Identification of the bacterium isolate from Mackerel Fish (Rastrelliger sp.) using 16S rRNA Gene

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Abstract. The use of probiotics in aquaculture can improve the quality and quantity of the fishes. One of potential probiotic bacterium from gastrointestine of mackerel fish (Rastrelliger sp.) isolated in this research were LUI-04. This study aimed to determine the species of bacterium isolate using nucleotide sequence homology compared to other microorganisms in GenBank. The 16S rRNA genes of LUI-04 isolate were analyzed. The results of electrophoresis showed the DNA band had a size of 1500 bp for amplification using Bact-27F and Uni-1492R primers, and about 250 bp to 500 bp with the Com-IF and Com-2R primers. The sequence of nucleotides was determined by Sanger method. The 16S rRNA sequence of the isolate and other bacteria in GenBank were compared with the Basic Alignment Search Tool (BLAST) to know the homology. Then, all of the sequences were aligned by ClustalW, and the phylogenetic tree was reconstructed with Neighbor Joining method using bootstrap analysis of 1000 replicates. The results showed that LUI-04 had a homology of 99% and the bootstrap value percentage of 86% with Bacillus aryabhattai B8W22 and four strains of Bacillus megaterium (strain QM B1551, NBRC15308, strain IAM 13418 and ATCC 14581). Therefore, the LUI-04 isolate belongs to Bacillus megaterium species.

Keywords: Aquaculture techniques, controlling pathogenic microbes, economic growth of fisheries, phylogenetic tree, potential probiotic bacteria.

1. Introduction
Aquaculture techniques in Indonesia at this time to the future have an important role for development of the fishery sector. This is because the technique is known to increase the economic growth of fisheries [1]. One of the techniques used is utilization probiotics as control agents [2]. The addition of probiotics in aquaculture can improve the quality and quantity of the products. Green methods use probiotic bacteria as a disinfectant and antibiotic in controlling pathogenic microbes. The use of this method in aquaculture is increasing rapidly compared to the use of chemicals [3]. Probiotic bacteria protect the host organism from pathogenic bacteria by producing antimicrobial compounds [4]. In addition, probiotic microbes have the ability to increase immunological and non-immunological defenses of the host organisms’ intestines, by increasing bowel permeability and maintaining the balance of microflora contained in the digestive tract [5].

Potentially probiotic bacteria can be found in several types of aquatic animals such as fish and shrimp. Some isolated probiotic bacteria include Lactobacillus plantarum, Bacillus sp., Streptococcus
lactis, Lactobacillus bulgaricus, Carnobacterium divergens, Vibrio pelagius, Pseudomonas fluorescens, Vibrio alginolyticus, Flavobacterium sp., Lactococcus lactis [2].

Exploration of potentially probiotic bacteria from fish in Indonesia is still very limited. One attempt to explore the probiotic bacteria is by isolating and identifying bacteria phenotypically and genotypically [6]. Screening or selection of potential bacteria probiotics performed with several stages of laboratory testing. Identification of bacterial species, antagonism testing of pathogenic bacteria, and a series of enzymatic tests are usually performed in probiotic selection [7]. Identification of potentially probiotic bacteria species using phenotypic analysis includes morphological observation and physiological activity assay [8, 9]. In addition to phenotypic analysis, genotypic analysis can also be used to determine the type of bacteria. 16S rRNA gene is the most appropriate gene used for bacterial genotypic analysis [5, 6, 10].

The genotypic analysis is usually performed using the 16S rRNA gene as a bacterial chronometer. Analysis of 16S rRNA is known to be more effective because the genes are universal in bacteria, have a conserved region and have a variable region that can be used to distinguish between bacteria [11]. According to Claridge et al. [12], the location of the same region in the 16S rRNA gene is at the beginning of the gene (base position 9-27), middle area (base position 515-531, 519-536), and the end (base position 1524-1541). So this part can be the region for analyzing comparison between groups of bacteria.

From the previous research, some potentially probiotic bacteria were isolated from mackerel fish (Rastrelliger sp.) intestine [13]. One of the bacterium isolate, LUI-04, was reported phenotypically belong to the genus Bacillus sp. [9]. Therefore, this study aimed to analyze the 16S rRNA gene of LUI-04 isolate and determine the species of the bacterium based on the molecular characters.

2. Materials and methods

The research was conducted in the Microbiology Laboratory of Department of Biology, and Biochemistry Laboratory of Department of Chemistry, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Banda Aceh.

