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16S rRNA Gen Analysis of Plastic Destruction Bacteries, South Sumatra, Indonesia

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ABSTRACT

Rivers are the main route for plastic entering the ocean, including the Musi River Estuary. Characteristics of bacteria that are able to degrade plastic waste through polymerase enzymes. The aim of this research is to determine the ability of bacterial isolates to degrade plastic and identify the types of bacteria that degrade plastic waste. This study used plastic bottles, nylon nets, and snack wrappers as objects for degradation measurement. Identification analysis of the 16S rRNA gene using universal PCR primers for bacteria in the form of forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3'). The type of bacteria with the highest percentage of degradation over 20 days, amounting to 7.75%, was *Bacillus amyloliquefaciens*. Identification of types of plastic degrading bacteria using 16S rRNA gene analysis showed 11 bacteria with 8 types including *Staphylococcus hominis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Acinetobacter baumannii*, *Acinetobacter variabilis*, *Shewanella* sp., *Micrococcus luteus*, and *Bacillus amyloliquefaciens*. The percentage of plastic degradation by bacteria is relatively small, so it is best to look for times where there is potential for bacterial growth.

Keywords: 16S rRNA gene analysis, bacteria, degradation, plastic.

INTRODUCTION

Its cheap, versatile, light and long-term use increases the use of plastic (Ali et al., 2023; Asiandu et al., 2020). Plastic waste is a problem on land and at sea (Abirami G et al., 2021), the main route for plastic entering the ocean is through rivers (Nurdhy, 2020), This is because the Musi River Estuary is influenced by various industrial, agricultural, aquaculture and residential activities (Rozirwan et al., 2021, 2024). Plastic has been used massively in recent times, even though it has many benefits, the high use of plastic creates plastic waste (Charnock, 2021). Indonesia is a developing country whose population uses a lot of plastic, adapting to the increasing demand for products from plastic producing industries (Fibriarti et al., 2021). Indonesia ranks 2nd in the world as a contributor to plastic waste into the sea after China with a range of 0.48–1.29 million metric tons of plastic per year (Sianturi et al., 2021). Current conditions include plastic waste contributing 60% to 80% of all waste in the ocean. Of the 64 million tons of waste from Indonesia thrown into the sea, 3.2 million tons are plastic (Syakti et al., 2017).

Rivers are waters that flow in an open system, so all waste from various human activities in the residential, agricultural and industrial areas around them enters. The consequences that occur from the entry of river waste, namely changes in chemical, physical and biological factors in the waters (Fitria et al., 2023; Rozirwan et al., 2023).

The linear nature of water flow means that rivers in the downstream area are a combination of various tributaries that transport water from upstream. The Musi River flows into dozens of other large and small rivers from Bengkulu and South Sumatra. The Musi River is around 720 km long and passes through the city of Palembang. Various human activities including the use of plastic entering rivers can have an impact on the organisms there (Galarza-Verkovitch et al., 2023). Plastic waste in macro and micro conditions can cause negative impacts if consumed by marine organisms through the food chain (Emmanuel-Akerele and Akinyemi, 2022; Fawcett et al., 2021). Bacteria have the ability to break down plastic naturally, which is called biodegradation (Alamer et al., 2023; Skleničková et al., 2022). It is necessary to use plastic waste degrading bacteria as a solution. Previously, there had been research on plastic-degrading bacteria in the Musi River, but the gene analysis was not carried out, so the types of bacteria with the ability to degrade plastic were not known, which was carried out by (Vianti et al., 2020), so further studies were needed regarding the types of plastic-degrading bacteria that were known from Gene analysis and previous research location coverage were only at the Musi River Estuary.

16S rRNA gene analysis can see the similarity of each bacterial species with the ability to approach 99% species similarity (Johnson et al., 2019). The advantages of using 16S rRNA analysis in bacterial identification are the high level of accuracy and effectiveness as well as the shorter time involved in the identification process compared to conventional methods (Akihary and Kolondam, 2020). The 16S rRNA gene sequence can be found in almost all types of bacteria and is a conserved region so it is used as a source of bacterial information (Janda and Abbott, 2007). The genotype test has many advantages, including being easier, more valid and faster (Ballard et al., 2020). It is necessary to carry out this research with the aim of determining the ability of bacterial isolates to degrade plastic and identifying the types of bacteria that degrade plastic waste.

