Optimization of medium composition for probiotic powder inoculum using the response surface methodology

Rohmatussolihat, R Ridwan, Y Widyastuti, N F Sari, R Fidryanto and W D Astuti

Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, 16911, Indonesia

E-mail: rohmatussolihat@gmail.com

Abstract. Probiotic as feed additives have been developed as alternatives to improve animal health and productivity. Probiotics are live microorganisms that confer health benefits to the hosts by improving intestinal microbial balance. The aims of this study was to optimize the formulation of probiotic powder inoculum from *Lactobacillus plantarum* TSD-10 BTCC 531 using Response Surface Methodology with Central Composite Design (RSM-CCD). The RSM-CCD was prepared using three variables, consist of; skim milk, maltodextrin and CaCO₃, with five-level combinations. The drying process of the probiotic powder inoculum used a freeze dryer for 16 hours. The *L. plantarum* TSD-10 BTCC 531 survival amounts were measured by the total plate count method. Statistical analysis showed that the viability of *L. plantarum* TSD-10 BTCC 531 was significantly affected by skim milk and CaCO₃. Estimated optimum conditions of the factors on the bacterial viability have consisted of 5% (w/v) skim milk, 1.547% (w/v) maltodextrin, 0.5% (w/v) CaCO₃, and with a maximum *L. plantarum* TSD-10 BTCC 531 number of surviving results at 1.945 x 10¹² CFU/g, which significantly increased by 34.9%. The results of the validation experiments showed that the cell number of probiotic reaches 2.17 x 10¹² CFU/ or 11.43% more than the predicted cell number of *L. plantarum* TSD-10 BTCC 531 from the regression model produced.

1. Introduction
Alternative development of antibiotic growth promoters in livestock has become a global concern in the field of animal nutrition after their ban in many countries [1]. Probiotics are live microorganisms which can survive and pass through the gastrointestinal tract providing beneficial effects in animal nutrition such as weight gain, development of beneficial intestinal microbiota and immune system enhancement in farm animals by improving absorption in the digestive system [2–4]. In terms of ruminant production systems, the efficacy of probiotics containing Lactic acid bacteria (LAB) has been studied mostly in pre-ruminants where they are reported benefits [5]. Many different strains and species of *Lactobacilli* and *Bifidobacteria* have been used probiotics commercially [6].

In recent years, probiotic products in powder form have been developed because they have several advantages including easy distribution, storage and increased product shelf life. Number of viable probiotic cells sufficient prerequisites for the success of its impact on animals [7]. To commercialize the probiotics, time-saving and cost-effective methods are needed to increase the yield of bacterial cells during production [8]. During the production process, the preparation of probiotic products requires reasonable cell stability. The main problem in powder probiotic production is the trial-and-
error process to select the optimal process conditions, to achieve the highest cell survival ratio after drying. The dried cells undergo environmental stresses like high cell density, vacuum (shear stress and oxygen) and milling (potentially cause heat stress) during production [9]. Among various techniques, drying methods are commonly used for the preservation and ease of handling of microorganisms [10]. Freeze drying is a conventional technology for preparing bacterial powder in industrial production, which can effectively remove water from bacterial samples. Freeze-drying technology is most preferred in probiotic production due to less inactivation, good rehydration and faster reduction [11] and has been widely applied to bacteria that exhibit high stability against low temperatures [10]. However, stress factors such as very low freezing temperatures or dehydration during freeze-drying can cause undesirable loss of viability for some probiotic strains. Due to this, a variety of cryoprotectants have been developed to increase the viability of probiotic bacteria during the freeze-drying procedure.

Cryoprotectant is one of the most important factors in improving probiotic viability during the freeze-dried process. According to Morgan et al (2016), the results of research that have been conducted have shown that the addition of a cryoprotectant can increase bacterial resistance during the freeze-drying process due to membrane and protein stabilization [12]. Protectants such as skim milk, maltodextrin, whey proteins, sugars, or other bio-polymers were studied mostly as combinations for synergistic protective effects with other protectants [10]. Skim milk can prevent cellular damage by stabilizing cell membrane constituents. It can also protect microbial cells from damage from the formation of ice crystals during the freezing process, because proteins in skim milk can form a viscous layer on the surface of cells, which can inhibit the growth of ice crystals by increasing the solution's viscosity and maintaining the structure of amorphous ice crystals near the cell [13]. Maltodextrin is now widely used in the food industry in the form of a bodying agent, coating, and carrier because it has good solubility, low viscosity at high solid content, and provides oxidative stability to encapsulated cell [14].

