Makara Journal of Technology

Volume 27 | Issue 3 Article 3

12-23-2023

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Recommended Citation

Dullah, Elvina Clarie and Farida Asras, Mohd Fazli (2023) "Isolation and Characterization of Caffeine-Degrading Bacteria from Coffee Plantation Areas in Malaysia," *Makara Journal of Technology*: Vol. 27: Iss. 3, Article 3.
DOI: 10.7454/mst.v27i3.1462
Available at: https://scholarhub.ui.ac.id/mjt/vol27/iss3/3

Makara J. Technol. 27/3 (2023), 93–98
doi: 10.7454/mst.v27i3.1462

Isolation and Characterization of Caffeine-Degrading Bacteria from Coffee Plantation Areas in Malaysia

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Abstract

Decaffeination by microbial degradation is currently the most optimal and low-cost approach, involving only microbial cells and/or their enzymes. The bacterium was characterized using a series of biochemical tests. Positive results were obtained from carbohydrate fermentation, citrate utilization, and catalase tests, while negative results were obtained from Voges-Proskauer (VP) and indole tests. Three different caffeine concentrations of 0.25%, 0.4%, and 2% were tested and measured through Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis. The highest caffeine reduction (89.25%) was found when 0.25% caffeine was used in the media. Only a small amount of caffeine was reduced to 0.4% and 2%, with 34.78% and 46.16%, respectively. Under microscopic observation, the shape of the isolated bacteria was rod bacilli and was stained with a pink color, which indicates Gram-negative bacteria. Comparing the results with previous research and observing the color of the pure culture, which revealed a yellow color, bacteria isolated from the coffee plantation area can be concluded to be a partially identified *Pseudomonas* sp.

Keywords: caffeine-degrading, coffee, Pseudomonas

1. Introduction

Europe has become the largest coffee consumer with almost 40% of the global consumption, followed by the US and Japan, which account for 24% and 10%, respectively [1]. Asian people, such as those in China and Japan, are originally tea drinkers. However, coffee consumers have continuously increased annually. The location of coffee plantation areas, which started in the 19th century, is in Malaysia. With its introduction, coffee was once the most important crop before the existence of rubber plantation.

Coffee contains a chemical stimulant known as caffeine. The percentage of caffeine in coffee generally depends on its genesis, brewing method, and others. Comparison results of roast and ground coffee with instant coffee revealed that the total amount of caffeine in roast and ground coffee is higher according to the range provided by the United States Food and Drug Administration. Apart from coffee, caffeine can also be found in teas, soft drinks, cocoa, chocolate drinks, and any other types of beverages. With more than 60 types of plants, caffeine naturally exists and can be extracted from the leaves, seeds, and fruits of plants. Coffee has been widely used as a beverage drink, but consumer awareness regarding the hidden effect of caffeine is lacking. In reducing its effect, decaffeinated coffee has become an alternative beverage for some people.

Caffeine degradation can generally be realized by either using conventional or microbial method. The conventional method is typically an ineffective technique in decaffeination due to the harmful and highly expensive chemical used in the method. By contrast, the microbial method has advantages over the conventional method because it uses microorganism systems without involving any chemicals. Different types of microorganisms, such as bacteria, yeast, and fungi, have been identified as potential agents in the microbial method; to date, further study is still being conducted on these microbes to enhance their decaffeination capability [2]. For this project, the targeted microbe will be the species that is highly capable of tolerating caffeine. As mentioned in previous reports, most targets on soil bacterial species of *Pseudomonas* can use caffeine as their sole source of carbon and nitrogen [3]. The isolation and identification of novel microbes are continuous processes. Therefore, this study was undertaken to explore the novel decaffeinating bacterial isolates.

2. Materials, Methods, and Media Preparation

A solution comprising the following mineral substances was prepared: 0.370 g/L potassium chloride (KCl), 0.205 g/L magnesium sulphate ($MgSO_4$), 0.710 g/L ferric sulphate ($Fe_2[SO_4]_3$), 0.205 g/L stearic acid ($Na_2HPO_4.12H_2O$), 80 mg/L calcium chloride ($CaCl_2$), 15 µg/L zinc sulphate monohydrate ($ZnSO_4.7H_2O$), 12 µg/L sodium molybdate