MATERIALS AND METHOD

Study area and sampling

This research was carried out in September 2021 – January 2022 taking water samples from the lower waters of the Musi River, South Sumatra (Figure 1). Sample analysis was carried out at the Microbiology Laboratory of the Biology Department, Sriwijaya University, the Oceanography and Marine Instrumentation Laboratory of the Marine Science Department, Sriwijaya University, and the Institute for General Aquatic

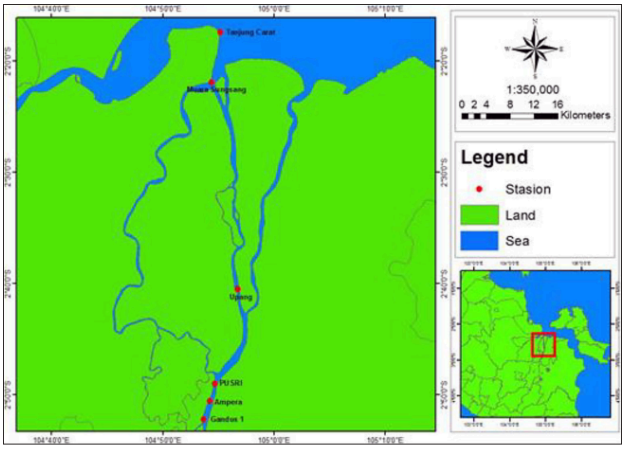

Figure 1. Map of sampling locations

Table 1. Coordinates of sampling stations

Station	Longitude	Latitude
Gandus	104°39'5704"	03°02'4954"
Ampera	104°45'4451"	02°59'3767"
Pusri	104°48'1529"	02°58'5950"
Upang	104°54'3714"	02°20'1519"
Muara Sungsang	104°56'4946"	02°36'3832"
Tanjung Carat	104°57'2870"	02°43'3199"

Fisheries Research and Fisheries Instructor, Palembang, as well as sending samples to PT. Genetic Science Indonesia.

This research used inoculating loop inoculum needles, inoculating needle inoculum needles, petri dishes, test tubes, test tube racks, test tube clamps, micropipettes and tips, Erlenmeyer flasks, glass beakers, measuring pipettes, dropper pipettes, measuring cups, tweezers, rubber bulb, analytical balance, autoclave, incubator, oven, water bath, light microscope, tip and micro tip, micro tube, hot plate, stirrer bar, vortex, tube rack, Laminar Air Flow (LAF), Bunsen burner, freezer, centrifuge, PCR (Polymerase Chain Reaction), UV-Transiliminator, gel documentation system, cotton, aluminum foil, laboratory coats, masks, latex gloves, tissue, labels, pens and books.

The materials needed were sample water, plastic bottles, nylon nets, snack wrappers, crystal violet solution, Lugol's solution, safranin solution, immersion oil, NaCl, distilled water, buffer solution, NA (nutrient agar) media, NB (nutrient broth) media. GT buffer, GB buffer, WI buffer, wash buffer, lysozyme, proteinase, elution buffer, 70% alcohol, 95% alcohol, ethanol, and gel electrophoresis. Water samples were taken using sterilized glass bottles

Environmental parameters

Water quality measured included salinity using a hand refractometer, current speed using a floating dredge, pH using a pH meter and temperature using a thermometer which was carried out directly at the research location (Melki et al., 2018a).

Bacterial isolation

Purification was carried out to obtain a single colony, which will later be used for microscopic observation and storage of bacteria. The streak planting technique aimed to isolate microorganisms from rejuvenating the culture into a new medium. Bacterial cultures were rejuvenated in slanted NA medium. The tube needle was heated over a Bunsen flame until it glows, then 1 tube of pure bacterial culture was taken aseptically. The loop needle was then scratched onto the slanted NA medium with a zig-zag movement. Bacterial cultures in NA medium were incubated in an incubator for 24 hours at a temperature of 37 °C.

Observing the growth characteristics of bacteria in the growth medium was a step in the identification process referring to namely colony shape, colony edges, colony color, colony elevation, and colony surface texture (Al-Kahtani et al., 2020; Li et al., 2019; Melki et al., 2018b). The gram staining method includes the process of preparing a smear of bacteria on a glass object, then applying a color solution, namely crystal violet, lugol, alcohol and safranin and observing under a microscope (Cappucinno and Sherman, 1999).

16S rRNA gene analysis

The extracted DNA was used as a template to amplify a segment of approximately 300 to 1,500 bp of the 16S rRNA gene sequence using Polymerase Chain Reaction (PCR). PCR product was purified to remove excess primers and nucleotides. Amplification of the 16S rRNA gene by PCR using universal prokaryotic specific oligonucleotide primers for bacteria, namely forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al., 1998).

