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Optimization of robusta green coffee fermentation by *Bifidobacterium bifidum* and *Lactobacillus plantarum* using response surface methodology

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Abstract

In vitro fermented robusta coffee is fermented coffee beans using lactic acid bacteria (LAB) isolated from the stomachs of civets. *In vitro* fermented robusta coffee was obtained by optimizing three variables, namely inoculum concentration, use of microorganisms *Bifidobacterium bifidum* and *Lactobacillus plantarum* and *in vitro* fermentation time using response surface methodology (RSM). This study aimed to produce fermented robusta coffee *in vitro* with optimal cupping test results using three variables that affect fermentation. The results of the RSM showed that the optimum cupping test results were 72.62, inoculum concentrations of 5% (w/v), *in vitro* fermentation time of 8 hrs and the ratio of *B. bifidum/L. plantarum* at a ratio of 1:0.

1. Introduction

Coffee is one of the most popular and widely consumed beverages worldwide. Coffee has a variety of flavours and aromas that can provide physiological and psychological effects beyond its nutritional benefits (Haile and Kang, 2019). Based on world coffee production data, Indonesia ranks fourth in 2019/2020 harvest year (ICO, 2021). Coffee has excellent potential as a driver of the Indonesian economy, especially for the export market (Kusmiyati and Fudholi, 2021). There are two types of coffee beans that have been most consumed, namely Robusta and Arabica coffee (Afriliana *et al*., 2018a). In addition to these two types of coffee, civet coffee is also one of the most popular coffees in the world (Ongo *et al.*, 2012). However, the rapidly growing demand for civet coffee is not supported by an adequate number of civet cattle to produce civet coffee (Muzaifa, 2019). This has resulted in the price of civet coffee soaring in the market (Jumhawan *et al.*, 2013)

Civet coffee, also known as Luwak coffee is known globally to have a different taste and aroma from the original coffee (Lopetcharat *et al*., 2016). Luwak coffee is produced from coffee cherries that have been digested by microorganisms found in the digestive tract of civet cats (*Paradoxurus Hermaphroditus*) (Jumhawan *et al*., 2016). The utilization of microorganisms from civet stomachs in coffee fermentation is an attempt to produce coffee with the characteristic taste of natural civet coffee (the most expensive coffee in the world) (Salengke *et al.,* 2019). The natural fermentation process for Luwak coffee has many limitations. The production process is inefficient and less hygienic because it can be contaminated with pathogenic bacteria like *E. coli* and *Salmonella* spp. Therefore, several studies have begun to develop a quality civet coffee production method without using the natural fermentation of civet animals, one of which is the *in vitro* fermentation method (Fitri *et al.*, 2019).

Luwak coffee production process with *in vitro* fermentation is a fermentation technique using lactic acid bacteria (LAB) isolated from the stomach of civet animals. The enzymatic activity of LAB in coffee fermentation affects the sensory and nutritional characteristics that can improve the quality of civet coffee (Muzaifa *et al.,* 2019). Currently, the processing of coffee beans is mainly done with a combination of lactic acid probiotic bacteria. The most used types of lactic acid probiotic bacteria are the *Lactobacillus plantarum* and *Bifidobacterium bifidum,* which are also used to treat various diseases causing drug-resistant pathogenic microbes (Mulaw *et al.*, 2019). Previous

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studies have demonstrated that LAB is acceptable for coffee fermentation without interfering with yeast development or shortening the fermentation duration (Pereira *et al.,* 2016). Therefore, researchers are interested in examining the potential of coffee beans combined with lactic acid probiotic bacteria with a fermentation scheme. In this study, *B. bifidum* and *L. plantarum* were obtained from the culture collection of the Laboratory of Biotechnological Processes at LPBII/ UFPR, Curitiba, Parana State, Brazil. They were evaluated for their quality in coffee fermentation. *Lactobacillus plantarum* LPBR01 has been previously shown to promote the dominance of LAB without interfering with the yeast growth and drastically reduce the time of fermentation.

