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Isolation and Identification of Lactic Acid Bacteria form Chinese Cabbage Waste by 16s rRNA Amplification using The Polymerase Chain Reaction (PCR) Method

Assyifa Junitasari*, Tina Dewi Rosahdi, Yuni Siti Lestari

Departement Chemistry, Faculty of Science and Technology, Universitas Islam Negeri Sunan Gunung Djati Bandung, Cibiru, Bandung, 40614, Indonesia

**Corresponding author: assyifajunitasari@uinsgd.ac.id*

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Abstract

Waste is material that is disposed of from sources of human and natural activities that do not yet have economic value. Physically, vegetable waste is perishable due to its high-water content, especially Chinese Cabbage waste. The purpose of this study was the isolation and identification of lactic acid bacteria from Chinese Cabbage waste. Isolation was carried out to obtain isolates of lactic acid bacteria from Chinese Cabbage waste. Then it was amplified using a Polymerase Chain Reaction (PCR) instrument. The results of chromosomal DNA amplification of bacterial isolates from mustard 1, mustard 2 and mustard 3 waste showed DNA bands with a size of ± 1200 bp, so it can be concluded that the process of amplification of the 16s rRNA gene fragment in mustard 1, mustard 2 and mustard 3 isolates was successful. The results of the 16s rRNA base sequence analysis showed that the mustard 1 isolate had a similarity index of 84.65% with *Bacillus* sp, the gene for the mustard 2 isolate had a similarity index of 84.09% with the Uncultured bacterium clone, and the mustard 3 isolate gene had a similarity index of 85.42% with Environmental 16s rDNA sequence.

***Keywords**:* Lactic acid bacteria; 16s rRNA gene fragment; PCR; gram staining; sequencing

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1. INTRODUCTION

Waste is material that is disposed of from sources of human and natural activities that do not yet have economic value. Waste is always synonymous with waste products or leftovers that are no longer suitable for use, either from food or animals. One of the biggest contributors to waste in life is market waste. The composition of market waste is more dominant than organic waste. Market waste is more waste from vegetables or fruit (Arihati et al., 2019). Waste that can be utilized from the waste of human activities is vegetable market waste.

Vegetable market waste is a collection of various kinds of vegetables that are not suitable for sale. Vegetable market waste is not treated properly which can cause environmental pollution. Physically, vegetable waste rots easily due to its high-water content, especially Chinese Cabbage waste. The utilization of Chinese Cabbage waste is isolated or obtained by lactic acid bacteria (LAB) (Pardosi et al.,2015).

Lactic Acid Bacteria (LAB) are facultative anaerobic bacteria that can live in a wide variety of habitats in nature such as fruits, vegetables, and meat products (Rahmiati et al., 2017). The contribution of lactic acid bacteria is used as a natural preservative for fermented products (Delvia et al., 2015). Lactic acid bacteria are referred to as food-grade microorganisms which are microbes that are not at risk to health because they do not produce harmful toxins but have good functions for health because lactic acid bacteria can naturally inhibit pathogenic microbes (Delvia et al., 2015).

In addition, lactic acid produced by lactic acid bacteria can lower the pH of the environment. A low pH can inhibit the contamination of spoilage microbes and kill pathogenic microbes, especially those in the

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body. The many benefits of active metabolites produced from lactic acid bacteria, so it is necessary to do research on the isolation of lactic acid bacteria from new sources such as Chinese Cabbage which has a high content of lactic acid bacteria to overcome problems in the production of lactic acid bacteria such as the number, the price is high and waste. Chinese Cabbage in the market is not piling up.

Mulyanto et al, (2009) in a study found an LAB of 2.1 x 1010 CFU in market vegetable waste consisting of cabbage and mustard waste. Then, in another study, Sukriani Kursia et al, (2019) succeeded in identifying LAB from Chinese Cabbage waste belonging to the family Lactobacillacaea, genus *Lactobacillus sp*.

