Response surface methodology for optimization of medium components for extracellular protease production by Enterococcus faecalis InaCC B745

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Abstract. Enterococcus faecalis InaCC B745 was isolated from dadih, a traditional fermented milk from Bukittinggi, West Sumatra. Previous study shows that the lactic acid bacteria was able to produce protease. In this study, protease production was optimized using response surface methodology (RSM) with central composite design (CCD). The total of four variables and five-level combinations namely: 0.4% (w/v) of skim milk, 0.2% (w/v) of yeast extract, 0.2% (w/v) of glucose, and 0.2% (w/v) of CaCO₃. Statistical analysis showed that the production of protease by E. faecalis InaCC B745 is significantly influenced by skim milk, yeast extract, glucose, and CaCO₃, combination of skim milk and yeast extract. The final concentrations of the optimized medium were 3% (w/v) skim milk, 1.5% (w/v) yeast extract, 0.89% (w/v) glucose, and 1.5% (w/v) CaCO₃. Under these conditions, the model predicted of protease activity was 60.637 U/ml or around 57.3% increased.

1. Introduction
Microbial proteases are among the most important class of hydrolytic enzymes, which constitute at least 60% of the total industrial enzyme market [1]. Proteases (EC 3.4.21-24) catalyze the hydrolysis of a peptide bond in a protein molecule which are used for numerous industrial applications such as laundry detergents, foods, pharmaceutical, leather preparations, protein recovery, and organic synthesis waste management [2].

Proteases are produced by wide range of microbes, including bacteria, molds, yeasts, and mammalian tissues [3]. Many reports of protease enzyme produce from bacteria, but only few report protease activities from E. faecalis. Sato et al. [4] reported that E. faecalis TUA2495L has an ability to produce extracellular proteinase with milk-clotting activity. Protease produced by LAB starter play an important role in the development of cheese flavor and texture [5]. Microbial enzymes production is highly influenced by several factors, such as type of C and N sources, metal ions, pH, temperature, inoculum size, and incubation time [6,7]. Components media were found to have an important contribution on extracellular protease production depending on the microorganism used. Medium components and their concentrations should be optimized [8].

Design techniques of statistical experimental are useful tools for the nutrient screening and its optimization. Response surface methodology (RSM) is a useful statistical approach performed for multiple regression analysis by using measurable data. This technique solves multivariate data, which is found from appropriately designed experiments to solve multivariate equation simultaneously [9]. The RSM is an efficient strategic experimental tool by which the optimal conditions of a multivariable
The factorial design and regression analysis in the RSM helps to evaluate the effective factors and to study the interaction between these factors. This method uses mathematical models to analyze the experimental data and to predict the relationship between the response and the variables [10]. Central composite design (CCD) was applied to optimize the medium constituents and explain the combined effects of medium constituents. The CCD are designed to estimate the coefficients of a quadratic model [9]. The application of statistical experimental design techniques in fermentation process development can improve product yield, reduce process variability, as well as reduce development time and overall costs [10].

The CCD method was chosen in this study because all variables are studied in five levels (\(-\alpha, -1, 0, +1, +\alpha\)) while Box and Behnken design (BBD) only at three levels (\(-1, 0, +1\)) [11]. In the CCD for each variable there are points outside the minimum and maximum range, in under similar conditions, often the number of tests is greater than Box-Behnken. The number of variables to consider in this method is 2 to 6 factors. Box and Behnken design have less design points when compare to axial points of CCD hence no experiments are more in CCD compared to BBD. The CCD also tests at extreme conditions and hence this gives better for quadratic models. In the BBD there is no point in the cubic vertex that creates the upper and lower bounds of each variable, that is, all the tested points are within a predetermined range, so that the beginning and end points of the interval are less accurate than they have other points.

From previous study, we have screened for 129 isolates producing protease, only one isolate Enterococcus faecalis InaCC B745 has ability to produce protease [unpublished]. The aims of this study was to optimize of medium for the production of protease by Enterococcus faecalis InaCC B745 using RSM.

2. Materials and Methods

2.1 Microorganisms

Enterococcus faecalis InaCC B745 is obtained from Indonesian Culture Collection (InaCC)-Indonesian Institute of Sciences (LIPI). The medium used in cultivating E. faecalis InaCC B745 was Tryticase Soy Yeast Extract [TSYE] broth (Merck, Jerman). Medium sterilized at 121 °C for 15 minutes in an autoclave. Preservation of isolates was carried out in sterile glycerol (150 ml. L\(^{-1}\)) and stored at -80 °C [12]. Those isolates are also stored in L-drying ampoules.