Therefore, Response Surface Methodology (RSM) have been become one of the most used optimizations approach to create the optimum conditions with a minimum number of experiments [15]. This study aimed to optimize the formulation of probiotic powder inoculum using Response Surface Methodology with Central Composite Design (RSM-CCD).

2. Materials and methods

2.1. Bacterial strain and culture conditions

*Lactobacillus plantarum* TSD-10 BTCC 531 was used in this study. This isolate was obtained from the Biotechnology Culture Collection, Research Center for Biotechnology-LIPI. The medium used in cultivating LAB and production was de Man Rogosa Sharpe (MRS) broth (Merck) [16]. Fifty microliters of *L. plantarum* TSD 10 BTCC 531 taken from glycerol stock were inoculated into 5 mL of sterile MRS broth media and incubated at 30°C for 24 h.

2.2. Preparation of cultures for production

The microorganisms, previously subcultures in MRS, were inoculated at 10% (v/v) in MRS broth and incubated at 30°C for 24 h. Cells production of *L. plantarum* TSD 10 BTCC 531 was conducted in 2 liter MRS broth media, incubated at 30°C for 24 hours. Cells were harvested by centrifugation at 9000 rpm, 4°C for 10 minutes.

2.3. Response surface optimization of medium composition for probiotic powder inoculum

Cells of *L. plantarum* TSD-10 BTCC 531 was added into media according to experimental design using CCD-RSM (table 1), stirred and frozen in a freezer -80°C. After freezing, the samples were dried using a freeze dryer for 16 hours.

Response Surface Method (RSM)-Central Composite Design (CCD) was used to optimized the medium for inoculum probiotik *L. plantarum* TSD-10 BTCC 531 powder. A CCD with three variables
consist of; skim milk, maltodextrin and CaCO₃, with five-level combinations were followed to
determine the response pattern and also to determine the synergy of variable [17]. According to this
design, 20 run were conducted containing six replications at the central point for estimating the purely
experimental uncertainty variance. Table 1 shows the coded and uncoded independent factors (Xi),
level and experimental design. The relationship of variables was analyzed by fitting a second order
polynomial equation to data obtained from the 20 runs.

**Table 1.** The experimental range of three variable studied using RSM-CCD in terms of actual and coded factors.

| Variables          | Level   |
|--------------------|---------|
| Skim milk (%)      | -1.68179 -1 0 1 18.405 |
| Maltodextrin (%)   | 0 1 2.5 4 5.023 |
| CaCO₃ (%)          | 0.159 0.5 1 1.5 1.84 |

The response surface analysis was based on the multiple linear regression taking into account the
main, quadratic and interaction effects, according to the following equation:

\[
Y = \beta_0 + \sum_i \beta_i X_i + \sum_{i,j} \beta_{ij} X_i X_j
\]

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

The number of colonies was calculated using the Total Plate Counting (TPC) method. A total of
0.5 grams of the dried sample was diluted in series 10⁻¹-10⁻¹⁰ using sterile distilled water.
A 200 μL from 10⁻⁷–10⁻¹⁰ dilution were transferred into a petri dish and added to MRS agar media,
incubated at 30°C for 48 hours. LAB colonies that grew were categorized as *L. plantarum* TSD-10
BTCC 531 which survived during drying process production. Furthermore, the validation has been
performed at the optimum condition.

3. Results and discussion

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
an influence on the production of probiotic powder inoculum from *L. plantarum* TSD-10 BTCC 531.
Response Surface Method (RSM)-Central Composite Design (CCD) was used to find the optimal
concentrations of these variables for production of probiotic powder inoculum from *L. plantarum*
TSD-10 BTCC 531. A CCD with three variables at five levels was followed to determine the response
pattern and also to determine the synergy of variables [18]. According to this design, 20 runs were
conducted containing six replications at the central point for estimating the purely experimental
uncertainty variance. A rotatable central composite design is one of the efficient central composite
designs, which has points which are equidistant from the center. Thirty experiments were performed to
examine the combined effect of five independent variables on probiotic powder inoculum. 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 [16]. In this study, the
*F*-value was 4.85 implied that could demonstrate the model was significant. The *P*-value (P>F, 0.0107) was significant that it could also prove the good fit of the model and there was only 1.07% chance that a model *F*-value could have occurred due to noise. The *F*-value indicated the significance of the quadratic model predicted by RSM and also established that the quadratic polynomial equation.
guiding this process was significant at 95% confidence level. 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 the equation also indicated a reasonable fit of the second-order model as an approximation to the true response [16]. The insignificant lack of fit (>0.05) also indicated the validity of the quadratic model for the present study.

