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Research Article
Efficacy of Using *Bacillus subtilis* Enzyme as a Caffeine Level Reducer in Cascara Robusta Coffee (*Coffea canephora* L.)

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Abstract

Background and Objective: An increase in the consumption of robusta coffee resulted in an increase in waste from coffee, one of the coffee wastes, namely coffee bean skins or cascara. Robusta coffee cascara contains 1-1.3 g of caffeine which causes side effects, such as insomnia and seizures etc. So this research aims to reduce the caffeine content in cascara by using *Bacillus subtilis*. Using optimum conditions and capabilities. **Materials and Methods:** The experiment was conducted from May to August, 2022 in the Pharmacy Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Indonesia. Before optimizing, cascara was extracted using the ultrasonic assisted extraction (UAE) method, validated by the High-Performance Liquid Chromatography (HPLC) method to determine caffeine content and a paired sample t-test was performed using Statistical Package for the Social Sciences (SPSS). **Results:** It showed that in validating the HPLC method, the wavelength of caffeine in cascara was 272 nm. The mobile phase was a mixture of methanol-water (adjust orthophosphate). The pH (2.4) (45:55), obtained the optimum decaffeination conditions at the concentration of bacteria *Bacillus subtilis* 6% and a long incubation time of 24 hrs resulted in a decrease in caffeine content of 51.3843±0.2503%. **Conclusion:** The results of the paired sample t-test indicate that the concentration of bacteria *Bacillus subtilis* and incubation time significantly influence caffeine levels.

Key words: Cascara, decaffeination, *Bacillus subtilis*, caffeine, RSHPLC

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cascara is the skin of coffee cherries that have changed color, starting from green to turning red when the coffee is ripe. Secondary metabolites contained in cascara are useful as antioxidants, inhibiting α-glucosidase and α-amylase enzymes so that they can be consumed by diabetics[1]. Increasing the amount of coffee consumption causes the production of coffee skin waste to increase, approximately 30-50% weight of the coffee produced. Coffee waste results in coffee skins, which account for 50 to 60% of the harvest[2]. As 100 g of cascara contains caffeine with a level of 1-1.3 g, besides that, it also contains other compounds, including quinic acid, malic acid, chlorogenic acid, tannins, hydroxamic acid, catechins and epicatechins[3,4]. Caffeine is the most frequently used substance with a central nervous system stimulant effect, but its consumption is most often due to the intake of drinks that contain it (coffee, tea, chocolate)[4]. The chemical formula is $C_8H_{10}N_4O_2$ (1,3,7-trimethylxanthine). It is a natural chemical found in the leaves, seeds and fruits of more than 63 plant species around the world. Caffeine belongs to a type of alkaloid. Alkaloids are basic nitrogen-containing chemicals found in plants. Alkaloids usually have a bitter taste and are frequently physiologically active in humans[5]. The side effects of caffeine ingestion can have several drawbacks, such as headache, gastrointestinal discomfort, tachycardia, insomnia, nervousness and tremors[6].

To reduce the side effects of caffeine, decaffeination can be done, one of which is using the bacteria *Bacillus subtilis*, which can produce cellulase and protease enzymes. These two enzymes can break cellulose and protein bonds in the cascara cell membrane, so the caffeine extraction process can run smoothly.

Based on this background, a study was conducted to determine *Bacillus subtilis* in optimum conditions and capabilities to lower caffeine levels.

MATERIALS AND METHODS

Study area: The experiment was conducted from May to August, 2022 in the Pharmacy Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Indonesia.

Materials: The materials used in this study robusta coffee cascara, *Bacillus subtilis* bacteria, caffeine (Sigma-Aldrich®), ethanol (Merck®), methanol HPLC grade (Fisher Scientific®), orthophosphoric acid (Merck®), water, nutrient agar, nutrient broth, drip activator, oven (Memmert®), HPLC instrument (Jasco®), column (Acquity®) C-18 RP (4, 6 µm × 150 mm, 5 µm) and 0.45 µm filter, incubator (Memmert®), vortex device (IKA®), Design Expert 11.1.2.0 Trial software, IBM Statistic 24 software, autoclave (Clinoclave®), sonicator (Branson®), Mc. Farland tube, photo-diode array (PDA) detector (Jasco® MD-4010), filter paper (Whatman®), micropipette (Gilson®) and pH meter (Ohaus®).

Methods

Withdrawal of the total caffeine compound: Cascara powder was extracted by the ultrasonic-assisted extraction method carried out 10 times. Weighed as much as 10 g of cascara powder, added 100 mL of solvent, namely 70% ethanol, then extracted for 30 min at 37°C[7,8]. Extraction results were injected into the HPLC system to obtain caffeine levels in the sample.

