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# Diversity of lactic acid bacteria isolated during fermentation of indigenous cassava obtained from Sumba, East Nusa Tenggara, Indonesia

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**Abstract.** *Hutajulu IBE, Kulla PDK, Retnaningrum E. 2021. Diversity of lactic acid bacteria isolated during fermentation of indigenous cassava obtained from Sumba, East Nusa Tenggara, Indonesia. Biodiversitas 22: 2561-2570.* Traditional fermented food products hold a great significance in the routine diet of Indonesian inhabitants. The present study investigated the diversity of lactic acid bacteria (LAB) found in fermented indigenous cassava, *ubi karet busuk*, produced in Sumba, East Nusa Tenggara, Indonesia. The isolated LAB was characterized for their phenotypic, biochemical, and molecular traits, which involved 16S rRNA gene sequencing, determination of lactic acid fermentation pathway, and measurement of β-glucosidase and tannase activity in the LAB strains. During fermentation, 15 LAB strains were obtained from the cassava. Among these, 93.33% strains were found to be homofermentative, whereas 6.73% strains displayed heterofermentative traits. BLASTn and phylogenetic analysis classified 15 LAB strains into six species, namely *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Enterococcus* sp. Among these, 10 LAB strains displayed β-glucosidase activity, with highest enzyme activity of 7.13 U/mL recorded in *L. lactis* F14IS3. Interestingly, two of these strains also showed expression of tannase, with F6IS1 strain displaying highest enzyme activity of 38.23 U/mL. Thus, the study results highlighted the suitability of these β-glucosidase and tannase-producing LAB strains to be used as a starter in the fermentation of cassava and other substrates such as vegetables, fruits, and legumes. These strains can also be utilized in animal feed, food, and beverage industries.

**Keywords:** β-glucosidase, homofermentative, tannase, phylogenetic analysis, 16S rRNA gene

## INTRODUCTION

In Indonesia, a variety of traditional fermented food products have been in use since ancient times. These fermented products constitute an integral part of daily diet of Indonesian people. Among these, *ubi karet busuk* is the most famous traditional fermented cassava produced in Sumba Island, East Nusa Tenggara, Indonesia. The production of *ubi karet busuk* uses cassava as a substrate and involves solid-state fermentation with natural microorganisms, present on the cassava and surrounding environment, for two weeks. This product is sun-dried to ensure long storage life. It displays distinct physical characteristics, such as chewy texture, black color, and dry nature. The process of fermentation prevents post-harvest deterioration of the cassava (Kulla and Retnaningrum 2019).

Among the various microorganisms involved in the fermentation process of *ubi karet busuk*, lactic acid bacteria (LAB) is the most common natural microbe that plays an important role in the inhibition of growth for both spoilage and pathogenic microorganisms, and thus act as major players in preventing the deterioration of the cassava. During the fermentation process, LAB has been reported to produce organic acid and various antimicrobial compounds, including hydrogen peroxide, antibiotics, reuterin, and bacteriocins, that are known to exert inhibitory effect on both the spoilage and pathogenic bacteria (Delpech et al. 2015; Langa et al. 2018; Nurhikmayani et al. 2019; Sapalina and Retnaningrum 2020; Retnaningrum et al. 2020). In addition to this, certain enzymes are also released during lactic acid fermentation process. These enzymes hydrolyze the chemical compounds present in the cassava to generate products with desired texture and enhanced flavor, while reducing the unsatisfactory and anti-nutritional components such as cyanogen and tannin (Freire et al. 2015)

During the fermentation of cassava, several LAB strains have been reported to produce tannase and β-glucosidase that act on cyanogen and tannins, respectively, to reduce their concentration (Acebrón et al. 2017; Liu et al. 2018; Kanpiengjai et al. 2019; Shang et al. 2019). Tannins are polyphenolic compounds that are divided into two categories, hydrolyzable tannins (HT) and condensed tannins (CT). Generally, tannins are naturally occurring water-soluble polyphenols with molecular weights in the range of 500-3000 Da, which is largely dependent on the chemical bonds possessed by proteins and polysaccharides. The tannins present in the cassava are responsible for its unpleasant taste and astringent aroma. In comparison to this, cyanogens are the toxic compounds present in cassava that

induce serious side-effects in the body, including vomiting, nausea, dizziness, stomach pain, weakness, headache, diarrhea, and sometimes even death (Nambisan 2011).

Sumba Island is known to have a long dry season of eight months (April-November) and a very short rainy season of four months (December-March). During the dry season, crop failure owing to drought conditions poses a serious challenge. Processing of cassava by fermentation is widely practiced by the inhabitants of Sumba Island and the resulting product, *ubi karet busuk*, provides food security during dry season. Currently, no information is available regarding the diversity of LAB present in *ubi karet busuk*. Thus, the present study aimed to investigate the diversity of LAB that plays an important role in the fermentation of *ubi karet busuk*. In particular, the properties of lactic acid fermentation were characterized and the production of tannase and β-glucosidase was measured in LAB strains. The study results would aid in identifying the strains that could be used as a starter in the fermentation of cassava and other food sources like fruits and vegetables.

## MATERIALS AND METHODS

### Sample, medium, and chemicals

The samples for *ubi karet busuk* were collected from the inhabitants of East Nusa Tenggara of Sumba Island, who were actively involved in the production of fermented cassava. Medium of Man Rogosa Sharpe Agar (MRSA) and Man Rogosa Sharpe Broth (MRSB) were used for isolating and characterizing the LAB. The Quick-DNA™ Fungal/Bacterial Miniprep Kit, Zymo Research was used to extract genomic LAB. Para-nitrophenyl-β-D-glucopyranoside (*p*NPG) and methyl gallate were utilized as substrates in the enzyme reaction to determine the activity of β-glucosidase and tannase in LAB strain, respectively.