Efforts to identify species of lactic acid bacteria can be done through phenotypic and genotypic examinations. The phenotypic method is considered less accurate because it relies on phenotypic expression under laboratory conditions and can lead to misidentification. Along with the advancement of molecular biology, the diversity of microorganisms can be studied by observing the 16s rRNA profile. This 16s rRNA profile is very specific for each microorganism so that it can help to identify microorganisms from the environment because it is more accurate and faster (Daniel et al.,2019).

Based on this description, a study was conducted to identify the species of lactic acid bacteria found in Chinese Cabbage waste with gram staining and continued with extracting the chromosomal DNA of bacterial isolates. The chromosomal DNA obtained was then amplified using the Polymerase Chain Reaction (PCR) instrument to obtain 16s rRNA gene fragments.

The choice of Chinese Cabbage waste is because the potential for Chinese Cabbage waste is quite large, namely, the amount is quite a lot found in the market, and Chinese Cabbage waste includes vegetables that are easily rotten and are not reused by farmers (Sasmita et al., 2018).

2. MATERIALS AND METHODS

Materials and Instrument

The materials used in this study were Chinese Cabbage waste from the traditional market of Taraju of waste disposal for 1-2 days was carried out on Tuesday and Saturday in the afternoon because that was the time for cleaning and collecting waste in the market after a day of trading activities, 2% technical sodium chloride, 0.5% glucose, distilled water, NB (Nutrient Broth) media, MRSA media (de Man Rogosa Sharpe Agar), and purple crystals. , iodine, 96% alcohol, 75% alcohol, safranin, 1x lysis buffer, proteinase K, Nuclease Free Water (NFW), agarose powder, 1x TAE buffer, red gel, loading dye, PCR master mix reagent, BactF1 forward primer, primer reverse UniB1, DNA marker 1 kb in size. The sample solution from DNA extraction was put into a 0.2 mL PCR tube with 1.5 L, 7.5 L of PCR master mix reagent, BactF1 forward primer (5'-AGA GTT TGA TC (G/C) TGG CTC AG-3 ') 1 L, Uni B1 reverse primer (5'-GGT TAC (G/C) TTG TTA CGA CTT-3') 1 L (Promega Corporation, USA) and 4.8 L NFW.

The instrument used in this study is the Polymerase Chain Reaction (PCR) T100 Thermal Cycler Bio-Rad.

Sample Preparation

The research sample used was Chinese Cabbage waste originating from the traditional market of Taraju, Tasikmalaya, West Java. Vegetable waste that has been taken, then washed, after washing with tap water then cut the Chinese Cabbage waste into small sizes and then weighed (Sasmita et al.,2015).

Lactic Acid Bacterial Fermentation

Sample fermentation was carried out by immersing the sample in an airtight glass fermentation container with 2% technical sodium chloride (w/w sample), 0.5% glucose (w/w sample), and boiling water until the sample was submerged for 24 hours (Sasmita et al., 2015).

Making MRSA and NB Media

MRSA media was prepared by dissolving 7.4 grams of MRSA powder into 100 mL of distilled water, while NB media was prepared by dissolving 1.3 grams of NB powder into 100 mL of distilled water. Each medium was heated until dissolved and sterilized using an autoclave at a temperature of 121 °C and a pressure of 1 atm for 15 minutes (Napitupulu et al., 2019).

Isolation of Lactic Acid Bacteria

A total solution of 1 mL was put into a test tube, 10-1 to 10-5 dilution was made, then 100 L of each dilution was taken and put in a petri dish to which 10 mL of MRSA (de Man Rogosa Sharpe Agar) media had been added,

incubated at 37°C. for 18 hours (Sasmita et al., 2018).

Isolate Purification

The isolates Lactic Acid Bacteria were purified using MRSA (de Man Rogosa Sharpe Agar) media with the streak plate method etched in a quadrant and incubated at 37°C for 18 hours (Sasmita et al., 2018).