Genes that had been amplified are separated using gel electrophoresis. Visualization used dye and detection on a UV-transilluminator. Detection result was documented using the gel documentation system (gel-doc) (Afreen et al., 2020; Nchedo Ariole and George-West, 2020). This research

sent the samples to PT. Genetic Science Indonesia to obtain DNA sequencing.

Data analysis

Equation to determine the percentage of plastic weight loss by cutting small pieces of plastic (Ahmed and Swargiary, 2021). In this study, observations were made for 20 days so that the degradation percentage was divided by 20 to obtain the daily degradation rate value, the equation used (Ali et al., 2023).

$$\%\ Degradation = \frac{wi - wf}{wi} \times 100\% \qquad (1)$$

where: wi – initial dry weight (g), wf – final dry weight (g).

The sequencing results are used to search for sequence similarities with existing databases via the Basic Local Alignment Search Tool (BLAST) using an online server (www.ncbi.nlm.nih.gov). Databases were used to compare 16S rRNA sequences and GenBank (Peker et al., 2019). Phylogenetics is a way to confirm the relationship of isolates which is then compared with other DNA sequences in GenBank data through a phylogenetic tree (Dissanayake et al., 2020).

RESULTS

Environmental parameters

According to the temperature data, there is no heat because water quality measurements were carried out before 12 o'clock. The range of temperature values from measurements in the waters of the Musi Hilir River, South Sumatra, was 24.72–26.08°C. Zubair et al. (2019) stated that the optimal temperature for bacteria to grow and develop was 25–37°C. Only two stations, namely Muara Sungsang and Tanjung Carat, had temperature data above 25°C. Le et al. (2019) stated that most aquatic biota were sensitive to changes in pH with an optimum range of 7–7.5. In general, pH values ranging from 7–9 indicate a good water system. The range of acidity values from measurements in the waters of the Musi Hilir River, South Sumatra, 5.36–5.67 can be seen in Table 2.

From the data presented, the Gandus, Ampera, Pusri and Upang locations had a salinity of 0 ppm, and Muara Sungsang, whose water was not fresh, has a salinity of 0.3 ppm. Meanwhile, the Musi water area which was closer to Bangka, the Tanjung Carat location, had a salinity of 1 ppm. This area was close to the estuary, the water conditions were influenced by the salt content of sea water originating from the Bangka Strait (Rozirwan et al., 2022). According to Nason, 1991) current speed was classified into 5 categories, namely very fast current (> 1 m/s), fast (0.5–1 m/s), medium (0.25–0.5 m/s), slow (0.1–0.25 m/s) and very slow (<0.1 m/s). This data explained that the current speed at the Gandus, Ampera, Upang, Muara Sungsang and Tanjung Carat locations was slow. Only the Pusri location had very slow current speeds. The range of current speed values resulting from water quality measurements in the lower Musi River region of South Sumatra was 0.029–0.192 m/s can be seen in Figure 10.

Isolation and morphological identification of bacteria

The bacterial isolate that should had been 12 became 11 because one of the bacteria from station 6 died. Two inoculations were carried out in an effort to grow station 6 bacteria with the fourth dilution characteristic of white color. In fact, the time period for bacterial rejuvenation from the discovery of a pure isolate has not reached one month. So, there were only 11 bacteria which will proceed to the genetic analysis stage of the bacteria to find out their type.

Table 2. Water quality results

Station	Temperature (°C)	Salinity (‰)	pH	Current speed (m/s)
Gandus	24.72	0	5.46	0.1
Ampera	24.76	0	5.42	0.111
Pusri	24.75	0	5.36	0.029
Upang	24.77	0	5.52	0.161
Muara Sungsang	25.94	3	5.67	0.192
Tanjung Carat	26.08	10	5.62	0.172

Table 3. Identification of bacterial morphology

No.	Species of Bacteria	Classification of Bacteria				
		Form	Texture	Elevation	Edge	Color
1.	*Micrococcus luteus*	Round	Dry	Convex	Wavy	Yellow
2.	*Bacillus amyloliquefaciens*	Round	Dry	Convex	Curved	Yellow
3.	*Pseudomonas aeruginosa*	Round	Slimy	Flat	Smooth	Beige
4.	*Shewanella* sp.	Round	Dry	Convex	Curved	Yellow
5.	*Staphylococcus hominis*	Round	Dry	Flat	Smooth	White
6.	*Acinetobacter variabilis*	Round	Slimy	Flat	Smooth	White
7.	*Acinetobacter baumannii*	Round	Slimy	Flat	Smooth	White
8.	*Acinetobacter* sp.	Round	Dry	Flat	Smooth	White

Twelve bacterial isolates obtained 11 gram-negative bacteria and 1 gram-positive bacteria, of which these gram-positive bacteria came from Tanjung Carat station.

