Isolation of Antibiotic-Producing Bacteria from Extreme Microhabitats in Mangrove Ecosystem

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Abstract. This study aims to isolate and identify the antibiotic-producing bacteria from extreme microhabitats in the marine ecosystem against fish pathogenic bacteria (V. alginolyticus, A. hydrophila, dan Pseudomonas sp). Microbes from samples were grown on NA and TSA media. Well grown colonies were examined for their ability to produce antibiotics against the pathogens by the streaking and paperdisc diffusion method on MHA. Potential isolates were identified through a series of phenotypic and genotypic test. A number of 4 antibiotic-producing bacteria were selected. DNA of the bacteria were extracted using DNA easy mini column and amplified by using Kappa PCR kit with 16S rDNA 1A and 16S rDNA2A primers. The PCR results were analyzed on a 1.5% agarose gel in Tris-acetat-EDTA (TAE) electrophoresisuffer (4 mM Tris-acetate and 1 mM disodium EDTA at pH 8.0). The electrophoresis process was carried out at 60 mA (100-120 v) for 30-45 minutes read by UV Transiluminator. Isolate N5 have a 99% homological similarity to Bacillus amyloliquefaciens strain SH20. Bacterial isolate X3 have a 100% homological similarity with Bacillus cereus strain NY180. Isolate S1.1 has a 97% homological similarity with Enterobacter hormaechei strain CGAPGPBBS-064. Isolate K2.2 has a 99% homological resemblance to Klebsiella pneumoniae strain MW-W 754.

1. Introduction
Antibiotics are chemical compounds produced by microorganisms, especially by fungi (now synthetically available) that can kill or inhibit the development of bacteria and other organisms. Since the beginning of the 20th century antibiotics as chemotherapeutic agents have been successful in fighting infectious diseases even though to this day infectious diseases are still the main cause of death worldwide.

Intensive aquaculture has caused the emergence of several bacterial diseases [1], which have led to increased use of antibiotics. Basically the use of these drugs is aimed at the treatment of diseases and improving the health status of fish and improving the quality of the environment [2]. However, the use of such drugs in commercial fish culture may increase the pressure on microbial flora and promotes the natural increase in antibiotic resistance [3].

Some groups of microorganisms can live in an extreme environments. Such environmental conditions provide tolerance, metabolic mechanisms, and cell resistance to that extreme environment. This capability can bring value and application in various fields of industry, such as food, agriculture, pharmacy and medicine, and biotechnology.

In marine ecosystems there are some extreme microhabitats that can be a source of new antibiotic-producing bacteria. This study aims to isolate and identify the antibiotic-producing bacteria from extreme microhabitats in the marine ecosystem against the fish pathogenic bacteria (Vibrio alginolyticus, Aeromonas hydrophila, dan Pseudomonas sp).
2. Methodology
Samples of 0.01-1.0 gr were taken from some extreme microhabitats (intestinal aquatic biota, reptiles and insects, decayed organic matter and others) in the mangrove ecosystem at the Marine Station, Riau University in Dumai, Riau. The samples were put into a sterile physiological solution, diluted and grown on NA and TSA media. Well grown colonies were selected and tested for their ability to produce antibiotics against pathogenic bacteria by the streaking and paperdisc diffusion method on MHA media. The test was proceeded to paperdisc diffusion method on Muller Hinton Agar media. Positive results is indicated by a clear zone around the disc paper.

Potential antibiotic-producing isolates are then identified through a series of phenotive and genotypic tests. Morphologically the color, shape, size, margin and elevation of the colonies were observed, gram stained. Physiological tests include catalase production, sulfide production (H₂S), sugar use, motility, citrate production, and methyl red test. A total of 4 isolates, considered as most potential one, were selected and identified molecularly. DNA of the bacteria were extracted using DNA easy mini column and amplified by using Kappa PCR kit with 16S rDNA 1A and 16S rDNA2A primers. The PCR results were analyzed on a 1.5% agarose gel in Tris-acetate-EDTA (TAE) electrophoresisuffer (4 mM Tris-acetate and 1 mM disodium EDTA at pH 8.0). The electrophoresis process was carried out at 60 mA (100-120 v) for 30-45 minutes read by UV Transilluminator [4].

