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Research Article

IDENTIFICATION OF GABA-PRODUCING LACTIC ACID BACTERIA FROM CINCALOK FERMENTATION BASED ON THE 16*S* RRNA AND *GAD* GENES

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ABSTRACT

Objective: The research to identify LAB using 16S rRNA potential as high produce GABA and design primer can amplify that gad gene.

Methods: Isolation genomic form LAB, molecular identification based 16S rRNA, design primer use primer3plus, and use application serial cloner to ensure the primer can amplify to target gene.

Results: Lactic acid bacteria that have been analyzed based on the 16 S rRNA gene have the highest similarity with *Weissella confusa* strain JCM 1093 with a similarity of 98.38%. the results of the analysis based on the gad gene using several primers that have been designed by researchers and based on existing references are unable to amplify the gad gene owned by *W. confusa.*

Conclusion: The results of the analysis based on the 16S rRNA gene for lactic acid bacteria were obtained by *Weissella confusa*. However, for the results of identification analysis based on the gad gene, the designed primers were unable to amplify the gad gene in *W. confusa.*

Keywords: Cincalok, Glutamate decarboxylase, Lactic acid bacteria, 16S rRNA.

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INTRODUCTION

Fermented foods in Indonesia are very diverse, from Sabang to Marauke seasoning and how to process fermented food obtained from our ancestors. Fermented foods such as Cincalok are fermented foods originating from West Kalimantan made from small shrimp. Shrimp has a nutritional content in the form of protein; this protein will later be used as a substrate in the fermentation process using microbes. One microbe plays a role, namely, lactic acid bacteria (LAB). One of the bakeries found by Achmad *et al.* [1] in Cincalok is *Lactobacillus* RED1.

Cincalok fermented food contains LAB which consists of various species. Research on the species that produce GABA requires identification. Identification of lactic acid bacterial species can be done in various ways through morphological characteristics, metabolites, phenotypes, and genotypes. Phenotypic identification is considered inaccurate, and there is a fear of misidentification. A combination of identification methods will provide better results, for example, a combination of morphological and genetic identification. In general, the identification of microbes uses molecular analysis of 16S and 23S rRNA [2]. The choice of 16S rRNA as a reference in identifying a microbe is because it has a base sequence of 1500–1550 base pairs. Furthermore, the analysis with 16S rRNA had a conservative base sequence, so that it can be a reference for making phylogeny trees [3].

Glutamate decarboxylase is considered to be responsible for the production of GABA through strains of GABA-producing lactic acid bacteria, such as Lactobacillus and Lactococcus. The *glutamate decarboxylase* has been isolated from various sources and its biochemical properties are known. Lactobacillus brevis had been isolated by GAD and determined its biochemical characterization. The research that has been done is to characterize GAD and clone the gad gene in *Lactococcus lactis* and found that *L. lactis* contains only one gad gene. Although GAD is widely distributed in BAL, the ability to produce GABA varies greatly among LAB [4].

The genetic identification method for LAB that is commonly used is the analysis of GAD gene sequencing. Glutamate decarboxylase enzyme (GAD) irreversibly via -decarboxylation of L-glutamic acid to produce GABA, which the enzyme depends on pyridoxal 5-phosphate. *Glutamate decarboxylase* is widely distributed in eukaryotes and prokaryotes. The research that has been done shows that GAD is present in LAB. Overall GABA can provide resistance to bacterial cells under acidic conditions and the GAD decarboxylation process has been combined with the energy synthesis found in *Lactobacillus* sp. Strain E1 [5].

This study aims to determine the species names of LAB and those previously isolated, tested biochemically and also analyzed for GABA content using the thin-layer chromatography method [6].