2.2. Experimental design

Response surface method (RSM)-central composite design (CCD) was used to optimize the medium for protease production from selected LAB. A CCD with four variables namely skim milk, of yeast extract, glucose, and CaCO\(_3\) at five levels was followed to determine the response pattern and also to determine the synergy of variables [10]. According to this design, 30 runs were conducted containing six replications at the central point for estimating the purely experimental uncertainty variance. Table 1 shows the coded and uncoded independent variables \((X_i)\), level and experimental design. The relationship of variables was analyzed by fitting a second order polynomial equation to data obtained from the 30 runs.

| Variables       | Code Values |
|-----------------|-------------|
| Skim milk (%)   | X\(_1\)     |
| Yeast Extract (%)| X\(_2\)     |
| Glucose (%)     | X\(_3\)     |
| CaCO\(_3\) (%)  | X\(_4\)     |

|             | -2 | -1 | 0  | +1 | +2 |
|-------------|----|----|----|----|----|
| Skim milk (%)| 0  | 1  | 2  | 3  | 4  |
| Yeast Extract (%)| 0  | 0.5| 1  | 1.5| 2  |
| Glucose (%)   | 0  | 0.5| 1  | 1.5| 2  |
| CaCO\(_3\) (%)| 0  | 0.5| 1  | 1.5| 2  |
The response surface analysis was based on the multiple linear regression considering the main, quadratic and interaction effects, according to the following equation:

\[ Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j \]  

(1)

Where \( \beta_0 \) is intercept or the scaling constant, while \( \beta_i, \beta_{ij} \) are regression coefficient.

2.3 Fermentation process

The modified medium of Sato et al. [4] was used for protease production contained (g/l): skim milk (variable), yeast extract (variable), CaCO\(_3\) (variable), CH\(_3\)COONa.3H\(_2\)O 2 g, MgSO\(_4.7\)H\(_2\)O 0.2 g, MnSO\(_4.4\)H\(_2\)O 0.01 g, FeSO\(_4.7\)H\(_2\)O 0.01 g and NaCl 0.01 g. Medium sterilized at 110 °C for 10 minutes in an autoclave. The amount of 5% (v/v) of inoculum was inoculated to production medium. The incubation condition were maintained at room temperature and agitation speed of 100 rpm for 48 hours. Cultured medium were centrifuged (Hitachi Himac CR 21G III, R15 A rotor) at 4 °C and 7840 x g for 15 minutes. The supernatant was collected for further analysis.

2.4 Proteolytic activity

The proteolytic activity [PA] of the enzyme was quantitatively measured by Arima et al. [12] method. The PA was measured using 0.5% solution of Hammerstein casein (0.02 M potassium phosphate buffer, pH 6.5) as the substrate. A 2.5 ml of the substrate solution was incubated with 0.5 ml enzyme solution at 35°C for 10 min, then the enzyme reaction was terminated with 2.5 ml 0.44 M Trichloroacetic acid (TCA). The precipitates formed were removed by centrifugation at 4°C and 7840 g agitation for 10 minutes. One milliliter of filtrat was added to 2.5 ml 0.55 M Na\(_2\)CO\(_3\) and 1 ml Folin ciocalteau (3 x). The mixture was incubated at 35°C for 20 min for colour development and the optical density at 660 nm was measured. One unit of the protease activity was defined as the amount of enzyme which released 1 μg of amino acid expressed as the tyrosine concentration per min under the above condition.

2.5 Statistical analysis

The results were analysed to extract the effects of factors and the analysis of variance (ANOVA) technique was applied to determine the statistically significant factors. All designs and calculations were conducted by Design Expert® 11 (Stat-Ease Inc., USA).

3. Results and Discussion

The CCD design of experiment and response results were given in Table 2. A polynomial coefficient for each term of the equation was determined through multiple regression analysis.

Summary of the analysis of variance [ANOVA] for the response surface quadratic model is given in Table 3. The F-value was used to check the statistical significance of equation [13]. In this study, the F-value was 16.169 implied that could demonstrate the model was highly significant. The P-value [P > F, 0.0001] was high significant that it could also prove the good fit of the model and there was only 0.0001 chance that a model F-value could have occurred due to noise. Since the model showed insignificant lack of fit, the response was sufficiently explained by the regression equation. The high P-value [>0.05] of lack of fit for regression in equation also indicated a reasonable fit of the second-order model as an approximation to the true response [13]. The generated regression relationship (Table 4) is given in equation was obtained as follows:

\[ Y = 38,553 + 9.24 X_1 + 3.37 X_2 + 3,716 X_3 + 2.99 X_4 + 2,576 X_1X_2 \]  

(2)
In the above equation, Y is the predicted response of PA, X₁, X₂, X₃, and X₄ are the coded values of the tested variables of skim milk, yeast extract, glucose and CaCO₃, respectively. The equation above took into account the quadratic, and interaction effects between the factors studied. The actual value of the factors studied was the value that can be input into the equation.

