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Isolation and Molecular Identification of Lactic Acid Bacteria from Robusta Coffee Fermentation and Antifungal Test against *Penicillium sp.*

Nurfitrah Salsabila, J.S., Siti Nur Jannah*, Nurhayati

Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia.

*Corresponding author (nurjannah.suroso@gmail.com)

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Abstract

Lactic acid bacteria (LAB) play an important role in robusta coffee fermentation by soaking coffee beans. Spontaneous fermentation of coffee beans can improve coffee quality by producing organic acids and volatile compounds. The ability of LAB to produce organic acids also has the potential as an antifungal compound. *Penicillium* is one of the predominant contaminants of coffee beans products in Indonesia. Coffee beans can become contaminated by *Penicillium* during various stages such as harvesting, processing, transport, and storage. To prevent contamination of coffee beans, new preservation methods involving biological agents, such as lactic acid bacteria, may be used instead of chemicals. The secondary metabolites produced by LAB can be utilized for bio preservation. Lactic acid bacteria in robusta coffee fermentation should be further explored and analyzed for antifungal activity against *Penicillium* sp., and molecularly identified to determine the spesies of lactic acid bacteria that have antifungal potential. The isolation method was carried out using multilevel dilution and spread plate on MRSA+$CaCO_3$ media. Identification was conducted through macroscopic and microscopic observation of cells and their characteristics, as well as molecular identification. Antifungal testing was performed using the agar well diffusion method. The results of LAB isolation yielded 12 LAB isolates with characteristics of round colony shape, gram-positive, rod and round cell shape, with negative catalase. The test results showed that isolates BKR 4, BKR 11, BKR 12 exhibited antifungal activity. BKR11 isolate was identified as *Leuconostoc mesenteroides*

Introduction

Robusta coffee is one of the plantation commodities in Indonesia. Post-harvest processing of coffee can be carried out using the wet method with spontaneous fermentation, by soaking the coffee beans for 12-48 hours. Fermentation is used by coffee farmers to efficiently remove mucilage layer adhering to the fruit before the storage and processing of coffee beans (Poltonieri and Rossi, 2016). Lactic acid bacteria in coffee fermentation consist of the genera *Leuconoctoc, Fructobacillus, Weissela, Lactobacillus, Pediococcus, Lactococcus, and Enterococcus* (De Mello Pereira, 2020).

Numerous studies have shown that the toxigenic fungi *Aspergillus* and *Penicillium* are natural coffee contaminants, and are present from the farm to the storage facility. (Silva et al, 2008; Rezende et al, 2013; Kusumaningrum et al, 2019). Coffee beans contaminated by fungi can affect the quality of the beverage in terms of flavor and aroma, but also pose a safety risk due to the production of toxic secondary metabolites, known as mycotoxins, which can affect health at high levels. Coffee beans contaminated by fungi can affect the quality of the beverage in terms of flavor and aroma, but also pose a safety risk due to the production of toxic secondary metabolites, known as mycotoxins, which can affect health at high levels. There is evidence that several species of *Penicillium* produce

ochratoxin A (OTA) that recognized as potentially harmful sources because their presence is found within coffee beans (Rezende et al, 2013; Moulia et al, 2014).

To prevent contamination of coffee beans, new preservation methods are needed to overcome consumer demand for product without chemical preservation. Biological agent such as lactic acid bacteria (LAB) may be used as preservatives post-harvest processing, instead of chemical. The secondary metabolites produced by LAB can used as bio preservation. Lactic acid bacteria produce a variety of antifungal compounds including organic acids, fatty acids, cyclic dipeptides, reutrines, and antifungal compounds that affect mold growth and mycotoxin production (Crowley *et al* 2013).

Lactic acid bacteria in robusta coffee fermentation should be explored further and analyzed for antifungal activity against *Penicillium* sp and identified molecularly to determine the spesies of lactic acid bacteria that has antifungal potensial. Detection of the antifungal activity of lactic acid bacteria in robusta coffee fermentation can determine the solution to *Penicillium* contamination.

Materials and Methods

Material

Robusta coffee fermented liquid taken at 20 h immersion obtained from Jambu District, Semarang Regency, de Man Rogosa (MRS) medium. Potato Dextrose Agar (PDA), $CaCO_3$, Agar, Gram stain, H_2O_2, NaOH, culture of *Penicillium* sp, aquadest, Ketokonazole, Agarose, cork borer, TAE, Kit Wizard Genomic (Promega), Lysozim, nuclease free water, My Taq™ HS Red Mix, Primer forward 27F (5'AGAGTTTGATCCTGGCTGAG-3') dan primer reverse 1492R (5'-GTTTACCTTACGACTT-3'), loading dye.

