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Optimum growth conditions of *Lactobacillus brevis* LIPI13-2-LAB131 in β-galactosidase enzyme production

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Abstract. *Indah AWN, Rohmatussolihat, Rahayu WP, Setyoningrum F, Priadi G, Afianti F. 2020. Optimum growth conditions of Lactobacillus brevis LIPI13-2-LAB131 in β-galactosidase enzyme production. Biodiversitas 21: 5403-5407.* Deficiency of β-galactosidase enzyme causes lactose to become undigested in gastrointestinal system, therefore the system needs further addition of external β-galactosidase. The sources β-galactosidase enzyme varies from plants, animals, and microorganisms. In industrial applications, microorganisms have become a considered potential source of β-galactosidase. *Lactobacillus brevis* LIPI13-2-LAB131 had high β-galactosidase enzyme activity, which was 7.93 U/mL. The aim of this research was to optimize the growth condition of *L. brevis* LIPI13-2-LAB131 in order to produce maximum β-galactosidase enzyme activity. This research consisted of performing optimization processes using design expert 7.0 (DX7) program with response surface methodology (RSM) and partial purification of β-galactosidase enzyme. The results of this research showed that the optimum growth conditions of *L. brevis* LIPI13-2-LAB131 were in 1.48% lactose level, incubation temperature of 34.91 °C, incubation time of 48.48 hours, and 2.83% inoculum size with desirability value of 0.839. The result of enzyme purification showed that value of β-galactosidase enzyme activity increased up to 22.88 ± 0.29 U/mL with purification yield of 11.65%.

Keywords: β-galactosidase, *L.brevis* LIPI13-2-LAB131, lactose, optimization, RSM

INTRODUCTION

Lactose intolerance is a condition where there is lack or insufficient amount of lactase enzyme in human body. Lactase is usually known as β-galactosidase, which is an enzyme that can breakdown lactose into simple sugars, namely into glucose and galactose. Lactose intolerance or deficiency of β-galactosidase causes lactose to become undigested and unabsorbed, therefore the increases fluid volume inside small intestine that enters into large intestinal tract, resulting in osmotic pressure differences. Unabsorbed lactose will then be utilized by colon microflora, resulting in short-chain fatty acids, carbon dioxide, hydrogen, and methane, and also causing one or more symptoms like diarrhea, stomach ache, bloated stomach, cramps, and nausea (Shaukat et al. 2010). Consumers with the mentioned symptoms reached up to 70% of world adult population (Chanalia et al. 2018). The sources of β-galactosidase enzyme are varied from plants, animals, and microorganisms (Soares et al. 2001). In industrial applications, microorganisms have become a considered potential source of β-galactosidase (Natarajaran et al. 2012). β-galactosidase in food industry is utilized to reduce the amount of lactose in milk, to prevent lactose crystallization, to increase sweetness, and to increase solubility of dairy products (Princely et al. 2013), and also to reduce lactose in whey as a byproduct from cheese production (Carevic et al. 2015).

β-galactosidase mostly are founded in lactic acid bacteria. According to Somkuti et al. (1998), lactic acid bacteria (LAB) become a focusing enzyme study because of three factors. First, people who suffer from lactose intolerance do not suffer from same symptoms when consuming fermented milk products that contain LAB, LAB is considered safe (Generally Recognized as Safe/GRAS), therefore the enzyme can be utilized without further purification (Valijevic and Jelen 2001). Second, several strains of LAB have probiotic activity such as increasing the digestion of lactose and the selected strains have been utilized in probiotic milk production (Vinderola and Reinheimer 2003). Third, probiotic bacteria have significant role in producing β-galactosidase with high activity (Chanalia et al. 2018). The consideration of selecting LAB for lactose hydrolysis was because of the ease of fermentation, high enzyme activity, and great stability (Picard et al. 2005). *L.brevis* is one of LAB that is mostly found in dairy products. Based on the previous research regarding β-galactosidase enzyme activity in several dairy products conducted by Indonesian Institute of Sciences (LIPI) (2018), *L. brevis* LIPI13-2-LAB131 had a high β-galactosidase enzyme activity, which was 7.93 U/mL. The activity of β-galactosidase enzyme is influenced by growth conditions, such as incubation temperature, incubation time, carbon source, and inoculum size. Therefore, optimization process is needed to determine the optimum condition for *L.brevis* LIPI13-2-LAB131 in producing β-galactosidase. Optimization is commonly done

using conventional method that requires a lot of treatments and a very long time. The alternative method that can be used for optimization process is a response surface methodology (RSM).

