on the interaction, the interaction between glucose concentration and pH, glucose concentration and temperature, the interaction between pH and temperature, and interaction between pH and yeast extract concentration have a significant effect on bacteriocin activity. The concentration of glucose, pH, and temperatur has a quadratic effect whereas the concentration of yeast extract does not give a significant effect (Table 2). This suggests that the content of yeast extract of MRS media is the optimum concentration hence the addition of yeast extract does not give a significant effect. In general, temperatures may also cause changes in the 3-dimen-sional structure of the enzyme (Subagiyo et al. 2015). Growth tends to run slowly at low temperatures because enzymes was less efficiently and lipids tend to freeze causing loss of membrane fluidity. The growth rate increases along with the increasing temperature and returns to decrease after until it reaches the optimum temperature.

Table 2: Results of variance analysis of bacteriocin optimization against *Salmonella typhymurium*

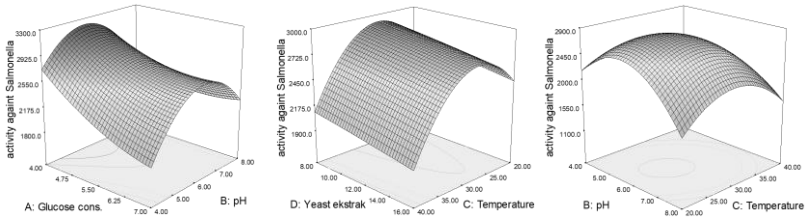
Source	*Sum of square*	*df*	*Mean square*	*F value*	*p-value*
Model	2.520E+007	14	1.800E+006	18.06	< 0.0001*
A-Glucose con.	1.661E+006	1	1.661E+006	16.67	0.0010*
B-Ph	143.67	1	143.67	1.441E-003	0.9702**
C-temperature	1.687E+006	1	1.687E+006	16.93	0.0009*
D-east extract	2.139E+005	1	2.139E+005	2.15	0.1636**
AB	4.841E+005	1	4.841E+005	4.86	0.0436*
AC	8.050E+005	1	8.050E+005	8.08	0.0124*
AD	74763.96	1	74763.96	0.75	0.4001**
BC	1.031E+006	1	1.031E+006	10.35	0.0058*
BD	6.423E+005	1	6.423E+005	6.44	0.0227*
CD	37.09	1	37.09	3.721E-004	0.9849**
A^2	5.189E+005	1	5.189E+005	5.21	0.0375*
B^2	1.100E+007	1	1.100E+007	110.31	< 0.0001*
C^2	7.250E+006	1	7.250E+006	72.74	< 0.0001*
D^2	782.98	1	782.98	7.855E-003	0.9305**
Residual	1.495E+006	15	99673.82		
Lack of Fit	1.174E+006	10	1.174E+005	1.82	0.2629**

*Significant ($p < 0.05$) ** not significant ($p > 0.05$)

The quadratic model that explains the data of bacteriocin activity against *S. typhimurium* is:

$$Y = 584.37891 - 883.88188X_1 + 900.36335X_2 + 288.48884X_3 - 227.37457X_4 + 57.98292X_1X_2 - 14.95333X_1X_3 + 11.39292X_1X_4 + 12.69531X_2X_3 + 25.04453\ X_2X_4 - 0.038062\ X_3X_4 + 61.13318\ X_1^2 - 158.28665\ X_2^2 - 5.14127\ X_3^2 - 0.33393X_4^2.$$

Coefficient X_1, X_2, X_3, X_4 show the concentration of glucose as carbon source (g/L) (X_1), pH (X_2), temperature (X_3) and yeast extract concentration as source N (g/L) (X_4). Plot surface response as the interaction between two factors (of the four tested factors) makes it easy to see the influence and interaction between factors. The relationship between glucose concentration, pH, temperature and concentration of yeast extract in the form of surface response is shown in Figure 2.



A B C

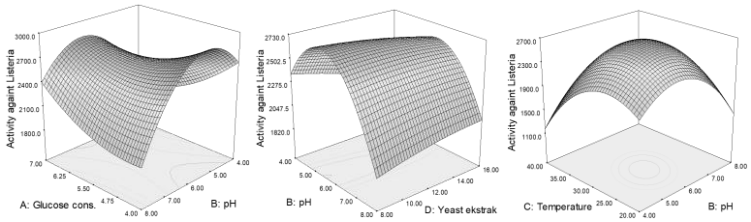
Figure 2. The three-dimensional graphs for the interaction between (A) yeast extract concentration and pH; (B) glucose concentration and temperature; (C) pH and temperature.

Optimization of bacteriocin production against *Listeria monocytogenes:* The optimization result is the response of bacteriocin activity against *L. monocytogenes*. The value regression coefficient of R-Squared (R^2) = 0.9066 shows that 90.66% of four factors affect the diversity of responses. Based on the analysis of variance, suggest that the temperature linearly gives a very significant effect on bacteriocin activity in inhibiting *L. monocytogenes*. In interaction of glucose concentration as carbon source and pH, the interaction between carbon source and temperature and interaction between pH and temperature indicate significant effect. Meanwhile the temperature and pH factors had significant effect quadratically on the response (Table 3). The result are visualized in the form of three dimensions graph, shown in Figure 3.

