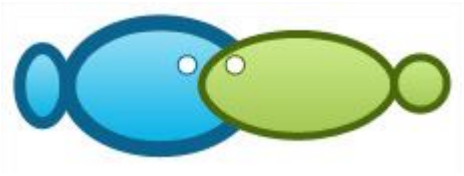

Antibacterial potential of heterotrophic bacteria isolated in Siak River estuary, Indonesia, against pathogens in fish

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Abstract. One of the major challenges of aquaculture is fish disease. However, heterotrophic bacteria are natural antibiotics capable of solving the problem. Heterotrophic bacteria are abundant worldwide, and use organic materials as a nutritional source. The aims of this research were to isolate and to identify heterotrophic bacteria from Siak River at water salinities of 5 ppt and 25 ppt. Selected bacterial isolates were examined for their ability to inhibit the growth of pathogenic bacteria (*Vibrio alginolyticus, Aeromonas hydrophila* and *Pseudomonas* sp.). Out of the 25 heterotrophic bacterial strains selected with the ability to act as anti-pathogenic bacteria in aquatic animals, only 8 have great potential to inhibit the growth of the three pathogenic bacteria. Through identification of heterotrophic bacteria with 16s rDNA technique and BLAST system search, it was discovered that three isolates (A11, A22, A23) were similar to *Kerstersia gyiorum* and one isolate (A14) was similar to *Enterococcus* sp. Four other isolates were not identified considering the fact that the species have not been registered in GenBank or there was a great possibility that they have not been identified prior to this study.
Key Words: heterotrophic bacteria, antagonistic, estuary, 16s rDNA.

Introduction. The quality of sea and coastal waters is strongly influenced by aquatic activities, as well as both domestic and industrial activities in the waters. The rate at which the population general community activities are increasing, all have a negative impact on water quality. Also, human activities in the watershed and coastal areas have the potential to directly or indirectly pollute the aquatic environment (Hamid et al 2020). Estuaries and coastal zones are becoming contaminated by various anthropogenic activities due to a quick economic growth and urbanization (Khan et al 2014). The anthropogenic pollution may affect marine and freshwater ecosystems from sewage, nutrients and terrigenous materials, crude oil, heavy metals and plastics (Hader et al 2020).

There are many settlements near the sea waters of Siak Regency, as well as the Siak River, which is one of the largest rivers in Riau, crossing the following regencies: Bengkalis, Rokan Hulu, Kampar and Pekanbaru City to Padang Strait. In addition to domestic waste, agricultural and industrial activities along the bank of Siak River such as crude palm oil (CPO) plant, plywood, pulp and paper and rubber processing plant contribute to the pollution of the river waters (Husnah et al 2009). Sixty percent of total waste polluting the Siak River originated from domestic waste (Adi 2008).

Various physical, industrial and anthropogenic activities, as well as sea transportation, all contribute to the high concentration of both organic and inorganic compounds that influence bacterial distribution and activity in water bodies (Santos et al 2013). Bacteria have an important role in various processes occurring in water columns. Heterotrophic bacteria, for example, have the capacity to utilize organic material in the

environment which is used as a carbon source for the nutrition and growth (Nursyirwani et al 2018). Heterotrophic bacteria have function as decomposer in water biogeochemical cycles and also to convert organic materials into simple inorganic components, which are returned to the soil and atmosphere as nutrients (Luo et al 2010). Heterotrophic bacteria which can be found in many marine habitats, such as in seawater and sponge have an ability to inhibit pathogenic microorganisms (Alekseevna et al 2013; Padmavathy et al 2015; Graça et al 2015).

Several studies have indicated that marine microbes have the capacity to produce natural products which exhibit a variety of biological activities such as antimicrobial, anti-tumor, anti-inflammatory and anti-cardiovascular agents (Xiong et al 2013). Our previous study also indicated that heterotrophic bacteria isolated from water samples of Siak River at salinity of 15 ppt and 27 ppt were able to inhibit the growth of pathogenic bacteria (*Vibrio* sp., *Aeromonas hydrophila* and *Pseudomonas* sp.). The bacteria species were *Bacillus safensis*, *Alcaligenes faecalis*, *Vagococcus fluvialis*, *Enterobacter* sp., *E. cloacae* and *Enterococcus* sp. (Nursyirwani et al 2018). The presence of pathogenic bacteria frequently results in disease in cultured fish, therefore, prevention is required (Feliatra et al 2012).