MATERIALS AND METHODS

Materials and equipment

Materials used in this research were an isolate of *L.brevis* LIPI13-2-LAB131obtained from Indonesian Culture Collection (InaCC) LIPI, De Man, Rogosa, Sharpe broth (MRSB) media (Merck, Germany), lactose (Fisher Scientific Company, USA), K_2HPO_4 (Merck, Germany), KH_2PO_4 (Merck, Germany), *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) (Thermo Fisher Scientific, USA), Na_2CO_3 (Merck, Germany), ammonium sulfate, dialysis membrane (11kDa), and distilled water.

Equipment used in this research were spectrophotometer UV-Vis 1800 (Shimadzu, Japan), incubator (Thermo Fisher Scientific, USA), autoclave (Raypa, Spain), centrifuge (Eppendorf, China), laminar airflow (TelstarBH-100, Spain), sonicator (Labsonic, Germany), analytical scale (Shimadzu, Japan), micropipette (Eppendorf, China), Bunsen burner, water bath, ice bath, Erlenmeyer flask, magnetic stirrer, and centrifuge tubes.

Conduction of optimization process

Optimization process design

The optimization of growth conditions in this research was using response surface methodology (RSM) with central composite design (CCD). The established parameters consisted of four factors, which were lactose level (X_1), incubation temperature (X_2), incubation time (X_3), and inoculum size (X_4) with enzyme activity of β-galactosidase as the response. Each factor had 5 level codes, specifically were -alpha, -1, 0, +1, and +alpha which can be seen in Table 1. Based on the combination of four variables and five levels of trial combinations, 30 designs of growth condition combinations were obtained.

Pre-enrichment of isolate

L.brevis LIPI13-2-LAB131 from glycerol at -80°C was inoculated aseptically in MRSB media and incubated at 30 °C for 24 hours. The purpose of this inoculation was for LAB to gradually adapt from liquid media. Inoculated media then was inoculated again aseptically in MRSB media and incubated at 30 °C for 24 hours. The purpose of repeated inoculation was to increase cell numbers of LAB.

Tabel 1. Combinations of four variables and five levels of trial combinations using *central composite design* (CCD)

Variables	Levels				
	-alpha	**-1**	**0**	**+1**	**+alpha**
Lactose (%)	0	0.75	1.50	2.25	3.00
Temperature (°C)	25	30	35	40	45
Time (hour)	0	24	48	72	96
Inoculum (%)	0.2	1.5	2.8	4.1	5.4

Extraction of intracellular enzyme from bacteria (Modification from Wang and Sakakibara 1997)

100 mL MRSB media from incubation process was centrifuged at 9000 rpm for 30 minutes at 10 °C. The centrifuged pellet then was diluted in buffer phosphate 0.1 M pH 7 with ratio 1:5 (w:v). The next step was cell breakdown by sonication for 5 minutes with pause in every 20 seconds. Sonication was conducted using 60 amplitudes and 0.5 cycles. Cell suspension then was centrifuged with 9000 rpm for 30 minutes at 10 °C. The supernatant made by the centrifugation was a crude extract of β-galactosidase enzyme.

Analysis of β-galactosidase enzyme activity (Modification from Marteu et al. 1990)

0.8 mL buffer phosphate 0.1 M pH 7 and 0.08 mL enzyme were inserted into test tube, and then incubated for 15 minutes at 37 °C. After the incubation had finished, 0.16 mL of *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) 4 mg/mL was added and incubated for 15 minutes at 37 °C. After the second incubation had finished, 0.8 mL of Na_2CO_3 1 M was added. The solution was analyzed with spectrophotometer UV-Vis at 420 nm. Enzyme activity (U/mL) was the amount of µmol o-nitrophenol (oNP) per minute per mL enzyme formed in test conditions (Al-Arrji et al. 2017). Enzyme activity then was calculated using following formula:

$$\text{Enzyme activity (U/mL)} = \frac{\mu\text{mol oNP}}{V \times t}$$

µmol oNP : amount of oNP formed from test result
V : volume of tested enzyme
t : incubation time

