Molecular systematic and phylogenetic analysis of indigenous bacterial isolates with potential as bioremediation agent based on 16S rRNA gene analysis

by Sri Darmawati

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Abstract. Liquid biomedical waste is a form of medical waste from community health centers (Pusat Kesehatan Masyarakat or Puskesmas) with high levels of health hazardous organic contaminants. Bioremediation is an alternative way to eliminate toxic components in liquid waste. A bacteria community that can be used as component of organic waste bioremediation is indigenous hydrolytic and non-pathogenic to low-pathogenic bacteria. From previous studies, 4 hydrolytic indigenous bacterial isolates with such characteristics were obtained from liquid clinical wastes of two health centers in Semarang City, namely H1, H3, H5 (from Puskesmas Halmahera), and T3 (from Puskesmas Tlogosari Kulon). This study aimed to reveal the molecular identity and kinship these bacterial isolates to understand more of their properties as consortium of bioremediation agent. Molecular identification and phylogenetic tree construction works were carried out based on 16S rRNA gene sequences. Sequences of 16S rRNA gene sequences were obtained by isolation and gene amplification using the PCR method followed by sequencing. Based on the results of molecular identification, the four isolates studied were in the same class, namely Gammaproteobacter with Phylum Proteobacter. H1 bacterial isolates have 98.01% similarity with Acinetobacter schindleri. H3 and H5 isolates share the same genus, Stenotrophomonas, with 99.79% similarity with *S. maltophiphila* and 97.69% with *S. acidaminiphila*, respectively. T3 isolate had a similarity of 98.85% with *Pararheinheimera aquatica* species, which was widely known as a potent bioremediation agent. The phylogenetic tree design with the MEGA 6 program showed that the H3 and H5 isolates had the closest kinship compared to the other two isolates, while the T3 isolates had the farthest relationship with the 3 other isolates. This is in line with the fact that T3 isolate was originated from a different location separating it from 3 other isolates.

1. Introduction

Community health center (Pusat Kesehatan Masyarakat or Puskesmas in Indonesia) is a facility provided by the regional government playing role as a first-level individual health service amenity to achieve the highest level of public health in the work area [1]. center produces both non-medical and medical wastes. Biomedical waste medical waste could be in the form of solids, liquids, sharp objects, laboratory waste and drug containers generated from human and animal health activities [2] center liquid domestic and biomedical waste. Liquid biomedical waste contains high levels of organic pollutants, so it could be treated biologically [3].

Bioremediation is a biological clean up or remediation process involves organisms including bacteria to reduce or eliminate pollutants in contaminated areas, which results in recovery to the original state without further disruption to the local environment [4-11]. The main biological agents in the bioremediation process are microorganisms and enzymes [12]. Hydrolases (hydrolytic enzymes) and oxidoreductases are the most explored enzymes for bioremediation purposes. Some examples of hydrolytic enzymes include protease, cellulase, esterase, lipase, phosphatase, cutaneous and amylase [13-14]. Hydrolytic bacteria having low level of pathogenicity or the non-pathogenic ones and can metabolize organic substances play important roles in accelerating the degradation process, so that it will reduce the possibility of pathogenic microorganisms multiplying, of infection and contamination due to these pathogenic microorganisms [15].

In previous study, Arifiani (2018) and Sabrina (2018) showed that there were 4 indigenous hydrolytic bacterial isolates H1, H3, H5 (Halmahera Health Center Semarang) and T3 (Tlogosari Kulon Health Center) which had low pathogenicity or non-pathogenic levels and ability to produce lipases and proteases have the potential as bioremediation agents for handling biomedical waste at health center [16-17]. However, the identity and taxonomic classification of the bacteria is unknown. Identification of these bacteria is needed before they can be used as a consortium, which is a mixture of several bacteria working synergic to degrade biomedical waste. It is widely reported that the use of a bacterial consortium gives better results compared to the use of a single isolate [18].

An effort that can be done to determine the taxonomic identity and classification of a microorganism is by molecular techniques. The advantage of molecular identification its ability to identify bacterial species more accurately and specifically. The 16S rRNA sequencing method is a way to identify microorganisms that can distinguish unique genotypic properties [12]. The product or the result of multiplying the 16S rRNA gene obtained using the Polymerase Chain Reaction (PCR) method is sequenced so that the nucleotide base sequence is obtained. The DNA sequence results data can be monitored through the Local Search Tool Basic (BLAST) at the National Biotechnology Information Center (NBI) and deposited in GenBank for access (www.ncbi.nlm.nih.gov). MEGA X software can connect relationship between living things through phylogenetic tree images. Although there are inadequate contamination and sensitivity problems, this 16S rRNA sequencing method is not affected by phenotypic variation or technological bias, and has the potential to reduce errors [13].

