The Effectiveness of Heterotrophic Bacteria Isolated from Dumai Marine Waters of Riau, Used as Antibacterial against Pathogens in Fish Culture

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Abstract. Heterotrophic bacteria have an important role as decomposer of organic compounds (mineralization) derived from industrial waste, decomposition of unconsumed feed, faecal, excretion of fish, and have the ability to inhibit the growth of pathogenic bacteria. We investigated the role of heterotrophic bacteria used as antibacterial against pathogens in fish culture. This research was conducted from January until March 2017. The phylogenetic of the isolated bacterial was determined by 16S rDNA sequences analysis. Antagonism test showed that the bacteria had the ability to inhibit the growth of pathogenic bacteria (Vibrio alginolitycus, Aeromonas hydrophila and Pseudomonas sp.) Three isolates (Dm5, Dm6 and Dm4) indicated high inhibition zones which were classified into strong category with the average from 10.5 to 11.8 mm toward V. alginolitycus. Other isolates were classified into medium and weak category. Based on DNA analysis of heterotrophic bacteria isolated from marine waters of industrial area and low salinity of estuarine waters twelve strains of bacteria were identified, and all had highest level of homology to Bacillus sp., one isolates has similarity to Enterobacter cloacae, other isolates to Clostridium cetobutylicum. Most of isolated bacteria obtained from the waters of industrial area due to it received much of nutrients that very influenced the growth of bacteria.
Keywords: heterotrophic bacteria, antagonism, pathogenic bacteria, 16S rDNA sequence, marine waters.

1. Introduction
Dumai marine waters is one of the Sumatera’s marine waters which is very crowded with navigation, industrial activity as well as residents’ bustle around its coast. The activity is seemed to be the main contributors of both organic and inorganic materials, causing pollution to the river mouth. Numerous physical processes, industrial activity and anthropogenic and marine transportation [1] influence the concentration of organic and inorganic chemical compound affecting the distribution and activity of
bacteria. Basically, marine microorganisms are as various as the ones exist in land. Organisms that can be classified into marine microbes are protista, cyanobacteria, bacteria, mold and viruses. These microorganisms have a critical role in the process occurring in sea water column [2]. In fact, bacteria are the organisms that are mostly huge in number and dissemination compared to other living things. The existence of bacteria population indicates sanitation, showing that the water has been polluted by waste. Generally, bacteria are facultative and heterotrophic, which allow them to live completely anaerobic condition.

Heterotrophic bacteria and other microorganisms are organisms having a crucial role in biogeochemical cycles within aquatic ecosystem for their ability to decompose and remineralize organic materials to simple inorganic material, which contains nutrition for phytoplankton, perifiton, and other aquatic microflora. Thus, bacterial activity in marine waters is very important [3]. The existence of heterotrophic bacteria is extremely affected by the entry of organic materials from the river mouth to Dumai marine waters.

This research aims to isolate and characterize heterotrophic bacteria using 16S rDNA sequences, and analyze their ability as an antibacterial agent toward pathogenic bacteria in mouth of River Mesjid and Dumai marine waters of Riau Province.

2. Materials and Methods
The research was conducted from January until March 2017. The sample was collected from four stations with different salinity, those were the mouth of River Mesjid, and marine waters of industrial area in Dumai of Riau Province. Heterotrophic bacteria was isolated in The Microbiology and Marine Biotechnology Laboratory, Marine Science Department, Faculty of Fisheries and Marine Sciences. The DNA identification was conducted in Molecular Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, Riau University, while the DNA purification and sequencing processes were performed by the Malaysian First Base sent by PT. Genetika Science Indonesia, West Jakarta.