METHODS

Sample and materials

This study used materials in the form of cincalok, MRSA media, $CaCO_3$, Phosphate Buffer Saline (PBS), ddH_2O, chelex, Dream Taq Green PCR Master Mix, agarose gel, aquades, Buffer TAE, primer *forward* 16S rRNA 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), primer *reverse* 16S rRNA 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), primer *forward gad* (5'- ATGGGCATCACTTATACTGG-3' dan 5'-CARGTNTGYTGGGARAA-3'), primer *reverse gad* (5'-TTTAGGTTGCGGATCACTAT-3', 5'-CGATCGGATAAATCGTAGAG-3' dan 5'-GGRTANACNARNCCRTAYTTRT'-3'), DNA *template, marker* (DNA *ladder*), alcohol 70%, *loading dye.*

DNA isolation and purification

DNA isolation using the Chelex method [7]. LAB isolates that produced the highest GABA were taken as much as 2 ose and put into a 1.5 mL microtube. The addition of 100µL ddH_2O to LAB. 1 mL of phosphate buffer saline (PBS) was added, and then incubated for 24 h at 4°C. Furthermore, the extraction was carried out by centrifugation at a speed of 12,000 rpm for 10 minutes. The results obtained are in the

form of natan and supernatant layers. The layer of the supernatant will be removed with a micropipette which aims to remove saponins without damaging the pellets.

The next step was adding 1 ml of ddH_2O and centrifuged at 12,000 rpm for 5 min, the supernatant was discarded. LAB was added with ddH_2O as much as 100 µL and 50 µL 20% chelex 100. The chelex solution was shaken so that the crystals could be lifted. Chelex should be added in cold conditions. The solution is heated on a hot plate for 10 min centrifugation again at a speed of 12,000 rpm for 10 min. The supernatant obtained was taken with a micropipette and separated from the pellets. The supernatant containing the DNA genome was transferred to another microtube. Samples are stored in the freezer at -20°C, if not used immediately. Quantitative DNA test to determine the purity and concentration of each DNA sample was measured using nanodrops.

Molecular identification of the 16S rRNA coding gene

Theprimersusedinthisstudywere27F(5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT -3'). DNA amplification by PCR was carried out using the GeneAmp PCR System 9700 thermal cycler. The initial stage of amplification began with the manufacture of PCR mix with a total volume of 25 µl consisting of 1 µL 27F primer, 1 µl 1492R primer, 12.5 µl Thermo Fisher Scientific's Dream Taq PCR, and 9.5 µL ddH_2O. This mixing is done on a microtube. PCR was performed starting from the predenaturation stage at 94°C for 4 min. 35 PCR cycles occurred on the separation of DNA strands (denaturation) at 94°C for 1 min, primary attachment (annealing) at 56°C for 1 min, and the extension stage at 72°C for 1 min. The amplification process was ended with a one-step extension at 72°C for 5 min and storage at a final stage of 4°C. DNA PCR products were separated by electrophoresis on 1% (wt/volume) agarose gel. The DNA sequencing results were sent to Macrogen through PT. Indolab Utama.

Molecular identification of the gad gene

Primers are designed using the Primer3Plus application and refer to references to identify the gad gene. The primary pairs are shown in Table 1.

The molecular identification of the gad gene requires a primary design; the primary design used is Primer3Plus. The primary design is needed to obtain a primer that can amplify DNA using the PCR method; in the primary design process an in silico stage is needed. *In silico*, the primary design with the help of computer software, in this study using Serial Cloner software.

A primer designed in this study to amplify the gad gene. Gad gene is a gene that plays a role in the process of forming GABA. The selection of molecular identification on the gad gene will be used to identify the species level based on the gad gene.

Sequencing

Data in the form of base sequences from forward and reverse primers that have been obtained from PT. Indolab processed using BioEdit. The sequencing results obtained will later be compared with other species in the NCBI application (http://blast.ncbi.nlm.nih.-gov) through the BLAST (Basic Local Alignment Search Tool) process.

Phylogenetic tree

The next stage after sequencing analysis, the data obtained will be aligned (Clustal W) making phylogenetic trees using MEGA X software, the method chosen by the neighbor-joining tree p-distance model.

RESULTS

Molecular identification of LAB based on 16S rRNA

CIN-1 isolate was able to produce the highest GABA concentration in the previous studies [6], then it will be identified molecularly based on analysis of the 16S rRNA gene. The primers used were 27F and 1492R primers, so that the amplification results were obtained as shown in Fig. 1. The sequences obtained from the 16S rRNA analysis were analyzed using a phylogenic tree and obtained in Fig. 2.