Isolation of Lactic Acid Bacteria

The study was conducted from November 2020-June 2021. LAB was isolated from Robusta coffee fermented liquid by preparing serial dilution of sampel, 1 mL of dilution was spread on de Man Rogosa Sharpe agar + 0,5% $CaCO_3$. The agar plates were incubated at 37 °C for 24 h. Colonies of LAB, identified by a clear zone around each colony were selected from MRS agar. The isolates that have been grown are then sub-cultured on new media using the streak plate method to obtain a single colony. The isolation was obtained by morphological characteristic (colony and cell morphology).

Antifungal Test with CFS

The size of inoculum fungi was adjusted $1x10^6$ spore/mL with counting chamber. The supernatants of LAB isolates were divided in two aliquots, one used its cell free supernatant (CFS) and the other neutralizes cell free supernatant (CFSN). Ketoconazole 2% was used as positive control dan MRSB was used as negative control. Fungal inhibition test was carried out by the well diffusion method. Suspension of *Penicillium* sp was inoculated with streak plate method with a cotton swab. Then holes were made on media using cork borer. Each well is filled with 40 µL supernatant (CFS & CFSN), positive control, and negative control. It was incubated at 37 °C for 48 hours. The inhibition zone has measured with caliper (Adeniyi et al, 2011)

Molecular identification

DNA isolation was performed using the procedure of the DNA isolation Kit (Wizard Genomic DNA from Promega). Amplification using PCR technique. DNA from selected bacteria as much as 2 µL was dissolved in PCR Mix containing 19 µL ddH_2O, My Taq HS Red Mix 25 µL, Primer forward 27F 2 µL, and Primer reverse 2 µL. Mixture it then inserted into PCR machine.

Amplification process wa conditioned at a initial denaturation 95 °C for 3 minutes, denaturation with 95 °C for 45 seconds, Annealing 54 °C for 1 minutes, Extension 72 °C for 1 minutes 30 seconds, post-extension with 72 °C for 10 minutes and 4°C for 5 minutes. The amplification process with 30 cycles. The PCR results of DNA sample were confirmed with using electrophoresis os 0,8% agarose gel. DNA sequencing uses the services of PT. Genetika Science Indonesia. Sequencing results used to find out similarities DNA sequence with other sequence in GeneBank (BLAST). Bacterial kinship is presented in the shape of phylogenetic tree with Mega X.

Results and Discussion

A total of 12 lactic acid bacteria isolates were isolated from fermented coffee robusta. These isolates were identified on the basis of morphological and physiological characteristics presented in Table 1. The morphology of the colony is almost identical and uniform with round shape, white, creamy, convex surface, flat edge, Gram positive, and negative catalase reaction. Lactic acid bacteria have the characteristics of belonging to the Gram positive group, non-motile, acid-fast, with rod or round shape (Du and Webb, 2011).

Table 1. Antifungal activities of LAB isolates

Isolat	Zone of inhibition (mm)±SD	
	CFS	CFSN
BKR1	0±0.0	0±0.0
BKR2	0±0.0	0±0.0
BKR3	0±0.0	0±0.0
BKR4	5.02±0.1	1.90±1.8
BKR5	0±0.0	0±0.0
BKR6	0±0.0	0±0.0
BKR7	0±0.0	0±0.0
BKR8	0±0.0	0±0.0
BKR9	0±0.0	0±0.0
BKR10	0±0.0	0±0.0
BKR11	11.10±1.8	5.77±1.2
BKR12	6.63±2.1	4.97±1.7
Ketoconazole	12.97±1.7	12.97±1.7

Antifungal activity was observed for LAB isolates against *Penicillium* sp by agar well diffusion method (Table 2). Isolates were active against *Penicillium* sp that isolate BKR4, BKR11, and BKR12 (Table 2). BKR11 isolate was found to have the most antifungal ability in inhibiting the growth of *Penicillium* sp. LAB produces a variety of antifungal compounds including organic acids, fatty acids, cyclic dipeptides, reutrine, and other compounds that affect mold growth and mycotoxin production (Crowley et al, 2013). Antagonistic effect of compound produced by LAB is product of carbohydrate metabolism and is safe to use for food preservation. The lactic acid produced lowers the pH which can inhibit the growth of various susceptible microorganisms (Perckzak, 2018). Lactic acid bacteria isolated from various fermentation sources showed the ability of lactic acid bacteria as antifungal agents to inhibit Penicillium (Aunsbjerg *et al* , 2014; Huh and Hwang, 2014; Xiong *et al*, 2014).