### Sampling and Isolation of LAB

Five samples of *ubi karet busuk* were collected during the fermentation process at pre-defined time intervals, particularly at 0, 2, 4, 6, and 14 days of incubation. Post sample collection, 10 g of the sample was suspended in 90 mL of sterile physiological solution, homogenized for 3 min, and serially diluted to obtain concentrations in the range of $10^{-5}$ to $10^{-8}$. Further, 0.1 mL of the diluted sample was inoculated onto Man Rogosa Sharpe Agar (MRSA) medium containing 1% (w/v) $CaCO_3$, using pour plate method. The plates were incubated at 37ºC for 48-96 hours. For each sample, colonies that formed clear zones were selected as LAB.

### Identification LAB strains based on phenotypic properties

In order to identify the phenotypic properties, the isolates were assessed for their morphological and biochemical traits. The morphological characteristics of LAB were evaluated on the basis of the morphology of the colonies as well as cells, which involved gram staining. Further, the biochemical characteristics of LAB were defined in terms of their motility and ability to produce catalase. Additionally, the fermentation profile of the isolates in Man Rogosa Sharpe Broth (MRSB) medium supplemented with various sugar sources such as glucose and pentose (xylose, arabinose and ribose) was also evaluated. The fermentation profile of these LAB strains was determined in terms of their fermentation products, like organic acids (lactic acid, acetic acid, propionic acid) and ethanol, which were analyzed using high-performance liquid chromatography (HPLC). The rise of the Durham tube carrying the $CO_2$ gas product towards the top of the culture was used to study the heterofermentative capabilities of the LAB strain in creating $CO_2$.

In order to monitor the fermentation profile of LAB strains, the bacterial isolates were first inoculated in MRSB medium supplemented with a variety of sugar sources for 48 hours to achieve desired cell density of $10^7$ CFU/mL. Following this, the LAB culture was heated to 80°C in a water bath, resulting in microorganisms and protein precipitation inactivation. The sample was centrifuged at 4000 rpm, 4°C for 10 min and 1.5 mL of the resulting supernatant was transferred to a fresh tube. The supernatant was further centrifuged at 20,800 × g for 3 min to obtain a clear solution. In order to detect the presence of lactic acid, acetic acid, propionic acid, and ethanol in the clear solution, HPLC analysis (Knauer) was performed on a Zorbax C-18 column (250 mm × 4.6 mm) with diode array detector at 210 nm and flow rate of 0.5 mL/min. The mobile phase used for HPLC separation comprised of 0.01 mol/L $KH_2PO_4$-$H_3PO_4$ (pH 2.6) and 3% methanol (v/v). To measure the production of organic acids, an external standard approach established by Vilanova and co-workers was used (Vilanova et al. 2014).

### Molecular identification of LAB strains using 16S rRNA gene

Genomic DNA of LABl strains was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit, Zymo Research, according to the manufacturer's instructions. Further, the extracted DNA was used to amplify 16S rRNA gene using universal 16S rRNA primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR amplification was performed in a thermal PCR cycler. The PCR products were separated on a 1% agarose gel by electrophoresis. The bands corresponding to PCR products were extracted, purified, and subjected to Sanger sequencing (Sanger et al. 1977). Further, the sequencing results were validated using Basic Local Alignment Search Tool for nucleotide (BLASTn) located on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). In addition to this, sequencing data were aligned using ClustalW software, and Kimura two-parameter (K2P) method was used to calculate the genetic differences (Kimura 1980). Maximum Likelihood method was used to establish phylogenetic relationships and a phylogenetic tree was generated. Bootstrap study involving 1000 resampling was used to estimate the confidence interval of tree topologies. MEGA X software was used for phylogenetic analysis (Kumar et al. 2018)

**Evaluation of β-glucosidase and tannase activity of LAB strains**

To evaluate β-glucosidase activity of LAB strains, para-nitrophenyl-β-D-glucopyranoside (*p*NPG) was used as substrate (Weagant et al. 2001). Each LAB strain was grown overnight in 250 mL MRSB medium and centrifuged at 6000 × g for 10 min at 4°C. The cell pellet was washed twice and re-suspended in 1 mL phosphate buffered saline (PBS). Further, β-glucosidase activity was determined by mixing 0.2 mL of the bacterial suspension containing $10^7$ CFU/mL and 0.4 mL of 5 mM *p*NPG. The sample was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 0.8 mL of 1 M $Na_2CO_3$ (Donkor and Shah 2008). The assay works on the principle that the presence of β-glucosidases in the sample catalyzes the conversion of *p*NPG to p-nitrophenol (*p*NP). Further, the amount of *p*NP released in the supernatant was measured at 405 nm using an ultraviolet-visible (UV-Vis) spectrophotometer. Here, one unit of enzyme activity was defined as the amount of β-glucosidase that produced 1 µmol of *p*NP per minute under the assay conditions (Otieno et al. 2006).

Next, the tannase activity of LAB strains was measured using methanolic rhodanine methodology previously described by Sharma et al. (2000). This method involves formation of a chromogen between gallic acid (released by the action of tannase on propyl gallate) and rhodanine (2-thio-4-ketothiazo-lidine). The assay was performed using bacterial culture containing $10^7$ CFU/mL. To prepare bacterial culture, one loop of LAB culture was taken on MRS agar plate, which was further transferred to 1 mL of substrate medium (pH 5.0) containing 33 mM $NaH_2PO_4$ and 20 mM methyl gallate. The substrate medium was further incubated at 37ºC for 24 h. Post incubation, the sample was alkalinized using an equal amount of saturated $NaHCO_3$ solution (pH 8.6), and it was exposed to the atmosphere for 1 h at room temperature. To prepare crude enzyme extract, LAB culture was centrifuged at 6000 × g for 10 min at 4°C. Further, the absorbance of the crude enzyme sample was measured using spectrophotometer at a wavelength of 530 nm (Mondal et al. 2001). One unit of tannase activity was defined as the amount of tannase that hydrolyzed 1 µmol of substrate tannic acid in one minute under the assay conditions.