dehydrate ($NaMoO_4$)(R&M), 11 μg/L manganese (II) sulphate ($MnSO_4$)(R&M), 10 μg/L copper (II) sulphate ($CuSO_4$), and 10 μg/L boric acid (H_3BO_4). All chemicals were purchased from Sigma Aldrich or stated individually in bracket. A solid screening medium (SSM) used for the isolation of caffeine-degrading bacteria was prepared by mixing the mineral solution with 2.5 g/L of coffee and 1.5% agar. The media was autoclaved at 121 °C for 10 min. A solid purifying medium (SPM) used for the screening of the caffeine-degrading bacteria was prepared similarly to SSM, with the exception of supplementing 4 g/L coffee. The liquid medium used in fermentation was prepared in six different concentrations of 2.0, 4.0, 6.0, 8.0, 10.0, and 20.0 g/L. The mineral solution was mixed to each coffee concentration and was autoclaved at 121 °C for 10 min.

Isolation and screening of caffeine-degrading bacteria. The soil samples were collected randomly from the coffee plantation area of Ranau, Sabah, Malaysia. Approximately 100 g of the soil was soaked in 1 L distilled water for 30 min. The solution containing microorganisms was obtained by filtration, and 10^1 to 10^{-6} times of dilution were then spread over the SSM surface in Petri dishes. The solution of 0.1 mL was spread on each dish, and the dishes were incubated at 30 °C for one to three days. The fast-growing single colony was observed within the incubation time. The colony was obtained with an inoculating loop and streaked on the SPM surface for screening. Colonies that grew normally on the SPM were then transferred to nutrient broth for characterization.

Morphological and biochemical identification of caffeine-degrading bacteria. Identification of caffeine-degrading microbe was realized using microscopic and biochemical tests. The heat-fixed smears and gram staining of 24 h young caffeine-degrading bacteria were performed and then directly observed under a microscope. Five tests included carbohydrate fermentation, MR-VP, indole, citrate utilization, and catalase tests [4]. Bergey's Manual of Systematic Bacteriology [5] was used as reference to classify the isolated caffeine-degrading bacteria based on their structural and functional characteristics by arranging the caffeine-degrading bacteria into a specific family order.

The heat-fixed smears of the isolated microbe were prepared and flooded with crystal violet. After 30 s, the slides were rinsed with water for 5 s. By using Gram's iodine mordant, the slides were then covered and left to stand for 1 min. The slides were rinsed again with water for 5 s and decolorized with a mixture of acetone from 30 s to 60 s. Afterward, the slides were rinsed with water for 5 s and directly counterstained with safranin for approximately 60 to 80 s. After final rinsing of the slides, they were blotted dry with bibulous paper, and the color of the Gram stains were observed under microscope using oil immersion.

Effects of culture condition on the growth. In evaluating the effect of caffeine concentration, the caffeine-degrading bacteria were inoculated in a mineral liquid medium with a supplement of different coffee concentrations of 2.0, 4.0, 6.0, 8.0, 10.0, and 20.0 g/L. The medium was then incubated in an incubator shaker at 25 °C for 24 h. The absorbance readings were taken after the incubation time using spectrophotometer and measured at OD_{600} [6].

Preparation of samples for Gas Chromatography–Mass Spectrometry (GC–MS). Fermentation of caffeine-degrading bacteria was performed by inoculating bacteria into mineral solutions supplemented with different coffee concentrations (0.25%, 0.4%, and 2.0%). The samples were prepared by taking the solution from each flask before fermentation and after 24 h of fermentation. A total of 1 ml of solutions taken from each flask was transferred into 2 mL of Eppendorf tube, and 1 mL of High-Performance Liquid Chromatography (HPLC)-grade methanol was added. The solutions were then centrifuged for 1 min and 130,000 rpm. The formation of pellet and two layers of supernatant was observed after centrifugation. Only the clear layer of supernatant (which was located at the top of the supernatant) was taken and transferred into the vial bottle. The same step was performed for the sample after 24 h of fermentation. The vial bottles were sealed and stored in the chiller before undergoing GC–MS analysis.

Gas chromatography–mass spectrometry analysis. Caffeine analysis was performed using a gas chromatography (GC) system combined with an internal source ion trap mass spectrometer under the electronic ionization mode. The carrier gas used was helium (99.999%), and the flow rate was 1.0 mL/min. The GC separation of caffeine was performed using a DB-5 capillary column (30 mm × 0.25 mm I.D., 1 μL film thickness). The temperature program was maintained during caffeine separation: 150 °C for 1 min; 20 °C/min to 230 °C for 20 min. The total time of analysis was 7 min. The injections were performed in the splitless mode at 250 °C. The trap, transfer line, and manifold temperature were set at 200 °C, 280 °C, and 50 °C, respectively. The mass range of scan spectra was 50 to 250 Da. Selective ion monitoring mode was employed to conduct all quantification experiments for caffeine. The utilized ions used for m/z confirmation of caffeine were 194, 109, 82, and 52 [7].

