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ISOLATION AND IDENTIFICATION OF MICROPLASTIC DEGRADING BACTERIA IN THE APHOTIC ZONE IN BAGANSIAPIAPI WATERS, RIAU

Sutan Bachar[1*], Feliatra[1], Nursyirwani[1]
[1]Department of Marine Science, Faculty of Fisheries and Marine,
Universitas Riau, Pekanbaru, 28293 Indonesia
*sutan.bachar2819@student.unri.ac.id

ABSTRACT

This study aims to isolate and identify microplastic-degrading bacteria from the aphotic zone in Bagansiapiapi waters, Riau Province. Seawater samples were taken from three different stations, namely the port area, the fisheries area, and the Rokan River estuary. Water quality parameters such as temperature, salinity, pH, current speed, brightness, and dissolved oxygen were measured to support environmental analysis. The method used in this study was a survey method, and the sampling location was around the Bagansiapiapi waters, Riau Province. A total of 9 samples were then taken to the laboratory for isolation and identification of microplastic-degrading bacteria. Bacterial isolation was carried out using Zobell Marine Agar media, morphological characterization and biochemical tests. The isolation results obtained 11 bacterial isolates with different morphological characteristics. The microplastic degradation test showed that three isolates, namely ISL1, ISL3, and ISL9, could degrade microplastics, with ISL1 showing a degradation percentage of 9.45%. Molecular analysis using 16S rRNA and BLAST techniques showed that the ISL1 isolate had a similarity of 99.93% with *Stutzerimonas stutzeri*. These results indicate the potential of native bacteria from the aphotic zone to be used in bioremediation efforts for microplastic pollution in waters.

Keywords: Microplastics, Degrading Bacteria, Aphotic Zone, *Stutzerimonas stutzeri*

1. INTRODUCTION

Environmental pollution due to plastic waste, especially microplastics, has become a significant problem threatening marine ecosystems globally. Bagansiapiapi, a coastal area in Riau Province located on the East Coast of Sumatra Island and directly connected to the Malacca Strait, is one of the areas vulnerable to this pollution. Community activities around the Rokan River estuary, which flows into the Bagansiapiapi waters, produce domestic and plastic waste that is not managed correctly, thus increasing the risk of microplastic accumulation in the aquatic environment.

Microplastics are pieces of plastic measuring less than 5 mm, not only physically dangerous to marine biota due to the potential for ingestion, but also as vectors for dangerous contaminants such as organic compounds and heavy metals. Over time, microplastic particles undergo biofilm colonization by marine microorganisms that can cause these particles to sink to the aphotic zone, the part of the ocean that does not receive sunlight. In this zone, certain microorganisms are known to be able to utilize microplastics as a carbon source, potentially starting the process of plastic biodegradation. Efforts to control microplastic pollution through a bioremediation approach, using plastic-degrading bacteria, are an interesting and environmentally friendly strategy.

Previous studies have identified various genera of bacteria, such as Bacillus,

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Pseudomonas, and Ralstonia, which can degrade plastic polymers[1]. However, data on the types of microplastic-degrading bacteria originating from the aphotic zone in Riau Sea waters, especially Bagansiapiapi, are still minimal.

Based on this background, this study aims to isolate and identify microplastic-degrading bacteria originating from the aphotic zone of Bagansiapiapi waters, and to test the ability of these bacterial isolates to degrade microplastics. It is hoped that the results of this study can contribute as basic data for the development of microbial-based bioremediation technology in the future and increase insight into the potential of marine microorganisms in overcoming the problem of microplastic pollution.

2. RESEARCH METHOD

Time and Place

This research was conducted from July to December 2024. The sampling location was in the waters of Bagansiapiapi, Riau Province.

Method

Sampling locations are determined by purposive sampling. Bagansiapiapi waters are used as a sampling location, determining the location of the sampling point through several considerations based on the source of pollution and water condition criteria. The research location is divided into three station points representing the research area. Station 1 is in the harbor area, Station 2 is in the fisheries area, and Station 3 is in the Muara area.

Procedures

Microplastic Samples

Microplastic samples in water are taken at each sampling point during high tide conditions. Water samples were taken using a Van Dorn at a depth of 2 m, as much as 100 L, and filtered using a plankton net. The filtered water is put into a sample bottle of 1 L. The sample bottle is labelled according to the sampling station, after which it is stored in an ice box, which is then analyzed at the Marine Chemistry Laboratory.