## RESULTS AND DISCUSSION

**Isolation and phenotypic identification of LAB isolated from *ubi karet busuk***

During the fermentation process, a total of six *ubi karet busuk* samples were collected, at pre-defined time points covering the initial period (0 days), middle period (2, 4, and 6 days), and final period (14 days). The collected samples were processed and inoculated on MRSA medium supplemented with 1% (v/v) $CaCO_3$. After incubation for 48-96 h, 23 bacterial colony strains showing clear zones on MRS agar plates were selected and predicted as LAB. The results for the isolated strains are summarized in Table 1. In general, the formation of the clear zone on the agar plates is contributed by the production of organic acids by bacterial strains, which are endowed with the ability to dissolve $CaCO_3$ into Ca-lactate present in the medium. As shown in Table 2, all colony bacteria displayed the same shape, margins, and internal colony structure; however, they differed in color and colony elevation. Further evaluation of the biochemical characteristics resulted in the classification of 15 (out of 23) LAB strains as gram-positive, catalase-negative, and non-motile (Table 3). These results were in agreement with the findings of Axelsson (2004).

LAB strains were further classified as homofermentative and heterofermentative, based on the by-products of the fermentation process. In the present study, homofermentative trait was found to be predominating and ~93.33 % LAB strains isolated from indigenous cassava were associated with homofermentative trait, whereas only 6.73% LAB displayed heterofermentative character. Among the isolated strains, F2IS1, F2IS2, F4IS4, F4IS5, F6IS1, F6IS2, F6IS3, F6IS4, F6IS5, F14IS3, F14IS4, F14IS5, F14IS6, and F14IS7 strains showed homofermentative characteristic, while the heterofermentative trait was observed only in F14IS1 strain. All LAB strains could ferment glucose and xylose as a substrate, but showed no activity toward arabinose or ribose. Generally, homofermentative LAB strains catabolize glucose and pentose sugar via Embden-Meyerhof and pentose phosphate pathways to produce lactic acid as major product, more than 90% (Sauer et al. 2017; Hatti-Kaul et al. 2018). The results of the present study were in concordance with previous studies that reported several genera and species of homofermentative LAB, including *Lactococcus*, *Enterococcus*, *Pediococcus*, *Lactobacillus delbruckii*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Streptococcus salivarius* (Grewal and Khare 2018; Hassan et al. 2019). In comparison to this, LAB displaying heterofermentative traits catalyzes lactic acid production and several other products, including acetic acid, propionic acid, ethanol, and $CO_2$. In previous studies, LAB genera of *Oenococcus* and species of *Weissella confuse*, *Weissella viridescens*, *Lactobacillus brevis*, and *Lactobacillus fermentum* were categorized as heterofermentative (González-Arenzana et al. 2014; Fusco et al. 2015; Huang et al. 2018; Fabien et al. 2019; de la Fuente et al. 2021).

**Table 1.** Bacterial colony strains isolated during the fermentation of indigenous cassava obtained from Sumba, which formed clear zones on MRS agar plates

| LAB strain isolated during period of fermentation time (days) | | | |
|---|---|---|---|
| **2** | **4** | **6** | **14** |
| F2IS1 | F4IS1 | F6IS1 | F14IS1 |
| F2IS2 | F4IS2 | F6IS2 | F14IS2 |
| F2IS3 | F4IS3 | F6IS3 | F14IS3 |
| F2IS4 | F4IS4 | F6IS4 | F14IS4 |
| F2IS5 | F4IS5 | F6IS5 | F14IS5 |
| F2IS6 | | | F14IS6 |
| | | | F14IS7 |

**Table 2.** Morphological characteristics of LAB strains obtained during the fermentation of indigenous cassava obtained from Sumba, Indonesia

| Strain | Morphology of bacterial colonies | | | | | Morphology of bacterial cells | |
|---|---|---|---|---|---|---|---|
| | Shape | Color | Margin | Elevation | Internal structure | Shape | Gram staining |
| F2IS1 | Circular | White | Entire | Flat | Smooth | Coccus | + |
| F2IS2 | Circular | White | Entire | Flat | Smooth | Coccus | + |
| F4IS4 | Circular | Grayish | Entire | Convex | Smooth | Coccus | + |
| F4IS5 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F6IS1 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F6IS2 | Circular | White | Entire | Flat | Smooth | Coccus | + |
| F6IS3 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F6IS4 | Circular | White | Entire | Flat | Smooth | Coccus | + |
| F6IS5 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F14IS1 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F14IS3 | Circular | White | Entire | Plateau | Smooth | Coccus | + |
| F14IS4 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F14IS5 | Circular | White | Entire | Convex | Smooth | Coccus | + |
| F14IS6 | Circular | White | Entire | Flat | Smooth | Coccus | + |
| F14IS7 | Circular | White | Entire | Convex | Smooth | Coccus | + |

