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Molecular identification 16S rRNA gene of active proteolytic lactic acid bacteria (LAB) isolated from kelengkeng (*Dimocarpus longan*) fruit

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Abstract. *Hidayat H, Haryadi W, Matsjeh S, Raharjo TJ. 2019. Molecular identification 16S rRNA gene of active proteolytic lactic acid bacteria (LAB) isolated from kelengkeng (*Dimocarpus longan*) fruit. Biodiversitas 20: 2222-2228.* Various fermentation food involves microorganisms, especially Lactic Acid Bacteria (LAB) which has beneficial properties for fermented food. The present study aims to do identification and characterization of active proteolytic LAB from kelengkeng *(Dimocarpus longan)*, a commonly found fruit in South East Asia. From ten LAB isolates, isolates K7 and K8 appeared as basil shape based on Gram staining and were chosen for further examination. The molecular identification of these selected isolates were investigated from the 16S rRNA analysis using universal primer 27F and 1525R. The K7 and K8 isolates showed resistance ability towards acidic environment (pH 2.0) and were examined for protease enzyme screening resulting 7.5 and 6 to clear zone, which was 17 and 12 mm. The result of the molecular characterization from the two isolates indicated that isolate K7 was identified as *Leuconostoc mesenteroides subsp suionicum strain* LT-38 while isolate K8 was identified as *Leuconostoc mesenteroides strain* C305.16.

Keywords: Active proteolytic, Lactic Acid Bacteria, *kelengkeng,* 16S rRNA gene

Abbreviations : LAB : lactic acid bacteria, PCR: polymerase chain reaction, NCBI : national center for biotechnology information, 16S rRNA: 16 svenberg ribosome-ribonucleic acid, PH: power of hydrogen, DNA: deoxyribonucleic acid, BLAST: basic local alignment search tool, BPB: Bromo Phenol Blue, MRS: de man, rogosa and sharpe, UV: ultraviolet, GF/C: glass fiber/ circle, TCA: tricarboxylic acid, SDS: sodium dodecyl sulfate, rpm: rotary per minute.

INTRODUCTION

Fermentation is a process of energy production in cells with the anaerobic condition. The process of fermentation technology can utilize microbes to change and increase the value of the raw material to a useful product. Fermented products can inhibit the growth of microorganisms (Yin et al. 2002). Various fermentation food involves microorganisms, especially Lactic Acid Bacteria (LAB). LAB is categorized as safe microorganism for food consumption because it has properties as food-grade microorganism. LAB can convert sugar to lactic acid that causing pH decrease and creating an acidic condition which can inhibit microbial growth (Candra et al. 2007). LAB produces some organic compounds such as organic acids, ethanol, hydroperoxide, and bacteriocin (Surono 2004). Generally, the microbes involved in the process, including LAB involved in fermentation include the genius genus *Lactobacillus, Lactococcus, Pediococcus,* and *Leuconostoc* (Soeharsono 2010). Biochemical test for LAB identification includes Gram staining, catalase test, gas production test from the glucose of which to determine the fermented and morphological cell (Yusmarini et al. 2016).

During the fermentation process of LAB, various kinds of the enzyme were produced to degrade the substrate, one of them is proteolytic enzymes. The presence of enzymes during the fermentation process will change the taste, texture, aroma, and will increase the nutritional value (Jamsari et al. 2013). The proteolytic enzyme help to hydrolyze the peptide bonds in protein to produces some of the simple compounds such as small peptides and amino acids (Kwok 1994). The results of the research above showed that LAB is a good proteolytic enzyme producer (Wikandari et al. 2012). Not all food nutrients can be properly absorbed in the intestinal wall. Therefore, the nutrients obtained from fruits are essential and highly needed (Nyanga et al. 2007). The acid fermentation of fruit involves two major classes, natural microflora like yeast and fermenting bacteria (indigenous) of mature fruits. Indigenous bacteria can be obtained from isolating certain bacteria on a material (Labeda 1990), for example, is ripe tropical fruit. During the fermentation process, fruits with high carbohydrate content are hydrolyzed into reducing sugar (Azizah 2013).