Molecular identification of LAB based on the gad gene

Primers for identifying gad genes in Cin-1 isolates were designed using primer3plus and based on reference (Table 1). Primers that have been designed were tested using the Serial Cloner application, with the experimental sample in the form of a gad gene from the bacteria *Lactobacillus brevis* BH2 glutamate decarboxylase (Figs. 3 and 4). The results of amplifying the gad gene using a primer that had been designed were unable to amplify the gad gene owned by *W. confusa* (Fig. 5).

DISCUSSION

Molecular identification of LAB based on 16S rRNA

Quantitative measurements and purity of DNA were carried out using a nanodrop 2000 UV-VIS spectrophotometer. According to Pelt-Verkuil *et al*. [8], DNA concentration was determined by absorbance measurement at 260 nm. The absorbance ratios of 260 nm and 280 nm (A260/280)

Table 1: The primary design used for the amplification of the GAD gene

Name	5-3 'sequence	Information
Gad-F	ATGGGCATCACTTATACTGG	Primer3Plus
Gad-R1	TTTAGGTTGCGGATCACTAT	Primer3Plus
Gad-R2	CGATCGGATAAATCGTAGAG	Primer3Plus
F3	CARGTNTGYTGGGARAA	Komatsuzaki *et al*., 2014
R6	GGRTANACNARNCCRTAYTTRTG	Komatsuzaki *et al*., 2014

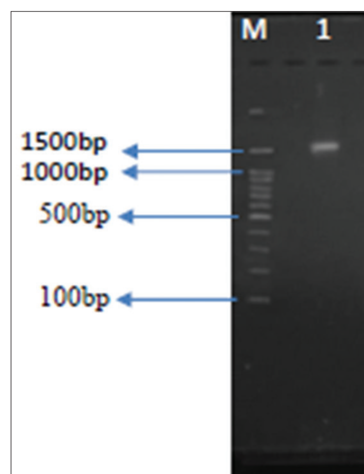

Fig. 1: Visualization of the amplification of the 16S rRNA gene encoding M. Marker 100 bp ladder, 1. CIN-1 isolate

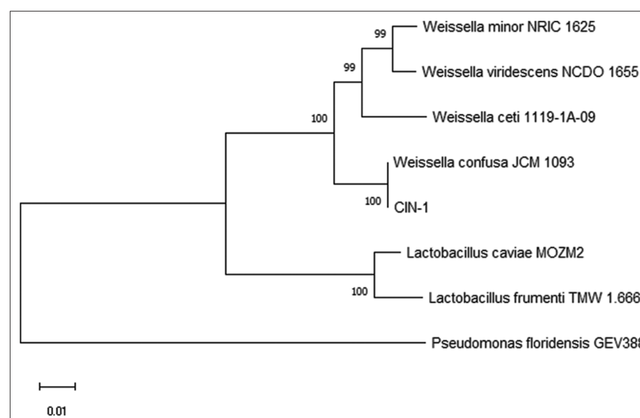

Fig. 2: Phylogenetic trees based on the 16S rRNA gene of CIN-1 isolates using Neighbor-Joining analysis and Bootstrap 1000 replicates phylogeny test

are commonly used to measure the contamination of dissolved DNA to RNA and protein. DNA purity was obtained from the absorbance ratio A260/280. According to Sambrook and Russell [9], A260/280 ratio <1.8 indicates protein contamination in the DNA extract obtained. Good DNA purity is indicated by the ratio A260/280 between 1.8 and 2.0.

The quantitative results of DNA extraction are shown in Table 2. The quantitative test of CIN-1 DNA extract shows a DNA concentration of 182.1 ng/µl with a DNA purity of 2.15 in the ratio A260/280. The results of the DNA purity and concentration analysis indicated that the DNA isolation method in this study was able to isolate DNA from CIN-1 isolates.