There are several variables in fermentation that play important roles in the success of the bioprocess industry besides the microorganisms used, such as inoculum concentration, urea concentration and fermentation time (Yang *et al.,* 2018; Hui *et al.*, 2020). Meanwhile, there is a method that can be developed to optimize the fermentation process using the response surface methodology (RSM). The RSM is a combination of mathematical and statistical techniques used in the optimization of the fermentation media to obtain an optimal response (Suhaimi *et al.,* 2018; Ching *et al.,* 2022). The use of RSM can display three-dimensional graphics using Minitab v17 software. Based on the previous research, the effect of time and temperature conditions for sorghum fermentation using RSM, the optimal temperature and time for optimal fermentation occurred at 34°C and 24 hrs to obtain the maximum content of tannins, flavonoids, phenolics and antioxidant activity, equipped with good microbial growth, decreased pH and high organic acid production (Adebo *et al.*, 2018). Hence, this study aimed to obtain the optimal cupping test results with variables of inoculum concentration, fermentation time and microorganism comparison using RSM. *In vitro* fermentation was carried out on robusta coffee beans using two types of microorganisms: *B. bifidum* and *L. plantarum.*

2. Materials and methods

2.1 Materials

Robusta coffee cherries were procured from plantations in Banyuanyar, Boyolali Village, Central Java, Indonesia. The fermentation microorganisms which are also LAB (*B. bifidum* and *L. plantarum)* were obtained from Gadjah Mada University. Other materials including molasses, de Man Rogosa Shape (MRS) agar, MRS broth and distilled water were purchased from Nitra Kimia, Darmstadt, Germany.

2.2 Methodology

The main ingredient, robusta coffee cherries were put into a pulper machine to remove the skin and then sun-dried for about 3 to 4 days to get a moisture content of less than 12%. This is because coffee beans with moisture above 12% are more prone to unwanted consequences such as microbial growth, fermentation, mycotoxin formation and alteration of sensory characteristics (Reh *et al.,* 2006; Derisma *et al.,* 2019). Dried coffee beans were fed into a huller machine to remove the horn skin and sort. Molasses was used as a starter culture and basic media such as MRS agar, MRS broth and distilled warer were used for bacterial growth.

2.3 Production of lactic acid bacteria inoculum in MRS medium

Bifidobacterium bifidum and *L. plantarum* were cultured onto MRS media at 37°C for 24 hrs. The production of LAB bacterial inoculum using MRS medium has been modified by referring to the previous study (Yang *et al.,* 2018) where the original LAB inoculum used was 20 mL of MRS broth at 37°C for 24 hrs. The modification was made by dissolving 5.2 g MRS broth in 100 mL distilled water and heated at 37°C for 16 hrs. The medium was sterilized at 121°C for 15 mins using an autoclave. The bacterial colonies of *B. bifidum* and *L. plantarum* were then inoculated separately in MRS broth media and incubated at 37°C for 24 hrs. The bacterial culture was ready to be inoculated on the fermentation medium

2.4 Starter culture production

The starter culture production has been modified by referring to the previous study (Martinez *et al.,* 2019). The modifications were the differences in the amount of molasses, temperature and time. In the previous study, the molasses used was 10 mL with a temperature of 30° C and incubation time of 48 hrs. In this study, 175 g of molasses was diluted in 1 L of distilled water, then heated and sterilized using an autoclave at 121°C for 15 mins. Subsequently, the cells were centrifuged and resuspended in distilled water (enough for inoculation without increasing the moisture of the coffee). The ratio of *B. bifidum* and *L. plantarum* at 0:1, 1:1 and 1:0 was incubated at 37°C for 24 hrs in cooled molasses media. After the bacteria had been incubated, the culture was used for *in vitro* fermentation (Kusmiyati *et al.,* 2020).

2.5 In vitro fermentation process

In vitro fermentation of robusta coffee beans was performed with different ratios of *B. bifidum* and *L. plantarum*. The huller was used to extract the coffee husk from the robusta beans (pulping). *Bifidobacterium*

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bifidum and *L. plantarum* were mixed with coffee beans in a fermentation tank with 5-15% (v/w) where v is the volume of inoculum (mL) and w is the weight of coffee beans. The *in vitro* fermentation time for coffee beans was 0 to 48 hrs and the incubation temperature was set at 38°C. After the fermentation process, the coffee beans were washed and dried in an oven at 50°C until the moisture content reached 10 to 12%. Subsequently, the robusta coffee beans were roasted in an oven at roasting temperature of 220°C for 20 mins. The robusta coffee was ground after roasting and was passed through a 20-mesh sieve size prior to the cupping test (Salengke *et al*., 2019).