Table 2 The CCD design of experiment and protease activity yield from *E. faecalis* InaCC B745

| No. | Skim milk (%) | Yeast extract (%) | Glucose (%) | CaCO₃ (%) | Protease activity (U/ml) |
|-----|---------------|------------------|-------------|-----------|-------------------------|
| 1   | 1             | 0.5              | 0.5         | 0.5       | 21.767                  |
| 2   | 3             | 0.5              | 0.5         | 0.5       | 32.514                  |
| 3   | 1             | 1.5              | 0.5         | 0.5       | 22.336                  |
| 4   | 3             | 1.5              | 0.5         | 0.5       | 48.768                  |
| 5   | 1             | 0.5              | 1.5         | 0.5       | 36.928                  |
| 6   | 3             | 0.5              | 1.5         | 0.5       | 45.327                  |
| 7   | 1             | 1.5              | 1.5         | 0.5       | 37.754                  |
| 8   | 3             | 1.5              | 1.5         | 0.5       | 49.791                  |
| 9   | 1             | 0.5              | 0.5         | 1.5       | 21.291                  |
| 10  | 3             | 0.5              | 0.5         | 1.5       | 40.855                  |
| 11  | 1             | 1.5              | 0.5         | 1.5       | 33.913                  |
| 12  | 3             | 1.5              | 0.5         | 1.5       | 57.849                  |
| 13  | 1             | 0.5              | 1.5         | 1.5       | 36.113                  |
| 14  | 3             | 0.5              | 1.5         | 1.5       | 47.092                  |
| 15  | 1             | 1.5              | 1.5         | 1.5       | 33.7969                 |
| 16  | 3             | 1.5              | 1.5         | 1.5       | 63.0725                 |
| 17  | 0             | 1                | 1           | 1         | 19.3324                 |
| 18  | 4             | 1                | 1           | 1         | 59.1449                 |
| 19  | 2             | 0                | 1           | 1         | 37.6667                 |
| 20  | 2             | 2                | 1           | 1         | 45.0290                 |
| 21  | 2             | 1                | 0           | 1         | 27.0000                 |
| 22  | 2             | 1                | 2           | 1         | 35.9275                 |
| 23  | 2             | 1                | 1           | 0         | 25.2609                 |
| 24  | 2             | 1                | 1           | 2         | 41.3623                 |
| 25  | 2             | 1                | 1           | 1         | 35.8841                 |
| 26  | 2             | 1                | 1           | 1         | 38.1594                 |
| 27  | 2             | 1                | 1           | 1         | 43.6232                 |
| 28  | 2             | 1                | 1           | 1         | 38.6667                 |
| 29  | 2             | 1                | 1           | 1         | 36.0580                 |
| 30  | 2             | 1                | 1           | 1         | 38.9275                 |

The high determination coefficient \(R^2 = 0.94\) proved the goodness fit of the model, suggesting that the sample variation of 94% for PA was attributed to the variable factors. The value of the adjusted determination coefficient [adjusted \(R^2 = 0.88\)] was also high, which recommended high significant of the model.
| Source               | Sum of Squares | df | Mean Square | Value F | Value P Prob > F | Significant |
|---------------------|----------------|----|-------------|---------|------------------|-------------|
| Model               | 3245.393       | 14 | 231.814     | 16.169  | < 0.0001         | Significant |
| $X_1$-Skim milk     | 2034.984       | 1  | 2034.984    | 141.938 | < 0.0001         |             |
| $X_2$-Yeast extract | 267.460        | 1  | 267.460     | 18.655  | 0.0006           |             |
| $X_3$-Glucose       | 325.879        | 1  | 325.879     | 22.730  | 0.0002           |             |
| $X_4$-CaCO$_3$      | 210.055        | 1  | 210.055     | 14.651  | 0.002            |             |
| $X_1X_2$            | 110.205        | 1  | 110.205     | 7.687   | 0.014            |             |
| $X_1X_3$            | 24.970         | 1  | 24.970      | 1.742   | 0.207            |             |
| $X_1X_4$            | 42.705         | 1  | 42.705      | 2.979   | 0.105            |             |
| $X_2X_3$            | 47.219         | 1  | 47.219      | 3.293   | 0.089            |             |
| $X_2X_4$            | 28.002         | 1  | 28.002      | 1.953   | 0.183            |             |
| $X_3X_4$            | 20.813         | 1  | 20.813      | 1.452   | 0.247            |             |
| $X_1^2$             | 12.256         | 1  | 12.256      | 0.855   | 0.369            |             |
| $X_2^2$             | 39.217         | 1  | 39.217      | 2.735   | 0.119            |             |
| $X_3^2$             | 44.608         | 1  | 44.608      | 3.111   | 0.098            |             |
| $X_4^2$             | 18.144         | 1  | 18.144      | 1.265   | 0.278            |             |
| Residual            | 215.058        | 15 | 14.337      |         |                  |             |
| Lack of Fit         | 175.694        | 10 | 17.569      | 2.232   | 0.1944           |             |
| Pure Error          | 39.363         | 5  | 7.873       |         |                  |             |
| Cor Total           | 3460.450       | 29 |             |         |                  |             |