In our previous study, LAB was isolated from fresh vegetables and fruit and tested for potential bioprotective agents against food-borne human bacterial pathogens for Golden Delicious apples and Iceberg lettuce (Rosalia et al. 2008). LAB have been extensively studied for their commercial value, food preservation, and health benefits. The industrial importance of LAB is based on their ability to ferment sugars readily into different metabolites and

provide an effective method for preserving fermented food products (Emerenini et al. 2013).

The identification of LAB based on carbohydrate fermentation patterns is unreliable and not accurate enough to distinguish closely related strains due to their similar nutritional requirements. Sequencing analysis of the 16S rRNA genes has been used to determine the diversity and dynamics of LAB in food (Nurmalinda et al. 2013). LAB is a small part of the autochthonous microbiota of vegetables and fruits. The diversity of the microbiota markedly depends on the intrinsic and extrinsic parameters of the plant matrix. Notwithstanding, the importance of spontaneous fermentation is to stabilize and preserve raw vegetables and fruits (Abu Saif 2016). The high biodiversity of the LAB gives great opportunities to get microorganism that can serve as an enzyme producer (Susilo 2017).

Furthermore, LAB could have a good influence on kelengkeng *(Dimocarpus longan)* flesh. Research on LAB as a producer of protease enzymes has been done on various foodstuffs (Triana et al. 2016). Up till now, research on molecular identification of proteolytic activity of LAB from kelengkeng *(Dimocarpus longan)* fruit is not yet reported. This paper, therefore, reports the molecular identification LAB isolated from kelengkeng fruit and further applied to get their active proteolytic activity.

The present study aims to identify, and characterization of active proteolytic LAB using 16S rRNA gene after that the bacteria gene obtained from kelengkeng *(Dimocarpus longan)* fruit were will be further sequenced.

Procedures

The flesh of kelengkeng *(Dimocarpus longan)* obtained from the traditional market, Yogyakarta.

Isolation of LAB from kelengkeng

The kelengkeng fruit fermentation process was done by peeling two pieces of riped kelengkeng fruit and placed on a sterilized banana leaf. It was heated sealed and allowed to stand for 36 hours and 48 hours at room temperature. The kelengkeng flesh as much as 1.0 g was taken and was mixed with 9 mL MRS Broth (Merck), then homogenized to obtain a multilevel dilution then incubated at 37 ºC for 24 hours. It was agar medium and incubated at 37 ºC for 24 hours. The bacteria colonies were purified identified macroscopically and microscopically. The macroscopic observation includes measuring size, form, elevation, edge shape, and color. Microscopic observation was conducted with Gram staining and then continued using a microscope with 40x magnification. □

Acid resistance test

The isolates obtained were subcultured into MRS broth supplemented HCl 0.1 M at different pH 2.0; 3.0; 4.0; 5.0 and 6.0 and incubated overnight at 37 ºC. The turbidity level of the overnight inoculated broth was observed at each pH. The isolate that resists to grown at pH 2.0 which contains LAB were then taken and grown into a liquid medium MRS broth, overnight to be tested for antimicrobial activity.

Antimicrobial activity test

The isolate bacteria at pH 2.0 were subcultured in MRS broth and incubated overnight at temperature 37 ºC. The filter paper used as a disc to isolate sterilized using an autoclave. 2 mm filter paper was used for the antimicrobial activity. The Perforator tool was also used for the test disc. *E. coli* was used as a test bacteria, and it was subcultured in MRS broth. After overnight incubation at 37 ºC, antimicrobial activity with using disc method. 20 mL Mueller Hilton (Merck) agar was prepared and smeared with *E.coli* by spreading in all parts of the medium using sterile cotton and then placed the filter paper on one side of the solid medium. As many as three drops of the LAB was dropped on a filter paper already put on one side of the solid medium and the other side of the antibiotic. The Antibiotics used are Erythromycin and Ampicillin. The antibiotics were used as a control, and it was Incubated at 37ºC for three days. Then observed and measured the diameter of the clear zone formed (Triana et al. 2006).

