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Screening Of Antibacterial Activity And Molecular Identification Of Lactic Acid Bacteria From Cabbage Fermentation On *Bacillus Cereus* Pathogenic Bacteria

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Abstract. Lactic Acid Bacteria (BAL) are often found naturally in food ingredients such as vegetables and fruits. Cabbage fermentation is one of the best sources of Lactic Acid Bacteria which contain antibacterial compounds such as bacteriocin, hydrogen peroxide, and organic acids. This study purpose are to isolate BAL, screen the antibacterial activity, and identification of molecular of selected isolates. This study was initiated with Lactic Acid Bacteria isolation from cabbage fermentation, followed by screening for antibacterial activity by disc diffussion method and identification of molecular isolates which having the highest antibacterial activity by PCR method. After isolation, 6 isolates were obtained: K31, K32, K33, K34, K35 and K36. The result of antibacterial activity test showed that K32 isolate had the highest activity against bacterium of *Bacillus cereus*. Molecular identification with PCR method and sequencing of amplification results showed that K32 isolates having 99% similarity level to *lactobacillus buchneri* JCM 115 strain. From the results of the study it can be concluded that cabbage fermentation contains Lactic Acid Bacteria which have antibacterial activity against *Bacillus cereus*.

Keyword : Cabbage Fermentation, Lactic Acid Bacteria, Antibacterial, *Bacillus Cereus*, PCR

1. Introduction

The use of various types of antibiotics may be selected not only to cure the infectious diseases but also to minimize the transmission. However, irrational use of antibiotics may cause various side effects to the users, including antibiotic resistance and normal flora changes in intestine. This encourages people to find other alternative treatments and switch from using chemical to natural ingredients for medicine. High biodiversity in Indonesia provides a great opportunity to obtain potential microorganisms to develop as the producer of secondary metabolite compound which provides various benefits for the treatment and prevention of a disease. Microbes have an important role as the producer of secondary metabolite compound due to their various advantages, such as having short life cycle, time and place efficiency, high productivity and facilitating us to conduct genetic manipulation (through microbial genetic engineering) or manipulation in the fermentation process (bioprocess engineering).

Lactic Acid Bacteria (LAB) are often naturally found in food materials, such as vegetables and fruit. One food material producing the lactic acid bacteria is cabbage. Cabbage is not only one local vegetable with high carbohydrate content but also substrate broken down by Lactic Acid Bacteria into the lactic acid compound. Cabbage fermentation may improve the secondary metabolites produced by lactic acid bacteria due to the optimization of enzymatic reactions during fermentation. The resulted fermentation products are ethanol, lactic acid and acetic acid [5]. LAB may produce lactic acid, hydrogen peroxide, and bacteriocin as the final product of the broken-down carbohydrate [1].

Polymerase Chain Reaction (PCR) is a process of enzymatic synthesis to in vitro amplify the nucleotides [6]. The PCR process is a repetitive cycle process, including denaturation, annealing, and extension made by the DNA polymerase enzyme. After the amplification process, it is followed with electrophoresis analysis and identification using the 16S rRNA gene marker. Gene 16S is a specified gene for prokaryotic species [2]. The previous research has been conducted by Yuni Nurisva

Maya Sari, *et al*. (2013) and is successfully to isolate, characterize and identify the lactic acid bacteria which have the antibacterial potentials obtained from the fermentation of yellow passion fruit (*Passiflora edulis var. Flavicarpa*). This research is conducted to obtain the lactic acid bacteria from the cabbage fermentation which has the antibacterial activity against *Bacillus cereus* and molecularly identifies the lactic acid bacteria from the fermentation of cabbage as the producer of secondary antibacterial metabolites. Antibacterial activity test is conducted using disc diffusion method. The Isolates having the greatest antibacterial activity are followed by the molecular identification using the 16S rRNA gene.

