Activity of heterotrophic bacteria from marine area of Siak District against pathogenic bacteria

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Abstract. The objective of this research was to isolate and to characterize heterotrophic bacteria in the marine area of Siak District of Riau Province based on sequence 16S rRNA and to examine the antagonism activity against pathogenic bacteria (*Vibrio* sp.,*Aeromonas*sp. and *Pseudomonas* sp.). Water samples were collected from two sea waters sites: waters with salinity of 15 ppt (station 1) and that at salinity of 27 ppt (station 2). Total heterotroph count in station 1 (7.04×10^7 cfu/mL) was higher than that in station 2 (6.04×10^7 cfu/mL). Antagonism test indicated that nine isolates (RM3, RM4, RM5, RM6, RM8, RM11, RL15, RL17 and RL 24) had potency in inhibiting the growth of tested pathogens.

1. Introduction

Siak District in Riau Province has coastal area which is close to Bengkalis District, and it is a relatively crowded area due to human activities in the settlements and industries. Various physical processess, industrial activities, antrophogenic and marine transportation contribute toorganic and anorganic concentrations which also influence bacterial distribution and activity [1].The inputs and processes will certainly influence carrying and supporting capacities of the coastal environment. In overload condition,the inputs will possibly result in pollution in the coastal area that is disadventagous to the people. Polluted seawaters in coastal area is a common effect due to input of polluting material in the environment. Microbial involvement can not be ignored in the polluted waters [2]. Marine microorganisms are basically variable as that occurs in terrestial areas. Marine microorganisms consist of protista, cyanobacteria, bacteria, fungi and virus. The microorganisms play important role in processes occuring in seawater columns.

Heterotrophic bacteria is a group of bacteria which uses organic materials in the environment as their nutrient sources. In marine biogeochemical cycles, heterotrophic bacteria have important roles in the degradation and remineralization of organic materials into simple anorganic components which are returned as mineral soil and atmosphere [3]. In addition, heterotrophic bacteria which can be found in many marine habitats, such as in water and sponge have an ability to inhibit pathogenic microorganisms [4, 5, 6].

Pathogenic bacteria are organism which cause negative effect to human. The presence of those bacteria such as Vibrio sp., Aeromonas sp. andPseudomonas sp. frequently results in disease in cultured fish, therefore,prevention is required [7]. Many researches have been performed to prevent pathogenic bacterial infection in fish, such as by using antimicrobial compounds. The chemical compounds can be derived from plants, animal or produced by microorganisms which are known as biopreservatives. Numerous studies have indicated that diverse marine microbes have the capacity to

produce marine natural products exhibiting a wide variety of biological activities such as antimicrobial, anti-tumor, anti-imflammatory and anti-cardiovascular agents [8].

The presence of pathogenic bacteria in polluted environment, and the possibility of heterotrophic bacteria produces antimicrobes, are to be the basic reasons of this research. Therefore, the objectives of this research was to examine activity of heterotrophic bacteria isolated from Siak seawaters against pathogenic bacteria (*Vibrio* sp., *Aeromonas* sp. and *Pseudomonas* sp.), and to identify the bacterial species by analysis the 16S rRNA sequences. Finding of this research can be scientific information for public especially in the use of heterotrophic bacteria to prevent bacterial fish diseases in aquaculture.

2. Materials and Methods

The research was conducted from May until September 2017. Microbial isolation and antagonism test were performed in Marine Microbiology Laboratory of the Faculty of Fishery and Marine, and bacterial molecular analysis was performed in the Genetic Laboratory of Biology Laboratory of the Faculty of Math and Natural Sciences in Universitas Riau. Bacterial DNA isolates was sequenced by PT. Genetika Science Indonesia in Jakarta Barat. The research was conducted by survey method.

2.1. Sampling location

Water samples were collected from two areas: Siak River Estuary area with the salinity of ±15 ppt (Station 1) and from the coastal waters in Desa Sungai Kayu Ara with the salinity ±27 ppt (Station 2). The sampling areas were located in Siak District of Riau Province, Indonesia (Figure 1).