The PCR results from bacterial isolates were sent to PT. Genetics Science, Jakarta for DNA sequencing in the 16S rRNA region. The results of sequencing of bacterial isolates were carried out by BLAST first to determine the correctness of the diagnosis results. Furthermore, the kinship relationship with phylogenetic tree was analyzed using the Maximum Parsimony method and Neighbor Joining with 1000 bootstrap resampling.

3. Result and discussion
Some extreme microhabitat samples from marine ecosystems have been taken. A total of 400 bacterial colonies were selected and screened against 3 (three) isolates of fish pathogenic bacteria through the streaking and paper diffusion method. Ten potential antibiotic producing bacteria were isolated and identified phenotypically. A total of 4 isolates, considered to be the most potential, were identified furthermore genotypically.

The total DNA extraction results from these four isolates were electrophoresed on 1% agarose gel. The extraction results show very clear DNA bands in columns C, D and A (Figure 1). The results of the amplified DNA samples were subsequently electrophoresed with 1% agarose gel and the results can be seen in Figure 2. This figure shows the size of PCR a single band products were close to 1500 bp according to the DNA marker. The size of this size is in accordance with the expected size of 16S rDNA bacterial genes, 1500-1600 bp.

In this study sequencing process was carried out by sequencing nitrogen bases using the ABI 3130 Genetic Analyzer machine. The 4 sequences of isolates used 24F primers: 5'-AGA TGA TGT CT-3' and 1541R: 5'-AAG GAG GTC ATC CAG CCG CA-3', carried out in one direction once on each primer used by cycle back and forth. Base pairs obtained after merging between fasta forward and reverse with sequen program on DNAstar, the total length of base pairs for each isolate is; Ni5 1056 bp, Xy3 1405 bp, S1.1 1479 bp, K2.2 1056 bp.

DNA sequencing technology has revealed information on genotypes of a number of organisms. The results of phylogenetic analysis of 4 selected isolates is presented in Table 1.
Figure 1. Electrophoresis result of DNA Total Extract

Remark:
M : Fragmen marker DNA 1 kb ladder
A : Isolat Ni₅
B : Isolat Xy₃
C : Isolat S₁₁
D : Isolat K₂₂

Figure 2. Electrophoresis result of PCR product on 1 % gel agarose

Remark:
M : Fragmen marker DNA 1 kb ladder
A : Isolat Ni₅
B : Isolat Xy₃
C : Isolat S₁₁
D : Isolat K₂
Table 1. Results of 16S rDNA bacterial isolates with the BLAST system

| Isolate | Species                           | Strain       | Kode Akses    | Homolog |
|---------|----------------------------------|--------------|---------------|---------|
| Ni5     | Bacillus amyloliquefaciens       | SH20         | KY362201.1    | 99 %    |
| Xy3     | Bacillus cereus                  | NY180        | MK215791.1    | 100 %   |
| S1.1    | Enterobacter hormaechei          | CGAPGPBBS-064| KY495222.1    | 97 %    |
| K2.2    | Klebsiella pneumonia             | MW-W 754     | KC835109.1    | 99 %    |

Isolates with similarity level of 16S rDNA more than 97% can represent at the same species level. The level of similarity of 16S rDNA sequences between 93-97% can represent identities at the genus level but differ at the species level. Whereas if under 93% it is possible that a new species whose sequence of nitrogen bases has not been included in the bank's gene data base [5].

Isolate Ni5. The results of 16S rDNA sequence homology from Ni5 bacterial isolates (isolated from decayed Nipa fruticans hump) had similarity with Bacillus amyloliquefaciens strain SH20 with a homology level of 99%. This means that the homology level is the same to the species level. From the homology results it can be seen that the molecular weight of 893 bp of nitrogen matches the pair of molecular weight of 895 bp of nitrogen contained in the bank's gene base data. Based on the results of the search for taxonomy listed at the National Center for Biotechnology Informatics (NCBI), the isolate is Bacillus amyloliquefaciens.

The ability of the isolate to produce antibiotics has been found by some researchers. Bacillus amyloliquefaciens B94 strain produces iturin A used as biocontrol to suppress the disturbing fungi of Rhizoctonia solani plants [6]. B. amyloliquefaciens is a species in the genus Bacillus which is a source of BamH1 restriction enzymes. This microbe also synthesizes natural barnase protein antibiotic, which is widely known as ribonuclease which forms a well-known complex as an antibiotic with selective activity against Bacillus anthracis.