The bacteria was isolated on medium Nutrient agar (NA, Merck, Cat.No. 1.05450.0500). For DNA isolation cultures of heterotrophic bacteria were grown in 4 mL of liquid Luria-Bertani medium (LB) at 37°C for 24 hours [4]. The cultures were then removed to 1.5 mL microtube and were centrifuged at a speed of 13,000 rpm (revolutions per minute) at 4°C for 2 minutes. The supernatant was discarded, the pellet was dried and was dissolved in 40 μL of Tris-EDTA (TE) buffer solution at pH 8, then 20 μL of lysozyme was added to the solution. The solution was inverted 3-5 times, and incubated for 10 minutes at 37°C. After incubation, 50 μL of 10% sodium dodecyl sulfate (SDS) was added, the mixture was gently rotated (20 rpm) for 5-10 minutes in a rotamix rotator. After that, 550 V of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the mixture, and the mixture was further rotated gently (20 rpm) for 10 minutes. The mixture was then centrifuged at 7,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new microtube, and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the tube. The mixture was gently rotated (20 rpm) in rotamix rotator for 10 minutes. The mixture was then centrifuged at 7,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new microtube and 3 M sodium acetate was added (40% volume) to the aqueous phase. Two times volume of 99% cold alcohol was then added to this solution to precipitate the DNA, and the solution was chilled at -20°C for 30 minutes. The precipitated DNA was centrifuged at 13,000 rpm and 4°C for 10 minutes, and the supernatant was discarded. The DNA pellet was washed in 1 mL 70% cold ethanol and centrifuged for another 10 minutes at 13,000 rpm and 4°C. The supernatant discarded, resulting DNA pellet was then air dried. The pellet was dissolved in 40 μL TE-buffer (pH 8) and 2.5 μL of RNase solution were added and homogenized using vortex mixer and incubated at 37°C for 1 hour. Finally, the DNA of probiotic candidate bacteria was ready to be used, or stored at -20°C.

2.1. Polymerase Chain Reaction (PCR).
The PCR reaction was performed by using 8 μL DNA of each probiotic candidate bacteria as template, and adding 23 μL of H2O, 4 μL of 10x Taq polymerase, 0.5 μL of 12 mM dNTP mix, 2 μL for each of universal primers consisting of forward primer 24F (5'AGAGTTTGATCCTGGCT -3') and reverse primer 1541R (5'AAGGAGGTGATCCAGCCGCA-3') and 0.5 μL Taq polymerase. PCR conditions were: warming up for 2 minutes at 94°C, followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 40 seconds at 50°C, and strand extension for 1 minute at 72°C.

2.2. Purification of DNA PCR fragments
After PCR reaction, the DNA obtained was separated in a 1% Agarose gel by electrophoresis. PCR DNA bands were purified from the Agarose gels using the Geneaid® Gel/PCR DNA Fragments Extraction Kit following instructions by the manufacturer.

2.3. Sequencing, BLAST and phylogenetic analysis.
Purified PCR products were sequenced with ABI 3130 XL Genetic Analyzer Applied Biosystem. Sequencing was done at the First Base Malaysia, from PT. Genetika Science Indonesia, West Jakarta. BLAST analysis was done using the tools and data of Gen Bank accessed at http://www.ncbi.nih.gov/blast. DNA sequences were aligned using CLUSTAL W 2.1. Phylogenetic trees were generated using the tools provided by CLUSTAL W, and trees were visualized using MEGA version 0.5 program. The data obtained from the isolation and identification of heterotrophic bacteria, and the activity against pathogenic bacteria (*Vibrio algynolyticus*, *Aeromonas hydrophila* and *Pseudomonas* sp.) were presented in tables and figures.

3. Result and Discussion

3.1. Water Quality Measurement
Water sample was collected from four stations, and each station sampling was determined at three sites of which the distance between them was 50 m (Figure 1). At the same time, water quality parameters were measured. Data of water quality parameters were presented in Table 1.
Table 1. Water quality condition in each sampling station of Dumai waters.