The tools used were incubator (Memmert), autoclave (Yamato Scientific), erlenmeyer flasks, hotplate, aluminium foil, bunsen lamp, petri dishes, dropper, digital scales (Sartorius TE 214S), measuring glasses 100 mL and 1 000 mL, measuring pump, chemical glasses, inoculation needles, centrifugation machine, laminar air flow cabinet, waterbath shaker, pH meter to make buffer solution, microtubes 1.5 mL; micropipette (Eppendorf), PCR tubes 2.5 uL; 50 uL; 10 uL to 100 uL; micropipette 100 uL to 1 000 uL (Eppendorf) and and the tips, Veriti 96 Well Thermal Cycler (Applied Biosystems) to amplify DNA, Owl Easycast BI (Thermo Scientific) for electrophoresis, EC200XL Power Supply (Thermo Scientific), Gel Omega Lum (Aplegeri) for visualization of electrophoresis and computer results.

The materials used in this study were the potentially probiotic bacterium LUI-04 isolate from mackerel fish (Rastrelliger sp.) collection of the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Trypticase Soy Broth (TSB) media, Trypticase Soy Agar (TSA) media, absolute alcohol, 70 % alcohol, aquadest, sterile aquabidest (ddH2O), 0.5M EDTA pH 8, Sodium Dodecyl Sulphate (SDS), Tris Base, HCl, glacial acetic acid, CH3COOK, chloroform, isoamyl alcohol, isopropanol, Taq DNA polymerase, dNTP mix, MgCl2, agarose, gel red, electrophoresis kit, 1 kb DNA Ladder (Thermo Scientific) marker, Bact27F and UniB1492R primer pair, Com1F and Com2R primer pair with nucleotide sequences can be seen in table 1.
Table 1. Nucleotide sequences of primers used.

| Primers      | Nucleotide sequences                          | References |
|--------------|-----------------------------------------------|------------|
| Bact27F      | 5′- AGAGTTTGATCATGGCTCAG-3′                   | [14,15]    |
| Uni1492R     | 5′GGTTACCTTGTTACGACTT-3′                     | [15]       |
| Com IF       | 5′- CAGCAGCGCGGTAAATAC-3′                    | [14]       |
| Com 2R       | 5′- CCGTCAATTTCTTATGTTT-3′                   | [14]       |

2.1. Regeneration of the bacterial isolate

Bacterial LUI-04 isolate in a glycerol stock culture was taken aseptically with a micropipette and spreaded in a Petri dish containing TSA media. Then incubated for 24 h to 48 h at 35 °C. Furthermore, a single bacterial colony that grew was inoculated on the TSB medium in Erlenmeyer flask. Comparison of media with Erlenmeyer size is 1:3. Then incubated in a waterbath shaker at 35 °C for 24 h.

2.2. Isolation of bacterial genomic DNA

Chromosomal DNA from the potentially probiotic bacterial LUI-04 isolate was isolated by chemically cell lysis method using buffers aimed to damage the cell wall without damaging the desired DNA. Cell pellets were suspended in 200 μL Tris-Cl buffer pH 8 and incubated at 37 °C for 1 h. The cells were then lysed by adding 200 μL of lysis buffer then incubated at 50 °C for 30 min. Lysis buffer contains SDS 2 % (w/v), 0.8 mg mL$^{-1}$ in 200 μL EDTA pH 8. Diluted microorganism cells were added by 150 μL of cold C solution and vortex-mixed for 10 s. Cold C solution was prepared by mixing 60 mL CH$_3$COOK 5 M and 11.5 mL glacial acetic acid in 28.65 mL sterile aquabidest. The mixture was then incubated on ice for 5 min. Thereafter, the solution was centrifuged at 6 000 g (times gravity) for 10 min. The resulting supernatant was transferred to a new centrifugation tube, and 300 μL of chloroform-isoamil alcohol (24:1) was added. The mixture was subsequently vortex-mixed and centrifuged at 16 000 g for 30 s. The addition of chloroform-isoamil alcohol was repeated twice. Further DNA was precipitated by adding 600 μL of isopropanol and incubated at room temperature for 1 h. Thereafter, the mixture was centrifuged at 16 000 g for 15 min. The resulting pellet was washed 3 times with 70 % ethanol. Subsequently suspended in 50 μL of sterile aquabidest. DNA was analyzed qualitatively with agarose gel electrophoresis. The DNA sample was stored in a freezer with a temperature of -20 °C.