This research aims to isolate and identify heterotrophic bacteria from Siak River at water salinities of 5 ppt and 25 ppt. Selected bacterial isolates were examined for their ability to inhibit the growth of pathogenic bacteria (*Vibrio alginolyticus, Aeromonas hydrophila* and *Pseudomonas* sp.).

Material and Method. This research was conducted between June and September 2018, involving a survey method in marine area and in the Siak River of Siak Regency. Water samples were collected at the marine area of 25 ppt (station 1 at the mouth of the Siak River) and 5 ppt (station 2 on the sea settlement area of Kayu Ara Village) (Figure 1). Station 2 was an industrial polluted coastal area in Desa Sungai Kayu Ara. Bacterial isolation was carried out at the Marine Microbiology Laboratory of the Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, while the DNA analysis was carried out at the Genetic Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences of University of Riau. The DNA sequencing process was sent to PT. Genetics Science Indonesia West Jakarta. The following water quality parameters were analyzed: pH, salinity, temperature, brightness, dissolved oxygen (DO), and current velocity.

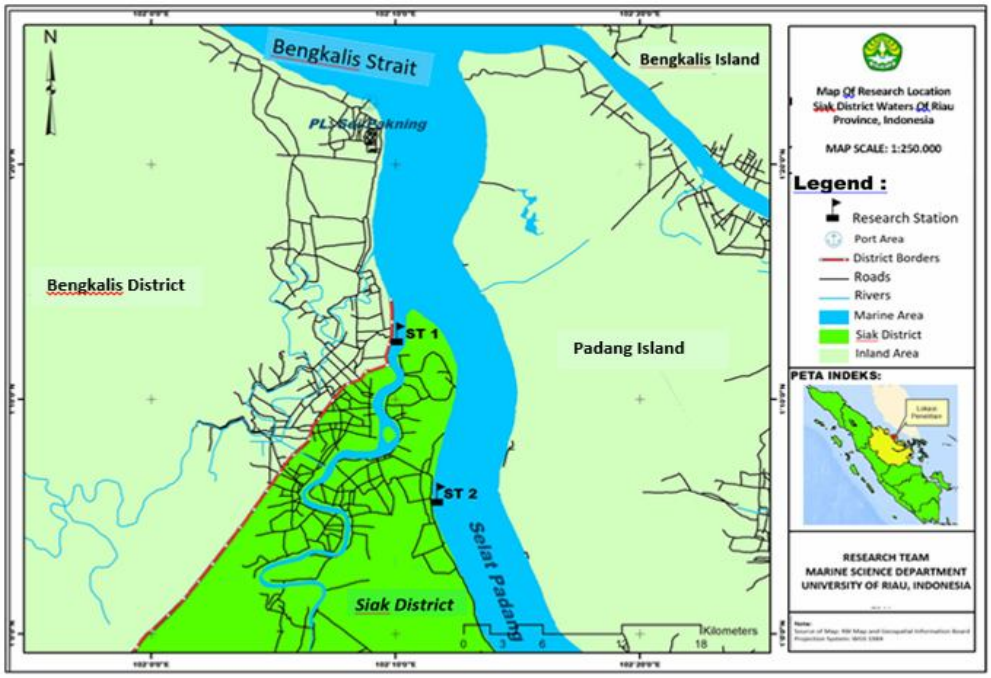

Figure 1. Map of research location in Siak District of Riau Province.

Bacterial isolation and identification. Heterotrophic bacteria was isolated and counted on nutrient agar (NA) using the total plate count (TPC). Water samples from each station were diluted in serial test tubes containing 0.9% of NaCl solution until the dilution level of 10^{-6}. Then 0.1 mL of dilution of 10^{-4}, 10^{-5} and 10^{-6} was spread on the nutrient agar (NA) in Petri dishes with the salinity 5 ppt and 25 ppt. All inoculated Petri dishes were incubated at 28^{o}C for 24 hours. Grown colonies were observed morphologically including the shape, size, texture and colour of colonies. Total colonies of each Petri dish were counted to calculate the total number of heterotrophic bacteria in waters of each station.

Bacterial isolates were selected and identified based on morphologic and biochemical characters. Morphological characters covered shape of cells, colour, size and type of colonies. While, biochemical observation included Gram staining, tests of catalase, methyl red, motility, production of indole and H_2S gas.