Validation of HPLC method determination of the mobile phase and maximum wavelength: A serial solution was prepared by weighing 50 mg of standard caffeine and then dissolving it in 50 mL of methanol, then diluted to 100 µg mL^{-1}. Then, series solutions were made from mother liquor with 1, 2, 4, 16, 32 and 64 µg mL^{-1} concentrations. Optimization of the mobile phase consisting of a mixture of methanol and water (adjusted orthophosphate pH 2.4) was carried out with different compositions, namely 10:90, 20:80, 30:70, 50:50 and 45:55 with a flow rate of 0.7 mL min^{-1}, injected with a series solution at the maximum wavelength and analyzed for the peaks produced[9]. After that, linearity, accuracy and precision tests were carried out.

Optimization of decaffeination with RSM: The decaffeination consisted of two samples: A sample without bacteria and a sample with bacteria. Isolate *Bacillus subtilis* rejuvenated first on nutrient agar, then incubated for 24 hrs at 37°C. Next, *Bacillus subtilis* was cultured in nutrient broth, then incubated at 40°C for 24 hrs in an incubator with a bacterial density based on Mc. Farland 3×10^8 cells/mL[10]. In samples with bacteria, 5 g of cascara powder was used and then sterilized by autoclaving at 121°C with a pressure of 1 atm for 15 min. The powder was added with 0.3% (v/w) activator drops and added *Bacillus subtilis* concentrations of 3, 6 and 9% (v/w). Then incubated for 24, 48 and 72 hrs at 35°C. Then, the fermented product was sterilized by autoclaving at 1 atm for 15 min at 121°C. The sterilized cascara powder was extracted using the UAE method using 70% ethanol as a solvent. For samples without bacteria, the process is the same as for samples with bacteria and only bacteria are not added to *Bacillus subtilis*. The decaffeination process is carried out based on the results of software analysis Design Expert 11.1.2.0 Trial using an experimental design central composite design (CCD), 12 decaffeinated treatment combinations were obtained. After optimization, a verification test was carried out.

Sample analysis: A total of 10 µL of liquid extract of the sample solution consisting of samples with bacteria and samples without bacteria, were analyzed by HPLC using methanol as mobile phase and water (adjusted orthophosphate pH 2.4) (45:55) with a flow rate of 0.7 mL min^{-1} in isocratic conditions for 7 min and a UV detector with a wavelength of 272 nm was used. Then, the result calculated levels of caffeine and decreased levels of caffeine. As well as a paired sample t-test using IBM Statistics 24 software.

RESULTS

Results of total caffeine: In withdrawing this compound, extraction was carried out 10 times, using 10 g powder and 70% ethanol as a solvent, the extraction process used the UAE method. The caffeine content was obtained as much as 0.4960%.

Results of determining the mobile phase and maximum wavelength: The results of optimizing the mobile phase can be seen in Fig. 1. Determination of the maximum wavelength of caffeine is carried out using a PDA detector and the aim is to obtain the maximum wavelength waves that can detect the caffeine compounds contained in the sample optimally. The results of measuring the wavelength of caffeine obtained are 272 nm, which can be seen in Fig. 2.

Results of linearity: The linear regression equation is obtained, namely:

$$y = 55109.5x+45213.7$$

Results of accuracy and precision: In the accuracy and precision test, 3 different concentrations were used, namely 7.5, 10 and 12.5 µg mL^{-1} three times. The accuracy results were expressed as percent recovery (recovery (%)). The resulting recovery (%) range is 100.5557-107.9545%.

Results of decaffeination optimization with RSM: The results of the RSM optimization analysis obtained an incubation time of 24 hrs with a 6% bacterial concentration. The results of the ANOVA test can show the best results with the lowest caffeine content. The results of decaffeination optimization can be seen in Table 1 and the results of the ANOVA test in Table 2.