Table 2. The CCD design of experiment and the cell number of L. plantarum TSD-10 BTCC 531.

| No. | Skim milk (%) | Maltodextrin (%) | CaCO₃ (%) | Cell number CFU/gr (x 10¹²) |
|-----|---------------|-----------------|-----------|-----------------------------|
| 1   | 5             | 1               | 0.5       | 1.9211                      |
| 2   | 15            | 1               | 0.5       | 0.8404                      |
| 3   | 5             | 4               | 0.5       | 1.3763                      |
| 4   | 15            | 4               | 0.5       | 1.0371                      |
| 5   | 5             | 1               | 1.5       | 1.4563                      |
| 6   | 15            | 1               | 1.5       | 0.4756                      |
| 7   | 5             | 4               | 1.5       | 0.7441                      |
| 8   | 15            | 4               | 1.5       | 1.0268                      |
| 9   | 1.591         | 2.5             | 1         | 1.0008                      |
| 10  | 18.409        | 2.5             | 1         | 0.3890                      |
| 11  | 10            | -0.0227         | 1         | 0.5052                      |
| 12  | 10            | 5.0227          | 1         | 1.0268                      |
| 13  | 10            | 2.5             | 0.159     | 2.2166                      |
| 14  | 10            | 2.5             | 1.841     | 0.4441                      |
| 15  | 10            | 2.5             | 1         | 1.5306                      |
| 16  | 10            | 2.5             | 1         | 1.6180                      |
| 17  | 10            | 2.5             | 1         | 1.6232                      |
| 18  | 10            | 2.5             | 1         | 1.2941                      |
| 19  | 10            | 2.5             | 1         | 1.3633                      |
| 20  | 10            | 2.5             | 1         | 1.2167                      |

Skim milk (X₁), CaCO₃ (X₃), interaction between skim milk and maltodextrin (X₁X₂) and quadratic model term (X₁² and X₂²) have been shown to significantly affect probiotic powder inoculum production (p<0.05). Meanwhile, maltodextrin, interaction between skim milk and CaCO₃ (X₁X₃), interaction between maltodextrin and CaCO₃ (X₂X₃) did not give a significant effect on probiotic powder inoculum production (p>0.05). This result is in conformity with another study proving that if the p value is below 0.05 then the model terms are preferred in representing the reliability of the results. The value of adequate precision is used to measure the signal noise ratio where the desired ratio value is greater than 4. Adequate precision obtained in this study is 8.2118 so that it significantly stated the suitability of the model and model could be used to navigate the design space. The relationship that transpired between those variables and number of cells are illustrated as a three dimensional representation and counter plot of the response surfaces shown in figure 1, 2 and 3. The generated regression relationship is given in equation was obtained as follows (according table 4):

\[ Y = 1.43 - 0.2304X₁ - 0.3261X₃ + 0.2506X₁X₂ - 0.2194X₁² - 0.1943X₂² \]

In the above equation, Y is the predicted response of a number of cells, X₁, X₂ and X₃ are the coded values of the tested variables of skim milk, maltodextrin 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. The high determination coefficient (R²=0.8135) proved the goodness fit of the model, suggesting that the sample variance of 81.35% in the production of probiotic powder inoculum was attributed to the variable factors.
Table 3. ANOVA for response surface quadratic.

| Source        | Sum of squares | df | Mean square | F-value | p-value |  
|---------------|----------------|----|-------------|---------|---------|
| Model         | 3.91           | 9  | 0.4347      | 4.85    | 0.0107  | Significant |
| X₁-Skim milk | 0.7251         | 1  | 0.7251      | 8.09    | 0.0174  |
| X₂-Maltodextrin | 0.0099       | 1  | 0.0099      | 0.1105  | 0.7464  |
| X₃-CaCO₃      | 1.45           | 1  | 1.45        | 16.19   | 0.0024  |
| X₁ X₂         | 0.5024         | 1  | 0.5024      | 5.60    | 0.0395  |
| X₁ X₃         | 0.0652         | 1  | 0.0652      | 0.7265  | 0.4140  |
| X₂ X₃         | 0.0044         | 1  | 0.0044      | 0.0489  | 0.8295  |
| X₁²           | 0.6938         | 1  | 0.6938      | 7.74    | 0.0194  |
| X₂²           | 0.5439         | 1  | 0.5439      | 6.07    | 0.0335  |
| X₃²           | 0.0004         | 1  | 0.0004      | 0.0044  | 0.9483  |
| Residual      | 0.8968         | 10 | 0.0897      |         |         |
| Lack of Fit   | 0.7463         | 5  | 0.1493      | 4.96    | 0.0518  | Not significant |
| Pure Error    | 0.1505         | 5  | 0.0301      |         |         |
| Cor Total     | 4.81           | 19 |             |         |         |