Gram Stain

The object glass was cleaned with 96% alcohol and then fixed on a silver lamp. The active isolates were taken aseptically and placed on an object glass evenly, then re-fixed on a silver lamp. The cold isolates were dripped with Gram A (crystal violet) 2-3 drops for 1 minute, then washed with distilled water and dried. Then it was dripped with Gram B (Iodine) and allowed to stand for 1 minute, washed with distilled water, and dried. The isolates were dripped with Gram C (96% alcohol) for 30 seconds, washed with distilled water, and dried. The isolate was dripped with Gram D (Safranin) for 45 seconds, then washed with distilled water. This observation was carried out by looking at the shape and color of the cells under a microscope with 1000 times magnification (Bibiana et al., 1994).

Chromosomal DNA Extraction

500 L of liquid culture was pipetted into a 1.5 mL microtube, then centrifuged for 1 minute at 12000 rpm. The precipitate was added with 200 L of 1x lysis buffer, 10 L of proteinase K, and 170 L of Nuclease Free Water (NFW), then incubated for 1 hour at 55 °C in a water bath and the enzyme was inactivated at 95°C for 10 minutes and centrifuged for 10 minutes. 3 minutes at a speed of 12000 rpm (Thoyibatun et al., 2012).

Agarose Gel Making

A total of 0.5 grams of agarose powder was dissolved in 50 mL of 1x TAE buffer with the help of a hotplate. The agarose solution was left to warm, then 5 L of gel red was added and poured into the agarose gel printer. The agarose gel was left to solidify, then put into the electrophoresis pan. 1x TAE buffer solution was poured into the pan until the gel was submerged (Thoyibatun et al., 2012).

16s rRNA Fragment Amplification

The initial stage of 16s rRNA analysis was carried out by extracting chromosomal DNA from selected bacteria which was then amplified by PCR technique. The sample solution from DNA extraction was put into a 0.2 mL PCR tube with 1.5 L, 7.5 L of PCR master mix reagent, BactF1 forward primer (5'-AGA GTT TGA TC (G/C) TGG CTC AG-3 ') 1 L, Uni B1 reverse primer (5'-GGT TAC (G/C) TTG TTA CGA CTT-3') 1 L and 4.8 L NFW. The mixture is put into the PCR apparatus, starting with a pre-separation temperature of 95 °C for 5 minutes, a separation temperature of 95 °C for 30 seconds, an attachment temperature of 60 °C for 30 seconds, a polymerization temperatur' of 72 °C for 1 minute and a final polymerization temperature of 72 °C. C for 4 minutes. The PCR process was carried out for 30 cycles (Thoyibatun et al., 2012).

Agarose Gel Electrophoresis

Verification of DNA extraction results was carried out by agarose gel electrophoresis. A total of 5 L of the DNA isolated solution was added to 1 L of loading dye and homogenized. The DNA marker was made from a mixture of 4 L NFW, 1 L DNA marker 1 kb size, and 1 L loading dye. The sample mixture and DNA markers were inserted into the wells on the agarose gel, then electrophoresed for 35 minutes with a voltage of 100 V/cm (Happy et al., 2018).

To verify the results of PCR amplification, agarose gel electrophoresis was also carried out. A total of 6 L of the amplified solution was added.

3. RESULTS AND DISCUSSION

Lactic Acid Bacterial Fermentation (LAB)

Fermentation is the process of chemical changes in organic substrates through the activity of enzymes produced by microorganisms (Suryani et al.,2017) so that they can produce a product using fermented microbial cultures. The fermentation process is carried out by putting it in an airtight jar, the sample used is 200 grams of Chinese Cabbage waste. The purpose of airtightness is because lactic acid bacteria are facultative anaerobes, the purpose of adding salt is as a selective inhibitor of interfering microorganisms, while glucose is added as an energy source in the fermentation process to produce products that have high economic value and pH

measurements are carried out (Sasmita et al., 2018). From the results of the measurement of the pH of the fermented Chinese Cabbage waste, the changes in the pH value can be seen in **Figure 1**. The decrease in the pH value can identify the presence of microbial activity in breaking down carbohydrates (Sasmita et al., 2018). The factors that influence the growth of lactic acid bacteria are fermentation time, acid pH, temperature, and oxygen.