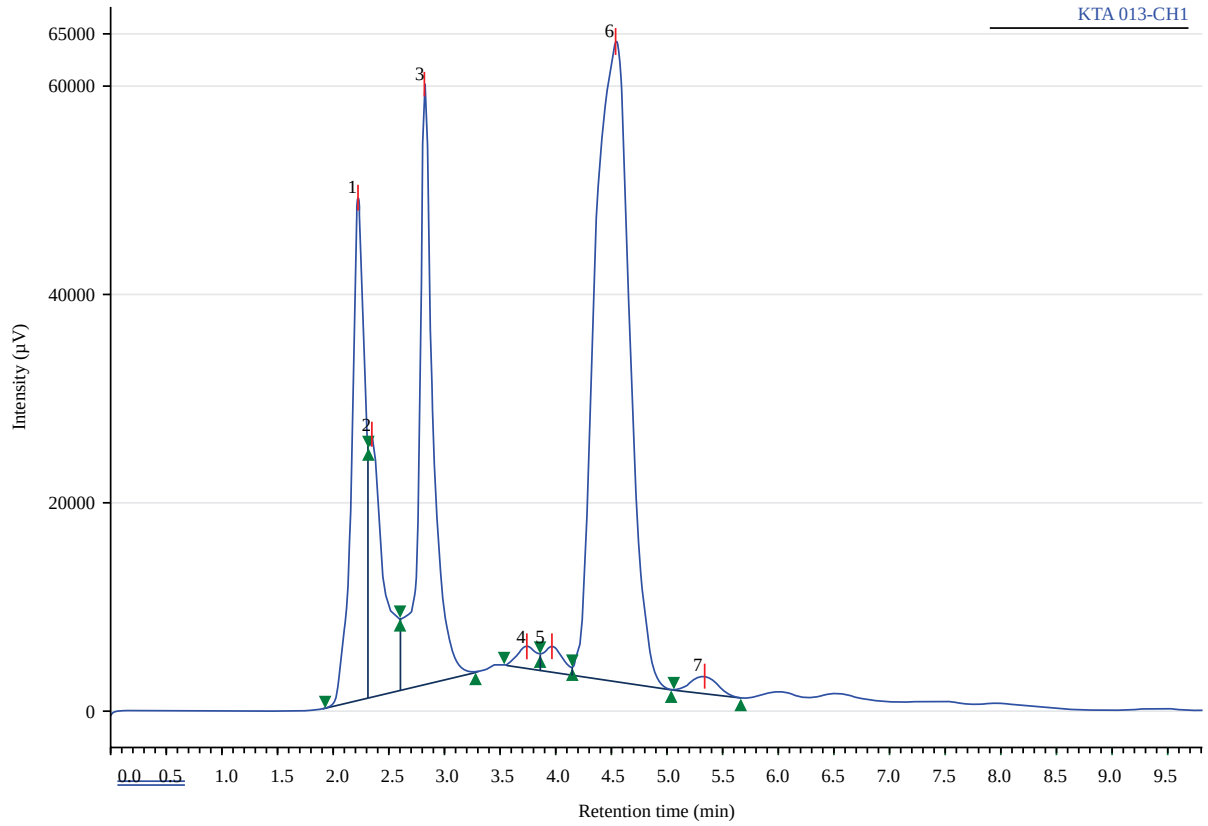

Fig. 1: Optimization of the mobile phase

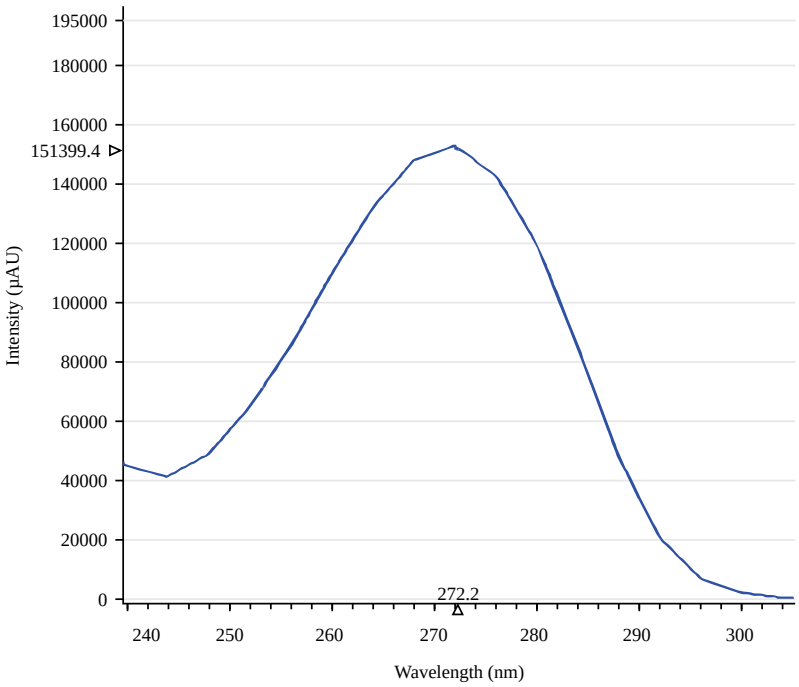

Fig. 2: Maximum wavelength of caffeine

Table 1: Optimization results of cascara decaffeination

Run	Incubation time (hrs)	Bacterial concentration (% v/b)	Caffeine levels (%)
1	72	3	0.313785
2	48	9	0.300001
3	72	6	0.265263
4	72	9	0.305994
5	38	3	0.307637
6	24	6	0.251377
7	48	6	0.259977
8	48	6	0.25356
9	48	6	0.257571
10	24	9	0.3024
11	48	6	0.251413
12	24	3	0.265263