Screening for proteolytic enzyme

The pure LAB was screened for proteolytic enzymes using a skimmed milk substrate (Rosiana 2009). As many as 20 mL, MRS agar supplemented with 1% skim milk was transferred into a petri dish and allowed to solidify, after that as many as three drops of the isolates were placed on one side of the agar which was then incubated at 37ºC for three days. The observation was made, and the size of the cleared zone was recorded (Triana et al. 2006)

Determining the protein level of proteolytic enzyme □

The inoculum was prepared by adding one osse needle isolate at 50 mL Nutrient broth and incubated in incubator shaker with an agitation speed of 120 rpm at 37 °C for 24 hours. The inoculum is then inoculated on Horikoshi production media in such a way until the final total OD in the production medium becomes 0.13/mL. The Horikoshi medium containing 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.02% $MgSO_4$, and 1% Na_2CO_3. Na_2CO_3 sterile was added to the medium after the sterilization process. Incubation is done in the incubator shaker with an agitation speed of 150 rpm at 37 °C with the variation of production time 6; 12; 18; 24; 30; and 36 hours (Rani et al. 2015). The proteolytic enzyme present in the production medium is separated from the isolated cell by centrifugation in cold conditions with a speed of 9500 rpm for 10 mins. Before centrifugation, the culture medium containing the enzyme was cooled at 10 ºC for about 1 hour. Supernatant filtered with glass fiber filter (Whatman GF/C) and sterilized with Corning sterile syringe filter 0.45µm. If the enzyme is not directly used for the analysis of enzyme activity, then added NaN_3 to a concentration of 1 mM solution into each supernatant solution

The crude extract enzyme was determined for proteolytic activity. As much as 1 mL crude enzyme extract was added to the casein substrate 0.65% (0.65 g casein into 100 mL buffer K- phosphate 0.05 M pH 7.5). The reaction mixture was incubated at 37 °C for 10 minutes. The termination of the reaction was done through addition of 5 mL reagent TCA 110 mM, and it was incubated more at 37

°C for 30 minutes. As much as 2 mL filtrate is separated with centrifugation at the speed of 10000 rpm for 10 minutes. After that, as much as 5 mL Na_2CO_3 and 1 mL reagent Folin Ciocalteau added to the filtrate and incubated at 37 °C for 30 minutes. Mixed absorbance was measured using a spectrophotometer at a wavelength of 578 nm. One unit of enzyme activity is defined as the number of enzymes needed to release 1µmol tyrosine on the casein substrate per minute. The protein content in the crude enzyme extract was determined using the Lowry method by bovine serum albumin as a standard.

DNA isolation

The LAB isolate that has been cultured in a nutrient broth liquid medium is then inserted into the tube Eppendrof I mL sterile and centrifuged at a speed of 14000 rpm for 3 minutes. The supernatant was discarded, and the cell pellet obtained was suspended again taken 500 mL of Ix TE and then added as many as 40 mL of 10% SDS (50 mL dH_20 and 50 mL of 20% SDS) and 5 mL proteinase-K was added, and vortex. Incubated at 37 °C for 60 minutes. Next, it was added PC (Phenol: Chloroform) 500 µl centrifuged for 3 minutes with speed of 14000 rpm and then, it was Discarded pellet and supernatant transferred to a new tube then added 50 µl sodium acetate 3 M, addition is 300 µl isopropanol cold. After that, it was centrifuged for 3 minutes at a speed of 14000 rpm. So that, DNA will precipitate. Next, it was discarded supernatant and Eppendorf dried. DNA pellet obtained washed with 1 ml ethanol 70% for 30 seconds. Next, it was discarded supernatant and DNA pellet dried with tube position inverted. After that, the DNA pellet suspended with 25 µL I x TE and incubated at 37 °C for 15 minutes that dissolves perfect and then saved at -20 °C. And then, gel electrophoresis is performed.