2.3. Amplification of the 16S rRNA gene using PCR

The 16S rRNA gene was amplified using two different primer pairs of Com1F-Com2R and Bact27F-Uni1492R. The reaction mixture was prepared in a sterile 200 μL PCR tube. The PCR reaction mixture consisted of 1 μL Com1F (20 pmol mL$^{-1}$) primer, 1 μL Com2R (20 pmol mL$^{-1}$) primer, 3 μL chromosomal DNA of the bacterial isolate, 1.25 μL Taq DNA polymerase, 5 μL buffer solution 10×, 0.4 μL dNTP mix (dATP, dGTP, dCTP, dTTP), 4 μL MgCl2, 34.35 μL ddH$_2$O that made the total volume was 50 μL. The reaction mixture for the Bact27F and Uni1492R primer pair was made in a different PCR tube. Annealing temperature conditions are listed in table 2.

Table 2. Temperature conditions for primer pairs annealing

| Bacterial isolate | Primer pairs                           | Temperature (°C) |
|-------------------|----------------------------------------|-----------------|
| LU 1-04           | Bact27F + Uni1492R                     | 51              |
|                   | Com1F + Com2R                          | 50              |

2.4. Electrophoresis of 16S rRNA gene amplicon

The results of the 16S rRNA gene amplification from PCR were electrophoresed using 1 % (w/v) agarose gel. The agarose gel was prepared by mixing 0.6 g of agarose in 60 mL of TAE 1× buffer. Agarose was heated to boil and let briefly at room temperature to remove the bubbles and then added 2 μL of gel red dye. Then molded and waited until solid. Buffer TAE 1× was made by diluting TAE
50× buffer from the stock solution. Buffer TAE 50x containing 10 mL 0.5 M EDTA pH 8, 5.71 mL glacial acetic acid, 2.42 g tris-base. All the material was diluted with aquadest in a 1 000 mL measuring flask. Each 5 μL amplified DNA was added by 1 μL of loading buffer 6x DNA loading dye and suspended on a parafilm sheet. DNA marker was made by adding 4 μL sterile aquabidest, 1 μL 1 kb ladder DNA marker and 1 μL loading buffer 6× DNA loading dye. Electrophoresis was carried out for 90 min with a current of 500 mA and a voltage of 45 V using TAE 1× buffer as carrier solution. The electrophoresis results were visualized using Gel Doc with a UV lamp device at a wavelength of 302 nm to 365 nm.

2.5. Phylogenetic analysis
The nucleotide sequences of the 16S rRNA gene with the two primer pairs were combined and analyzed using the SeqMan program in DNA STAR. Sequences of the isolate were compared with the nucleotide sequences of other microorganisms in GenBank using Basic Alignment Search Tool Nucleotide (BLAST-N). BLAST-N is accessible on the National Center for Biotechnology Information (NCBI) website. Then the phylogenetic tree was reconstructed with MEGA 6 software, using the Neighbor Joining reconstruction method with a bootstrap test of 1 000× replications.

3. Results and discussion
Based on figure 1, the results of regeneration of bacterial isolate showed fairly good colony growth and has its own colonic characteristics. The colonies of the LUI-04 is large round with wavy edge and cream color. Growth of bacterial colonies is relatively rapid during 24 h of incubation, this can be proved by the density of colonies that grew on TSA media. The bacterial incubation process was carried out for 24 h to 48 h at 35 °C. Such incubation conditions can provide optimum growth for potentially probiotic bacteria. Based on previous research conducted by Safrida et al. [13], the isolates had fairly good colony growth at 30 °C to 37 °C for 24 h. Barman et al. [5] states that probiotic bacteria can grow at a temperature range of 30 °C to 37 °C. Based on Feliatra et al. [10] research, some probiotic bacteria from the digestive tract of fish can still grow below 30 °C, but cannot grow at 45 °C.