Data analysis

The data from enzyme activity tests were analyzed using ANOVA DX7 program. The data that were evaluated consisted of 4 criteria which were response model (must be significant), lack of fit (must not be significant), adjusted R^2 and predicted R^2 (difference smaller than 0.2), and adequate precision (bigger than 4). Then, the interpretation of mathematical model for completed responses was conducted by using contour curve or 3D. After performing ANOVA test, the determination of factors and response criteria was done. Optimization was done to obtain the combinations with predicted score value and suitable response with the desired value. The optimum combination was the combination with maximum desirability. Desirability value shows the achievements of each response according to the expected criteria on chosen formula (model was better if desirability value close to 1.0).

Optimization result verification

Combinations of optimum treatments were verified; thus, the results were actual response variables. The performed experiment and measurement of β-galactosidase enzyme activity were repeated directly using the

combination of optimum treatments, so that the results were actual response variables. Verification was done by measuring repeatedly three times. Data result of verification was used as evidence that the established factors were actually giving response values in range values that have been set by design expert 7.00 (DX7) program. The verification results were in the 95% confidence interval (CI) range and 95% prediction interval (PI) range. The 95% CI value indicated that 95% of data measurement averages were in the mentioned range value, while 95% PI value indicated that 95% of measured data responses were in the mentioned range (Verschuuren 2014).

Partial purification of verified crude enzyme (Scopes 1987; Pal et al. 2013)

Precipitation by using Ammonium Sulfate (Scopes 1987)

Crude enzyme from β-galactosidase was precipitated using ammonium sulfate. The concentration of the added ammonium sulfate was 60%. Ammonium sulfate was added slowly while being agitated using magnetic stirrer until ammonium sulfate was dissolved and reached the desired concentration. The result of precipitation then was stored overnight at 4 °C to prevent damage protein. The precipitated enzyme then was centrifuged using 9000 rpm speed for 30 minutes at 10 °C. The pellet from centrifugation step then was diluted using buffer phosphate 0.1 M pH 7. The concentration of ammonium sulfate needed to precipitate the enzyme was calculated using following formula:

$$\text{Ammonium sulfate concentration (g/L)} = \frac{\text{Crude enzyme volume}}{1000} \times Y$$

Y is a tabulation value from the percentage of needed ammonium. Y value for 60% ammonium sulfate is 390.

Dialysis (Pal et al. 2013)

Solution of precipitated enzyme by using ammonium sulfate was dialyzed using buffer phosphate 0.025 M pH 7. The enzyme was dialyzed at 4 °C for 24 hours using dialysis membrane with size 11 kDa. Buffer phosphate was changed 3 times every 2 hours.

Measurement of β-galactosidase activity and yield

The measurements of purified β-galactosidase activity have consisted of measurement of enzyme activity and its specific activity. The measurement of enzyme activity was using modification from Marteu et al. (1990) method, while specific activity was determined by measuring protein content of the enzyme. The determination of enzyme protein content was done by the addition of 2 mL of Bradford solution into 40 µL of purified β-galactosidase. The solution then was mixed and settled for 5 minutes and measured its absorbance at 595 nm wavelength. The specific activity was calculated using following formula:

Enzyme specific activity (U/mg protein) = Enzyme activity (U/mL)/ Protein (mg/mL)

Measurement of enzyme yield was done by calculating the ratio of total of purified enzyme activity and crude enzyme activity using following formula:

$$\text{Enzyme yield (\%)} = \frac{\text{Total of purified enzyme activity (U)}}{\text{Total of crude enzyme activity (U)}} \times 100$$

RESULTS AND DISCUSSION

Enzyme activity of β-galactosidase from *Lactobacillus brevis* LIPI13-2-LAB131

The enzyme activity values of β-galactosidase produced by *L.brevis* LIPI13-2-LAB131 ranged between 0.11 – 22.94 U/mL. The determined results of β-galactosidase enzyme activity were inserted into design expert 7.00 (DX7) program. The suggested model by the program was a quadratic model. Best model that described the response data was selected for analysis of variance (ANOVA). The result of analysis of variance for model could be stated as significant. The quadratic effects of lactose level, temperature, and time significantly influenced β-galactosidase enzyme activity. The linear effect of each factor and interaction between factors had no significant effect. Based on the analysis of variance of quadratic regression coefficient (R^2), β-galactosidase enzyme activity value was 0.96. The value showed that four factors influenced the variety of responses by 96% while the rest were influenced by other factors.