2. Research Methods

2.1 Materials

The equipment used includes glassware, micro pipette (Neson®), microtip, needle Ose, bunsen burner, autoclave (Hirayama Hiclave HVE-50®), incubator (Memmert®), oven (Memmert®), Laminar Air Flow (LAF), hot plate (Akebono®), vortex, microscope, UV transiluminator, pH indicator, refrigenerator microsentrifuge (PerfectSpin 24 Plus®), thermo cycler PCR (Tanach RAY-MG48®), electrophoresis (Mupid EXU®), UV transilluminator (Genesys 20®), Rotary shaker (Eyela®) Samples were Vegetable Cabbage (*Brassica oleracea var. Capitata*), NaCl 0.85% , crystal violet, iodine solution, safranin, Tris-EDTA buffer solution, Kit Promega, GoTaq Green Master mix, Nuclei Free Water, Ethidium bromide, TAE 1x, isopropanol, agarose gel 1% (b / v), Medium DeMann Rogosa Sharpe Agar (MRSA), DeMann Rogosa Sharpe Broth (MRSB), Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), Nutrient Broth (NB), Nutrient Agar (NA), *Bacillus Cereus*.

2.2 Sample preparation and plant identification

Plant samples were taken from the Bekasi area and then identified at the Herbarium Bogoriense, Botanical Field of the LIPI Research Center, Cibinong-Bogor.

2.3 The isolation of lactic acid bacteria from cabbage fermentation

Thoroughly wash and finely slice the cabbage. Put the finely cabbage slices into a fermentor and then immerse in salt solution 3% for 3 days and tightly close until reaching pH 4. Aseptically take 1 ml of the fermentation as and then make a series of dilution 10^{-1} to dilution 10^{-7} in sterile NaCl solution 0.85% and then vortex the results. Take 0.1 ml of Each dilution series and then inoculated them in solid MRSA medium on petri dishes using the distributive method. Incubate at 37 ^{0}C for 48 hours until a growing colony is obtained [16].

2.4 Characterization and Gram staining

The macroscopic and microscopic observations are made. The macroscopic observation shows the morphological characterization of lactic acid bacteria colonies includes colony pigmentation, colony shape, colony elevation, colony surface, and colony consistency. Meanwhile, the microscopic observation includes the cell shape and color with Gram staining. Gram staining starts by putting the bacteria on glass objects. Add 1 drop of crystal violet solution for 1 minute and then wash with the running water and then dry. Add 1 drop of lugol solution, let it stand for 1 minute, wash with water, and then dry. Wash the observed objects (in Indonesia known as *preparat*) with alcohol 96% until the dye is faded away from the *preparat*, then rinse with water and let it dry. The last stage is conducted by giving 1 drop safranin and then let it stand for 30 seconds. [10]

2.5 Cell-free supernatant preparation from lactic acid bacteria

The culture in the MRSA slant medium was inoculated with 1 Ose into 10 mL MRSB, then incubated for 24 hours at 37^0 C centrifuged at 3000 rpm for 10 minutes [15].

2.6 The qualitative screening on Antibacterial activity of Lactic Acid Bacteria

The screening is conducted using the disc diffusion method on the tested bacteria of *bacillus cereus*. Inoculate 10.1 mL of Bacillus cereus bacteria and then homogeneously mix with 20 ml Mueller Hinton Agar (MHA) in Petri dishes. In solid media, put the immersed disc paper in lactic acid bacteria suspension and then use the disc paper containing ciprofloxacin 5 µg (CLSI) to compare the positive control. Furthermore, incubate the bacteria at 37 ^{0}C for 24 hours. Finally, observe the antibacterial activity whether or not there are inhibitory zones around the disc paper [9].