B. amyloliquefaciens GA1, a type of bacteria that has four gene groups capable of synthesizing biocontrol agents; cyclic lipopeptide surfactin, iturin A and fengycin and bacillibactin iron-siderophore. In addition to the non-ribosomal peptides synthesized, three additional gene groups directing the synthesis of macrolactin, bacillaene and difficidin polyrolide antibodides are also identified [7].

Isolate Xy3. The results of 16S rDNA sequence homology from Xy3 bacterial isolates (isolated from decayed X. granatum stems) have similar homologies with Bacillus cereus strain NY180 with a homology level of 100%. From the results of homology, it can be seen that the molecular weight of 720bp nitrogen matches the pair of 720bp nitrogen molecular weight contained in the bank's gene base data. Based on taxonomy listed at the National Center for Biotechnology Informatics this isolate is also Bacillus cereus.

Similar results were reported by a researcher [8] who isolated the bacteria from the oyster (Saccostrea cucullata). It was reported Bacillus cereus strain SU12 as a protease producer. These bacteria are actually enterotoxin-producing pathogens that can inhibit the growth of other microbes [9].

B. cereus is a spore-forming bacterium that produces toxic material that are usually found in the environment (for example soil) and various foods. Spores can survive in extreme environments including temperature. Gram-positive, motile, spore forming, rod-shaped or bacil. These species include B. anthracis, B. cereus, B. mycoides, B. thuringiensis, B. pseudomycides and B. weihenstephanensis [10][11].

Genomic sequence data show that B. anthracis, B. cereus and B. thuringiensis have very close affinity [12] with a sequence of 16S rRNA genes sharing more than 99% similarity [13].

Isolate S1.1. The results of the 16S rDNA sequence homology of bacterial isolates S1.1 (isolated from ant Hymenoptera) have homological similarities with Enterobacter hormaechei strain CGAPGPBBS-064 with a homology level of 97%. From the results of the homology analysis, it can be seen that the molecular weight of 889 bp nitrogen matches the pair of 919 bp nitrogen molecular weight found in the genebank data base. Based on taxonomy listed at the National Center for Biotechnology Informatics (NCBI) this isolate was Enterobacter hormaechei. These bacteria are
pathogens that are often found in soil, water, sea and food ingredients. *E. hormaechei* is the Enterobacter group producing an extended-spectrum beta-lactamase (ESBL). This enzyme is inhibiting and even killing other bacteria [14].

Isolates K2.2. The results of 16S rDNA sequence homology from bacterial isolates K2.2 (obtained from the crab; *Scylla serrata* digestive tract) have similar homologies with *Klebsiella pneumoniae* strain MW-W 754 with homology levels of 99%. From the results of homology, it can be seen that the molecular weight of 738bp of nitrogen matches the pairing of the molecular weight of 740 bp of nitrogen contained in the bank’s gene base data. Based on the results of the search for taxonomy listed at the NCBI this isolate is *Klebsiella pneumoniae*.

*K. pneumoniae* is a non-motile, non-sheathed gram negative bacterium, facultative anaerobes, found as a normal flora in the mouth, skin and intestines. The *Klebsiella* species shows mucoid growth, non motile and usually gives positive results for decarboxylase, lysine and citrate tests. This bacterium forms capsules, but do not form spores, and facultative anaerobic bacteria. The results of this study are quite interesting considering that bacteria are pathogenic bacteria and cause serious infections in humans [15]. Their presence will be very serious if the condition of the host is weak. So far the author has not found a reference that states that this bacterium is capable of producing antibiotics.

4. Conclusions and Suggestions

Microhabitats, namely the digestive tract of crabs and mangrove ant, and decayed stem of *X. granatum* and *N. fruticans* can be used as a source of antibiotic-producing bacteria to fish pathogens *A. hydrophila*, *V. alginolyticus*, *E. tarda* and *E. coli*. The bacteria phenotypically and genotypically identified as *B. amyloliquefaciens*, *B. cereus*, *E. hormaechei*, and *Klebsiella pneumonia*. It is suggested that isolates can be studied further and tested for implementation in the field.

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