Lack of fit test and coefficient determination (predicted R-squared, adjusted R-squared) showed that there was conformity between distributed data and the model. The model also had an ideal adequate precision value (Adequate Precision > 4). Based on the obtained result, lack of fit value of 0.9984 showed that it was not significant. The data result had been tested by determination coefficients which included predicted R-squared and adjusted R-squared. The smaller difference is better. The result of differences between predicted R-squared and adjusted R-squared was 0.01. The adequate precision value was 12.03. The quadratic model that explained the data of β-galactosidase enzyme activity from *L. brevis* LIPI13-2-LAB131 was:

$$Y = -274.88563 + 23.74835X_1 + 13.25613X_2 + 0.88285X_3 + 17.54064X_4 + 0.050500X_1X_2 - 0.012396X_1X_3 - 0.22372X_1X_4 - 3.95312E\text{-}003X_2X_3 - 0.073365X_2X_4 + 0.016446X_3X_4 - 8.20093X_1^2 - 0.18522X_2^2 - 7.96965E\text{-}003X_3^2 - 2.73182X_4^2$$

Y coefficient was described as enzyme activity of β-galactosidase. X_1, X_2, X_3, X_4 were described as lactose level or as the carbon source, time, temperature, and inoculum size. The equation model above showed that enzyme activity of β-galactosidase increases with the increasing of lactose level, temperature, time, and inoculum size, lactose level-temperature interaction, and time-inoculum size interaction. Enzyme activity of β-galactosidase decreases with the increasing of lactose level-time interaction, lactose level-inoculum size interaction, temperature-time interaction, temperature-inoculum size

interaction, and also quadratic interactions between lactose level, temperature, time, and inoculum size.

Optimum growth condition of *Lactobacillus brevis* LIPI13-2-LAB131

Based on the result of optimization in DX7 program, a formula for growth conditions was obtained with desirability value of 0.839. Desirability value is a parameter that indicates the best optimization result with range between 0-1.0. The closer to 1.0 value means the recommended solution by the program is more to suitable fulfill the desired criteria, as well as to set objectives (Myers et al. 2009). The growth combinations were chosen by the program for enzyme activity of β-galactosidase were 1.48% lactose, incubation temperature of 34.91°C, incubation time of 48.48 hours, and inoculum size of 2.83%. These conditions are in line same with previous study. According to Holt (2000), optimum temperature for the growth of *Lactobacillus* was 30-40°C. Based on Mahoney (2004), lactose could act as an inducer in β-galactosidase production. A study by Carevic et al. (2017) mentioned that β-galactosidase enzyme activity in *Lactobacillus acidophilus* increased with the addition of 1.48% lactose. Carevic et al. (2015) stated that enzyme activity of β-galactosidase increased gradually when the fermentation started and would achieve maximum activity on stationary phase after two days, but then experienced a decrease in activity afterward. Carevic et al. (2015) also mentioned that the optimum time to achieve maximum β-galactosidase enzyme activity was 48 hours. Based on the research conducted by Carevic et al. (2017), there was effect between inoculum size and β-galactosidase enzyme activity. β-galactosidase enzyme activity of *Lactobacillus acidophilus* increased by the addition of 2.8% inoculum size. Figure 1 showed a three-dimensional surface curve regarding relation between the combination of lactose level and temperature with desirability value.

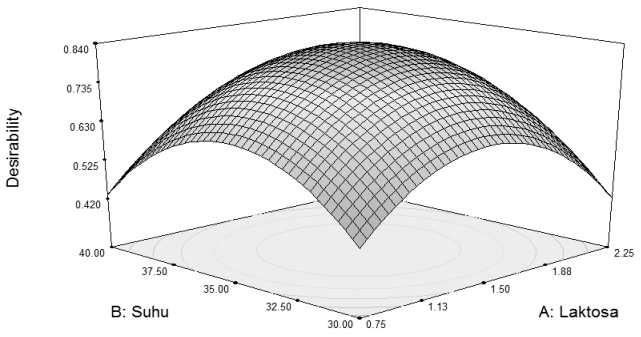

Figure 1. Three-dimensional surface curve of the relationship between combinations of temperature and lactose level (X-axis) with desirability value (Y-axis)

Verification result

Verification was conducted at the point with highest desirability value. Verification result showed the predicted value of β-galactosidase of the selected formula. Table 1 showed the summary of verification results, predicted value, 95% CI range, and 95% PI range.