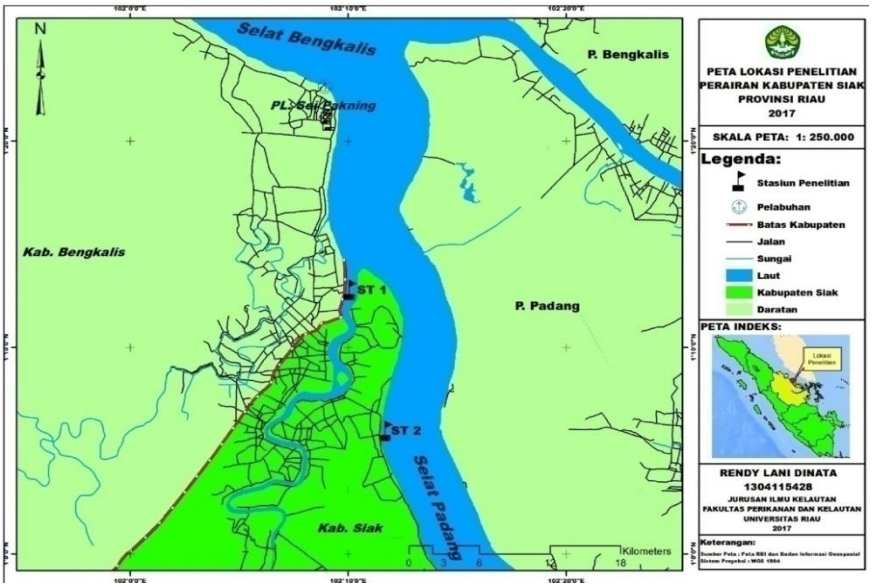

Figure 1. Map of Research Location in Siak District of Riau Province.

2.2. Bacterial isolation and identification

Water sample of each station was diluted in a dilution serial of test tube containing 0.9 % of NaCl solution until the dilution level of 10^{-6}. A volume of 0.1 mL of each dilution of 10^{-4}, 10^{-5} and 10^{-6} was spread onto nutrient agar (NA, Oxoid)) in petridishes with the salinity 15 ppt and 27 ppt. All inoculated petridishes were incubated at 28^{o}C for 24-48 hours. Grown colonies were observed morphologically including the shape, size, texture and colour of colonies. Number of colonies were counted to calculate the total number of heterotrophic in waters of each station.

Selected bacterial isolates were identified based on morphologic and biochemical characters. Morphologic characters observed were 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.

2.3. Antagonism test

Acitivity of bacterial isolates against pathogenic bacteria (*Vibrio* sp.,*Aeromonas*sp. and *Pseudomonas* sp.)was examined by using diffusion agar method following the procedure of Wolf and Gibbons [9].A volume of 25 µL of each pathogenic bacteria suspension in nutrient broth (NB) was inoculated and spread onto NA medium. Meanwhile, 5 µL of suspension of each heterotrophic bacterial isolate was dropped on sterile paper disc. In addition, Amoxan ®500g solution and NB were used as positive and negative controls, respectively. Each treatment was performed in triplicate. All treatments were incubated at 28^{o}C for 24 hours. Heterotrophic bacterial isolates containing antibacteria were indicated from inhibition zones appeared around the paper disc. Diameter of the inhibiton zones were measured using a caliper.

2.4. 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 analized descriptively and compared to previous related and similar researches.

3. Results and Discussion

3.1. Water quality parameters of sampling sites

Condition of water quality is important for the growth of living organisms including bacteria. Water quality parameters observed in this research were water temperature, pH, salinity, water transparancy, dissolved oxygen (DO) and current velocity (Table 1).

Table1. Water quality condition of sampling sites in Siak Regency, Riau Province

Water quality parameters	Sampling sites		Water Quality Standard of KepMen LH No. 51 in 2004
	1	2	
Coordinate points	01^o 13’ 2,3” N 102^o 10’ 24,4” E	01^o 05’ 59,8” N 102^o 12’ 19” E	-
pH	6.00	6.50	7.00 – 8.50
Salinity (ppt)	15.00	27.00	Natural
Temperature (^{o}C)	30.00	31.00	Natural
Transparancy (cm)	6.50	23.50	Natural
DO (mg/L)	6.88	4.24	>5
Current velocity (m/s)	0.20	0.70	-

Note: Sampling site 1: Siak River Estuary; Sampling site 2: Marine waters of Desa Kayu Ara