Taking and Handling Samples of Microplastic-Degrading Bacteria

Sampling is done by taking seawater using a Van Dorn, putting it into a 250 ml sample bottle, and then labelling it. Furthermore, the sample is put into an ice box to keep it in its actual microbiological condition during the trip to the Marine Microbiology Laboratory for analysis[2].

Measurement of Water Quality

At the time of sampling, water quality measurements were taken, including temperature, salinity, pH, current speed, and water transparency, to provide an overview of the condition of the waters during the study. Water quality parameters measured include temperature, salinity, pH, current velocity and water transparency. Measurement of water parameters is done 3 times at each station in situ.

Microplastic Analysis

A 500 mL water sample was filtered, and 200 mL of 30% NaCl was added, stirred and allowed to stand for one night. After that, 10 mL of 30% H_20_2 was stirred for 5 minutes and left for 2 nights. The sample solution was filtered using the vacuum pump method and the filter paper in a desiccator for 1 night and analyzed using an Olympus CX 21 microscope. The addition of hydrogen peroxide aims to degrade the filtered organic matter in the sample, so that only microplastics will be observed[3].

The following is the formula for Microplastic Abundance :

$$C = \frac{n}{v}$$

Description:

C = microplastic abundance (Number of particles/m^3)

n = number of microplastic particles

Isolation and Purification of Bacteria

At the isolation stage, seawater samples brought to the Marine Microbiology

Laboratory were homogenized using a vortex, then put into physiological solution (NaCl 0.9%). Then, dilution is carried out using the serial dilution method by taking 1 ml of the sample and then putting it in the first test tube containing 9 ml of physiological solution, so that a 10^{-1} dilution is obtained from the dilution, 1 mL is taken again and then put into a second test tube containing 9 mL to get a 10^{-2} dilution, and so on until a 10^{-6} dilution[4]. Inoculation was carried out on Zobell Marine Agar media by taking 0.1 ml of samples from each dilution and spreading them on a Petri dish containing Zobell Marine Agar L rod. All were incubated in petri dishes with the cup upside down for 24 - 48 hours at 37°C.

Bacterial purification was carried out using the streak plate method. Based on the results of bacterial cultivation, each Petri dish contained several bacterial colonies that showed different morphologies. The bacterial colonies were streaked on Zobell Marine Agar media, then incubated at room temperature for 1 × 24 hours.

Morphological Observation and Calculation of Bacteria

Colonies that grew on Zobell Marine Agar media were directly observed for their morphology. Morphology observed included color, colony shape, size, periphery and elevation of colonies. In addition, bacterial morphology was observed through Gram staining on every one of the different colonies from Zobell Marine Agar.

Gram Staining and Form of Bacteria

A Gram staining test is carried out in each colony to distinguish whether the bacteria are Gram-positive or Gram-negative. Gram-positive bacteria are bacteria that retain purple dye during the Gram staining process. This type of bacteria will be blue or purple under a microscope, while gram-negative bacteria will be red. The difference in classification between the two types of bacteria is mainly based on differences in the structure of the bacterial cell wall

Oxidase Test

Bacterial colonies taken from a 24-hour-old oblique agar culture using an ose needle, then inoculated on filter paper that has been dripped with oxidase reagent (tetramethyl-phenylenediamine). Oxidase reagent was dripped on bacteria as much as 1-2 drops of observation was done for about 10-15 seconds if a purple/blue color appears on the reagent wash, the bacteria produce the oxidase enzyme, otherwise if there is no color change in the oxidase reagent wash, it means the bacteria do not produce the oxidase enzyme.

Catalase Test

The catalase test was carried out by taking a bacterial colony using a sterile ose needle, then applying it to an object glass. Then, a drop of 3% H_2O_2 solution, as much as one drop, is observed. In bacteria that are positive for catalase, O_2 gas bubbles will be seen. Positive catalase test results indicate that bacteria can form O_2 by producing the enzyme catalase through the degradation of hydrogen peroxide carried out by bacteria. If gas bubbles are not formed, it indicates that catalase is negative.

Motility Test

The motility test was carried out by taking a bacterial isolate using an ose needle and stabbing it up to 2/3 on SIM (Sulfide Indol Motility) media. Then, it was incubated for 24 hours or more. If the growth of bacterial colonies appears to spread to the side outside the puncture, the bacteria are motile, whereas if the growth of bacterial colonies is only in the puncture area, the bacteria are non-motile.