2. Methods

2.1. Bacterial Isolate

The H1, H3, H5, and T3 bacterial isolates obtained from previous study were sub-cultured the Nutrient Agar (NA) medium [16-17]. Sub-cultured colonies on which did not show contamination were used for further step [19].

2.2. Isolation of DNA by Chelex Method

A loop full of bacterial cells from pure colonies grown on NA was mixed with 50-100 µl ddH_2O in 1.5 mL Eppendorf. As much as 1µl saponin 0.5% was then added to bacterial cell suspension, which then mixed with vortex. The mixture was allowed to stand for 1 night at 4 ° C. Samples were later centrifuged at 12000 RPM for 5 min, then the supernatant was discarded. As much as 100 µL ddH_2O and 50 µL telex 20% were then added to the pellet, and the mixture was placed in iso-temp block heater for 10 min at 95 ° C. After the first 5 min, the sample was vortexed. Next, the sample was centrifuged at 12000 rpm for 5 min. The supernatant was moved into a clean and sterile Eppendorf. DNA concentration was measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific) at λ of 260 nm and 280 nm. The results of DNA extraction were stored in a freezer at -20°C [20].

2.3. Polymerase Chain Reaction (PCR) 16S rRNA

The next step is DNA amplification. Primer 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3' was added 1 µl of each to a 0.2 mL-Eppendorf base. DNA template was added as much as 3 µL, ddH2O as much as 7.5 µL and master mix Bioline 12.5 µL. Then the thermocycler is set with the initial stage or pre-denaturation 96°C for 1 minute, denaturation 96°C for

15 seconds, annealing 55°C for 15 seconds, elongation 72°C for 10 seconds, extension 72°C for 12 minutes, with cycle 34 times and cooling down to 4°C [20].

2.4. Electrophoresis

Agarose powder weighed as much as 0.3 g put into a 100 ml Duran bottle dissolve with 30 ml TAE buffer, microwaved for 3-5 minutes with high temperature (150°-200°C), wait until slightly cool, the solution gel is put into a gel mold. Gel inserted into the electrophoresis chamber, then TAE buffer added to inundate the gel. Then the marker and PCR product were put into each of the different wells as much as 5µl. The power supply set for 25 minutes with a voltage of 100 volts. After the gel taken and soaked with EtBr for 15 minutes in the dark place, the gel placed in the UV to the the PCR product [19].

2.5. Sequencing DNA

The PCR products sent to PT. Genetika Science Jakarta for 16S rRNA sequence analysis. through the *Basic Local Alignment Search Tool* (BLAST) were deposited to GenBank to get access number [20].

2.6. Phylogenetic Tree Construction

Making phylogenetic trees with 16S rRNA sequences using MEGA X software [21].

3. Result

The four bacterial isolates were successfully sub-cultured in NA. The bacterial isolate subcultures aimed to make bacterial cells pure from any other contaminating cells, so they could be processed to DNA isolation [19]. The results of the subcultures of the four isolates are shown in Table 1.

Table 1. Morphology of Colonies in Isolate Subculture

Isolate	Shape	Color	Size (cm)	Edge	Elevation
H1	Round	Murky white	0.2	Entire	Convex
H3	Round	Yellow	0.1	Entire	Convex
H5	Round	Bright yellow	0.2	Entire	Convex
T3	Round	White	0.25	Entire	Raised

Morphology of the sub-cultured colonies were the same with those reported by the previous study. Therefore, the purity of the colonies could be confirmed without any contamination.

Genomic DNA isolation of the four bacterial isolates was carried out using chelex method. This method aimed at extracting DNA by separating proteins and other cellular material from DNA molecules [22]. The purity level and concentration of genomic DNA isolation results from the four bacterial isolates using NanoDrop 2000 could be seen in Table 2.