| Station | Coordinate     | Time (WIB) | pH  | Salinity (ppt) | Transparency (cm) | Temperature (°C) | Current velocity (m/s) |
|---------|----------------|------------|-----|----------------|-------------------|------------------|------------------------|
| 1       | N 01° 42'09.717'' W 101°22'38.745'' | 10.16 | 6.5 | 10             | 49.5              | 27               | 0.13                   |
| 2       | N 01°42'13.154'' W 101°22'44.457'' | 10.00 | 6.5 | 20             | 52.5              | 28               | 0.19                   |
| 3       | N 01°43'38.500'' W 101°23'06.906'' | 11.40 | 6.5 | 25             | 93.5              | 29               | 0.18                   |
| 4       | N 01°41'57.706'' W 101°24'30.942'' | 13.43 | 7   | 30             | 60                | 30               | 0.34                   |

Condition of water quality measurement was as follows: samples were collected from 08:00 am until 14:00 pm; pH value ranged from 6.5 to 7.0; water salinities at station 1, 2, 3 and 4 were 10‰, 20‰, 25‰ and 30‰, respectively; water temperatures were 27-30°C, and the current velocities range was 0.13-0.34 m/s (Table 1). This area is located in the east part region of Sumatera island which is directly opposite to Rupat island.

Dumai city is located at the east coast of Sumatera island. This area integratedly develops its coastal area as a productive region for growing and cultivating its primary export commodity fish such as grouper, white snapper, crabs and pomfret through the restoration function of mangrove area. Dumai also has a harbour that can be functioned as a transit area for traveling to neighboring countries, such as Singapore and Malaysia. Besides, Dumai marine waters is considered as a place of industrial activity for several companies such as Pertamina UP II Dumai, PT. Patra Dock, PT. Chevron Pasifik Indonesia, PT. Sarana Sawitindo, and palm oil refinery Bukit Kapur Raksa and PT. Splar Cooperation.
3.2. Bacteria isolation

After being cultured and purified repeatedly, 14 different bacteria isolates were selected based on morphological characters from each bacterial colony, and the ability to produce antibacteria towards the pathogenic bacteria. The isolates were DM1, DM2, DM3, DM4, DM5, DM6, DM7, DM8, DM9, DM10, DM11, DM12, DM13 and DM14. The morphological characteristic of those isolates were presented in Table 2.

Table 2. Morphological characteristics of 14 bacterial isolates from Dumai waters

| Isolate Name | Plate No | Diameter (cm) | Color           | Colony Form                  | Border           | Surface         |
|--------------|----------|---------------|-----------------|------------------------------|-----------------|-----------------|
| DM1          | 1.3 (-5)1| 0.4           | Yellowish white | Round                        | Waved smooth     | Even            |
| DM2          | 2.2 (-5)1| 0.5           | White           | L-shape                      | Curved relief    |                 |
| DM3          | 1.1 (-5)2| 1.5           | White           | Irregular                    | Smooth           | Even            |
| DM4          | 2.2 (-6)1| 1.4           | White           | L-complex form               | Irregular        | Relief           |
| DM5          | 2.2 (-4)1| 1.8           | Yellowish white | Circular with relief edge    | Wavy             | Bumpy           |
| DM6          | 2.1 (-5)3| 1.2           | Yellowish white | Rhizoid L-complex            | Branched         | Drop-like       |
| DM7          | 2.2 (-4)1| 1.8           | Yellowish white | Having Rhizoid Thread        | Wavy             | Bumpy           |
| DM8          | 20 (-6)2 | 1.9           | White           | Wrinkled                     | Curved           | Even            |
| DM9          | 20 (-5)2 | 1.4           | White           | Irregular                    | Wavy             | Bumpy           |
| DM10         | 20 (-4)3 | 0.7           | White           | Irregular                    | Wavy             | Even            |
| DM11         | 20 (-5)3 | 1.0           | White           | Round with reef edge         | Wavy             | Even            |
| DM12         | 30 (-5)2 | 1.2           | Red             | Round                        | Smooth           | Even            |
| DM13         | 30 (-6)3 | 0.9cm         | White           | Round                        | Irregular        | Even            |
| DM14         | 30 (4)3  | 1.3cm         | White           | Round                        | Smooth           | Even            |

The observation result indicated that the colony size varies between 0.4-1.9 cm which varied from diverges to white, yellowish white, and even red (DM12). The colony shape varied from round, irregular, and even coral-like edge. As for the border, there were also various like waved smooth and irregular. The colony surfaces were diverse to even, relief, and bumpy. This indicated that the colonies were indeed distinctive from each isolate to others (Table 2).