Antagonistic activity test of bacteria. The antagonistic activity test of the pathogenic bacteria was carried out by disk diffusion method, proposed by Wolf & Gibbons (1996). About 1 mL of the purified pathogenic bacteria (*V. alginolyticus, A. hydrophila* and *Pseudomonas* sp.) were planted into NA media and homogenized. It was allowed to condense after which a disc paper was inserted into it, dripped with Amoxan 500 g, which is an antibiotic solution, as a positive control while a disc paper with 0.5 µL nutrient broth (NB) media was inserted as a negative control. Then, paper discs with 0.5 µL isolate suspension was dipped into the culture 3 times, repeatedly and then all cultures were incubated at 28^{o}C for 24-48 hours. The filtrate containing antibacterial substances inhibited the growth of the pathogenic bacteria as evidenced by the presence of clear zones around the disc. Also, the amount of antibacterial activity is determined by measuring the diameter of this clear zone.

Identification of bacteria with 16s rRNA technique. The molecular identification of the bacteria was carried out using the 16S rRNA technique in which the samples of existing bacteria were first rejuvenated in the NB media (Feliatra et al 2016). This process commenced with the isolation of heterotrophic bacterial DNA, then electrophoresis to know the total DNA of the bacteria. Upon successfully obtaining the DNA band, it was subjected to PCR process in order to replicate the DNA bands up to ±1500 BP.

These DNA isolates which have been through the PCR process were then sent to First base - Singapore for purification and sequencing processes. The sequencing results were then subjected to BLAST analysis to know the bacterial species obtained through research on the NCBI GenBank site. Finally, these were determined at the homology level of the bacterial sequence listed on the site.

Data analysis. Data of water quality parameters of sampling sites, heterotrophic bacterial isolates, antagonism against pathogens and identified bacterial species were presented in tables and figures. The data were then analyzed descriptively and compared to previous related and similar researches.

Results and Discussion. Results of the measurement of water quality in the research locations are shown in Table 1. The data shows that the quality of waters in the sampling locations such as pH, temperature and DO are still relatively normal and support the growth of heterotrophic bacteria, although the pH values were lower than data from KepMen LH of Indonesian Republic No. 51 in 2004 for the life of marine organisms those were 7.00-8.50. However, other parameters (water salinity, temperature and transparency) were in natural condition.

Table 1

Water quality at the sampling location

Parameter	*Station*	
	1	*2*
Coordinates	102°11'31,1"E - 01°06'13,9" N	102°10'104"E - 01°11'209"N
pH	6.5	6
Salinity	25 ppt	5 ppt
Temperature	29°C	28°C
Brightness	17.5 cm	15 cm
DO	8 mg L^{-1}	6 mg L^{-1}
Current velocity	0.35 m s^{-1}	0.45 m s^{-1}

Then, the bacterial growth in the NA media were calculated based on the number of colonies using the TPC method and the results are shown in Table 2.

Table 2

The average number of heterotrophic bacterial colonies from both stations

Station	*Average number of bacteria (cfu mL^{-1})*
1	2.3 x 10^7
2	1.8 x 10^7

Based on the results of the calculations shown in Table 2, it is evident that the average number of heterotrophic bacteria at the mouth of the Siak River is higher than that of the sea settlement area of Kayu Ara Village. This implies that the number of heterotrophic bacteria is more prevalent in the estuary, which is presumably due to accumulation of organic materials from the transportation by the Siak River.

Based on the result of antagonistic activity test (Table 3), it was seen that not all isolates were able to produce antimicrobial substances which could inhibit bacterial growth. Eight best isolates capable of inhibiting the growth of the three pathogenic bacteria were isolates A11, A12, A14, A19, A21, A22, A23 and A25. Although each isolate indicated difference in inhibitory activity, isolate A25 was the most capable in inhibiting the growth of the three pathogenic bacteria (*V. alginolyticus, A. hydrophila* and *Pseudomonas* sp.). The isolate 25 was obtained from station 2 which is located in the settlement area in Kayu Ara village. The difference in inhibitory zones was as a result of the variation in the antibacterial ability of the bacteria, depending on the compound produced.

After the analysis on DNA sequence of isolates followed by the result of BLAST system, three isolates (A11, A22 and A23) were grouped into genus *Kerstersia* and one isolate (A14) was *Enterococcus* sp. Isolates A11, A22 and A23 were identified as *Kerstersia gyiorum*. However, only isolate A11 had the highest homology > 97% which means that the isolate of same species to *K. gyiorum*. While, other isolates had homology < 97% which means the isolates only have same genus (Tindall et al 2010). Isolates A12, A21 and A25 with homology 79%, 86% and 83% respectively, were obtained from uncultured bacterium, presumably this is because this species has not been registered in GenBank or there is a possibility that it has never been identified before as shown in Table 4.