Polymerase Chain Reaction amplification

In the present study, bacterial universal 16S rRNA primers 27F (5'-AGAGTTTGAATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3') (Habibi 2017) were used for identification of isolates at the species level. The total 25 µL PCR mix consisting of IlustraTM ready to Go PCR bead (GE Healthcare), 2 µL bacterium DNA, 2 µL forward dan reverse respectively with concentration 10 pmol, and 19 µL NFW. After that, PCR mix put in PCR thermal cycler with the condition were as follows: before denaturation at 95 °C for 5 min, followed by 35 cycles, consist of denaturation step for 1 min at 95 °C, annealing step for 1,5 min 50 °C, elongation step for 1 min at 72 °C, and final elongation for 10 min at 72 °C. DNA fragment from amplification product analyzed on the electrophoresis gel agarose with a concentration of 2.5% at 100 Volt for 40 min. The PCR products were sequenced, the sequence was analyzed using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Multiple-Alignment Analysis which is accessed by internet with entering the sequence of nitrogen bases that have been obtained from sequencing process. The result from analyzing sequences showing kind of species, but the top is target species with identification percentage similarity highest.

RESULTS AND DISCUSSION

Isolation of LAB from kelengkeng

The isolation of LAB from kelengkeng was obtained as many as 10 LAB, namely K1-K10. All obtained isolates were calculated the number of the colonies using a colony counter, the result calculation of the number of the colony for each isolate. The result of fermentation has been dilution 10^{-7}, from the ten isolates of bacterial colonies and obtained number of colony for each sample were K1 of 54.10^7 CFU/ mL, K2 of 46.10^7 CFU/mL, K3 of 34.10^7 CFU/ mL, K4 of 68.10^7 CFU/ mL, K5 of 77.10^7 CFU/ mL, K6 of 45.10^7 CFU/ mL, K7 of 133.10^7 CFU/ mL, K8 of 87.10^7 CFU/ mL, K9 of 37.10^7 CFU/ mL and K10 of 63.10^7 CFU/ mL. The total number of these colony for each isolates were included as probiotics for food because according to Food and Agriculture Organization (FAO) and World Health Organization (WHO) which stated that the number of colonies of probiotic bacteria in a food must have a colony count of 10^6 - 10^8 CFU/mL (Figure 1). The factors that influence bacterium growth include time, immune response, nutrition, and antibiotics (Barrow 1992). Further two samples with the highest number of colony namely K7 isolate and K8 isolate were used for further approval.

The identification process of LAB from isolates K1 until K10 were done macroscopically by looking at morphological form from the isolates (Figure 2). It was observed that morphology of the sample was LAB Isolate, white and round. Results obtained a microscopic form of bacteria such as Basil (stem) with Gram-positive bacteria. Gram-positive bacteria have a layer of peptidoglycans (molecules consist of amino acids and sugars) which is thick, namely between 20-80 nm and consist of 60-100% peptidoglycan (Table 1).

Gram-positive bacteria have thick cell walls and cell membranes and do not have an outer membrane (Figure 3). Fifty strains of LAB were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequence (16S rRNA) analysis, Species belonging to *Lactobacillus* genus were the predominant LAB in all fruit pulp processing byproducts. The average congruency between the MALDI-TOF MS and 16S rRNA in LAB species identification reached 86%. Isolates of *L. plantarum*, *L. brevis*, *L. pentosus*, *L. lactis*, and *L. mesenteroides* were identified with 100% congruency. MALDI-TOF MS and 16S rRNA analysis presented 86 and 100% efficiency of LAB species identification. Moreover, MALDI-TOF MS and 16S rRNA analysis revealed high efficiency and congruency for LAB species identification, and the selected *Lactobacillus* strains may be candidates for further investigation of new probiotic strains (Garcia et al. 2016).

Table 1 shows the type of bacteria produced from the isolated sample was done microscopic process using a Gram test method. From the Gram Test Result obtained a microscopic form of bacteria such as Basil (stem) with Gram-positive bacteria (Figure 2). Gram-positive bacteria is a bacteria that has a layer of peptidoglycans (molecules consist of amino acids and sugars) which is thick, namely between 20-80 nm and consist of 60-100% peptidoglycan.

Gram-positive bacteria have thick cell walls and cell membranes and do not have an outer membrane because 90% of cell walls are composed of peptidoglycan which absorbs crystal violet not safranin while the rest consist of molecules called teichoic acid (Figure 3).