3.2. Antagonism of bacteria isolates

Results of antagonism test conducted in triplicate were presented in Table 3. Each bacterial isolate had an ability to inhibit the growth of pathogenic bacteria, indicated by the appearance of clear zone around the paper disc. There were three isolates (DM4, DM5 and DM6) demonstrated strongest inhibition power of 10.5-11.8 mm clear zone toward V. algynolyticus (Table 3). Other three bacteria isolates indicated the biggest clear zone toward pathogen A. hydrophila were DM14, DM8 and DM5 with 5.8-7.8 mm clear zone (Table 3). Regarding to pathogen Pseudomonas sp., three isolates (DM1, DM11 and DM13), indicated clear zones of 6.5-7.6 mm (Table 3).

According to Pratama [5] when the diameter of inhibition zone is more than 20 mm, the response classification of bacterial growth inhibition will be intense. In addition, when the diameter of inhibition zone is in the range of 10-20 mm, the response classification of bacteria growth inhibition is still categorized intense. Yet, when the diameter of inhibition zone is merely around 5-10 mm, the response of bacteria growth inhibition is medium, and if it is less than 5 mm, the response will be weak. To conclude, this research found there were only three bacterial isolates that were powerful toward pathogen V. algynolyticus, and the rests showed medium and weak response.

Antimicrobial test of bacteria isolated from Dumai waters indicated that one potential isolate (DM11) in inhibiting pathogenic bacteria V. algynolyticus (9.6mm), A. hydrophila (5.6mm) and Pseudomonas sp (6.8mm) at medium level in comparison to other 13 isolates. Meanwhile, the isolate
DM5 performed the largest clear zone toward *V. alginolyticus* (11.8 mm). Then, isolate DM14 showed the largest clear zone against *A. hydrophila* (7.8 mm), and finally, isolate DM1 had the largest clear zone toward *Pseudomonas* sp. (7.5 mm) as shown in Table 3.

The heterotrophic bacteria inhibited pathogenic bacteria (*V. alginolyticus*, *A. hydrophila* and *Pseudomonas* sp.) by the production of antibiotic products, bacteriocin, or certain organic acid. This finding was in agreement to Verschuere et al. [6] who noted that microbial population can actually release the chemical materials having bactericidal or bacteriostatic capacity, which can prevent the growth of other bacteria by several factors, such as antibiotic production, bacteriocin, siderophore, lysozyme, protease, and hydrogen peroxide. In addition, it can also influence pH of media by generating certain organic acid. The antibacterial agent such as lactic acid and bacteriocin contained by probiotic bacteria were able to inhibit the development of pathogenic bacteria [7]. This occurs due to the antibacterial agent could decrease or lower the pH, thus the pathogenic bacteria are difficult to survive [8].

Beeneva et al. [9] performed a research on heterotrophic bacteria in several marine waters, and discovered 68.9% isolates from temperate zone and 56.76% of Vietnamese strain showed antimicrobial activity. The strains showing the greatest activity were of the tropical origin.