Table 3

Diameter of the isolate inhibitory zone of heterotrophic bacteria against pathogenic bacteria

Isolate code	Diameter of inhibition zone (mm)													
	Vibrio algynoliticus					Aeromonas hydrophila					Pseudomonas sp.			
	(+)	R1	R2	R3	Mean of R	(+)	R1	R2	R3	Mean of R	(+)	R1	R2	R3	Mean of R
A1	2	0	0	0	0	3	2	4	7	4.3	3	0	0	0	0
A2	13	0	0	0	0	6	8.5	10	10	9.5	3.5	0	0	0	0
A3	15	13	10	8	10.3	11	0	0	0	0	11	0	0	0	0
A4	6.5	5	2.5	3.5	3.6	5	4	8	5.5	5.8	6	0	0	0	0
A5	12.5	7	11.5	4	7.5	11	8	10	11	9.6	6	0	0	0	0
A6	14	0	0	0	0	2	0	0	0	0	3	0	0	0	0
A7	14	0	0	0	0	9	0	0	0	0	4	7.5	6.5	4.5	6.2
A8	14	13	12	12.5	12.5	10	0	0	0	0	3	0	0	1	0
A9	6.5	0	0	0	0	4	4	4	6	3.3	6	0	0	0	0
A10	14	0	0	0	0	11	15	11	9	11.6	8	7	6	8	7
A11	9	7	5	6	6	6	5	5.5	4	4.8	11	8	4	5	5.6
A12	11	9	6	6	7	11	13	11.5	12.5	12.3	4	8.5	8	5	7.2
A13	9	4	5	6.5	5.2	5	6	5.5	8.5	6.6	6	0	0	0	0
A14	7	10	8	8.5	8.8	16	13	8.5	8	9.8	3	5	5	5	5
A15	8	4.5	4	4	4.2	6	3.5	2	2.5	2.6	8	0	0	0	0
A16	9	2	2.5	3.5	2.6	11	0	3	5.5	2.8	6	0	0	0	0
A17	13	0	0	0	0	7	8	10.5	17.5	12	11	11	9	9	9.6
A18	9	0	0	0	0	7	0	0	0	0	16	0	0	0	0
A19	7	14	6.5	11	10.5	5	5	15	11	10.3	11.5	7	6.5	5.5	6.3
A20	17.5	8	9	7	8	3	4	4	4	4	7	0	0	0	0
A21	11	8	7	13	9.3	14	12	12	7	10.3	5	9.5	10	10	9.8
A22	5	8	5	6	6.3	10	11.5	12	13	12.2	6	7	7	5	6.3
A23	12.5	8	7	6	7	16	6.5	12	18	12.2	6	8.5	11	11	10.2
A24	2	0	0	0	0	2	0	0	0	0	2	0	0	0	0
A25	4	14.5	14.5	14.4	14.5	11	14.5	14	13	13.8	12	13	5.5	8	8.8

Isolates 1-12 = isolates from station 2; 13-25 = isolates from station 1; R = repetition; (+) = positive control (Amoxan 500 g).

Table 4

Types of heterotrophic bacteria based on the search for 16S rDNA sequences of isolate bacterial using the BLAST system

Isolate	*Species*	*Strain*	*ID*	*Query coverrage*	*Homology*
A11	*Kerstersia gyiorum*	S7	Km884887.1	100%	99%
A12	Uncultured bacterium	Clone BXHB27	Gq480076.1	79%	79%
A14	*Enterococcus* sp.	F2B1	KY486242.1	99%	91%
A19	Uncultured *Kerstersia* sp.	Clone OTU-13-ABB	JQ-624321.1	99%	90%
A21	Uncultured bacterium	Clone SB-11	JX-000034.1	94%	86%
A22	*Kerstersia gyiorum*	S7	KM884887.1	60%	93%
A23	*Kerstersia gyiorum*	S7	KM884887.1	94%	95%
A25	Uncultured bacterium	Clone HCA24	EU723864.1	78%	83%

Based on the BLAST system search results, phylogenetic tree was then constructed to see the relationship between bacterial isolates based on similarities and differences in genetic characteristics as shown in Figure 1.