Table 2. Bacterial Genomic DNA Purity and Concentration Data

Isolate	Nucleic Acid Concentration (µg/ml)	A260	A280	Absorbance Ratio
H1	36.3	0.726	0.346	2.10
H3	191.2	3.824	2.111	1.81
H5	2.6	0.051	0.069	0.75
T3	56.6	1.131	0.651	1.74

The 16S rRNA PCR was successfully carried out using the isolated genomic DNA as templates. In general, PCR is a technique for enzymatic amplification or propagation of DNA using primers from specific base sequence pieces [23]. The PCR product was then examined for the presence of the 16S rRNA gene by checking ~1500-bp size amplicons on electrophoresis gel, a technique based on the

movement of charged molecules in a stable matrix media under the influence of an electric field. The most commonly used media is agarose gel [24-25]. Gel electrophoresis results confirming the obtained 16S rRNA gene fragments from all bacterial isolates are shown in figure 1.

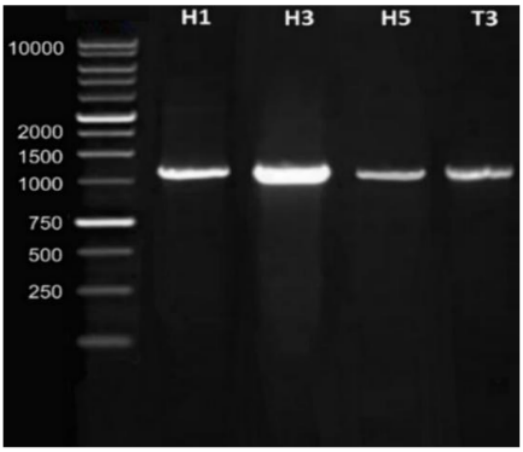

Figure 1. Electrophoresis Visualization with UV Transluminator

For DNA sequencing step, the 16S rRNA gene sequences obtained from forward and reserve primers were aligned [26]. The sequence editing step was carried out with MEGA X software. The 16S rRNA gene sequences were then checked for their similarity or homology to the 16S rRNA sequences belonging to other bacteria present in GenBank with the Basic Local Alignment Search Tools (BLAST) program at www.ncbi.nih.gov.blast. The BLAST results of 16S rRNA DNA sequences of 4 bacterial isolates could be seen in Table 3.

MEGA software created by Masatoshi Nei in 1993 was used to construct phylogenetic trees. The algorithm used in our work was neighbour-joining, which combines sequences to define predicted tree branches and to calculate branch lengths from trees [26]. The constructed phylogenetic tree of the four bacterial isolates studied could be seen in Figure 2.

Table 3. The BLAST results16S rRNA DNA sequences of 4 bacterial isolates

Isolate	*Accession Number*	Size of nucleotide acid (bp)	Proximity of species	Percentage of similarity (%)
H1	LC482255	1405	*Acinetobacter schindleri*	98.01
H3	LC482254	1416	*Stenotrophomonas maltophilia*	99.79
H5	LC482253	1393	*Stenotrophomonas acidaminiphila*	97.69
T3	LC482252	1389	*Pararheinheimera aquatica*	98.85

