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Research Article

Identification by 16S Ribosomal RNA Gene Sequencing of Lactic Acid Bacteria Producing Antibacterial Agents from Langsat Fruit (*Lansium domesticum*) in North Minahasa District, North Sulawesi

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ABSTRACT

Fourty two strains Lactic Acid Bacteria (LAB) were isolated from Langsat fruit (*Lansium domesticum*) samples obtained from 4 districts in Minahasa, North Sulawesi. 12 isolates of lactic acid bacteria were isolated from Langsat fruit in North Minahasa District. Lactic acid bacteria have the ability to inhibit the growth of pathogenic bacteria and putrefactive bacteria. The aim of this study was to identify LAB isolates producing antibacterial agents from Langsat fruit at North Minahasa District based on identification genotypic. Identification genotypic isolat LMU7 from Langsat fruit was based on 16S rRNA gene sequencing Method. One Isolate (LMU7) had a great ability to inhibit the growth of pathogenic bacteria and putrefactive bacteria. The 16S ribosomal RNA gene from one isolate could be amplified by PCR (Polymerase Chain Reaction) and show a single band on a 2% (w/v) agarose gel. Identification by 16S Ribosomal RNA gene showed that one isolate LAB producing antibacterial agents were identified as *Lactobacillus plantarum* with a similarity index of approximately 99.93%.

Keywords: Langsat Fruit; Antibacterial; LAB; 16S rRNA gene; Identification

INTRODUCTION

North Minahasa District is located in North Sulawesi Province. One of the plantation resources in the North Minahasa area is Langsat fruit (*Lansium domesticum*). Langsat fruit is endemic to North Sulawesi and very popular with the people of North Sulawesi. These fruits have a combination of sharp, sour and sweet flavors, and taste very similar to grapes. Some people feel the taste is similar to grapefruit. This sour taste actually indicates the many benefits of langsat fruit for the health of the body. This can be seen from the content of Langsat fruit which is rich in many important elements such as protein, carbohydrates, fat, minerals, vitamins and abundant dietary fiber. Rich in vitamin A, thiamine and riboflavin, which are necessary for many body functions [1].

Naturally, lactic acid bacteria are found in various habitats such as fruits, fermented foods, vegetables and the human digestive tract. So far it is known that lactic acid bacteria are not pathogenic and safe for consumption so that it can be used to increase human health. Lactic acid bacteria can be used as a natural preservative because they produce antibacterial compounds such as organic acids, bacteriocins, hydrogen peroxide, diacetyl [2]. The use of lactic acid bacteria as a natural preservative can be in the form of starter cultures or metabolites produced by lactic acid bacteria.

The availability of lactic acid bacteria from domestic fruit sources is still lacking, so LAB exploration is needed to increase the collection of LAB isolates. Genotypic identification of lactic acid bacteria is used to determine the identity of LAB accurately and reliably. The method used for genotypic identification was 16S rRNA sequencing because it has high separability at both the species and intraspecies level [3]. Extraction of DNA is a basic principle in molecular analysis and it is one of the success factors in DNA amplification that is used in the analysis of genetic characters [3]. Polymerase Chain Reaction (PCR) and phylogenetic analysis based on 16S rRNA gene sequences have been used for successful identification of isolates from various fermented food products [4]. This molecular approach allows Lactobacillus species to be identified reliably [5]. The aimed of this research was to identify LAB isolates producing antibacterial

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agents from Langsat fruit at North Minahasa District by 16S Ribosomal RNA gene sequencing.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

Langsat fruit were collected from North Minahasa District. LAB was isolated from sample of Langsat fruit. Ten grams of samples were taken aseptically and homogenized in 90 mL of NaCl solution. Serial dilutions up to 10-7 were prepared and appropriate dilutions were plated onto de Man Rogosa and Sharpe Agar supplemented with $CaCO_3$ 1%, Na Azida and cyclohexamide. All plates were incubated at 37°C for 48 h. Only lactic acid producing bacterial colonies was selected. This can be observed from clear zones around the colonies which indicated the dissolving of $CaCO_3$ by an acid [6]. Colonies with different morphology were counted, picked up and purified by restreaking on the same medium. Cell morphology, Gram staining and catalase test, motility, non-spore forming were performed as a preliminary screening for lactic acid bacteria [7]. The selected LAB was maintained as stock cultures at -80°C in 10% skim milk and 20% glycerol.

Screening of lactic acid bacteria for antimicrobial activity

The antimicrobial activity of LAB (Culture) against *Escherichia coli* ATCC 35218, and *Stapylococcus aureus* ATCC 25923 was performed by the well diffusion assay [8]. The antimicrobial activity was determined by measuring the clear zone around the wells.

Identification of lactic acid bacteria isolates

The isolated LAB showing the highest antimicrobial activity was identified based on genotypic characterization. Genotypic characterization was carried out by comparison of the 16S rDNA sequence.

Extraction of DNA LAB isolate

Identification of bacterial isolates was carried out molecularly based on genetic analysis partial on 16S rRNA. Extraction of DNA was carried out using the GES method modified [9].

16S rRNA gene amplification

PCR amplification on 16S rRNA using Primer 27 F:5`-AGA GTT TGA TCC TGG CTC AG-3` and Primary 1492 R:5`-GGT TAC CTT GTT ACG ACT T-3` [10]. The condition of amplified gene fragment: pre-denaturation of the target DNA at 96°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 51.5°C for 1min and 30 sec and primer extension at 68°C for 8 min. PCR was completed with 10 min elongation at 68°C followed by cooling to 4°C [11]. PCR product purification was carried out by using the PEG precipitation method [12].

Electrophoresis

PCR product was visualised by electrophoresis on a 2 % (w/v) agarose gels, stained with ethidium bromide in the presence of a 1 kb ladder. The parameters for the electrophoresis were 90 V for 30 min.