**Table 3.** Biochemical characteristics of LAB strains isolated during fermentation of indigenous cassava obtained from Sumba, Indonesia

| Strain | Catalase | Motility | Fermentation products of LAB strains (lactic acid, acetic acid, propionic acid, ethanol, $CO_2$) in various sugar (mg/mL) | | | | Fermentation type |
|---|---|---|---|---|---|---|---|
| | | | Glucose | Xylose | Arabinose | Ribose | |
| F2IS1 | − | Non-motile | LA (32) | LA(75) | − | − | Homofermentative |
| F2IS2 | − | Non-motile | LA (50) | LA(60) | − | − | Homofermentative |
| F4IS4 | − | Non-motile | LA (30) | LA(50) | − | − | Homofermentative |
| F4IS5 | − | Non-motile | LA (40) | LA(60) | − | − | Homofermentative |
| F6IS1 | − | Non-motile | LA (60) | LA(70) | − | − | Homofermentative |
| F6IS2 | − | Non-motile | LA (45) | LA(65) | − | − | Homofermentative |
| F6IS3 | − | Non-motile | LA (35) | LA(55) | − | − | Homofermentative |
| F6IS4 | − | Non-motile | LA (43) | LA(59) | − | − | Homofermentative |
| F6IS5 | − | Non-motile | LA (38) | LA(57) | − | − | Homofermentative |
| F14IS1 | − | Non-motile | LA (40); AA (20); Eth (15); G (+) | LA (40); AA (20); Eth (15); G (+) | − | − | Heterofermentative |
| F14IS3 | − | Non-motile | LA (80) | LA(75) | − | − | Homofermentative |
| F14IS4 | − | Non-motile | LA (48) | LA(50) | − | − | Homofermentative |
| F14IS5 | − | Non-motile | LA (58) | LA(60) | − | − | Homofermentative |
| F14IS6 | − | Non-motile | LA (52) | LA(65) | − | − | Homofermentative |
| F14IS7 | − | Non-motile | LA (48) | LA(57) | − | − | Homofermentative |

Note: +: positive result, ⁻: negative result, LA: lactic acid, AA: acetic acid, PA; propionic acid, Eth: ethanol, G: $CO_2$ gas

Lactic acid processing, especially by homofermentative LAB strains, finds wide applications in various industries, including food, beverage, cosmetic, pharmaceutical, and chemical industries. In addition to this, lactic acid production can be successfully carried out using a variety of inexpensive and abundant substrates, including food waste, starchy biomass and sugar plant wastes, dairy waste, and industrial waste (Sauer et al. 2017; Baruah et al. 2018; de Oliveira et al. 2018; Hatti-Kaul et al. 2018; Lopez-Gomez et al. 2019).

**Molecular identification of LAB isolated from *ubi karet busuk* using amplification of 16S rRNA gene**

To further confirm the results of phenotypic analysis, molecular identification of 15 LAB strains was performed using PCR amplification of 16S rRNA gene. The 16S rRNA gene was amplified using universal primers 27F and 1492R. As shown in Figure 1, all bacterial isolates displayed strong amplification results and PCR products with size ~1500 bp were detected.

Nucleotide sequencing data for 16S rRNA gene is the most suitable method for identifying microorganisms and establishing phylogenetic relationships among all microorganisms present on earth. Furthermore, the use of 16S rRNA gene for identification purposes ensures accuracy due to certain unique characteristics. Importantly, this gene can be easily found in all organisms and its genetic sequences are moderately conserved, with conserved, variable, and hyper-variable regions. Besides this, 16S rRNA gene is only 1500 bp in length, making it

relatively simple to sequence and large enough to provide significant information required for the identification and phylogenetic analysis (Clarridge 2004).

The 16S rRNA gene sequences, obtained using Sanger sequencing, were compared with database at NCBI using Basic Local Alignment Search Tool Nucleotide (BLASTn, www.ncbi.nlm.nih.gov). According to BLASTn results, significant similarity was observed between both databases, with similarity values in the range of 96.21-99.79 % (Table 4). In accordance with findings of Drancourt et al. (2000), the bacteria were classified into the same genus when similarity values were > 99%. For similarity values < 97 %, the bacteria were assigned into the same genus. As shown in Table 2, the identification process classified 15 LAB strains into six species, including *Enterococcus faecium, E. faecalis*, *E. casseliflavus*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Enterococcus* sp. Among 15 LAB strains, F2IS1 strain was identified as *E. faecium*, while both F2IS2 and F6IS1 strains were identified as *E. faecalis*. LAB strains F4IS4, F4IS5, F6IS4, F6IS5, F14IS5, and F14IS6 could be classified as *E. casseliflavus*. Strains F6IS2, F6IS3, F14IS4, and F14IS7 belonged to *Enterococcus* sp. Further, the strains F14IS1 and F14IS3 were identified as *L. mesenteroides* and *L. lactis*, respectively.

Evaluation of diversity of LAB species isolated from the samples collected during the production of *ubi karet busuk* showed the dominance of *E. casseliflavus*. Among the isolated strains, 40% of strains belonged to *E. casseliflavus*, followed by *Enterococcus* sp. (26.67 %) and *E. faecalis* (13.33%). In comparison to these, very few LAB strains (6.67%) were categorized as *E. faecium, L. mesenteroides* and *L. lactis*. These results were quite contrasting as compared to previous reports. In a previous study, *E. casseliflavus* was isolated from the intestinal tract of healthy rainbow trout (*Oncorhynchus mykiss*). *E. casseliflavus* was reported to display probiotic properties, such that the administration of *E. casseliflavus* to fish improved their resistance against *Streptococcus iniae* via immunomodulatory mechanisms. The study reported a significant increase in serum levels of lysozyme and IgM upon *E. casseliflavus* administration (Safari et al. 2016). Several other studies reported isolation of *E. casseliflavus* from a variety of food sources, including curd and sausage samples. These studies reported the production of bacteriocins by this strain. Use of *E. casseliflavus* inhibited the growth of *Listeria monocytogenes* and *Pseudomonas aeruginosa*, highlighting its suitability to be used as a food bio-preservative (Esposti et al. 2018; Indira et al. 2018). In a recent study, Ng et al. (2020) provided evidence for the isolation of *E. faecium* strains from a variety of food products and organic waste. These results further suggested that this bacterial strain might aid in the development of antimicrobials to inhibit the growth of *Streptococcus mutans* and *Candida albicans*.