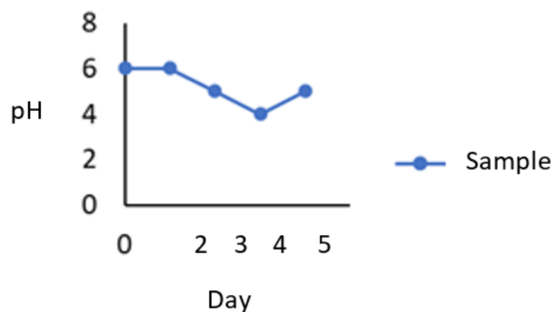

Figure 1. Results of pH measurement of chinese cabbage waste fermentat

On day 1 the pH did not increase because there was no microbial activity to decompose carbohydrates. On the 2nd day, the pH becomes acidic, namely pH 5 due to the presence of microorganisms that develop. On the 3rd day, the pH becomes more acidic due to the increasing number of microorganisms that multiply. On the 4th day, the pH rose again due to the long storage time and a lot of disturbing impurities such as fungi that affect the fermentation process, on the 4th day the fermentation did not produce lactic acid. Lactic Acid Bacteria (LAB) grew in the acidic pH range so that the 3rd-day fermentation samples were isolated and identified. On the 3rd day, it was isolated because at pH 4 it was good for the growth of lactic acid bacteria.

Isolation of Lactic Acid Bacteria

Isolation of lactic acid bacteria is a method of purifying isolates using selective media de Men Rogrosa Sharpe Agar (MRSA) repeatedly so that a single medium is obtained (Sasmita et al., 2018). MRSA is a selective medium that only grows and isolates lactic acid bacteria that grow in colonies (Ningsih et al., 2018). The colonies that will be identified are only milky white and shiny, the shape of the colonies is round and flat because lactic acid bacteria isolates grown in an MRSA medium and incubated showed white and shiny bacterial colonies.

The samples taken were the result of fermentation on the 3rd day because the pH decrease in the lactic acid bacteria samples was on the 3rd day. Isolation was carried out using a dilution technique up to 10-5 and incubated at 37 °C for 18 hours. The isolation results were obtained at a dilution of 10-2. The isolate 10-2 was then purified by the quadrant streak method on solid MRSA media and incubated at 37 °C for 18 hours (Ningsih et al., 2018).

Gram stain

The isolates of lactic acid bacteria from Chinese Cabbage waste were then characterized for their cell morphology by gram staining. Gram staining uses 4 types of dyes, the first is the addition of gram A dye (crystal purple) which functions as a primary dye (Fajriani et al., 2018). The isolates were allowed to stand for a few minutes, then rinsed with distilled water which aims to clean the dyes that do not react with the bacterial cell walls. The second dye was added with gram B dye (iodine) as a color enhancer for purple crystals. The third dye gram C dye (alcohol-acetone) serves to dissolve the fat contained in the bacterial cell wall. While the last dye gram D (safranin) has a function as a secondary dye (Fajriani et al., 2018).

Each time the dye was added, the mustard green waste bacterial isolate was allowed to stand for a few minutes to absorb each dye and react with the bacterial cell wall perfectly. Rinsing with distilled water was carried out every time adding another dye to avoid mixing the dyes and removing the remaining dyes that did not react with the bacterial isolates. Bacterial isolates that had been gram-stained were then observed using a 1000x magnification microscope by adding immersion oil. Immersion oil is oil that is used to smear on the microscope. Oil immersion serves to clarify objects and protect the microscope. Immersed oil has a high index of refraction compared to without immersion oil.