2.6 In vitro fermentation optimization using response surface methodology

This study was designed as a Box Behnken design (BBD) through RSM with three parameters: the inoculum concentration, *in vitro* fermentation time and the ratio of microorganisms used. Three-dimensional curves were used to test the validity of the effect of variables on the results obtained. The coefficient on the empirical model was estimated using multivariate regression analysis, where the suitability of the empirical model with experimental data can be determined from the coefficient of determination (R^2). The regression equation obtained was used to determine the most optimal response conditions. In general, the RSM regression equation is shown in Equation (1) and (2).

From the initial formulation with 3 factorials, the linear regression equation becomes:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \varepsilon \quad (1)$$

where, y is for the dependent variable (observed response), k is for factorial, β is for parameter values, x_i

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \quad (2)$$

is for independent variables and the residual (error) component (Bas and Boyaci, 2007). Meanwhile, the parameters used were inoculum concentration (x_1), fermentation time (x_2) and microorganism ratio (x_3). Table 1 shows the coded values of the three parameters used in this study. The lowest code value was -1, the median value was 0 and the highest value was 1. The results for RSM data processing were obtained with the established three parameters, which had 15 runs of a combination of the three variables with three centre points. The ideal outcome for *in vitro* fermented robusta coffee was established after 15 runs of a cupping test were completed. Every run consists of two replicates and its average standard deviation is 0.769.

Table 1. The codification of the research variables.

Factor	Original value		Coded value	
	Low	High	Low	High
Inoculum Concentration (%)	5	15	-1	1
Fermentation Time (hrs)	8	48	-1	1
Ratio *B. bifidum/L. plantarum*	-1	1	-1	1

2.7 Cupping test analysis

Cupping test analysis was carried out on the roasted and ground robusta coffee to determine the quality of the coffee products. Four panelists were invited and performed the cupping test analysis by referring to the Specialty Coffee Association of America (SCAA, 2015) method. The cupping test indicators include flavour, aftertaste, acidity, body, balance, uniformity, cups clean, sweetness, defects and overall (Ribeiro *et al*., 2018). The range of cupping test results was evaluated on a scale of 6 to 10 in increments of 0.25 and the sum of the ten cupping test indicators was the final score for each sample (Salengke *et al*., 2019). The total cupping test ranges from 50-100, the non-special category has a score of <80; the special category (very good) with a score between 80-84.99; the special category (excellent) with scores between 85-89.99 and special category (very excellent) with scores between 90-100 (Afriliana *et al.*, 2018). Furthermore, the results of the cupping test of the coffee were categorized into three groups: dislike, neutral and like (Stokes *et al.*, 2017).

2.8 Statistical analysis

The data obtained based on the statistical design were analyzed using Minitab v.17 software and analysis of variance (ANOVA) to test the significance.

2.9 Response surface plot analysis

The response surface plot in this study was needed to determine the response value and the desired operating conditions. In a contour plot, the response surface is viewed as a two-dimensional plane where all points having the same response are connected to produce a constant response contour line. Measurement of the response surface plot in this study was done with three main variables, namely fermentation time, *B. bifidum/L. plantarum* ratio and inoculum concentration.

3. Results and discussion

3.1 Cupping test optimization

Based on Table 2, the results show that the highest cupping test value was 72, obtained at 5% inoculum concentration (w/v) with fermentation time of 28 hrs and at 10% concentration (w/v) with fermentation time 8 hrs. The ratio of the microorganism *B. bifidum/ L. plantarum* was at 1:0. For the assessment of coffee cupping (or often referred to as coffee tasting), assessment options were provided for panelists with the following divisions:

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Table 2. Total cupping test of *in vitro* fermented civet coffee on 15 runs obtained from the study design using Minitab v.17.

Inoculum Concentration (%)	Fermentation Time (hrs)	Ratio *B. bifidum/ L. plantarum*	Total Cupping Test	Standard deviation
10	28	1:01	69.25	0.769
10	8	0:01	68.75	0.781
15	28	0:01	67.38	0.747
10	28	1:01	70.22	0.804
5	48	1:01	69.25	0.768
10	28	1:01	68.28	0.763
10	8	1:00	72.5	0.782
15	8	1:01	70.88	0.758
15	48	1:01	66.5	0.805
5	8	1:01	70.38	0.776
10	48	1:00	69.88	0.732
15	28	1:00	70	0.748
5	28	0:01	67.25	0.787
5	28	1:00	72.38	0.738
10	48	0:01	65.88	0.777
				p-value = 0.004

the most delicious taste is close to 90-100, quite good 70 -80, normal 50-60, tasteless 30-40, quite bad 0-20. Based on SCAA Protocols, these are rated on a 16-point scale representing levels of quality in quarter-point increments between numeric values from 6 to 9 (Specialty Coffee Association of America, 2015). The unique taste for 2 conditions has met the highest taste: (i) 10% concentration (w/v) with fermentation time of 8 hrs has a score of 72.50, representing a good but bitter coffee; (ii) 5% concentration (w/v) with fermentation time 28 hrs has a score of 72.38, indicating it has a more fragrant and acidity coffee.