Additionally, several other LAB species, including *E. faecalis, L. mesenteroides*, and *L. lactis* have been successfully utilized in the fermentation of milk to produce functional drinks containing bioactive and antimicrobial compounds (Borges et al. 2019; Graham et al. 2019; Bragason et al. 2020). Use of *L. mesenteroides* during fermentation of milk resulted in the production of a functional drink endowed with antimicrobial activity against *Listeria monocytogenes*. This functional drink also contained bioactive compounds in the form of conjugated linoleic acid (CLA), which were found to be useful in preventing and inhibiting various cardiovascular diseases (Borges et al. 2019). In a recent study, Graham et al. (2019) discovered that *E. faecalis* could ferment milk, resulting in products rich in phenolic compounds. These products displayed antioxidant activity and inhibited enzymatic activity of angiotensin-converting enzyme (ACE) and α-glucosidase. Interestingly, both these enzymes are known to be associated with hypertension and hyperglycemia. In another study, Bragason et al. (2020) found that *L. lactis* could also catalyze the fermentation of milk, and the resulting products inhibited the growth of three pathogenic bacteria, namely *Salmonella typhimurium*, *Klebsiella pneumonia*, and *Escherichia coli* O157:H7.

In the present study, phylogenetic analysis was performed using the maximum likelihood method based on 16S rRNA gene sequences of bacteria. This method allows precise determination of the evolutionary history and relationships among bacterial species (Woese 1987). Following this, the sequencing data for each LAB strain was constructed using MEGA X software, while ClustalW was used for sequence alignment. ClustalW alignment is basically aimed at arranging each sequence in a homologous position. Gregory et al. (2008) previously proposed that the selection of one or more outgroups by the distance method could possibly help in localizing/identifying the root of the phylogenetic tree. In the present analysis, *Escherichia coli* U 5/41 strain was selected and used as an outgroup.

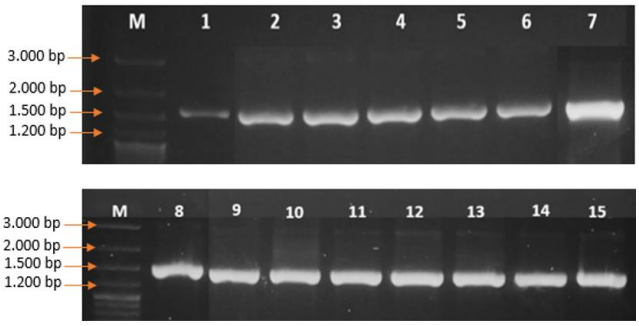


**Figure 1**. Results for PCR amplification of LAB strains isolated from indigenous cassava, performed using primers 27F and 1492R. M: Marker DNA 100-300 bp; 1: F14IS1 strain, 2: F14IS3 strain, 3: F14IS4 strain; 4: F14IS5 strain; 5: F14IS6 strain; 6: F14IS7 strain; 7: F4IS4 strain; 8: F2IS2 strain; 9: F6IS2 strain; 10: F6IS3 strain; 11: F6IS4 strain; 12: F6IS5 strain; 13: F2IS1 strain; 14: F4IS5 strain; and 15: F6IS1 strain.