The results of the microscopic identification of lactic acid bacteria isolates from Chinese Cabbage waste showed that the isolates were gram-positive bacteria. Lactic acid bacteria are gram-positive bacteria because they bind crystal violet (Gram A) so that they appear purple. The results of cell morphology observations of lactic acid bacteria with gram staining can be seen in **Table 1**.

Table 1. Morphological characteristics of LAB cells from cabbage waste

No	Isolate	Cell Shape	Gram
1	Chinese cabbage 6	Cocci	positive
2	Chinese cabbage 5	Basil	positive
3	Chinese cabbage 4	Basil	positive
4	Chinese cabbage 3	Basil	positive
5	Chinese cabbage 2	Cocci	positive
6	Chinese cabbage 1	Basil	positive

The addition of 96% alcohol and acetone in gram-positive bacteria has a dehydrating effect on the cell wall, causing the cell wall pores to shrink (Bartholomew et al., 2014). The process of adding alcohol and acetone is also known as decolorization. This process is very important and must be timed properly. Otherwise, the crystal violet stain will be removed from the gram-positive cells. If the decolorizing agent is left on the cells for too long, gram-positive organisms will appear to be gram-negative. The addition of safranin will make the cell walls of gram-negative bacteria that undergo decolorization turn red because it binds to the safranin dye which has a positive charge. Meanwhile, in Gram-positive bacteria, safranin dye cannot bind to the cell wall which is firmly attached to the layered cell wall (Bartholomew et al., 2014). So that the result of gram staining will make gram-positive bacteria purple and gram-negative bacteria red.

Chromosomal DNA Extraction

The liquid cultures that have been incubated are centrifuged. Centrifugation aims to separate bacterial cultures with NB (Nutrient Broth) media based on differences in specific gravity. Bacterial culture settles at the bottom because it has greater specific gravity than NB (Nutrient Broth) media. The precipitate obtained is then added with a lysis buffer which functions to break down the bacterial cell wall without damaging the bacterial DNA. The lysis buffer used was a mixture of Tris-HCl, EDTA, and Tween-20. Tris-HCl is a buffer that has the function to maintain the optimum pH of the activity of the proteinase K enzyme. EDTA in the lysis buffer has the function of preventing denaturation of the DNA structure where EDTA is a chelating agent for cofactors in the DNAse enzyme. Tween-20 has a function as a lipid solvent on cell membranes so that cell membranes are stabilized (Handoyo et al., 2000).

The bacterial culture precipitate was added with proteinase K. The addition of proteinase K functions as a peptide bond breaker from bacterial cell proteins. Furthermore, NFW (Nuclease Free Water) was added. NFW serves to dissolve. After the addition of these substances, incubation was carried out in a water bath to optimize the work of the proteinase K enzyme at a temperature of 55 °C. At this temperature, the activity of the proteinase K enzyme increases so that the process of breaking the peptide bond will work optimally. The mixture was then incubated again at 95 °C to inactivate the proteinase K enzyme. At 95 °C the action of the proteinase K enzyme was inhibited so that it could not break the peptide bond.

The enzyme inactivation process only lasts for 5 minutes, because the temperature is too high for too long it is feared that it will damage the target DNA. Centrifugation aims to separate the bacterial culture deposits that are not isolated from the isolated solution. Centrifugation will make it easier to take the isolated solution containing bacterial chromosomal DNA.