3. Results and Discussions

Bacterial isolation. After bacterial isolation from the soil of coffee plantation areas, seven colonies were observed on the SSM with 0.25% of coffee concentration, as shown in Figure 1. These colonies displayed similar appearances that were circular in shape and white in color. Bacteria will grow within 24 h. However, isolated bacteria have a slow growth rate, and the fast-growing

bacteria can only be observed after 3 days. The growth might be slow due to the inhibition from caffeine or the time needed in caffeine uptake by the bacteria as its sole source of nitrogen. The seven colonies were then further purified in the SPM with a coffee concentration of 0.4%, and bacterial growth was observed in high concentrations of coffee, as shown in Figure 2. The screening result revealed that only one plate displayed bacterial growth, while the six remaining plates had no bacterial growth even after increasing the incubation time. This finding indicates that the isolated colonies might belong to the same genus but different species.

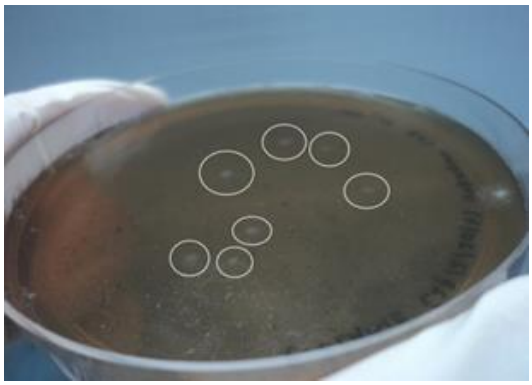

Figure 1. Bacterial Isolates on SSM

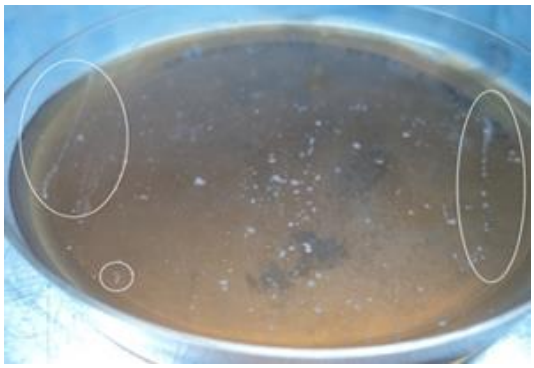

Figure 2. Bacterial Isolates on SPM

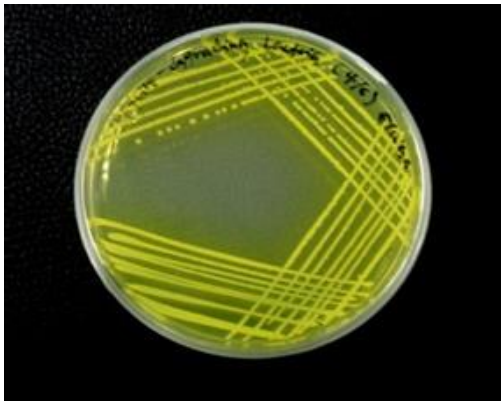

Figure 3. Partially Identified *Pseudomonas* sp.

Morphological and biochemical identification. A series of morphological biochemical reactions was performed to identify the isolated caffeine-degrading bacteria. Morphological observation can help narrow down the identification, and biochemical test can help distinguish unknown and known bacteria. Different genera have specific metabolic characteristics and various capabilities in utilizing carbohydrates, amino acids, and protein [8]. Therefore, biochemical test can be used in differentiating and identifying different genera of bacteria.

The result of the morphological observation revealed that the isolated caffeine-degrading bacteria from the soil of coffee plantation area partially identified as *Pseudomonas* species. This finding is due to the appearance of partially identified *Pseudomonas* species when grown on nutrient agar plate (Figure 3); these species are nonmotile and yellow in color. Microscopic observations showed that these bacteria are rod bacilli in cell shape and revealed a pink color for Gram staining (Figure 4). Table 1 summarizes the morphology results of bacterial isolates.