| Isolat | Inhibition Zone Diameter (mm) |
|--------|-----------------------------|
|        | *V. alginolyticus* | *A. hydrophila* | *Pseudomonas* sp. |
| (+) U₁  | U₂  | U₃  | R  | (+) U₁  | U₂  | U₃  | R  | (+) U₁  | U₂  | U₃  | R  |
| DM1     | 4   | 2   | 2   | 1   | 1.6  | 1.5  | 1   | 3   | 4   | 2.6  | 8   | 1   | 9   | 12.5 | 7.5 |
| DM2     | 1.5 | 8   | 9.5 | 8   | 8.5  | 3    | 4.5 | 3   | 5   | 3.6  | 6.5 | 1   | 3   | 1.5  | 1.8 |
| DM3     | 8   | 6.5 | 8   | 4.5 | 6.3  | 2    | 2   | 2   | 1.5 | 1.8  | 5.5 | 5   | 5   | 6.5  | 5.5 |
| DM4     | 5.5 | 3.5 | 15  | 13  | 10.5 | 5    | 2   | 3   | 3   | 2.6  | 6   | 3   | 2.5 | 3   | 2.8 |
| DM5     | 2   | 10  | 12.5 | 13  | 11.8 | 6    | 6   | 6   | 5.5 | 5.8  | 7   | 3   | 3   | 6.5  | 4.1 |
| DM6     | 5.5 | 4   | 13  | 17  | 11.3 | 6    | 2   | 3.5 | 3.5 | 3   | 3   | 5   | 5.5  | 5.5  | 5.3 |
| DM7     | 3   | 6   | 6   | 0.5 | 4.1  | 5    | 0   | 2   | 3   | 2.5  | 6   | 1   | 11  | 7    | 6.3 |
| DM8     | 5   | 5   | 5.5 | 5   | 5.1  | 4    | 5   | 6.5 | 8   | 6.5  | 8   | 4   | 3   | 4    | 3.6 |
| DM9     | 12  | 11  | 12  | 9.5 | 10.8 | 3.5  | 7   | 3   | 3   | 4.3  | 5.5 | 4   | 5   | 4.5  | 4.5 |
| DM10    | 4.5 | 5   | 5   | 4   | 4.6  | 3    | 2.5 | 4.5 | 5   | 4   | 10.5 | 3   | 7   | 3    | 4.3 |
| DM11    | 14  | 12  | 11  | 6   | 9.6  | 6    | 2   | 6   | 9   | 5.6  | 7.5 | 4   | 7.5  | 9    | 6.8 |
| DM12    | 5   | 2.5 | 2.5 | 3   | 2.6  | 5    | 2.5 | 4   | 4.5 | 3.6  | 10.5 | 3.5 | 4   | 2.5  | 3.3 |
| DM13    | 2   | 0   | 4   | 5   | 3    | 3    | 2.5 | 4   | 5.5 | 4   | 2   | 6   | 9.5  | 4    | 6.5 |
| DM14    | 2   | 3.5 | 7   | 2.5 | 4.3  | 13   | 10  | 6.5 | 7   | 7.8  | 1   | 0   | 1   | 1    | 0.6 |

The result above have been multiplied by 6 mm paper disc. Information: (+): positive control; (0): U₁: 1st recurrent; U₂: 2nd recurrent, U₃: 3rd recurrent,; R: average
Figure 2. The result of Total DNA Electrophoresis of bacteria isolate in 1.2% agarose. DNA marker was 1kb ladder.

Figure 3. PCR Product of DNA Electrophoresis

From the electrophoresis shown in Figure 3, the size of PCR was going on for 1500 bp. This great size was as the expected result from the 16s rRNA genes. Sabdono stated that bacteria isolate amplification possessing single band revealed that primer used was specific one to be applied to amplify the 16S rRNA of bacteria. The 16S rRNA amplification had become a standard to study phylogenetic and diversity of marine microorganism.

From the sequence analysis of bacteria isolate that had been Blasted in Table 4, it was found that there were several bacteria having higher level compared to other kind of bacteria. There was a possibility of close relationship of each isolate such as the DM1 isolate which had 97% homology rate to strain ASK16 *Bacillus cereus*, DM2 indicated 96% homology rate to strain A C *Bacillus toyonensis*, OPR1150Xg of DM3 had 94% homology rate to strain F1-8*Pseudozyma pseudomycoides*. Furthermore, the association was as well occurred to DM4 which had 97% homology rate to strain SBFW51 *Bacillus cereus*, DM5 possessed 97% homology rate to strain OPP5 3-2 *Bacillus cereus*, DM6 had 96% homology rate towards strain SBFW5S *Bacillus cereus*, and DM7 which had 97% homology rate towards strain B4 *Bacillus cereus*.