Indole Test

The indole test was carried out by inoculating bacteria into SIM media and then incubating at 37°C for 24 hours. Bacteria were given five drops of Kovacs reagent solution and observed for colour changes. If a red ring is formed, the bacteria can form indole from the tryptophan

molecule, while if no red ring is formed, the bacteria cannot form indole.

Sulfide Test

Bacteria cultured on Tryptic Soy Agar (TSA) media were taken 1-2 ose and scratched into the test media, namely SIM media. Furthermore, bacteria incubated for 24 hours at 28°C are observed for changes. The media turns blackish, indicating the bacteria produce H_2S, while the media does not turn blackish, indicating the bacteria do not produce H_2S.

MR Test

The methyl red test was conducted by taking a bacterial colony using an ose needle, inoculating into a test tube containing 10 ml of MR-VP (Methyl-Red Voges-Proskauer) media. Then, all tubes were incubated at 37°C for 24-48 hours, and a control tube containing MR-VP media without any bacterial colonies was included. Drops of methyl red indicator were added to the test tube and observed for changes in microplastic-degrading bacteria. The formation of red colour characterises the results of bacteria that convert glucose into pyruvate / positive. While bacteria that do not convert glucose into pyruvate / negative are characterized by the formation of a yellow color in the media.

Degradation Test

The microplastic waste biodegradation test was determined from the reduction percentage of LDPE plastic samples used by degrading bacteria through several stages[4].

Plastic waste samples measuring 1 cm x 1 cm were weighed initially, washed with sterile distilled water and sprayed with 70% alcohol. Next, the plastic sample is put into a glass sample bottle containing 50 mL of TSB media aseptically. Then, as many as two loops of bacterial isolates were inoculated into the media and shaken at room temperature for one month.

After one month on the shaker, the plastic waste samples were washed with sterile distilled water and sprayed with 70% alcohol. Plastic waste samples were dried and weighed at the final weight. Plastic pieces were weighed using an analytical balance.

The percentage of degradation of plastic waste samples by bacteria was calculated using the formula. Next, take the media that has grown colonies, and dilute them with a physiological solution from 10^{-1} to 10^{-8}. Then, it was planted on Plate Count Agar media with 0.1 mL of dilution, put into a Petri dish containing PCA solid media, and levelled using an L rod. Then incubate for 24-48 hours at 37°C. Count the bacteria after 24-48 hours, then calculate using the formula.

Molecular Analysis of Bacteria with 16S rRNA and DNA Sequencing Techniques

PCR (Polymerase Chain Reaction) method and 16S rRNA gene sequencing are important techniques in molecular-based identification and characterization of microorganisms. The 16S rRNA gene was chosen because it has conservative and variable regions that allow phylogenetic recognition of various bacterial species. Amplification of this gene was carried out using universal primers that are widely used, namely primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') as the forward primer and primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as the reverse primer. PCR reactions are usually carried out in a total volume of 25–50 µL consisting of a mixture of template DNA (\~10–100 ng), each primer with a concentration of 0.5 µM, dNTP of 200 µM, PCR buffer, $MgCl_2$ (1.5–2.5 mM), and Taq DNA polymerase enzyme of 1–2 units.

Thermal conditions of PCR include initial denaturation at 94–95°C for 2–5 min, followed by 30–35 cycles of denaturation at 94–95°C for 30 s, annealing at 50–60°C for 30 s (usually 55°C), and elongation at 72°C for 1–2 min. The reaction is terminated with a final elongation at 72°C for 5–10 min. After PCR, the products are examined by agarose gel electrophoresis and purified using column or enzymatic methods before

sequencing. Sequencing is generally performed using the Sanger method using primers 27F and 1492R. The sequencing results are then analyzed using software such as Chromas or BioEdit and compared with databases such as NCBI BLAST to identify the species of microorganisms based on the similarity of the 16S rRNA gene sequence. The sequencing data is converted into Paste using Bioedit software.

Data Analysis

Observation data, such as water quality, bacterial morphology, bacterial count, and microplastic degradation test, were presented in tables and figures. Differences in the number of bacteria between research stations were analyzed using a One-Way ANOVA (Analysis of Variance) test. The data obtained from the sequence results were analyzed using the BLAST (Basic Local Alignment Search Tool) technique, namely matching the DNA sequence of the test bacteria with the DNA sequence of bacteria on the website https://www.ncbi.nih.nlm.gov/ with MEGA 6 and Bioedit applications. Furthermore, the data were analyzed descriptively, and the results of observations were explained in detail and clearly in the discussion with literature studies and previous research results.