Table 2. Quantitative analysis of DNA

Sampel DNA	A260	A280	A260/A280	Konsentrasi (ng/µL)
BKR 11	3.499	1.611	2.17	174.9

The BKR11 isolate was identified molecularly. The results of the quantitative analysis of DNA can be seen in Table 2 showed a value of 2.17 which indicates that the DNA was contaminated with RNA. DNA sample should have an absorbance ratio of 1.8-2.0. If the ratio is above 2.0, the sample contains a lot of RNA. A260/280 ratio below 1.8 indicates protein or phenol contamination (M O'Neill et al, 2011)

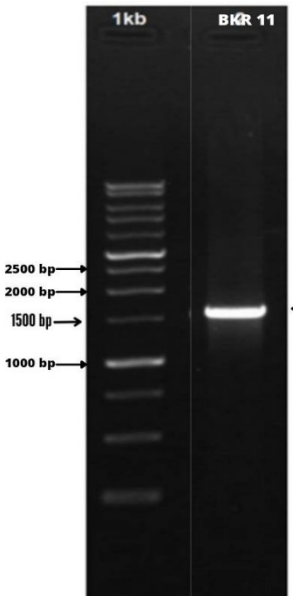

Figure 1. Qualitative analysis of DNA

DNA was amplified using Universal Primer 27F dan 1492R as pair primer. The amplified DNA was analyzed qualitatively using electrophoresis can be seen in figure 1. The results of qualitative analysis with 0.8% agarose obtained thick DNA bands with a size of about 1550 bp. Identification based on highly conserved genes such as 16S rRNA usually uses long sequences around 500 - 1500 bp (Janda & Abbott, 2007).

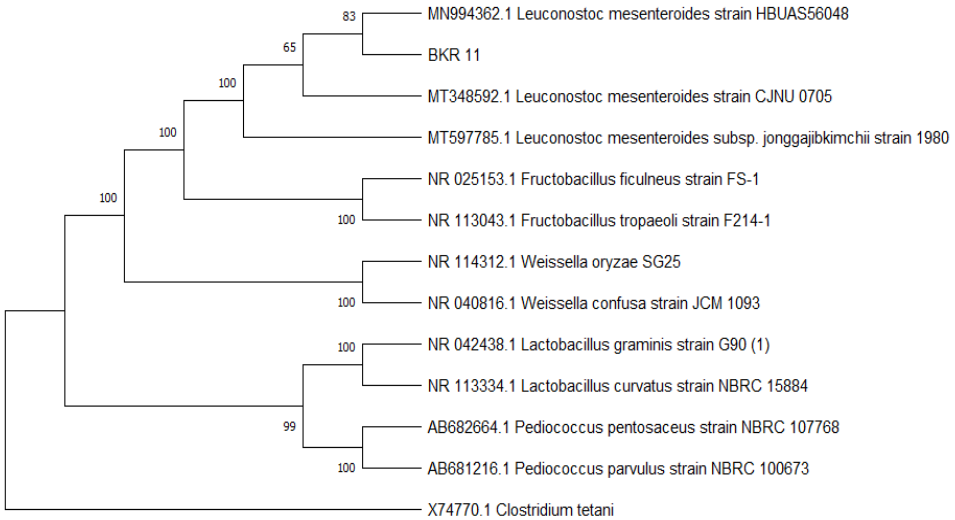

Figure 2. Phylogenetic tree of BKR 11

Blast analysis BKR11 isolate has 99,86% similarity with *Leuconostoc mesenteroides*. The result of analysis of genetic joining phylogenetics trees (neighbor joining) can be seen in figure 2. The phylogenetic tree by neighbor joining method bootstrap consensus identified BKR11 isolate as *Leuconostoc mesenteroides*. Isolates identified through the same branch as the reference strain in GenBank data base. Based on phylogenetic trees which includes several GenBank sequence data as comparison, it showed that isolate BKR11 was in same branch and node as *Leuconostoc mesenteroides* strain HBUAS56049 with

boostrap value of 83%. *Leuconostoc mesenteroides* is a bacterium with the form of cocci, Gram positive, catalase negative, facultative anaerobic organisms, heterofermentative. *Leuconostoc mesenteroides* plays an important role in the fermentation of foods such as kimchi, sauerkraut, and milk leading to the production of organic acids and aromatic compounds (Zubaidah *et al*, 2020).

Conclusion

The results of LAB isolation obtained 12 LAB isolates that isolate BKR11 potensial as antifungal agent that can inhibit *Penicillium* sp. Phylogenetic analysis showed that the BKR11 isolate was 99,86% similar to *Leuconostoc mesenteroides*.

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