The selected verification result indicated that enzyme activity of β-galactosidase was inside of 95% PI range. This value showed that 95% of the measured sample response data were in the interval and the actual test data were still in the model prediction range, and indicated that the model could be used to predict all four responses. The compatibility between predictions and measurement results implied that the applied model was verified and consistent.

Partial purification of β-galactosidase enzyme result

The partial purification process was conducted on crude enzyme of *L. brevis* LIPI13-2-LAB131. Partial purification was conducted in order to purify the enzyme so that the result activity value is higher and also to calculate the yield enzyme. The first step of this process was by precipitation of protein using ammonium sulfate. The selection of ammonium sulfate salt for protein precipitation was because of its high solubility properties and economic cost. In addition, generally, it does not influence the protein structure in certain concentrations (Beynon and Bond 2001 in Fikri 2013). The principal of protein precipitation was based on salting in and salting-out protein solubility. The initial addition of ammonium sulfate at low level will increase the solubility of protein. At increased concentration, the solubility of protein decrease until precipitation occurs (Grogan 2009). Precipitation occurs because the ionic strength of salt is stronger, so it is more able to bind water. This causes the attraction between protein becomes higher, thus resulted in protein precipitation (Fatchiyah et al. 2011).

The next step of enzyme purification was dialysis. This process was carried out to remove salt ions from the precipitation process using ammonium sulfate and other smaller impurity particles. The dialysis was done by using dialysis membrane with 11 kDa size. The selection of 11 kDa membrane size was based on the size of dialyzed enzyme. According to Gheytanchi et al. (2010), size of β-galactosidase particle produced from isolated *Lactobacillus* strain from milk was approximately 116 kDa. According to the mentioned study, membrane of 11 kDa size could be utilized for β-galactosidase dialysis because the size was smaller than the dialyzed enzyme. The utilized membrane must be ensured to remove other particles such as ions from ammonium sulfate salt, but did not allow enzyme to come out of the membrane during dialysis process. Table 2 showed the results of the partial purification process carried out on the crude enzyme produced by *L. brevis* LIPI13-2-LAB131 based on the verification result.

Table 1. Verification result of β-galactosidase enzyme activity response

Response	Prediction	Actual	95% CI low	95% CI high	95% PI low	95% PI high
Enzyme activity (U/mL)	19.27	15.11 ± 0.24	17.29	21.26	14.02	24.52

Note: CI: confidence interval; PI: prediction interval

Table 2. Measurement results of specific activity and yield of β-galactosidase

Purification step	Protein (mg/mL)	Total activity (U)	Enzyme activity (U/mL)	Specific activity (U/mg)	Purity	Yield (%)
Crude enzyme	1.09 ± 0.36	196.38	15.11 ± 0.24	13.92	1.00	100.00
60 % Ammonium sulfate	1.12 ± 0.37	24.59	18.92 ± 0.31	16.85	1.21	12.52
Dialysis	1.24 ± 0.31	22.88	22.88 ± 0.29	18.47	1.33	11.65

Note: Measurement were made 3 times repetition

The increase of enzyme activity after precipitation was caused by the decrease of contaminants that inhibited the active side of enzyme from binding substrate (Wardani et al. 2012), it was also the same case with dialysis. When the dialysis was carried out, ions from ammonium sulfate salt and other ions that potentially inhibit the enzyme activity were removed through diffusion process, thus the resulted enzyme activity from dialysis would increase. Based on precipitation 60% ammonium sulfate, the yield of β-galactosidase was 12.52%. That value was not good so could be suggested to test other saturation degrees, such as 80%, or by doing optimization on 0-30%, 30-60%, and 60-100%. Based on the dialysis of crude enzyme, the yield of β-galactosidase was 11.65%. This value was lower than research conducted by Khusniati et al. (2015) by using *Lactobacillus plantarum* B123 that produced β-galactosidase with 14.01% yield.

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