3. RESULT AND DISCUSSION

Water Quality

Bagansiapiapi water quality measurements were carried out on July 6, 2024. The measurement of three sampling stations aims to determine whether the waters of Bagansiapiapi are in good condition. The results of Bagansiapiapi water quality measurements can be seen in Table 1.

Table 1. Bagansiapiapi waters quality condition

Station	Water Quality Parameters					
	Temperature (°C)	Salinity (ppt)	pH	Current (m/s)	Transparency (cm)	DO (mg/L)
1 (Harbor)	31	5	5,58	0,8	0	2,4
2 (Fishing area)	33,9	10,3	5,12	0,5	10	2,7
3 (Rokan River estuary)	31,3	15	5,74	0,4	23,8	2,4

Data in Table 1 indicate values of water parameters in Bagansiapiapi. The average temperature value ranges from 31 to 33.9°C. The water temperature in Bagansiapiapi is still in the normal range and supports marine organisms' metabolic processes and growth. The optimal temperature for bacterial growth itself is in the range of 25-35°C[5].

The average value was obtained between 5-15 ppt in the salinity measurement. The measurement of salinity levels at the three stations showed relatively uniform results, without significant differences. The salinity range still supports optimal bacterial growth. Heterotrophic bacteria can survive in low salinity conditions due to their ability to produce secondary metabolites[6].

The average value of pH (acidity) is between 5.12 and 5.74. Referring to the Decree of the Minister of Environment No. 51 of 2004, the pH level of waters in Bagansiapiapi is below the recommended quality standard for the survival of marine biota. Although acidic, this condition can still support microbial growth, because in general, microorganisms can grow in the pH range between 3 and 6[7].

The value of the current velocity obtained on average is 0.4-0.8 m/s. Measurement of surface current velocity at three stations shows an almost uniform average. Surface currents are influenced by

wind, but their influence decreases with increasing depth. Currents generated by wind and tides play a role in the distribution of microplastics. Strong currents spread them, while weak currents cause accumulation. Currents are also critical in spreading nutrients that support the growth of plankton and bacteria.

Measurement of the level of water transparency of the average value varies between stations, ranging from 0 to 23.8 cm. Measurement of the brightness level at the three stations shows differences in the brightness level at each station. The brightness level of Bagansiapiapi waters is generally classified as poor, where mud substrates dominate the waters. The low level of brightness was caused by the large supply of dissolved particles, organic and inorganic matter through the flow of run off from the mainland through the Rokan River which caused high levels of turbidity and DO (dissolved oxygen) found quite different average results at each station, ranging from 2.4-2.7 (mg/L) each observation station shows the DO concentration of marine waters below the quality standards for marine biota.

Microplastic Analysis

The results of the microplastic abundance analysis that has been carried out can be seen in Table 2.

Table 1. Average microplastic abundance in Bagansiapiapi Waters

Station	Microplastic abundance (Particles/m³)
I	0,08
II	0,08
III	0,05

Table 2 shows that the average abundance of microplastics at the harbor station and fishing area is 0.08 particles/m^3, while the estuary station has an average of 0.05 particles/m^3. In this study, the types of microplastics found in the seawater of the aphotic zone of Bagansiapiapi waters are only microplastics of the fragment, fiber, film and pellet types. The abundance of microplastics is closely related to the population density in an area. Population growth has the potential to increase microplastic pollution in the environment. Therefore, human activities in coastal areas and various environmental factors have an important role in determining the level of abundance of microplastics detected.

Bacterial Isolation

Based on the results of repeated culture and purification, 11 different bacterial isolates were obtained based on colony morphology identification, namely ISL1, ISL2, ISL3, ISL4, ISL5, ISL6, ISL7, ISL8, ISL9, ISL10 and ISL11. More details can be seen in Table 3 for morphological observations of the bacteria.