In the previous study, the increase in the total cupping test value was caused by organic acids, amino acids and reducing sugars produced (Borem *et al.*, 2016). These acidic compounds can improve the taste of civet coffee and fermentation time is a determining factor in achieving coffee quality (Jumhawan *et al*., 2013; Ribeiro *et al*., 2018). A longer fermentation process can trigger a sour and sweet taste because the natural bacteria in coffee converts malic acid into lactic acid which gives it a sweet taste (Mota *et al*., 2020). Succinic and malic acids have an impact on the sensory qualities of coffee beverages (Silva *et al*., 2013). Therefore, it can be inferred that the fermentation process will affect the acidity of the coffee which gives it a unique taste between sweet-sour-bitter.

The increase in the total cupping test value was also influenced by the ratio of the microorganism *B. bifidum/ L. plantarum.* This was in agreement with the results achieved in the ratio 1:0 in which the *B. bifidum* was only added during the *in vitro* fermentation process had greater cupping test values. This inferred that *B. bifidum* has good therapeutic and nutritional properties to produce thiamine, riboflavin, vitamin B6 and vitamin K (Moriya *et al.,* 2006). Inoculum concentration did not affect the total cupping test results of *in vitro* fermented civet coffee samples.

Meanwhile, the lowest cupping test value of 65.88 was obtained under conditions of 10% inoculum concentration, 48 hrs of fermentation time and a 0:1 ratio of *B. bifidum* and *L. plantarum* co-culture. The decrease in the total cupping test value was caused by the longest fermentation time and ratio of the microorganism *B. bifidum/L. plantarum* at a ratio of 0:1. High inoculum concentration and high ratio of *L. plantarum* for *in vitro* fermented robusta coffee samples can increase the sour taste. In the previous study, the sour taste of *in vitro* fermented civet coffee is caused by phosphoric acid, chlorogenic acid, quinic acid and aliphatic acid (Bekedam *et al.,* 2006). High levels of chlorogenic acid can reduce the taste of *in vitro* fermented civet coffee because the acid produces an astringent and bitter taste in coffee drinks (Jumhawan *et al.*, 2013).

3.2 Processing data using response surface methodology

The coefficient of determination on the cupping test value has been estimated using Minitab v.17 as summarized in Table 3. Based on the results from the RSM analysis, the regression equation was obtained and as shown in Equation (3):

$$y = 70.02 + 0.115x_1 + 0.0125x_2 + 3.10x_3 + 0.000{x_1}^2 - 0.000{x_2}^2 + 0.000{x_3}^2 - 0.00813x_1x_2 - 0.125x_1x_3 + 0.0031x_2x_3 \quad (3)$$

The coefficient of determination (R^2) is the total value of variation represented by the test results. This

value is important in determining the optimum variable of robusta coffee. The value of R^2 for the total cupping test was 0.9651, indicating 96.51% of the total variability of the cupping test efficiency. The value of the R^2 has a range of $0<R^2<1$. The R^2 which is close to 1 can be used to predict the response effectively (Behrouzian *et al.*, 2016). The R^2 value produced in this study meets the range of R^2, which is $0<0.9651<1$. Linear model with R^2 (43.54%) had lower R value than that of quadratic model (R^2 = 96.51%). When compared to the linear model, the quadratic model could closely approximate the outcomes of the cupping test. The panelists opted for a moderate flavour over one that was more bitter or sweet.

Table 3. Estimated regression coefficients for cupping test using BBD.