Table 2: ANOVA test results of cascara decaffeination

Parameter	Result
p-value (model)	<0.0001
p-value (lack of fit)	0.5135
Linearity (R^2)	0.9915
Adequate precision	27.3417

The ANOVA test also produces an equation to determine the response of caffeine levels when affected by incubation time and bacterial concentration *Bacillus subtilis*:

$$y = 0.441703+9.87496E\text{-}06X_1 -0.063047X_2 - 0.000033X_1X_2 + 4.16086E\text{-}06X_1^2 + 0.005322X^2$$

Where:

X_1 = Incubation time
X_2 = Concentration bacteria
X = Coefficient 1

The X coefficient 1, X_2 shows the magnitude of the increase or decrease in the value of Y. If the coefficient of X is negative, then there is a decrease in the value of Y, whereas if the coefficient of X is positive, then there is an increase in the value of Y. In the equation above, the constant value at X_1X_2, a negative value, indicates that the interaction between variables does not affect the caffeine content obtained. The results of the model equation can be seen in the form of a 3D (three-dimensional) graphic in Fig. 3.

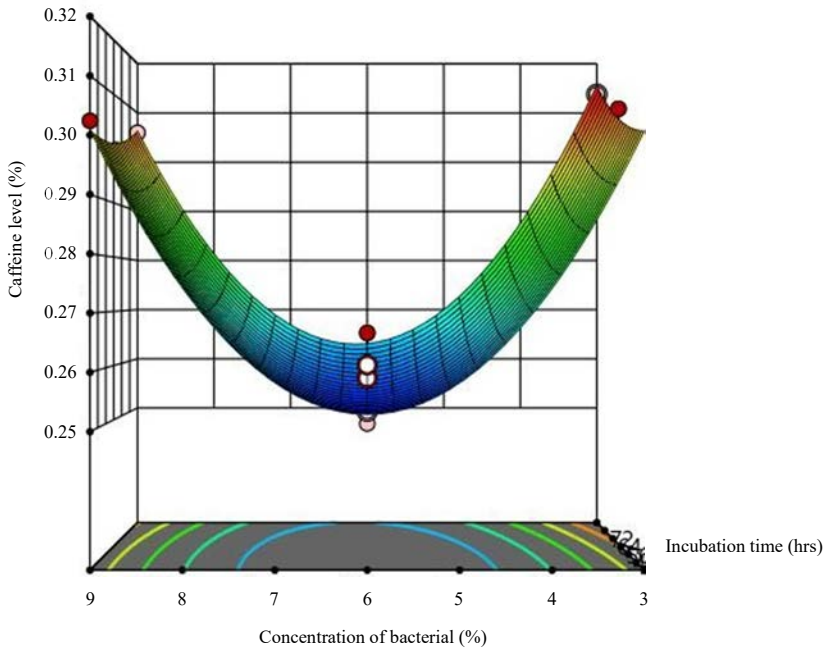

Fig. 3: 3D interaction between incubation times and bacterial concentration in caffeine levels

The verification test results obtained an accuracy value of 101.676% for caffeine content so that it could be stated that the model suggested by RSM was appropriate. Decreasing levels of caffeine has an average of 51.3843±0.2503%. Next, the paired sample t-test was carried out and obtained a significance value (2-tailed) <0.05, which was equal to 0.002, so it can be stated that the incubation time and the concentration of bacteria *Bacillus subtilis* had a significant influence on caffeine levels.

DISCUSSION

Based on the caffeine content obtained, the results differ from the research by Lestari *et al.*[11], which is 1-1.3 g in 100 g of cascara. One factor affecting the results obtained is the difference in the place of cultivation of robusta coffee plants. Other factors that can influence include extraction method, extraction temperature, extraction time and drying method of cascara[12].

The mobile phase optimization in Fig. 1 was carried out to determine the separated compounds in the liquid extract. The separation of compounds in the HPLC system was influenced by several factors, one of which is the bonding of the compound to the stationary phase.