**Table 4.** BLASTn results for LAB strains isolated from *ubi karet busuk*

| Strain | Species of LAB homolog | Identity (%) | Accession number | Isolate sources |
|---|---|---|---|---|
| F2IS1 | *Enterococcus faecium* strain DSM 20477 | 99.58 | NR_114742.1 | Culture collection |
| | *Enterococcus faecium* strain NBRC 100486 | 99.51 | NR_113904.1 | Culture collection |
| | *Enterococcus faecium* strain ATCC 19434 | 99.51 | NR_115764.1 | Culture collection |
| F2IS2 | *Enterococcus faecalis* strain NBRC 10048 | 99.65 | NR_113901.1 | Culture collection |
| | *Enterococcus faecalis* strain ATCC 19433 | 99.59 | NR_115765.1 | Culture collection |
| | *Enterococcus faecalis* strain LMG 7937 | 99.38 | NR_114782.1 | Culture collection |
| F4IS4 | *Enterococcus casseliflavus* strain NBRC 100478 | 99.72 | NR_104560.1 | Plant material |
| | *Enterococcus casseliflavus* strain NCIMB 11449 | 99.72 | NR_119280.1 | Culture collection |
| | *Enterococcus gallinarum* strain LMG 13129 | 99.52 | NR_104559.2 | Chicken intestine |
| F4IS5 | *Enterococcus casseliflavus* strain NBRC 100478 | 99.72 | NR_104560.1 | Plant material |
| | *Enterococcus casseliflavus* strain NCIMB 11449 | 99.72 | NR_119280.1 | Culture collection |
| | *Enterococcus gallinarum* strain LMG 13129 | 99.52 | NR_104559.2 | Chicken intestine |
| F6IS1 | *Enterococcus faecalis* strain NBRC 100480 | 99.50 | NR_113901.1 | Culture collection |
| | *Enterococcus faecalis* strain ATCC 19433 | 99.45 | NR_115765.1 | Culture collection |
| | *Enterococcus faecalis* strain LMG 7937 | 99.24 | NR_114782.1 | Culture collection |
| F6IS2 | *Enterococcus casseliflavus* strain NCIMB 11449 | 96.32 | NR_119280.1 | Culture collection |
| | *Enterococcus casseliflavus* strain NBRC 100478 | 96.25 | NR_104560.1 | Plant material |
| | *Enterococcus gallinarum* strain LMG 13129 | 96.21 | NR_104559.2 | Chicken intestine |
| F6IS3 | *Enterococcus casseliflavus* strain NBRC 100478 | 98.93 | NR_104560.1 | Plant material |
| | *Enterococcus casseliflavus* strain NCIMB 11449 | 98.93 | NR_119280.1 | Culture collection |
| | *Enterococcus gallinarum* strain LMG 13129 | 98.68 | NR_104559.2 | Chicken intestine |
| F6IS4 | *Enterococcus casseliflavus* strain NBRC 100478 | 98.31 | NR_104560.1 | Plant material |
| | *Enterococcus casseliflavus* strain NCIMB 11449 | 98.31 | NR_119280.1 | Culture collection |
| | *Enterococcus gallinarum* strain LMG 13129 | 98.01 | NR_104559.2 | Chicken intestine |
| F6IS5 | *Enterococcus casseliflavus* strain NBRC 100478 | 98.57 | NR_104560.1 | Plant material |
| | *Enterococcus casseliflavus* strain NCIMB 11449 | 98.57 | NR_119280.1 | Culture collection |
| | *Enterococcus gallinarum* strain LMG 13129 | 98.33 | NR_104559.2 | Chicken intestine |
| F14IS1 | *Leuconostoc mesenteroides* strain ATCC 8293 | 99.59 | NR_074957.1 | Fermented olives |
| | *Leuconostoc mesenteroides* subsp. dextranicum strain NBRC 100495 | 99.59 | NR_113911.1 | Culture collection |
| | *Leuconostoc mesenteroides* strain JCM 6124 | 99.59 | NR_113251.1 | Fermenting olives |
| F14IS3 | *Lactococcus lactis* Strain NBRC 100933 | 99.72 | NR_113960.1 | Culture collection |
| | *Lactococcus lactis* Strain NCDO 604 | 99.72 | NR_040955.1 | Culture collection |
| | *Lactococcus lactis* subsp. hordniae strain NBRC 100931 | 99.59 | NR_113958.1 | Leafhopper, Hordnia circellata |
| F14IS4 | *Enterococcus casseliflavus* strain NCIMB 11449 | 97.18 | NR_119280.1 | Culture collection |
| | *Enterococcus casseliflavus* strain NBRC 100478 | 97.11 | NR_104560.1 | Plant material |
| | *Enterococcus gallinarum* strain LMG 13129 | 96.98 | NR_104559.2 | Chicken intestine |
| F14IS5 | *Enterococcus casseliflavus* strain NBRC 100478 | 99.79 | NR_104560.1 | Plant material |
| | *Enterococcus casseliflavus* strain NCIMB 11449 | 99.79 | NR_119280.1 | Culture collection |
| | *Enterococcus gallinarum* strain LMG 13129 | 99.59 | NR_104559.2 | Chicken intestine |
| F14IS6 | *Enterococcus casseliflavus* strain NCIMB 11449 | 99.72 | NR_119280.1 | Culture collection |
| | *Enterococcus casseliflavus* strain NBRC 100478 | 99.65 | NR_104560.1 | Plant material |
| | *Enterococcus gallinarum* strain LMG 13129 | 99.52 | NR_104559.2 | Chicken intestine |
| F14IS7 | *Enterococcus casseliflavus* strain NCIMB 11449 | 97.37 | NR_119280.1 | Culture collection |
| | *Enterococcus casseliflavus* strain NBRC 100478 | 97.30 | NR_104560.1 | Plant material |
| | *Enterococcus gallinarum* strain LMG 13129 | 97.16 | NR_104559.2 | Chicken intestine |

Further, Tamura-Nei + Gamma (TN93+G) model was chosen as the optimal phylogenetic parameter for transition rates among sites, based on the Bayesian information criterion. In particular, TN93 + G model was chosen owing to the lowest values for Bayesian information criterion (BIC) and Akaike information criterion (AICc) (Nishimaki and Sato 2019). This Maximum likelihood analysis was performed on the basis of the nucleotide substitution model, involving partial elimination of gaps or missing data.

Therefore, bootstrap analysis of 1000 replicates was performed to evaluate the phylogenetic tree topology. The use of bootstrap was particularly critical as it measured the reliability of phylogeny tree, resulting in trees with variable truth support. According to Holmes et al., a very similar sequence could be placed as a neighbor outside of the branches and connected in a common branch (Holmes 2003). As shown in Figure 2, 15 LAB strains isolated from the indigenous cassava were clustered into five clades of *E. faecium*, *E. faecalis*, *E. casseliflavus*, *L. lactis*, and *L. mesenteroides* with bootstrap values > 70%.