This step aims to optimize the ability of bacterial growth in acidic conditions. The result of resistance test obtained shows that the isolates K7 and K8 resist acid condition at pH 2.0 (Figure 4). The isolates K7 and K8 grow optimally at pH 2.0 present in the medium, because the main requirement of the isolate is probiotic since it has a high tolerance of stomach acid and bile salts in the digestive tract. So that, organisms that are not acid resistant unable develop normally (Cullimore 2000). The acidic condition that must be passed of probiotics bacteria in the digestive tract started from the stomach; namely, the bacteria must be able to survive against very low pH for at least 90 minutes. Further, the probiotics bacteria get into the intestinal tract with pH 6.0 that is a place secreted bile salts (Surono 2004). Besides, probiotic bacterium can produce useful substances, can reduce pathogenic microbial populations, improve health and immunity (Ziemer 1998).

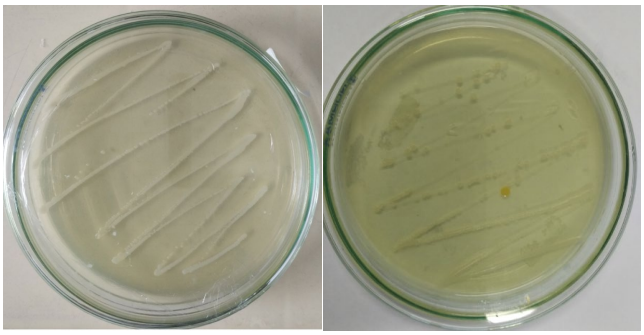

Figure 1. Sample of colony A. K7 Isolate, B. K8 isolate

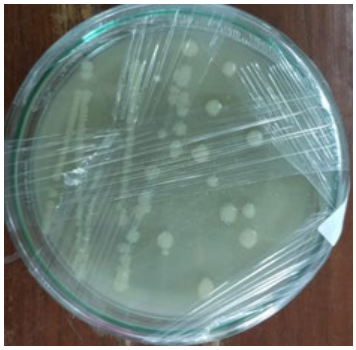

Figure 2. Bacterial colonies of the K7 isolate

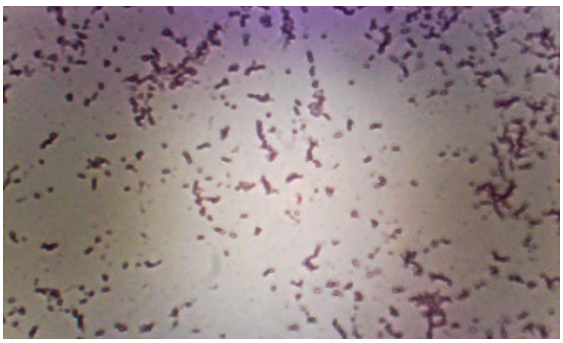

Figure 3. Gram staining results of K7 Isolate

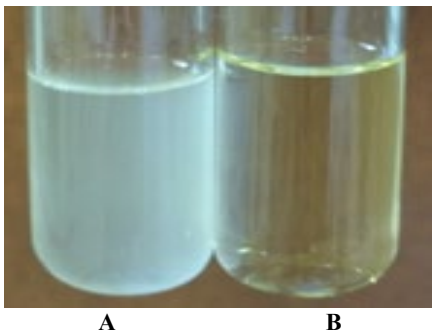

Figure 4. Acid resistance test of: A. K7 Isolate, B. Control

Table 1. Observation of bacterial morphology

Isolates	Size	Form	Elevation	Edge Shape	Color	Gram Staining
K1	Small	No Regular	Concave	Lobate	Yellowish white	Bacillus (gram-positive)
K2	Small	Regular	Concave	Full round	Yellow	Coccus (gram-positive)
K3	Big	Regular	Convex	Full round	White	Coccus (gram-positive)
K4	Small	No Regular	Convex	Lobate	Yellowish white	Bacillus (gram-positive)
K5	Small	No Regular	Concave	Full round	White	Coccus (gram-positive)
K6	Big and small	No Regular	Concave	Full round	Yellow	Bacillus (gram-positive)
K7	Big and small	Regular	Convex	Full round	White	Bacillus (gram-positive)
K8	Big	Regular	Convex	Lobate	White	Bacillus (gram-positive)
K9	Big	No Regular	Concave	Full round	Yellow	Coccus (gram-positive)
K10	Big and small	Regular	Convex	Full round	White	Coccus (gram-positive)