Tabel 3 Results of analysis variance of bacteriocin optimization for *Listeria monocytogenes*

Source	*Sum of Squares*	*df*	*Mean Square*	*F Value*	*p-value*
Model	2.416E+007	14	1.726E+006	10.39	< 0.0001*
A-Glucose conc.	52316.81	1	52316.81	0.32	0.5828**
B-pH	1.166E+005	1	1.166E+005	0.70	0.4151**
C-temperature	9.592E+005	1	9.592E+005	5.78	0.0296*
D-yeast extract	25637.34	1	25637.34	0.15	0.6999**
AB	1.429E+006	1	1.429E+006	8.61	0.0103*
AC	1.727E+006	1	1.727E+006	10.40	0.0057*
AD	2.252E+005	1	2.252E+005	1.36	0.2623**
BC	7.681E+005	1	7.681E+005	4.63	0.0482*
BD	2.623E+005	1	2.623E+005	1.58	0.2280**
CD	35814.23	1	35814.23	0.22	0.6490**
A^2	7.082E+005	1	7.082E+005	4.27	0.0566**
B^2	1.005E+007	1	1.005E+007	60.55	< 0.0001*
C^2	7.936E+006	1	7.936E+006	47.81	< 0.0001*
D^2	26405.28	1	26405.28	0.16	0.6956**
Residual	2.490E+006	15	1.660E+005		
Lack of Fit	1.248E+006	10	1.248E+005	0.50	0.8341**
Pure Error	1.242E+006	5	2.484E+005		

*Significant ($p < 0.05$) ** not significant ($p > 0.05$)



A B C

Figure 3. The three-dimensional graphs for the interaction between glucose concentration and pH (A); pH and yeast extract concentration (B); pH and temperature (C).

The quadratic model that explains the data of bacteriocin activity against *S. typhimurium* is:

$$Y = 2742.87954 - 519.94367X_1 + 712.76903X_2 + 343.30094X_3 + 15.62550X_4 + 99.60728X_1X_2 - 21.90393X_1X_3 - 19.77465X_1X_4 + 10.95544X_2X_3 + 16.00356X_2X_4 + 1.18279X_3X_4 + 71.41707X_1^2 - 151.34724X_2^2 - 5.37911X_3^2 - 1.93921X_4^2$$

Coefficient X_1, X_2, X_3, X_4 show the concentration of glucose as carbon source (g/L), pH, temperature and yeast extract concentration as N source (g/L), respectively.

Validation of optimum conditions: Based on the predicted model, The bacteriocin activity in inhibiting *S. typhimurium* and *L. monocytogenes* was

3405.64 AU/mL and 2863.5 AU/mL respectively with the addition of 4 g/L glucose, pH of 5.3, temperature of 30ºC and yeast extract concentration of 8.11 g/L. The optimization treatment of bacteriocin production was re-verified for six repetitions. The result of re-verification shows that bacteriocin activity was 3136 AU/mL for activity against *S. typhimurium* and 2426 AU/mL for bacteriocin activity against *L. monocytogenes***.** This value is categorized in the 95% of the maximum bacteriocin activity confidence interval. According to Xu *et al*., (2008) the results of model prediction verification with repeatability accuracy of more than 90% indicates the appropriate use of the model for optimization; however, the rest is infl-uenced by other variables in the media cultivation is not examined (Spolaore et al. 2006). The opti-mum concentration of carbon source, pH, tempe-rature and concentration of nitrogen source proved to increase bacteriocin production against *S. Typh-imurium* by 2.12 times and to *L. monocytogenes* of 1.88 times than that before optimization (**Fig-ure 4**). The optimum condition for the production of bacteriocin is by the application of 4 g/L glu-cose, 8.11 g/L yeast extract, pH of 5.3 and tempe-rature of 30ºC.

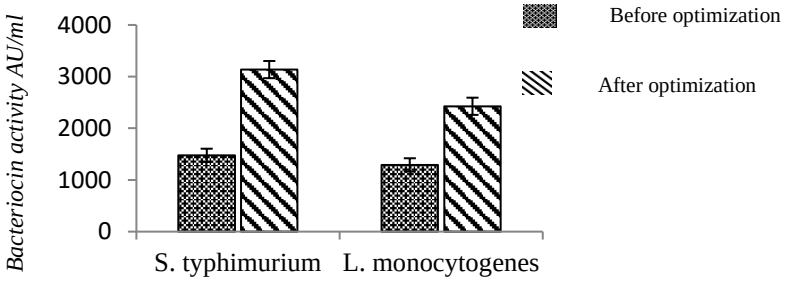


Figure 4. Comparison of bacteriocin activities before optimization and after optimization

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