Sequencing alignment of the 16S rRNA gene

PCR product purification continued with a sequencing cycle. For sequencing, the primer used is Primer 27F:5`-AGA GTT TGA TCC TGG CTC AG-3` and Primary 1492 R:5`-GGT TAC CTT GTT ACG ACT T-3`. The results of the sequencing cycle were re-purified by Ethanol purification method. Analysis of nitrogen base sequence readings using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems).

The raw data from the sequencing is then trimmed and assembled using BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence data that has been in the next assembling was in BLAST with genomic data that had been registered in DDBJ/DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) or NCBI/National Center for Biotechnology Information. (http://www.ncbi.nlm.nih.gov/BLAST/) to determine taxon/species that have Homology/similarity molecularly.

Phylogenetic analysis

The phylogenetic analysis, sequences LMU7 strain was aligned by using Mega X [13]. The phylogenetic tree of the 16S rDNA sequences was constructed by the neighborjoining algoritm [14]. The root position on the unrooted tree was estimated by using *Bacillus subtilis* ATCC 21331 as the outgroup strain.

RESULTS AND DISCUSSION

The identification of LMU7 isolate to determine the strain carried out based on the 16S rRNA gene. The first step is amplification using the PCR method. The amplification process is carried out to obtain more copies of the 16S rRNA gene for the next step, the sequencing process. Analysis of sequencing results begins with aligning the base sequence of the 27F forward and the reverse 1492R. PCR of the 16S rRNA gene LMU7 isolate provides an amplicon of about 1400 bp (Figure 1) [15]. It shows that evolutionary history of organisms can be identified by the neighbor join method.

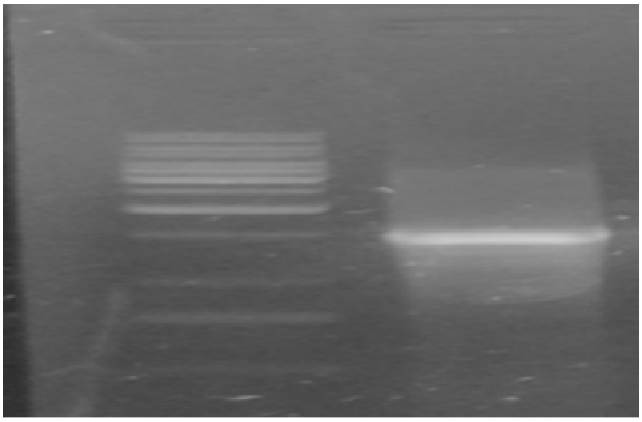

Figure 1: Agarose gel (2%) electrophoresis showing amplified 16S rRNA gene of LMU7 isolate. M, DNA marker; 1, PCR product of LMU7 isolate.

Organisms in the same taxa usually clustered together in the phylogenetic tree and has a better bootstrap value [16]. In this study phylogenetic tree construction to measure and determine evolutionary distances. A total of 11 nucleotide sequences and Codon positions 1+ 2+ and 3+ were not codes considered, using MEGA X as reported by for evolutionary analysis [17].

The result of DNA sequencing was analyzed using NCBI BLAST (Figure 2). According to 16S rRNA gene sequencing was used to see the similarities between isolates that were already available in GenBank [18]. This is one of the molecular detection methods which is ideal for knowing the kinship relationship between bacteria because the nucleotide sequence of the 16S rRNA is a gene present in all microbes and is indispensable in maintaining life. The 16S rRNA gene was used to identify LMU7 isolate belonging to the genus *Lactobacillus* and to form clusters with *Lactobacillus plantarum* strain DP1T.

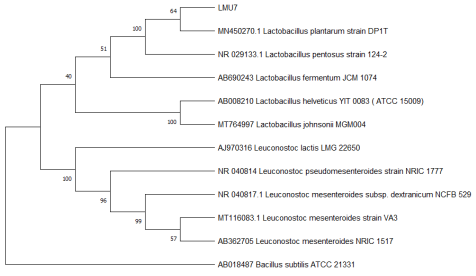

Figure 2: Phylogenetic tree showing the relationship between strain LMU7 and representatives of the genus Lactobacillus and Leuconostoc based on 16S rDNA sequences. The scale bar indicates 10 nucleotide substitution per 100 nucleotides in 16S rRNA gene sequences.

The cluster is recovered in a 100% bootstrap analysis. *Lactobacillus sp.* is widely described as a probiotic [19]. This also found that *Lactobacillus plantarum* in fermented milk product [20]. LAB is a LAB that is used as a starter culture in the fermentation of fish, meat, vegetables, fruit and milk which induces a distinctive taste change, improves hygiene and extends the life of the product [21].

Another species with the closest relationship apart from *Lactobacillus plantarum* whose nucleotide sequence was parallel or at least 100% of the observed query coverage was *Lactobacillus pentosus* 124-2 at 99.79% similarity index to LMU7 isolates. *Lactobacillus fermentum* strain JCM 1074 has 90.04% similarity with the LMU7 isolate while *Lactobacillus jhonsoni* MGM 004 has similarities 89.67 with LMU7 isolates. The results of the 100% alignment query showed significant alignment, which means that the search order in this study was identical to the genus identified, even at the species level [22].

CONCLUSION

Identification based on the 16s rRNA gene shows that the LMU7 isolate, which was isolated from Langsat fruit (*Lansium domesticum*) in North Minahasa District and has the ability to produce antibacterial compounds, as a species within the genus *Lactobacillus* and as a strain belonged to members of species *Lactobacillus plantarum*. Phylogenetic analysis showed that the *Lactobacillus plantarum* strain LMU7 was 99.93% similar to *Lactobacillus plantarum* DP1T.

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