The next step is the agarose electrophoresis process from the chromosomal DNA extraction solution. TAE 1x buffer was used as agarose solvent. TAE 1x buffer functions as a conductor of electricity because it has strong ionic power. In the manufacture of agarose, a red gel is added. Gel red serves as a dye that will help the DNA visualization process (Francois et al., 2010). The solution resulting from the extraction of bacterial chromosomal DNA was added with loading dye. Loading dye functions as a ballast so that the isolated solution comes out of the well regularly. In addition to the solution extracted from DNA, DNA markers were also inserted into the agarose well. A marker is a specific DNA segment of known size. The marker serves to determine the size of the isolated DNA and as a marker of the position of the migrating DNA molecule to determine the approximate size of the bases. The markers used for electrophoresis have a size of 1kb or 10,000 bp. This electrophoresis was carried out for 35 minutes at a voltage of 100 V/cm. These timing and voltage settings affect the speed at which DNA migrates. The higher the voltage and the faster the electrophoresis time, the DNA molecules will migrate faster, and the DNA band separation process will not be optimal

(Francois et al., 2010). The results of the electrophoresis were visualized using a DNA UV lamp. The results of the electropherogram of six bacterial isolates can be seen in **Figure 2**.

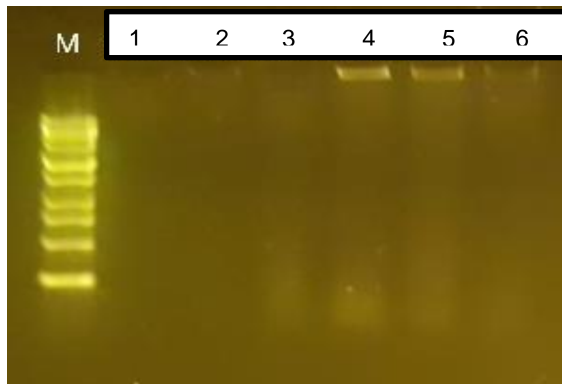

Figure 2. Electrophorogram of LAB Chromosomal DNA from Cabbage Waste M= DNA size marker, mustard 6= isolate 6, mustard 5 = isolate 5, mustard 4 = isolate 4, mustard 1 = isolate 1, mustard 2 = isolate 2, mustard 3 = isolate 3

The results of electrophoresis of chromosomal DNA of lactic acid bacteria isolates from Chinese Cabbage waste showed the formation of DNA bands in the samples of mustard 4, mustard 1, mustard 2, and mustard 3, but in the sample of mustard 3 the DNA band formed was thin compared to other samples due to the small content of DNA in the sample of mustard green (Widyastuti et al., 2017). While in the samples of mustard 5 and mustard 6, no band was formed due to the very little DNA content in the sample. The cause of the low DNA content in the sample is due to the presence of other proteins or there are cell parts that are included during DNA isolation. In samples of mustard 5 and mustard 6, we get DNA with a very small band concentration (Faatih et al., 2009). Noer and Gustiananda (2017), stated that the greater the template concentration, the brighter and thicker the DNA bands will be, but too high a template concentration will also result in the formation of smeared bands, on the other hand, too low a template concentration will cause the formation of bands that are too thin to be able to be detected. detected by agarose gel electrophoresis.

The band is separated above the 10,000 bp region, this indicates that the presence of molecules with a size of more than 10,000 bp was detected. The delay in the migration rate of the molecule in the 10,000 bp region indicates that the molecule is a bacterial chromosomal DNA molecule. Bacterial chromosomal DNA molecules have a size of more than 10,000 bp, namely 21,000 bp-23,000 bp (Thoyibatun et al., 2012). From the results of mustard 4, mustard 1, mustard 2, and mustard 3, it can be said that the extraction of chromosomal DNA of lactic acid bacteria was successful.

16s rRNA Fragment Amplification

The results of the chromosomal DNA extraction were then amplified. The amplification of the 16s rRNA fragment was carried out using a Polymerase Chain Reaction (PCR) instrument. PCR is an enzymatic method for exponentially amplifying a certain nucleotide sequence in vitro. PCR takes place in several repeated temperature cycles and each cycle consists of 3 stages (Handoyo et al., 2000). The optimum cycle range in the PCR process is as much as 30-35 cycles, depending on the polymerase enzyme, the number of templates, and so on.