Term	Coef.	SE Coef.	T
Constant	69.250	0.354	195.77
X_1-Inoculum	-0.563	0.217	-2.60
X_2-Time	-1.375	0.217	-6.35
X_3-Ratio	1.938	0.217	8.94
X_1-Inoculum* X_1-Inoculum	-0.000	0.319	-0.00
X_2-Time* X_2-Time	-0.000	0.319	-0.00
X_3-Ratio* X_3-Ratio	0.000	0.319	0.00
X_1-Inoculum* X_2-Time	-0.813	0.306	-2.65
X_1-Inoculum* X_3-Ratio	-0.625	0.306	-2.04
X_2-Time* X_3-Ratio	0.063	0.306	0.20
S = 0.612691			
R^2 = 0.9651; R^2 (adjusted) = 0.9023; R^2 (predicted) = 0.9215			

Overall, results deduced that shorter fermentation time would have bitter taste because of the chlorogenic acid in coffee. On the other hand, longer fermentation time would have sour taste because of the malic and succinic acids. Due to the naturally occurring bacteria in coffee that transform malic acid into lactic acid, it would then be sweetened (Mota *et al*., 2020). This showed that the variables could be explained well by the model so that the optimization in determining the optimum variable for *in vitro* fermentation of robusta coffee using the RSM method was considerably effective.

3.3 Statistical analysis

The statistical significance of the quadratic polynomial model equation was evaluated by the ANOVA test as demonstrated in Table 4. ANOVA results explained the responses of the three study variables: X_1 (inoculum concentration), X_2 (fermentation time) and X_3 (*B. bifidum/L. plantarum* ratio). The square term and an interaction term for research variables (X_1, X_2 and X_3) were not significant (p = 1.00 and p = 0.095) at 95% probability level ($p<0.05$), while the linear term was significant at 95% probability level. As for the results of the lack of fit, they were not significant (p = 1.00), showing minimum error and suitability of a model. All research variables were significant at the 95% probability level. A previous study on the ANOVA test had explained the response of the three dependent variables: Y_1 (pH), Y_2 (anti-lipase activity) and Y_3 (anti-adipogenic activity) in optimizing the anti-obesity effect of fermented milk by *L. plantarum* Q180 with RSM (Park *et al.,* 2014). In their study, the square term and an interaction term for the dependent variables (Y_1, Y_2 and Y_3) were not significant at the 95% probability level. In this study, the square term and an interaction term were also applied for the dependent variable of *in vitro* fermentation process of robusta coffee and found to be significant.

3.4 Response surface plot

Figure 1 shows the cupping test results of the response contour plot which are influenced by fermentation time and inoculum concentration. Figure 1a

Table 4. Analysis of variance for cupping test

Source	DF	Adj SS	Adj MS	F	P
Regression Model	9	51.9063	5.7674	15.36	0.004
Linear	3	47.6875	15.8958	42.34	0.001
X_1-Inoculum	1	2.5313	2.5313	6.74	0.048
X_2-Time	1	15.1250	15.1250	40.29	0.001
X_3-Ratio	1	30.0313	30.0313	80.00	0.000
Square	3	0.0000	0.0000	0.00	1.000
X_1-Inoculum* X_1-Inoculum	1	0.0000	0.0000	0.00	1.000
X_2-Time* X_2-Time	1	0.0000	0.0000	0.00	1.000
X_3-Ratio * X_3-Ratio	1	0.0000	0.0000	0.00	1.000
Interaction	3	4.2188	1.4063	3.75	0.095
X_1-Inoculum* X_2-Time	1	2.6406	2.6406	7.03	0.045
X_1-Inoculum* X_3-Ratio	1	1.5625	1.5625	4.16	0.097
X_2-Time* X_3-Ratio	1	0.0156	0.0156	0.04	0.846
Error	5	1.8770	0.3754		
Lack of Fit	3	0.0000	0.0000	0.00	1.000
Pure Error	2	1.8770	0.9385		
Total	14	53.7832			

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displays the response surface plot, demonstrating the range of lower inoculum concentrations in order to produce the best overall cupping results in the dark red area. The high total cupping test value in this study was caused by organic acids, amino acids and reducing sugars produced from the *in vitro* fermentation process for 48 hrs which was also discovered by the previous study (Sudarma *et al.*, 2019). These acidic compounds can improve the taste of fermented civet coffee *in vitro* (Jumhawan *et al.*, 2013). Fermentation time is a determining factor in achieving coffee quality, if the fermentation is too short or too long, it will make the coffee have a low cupping test (Muzaifa *et al.,* 2018).

Figure 1b depicts that fermentation using *B. bifidum* had performed the greatest overall best cupping results. This suggested that the *B. bifidum* microbe is more preferable to include in the *in vitro* fermentation process since it has good nutritional content and therapeutic properties (Moriya *et al.,* 2006). When the microorganism *B. bifidum* was merely added during the *in vitro* fermentation process, good nutrition from the microorganism *B. bifidum* would also be beneficial for the health of consumers who drink coffee.