Data in Table 1 indicates that all water quality parameters, except DO, in sampling site 2 are higher than those in site 1. Sampling site 1 (Siak river estuary) received more inland run off, particularly from activities of people living in villages along the Siak river. While, sampling site 2 was relatively opened coastal area and far from human activities. The water quality condition of these two areas, especially the pH and DO values were slightly lower in comparison to data from KepMen LH of Indonesian Republic No. 51 in 2004 [11] for the life of marine organisms. The lower DO value in Station 2 compared to Station 1. Similar finding was also reported from the Ennore Estuary Raj et al., 2013)[12] which had higher DO value (5.3±0.65 ppm) compared to Ennore Coastal water (6.7±0.91 ppm)

3.2. Heterotrophic bacterial counts

From the bacterial total counts of the two sampling sites, the higher heterotrophic counts was obtained from water samples of Siak estuary than that of marine waters of Sungai Kayu Ara (Table 2).This might due to the input and accumulation of organic matters from the urban living in villages along the Siak River which is finally deposited in the estuary. Several researches had reported the abundances of heterotrophic bacteria in estuary and coastal water areas.The highest heterotrophic bacterial abundance was observed in the central coastal Bay of Bengal India that received urban sewage from the major city [13]. Moreover, in dynamic estuaries, diverse microbial communities are formed during mixing of fresh and marine water masses [14]. Higher microbial populations in coastal waters were observed throughout the year irrespective of seasons due to the intensive anthropogenic activities such as discharge of industrial and domestic sewage [15].

Table 2.Average total heterotrophic counts in Siak River estuary and marine waters of Desa Kayu Ara.

Sampling sites	**Average bacterial counts (cfu/mL)**
1. Siak River estuary (RM)	7.04×10^7
2. Marine waters of Desa Kayu Ara (RL)	6.46×10^7

Note: The average values are of triplicate samples.

Number of heterotrophic bacteria and acitivities in waters are controlled by the presence of organic matter as the nutrition sources and various hydrobiological factors. The distribution depends on changes in water temperature, salinity and physicochemical parameter. Present research found that current velocity in Siak River Estuary was lower (0.20 m/s) than that in Desa Kayu Ara coastal water (0.70 m/s). The lower current velocity could result in more organic matter accumulate in the estuary compare to the coastal waters. On another hand, lower DO value was observed in the coastal waters of Desa Kayu Ara. This could be due to higher discharge of domestic sewage and industrial acitivities containing organic matters in addition to higher water transparancy which increased the decomposition rate which resulted in decrease in the DO value compared to the Siak River Estuary. The value of dissolved oxygen is remarkable in determining the water quality criteria of an aquatic ecosystem. The dissolved oxygen is regulator of metabolic activities of organisms and thus governs metabolisms of the biological community as a whole and also acts as an indicator of trophic status of the water body [16].

3.3. Bacterial morphology and biochemical characteristics

Nine out of 25 heterotrophic bacterial isolates were selected for the antagonistic test against three pathogenic bacteria. The isolates were then observed for the morphology and biochemical characters as presented in Table 3 and Table 4.

Biochemical tests (Table 4) indicates there were variation in characters of the nine isolates. Seven of nine bacterial isolates were Gram positive. All isolates produced catalase, eight isolates were motile, one isolate produced indole, but H_2S was not produced. Positive results on methyl red and citrate tests were indicated by two and four isolates, respectively. All isolates were able to ferment carbohydrate (glucose, lactose and sucrose) as carbon sources to produce acid.

Table 3. Morphology characters of heterotropic bacteria isolated from the Siak River estuary and marine waters of Desa Kayu Ara

Isolate code	Colony diameter (cm)	Colony colour	Shape of colony	Colony edges	Colony elevation
RM3	1.0	White to yellowish	Coccus	Smooth	Concave
RM4	0.9	White	Coccus	Smooth	Concave
RM5	0.7	White to yellowish	Coccus	Smooth	Concave
RM6	0.8	White to yellowish	Irregular and spread	Wavy	Concave
RM8	0.9	White	Coccus and spread edges	Branched	Concave
RM11	1.1	White	Coccus and spread edges	Branched	Datar
RL15	1.0	White to yellowish	Coccus and elevated edges	Smooth	Concave
RL17	0.9	White to yellowish	Coccus and elevated edges	Wavy	Sunken
RL24	1.1	White to yellowish	Coccus arnd elevated edges	Smooth	Sunken

Note: RM, isolate from Siak River estuary; RL, isolate from marine waters of Desa Kayu Ara

Table 4. Biochemical characters of heterotropic bacteria isolated from the Siak River Estuary (RM) and marine waters of Desa Kayu Ara (RL)