The components needed in the PCR process are DNA templates (DNA samples), master mix, primers, and NFW. The DNA template is the result of the extraction of bacterial chromosomal DNA which serves as a template for the formation of the same new DNA molecule. Primers are oligonucleotides whose base sequences are designed to be complementary to specific regions of the template DNA. There are two types of primers used in the amplification process by PCR. Primers also function as a barrier for target DNA fragments to be amplified. The forward primer is before the target and the reverse primer is after the target.

In addition to the primer, another component required for the amplification process is the master mix reagent. Master mix is a mixture containing DNA Taq polymerase, dNTP, $MgCl_2$, and reaction buffer. DNA Taq polymerase is derived from the bacterium Thermus aquaticus (Taq) which is a thermostable enzyme used in the denaturation stage. dNTP serves as a source of nucleotides used in the DNA elongation step. MgCl2 functions as a cofactor to stimulate DNA polymerase. The reaction buffer at the optimal concentration serves for efficient amplification of template DNA by PCR (Promega, 2012). The addition of NFW to the amplification process by PCR serves as a solvent for the materials mentioned above.

The amplification process by PCR begins with an initial denaturation at 95 °C for 5 minutes which serves to separate the long initial DNA chain so that it is completely denatured. Denaturation at a temperature of 95 °C for 30 seconds is a step to separate double-stranded DNA into single chains by breaking the hydrogen bonds in the DNA chain. The temperature and time used at this stage are general temperatures for the denaturation process. If the denaturation temperature is too high, it will reduce DNA polymerase activity which has an impact on PCR efficiency and can damage the DNA template. Meanwhile, if the denaturation temperature is too low, the DNA template denaturation process will be imperfect. The next stage is annealing or primer attachment to single-stranded DNA. This stage is carried out at a temperature of 60 °C for 30 seconds, and the annealing temperature (Handoyo et al., 2000) is determined based on the calculation of the melting temperature (Tm) namely (Tm-5) °C to (Tm+5) °C (Handoyo et al., 2000).The ideal annealing temperature is 52 °C - 65°C, an annealing temperature that is too high can cause oligonucleotide primers to adhere imperfectly, while an annealing temperature that is too low can cause annealing of non-specific primers. This can result in the amplification of unwanted DNA segments.

The amplified 16s rRNA gene fragment was then subjected to an agarose electrophoresis process to determine the amplification results. The electropherogram of the amplification process can be seen in **Figure 3**.

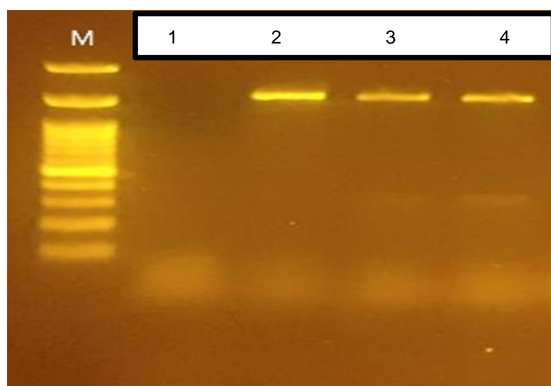

Figure 3. Electrophoresis Results of Gene Fragment 16s rRNA= DNA size marker, mustard 4= isolate 4, mustard 1= isolate 1, mustard 2= isolate 2, mustard 3= isolate 3

From the results of electrophoresis in **Figure 3** on the isolates of mustard 1, mustard 2, and mustard 3, there are separate bands around the 1,200 bp marker. This indicates that the amplified gene fragment has a size of ± 1,200 bp. The results of electrophoresis on these two isolates are by the theory which states that the bacterial 16s rRNA gene fragment molecule has a size of about 1000-1,500 bp, so it can be concluded that the amplification process of the 16s rRNA gene fragment in mustard 1, mustard 2 and mustard 3 isolates was successful. While the results of amplification of the chromosomal DNA of other isolates, namely mustard 4, showed no separated bands. Because of the process of amplification of the 16s rRNA gene fragment in the DNA of mustard 4, the bacterial isolate was not successful. For mustard 5 and mustard 6, 16s rRNA was not followed by amplification because the chromosomal DNA extraction process did not contain a band.