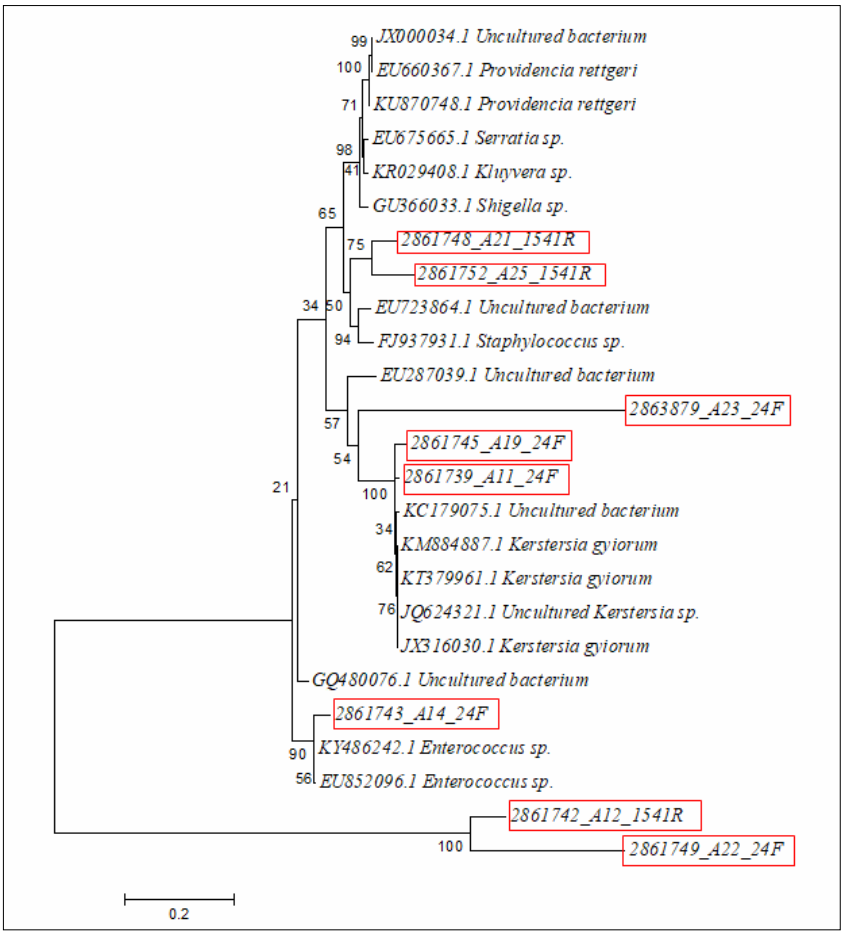

Figure 1. Phylogenetic tree of heterotrophic bacteria.

Discussion. Based on the BLAST analysis, three isolates were discovered with high homologous values equal to 93% and above. The three species were close to those in the world GenBank. The three isolates, A11, A22 and A23, were similar to *K. gyiorum* with the homology values of 99%, 93% and 95%, respectively. One isolate has a homologous value of 91% to *Enterococcus* sp. The rest (isolates A19, A21, A25) have homologous values less than 91% which are believed to be new isolates. These could be native Indonesian isolates or bacteria yet to be identified or not yet registered in the GenBank. From the homologies value, only isolate A11 has value > 97%. Some previous studies

stated that if two strains sharing less than 97% 16S rRNA gene sequence similarity are not members of the same species (Amann et al 1992; Fox et al 1992; Martinez-Murcia et al 1992). Lee et al (2010) suggested that 97% identity based on 16S rRNA gene sequence could be a threshold value for defining a bacteria species.

In general, *K. gyiorum* is a type of bacteria often associated with infection in humans. Hence, it is mostly isolated from human clinical samples obtained from ear infections, foot injuries, sputum, urine and feces, as well as from wastewater (Deutscher et al 2014). The availability of many settlements with different activities within the Siak River estuary and the Siak sea watershed could cause the presence of these bacteria, mainly from household and human wastes. Additionally, the uncultured *Kerstersia* Clone OTU-13-ABB strain has some similarities with A19 isolates which was isolated from a wastewater treatment plant. *K. gyiorum* is classified as follows; Kingdom: Bacteria, Phylum: Proteobacteria, Class: Beta Proteobacterium, Order: Burkholderiales, Family: Alcaligenaceae, Genus: *Kerstersia*, Species: *Kerstersia gyiorum* (NCBI 2017b).