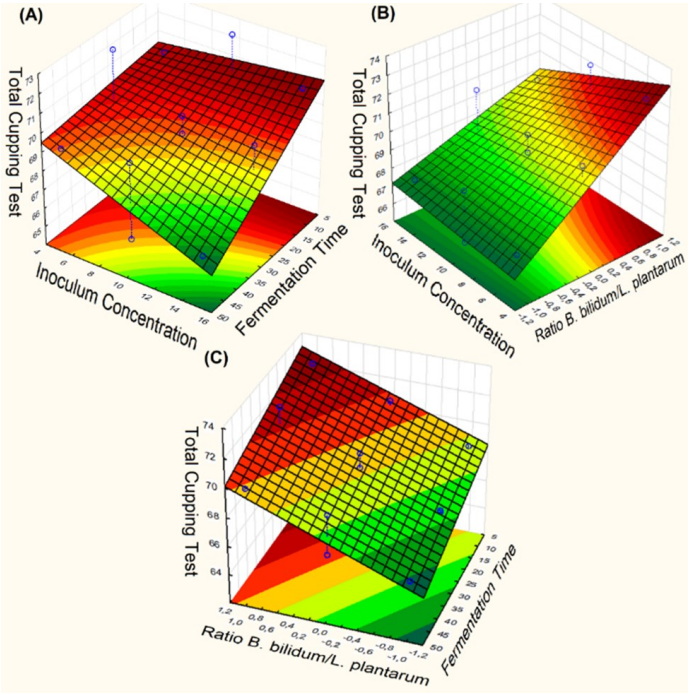

Figure 1. Response surface plot of cupping test content from inoculum concentration, ratio of *B. bifidum/ L. plantarum* and fermentation time based on the results obtained from the Box-Behnken experimental design.

Figure 1c displays the response surface plot, which demonstrates the range of lower fermentation time could produce the best overall cupping results in the dark red area. The decrease in the value of the total cupping test at the time of *in vitro* fermentation from 32 to 48 hrs was due to the increase in acid levels during *in vitro* fermentation process (Sudarma *et al.,* 2019). During *in vitro* fermentation process, the sucrose content in coffee beans will be broken down into glucose and fructose, which are further converted into organic acids and alcohol (Pereira *et al.,* 2016). Several researchers have indicated that the acidity of coffee is caused by the presence of organic acids including phosphoric acid, quinic acid, aliphatic acid and chlorogenic acid in roasted civet coffee beans (Ribeiro *et al.,* 2011). Chlorogenic acid affects the bitterness of coffee thus high content of chlorogenic acid in coffee can produce undesirable flavours (Muzaifa *et al.*, 2018).

The optimum total cupping test obtained from the RSM calculation was 72.62 at a fermentation time of 8 hrs, inoculum concentration of 5% and a ratio of 1.0 for bacteria used (*B. bifidum/L. plantarum*). The results of the static quadratic model showed that 8 hrs is the appropriate time for fermentation to produce the best cupping test results. This was because the longer the fermentation time, the more the flavour will change to be sour and reduce the bitter taste of coffee. Table 5 summarizes the data validation from experimental and data predictive from RSM. Results depict that the optimum variables were validated with a low experimental error (average 0.40 %).

4. Conclusion

In this study, the RSM method was effective in optimizing the total cupping test analysis for fermented robusta coffee *in vitro*. The length of the fermentation process is more important in creating a distinct flavour. The results of the RSM showed that the optimum variables were with inoculum concentrations at 5% (w/v), *in vitro* fermentation time at 8 hrs and the ratio of *B. bifidum/L. plantarum* at 1:0. The optimum variable resulted in a total cupping test of 72.62. A significant

Table 5. Validation of data from experimental and data predictive from RSM.

Factor			Response Total Cupping Test			
Inoculum Concentration (%)	Fermentation Time (hrs)	Ratio *B. bifidum/ L. plantarum*	OV	PV	R	Error (%)
5	8	1:00	72.81	72.62	0.19	0.26
5	8	1:00	72.49	72.62	0.12	0.17
5	8	1:00	72.06	72.62	0.56	0.77
					Average	0.40

OV: Observed value, PV: Predicted value, R: Residual

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regression model and a high coefficient of determination (R^2) of 0.9651 allow the RSM method to be used as an effective optimization of the total cupping test for fermented robusta coffee *in vitro*.

Conflict of interest

The authors declare no conflict of interest.

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