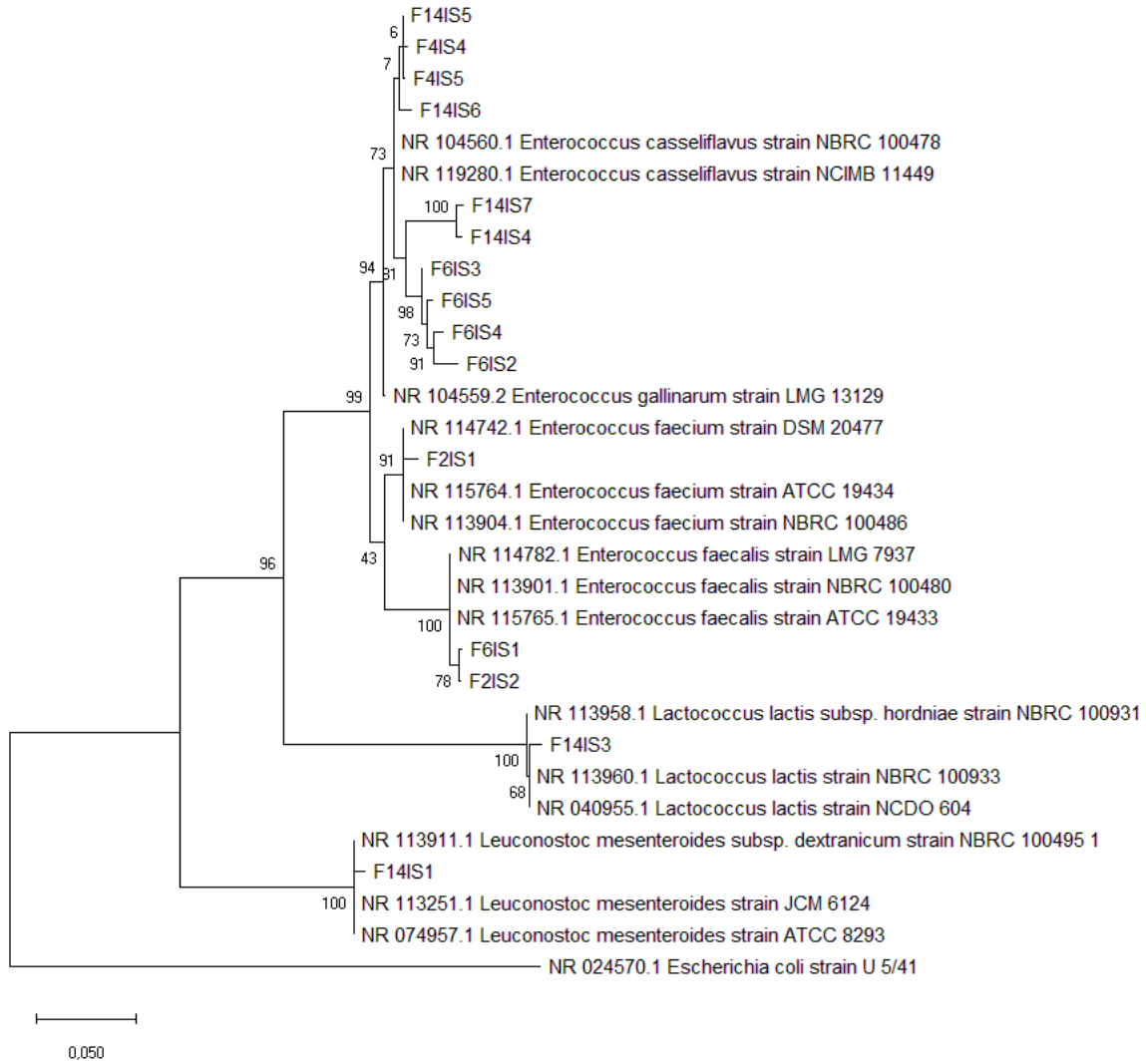


**Figure 2.** Phylogenetic tree showing the relationship between 15 LAB strains and 16S rRNA gene sequences obtained from NCBI database using Maximum Likelihood analysis with bootstrap of 1000 replication

In 91% of the bootstrap replications, F2IS1 strain was located in the same cluster and appeared as a descendant to *E. faecium* strain DSM 20477, *E. faecium* strain NBRC 100486, and *E. faecium* strain ATCC 19434. The results of the analysis were in accordance with the BLASTn results, which showed that the F2S1 strain was characterized by an identity score of 99.51-99.58 with *E. faecium* strain. In comparison to this, F2IS2 and F6IS1 strains clustered with *E. faecalis* strain LMG 7937, *E. faecalis* strain NBRC 100480, and *E. faecalis* strain ATCC 19433, with bootstrap value of 100%. These findings were consistent with the results of BLASTn analysis, where these strains showed identity scores in the range of 99.65-99-99.24 with *E. faecalis*. LAB strains including F4IS4, F4IS5, F6IS2, F6IS3, F6IS4, F6IS5, F14IS4, F14IS5, F14IS6, and F14IS7 were located as the closest neighbors to *E. casseliflavus* strain NBRC 100478 and *E. casseliflavus* strain NCIMB 11449, with bootstrap value of 73. The results of this phylogenetic analysis were found to be in concordance with BLASTn results, where the strains displayed identity score values in the range of 96-99% with *E. casseliflavus*. The LAB strains F14IS5 and F14IS6 constituted one clade and showed identity value of 99% with *E. casseliflavus* strains. Similarly, LAB strains F6IS3, F6IS4, and F6IS5 formed one clade and were characterized by an identity value of 98% with *E. casseliflavus* strains. Further, F14IS4 and F14IS7 strains produced one clade and showed identity value of 97% with *E. casseliflavus* strain. In comparison to these, F6IS2 strain showed lowest identity value of 96% with *E. casseliflavus* strain.

The results of the analysis showed that F14IS3 strain clearly clustered within the clade of *L. lactis* subsp. *hordniae* strain NBRC 100931, *L. lactis* strain NBRC 100933, and *L. lactis* strain NCDO 604, with a bootstrap value of 100%. In comparison to this, F14IS1 strain clustered with *L. mesenteroides* subps. Dextranicum strain NBRC 100495, *L. mesenteroides* strain JCM 6124, and *L. mesenteroides* strain ATCC 8293, with a bootstrap value of 100%. The phylogenetic analysis for F14IS3 and F14IS1 strains was found to be in concordance with the findings of BLASTn, where identity scores of 99.72% and 99.59% were recorded with *L. lactis* and *L. mesenteroides*, respectively.