![Figure 1. The growth of LUI-04 isolate on TSA (Trypticase Soy Agar)](image)

The DNA electrophoresis results in figure 2 showed that the DNA bands of the LUI-04 isolates were above the size of 10 000 bp when compared to the 1 kb ladder DNA marker (size 250 bp to 10 000 bp). DNA bands above 10 000 bp indicated that the chromosomal DNA was successfully isolated and the isolated DNA was not damaged. Damaged DNA in the form of broken and degraded DNA by nuclease enzymes can be marked by the size of DNA bands below 10 000 bp. Nuroniyah and Putra [16] reported that the pattern of the bands of electrophoresis from bacterial genomic DNA using 250 bp to 10 000 bp DNA marker is in the area above 10 000 bp. According to Ntushelo [11], bacterial DNA molecules have size > 10 000 bp, which ranges from 21 000 bp to 23 000 bp. The DNA that has been known for its good quality can be then used for the 16S rRNA gene amplification steps.
Figure 2. The genomic DNA electrophoresis of LUI-04 isolate (M = Marker 1 kb DNA Ladder; K = negative control (ddH2O); 1 = DNA of LUI-04 isolate).

Based on the electrophoresis results in figure 3, the LUI-04 isolate amplified with the Bact27F - Uni1492R primer pair and the Com1F - Com2R primer pair showed the presence of fixed bands. The isolated LUI-04 DNA bands were amplified with Bact27F and Uni1492R primer pair separated in areas parallel to the 1500 bp DNA marker size. The DNA bands of LUI-04 isolate using the Com1F and Com2R primer pair were between the sizes of 250 bp to 500 bp DNA marker size. Based on Baker et al. [14] research, amplification with the Com1F and Com2R primers was performed on a small sub-unit fragment of 16S rRNA. The amplification product generated by these primers was about 400 bp size. As for Bact27F and Uni1492R primers can amplify the 16S rRNA gene intact with an amplification product about 1 500 bp size. Several other studies have also reported that the 16S rRNA gene produces a single band of about 1 500 bp to 1 600 bp [5, 10, 16,]. The result of the amplification of 16S rRNA gene of LUI-04 isolate have obtained intact 16S rRNA gene with size close to 1 500 bp. Based on that, it can be concluded that the amplification process of 16S rRNA gene from the LUI-04 isolate was successfully performed.

Figure 3. The amplified 16S rRNA genes using Bact1F-Uni1492R primer pair and ColF-Com2R primer pair (M = Marker 1 kb DNA ladder; 1 = negative control (ddH2O); 5 & 6 = LUI-04 isolate amplified with primers Bact27F and Uni1492R; and 8 = LUI-04 isolate amplified with the primers Com1F and Com2R).

From the result of LUI-04 isolate phylogenetic tree reconstruction in figure 4, it can be seen that the LUI-04 isolate formed monophyletic group with four strains of Bacillus megaterium, three strains of Bacillus flexus, and one strain of Bacillus aryabhattai. Based on the phylogenetic tree, the LUI-04 isolate has the closest kinship with two bacterial strains of two Bacillus genera, i.e. the Bacillus megaterium strain QM B1551 and the Bacillus megaterium strain IAM 13418. The bootstrap values obtained at branch formation were 86 %. Based on this, it can be concluded that LUI-04 isolate has a close kinship with both strains of Bacillus megaterium. Based on sequences alignment from BLAST application (in supplementary data), the LUI-04 isolate has 99 % homology with strain Bacillus aryabhattai strain B8W22 and four strains of Bacillus megaterium (strain QM B1551, strain NBRC15308, strain IAM 13418 and strain ATCC 14581). According to the data above, it can be concluded that the LUI-04 isolate identified as Bacillus megaterium.
Figure 4. Phylogenetic tree of LUI-04 isolate

This genotypic analysis results using the 16S rRNA gene of LUI-04 isolate gave similar results with the phenotypic analysis of LUI-04 isolate. Previous research conducted by Yulvizar [9] reported that LUI-04 isolate phenotypically belongs to the genus *Bacillus* sp. From several studies, Watson et al. [4] concluded that bacteria from the *Bacillus* genus originating from the marine environment have the ability as probiotic agents for animals. Bacteria from this genus act as antipathogens to *Aeromonas hydrophila* and *Vibrio harveyi*. Cruz et al. [17] stated that several species of the *Bacillus* genus isolated from the marine environment act as growth promoters, antipathogens, improve water quality, help digestion of nutrients, and improve reproduction in the aquaculture system.

4. Conclusions

Based on the results of this study, it can be concluded that the LUI-04 isolate belongs to the *Bacillus megaterium* species, since it has the closest kinship with four strains of *Bacillus megaterium* (strain QM B1551, strain NBRC15308, strain IAM 13418 and ATCC strain 14581) with 99 % homology sequence and 86 % bootstrap value.

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