Isolate code	Gram staining	Catalase production	Motility	Indole	H_2S	TSIA			**Methyl Red**	**Citrate**
						G	L	S		
RM3	+	+	+	-	-	+	+	+	-	-
RM4	+	+	+	-	-	+	+	+	+	-
RM5	+	+	+	-	-	+	+	+	-	-
RM6	+	+	-	+	-	+	+	+	-	+
RM8	+	+	+	-	-	+	+	+	+	+
RM11	+	+	+	-	-	+	+	+	-	+
RL15	-	+	+	-	-	+	+	+	-	+
RL17	+	+	+	-	-	+	+	+	-	-
RL24	-	+	+	-	-	+	+	+	-	-

Note: TSIA = triple sugar iron agar; G = glucose; L = lactose; S = sucrose

3.4. Antagonism of heterotrophs against pathogenic bacteria

Antagonistic activity of nine heterotrophic bacterial isolates against pathogens (*Vibrio* sp., *Aeromonas* sp.and *Pseudomonas* sp.) observed from the diameter of zone inhibition was presented in Table 5. The data indicated that the ability of heterotroph isolates in inhibiting the growth of each of the pathogens is different.

Table 5. Antagonism of heterotrophic bacterial isolates from Siak River estuary (RM) and marine waters of Desa Kayu Ara (RL) against pathogens

Isolate code	Diameter of inhibition zone (mm)														
	Vibrio sp					*Aeromonas*sp					*Pseudomonas* sp				
	(+)	R1	R2	R3	Aver.	(+)	R1	R2	R3	Aver.	(+)	R1	R2	R3	Aver.
RM3	9.0	10.0	8.0	9.0	**9.0**	12.0	11.0	10.0	11.5	**10.8**	7.0	9.0	9.0	11.0	**9.6**
RM4	16.0	11.0	10.0	11.0	**10.6**	5.5	6.5	6.5	8.5	**7.1**	4.0	5.0	4.0	3.5	**4.1**
RM5	16.0	8.0	5.0	5.0	**6.0**	6.5	12.0	10.5	8.0	**10.1**	7.0	9.0	6.0	8.0	**7.6**
RM6	6.0	11.0	8.0	7.0	**8.6**	6.7	20.0	19.0	15.0	**18.0**	4.0	5.0	6.0	7.0	**6.0**
RM8	6.0	14.0	16.0	14.0	**14.6**	6.0	14.0	13.0	14.0	**13.6**	6.0	10.0	12.0	13.0	**11.6**
RM11	8.0	14.5	15.0	16.5	**15.3**	6.0	14.5	12.5	15.0	**14.0**	6.0	9.5	12.5	14.0	**12.0**
RL15	11.0	13.0	15.0	15.0	**14.3**	6.5	6.0	10.0	16.0	**10.6**	5.0	20.0	16.0	14.0	**16.6**
RL17	4.0	0.4	0.5	1.0	**0.6**	7.5	1.0	0.8	0.7	**0.8**	3.0	8.0	9.0	8.5	**8.5**
RL24	11,5	12.0	13.0	16.0	**13.6**	6.0	8.0	10.0	15.0	**11.0**	5.0	19.0	17.0	13.5	**16.5**

Note: R1, R2 and R3 = replications 1, 2 and 3; Aver. = average value

The highest zone inhibition against *Vibrio* sp. was indicated by isolate RM11, followed by isolates RM8, RL15 and RL24. While isolate RM6 showed the highest inhibition against *Aeromonas* sp. followed by isolates RM11, RM8 and RL24. Then, isolate RL15 showed the highest antagonism activity against *Pseudomonas* sp. followed by isolates RL24, RM11 and RM8. Based on the ability to inhibit the growth of pathogens, isolates RM8, RM11, RL15 and RL24 were heterotrophic bacteria that had inhibition response categorized into strong with the values ranged from 10,6 – 18 mm. Meanwhile, isolates RM3, RM4, RM5, RM6 and RL17 were heterotrophic bacteria that categorized into weak and medium responses with the diameter values ranged from 0,6 – 10,8 mm against the pathogens.