Table 4. Regression coefficients & statistical significance from Response Surface Quadratic.

| Factor          | Coefficient Estimate | df | Standard Error | 95% CI Low | 95% CI High |
|-----------------|----------------------|----|----------------|------------|-------------|
| Intercept       | 1.43                 | 1  | 0.1221         | 1.16       | 1.71        |
| X₁-Skim milk    | -0.2304              | 1  | 0.0810         | -0.4110    | -0.0499     |
| X₂-Maltodextrin | 0.0269               | 1  | 0.0810         | -0.1536    | 0.2075      |
| X₃-CaCO₃        | -0.3261              | 1  | 0.0810         | -0.5066    | -0.1455     |
| X₁ X₂           | 0.2506               | 1  | 0.1059         | 0.0147     | 0.4865      |
| X₁ X₃           | 0.0902               | 1  | 0.1059         | -0.1457    | 0.3261      |
| X₂ X₃           | 0.0234               | 1  | 0.1059         | -0.2125    | 0.2593      |
| X₁²             | -0.2194              | 1  | 0.0789         | -0.3952    | -0.0436     |
| X₂²             | -0.1943              | 1  | 0.0789         | -0.3700    | -0.0185     |
| X₃²             | 0.0052               | 1  | 0.0789         | -0.1705    | 0.1810      |

Three-dimension response surface and contour plot were made to investigate the relationship between different variables and response, in order to obtain the optimal probiotic powder inoculum from conditions that would maximize the yield of cell number of *L. plantarum* TSD-10 BTCC 531. Figure 1 shows the response surface plots as function of skim milk and maltodextrin interaction on cell number of *L. plantarum* TSD-10 BTCC 531. Figure 1 shows that the cell number decrease with increase in reaction skim milk. Figure 1 also shows that the cell number increase with increase in maltodextrin reaction.
Figure 1. (A) Three-dimensional (3D) response surface and (B) contour plot showing the effect of skim milk, maltodextrin and their mutual interaction.

Figure 2 shows the response surface plots as function of skim milk and CaCO$_3$ interaction on the cell number of *L. plantarum* TSD-10 BTCC 531. Figure 1 shows that the cell number decrease with increase in reaction CaCO$_3$ and also shows that the cell number decrease with increase in skim milk reaction.

Figure 2. (A) Three-dimensional (3D) response surface and (B) contour plot showing the effect of skim milk, CaCO$_3$ and their mutual interaction.
Figure 3. (A) Three-dimensional (3D) response surface and (B) contour plot showing the effect of maltodextrin, CaCO3 and their mutual interaction.

Figure 3 shows the response surface plots as function of maltodextrin and CaCO3 interaction on the cell number of L. plantarum TSD-10 BTCC 531. Figure 3 shows that the cell number decrease with increase in reaction CaCO3 and also shows that the cell number decrease with increase in CaCO3 reaction.

The experiment produced a regression model that could be used to optimize probiotic powder inoculum. In accordance with the calculation of the regression model, skim milk, maltodextrin and CaCO3 were 5, 1.547 and 0.5 %, respectively, with a maximum predicted cell number of 1.945 x 10^{12} CFU/gr, which is increased by 34.98%. The results of the validation experiments show that the cell number of probiotic reaches 2.17 x 10^{12} CFU/gr or 11.43% more than the predicted cell number of L. plantarum TSD-10 BTCC 531 from the regression model produced.

4. Conclusion
The production of probiotic powder inoculum from L. plantarum TSD-10 BTCC 531 is significantly influenced by skim milk (X1), CaCO3 (X3), interaction between skim milk and maltodextrin (X1X2) and quadratic model term (X1² and X2²). Optimum medium composition for the production of probiotic powder inoculum from L. plantarum TSD-10 BTCC 531 is as follows: 5% skim milk, 1.547% maltodextrin, 0.5% CaCO3. According to the model developed, maximum cell number of probiotic powder inoculum from L. plantarum TSD-10 BTCC 531 of 1.945 x 10^{12} CFU/g, which is increased by 34.98% when optimum condition were used.

Acknowledgments
The authors would like to acknowledge the Center of Excellent (PUI) 2018–2019 KEMENRISTEK and Program Pengembangan Teknologi Industri (PPTI) 2019–2020 for the financial support and Ditta Putri for her assist.