Biodegradation

There were three types of plastics used, namely polyethylene terephthalate (PET) plastic bottles, low density polyethylene (LDPE) snack food wrappers, and nylon nets. These degradation test samples were chosen because they are often used by the public and have characteristics that are easier to degrade. The data shows that nylon net plastic degrades a lot due to its characteristics that have particles that are not as dense as plastic bottles and snack food wrappers. The use of new plastics must have a strong binding force between particles.

Table 4. Grams of bacteria

No.	Types of Bacteria	Gram
1.	*Micrococcus luteus*	Negative
2.	*Bacillus amyloliquefaciens*	Negative
3.	*Pseudomonas aeruginosa*	Negative
4.	*Shewanella* sp.	Negative
5.	*Staphylococcus hominis*	Negative
6.	*Acinetobacter variabilis*	Negative
7.	*Acinetobacter baumannii*	Negative
8.	*Acinetobacter* sp.	Positive

The plastic decomposition process is very slow. It takes hundreds of years for plastic to degrade into microplastics and nanoplastics through various physical, chemical and biological processes proposed (Dalimunthe et al., 2021). Within 20 days, plastic degradation had occurred, but with a small amount. It was proven that degradation resulted in the final weight of the plastic being reduced and for the final weight of the plastic not being reduced, a biofilm had actually been formed. Biofilm formation on plastic surfaces is a way for bacteria to degrade plastic. Biofilm formation as stated by (Al-Kadmy et al., 2023) bacteria will change the color of the colony and it can be seen in this study that around the plastic there is a brown liquid. Biofilms filled with bacteria on plastic surfaces show selectivity to the type of polymer and tend to colonise the type of polymer they adhere to

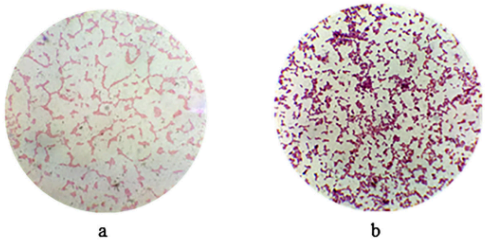

Figure 2. Bacterial gram colour, (a) Gram negative; (b) Gram positive

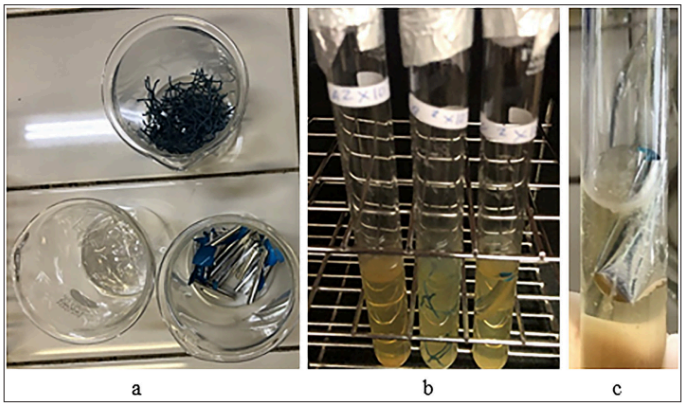

Figure 3. Biodegradation of plastics, (a) plastic type; (b) bacteria break down plastic in nutrient broth; (c) biofilm of plastic surfaces

(Birami et al., 2022). The degradation process by bacteria can lead to the formation of pits and cracks on the plastic surface (Wu et al., 2022). Ahmed and Swargiary (2021) stated that polymer degradation activity depends on the conversion of polymers into oligomers and then into monomers by enzymes produced by microbes. Buchholz et al. (2022) said that these enzymatically digested materials are used by microbes as a carbon source. The production of proteolytic enzymes by microorganisms is a very complex regulated process, depending on the stage of microbial growth.