2.7 The isolation of Genomic DNA Lactic Acid Bacteria with the Highest Antibacterial Activity

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). A total of 1 ml of liquid culture was put into a 1.5 ml micro tube. Centrifuged at a speed of 14.000 rpm for 3 minutes. Cell pellets were taken and added 480 µl EDTA 50 mM with pH 8.0 and 120 µl Lysozim 10 mg / ml, homogenized and incubated at 37^{0}C for 1 hour. Then centrifuged at 14.000 rpm for 2 minutes. The supernatant was removed, in cell pellets added 600 µl of buffer Nuclei lysis solution was homogenized and incubated at 80^{0}C for 5

minutes. After lysis solution is left at room temperature. In the solution, 3 µl of RNase is added and incubated at 37^0C for 15 minutes. Then 200 µl was added. Protein precipitation solution was extracted for 20 seconds and incubated in cold temperature for 15 minutes. Then centrifuged at 14.000 rpm for 3 minutes.

The supernatant was taken and transferred to a new micro-tube containing 600 µl of isopropanol p.a. the tube is turned several times, until there is a fine DNA thread, then centrifuged at 14.000 rpm for 2 minutes to precipitate the DNA. The supernatant was discarded and the pellet was washed by adding 600 µl of 70% ethanol, then centrifuged at 14.000 rpm for 2 minutes. The DNA pellets are then dried and dissolved by adding 100 µl of DNA rehydration solution, after which they are incubated at 4^0C for overnight. The results of genomic DNA isolation were then analyzed by agarose gel electrophoresis 1%.

2.8 DNA Amplification With PCR

The bacterial genome amplification process is carried out based on the protocol contained in the Maxima Hot Start Green PCR Master Mix (2X) using 27F and 1492R primers (Jinbo 2008). A total of 30 µl Maxima Hot Start PCR master mix (2X) was put into 0.5 ml microtube. Nuclease free water is added as much as 9.5 µl, then the mixture is resuspended until it dissolves completely by homogenizing. Furthermore, the homogeneous mixture added 27f primer and 1492r primer as much as 1 µl respectively. Then 1 µl of DNA is added and homogenized. PCR reaction using Thermal Cyclers PCR (Bio-Rad, UK) with first pradenaturation temperature of 94^0C for 90 seconds, followed by 30 cycles consisting of temperature denaturation of 95^0C for 30 seconds, primary attachment temperature of 50 ° C for 30 seconds and extension of temperature 72^0C for 90 seconds. After 30 cycles, the final extension phase was followed at 72^0C for 5 minutes and cooling at 4^0C for 20 minutes [4].

2.9 Sequencing of Gen 16S rRNA

The sample was put into a 0.2 ml dry and sterile micro tube, then sent to Eijkman Molecular Biology Institute, Jakarta Indonesia for further purification and sequencing.

2.10 Identification of 16S rRNA lactic acid bacteria gene.

DNA sequenced were analyzed with Bioedit program. The DNA sequence obtained is compared with the database sequence at the nBLAST site (http: /www.blast.ncbi.nlm.nih.gov/). After the results obtained compared with data on GeneBank.

3. Result And Discussion

3.1 Isolation And Characterization Of Morphology Of Lactic Acid Bacteria

Cabbage fermentation is aseptically conducted in anaerobic conditions. The fermentation process decreases the pH due to the formation of lactic acid produced by lactic acid bacteria that the solution has acidic pH. The lactic acid bacterial isolation is conducted using a multilevel dilution method from dilution 10^{-1} to dilution 10^{-7}. This study results in six selected isolates: K31, K32, K33, K34, K35, and K36. Isolate K31, K32, and K33 are obtained from the isolation of dilution 10^{-5}. Isolate K34 and K35 are obtained from the isolation of dilution 10^{-6}. Isolate K36 is obtained from the isolation of dilution 10^{-7}. Those isolates are selected based on the best morphology of lactic acid bacteria: round, convex, milky white color, shiny, and have clear edges. For the microscopic staining test, the produced lactic acid bacteria are in purple color with bacillus and coccus shape. The isolation result of lactic acid bacteria may be seen in Figure 1 below.