The results of 16S rRNA gene amplification were analyzed by agarose gel electrophoresis. The marker used to compare the size of the DNA in the amplification results was 100 bp ladder. The results of 16S rRNA gene amplification from CIN-1 DNA showed the presence of DNA bands with a length range of 1500 bp in the agarose gel electrophoresis results (Fig. 1). According to Patel [10], the 16S rRNA gene has a length of 1522 bp in bacteria and can be used to determine the phylogeny and taxonomy of bacteria. The DNA amplification product is followed by a sequencing process.

Sequencing of CIN-1 DNA amplification aims to obtain information on the base sequence of the target DNA amplified by PCR with a certain length. The sequencing data obtained are aligned using the Basic Local Alignment Search Tools (BLAST) on the NCBI website. The program shows areas of local similarity between the sequences examined with a stored sequence information database to calculate statistical significance of match.

The result of CIN-1 isolate sequencing had a Query Length of 1362 bp. The BLAST results showed that the CIN-1 isolate had the highest similarity to Weissella confusa strain JCM 1093 with a similarity of 98.38% (Table 3). The percentage identification value above 97.5% can be stated as having a similarity at the species level, while the percentage identification value above 95% can be stated as a similarity at the genus level [11].

Table 2: DNA quantification results at OD 260/280

S. No.	Sample	DNA concentration ng/µl	A260/280
1.	CIN-1	182.1	2.15

Table 3: Homologous sequences of alignment results with 16S rRNA from CIN-1 bacterial isolates

Description	Max Score	Total Score	Query Cover (%)	E value	Per. ID (%)
Weissella confusa JCM 1093	2412	2412	100	0.0	98.38
Weissella cibaria II-I-59	2357	2357	100	0.0	97.58
Weissella oryzae SG25	2209	2209	98	0.0	96.19
Weissella hellenica NBRC 15553	2187	2187	98	0.0	95.77
Weissella paramesenteroides NRIC 1542	2187	2187	98	0.0	95.84
Weissella bombi R-53094	2180	2180	98	0.0	95.62
Weissella soli Mi268	2169	2169	99	0.0	95.41
Weissella viridescens NRIC 1536	2165	2165	100	0.0	95.08
Weissella diestrammenae ORY33	2145	2145	99	0.0	95.50
Weissella uvarum B18NM42	2137	2137	100	0.0	94.71

The alignment result also shows the same max score and total score, namely, CIN-1 of 2412. E-value 0.0 indicates a significant alignment. The relationship between species was analyzed using phylogenetic tree development using the MEGA X program. Phylogenetic trees of CIN-1 isolates were analyzed using the Neighbor Joining (NJ) method (Fig. 2).

These results indicated that the CIN-1 isolate had a bootstrap value of 100 with Weissella confusa JCM 1093. The bootstrap value of 100 indicated that in 1000 replications the same 1000 trees were formed. According to Hidayat and Pancoro [12], bootstrap analysis is a method that tests how well the model data set is and this value is usually located in the branches of a phylogenetic tree. Bootstrap value categories include high (>85%), moderate (70–85%), weak (50–69%), or very weak (<50%) [13]. A phylogenetic tree with a 0.01 scale bar shows a genetic distance with a change in nucleotides of 1 base in every 100 bp. Based on the observation result, CIN-1 isolate bacteria have stem cell morphology and Gram stain is Gram positive, and the catalase test is catalase negative. According to Fusco *et al*. [14], W. confusa are Gram-positive and catalase Gram-negative bacteria. The cells of these bacteria are short rods. These bacteria are heterofermentative and produce D and L lactic acid enantiomers when fermenting glucose. This bacterium is able to grow at temperatures of 45°C in several strains. Research that has been conducted by Thapa *et al*. [15], W. confusa can be found in fish-based traditional foods from the East Himalayas and other studies from [16], these bacteria are also found in fermented foods from freshwater fish originating from Thailand.