Moreover, the homology of DM8 isolate was similar to strain ACOPR1ISOXg *Bacillus toyonensis* with 95% homology rate and had 1541bp base length. It implies that the homology rate was only similar at the genus level and was different at species level. Based on a research *B. toyonensis* forming spores, was appropriate to be used as an active element of
TOYOCERIN additive. The notion is well supported by Jimenez et al., [10] on his research declaring that the TOYOCERIN has shown inhibition toward the growth of pathogenic bacteria.

As for DM9 isolate, it has homology similar to strain Y37 B. subtilis, with 96% homology rate and 1609bp base length. Implicitly, its homology rate is identical merely until the genus level but not at the species level. As noted by Pant et al., [11], B. subtilis looks like a trunk and has the ability to compose endospore. This bacterium has also play a role in health sector to yield bacitracin antibiotic, and in industrial sector to produce bacterialamylase or other materials that could modify extract, glue papers, and to release glue for textile. The B. subtilis bacteria can be found in either river or marine sediment [12].

Then, the DM10 isolate was known for its similar trait to strain Enc-3 Enterobacter cloacae with 99% homology rate and 1469 bp. This indicated that the homology rate was identified until the species level. According to Lina et al. [13] Enterobacter cloacae lived in diverse environment, both in land and water (soil and food waste). This strain lives as a microflora commensal within human and animal intestines, and play a fundamental role as a pathogen for plants and insects.

Next for the DM11, its homology was the same to strain S512 Clostridium acetobutylicum, having 96% homology rate and base length as 1542 bp. Unlike DM10, the homology level was similar merely until the genus level. Clostridium acetobutylicum was a commercial bacterium, belongs to Clostridium genus. This bacterium was a trunk-like Gram positive and mostly found in main land, thought it could also be found in various circumstances. According to Logan and Devos [14], Clostridium acetobutylicum could be found in land, river sediment, wells, intestines, cattle excrement, dogs and human feces.

Bacillus cereus exists in DM12 and DM13 isolates. The homology of DM13 isolate was similar to strain 4PLGES B. cereus with 99% homology and 756 bp base length. In other words, the homology rate was similar until the species level. Meanwhile, F isolate homology was similar to strain ML267 B. cereus with 96% homology rate and 1517 bp length. Similar to DM11, the homology level was similar onli until genus level.

The primary habitat of B. cereus is the surroundings and the canal of digestion. Water and soil have a significant function for this bacterium in polluting food. This bacterium can also adhere to shoes, clothes, skin or through air and dust [15].

According to Khetan [16], the B. cereus bacterium is one of pathogenic agents possessing great potential to be utilized as a biological control. The bacterium has particular host sharmless to pests’ natural enemies and other non-target organisms, which are easily disjointed by the environment, and its pathogenicity can be improved using genetic engineering technique. The B. cereus bacterium is a probiotic bacterium having the ability to hinder the pathogenic bacteria growth as Vibrio sp and Aeromonas sp [17].

The DM14 isolate has homological resemblance to strain C17 B. thuringiensis with 97% homology rate and 1546 bp base length. Its homological rate is identical until the species level. B. thuringiensis bacterium is a heterotrophic bacterium living in established and fine environment. As stated by Lantang and Runtuboi (2012), B. thuringiensis exists in natural realm and could also be discovered in various habitats, like soil, water, and mud.

B. thuringiensis could produce 2 types of toxin: crystal toxin (Crystal, Cry) and cytolytic toxin (cytolytic, Cyt). Cytotoxin could fortify the Cry toxin so that it is considerably used to develop the effectiveness in controlling the insects. Several studies have been conducted to isolate and purify the bacteriocin from Bacillus sp generated by B. cereus, and toxin yielded by B. thuringiensis [19].