The research conducted by Halaburgi & Karegoudar (2016) showed the benefits of *Kerstersia* bacteria, isolated from coconut fiber, as being able to degrade and change carcinogenic Amarant and Azo dyes into non-toxic compounds.

In addition, six isolates originated from station 1 with high salinity, have better antagonistic abilities compared to the two others, that is, isolates, 11 and 12, which were derived from station 2 with 5 ‰ salinity. The clear zones produced by those from station 2 are also relatively smaller in relation to the isolates from station 1. This research shows that *Kerstersia* bacteria, which are pathogenic in nature, have the potential of antibacterial effect against other pathogenic bacteria such as *V. algynoliticus, A. hydrophila* and *Pseudomonas* sp. with medium to strong inhibitory power.

Enterococcus sp. F2B1 strain in NCBI (2017a) are bacteria isolated from fish with classification; Kingdom: Bacteria, Division: Firmicutes, Class: Bacilli, Order: Lactobacillales, Family: Enterococcaceae, Genus: *Enterococcus.* Also, these bacteria do not form spores, are facultative anaerobes, Gram-positive, coccus, ovoid-shaped with diameters of 0.5-1 µm. According to Garcia-Martinez et al (2011), *Enterococcus* is usually single, in pairs or in the form of short chains. These bacteria grow widely within a temperature range of 10-45^{o}C, pH 4.6-9.9 and with high presence of sodium concentrations, chloride and bile salts (Holzapfel & Wood 2012).

In addition, the *Enterococcus* genus includes species which are highly beneficial, such as *E. faecium* and *E. faecalis* which are probiotic bacteria. *E. faecalis* on its own is used in traditional Mediterranean cheese and other fermented foods such as sausages, olives and vegetables (Moreno et al 2006). According to Fritzenwanker et al (2013), enterococci have been used as probiotics to improve intestinal microbial balance.

Furthermore, this study shows that *Enterococcus* sp. are capable of inhibiting the growth of all the three pathogenic bacteria - *V. algynoliticus, A. hydrophila,* and *Pseudomonas* sp.. Likewise, a study conducted by Nugraha (2013) discovered that *Enterococcus* bacteria are capable of suppressing the growth of coliform bacteria, one of which is *Vibrio* bacteria.

The results also showed the diameters with which the three pathogenic bacteria were inhibited: *V. alginolyticus*, within 6.3-10.5 mm; *A. hydrophila* within 4.8-12.6 mm and *Pseudomonas* sp. within 5.6-10.2 mm. Additionally, the heterotrophic bacteria tested with pathogenic bacteria - *V. alginolyticus*, *A. hydrophila* and *Pseudomonas* sp. formed barriers. These heterotrophic bacteria produce antibiotic products, bacteriocins and certain organic acids. This is consistent with the results of the research conducted by Verschuere et al (2000), which stated that microbial populations have the capacity to release chemical substances with bactericidal or bacteriostatic abilities, hence, inhibiting the growth of other microbial populations. Generally, this ability to inhibit the growth of other bacteria is due to several factors, which include; the production of antibiotics, bacteriocins, siderophores, lysosomes, proteases and hydrogen peroxide. Also, it produces certain organic acids, thereby affecting the pH of its media. Research conducted by Fauziah et al (2015) also revealed that antibacterial agents such as lactic acid and bacteriocin present in probiotic bacteria have the capacity to inhibit the growth of pathogenic bacteria. This is because antibacterial agents reduce the pH thereby making

the media a difficult environment for the survival of pathogenic bacteria (Tambekar & Bhutada 2009).

Conclusions. This study shows that 8 out of the 25 isolates of heterotrophic bacteria have the ability to inhibit the growth of pathogenic bacteria in fish. Three of the isolates (A11, A22, A23 had homologues close to that of of *K. gyiorum*, one isolate (A14) was categorized into *Enterococcus* sp. and four other isolates that have not been identified in the GenBank, which are thought to be specific Indonesian heterotrophic bacteria. These have not been registered in the world GenBank because their homologous levels are less than 90%. The eight identified isolates have the potential to act as antibacterial agents against pathogenic bacteria within the medium (5-10 mm) to strong (10-20 mm) categories. Furthermore, isolates originating from station with high salinity have higher antibacterial potentials compared to those obtained from the station with low salinity.

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