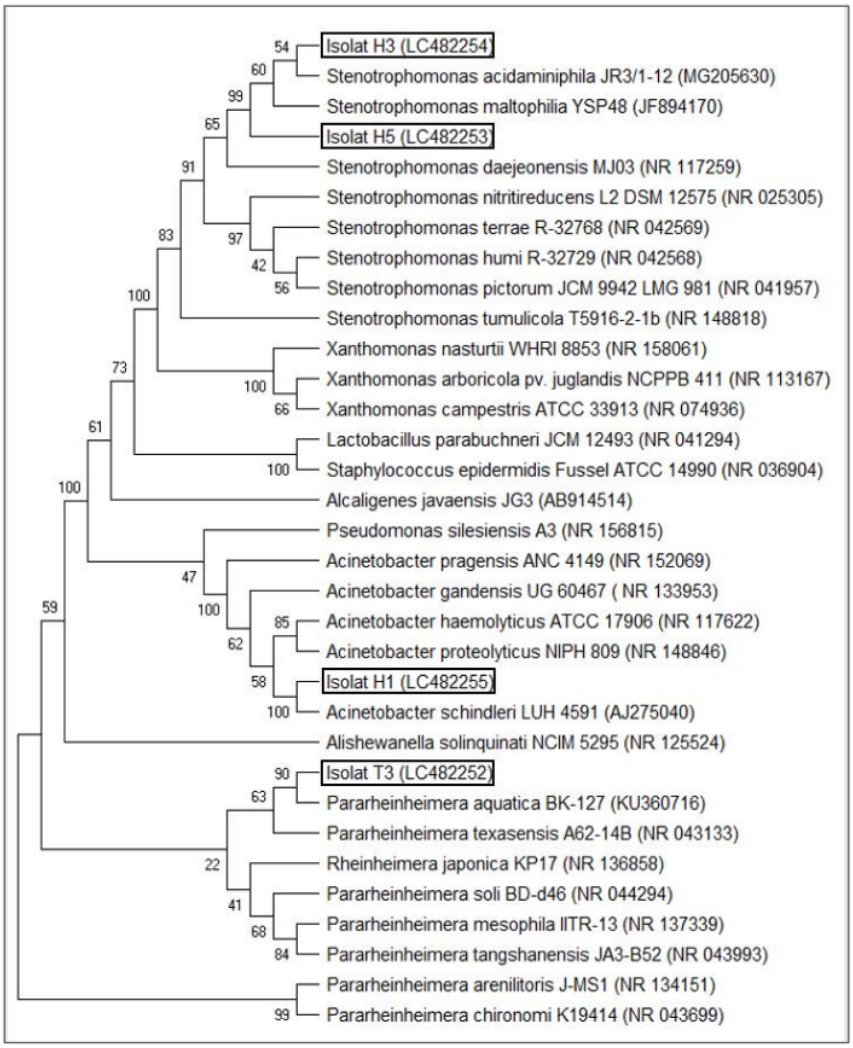

Figure 2. A Phylogenetic tree method of neighbour-joining bacterial isolates that have the potential as a bioremediation agent are isolates H1, H3, H5 and T5 analysed bootstrapping with 1000 repetitions.

4. Discussion

Bacteria that can be used as bioremediation agents are those with a low level of pathogenicity to non-pathogenic and capable of producing enzymes that have an important role in the degradation process [2]. Research by Arifiani (2018) and Sabrina (2018) obtained 4 bacterial isolates that have the potential as bioremediation agents.

4.1. DNA Isolation, PCR and Electrophoresis

Purity and concentration data of isolated DNA, which will be used as PCR tempate (Table 1) show difference levels. Ideally, the purity level of DNA template has A260/280 ratio of 1.8 - 2.0 [27]. DNA template of bacterial isolate H5 has a A260/280 ratio of 0.75 (which means out of the ideal range),

with a nucleic acid concentration of 2,6 μ g/ml. This low ratio value is likely due to protein contamination due to low quality of purification step. Such contamination seemed to affect the electrophoresis results after PCR in Figure 1 causing the H5 isolate DNA band to appear thinner than those of other isolates. The thickest DNA band of H3 isolate is visible. The thicker and collected DNA band shows a high level of concentration and the condition of the total DNA isolated is still intact [28].

4.2. Basic Local Alignment Search Tools (BLAST)

The 16S rRNA gene generally has a nucleotide base length of about 1500 bp [29]. Sequencing results showed a range of nucleotide base sizes from 1393 to 1416 bp. According to Schlaberg et al., 2012 bacteria with a similarity in nucleotide base sequence ≥ 97% to < 99% are bacteria that refer to the same species, < 97% indicate they are identical to comparative bacterial species and more conservative than they are new species, < 95% identified the bacteria as a new genus [30]. The BLAST results in table 3 show that Isolate H1 has a similarity of 98.01% with *Acinetobacter schindleri*. This shows that the H1 isolate was the same species as *Acinetobacter schindleri*. Isolate H3 has 99.79% similarity with *Stenotrophomonas maltophilia* which shows that H3 is the same species as *S. maltophilia.* Isolate H5 has 97.69% similarity with *S. acidaminiphilia* showing that H5 is the same species as *S. acidaminiphilia*. T3 isolates have a similarity of 98.85% with *P. aquatica* showing T3 is the same species as *P. aquatic*

4.3. Family Relation

Phylogenetic tree on the Figure 2 was constructed using neighbor-joining in which the sequences which, when combined, will provide the best estimate of the length of the branch closest to representing the real distance between sequences [26] and analyzed the bootstrap with 1000 repetitions. The greater the bootstrap value, the higher the confidence level of the topology resulting from phylogenetic tree reconstruction [31-32]. According to Hall (2001), the bootstrap branching value of 95% is a grouping value that can be trusted and the bootstrap value of 25% is a value that cannot be trusted [33]. Relationships that are not close to closeness can be caused by gaps in the form of dotted lines in the alignment results [32].