An anaerobic test was conducted to determine the type of respiration of the isolated bacteria. The result showed a growth of isolated bacteria in anaerobic and aerobic conditions. Therefore, the isolated caffeine-degrading bacteria performed a facultative anaerobic respiration (Figure 5).

Figure 4. Microscopic Observation of Caffeine-Degrading Bacteria at 100x Magnification

Table 1. Morphology of Bacterial Isolate

Test Performed	Characteristics of Isolate
Cell Shape (Microscopy and Gram Staining)	Rod Bacilli (Gram Negative)
Configuration	Punctiform
Margin	Entire
Elevation	Raised
Surface	Moist
Colony color	Yellow
Opacity	Opaque
Spore (s)	-
Motility	Non-Motile
Anaerobic Growth	Can Grow in Anaerobic Condition

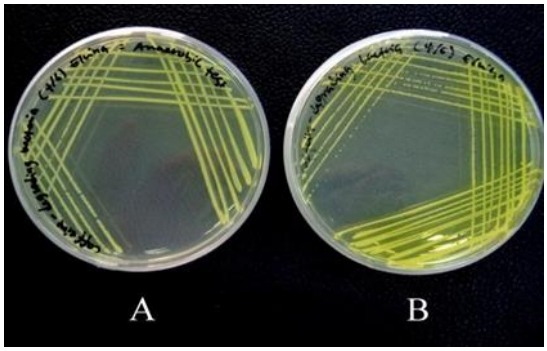

Figure 5. Anaerobic Test (A) Growth Under Anaerobic Condition; (B) Growth Under Aerobic Condition

Table 2. Biochemical Test Result

Biochemical Test	Isolated Caffeine-Degrading Bacteria
Glucose Fermentation	+
Lactose Fermentation	+
Voges-Proskaeur	-
Indole Production	-
Citrate Utilization	+
Catalase	+

"+" positive for test, "-" negative for test

Pseudomonas sp. is classified as an aerobic organism, but some *Pseudomonas* sp. is considered a facultative anaerobe because of its characteristics as pathogenic bacteria, as stated by Itzhak [9]. Moreover, comparing the growth of isolated bacteria in aerobic and anaerobic conditions, they can grow well in aerobic condition because their observed appearance in the aerobic plate is more opaque compared with the growth in the anaerobic plate.

A series of biochemical tests, such as carbohydrate fermentation (lactose and glucose fermentation), Voges-Proskaeur, indole, Simmon's citrate, and catalase tests, was conducted to further characterize the isolated caffeine-degrading bacteria. Different genera of bacteria have unique enzymes that allow them to undergo different biochemical tests [8]. Table 2 shows that the isolated bacteria can ferment lactose and glucose, yielding a positive result for the carbohydrate test. The positive result, where the color turned from red to yellow, can only be obtained from bacteria that can grow in anaerobic conditions because they can break down the carbohydrates for energy production during fermentation. Hence, the carbohydrate test confirmed that facultative anaerobic bacteria are caffeine-degrading bacteria.

Voges-Proskaeur and indole tests both provide negative results. The Voges-Proskaeur test is used to detect the production of acetylmethylcarbinol acetoin, which is a natural product formed from pyruvic acid during glucose fermentation. Meanwhile, the indole test aims to observe tryptophanase in the bacteria. The negative result for Voges-Proskaeur test displayed no change in color. Meanwhile, the indole test revealed the absence of a "cherry-red ring" formation, which indicates a negative result, as shown in Figure 6. Previous study also indicated the same result for these tests, wherein the isolated strain did not produce acetylmethylcarbinol acetoin [6]. Moreover, *Pseudomonas* sp. did not produce the enzyme tryptophanase, revealing a negative indole test.

A positive results was obtained for citrate and catalase tests (Table 2). The citrate test is often used in the identification of Gram-negative pathogenic bacteria. A positive result was recorded for this test after the color of the citrate medium changed from green to blue. The isolated caffeine-degrading bacteria were observed as Gram-negative. Thus, these bacteria can utilize citrate as a sole carbon and energy source for growth in Simmon's citrate agar. The result of the catalase test will be positive only for aerobic bacteria, where bubble formations were observed after 3% hydrogen peroxide was dropped on a loop full of bacteria that have been transferred on a microscopic slide. This observation can be attributed to the breakdown process of hydrogen peroxide into water and oxygen produced during aerobic respiration. This test result supports the partially identified caffeine-degrading bacteria, namely *Pseudomonas* sp., as a facultative anaerobic bacterium.