Molecular identification of LAB based on the gad gene

The primary design obtained from the application of Primer3Plus is the primary gad-F 5'-ATGGGCATCACTTATACTGG-3 'and the reverse gad-R1 5'-TTTAGGTTGCGGATCACTAT-3' and gad-R2 5'-CGATCGGATAAATCGTAGAA-3 'primer. Gad-F, gad-R1, and gad-R2 primers will attach to bases 634, 1386, and 1193, respectively, on the Lactobacillus brevis BH2 glutamate decarboxylase DNA. This gad-F primer has a base length of 20 bp, Tm 55 oC, and GC 45%. The reverse gad-R1 primer has a base length of 20 bp, a Tm of 54.9 oC, and a GC of 45%, while the primary gad-R2 has a base length of 20 bp, Tm of 55 oC, and GC of 45%.

Other primers adjusted according to the journal [17] are forward primers with code F3 5'-CARGTNTGYTGGGARAA-3 'and reverse primers with code R6 5'-GGRTANACNARNCCRTAYTTRTG-3'. Primary F3 has a base length of 17 bp and Tm of 45.1°C while primer R6 has a length of 23 bp and Tm of 53.9°C.

Software used in *in silico*, namely, Serial Cloner. The Lactobacillus brevis BH glutamate decarboxylase DNA sequence was opened through the software. The *in silico* results in the software using gad-F primer as forward primer and gad-R1 as reverse obtained 753 bp PCR products. The amplification results of the two primers are shown in Fig. 3. The results of *in silico* PCR products using F-3 primer and R-6 primer on Lactobacillus brevis BH glutamate decarboxylase obtained 526 bp PCR products are shown in Fig. 4.

The amplification of the gad gene using PCR with primary variations did not produce results even though *in silico* could be done. Result electrophoresis is shown in Fig. 5.

Primers that cannot amplify DNA can be caused by several factors. According to Borah [18], a good primer has a base length between 18 and 30 oligonucleotides, with this length expected to bind the template at a specific annealing temperature. If the primer is too short it can affect the specificity of the primer so that the primer will have difficulty sticking to the template. The percentage of GC should be in the range of 40–60%, a percentage of GC that is too low can reduce the efficiency of the PCR process because the primers cannot compete to stick to the