The parameters for optimizing the mobile phase can be seen by separating caffeine compounds from peak shapes. Good results occurred in the mobile phase composition with a ratio of 45:55 and a peak detected at a wavelength of 272 nm (Fig. 2). Determination of the maximum wavelength of caffeine is carried out using a PDA detector and the aim is to obtain the maximum wavelength waves that can detect the caffeine compounds contained in the sample optimally.

The purpose of making a standard curve is to see the suitability between the measured response and the concentration of the analyte. The result coefficient value (R^2) of 0.9998 meets the requirements for a good linearity value $\geq$0.99.

In the accuracy and precision test, the result of recovery (%) meets the requirements, namely 85-110%[13]. Precision is carried out to see the closeness between a series of analyses obtained from several measurements of the same sample, the results of precision test measurements are expressed in percent relative standard deviation (RSD (%)), resulting in a range of RSD (%) of 1.6518, 1.6057 and 1.2126% have fulfilled the requirements, namely $\leq$2%[14].

In decaffeination optimization with RSM before optimizing decaffeination, a preliminary test is carried out, the goal is to be able to determine the factors that have the most influence on the response as a basis for determining the right factors in optimizing RSM. Optimization was done to determine the optimum conditions of incubation time and bacterial concentration of *Bacillus subtilis* in reducing caffeine levels. After optimizing the cascara decaffeination, a design expert carried out the ANOVA test.

The ANOVA test results in Table 2 were stated as significant and the model has compatibility with the variables and responses. The ANOVA test also uses the mismatch equation model (lack of fit) to measure the discrepancy between the treatment and the model, the test lack of fit generated 0.5135. These results exceeded the $p<0.05$ until the test results lack of fit was insignificant. The coefficient of determination (R^2) of 0.9915 indicates that the variable bacterial concentration and incubation time have an effect of 99.15% on the overall caffeine content and other factors influence 0.85%. The model from the optimization test is said to be good if it has an R value>75%, from the optimization test results obtained, the value of R^2 was as much as 99.15% until the conditions have been met and a good model has been obtained with showed high levels of linearity ($R^2>0.9805$)[15]. Score Adequate precision: The ANOVA test measures the signal-to-interference ratio while analyzing caffeine levels. Value terms adequate precision that can be used in an analysis is >4. The results of the study obtained an adequate precision of 27.3417.

The resulting 3D graph (Fig. 3) is quadratic with an upward opening, indicating that the model used is optimum, marked by the presence of an optimum point in the middle of the graph. In addition, the 3D graph in the form of a quadratic indicates that there is an interaction between incubation time and bacterial concentration. After the optimization test was carried out, a verification test was carried out and the aim was to confirm the solution suggested by RSM.

The process of decreasing caffeine levels occurs because the cascara cell walls are composed of cellulose. The cascara cell membrane is composed of 40% fat, 52% protein and 8% carbohydrates, then broken down by enzymes sourced from *Bacillus subtilis*, namely cellulase enzymes and protease enzymes, so that caffeine can dissolve and exit the cell membrane[16].

This finding can be developed for further research, for example, the test of protease enzyme activity and cellulase enzyme activity to determine the amount of protease enzyme and cellulase enzyme produced by *Bacillus subtilis* and decaffeinated with other microorganisms, such as fungi testing yeast to see the difference in the response given.

CONCLUSION

The optimum conditions for cascara robusta coffee decaffeination were obtained using the RSM method at bacterial concentrations of *Bacillus subtilis* 6% with a long incubation time of 24 hrs. A decrease in caffeine content was obtained in the decaffeinated cascara with an average of 51.3843±0.2503%, so it can be stated that the effect of giving bacteria *Bacillus subtilis* and differences in incubation time significantly affect the decrease. This information can be expanded upon for additional research, such as measuring the amounts of protease and cellulase enzymes produced by *Bacillus subtilis* and decaffeinated with other microorganisms, like fungi testing yeast to observe the variation in response.

SIGNIFICANCE STATEMENT

The purpose of this research was to decaffeinate robusta coffee cascara (*Coffea canephora* L.) with the help of bacteria *Bacillus subtilis* and determine the optimum concentration and incubation time of *Bacillus subtilis* in reducing caffeine levels. Current findings suggested that the effect of giving bacteria *Bacillus subtilis* and differences in incubation time significantly affect the decrease in caffeine levels with the optimum conditions for cascara robusta coffee decaffeination at bacterial concentrations *Bacillus subtilis* 6% with a long incubation time of 24 hrs. Caffeine content decreased in the decaffeinated cascara with an average of 51.3843±0.2503%. This finding warrants further research to determine the amount of protease and cellulase enzymes produced by *Bacillus subtilis* and that interact with other microorganisms.

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