Table 2. Morphology of bacterial isolates

No	Isolate name	Diameter (cm)	Color	Colony form	Edge	Surface
1	ISL 1	0.2	Pale yellow	Stringy	Curved	Flat
2	ISL 2	0.1	Yellow	Round	Slippery	Like a Drop
3	ISL 3	0.8	Yellow	Round	Slippery	Like a Drop
4	ISL 4	0.2	White	Round	Slippery	Convex
5	ISL 5	0.2	White	Round	Slippery	Like a Drop
6	ISL 6	0.6	Deep yellow	Round	Slippery	Like a Drop
7	ISL 7	0.5	White	Stringy	Curved	Flat
8	ISL 8	0.6	Pale yellow	Stringy	Curved	Flat
9	ISL 9	0.8	Yellow	Round	Slippery	Like a Drop
10	ISL 10	0.3	Pale yellow	Round	Slippery	Like a Drop
11	ISL 11	0.1	White	Round	Slippery	Like a Drop

The morphological differences between the 11 bacterial isolates. Colony diameter ranged from 0.1 to 0.8 cm. The colonies obtained by three isolates have a filamentous shape, and eight isolates have a round shape. Seven isolates have yellow colony color (concentrated and pale) and four isolates have white color. Observation of the shape of the colony surface found seven isolates to have a curved surface, three isolates to have a flat surface, and one isolate to have a convex surface. Observation of the shape of the edges obtained eight smooth isolates and three curved isolates.

Number of Bacteria

The number of bacterial cells in this study tended to show very different results between stations. The highest number of bacterial cells was seen at the harbor station, with an average number of bacteria of 13.6 $\times 10^4$ (CFU/mL), while the lowest number of bacterial cells was found at the estuary station, with an average number of bacteria of 0.2 $\times 10^4$ (CFU/mL) (Table 4).

Table 4. The total number of bacteria in each station

Station	Bacterial count (CFU /mL)
I	13.6 $\times 10^4$
II	0.6$\times 10^4$
III	0.2 $\times 10^4$

Description: CFU = Colony Forming Unit

Biochemical Characteristics

Biochemical tests greatly assist the identification process. Bacteria are identified by observing the biochemical reactions of the bacteria to several test media. The results of the biochemical tests of each bacterial isolate can be seen in Table 5.

Table 5. Biochemical characteristics of bacterial isolates

Isolate Code	Biochemical Test						
	Gram staining	Catalase	Oxidase	Motility	Indole	Methyl red	H_2S test
ISL 1	- (bacilli)	+	+	+	-	+	-
ISL 2	+ (coccus)	-	+	-	-	+	-
ISL 3	+ (coccus)	+	+	-	+	+	-
ISL 4	+ (coccus)	+	+	-	-	-	-
ISL 5	+ (coccus)	+	+	-	-	+	-
ISL 6	+ (coccus)	+	+	-	-	-	-
ISL 7	+ (bacilli)	+	+	-	-	+	-
ISL 8	+ (bacilli)	+	+	-	-	+	-
ISL 9	+ (coccus)	+	+	-	-	+	-
ISL 10	+ (bacilli)	+	+	-	-	+	-
ISL 11	+ (bacilli)	+	+	-	-	+	-

Note: + = Test is positive; - = Test is Negative

Based on Table 5, the results of the Gram staining test showed that ten isolates were Gram-positive and one isolate was Gram-negative. Based on Gram staining, six were Coccus and five were Bacilli. In the catalase test, ten isolates were positive and one was negative. The oxidase test showed positive results for all isolates. The motility test showed one isolate was positive and ten isolates were negative. The ISL3 indole test showed positive results while the other ten isolates showed negative results. For the methyl red test, there were nine positive isolates; two isolates were negative, and the sulfide test showed that all isolates were negative.

Degradation of Microplastics

The data from the microplastic waste degradation test results after being shaken for 30 days can be seen in Table 6.

Table 6. Degradation rate of bacterial isolates

Isolate Name	Average degradation (%)
ISL 1	9.45
ISL 2	-
ISL 3	11.63
ISL 4	-
ISL 5	-
ISL 6	-
ISL 7	-
ISL 8	-
ISL 9	9.22
ISL 10	-
ISL 11	-

They were found to have the potential and were able to reduce the weight of microplastic waste. The three isolates are: ISL1, ISL3, and ISL9. Among the three isolates, ISL3 can degrade microplastic waste well because it can reduce the weight of plastic waste by more than 10% after 30 days of incubation. ISL1 and ISL9 could only degrade +/- 9%. No weight reduction was found in microplastic waste for ISL2, ISL4, ISL5, ISL6, ISL7, ISL8, ISL10, and ISL11 after the degradation test was carried out.