The isolates that have potential as LAB were taken and done acid resistance test. The results from K7 isolate with the acid resistance test at pH 2.0 until pH 6.0, which is suitable for the condition of the small intestine (Triana et al. 2006). K7 isolate was looked more turbid than controls because K7 isolate containing organic acids of fermentation product is the hydrolysis from fatty acids and also as the result of bacterial activity (Jamsari et al. 2013). It was proved that K7 isolate from sample has the ability as a probiotic. Besides, K7 and K8 isolate were carried out to antimicrobial activity test.

Antimicrobial activity was done using a diffusion method with *E. coli* as a test bacteria. Isolate K7 and K8 were tested in pathogenic bacteria will be observed clear zone formed with using controls. The controls used in this antimicrobial test were erythromycin and ampicillin as a sensitivity test (Figure 5). The results of antimicrobial activity test (Figure 5) for samples K7 and K8 at 24 hours, 48 hours, and 72 hours show a clear zone. K7 samples were obtained at each treatment time of 12 mm, 16 mm and 18 mm while samples K8 shows clear zone with 9 mm, 12 mm, and 13 mm. Results of clear zone obtained from both samples are in strong category because the measurement of antibiotic-antibacterial strength using the Davis Stout method for the results as obtained above is classified into the strong category, so effective to be used as an antimicrobial in inhibiting the growth of pathogenic *E. coli* bacteria for 72 hours (Habibi 2017). The clear zone formed because the presence of bacteriocins that inhibit the growth of pathogenic bacteria so that a clear zone is formed around the colony, this indicates showing that the LAB of the isolate sample is can be one of the requirements in the development of probiotics (Surono 2004).

Proteolytic enzyme activity

The activity of the proteolytic enzyme test was done qualitatively and quantitatively tests. Isolate K7 and K8 had a very high ability in remodeling the protein into a peptide compound and amino acids that are soluble in the medium (Table 2).

Milk is an appropriate medium for microbial growth because it is rich in nutrients; skim milk was added to the growth medium as an enzyme-substrate. Skim milk contains milk proteins such as phosphoproteins binding to calcium forming calcium salts (calcium calcinate) called casein (Habibi 2017). The quantitative test is using spectrophotometric UV-vis method (Table 2). The measurement of protein content with using the Lowry method, BSA standard curves are made with variations of BSA concentrations. The absorbance obtained from each concentration plotted as ordinate, while concentration as an axis. So that, obtained regression equation Y = 0.02415 + 0.0008354X. After substitution, the protein content of each isolate K7 and K8 were obtained is 2.19757 mg/mL and 1.92345 mg/mL at a wavelength of 578 nm. The activity of the proteolytic enzyme obtained is 6.18243 unit/mL and 1.235 unit/mL. The specific activity of the proteolytic enzyme produced is 2.813304 unit/mg and 0.64207 unit/mg. The existence of differences in the value of proteolytic enzyme activity qualitatively and quantitatively caused by several factors among others different types of microorganisms, isolate growth rate in a solid and liquid medium, and number of inoculums given on both media (Rosiana 2009).

The result from screening proteolytic enzyme from the K7 and K8 Isolates obtained a clear zone for 72 hours of 15 mm and 12 mm with a colony diameter of 2 mm (Table 2). This result showed that both samples could produce proteolytic enzymes. Casein is a large molecule, insoluble in water, and can form colloids. Casein will be hydrolyzed into peptides and soluble amino acids because of the availability of proteolytic enzyme activity, a namely proteolytic enzyme. The result of the enzyme activity obtained from K7 and K8 samples amount 7.5 and 6. Proteolytic enzymes catalyze casein degradation by severing the CO-NH peptide bond with the entry of water to the molecules into amino acids like tyrosine (Rosiana 2009). The loss of casein particles in skim milk medium is indicated by the formation of a clear zone around bacterial colonies.

Identification of genes 16S rRNA

The extraction of DNA and amplification using the 16S rRNA gene was done using universal primer namely 27F (5'-AGAGTTTGAATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3') is shown in (Figure 6) the results are around 1500 bp. This results showing that the product generated was LAB as a target. The PCR products obtained were then sequenced.