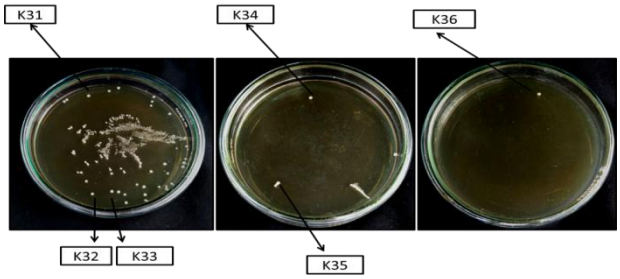

Figure 1. Isolation result of Lactic Acid Bacteria derived from the Cabbage Fermentation:
a. Dilution10^{-5}, b. dilution 10^{-6}, c. dilution 10^{-7}

3.2 Qualitative screening of antibacterial activity

The antibacterial activity testing result of lactic acid bacteria may be observed based on the formed clear zones around the disc. The measurements are made based on the horizontally and vertically produced clear zone diameter [17]. The formed clear zone shows that lactic acid bacteria have the inhibitory activity to the growth of the tested positive bacteria of *Bacillus cereus*. The Control Test is conducted using ciprofloxacin as a broad-spectrum antibacterial positive control which is able to inhibit both gram-positive and negative bacteria [8]. Davis & Stout (2009) divide antibacterial power into four categories: those with weak inhibition (<5 mm), moderate inhibition (5-10 mm), strong inhibition (10-20 mm), and very strong inhibition (> 20 mm).

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Table 1. Antibacterial activity screening result of Lactic Acid Bacteria against *Bacillus cereus*

Isolate Code	Fermentation Day						
	1	2	3	4	5	6	7
	Inhibition Zone (mm)						
K31	8.417	9.608	8.708	9.867	9.375	10.258	10.167
K32	10.1	9.692	7.958	9.158	9.733	8.783	11.8
K33	7.717	10.2	8.375	9.342	9.567	9.825	11.492
K34	8.15	9.333	9.217	9.467	9.583	9.925	10.817
K35	8.717	9.475	9.858	9.467	9.4	10.583	11.517
K36	8.675	9.817	9.075	8.242	6.983	6.517	10.375

Based on Table 1, it shows that the isolates of lactic acid bacteria have the relatively moderate to strong inhibitory power against *Bacillus cereus*. The research result shows that isolate K32 has the highest antibacterial activity against the tested bacteria. Isolate K32 is selected for further tested with PCR method to determine the type of K32 bacterial isolates which has the highest antibacterial activity against *Bacillus cereus*.

3.3 DNA Amplification of isolate K32 with PCR method

The amplification of 16S rRNA gene is conducted to lactic acid bacteria isolate K32 using PCR method and primary 27F (5'-AGAGTTTGATCCTGGCTCAG- 3') and primary 1492R (5'-GGTTACCTTGTTACGACTT-3'). Primary 27F is the forwarded primer attaching to the end of the targeted DNA strand of 5' which has previously decomposed, while the primary 1492R is the reverse primer attaching to the other end of DNA single chain of 5'. The PCR result presented in Figure 2 shows positive result as there are DNA fragments.