Table 3. ANOVA for response surface quadratic

| Factor               | Coefficient Estimate |
|---------------------|----------------------|
| Intercept           | 38,553               |
| $X_1$-Skim milk     | 9,240                |
| $X_2$-Yeast extract | 3,370                |
| $X_3$-Glucose       | 3,717                |
| $X_4$-CaCO$_3$      | 2,990                |
| $X_1X_2$            | 2,577                |
| $X_1X_3$            | -1,297               |
| $X_1X_4$            | 1,586                |
| $X_2X_3$            | -1,766               |
| $X_2X_4$            | 1,275                |
| $X_3X_4$            | -1,188               |
| $X_1^2$             | 0,660                |
| $X_2^2$             | 1,188                |
| $X_3^2$             | -1,283               |
| $X_4^2$             | -0,821               |
CaCO$_3$ interaction on the PA yield of *E. Faecalis* InaCC B745. Figure 1 shows that PA increase with increase in reaction skim milk and yeast extract (Fig. 1a), reaction glucose and skim milk (Fig. 1b), reaction CaCO$_3$ and skim milk (Fig 1c), reaction CaCO$_3$ and glucose (Fig 1d), reaction CaCO$_3$ and yeast extract (Fig. 1e), reaction glucose and yeast extract (Fig. 1f).

In order to characterize how the significant variables affect the responses, we studied to improve the composition of the medium by comparing different levels of several variables that were found to have influence on protease production by *E. faecalis* InaCC B745. A CCD with four variables at five levels was followed to determine the response pattern and also to determine the synergy of variables.
A rotatable central composite design is one of the efficient central composite designs, which has points which are equidistant from the centre.

One of the factors that influenced the protease activity of bacteria was the content of the media that used as nutritional sources. The selection of four variables based on nutritional requirements for growth and substrates for the production of protease enzymes from E. faecalis InaCC B 745. Skim milk contained many nutrients such as casein, calcium, potassium, magnesium, phosphorus, and others. The content of casein was phosphor protein that can bind with calcium to formed calcinate. Skim milk was found to have significant effects on the production of the extracellular protease from Bacillus cereus strain CA15 [13]. Yeast extract as nitrogen sources for growth of bacteria. Glucose as supplementary carbon sources for growth of bacteria. CaCO₃ as metal ions sources [14].

The experiment produced a regression model that could be used to optimize protease enzyme production. In accordance with the calculation of the regression model, skim milk, yeast extract, glucose and CaCO₃ were 3, 1.5, 0.89 and 1.5%, respectively, with a maximum predicted protease yield of 60.637 U/ml, which is increased by 57.3%. The PA from E. faecalis InaCC B745 was higher than that of the PA from E. Faecalis TUA2495L (4.1 U/ml) [5].

4. Conclusion
From the ANOVA results, the P-value of all variables less than 0.05 indicate model terms are significant. The production of protease by E. Faecalis InaCC B745 is significantly influenced by skim milk, yeast extract, glucose, and CaCO₃, and a combination of skim milk and yeast extract. Optimum medium composition for the production of protease is as following: 3% skim milk, 1.5% yeast extract, 0.89% glucose and 1.5% CaCO₃. According to the model developed, maximum protease produced having an activity of 60.637 U/ml compare with only 38.55 U/ml when 2% of skim milk, 1% of yeast extract, 1% of glucose and 1% CaCO₃ were used. For next study production, purification, and characterization of protease from E. Faecalis InaCC B745 are needed.

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