Analysis of the 16s rRNA. Fragment Gene Sequence

Gene sequence analysis was performed at Macrogen Korea. The PCR product sequencing step was carried out to determine the sequence of nucleotide bases (Bunga et al., 2018). DNA sequencing is the process of determining the base sequence of DNA. This process uses the principle of an enzymatic DNA polymerization reaction (Bunga et al., 2018). The sequence of DNA bases is referred to as a DNA sequence, which is the most basic information of a gene or genome because it contains the instructions needed for the formation of the body of living things. This sequencing was carried out using the dideoxy sanger method.

The results of the sequencing are then entered into the Bioedit program. Bioedit program is a program that is used to view the chromatogram of the sequencing results. The chromatogram has a different color depending on the type of nucleotide it represents. The different colors represent a specific pyrimidine base and a purine base, respectively. Pyrimidine bases consist of thymine (red) and cytosine (blue) while purine bases consist of adenine (green) and guanine (black) (Bungol et al., 2014).

Intact genes that have been analyzed by the Bioedit program are then uploaded to the BLAST tracking program on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The search results found that the Sawi 1 isolate gene had a similarity index of 84.65% with *Bacillus sp*, the

Sawi 2 isolate gene had an 84.09% similarity index with an Uncultured bacterium clone, and the Sawi 3 isolate gene had a similarity index of 85.42%. with the Environmental 16s rDNA sequence.

The characteristics of the sequences from the gene bank that are most like the DNA sequences obtained are the same Max Score and Total Score, Querty Coverage is close to 100%, E-value is close to 0, and Max Ident is close to 100%. Phylogenetics is phylogeny which compares the equivalent genes of several species to reconstruct the genealogic tree of several species and find out who is relatively closely related to others.

The results of the phylogenetic tree analysis of genetic distances (neighbor-joining) for the numbers contained in each tree branch show the bootstrap values of mustard 2 and mustard 3 isolates. the same branch or node (genus) as the Uncultured bacterium clone with a bootstrap value of 61. For isolates of mustard bacteria 3 located in the same branch or genus as the Uncultured bacterium clone with a bootstrap value of 42. The bootstrap value indicates close kinship if have the same value. For mustard 1 isolate, no phylogenetic tree was made because the similarities were only two species, while for a phylogenetic tree to be made there must be at least three similar species.

The results of the analysis of the sequence of the 16s rRNA gene fragment from the mustard 1 isolate had a similarity of 84.65% with *Bacillus sp*, the Sawi 2 isolate had a similarity index of 84.09% with the Uncultured bacterium clone, while the mustard 3 isolate had a similarity index of 85.42%. with the Environmental 16s rDNA sequence. The results of the three isolates did not all show the same species because the results of the isolates were not pure.

4. CONCLUSIONS

The pH of fermentation from chinese cabbage waste on days 0 and 1 is pH 6, on day 2 is pH 5, on day 3 is pH 4 and on day 4 is pH 5. The morphological characteristics of the isolated LAB isolates found in chinese cabbage waste, namely in mustard 6 and mustard 2 were cocci, while in mustard 1, mustard 3, mustard 4, and mustard 5 were bacilli. The results of chromosomal DNA extraction of LAB isolates of lactic acid bacteria from chinese cabbage waste showed the formation of DNA bands in samples of mustard 4, mustard 1, mustard 2, and mustard 3, but in sample mustard 3 the DNA band formed was thin compared to other samples. While the samples of mustard 5 and mustard 6 did not form a band. The results of amplification of the 16s rRNA fragment of LAB isolates on mustard 1, mustard 2, and mustard 3 isolates showed bands, which means it was successfully carried out. Meanwhile, mustard 4 shows that there is no separated band, which means it didn't work. Mustard 1 isolate has a similarity index of 84.65 % with *Bacillus sp*.

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