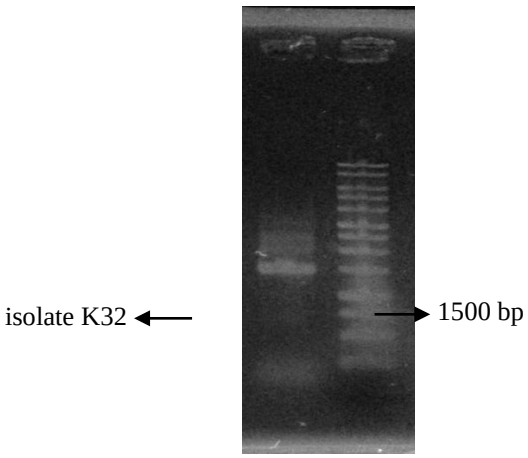
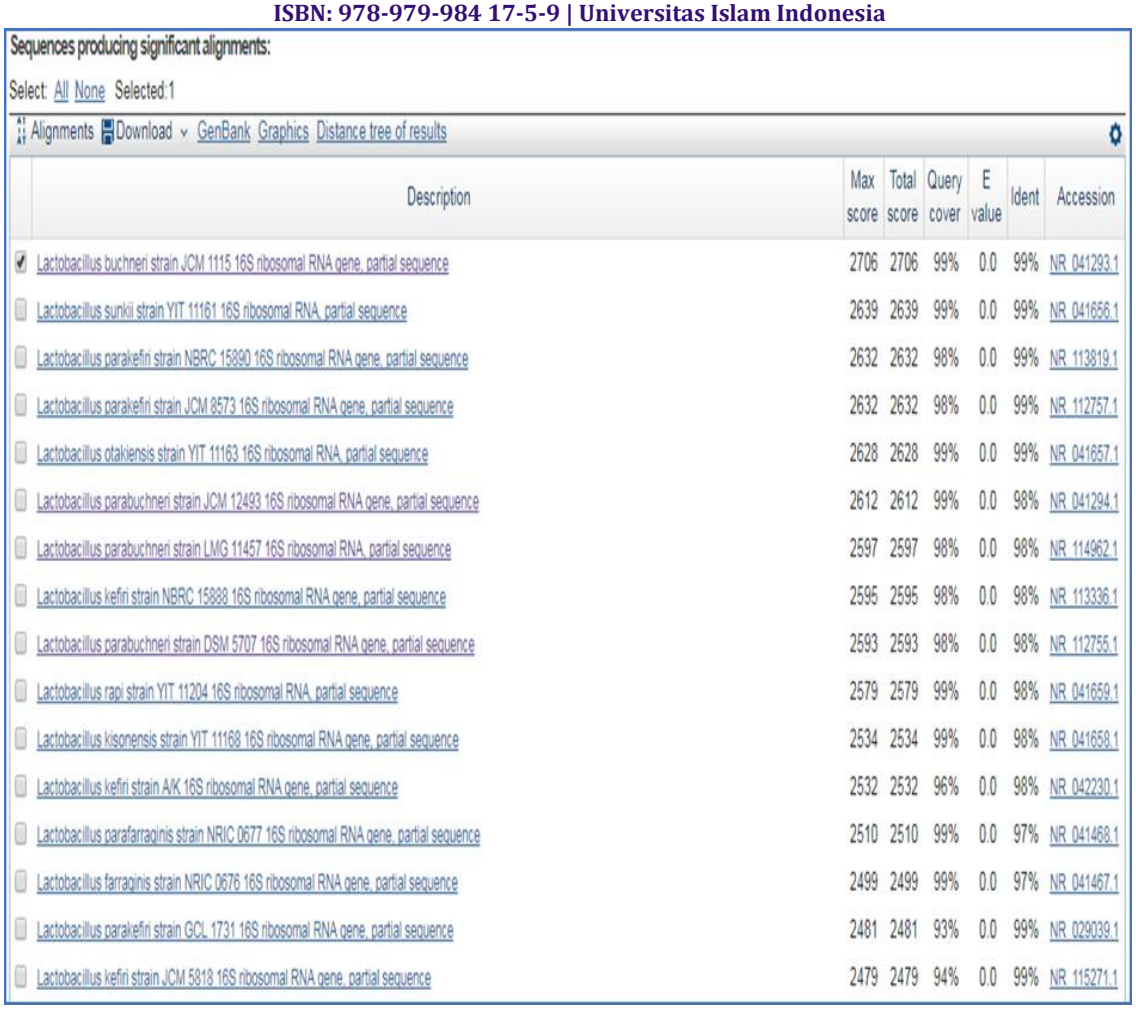


Figure 2. Electrophoresis Results of Amplicon Isolate K32 from The Cabbage Fermentation: 1. DNA Template, 2. DNA Ladder Bennech 1 Kb

The successfully conducted amplification process is characterized by the presence of DNA fragments on agarose gel, shown in Figure 2. The magnitude of the DNA fragment is ranging in1500 bp consistent with that of 16S rRNA gene [2]. The electrophoresed amplicon result is further sequenced to determine the nucleotide base sequence. The sequence data processing result is analyzed using the online NBLAST program on NCBI website (http://blast.ncbi.nlm.nih.gov/) to compare the sequenced data (query) from this research result with the DNA sequences from various parts of the world deposited and published in DNA or gene banks.