From Table 4, it is stated that the data on the percentage of weight loss that is able to degrade nylon-type plastics is highest at 7.75% for *Bacillus amyloliquefaciens*, followed by *Staphylococcus hominis* and *Pseudomonas aeruginosa* bacteria at 6.625%. *Pseudomonas* sp. and *Bacillus amyloliquefaciens* are able to degrade LDPE (Han et al., 2022). *Bacillus* is a contributor to plastic degradation (Wu et al., 2022). The ability of bacteria in the degradation process does not occur in all types of plastic. Even though the growth of bacteria in the plastic degradation process is carried out by the same environmental factors by using Nutrient Broth (NB) media which has liquid properties so that it can cover the entire surface of the plastic, then bacteria can be seen growing in the Nutrient Broth (NB) media, characterized by the cloudiness of the media. Bacteria can utilise plastics as their carbon source (Wu et al., 2022), bacteria convert complex polymers into simple monomers through enzymatic degradation as part of metabolism. Microplastics and nanoplastics are generated from macroplastics through physical processes or multi-stage biodegradation (Jaiswal et al., 2022; Wu et al., 2022). Microorganisms in bioflms are sometimes able to catalyse the mineralisation of part or all of the plastic into energy, biomass and inorganic molecules such as carbon dioxide, water, methane, hydrogen and ammonia (Gerritse et al., 2020). Biodegradation of intermediate and final products will not cause secondary pollution and the production of non-toxic compounds (Ji et al., 2022; Xiang et al., 2023). This is in contrast to (Tong et al., 2022; Wu et al., 2022), that stated the toxicity of secondary microplastics and nanoplastics formed during plastic biodegradation is unknown. Biodegradation produces tremendous secondary microplastics and may pose a threat to the environment before reaching full degradation and a major risk of microplastic pollution from biodegradable plastics (Bao et al., 2022; Wei et al., 2022). It is necessary to know the exchange of plastics with the atmosphere and hydrosphere, and the possible transfer mechanisms (Sridharan et al., 2021).

Currently, most research on plastic biodegradation is only at the laboratory scale. There are many challenges in the form of factors affecting

Table 5. Plastic degradation test

No.	Plastic type	Bacterial isolate	Initial weight (g)	Final weight (g)	Weight difference (g)	Loss percentage (%)
1.	Bottle	*Staphylococcus hominis*	0.08	0.08	0	0
	Nets		0.08	0.0747	0.0053	6.625
	Wrap		0.05	0.05	0	0
2.	Bottle	*Acinetobacter* sp.	0.08	0.08	0	0
	Nets		0.08	0.0755	0.0045	5.625
	Wrap		0.05	0.0485	0.0015	3
3.	Bottle	*Pseudomonas aeruginosa*	0.08	0.08	0	0
	Nets		0.08	0.0747	0.0053	6.625
	Wrap		0.05	0.0492	0.0008	1.6
4.	Bottle	*Acinetobacter baumannii*	0.08	0.08	0	0
	Nets		0.08	0.0755	0.0045	5.625
	Wrap		0.05	0.0497	0.0003	0.6
5.	Bottle	*Bacillus amyloliquefaciens*	0.08	0.08	0	0
	Nets		0.08	0.0738	0.0062	7.75
	Wrap		0.05	0.05	0	0
6.	Bottle	*Staphylococcus hominis*	0.08	0.08	0	0
	Nets		0.08	0.0760	0.004	5
	Wrap		0.05	0.05	0	0
7.	Bottle	*Micrococcus luteus*	0.08	0.08	0	0
	Nets		0.08	0.0751	0.0049	6.125
	Wrap		0.05	0.05	0	0
8.	Bottle	*Acinetobacter variabilis*	0.08	0.08	0	0
	Nets		0.08	0.0760	0.004	5
	Wrap		0.05	0.05	0	0
9.	Bottle	*Shewanella* sp.	0.08	0.08	0	0
	Nets		0.08	0.0765	0.0035	4.375
	Wrap		0.05	0.05	0	0
10.	Bottle	*Pseudomonas aeruginosa*	0.08	0.08	0	0
	Nets		0.08	0.0753	0.0047	5.875
	Wrap		0.05	0.05	0	0
11.	Bottle	*Acinetobacter baumannii*	0.08	0.08	0	0
	Nets		0.08	0.0769	0.0031	3.875
	Wrap		0.05	0.0496	0.0004	0.8

degradation such as improving microbial degradation ability, increasing colonisation is complicated, especially in large bodies of water (Li et al., 2023). It is not easy to measure the rate of plastic degradation in the marine environment even when using methods (Gerritse et al., 2020). Strict degradation conditions are difficult to achieve in the natural environment, biodegradation will last longer than the period expected under strict laboratory conditions (Bao et al., 2022). Plastic degradation takes decades or even hundreds of years in the wild (Lin et al., 2022).