Several previous researches reported that marine heterotrophic bacteria had antibacterial potential against microorganism pathogens. Alekseevna *et al*. [4] found 68.97% of isolates from temperate zone and 56.76% of tropical zone showed antimicrobial activity, and the most active strains belonged to genera Pseudomonas and Pseudoalteromonas. From the coastal locations in Gulf of Mannar Region, Tamilnadu, India, it was reported five bacterial strains identifed as Marinonascens sp., Bacillus sp., Mesophilobacter sp., Alteromonas sp. and Marinococcus sp. showed antagonistic activity against 17 multi drug resistant pathogens including Gram positive, Gram negative and fungi [15]. Genus Bacillus (Bacillus sp. HS1, B. subtilis HS2, B. amyloliquefaciens HS6) isolated from Mediterranean Sea sponge, Spongionella gracilis, had the highest proportion of antimicrobial activity against two pathogenic bacteria (Staphylococcus aureus, Klebsiella pneumoniaer) and fungi Candida albicans [17].

The ability of heterotrophic bacteria to inhibit the growth of pathogens indicated that the bacteria could produced secondary metabolitesor bioactive compounds. Generally, the ability to inhibit other bacterial growth might be due to any of factors such as production of antibiotics, bacteriocins, sidephores, lisozymes, proteases and hydrogen peroxideor from production of organic acids influencing medium pH. In addition, antibacterial agents were able to lowering medium pH, so that it is difficult for pathogenic bacteria to survive [18].

Current research found that *Enterobacter* sp., *E. cloaca* and *Enterococcus* sp. had antagonistic activity against pathogens (*Vibrio* sp., *Aeromonas* sp., *Pseudomonas* sp.).This bacterial species had also been found in Dumai marine waters of Riau Province, Indonesia, however, it performed a weak antagonistic activity against bacterial pathogens [19]. Enterobacter cloaca strain GH1 (ac: JF261136.1) isolate from the Red Sea alga *Cystoseira myrica* had been reported inhibiting pathogenic bacteria and H5N1 and NDV viruses. In addtition, antioxidant activity was also detected in the bacterial extract from which diketopiperazines derivatives were isolated [20].

Enterobacter cloacae is a species of genus Enterobacter, Gram-negative, facultative anaerobic, rod-shaped, non- spore-forming bacteria belonging to the family Enterobacteriaceae [21].The maximum growth of E. cloacae was obtained at 30°C, pH 7, with the addition of maltose and KI to the media [20]. The E. cloacae species comprises an extremely diverse group of bacteria that has been found in diverse environments,ranging from plants to soil to humans [22]. Enterobacter is also found in marine environment. Enterobacter from sponge Dysidea granulosa showed significant antibacterial activity against clinical bacterial pathogens Staphylococcus aureus, Escherichia coli, Salmonella typhi, Klebsiella pneumoniea and Streptococcus sp. [23]. Enterobacter sp. ST3 from the marine dinoflagellate Scrippsiella trochoidea, and found it has the ability to produce short-chain quorum sensing-based acyl-homoserine lactone (AHL) signal [24].

Enterococci are Gram positive bacteria as important members of gut communities in many animals. They are classified as lactic acid bacteria (LAB) as they carry most of the phenotypes of the other components of the group such as Gram positive, catalase negative and the ability to convert glucose into lactic acid as mainproduct (homofermentative) of primary metabolism [25].

Enterococci are members of the intestinal microbiota of healthy humans and animals and can be released into the environmental sources such as soil and surface water by human and animal fecal material [26]. This research found the bacteria in water samples collected both from coastal marine and estuary areas. This indicates the widely distribution in a variety of environmental habitats. Species from the genus Enterococcus have also been used as human probiotics because they can survive and compete in the gastrointestinal tract [27]. The Enterococcus antagonistic capacity allows control of undesirable bacteria in foods [28]. The *E. faecalis* and *E. faecium* species are the most important within the Enterococcus genus, because they are currently the only species used to produce probiotics intended for human and animal consumption [29].

The ability to inhibit pathogenic bacteria, low resistance to antibiotics and absence of virulence factors make some of Enterococcus faecalis strains characterized in the present study promising for exploitation in other applications such as probiotics in aquaculture [30]. The bacteria grow in aerobic- and anaerobic condition and in widely temperature range ((10-45oC), pH 4.6-9.9 and at high NaCl and bile salt concentrations [31, 32].

4. Conclusion

Four of nine heterotrophic bacteria isolated from the Siak River estuary and coastal waters of Desa Sungai Kayu Ara of Riau Province have potency in inhibiting the growth of pathogenic bacteria. Four isolates indicated high antimicrobial activity. Those were isolate RM6 identified as *Enterobacter* sp., isolate RM11 as *Enterococcus* sp., isolates RL15 and RL24 has similarity to *Enterobacter cloacae*.

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