From the end results, it can be understood that the fourteen heterotrophic isolate bacteria gotten from Dumai marine waters are genus from Bacillus bacterium. The bacteria of Bacillus, Bifidobacteria, Pseudomonas, Lactobacillus and Micrococcus genus have been proved useful and could dwell both inside and outside a normal organism, and are also believed to be beneficial probiotic bacteria [20]. The bacteria were also assumed to have ability in inhibiting the pathogenic bacteria growth, which was known based on the antagonism test result, showing the existence of clear zone
around the disk. This is also indicated in a research conducted by Suwardi [21]. The bacteria resulting antimicrobia are Bacillus sp., A1 and A2. To find out the characteristics of antimicrobial producer bacteria, morphology, physiology, and analysis of protein were carried out using SDS-PAGE. The morphology observation exposes that the colony has a circular shape with medium size and convex surface. The color of the colony is white and that its edge is even. Further, from the Gram staining and BA1 and BA2 bacteria, the morphological traits are obtained. The bacteria appear as trunk bacteria cell and possess Gram positive traits (purple). As in the spora staining, BA1 and BA2 are identified to have oval shape positioned in the middle of the bacterium. Russell et al. [22] conducted a study on inland waters using 16s RNA technique and found that 3 bacteria are able to respire nitrate and oxygen. The cultures represent members of abundance phyla, as determined by amplicon sequencing of environment DNA extract, and allow for further studies into geothermal factors impacting life in the deep sub surface.

Table 4. The channel accessed from Gen Bank database used in determining the phylogenetic relationship towards bacteria isolate

| Isolate | Species                    | Strain | Access Code | Homology |
|---------|----------------------------|--------|-------------|----------|
| DM1     | Bacillus cereus            | ASK16  | KY750685    | 97%      |
| DM2     | B. toyonensis              | ACOPR1150Xg | KY750686  | 96%      |
| DM3     | B. pseudomycoides          | FI-8   | KY750687    | 94%      |
| DM4     | B. cereus                  | SBFW51 | KY750688    | 96%      |
| DM5     | B. cereus                  | OPP5 3-2 | KY750689  | 97%      |
| DM6     | B. cereus                  | SBFW55 | KY750690    | 96%      |
| DM7     | B. cereus                  | B4     | KY750691    | 97%      |
| DM8     | B. toyonensis              | ACOPR11ISOXg | KX129781 | 95%      |
| DM9     | B. subtilis                | Y37    | KF641801    | 96%      |
| DM10    | Enterobacter cloacae       | Enc-3  | KU747082    | 99%      |
| DM11    | Clostridium cetobutylicum  | S512   | JQ086380    | 96%      |
| DM12    | B. cereus                  | 4PLGES | LC215052    | 99%      |
| DM13    | B. cereus                  | ML267  | KC692161    | 96%      |
| DM14    | B. thuringiensis           | C17    | LC146717    | 97%      |

From the BLAST analysis, a Phylogenetic tree is created. The tree functions to connect node and is considered as a taxonomy unit, like species or genetrees’ root is the oldest part of all analyzed organisms. The Alignment sequence of sample from Gen Bank database is undertaken by using Mega 0.6 and Clustal W programs (Figure 2). Majority of the 14 observed isolates had particular relationship to Bacillus which is different from one isolate to another. For instance, DM1 and DM2 isolates have closer association. In contrast, isolates DM12, DM5, DM14, DM10 and DM11, had closest relationship to Bacillus sp. However, all isolates had close relationship to Bacillus sp. (Figure 4).
4. Conclusion
From the research results, it can be identified that the four heterotrophic bacteria isolates indeed have relationship to *Bacillus* sp. All isolates had homology more than 90%. In addition, the fourteen heterotrophic bacteria have an ability to inhibit the growth of pathogenic bacteria for fish. In the antagonism test, the inhibition zones showed by isolates DM5, DM6, and DM4 were highest towards *V. alginolyticus*. Meanwhile, other isolates had medium and weak inhibition zone. Isolate DM11 was to be the best isolate in inhibiting the growth of the three pathogenic bacteria, *V. alginolyticus* (9.6mm), *A. hydrophila* (5.6mm) and *Pseudomonas* sp. (6.8mm), in comparison to other 13 isolates.

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