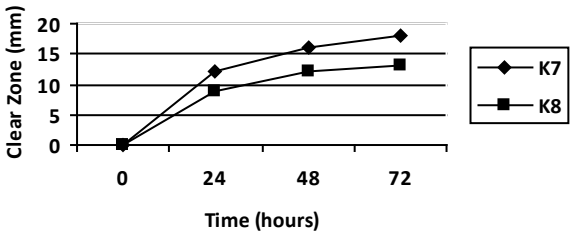

Figure 5. Antimicrobial activity from K7 and K8 isolates

Table 2. Screening proteolytic enzyme

Isolate	The diameter of the colony	Clear zone 72 hours	Enzyme activity
K7	2 mm	15 mm	7.5
K8	2 mm	12 mm	6

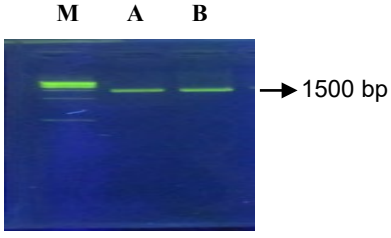

Figure 6. Product PCR isolate of M. Marker; A. K7 isolate and B. K8 isolate

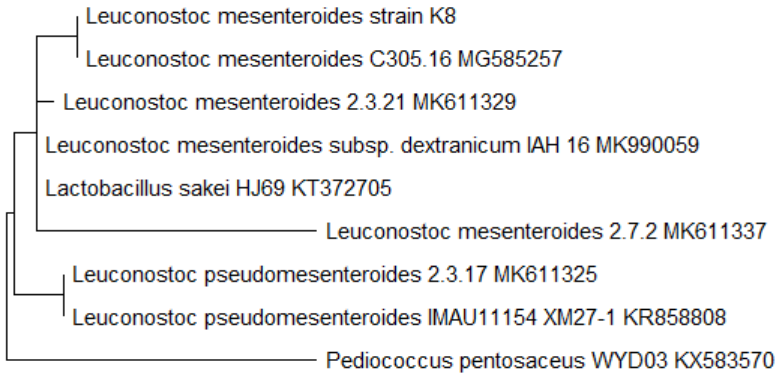

Figure 7. Phylogenetic tree isolates of K8 from kelengkeng fruit

Based on the results of analysis 16S rRNA gene sequence using a database from BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Multiple-Alignment Analysis. The BLAST finds regions of similarity between biological sequences. The program compares nucleotide sequences to sequences databases and calculates the statistical significance. The results obtained by the percentage of bacterial identity samples of kelengkeng fruit K7 isolate of 99% with strain *L. mesenteroides subsp suionicum strain* LT-38 and K8 sample has a bacterial identity percentage of 96% with *L. mesenteroides strain* C305.16 (Figure 7). *Leuconostoc* is one type of LAB that belongs to heterofermentative group because it has ability to utilize glucose in metabolic process of cells besides generating lactic acid, it also producing acetic acid, ethanol and carbon dioxide (Qonita 2018). In addition, *L. mesenteroides* is a bacterium that has ability producing an extracellular enzyme called dextransucrase.

In conclusion, the results of LAB identification of kelengkeng fruit was shaped basil with the number of colonies in samples K7 and K8 obtained as much as 133.10^7 CFU/mL and 87.10^7 CFU/mL. The total number of these colonies is the criteria for as food for probiotics. The proteolytic active enzyme and activity of specific enzyme obtained a result as much as 1.235 unit/mL and 0.64207163 Unit/mg for a sample of K7 and 6.18243 Units/mL and 2.813304 Units/mg for a sample of K8. The sequence of isolate genes from kelengkeng (*Dimocarpus longan*) fruit showed that the bacteria species contained from K7 and K8 isolates were *L. mesenteroides strain* K7 and *L. mesenteroides strain* K8. They have the highest similarity with strain LT-38 (AP017935.1) and C305.16 (MG585257.1), but, not strain LT-38 nor C305.16. Hence, the identity of K7 and K8 isolates probably members of *Leuconostoc* genus.

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