Effect of culture condition on the growth. The acceleration growth of caffeine-degrading bacteria can be observed from the concentration of 2.0 to 10.0 g/L in the medium, as shown in Table 3. However, sudden deceleration of growth was observed when the coffee concentration was further increased to 20.0 g/L.

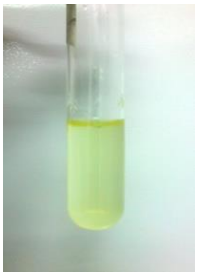

Figure 6. Indole Test Result

Table 3. Addition of Coffee Concentration

Coffee concentration (g/L)	OD_{600}
2.0	0.006
4.0	0.036
6.0	0.041
8.0	0.055
10.00	1.493
20.00	0.083

This finding indicates that the isolated caffeine-degrading bacteria can grow in high caffeine concentrations. However, the growth may occur slowly due to the presence of caffeine. A previous study stated that some caffeine-degrading bacteria, such as *Pseudomonas* sp. isolated from the soil, contain high caffeine compositions that could tolerate and survive in the media supplemented with caffeine at initial concentrations of 10, 15, and 20 g/L [10, 11]. In this study, the isolated caffeine-degrading bacteria might have the same characteristic and capability in tolerating high caffeine concentrations if further study is conducted on this strain. The isolated bacteria can grow in the medium with the presence of caffeine. Therefore, this phenomenon indicates that caffeine-degrading bacteria use and metabolize caffeine as their sole nitrogen and carbon sources due to the absence these sources in the medium. This finding is also supported by the previous research performed by Fan [6]. This research proposed that isolated bacteria can utilize caffeine as their non-priority nitrogen and carbon sources, which may take time to enable their metabolic route (s) to convert the caffeine into some compounds that can be used for the bacterial growth.

Confirmation of the presence of caffeine compound by GCMS. GC–MS was used to measure the concentration of caffeine compound from the fermentation process. The quantification can be based on peak areas from mass chromatograms, where the peak area is related to the quantity of the compound. The presence of caffeine in the medium was confirmed by the results of GC–MS for different coffee concentrations, which showed a peak for the caffeine compound. The result in Table 4 reveals that the peak area for the fermentation sample containing 0.25% and 0.4% of coffee was initially 100 before the incubation. The values were decreased after 24 h of incubation, where the peak values were 10.75 and 65.22 for 0.25% and 0.4%, respectively. For the fermentation of the medium supplemented with 2% coffee, the initial peak area was 87.55 and the value was decreased to 47.14 after incubation. Overall data obtained from the GC–MS result indicate that caffeine reduction might be related to caffeine degradation by the isolated bacteria.

The caffeine reduction in media of different coffee concentrations was calculated based on the peak area percentage produced before and after incubation (Table 5). In media containing 0.25%, the caffeine reduction was 89.25%, which was the highest caffeine reduction compared with the two other media (34.78% and 46.16% caffeine reduction with 0.40% and 2% coffee concentrations, respectively). Fan [6] reported that the efficiency of caffeine degradation was also affected by the temperature: a high temperature leads to the low efficiency of bacteria in caffeine degradation. Moreover, the results of Fan [6] revealed that the most favorable incubation temperature for bacteria is approximately 30 °C.

Table 4. GC–MS Result Before and After Incubation

Concentration of Coffee in the Sample (%)	% Peak area (Before incubation)	% Peak area (After incubation)
Control (0.25)	65.79	-
0.25	100	10.75
Control (0.40)	100	-
0.40	100	65.22
Control (2.00)	70.0	-
2.00	87.55	47.14

*Incubation time was 24h

Table 5. Percentage of Caffeine Reduction

Concentration of Coffee in the Sample (%)	Caffeine Reduction (%)
0.25	89.25
0.40	34.78
2.00	40.14

Further tests and analyses of caffeine reduction must be conducted to confirm whether isolated bacteria are responsible for caffeine degradation in coffee. Different incubation temperatures for media with various caffeine concentrations can be studied to observe the most efficient condition for bacteria in realizing caffeine degradation.

4. Conclusions

The isolated bacteria have been identified as *Pseudomonas* sp. based on the morphological and biochemical tests. Moreover, the bacteria displayed the decaffeination capability based on the GC–MS results.

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