Bacterial DNA electrophoresis

Electrophoresis produced a DNA band in well 1 with a 100 bp DNA marker, well 2 a positive control, well 3 a negative control, and the other wells with bacterial codes J1FR to J11FR. The condition of the DNA extracted shows that there is a similarity in sequence between the sample and the primer. Using this primer produces eleven DNA bands with a size of 400 bp. The band obtained was the result of primer and DNA template amplification (Eashur and Jasim, 2022). The

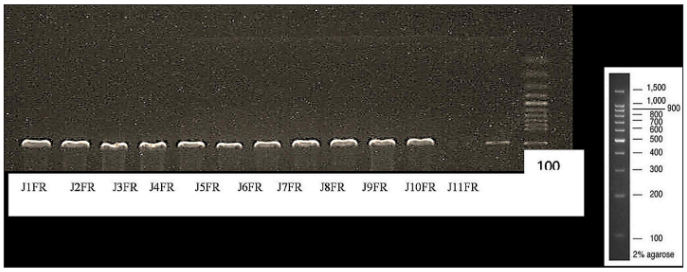

Figure 4. DNA band

Table 6. Bacterial phylogenetic tree barcode

No.	Types of Bacteria	*Barcode*
1.	*Staphylococcus homonis* J1FR	MT585539
2.	*Staphylococcus homonis* J6FR	KY490691
3.	*Acinetobacter baumannii* J11FR	MW255146
4.	*Acinetobacter baumannii* J4FR	EU661695
5.	*Acinetobacter variabilis* J8FR	KY703395
6.	*Acinetobacter* sp. J2FR	MG231214
7.	*Pseudomonas aeruginosa* J3FR	MK719968
8.	*Pseudomonas aeruginosa* J10FR	MZ379421
9.	*Shewanella* sp. J9FR	MG594823
10.	*Bacillus amyloliquesfaciens* J5FR	MN640843
11.	*Micrococcus luteus* J7FR	EU071591

intensity of the DNA band resulting from amplification by primers is greatly influenced by the purity and concentration of the template DNA.

Phylogenetics of plastic degrading bacteria

Genetic barcodes of bacterial types obtained from the NCBI BLAST software showed the highest level of relationship reaching more than 99% which can be seen in Table 6. Phylogenetics is a way to confirm the relationship of isolates which is then compared with other DNA sequences in GenBank data through a phylogenetic tree (Delacuvellerie et al., 2021). Gene families are found in organisms or groups of organisms, phylogenetic relationships between genes can predict the possibility of having equivalent functions (Kapli et al., 2020). The results of this phylogenetic tree will show how close the isolates are as shown by the relationship distance. The greater the relationship value, the greater the similarity of the isolate to the sequence in GenBank data.

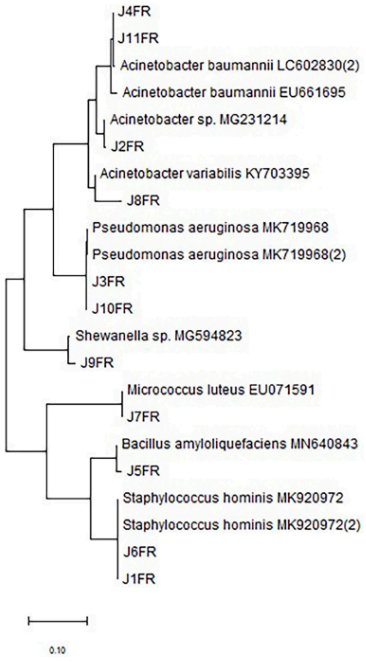

Figure 5. Phylogenetic tree

CONCLUSIONS

This research concluded that the ability of the type of bacteria that had the highest ability to degrade plastic was *Bacillus amyloliquefaciens* for

20 days with a loss percentage of 7.75%. Identification of types of plastic degrading bacteria using 16S rRNA gene analysis showed 11 bacteria with 8 types including *Staphylococcus hominis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Acinetobacter baumannii*, *Acinetobacter variabilis*, *Shewanella* sp., *Micrococcus luteus*, and *Bacillus amyloliquefaciens*. This research used 20 days as the degradation test time which resulted in a small percentage of plastic weight loss. It is best to look for a potential time to test plastic degradation by bacteria. Need to find out if plastic biodegradation can form toxic secondary microplastics or if plastic can be completely degraded.

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