References
[1] Grashorn M A 2010 Use of phytobiotics in broiler nutrition – an alternative to infeed antibiotics J. Anim. Feed Sci. 19 338–47
[2] Wang H, Ni X, Qing X, Zeng D, Luo M, Liu L, Li G, Pan K and Jing B 2017 Live probiotic Lactobacillus johnsonii bs15 promotes growth performance and lowers fat deposition by
improving lipid metabolism, intestinal development, and gut microflora in Broilers \textit{Front. Microbiol.} \textbf{8} 1073

[3] Dowarah R, Verma A K, Agarwal N and Singh P 2018 Efficacy of species-specific probiotic \textit{Pediococcus acidilactici} i FT28 on blood biochemical profile, carcass traits and physicochemical properties of meat in fattening pigs \textit{Res. Vet. Sci.} \textbf{117} 60–4

[4] Puphan K, Soraprapong P, Uriyapongson S and Navanukraw C 2015 Screening of lactic acid bacteria as potential probiotics in beef cattle \textit{Pakistan J. Nutrition} \textbf{14} 474–79

[5] Seo J K, Seon-Woo K, Kim M H, Santi D, Kam D K and Jong K H 2010 Direct-fed microbials for ruminant animals \textit{Asian-Aust. J. Anim. Sci.} \textbf{12} 1657–67

[6] Sanders M E and Klaenhammer T R 2001 Invited review: the scientific basis of \textit{Lactobacillus acidophilus} NCFM functionality as a probiotic \textit{J. Dairy Sci.} \textbf{84} 319–31

[7] Simon O 2005 Micro-organisms as feed additives–probiotics \textit{Advances in Pork Production} \textbf{16}

[8] Hwang C F, Chang J H, Houng J Y, Tsai C C, Lin C K and Tsen H Y 2012 Optimization of medium composition for improving biomass production of \textit{Lactobacillus plantarum} P106 using the taguchi array design and the Box-Behnken method \textit{Biotechnol. Bioprocess. Eng.} \textbf{17} 827–34

[9] Ananta E, Heinz V and Knorr D 2004 Assessment of high pressure induced damage on \textit{Lactobacillus rhamnosus} GG by flow cytometry \textit{Food Microbiol.} \textbf{21} 567–77

[10] Reid A, Champagne C P, Gardner N, Fustier P and Vuillemard J C 2007 Survival in food systems of \textit{Lactobacillus rhamnosus} R011 microentrapped in whey protein gel particles \textit{J. Food Sci.} \textbf{72} 31–7

[11] Chen H, Tian M, Chen L, Cui X, Meng J and Shu G 2019 Optimization of composite cryoprotectant for freeze-drying \textit{Bifidobacterium bifidum} BB01 by response surface methodology \textit{Artificial Cells, Nanomedicine, Biotechnology} \textbf{47} 1559–69

[12] Morgan, Herman N, White P A and Vesey G 2006 Preservation of micro-organisms by drying: a review \textit{J. Microbiol. Methods} \textbf{66} 183–93

[13] Carvalho A S, Silva J, Ho P, Teixeira P, Malcata F X and Gibbs P 2004 Effects of various sugars added to growth and drying media upon thermostolerance and survival throughout storage of freeze-dried \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus} \textit{Biotechnol. Prog.} \textbf{20} 248–54

[14] Mehnoush A, Mustafa S and Yazid A M M 2012 Optimization of freeze drying conditions for purified pectinase from mango (\textit{Mangifera indica} cv. Chokanan) peel \textit{Int. J. Mol. Sci.} \textbf{13} 2939–50

[15] Han L, Pu T, Wang X, Liu B, Wang Y, Feng J and Zhang X 2018 Optimization of a protective medium for enhancing the viability of freeze-dried \textit{Bacillus amyloliquefaciens} B1408 based on response surface methodology \textit{Cryobiology} \textbf{81} 101–06

[16] De Man J C, Rogosa M and Sharpe M E 1960 A medium for the cultivation of \textit{Lactobacilli J. Oppl. Bact.} \textbf{23} 130–35

[17] Coman G and Bahrim G 2011 Optimization of xylanase production by \textit{Streptomyces} sp. P12-137 using response surface methodology and central composite design \textit{Ann. Microbial.} \textbf{61} 773–79

[18] Jose P A, Sivakala K K and Jebakumar S R D 2013 Formulation and statistical optimization of culture medium for improved production of antimicrobial compound by \textit{Streptomyces} sp. \textit{JAJ06 Int. J. Microbiol.} 1–9