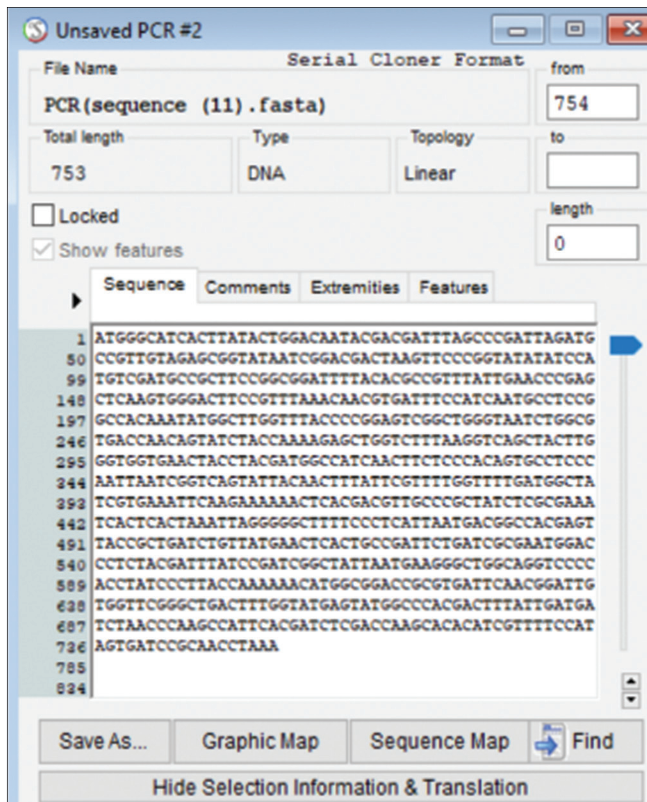

Fig. 3: Results of *in silico* PCR products from gad-F and gad-R1 primers

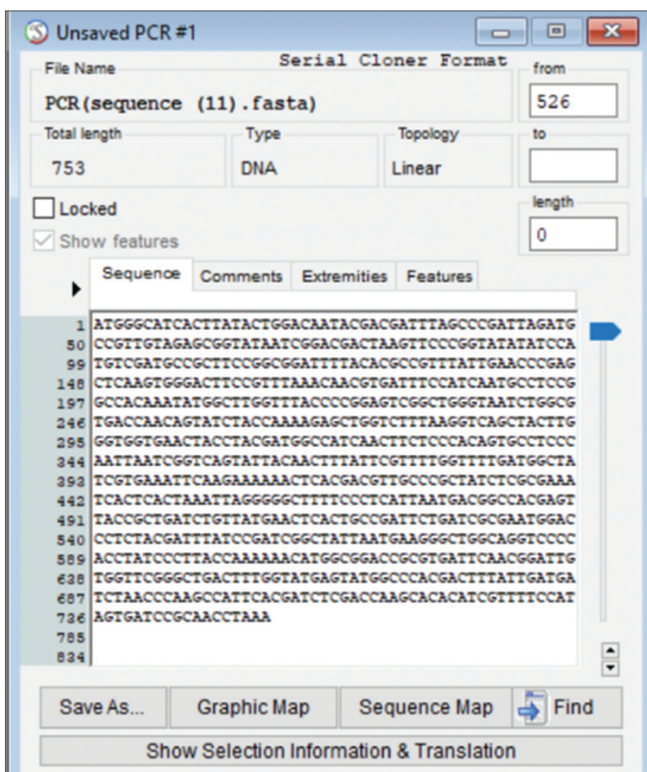

Fig. 4: Results of *in silico* PCR products from primers F3 and R6

template. According to Sulistyaningsih [19], the best Tm for forward and reverse primers has a range between 2 and 4°C. Too high a Tm can result in a low PCR product.

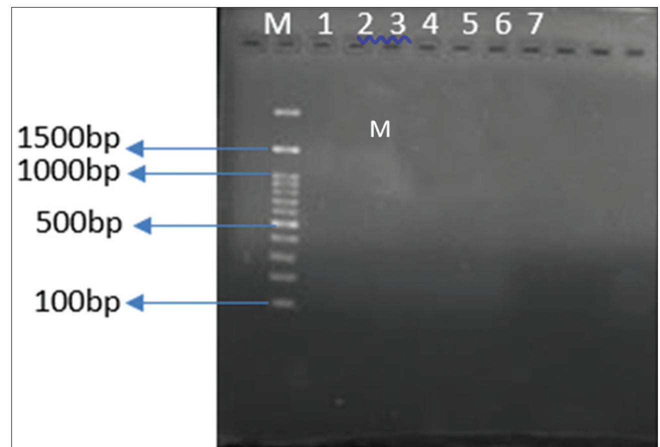

Fig. 5: Visualization of the PCR product of the gad M gene: 100 bp marker, 1: gad-F and gad-R1, 2: gad-F and R-2, 3: F3 and R6, 4: gad-F and R6, 5: gad-F and R6, 6: F3 and gad-R1, 7: F3 and gad-R2

In addition, the cause of failure of the gad gene amplification is in the selection of bacteria *in silico* which is different from the identification results of the 16S rRNA coding gene. Initially, the bacteria found were thought to be bacteria from the genus Lactobacillus, but the identification of the 16S rRNA gene coding for the bacteria was obtained from the *Weisella confusa* species. This difference causes the designed primers to not amplify the gad gene in the genus Weissella.

CONCLUSION

LAB isolates with the highest GABA concentration, identified using 16S rRNA, were similar to species *Weissella confusa*. Analysis based on the gad gene with several primers that had been designed, was not able to amplify the gad gene owned by *W. confusa*. The need to design specific primers according to the targeted species, by considering several good primer requirements, namely melting temperature (Tm), percentage of G and C (%GC), 3′dimer, stability, repeats, and hairpins.

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AUTHORS' CONTRIBUTIONS

Adhitya Naufal Pribadhi conducts research and writes journal with help of Endang Kusdiyantini and Siti Rejeki Ferniah.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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