Sequences producing significant alignments:

Select: All None Selected:1

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactobacillus buchneri strain JCM 1115 16S ribosomal RNA gene, partial sequence	2706	2706	99%	0.0	99%	NR_041293.1
Lactobacillus sunkii strain YIT 11161 16S ribosomal RNA, partial sequence	2639	2639	99%	0.0	99%	NR_041656.1
Lactobacillus parakefiri strain NBRC 15890 16S ribosomal RNA gene, partial sequence	2632	2632	98%	0.0	99%	NR_113819.1
Lactobacillus parakefiri strain JCM 8573 16S ribosomal RNA gene, partial sequence	2632	2632	98%	0.0	99%	NR_112757.1
Lactobacillus otakiensis strain YIT 11163 16S ribosomal RNA, partial sequence	2628	2628	99%	0.0	99%	NR_041657.1
Lactobacillus parabuchneri strain JCM 12493 16S ribosomal RNA gene, partial sequence	2612	2612	99%	0.0	98%	NR_041294.1
Lactobacillus parabuchneri strain LMG 11457 16S ribosomal RNA, partial sequence	2597	2597	98%	0.0	98%	NR_114962.1
Lactobacillus kefiri strain NBRC 15888 16S ribosomal RNA gene, partial sequence	2595	2595	98%	0.0	98%	NR_113336.1
Lactobacillus parabuchneri strain DSM 5707 16S ribosomal RNA gene, partial sequence	2593	2593	98%	0.0	98%	NR_112755.1
Lactobacillus rapi strain YIT 11204 16S ribosomal RNA, partial sequence	2579	2579	99%	0.0	98%	NR_041659.1
Lactobacillus kisonensis strain YIT 11168 16S ribosomal RNA gene, partial sequence	2534	2534	99%	0.0	98%	NR_041658.1
Lactobacillus kefiri strain A/K 16S ribosomal RNA gene, partial sequence	2532	2532	96%	0.0	98%	NR_042230.1
Lactobacillus parafarraginis strain NRIC 0677 16S ribosomal RNA gene, partial sequence	2510	2510	99%	0.0	97%	NR_041468.1
Lactobacillus farraginis strain NRIC 0676 16S ribosomal RNA gene, partial sequence	2499	2499	99%	0.0	97%	NR_041467.1
Lactobacillus parakefiri strain GCL 1731 16S ribosomal RNA gene, partial sequence	2481	2481	93%	0.0	99%	NR_029039.1
Lactobacillus kefiri strain JCM 5818 16S ribosomal RNA gene, partial sequence	2479	2479	94%	0.0	99%	NR_115271.1

Figure 3. The 16S rRNA Gene BLAST Nucleotide Result Description of Lactic Acid Bacterial isolate K32 from the Cabbage Fermentation

The result of sequence data analysis presented in Figure 3 shows that the bacterial isolate K32 from the cabbage fermentation has similar nucleotide base sequence with the query coverage value of 99% with *Lactobacillus buchneri* bacterial strain JCM 115. Query coverage is the percentage of nucleotide lengths consistent with the contained database in BLAST. The obtained identity percentage is 99%, stating that isolate K32 has 99% nucleotide base similarity with the existing database in Gene Banks [12]. If homology has the percentage of ≥ 97%, it can be confirmed as a species. However, if the homology is less than 97%, the isolate is probably a new species or species which cannot be confirmed yet [7].

4. Conclusion

From this study successfully isolated 6 lactic acid bacteria from cabbage fermentation. Of the six isolates obtained by K32 isolates, the greatest inhibition against *Bacillus cereus* bacteria. The results of molecular identification of K32 isolates using 16S rRNA gene sequence analysis had a 99% similarity with *Lactobacillus buchneri* strain JCM 115.

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