**Table 5**. Comparison of β-glucosidase and tannase activity of LAB strains isolated during fermentation of indigenous cassava produced in Sumba, Indonesia

| Strain | Species | Enzyme activity (U/mL) | |
|---|---|---|---|
| | | β-glucosidase | Tannase |
| F2IS1 | *Enterococcus faecium* | 6.23 | – |
| F2IS2 | *Enterococcus faecalis* | 5.23 | 35.45 |
| F4IS4 | *Enterococcus casseliflavus* | – | – |
| F4IS5 | *Enterococcus casseliflavus* | 4.29 | – |
| F6IS1 | *Enterococcus faecalis* | 5.12 | 38.23 |
| F6IS2 | *Enterococcus* sp. | – | 25.79 |
| F6IS3 | *Enterococcus* sp. | 4.33 | – |
| F6IS4 | *Enterococcus casseliflavus* | 4.35 | – |
| F6IS5 | *Enterococcus casseliflavus* | – | – |
| F14IS1 | *Leuconostoc mesenteroides* | 7.82 | – |
| F14IS3 | *Lactococcus lactis* | 7.13 | – |
| F14IS4 | *Enterococcus* sp. | – | – |
| F14IS5 | *Enterococcus casseliflavus* | 4.31 | – |
| F14IS6 | *Enterococcus casseliflavus* | 4.15 | – |
| F14IS7 | *Enterococcus* sp. | – | – |

**β-glucosidase and tannase activity of LAB strains**

The presence of β-glucosidase and tannase activity was evaluated in 15 LAB strains isolated from *ubi karet busuk*. The results for the enzymatic activity are summarized in Table 5. Among these, 10 LAB strains, including *E. faecium* F2IS1, *E. faecalis* F2IS2, *E. casseliflavus* F4IS5, *E. faecalis* F6IS1, *Enterococcus* sp. F6IS3, *E. casseliflavus* F6IS4, *L. mesenteroides* F14IS1, *L. lactis* F14IS3, *E. casseliflavus* F14IS5, and *E. casseliflavus* F14IS6 showed β-glucosidase activity in the range of 7.13-4.15 U/mL. In particular, *L. lactis* F14IS3 displayed highest β-glucosidase activity, while *E. casseliflavus* F14IS6 showed lowest β-glucosidase activity. Additionally, *E. faecalis* F2IS2 and *E. faecalis* F6IS1 also produced tannase, with enzyme activity of 34.45 and 38.23 U/mL, respectively. In comparison to these, *Enterococcus* sp. F6IS2H exclusively produced tannase, with enzyme activity of 25.79 U/mL. Previous studies reported β-glucosidase activity of 24.5, 3.96, 6.72, and 2.7 U/mL for LAB species *Lactobacillus* sp., *L. acidophilus*, *L. rhamnosus*, and *L. mesenteroides*, respectively (Strahsburger et al. 2017; Liu et al. 2018; Zhu et al. 2019). In comparison to this, tannase activity of 6.26, 32.58, and 4.2 U/mL have been previously reported for LAB species *L. plantarum* isolated from pickled cabbage, *E. faecalis* isolated from goat feces, and *E. cloacae* isolated from gut of mole crickets (*Gryllotalpa krishnani*), respectively (Rodríguez et al., 2008; Goel et al. 2011; Govindarajan et al. 2019). All these findings suggested the suitability of β-glucosidase and tannase producing LAB strains to be utilized as a starter in the fermentation of cassava and other substrates such as vegetables, fruits, and legumes (Sáez et al. 2017; Li et al. 2018; Liu et al. 2018; Zhu et al. 2019). Furthermore, these two enzymes find wide applications in food, beverage, and animal feed industries. In particular, these enzymes can be utilized in the clarification and de-bittering of refreshing drinks, removal of anti-nutritional factors (ANF), and production of bioactive metabolites in animal feed and food industries (Yao et al. 2014; Aharwar and Parihar 2018).

In the present study, 15 LAB strains were isolated during the fermentation of *ubi karet busuk*, the most famous traditional fermented cassava produced in Sumba Island, East Nusa Tenggara. Homofermentative trait was found to be predominating among these LAB strains, where 93.33% strains displayed homofermentative trait, while 6.73% strains were associated with heterofermentative characteristic. According to BLASTn and phylogenetic analysis, these LAB strains were classified into six species, namely *E. faecium*, *E. faecalis*, *E. casseliflavus*, *L. mesenteroides*, *L. lactis*, and *Enterococcus* sp. In particular, 40% of these LAB strains were characterized as *E. casseliflavus*, followed by 26.67 % identified as *Enterococcus* sp., and 13.33% as *E. faecalis*. In comparison to these, only 6.67% of strains were classified as *E. faecium*, *L. mesenteroides*, and *L. lactis*. Interestingly, β-glucosidase activity was detected in 10 LAB strains, with *L. lactis* F14IS3 displaying highest β-glucosidase activity of 7.13 U/mL. Among these 10 LAB strains, F2IS2 and F6IS1 strains also expressed tannase, with F6IS1 displaying highest enzyme activity of 38.23 U/mL. Thus, the present study results provided evidence for the production of β-glucosidase and tannase by the LAB strains isolated from *ubi karet busuk*, highlighting their suitability to be utilized as a starter in the fermentation of cassava and other substrates like vegetables, fruits, and legumes. These strains can also be utilized in various processes in the animal feed, food, and beverage industries.

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