Bacteria generally utilize enzymatic systems both outside and inside cells to decompose microplastics. In the degradation process, bacteria secrete extracellular hydrolytic enzymes such as xylanase, lipase, chitinase, and protease, which are important in breaking down microplastics[8].

BLAST Analysis of Bacterial Isolate DNA Sequencing Results

DNA sequencing was performed on ISL 1 isolate using BioEdit software in this study. The paste results can be seen in the BLAST Results ISL 1 has a percentage of homology that has similarities with the bacteria *Stutzerimonas stutzeri* strain 15.1' with 99.93% homology (Table 7).

Table 7. Bacterial BLAST Results

NCBI Code	Species Name	Sequence Length (bp)	Homology Percentage
KU236477.1	*Stutzerimonas stutzeri*	2566	99.93%
KT380584.1	*Pseudomonas stutzeri*	2566	99.93%
OQ561303.1	*Stutzerimonas stutzeri*	2566	99.93%
MG262445.1	*Stutzerimonas stutzeri*	2566	99.93%
OR455405.1	*Stutzerimonas stutzeri*	2566	99.93%
OR225135.1	*Stutzerimonas stutzeri*	2566	99.93%
ON624167.1	*Pseudomonas sp.*	2566	99.93%
HQ105013.1	*Pseudomonas sp.*	2566	99.93%
HQ105008.1	*Pseudomonas sp.*	2566	99.93%
MT356167.1	*Stutzerimonas stutzeri*	2566	99.93%

Based on the BLAST results, ISL 1 has similarities with several bacteria from the genus Stutzerimonas. For more details, see Figure 4. Several studies have shown that the bacteria *S.stutzeri* can degrade microplastics. Identified seven bacterial isolates that have the potential to degrade microplastics using the 16S rRNA gene, namely *Bacillus sporothermodurans*, *Cytobacillus firmus*, *Rossellomorea vietnamensis*, *S. stutzeri*, *Dyadobacter jejuensis*, *Rhodococcus* sp, and *Achromobacter* sp[9]. Howard also reported that *S. stutzeri* (formerly *Pseudomonas stutzeri*) can degrade polyester10. Meanwhile, Yang[11] found that *S. stutzeri* can reduce plastic weight by up to 6.5%. The phylogenetic tree in Figure 4 was analyzed using the MEGA (Molecular Evolutionary Genetics Analysis) software version 11 by aligning the DNA sequences of bacterial isolates.

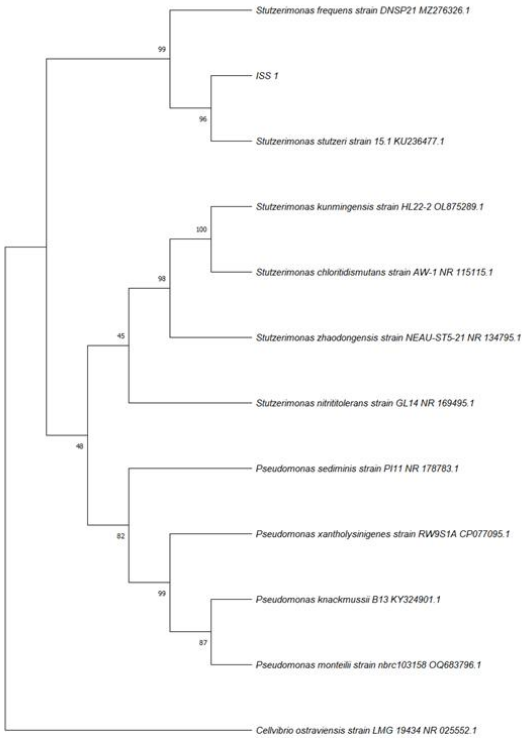

Figure 1. Phylogenetic tree of ISL1 constructed by using the Neighbour-Joining method (Unrooted Tree)

4. CONCLUSION

Plastic-degrading bacteria that can be isolated are 11 isolates. The degradation results found that three isolates were able to degrade microplastics, namely ISL1 (9.45%), ISL3 (11.63%), and ISL9 (9.22%). Isolate 1 is an isolate that can degrade microplastics by 9.45%. The results of identification using 16s rRNA analysis showed that ISL 1 has a base sequence similarity (homology) of 99.93% with *S.stutzeri*, so that it can be said to still be in the same genus, namely *S. stutzeri.*

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