H1 isolates have the same branch as *Acinetobacter schindleri* with a bootstrap value of 100%, indicating H1 isolates have a very close relationship with *A. schindleri.* The results of BLAST H1 isolates also showed a similarity of 98.01% with *A. schindleri*. *A. schindleri* is derived from the genus *Acinetobacter*. This bacterium was commonly isolated from human clinical specimens [34-35], and widely known as opportunistic pathogenic bacterium [36]. This indicates that these bacteria have little possibility to be used as bioremediation agents for health center liquid waste due to their pathogenicity.

Isolate H3 and *Stenotrophomonas acidaminiphilia* are in the same branch with a bootstrap value of 54% showing a close relative relationship with *S. acidaminiphilia.* There is also a branch that is connected with H3 isolate and *S. acidaminiphilia* namely *S. maltophilia* with a bootstrap value of 60%. This shows that *S. maltophilia* has a close relationship with H3 isolates and *S. acidaminiphilia*. The BLAST results showed 99.79% H3 isolates of the same species as *S. maltophilia. S. maltophilia* comes from the genus *Stenotrophomonas*. This bacterium is a Gram-negative bacterium in the form of bacilli, aerobics, non-fermenting sugar and can produce the enzyme lysine. This bacterium is an opportunistic pathogen [37].

The H5 isolate was connected to the *S. maltophilia* branch which was also related to the isolate H3 branch and *S. acidaminiphilia* with a bootstrap value of 99%. This means that the H5 isolate has a very close relationship compared to that of one grandmother with the other three bacteria. The BLAST results also showed that the H5 isolate shared 97.69% similarity with *S. acidaminiphilia. S. acidaminiphilia* is a bacterial species belonging to the genus *Stenotrophomonas*. Its main reservoir is soil and plants [38]. This Gram-negative bacterium was also isolated from industrial wastewater in Mexico with bacilli form, aerobics, and yellow colonies [39]. This bacterium is known for its ability to degrade polycyclic hydrocarbons, a group of organic compounds, which are toxic to marine biota [40]. This bacterium is possible to be used as a bioremediation agent for its ability to reduce polycyclic hydrocarbons, yet, it still might expose toxins to the environment.

Isolate T3 has the same branch as *Pararheinheimera aquatica* with a bootstrap value of 90% which shows that it has a close relative relationship *with P. aquatica. P. aquatica* is a Gram-negative, aerobic bacterium with bacilli form. This bacterium was previously isolated from freshwater aquaculture ponds in Taiwan when filtering bacteria for antimicrobial compounds. This bacterium arises due to the generation of the activity of the enzyme L-lysine oxidase, an antimicrobial substance, which is used to make inhibitory zones in pathogenic bacteria, one of which is *Staphylococcus aureus* [41]. This bacterium has potential to be used as a bioremediation agent because it has ability produces the L-lysine enzyme which is a family of oxidoreductase enzymes. The oxidoreductase enzyme is one of the enzymes that play an important role in bioremediation of biomass [2].

Of the four bacterial isolates, isolates H3 and H5 had the closest relation compared to the other two isolates. Both isolates were from the Halmahera Health Center Semarang and are connected to the branching line which is likened to a grandmother's brother. There is also a branch connecting H1 isolate to H3 and H5 isolates, but this branch is located very far away. Although H1, H3, and H5 isolates were taken from the same place, the results showed that H1 isolates were bacteria with different genera and were closely related to isolates H3 and H5. T3 isolate has the most distant relationship with three other isolates. This is in line with the fact that T3 isolate was originated from a different location separating it from 3 other isolates.

5. Conclusions

Four indigenous, hydrolytic bacterial isolates, T3, H1, H3, and H5 were successfully identified using PCR. Isolate T3 molecularly identified as *Pararheinheimera aquatic*, while the 3 other isolates, H1, H3 and H5 were identified as *Acinetobacter schindleri*, *Stenotropomonas acidaminiphilia*, and *S. maltophilia*, respectively. Among the 4 studied isolates, H3 and H5 have the closest kinship, yet the T3 isolate has the farthest relationship with the 3 other isolates.

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