

ANTIBACTERIA ACTIVITY OF GASTROPOD ASSOCIATION BACTERIA FROM MANGROVE ECOSYSTEM AGAINST *BACILLUS CEREUS* AND *ESCHERICHIA COLI* AND IT'S POTENCY OF APPLICATION FOR BELANAK FISH (*MUGIL SUBVIRIDIS*)

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Abstrak

Bakteri asosiasi gastropoda dari ekosistem mangrove memiliki potensi sebagai antibakteri yang dapat dimanfaatkan dalam produk perikanan. Kontaminasi *Bacillus cereus* dan *Escherichia coli* dapat menyebabkan pembusukkan pada daging ikan. Penelitian ini bertujuan untuk mengidentifikasi bakteri asosiasi yang dapat menghambat *B. cereus* dan *E. coli*, serta potensinya dalam menghambat bakteri kontaminan pada daging ikan Belanak (*Mugil subviridis*). Pengambilan sampel di *Mangrove Education Park*, Kecamatan Tugu, Semarang. Uji antibakteri dilakukan di *Laboratorium Tropical Marine Biotechnology*, Universitas Diponegoro. Prosedur penelitian adalah isolasi bakteri, uji antibakteri, identifikasi secara biokimia, dan uji aplikasi potensi terhadap ikan Belanak. Gastropoda yang didapat adalah *Certhideopsilla alata*, *Certhidea quoyii*, *Cassidula aurisfelis*, *Cassidula nucleus*, dan *Telescopium telescopium*. Hasil isolasi bakteri sebanyak 61 isolat. Kultur yang paling menghambat pertumbuhan patogen adalah GMT 3.2.5 dan GMT 4.1.3 (8,48 mm dan 7,84 mm). Karakterisasi menunjukkan GMT 3.2.5 dan GMT 4.1.3 lebih dekat pada genus *Micrococcus* sp. dan *Bacillus* sp. Supernatan GMT 3.2.5 dan GMT 4.1.3 dapat menghambat bakteri kontaminan ikan Belanak dengan jumlah koloni 1150×10^3 dan 265×10^3 CFU/ml. Jumlah koloni lebih sedikit dibanding tanpa perendaman yaitu 2312×10^3 CFU/ml.

Kata kunci: Bakteri Asosiasi Gastropoda, Aktivitas Antibakteri, Pembusukan Ikan

Abstract

Gastropod association bacteria from mangrove ecosystem have a great potency as antibacterial. The contamination of *Escherichia coli* and *Bacillus cereus* cause damage in fish product. The aims of this research are to identify gastropod association bacteria that can inhibit *E. coli* and *B. cereus* growth, as well as the contamination bacteria in Belanak (*Mugil subviridis*). Gastropods sample *Certhideopsilla alata*, *Certhidea quoyii*, *Cassidula aurisfelis*, *Cassidula nucleus*, *Telescopium telescopium* were collected at Mangrove Education Park, Tugu, Semarang. The research procedure was isolation, antibacteria test, biochemical identification, and the potency to inhibit contaminant bacteria in Belanak fish (*Mugil subviridis*). There were 61 bacteria isolated. Thirteen isolates were able to inhibit *E. coli* and eight to *B. cereus*. Supernatant of GMT 3.2.5 and GMT 4.1.3 have the most widely inhibition zone, (8,48 mm and 7,84 mm). Biochemistry identification shown that GMT 3.2.5 and GMT 4.1.3 has identic characteristic with genus *Micrococcus* sp. and *Bacillus* sp. Total plate count (cfu/ml) of bacteria from Belanak fish flesh that soaked by *Micrococcus* sp. GMT 3.2.5 and *Bacillus* sp. GMT 4.1.3 supernatant were 115×10^4 and 265×10^3 colony. The colony were less than negative control (2312×10^3 colony). The association bacteria can inhibit contaminant bacteria in Belanak fish.

Keywords: Gastropod Association Bacteria; Antibacterial Activity; Fish Decomposition

INTRODUCION

Gastropod association bacteria have great potency for antibacterial activity. The previous study showed that the association

bacteria from gastropods *Conus miles*, *Stramonita armigera*, and *Cymbiola vespertillo* can inhibit Multi Drug Resistant bacteria growth [1]. Association bacteria synthesized the antibacterial compound from

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its host [2]. Gastropod were one of the important sources to produce bioactive compound that have antibacterial activity [3]. Antibacterial is important compounds of the host defence system of many animal species [4].

Bacillus cereus and *Escherichia coli* are bacteria that live in any condition and environment. *Bacillus cereus* and *Escherichia coli* could contaminate fish product. *Bacillus cereus* and *Escherichia coli* cause rapid deterioration and decomposition of fish products. These contaminant bacteria degrade protein from the fish for its metabolism [5]. Contamination occurred during fish processed, there were from the unhygienic environment, device, or workers [6]. Contaminated fish consumed by humans can cause poisoning in humans.

The aims of this research are to determine gastropod association bacteria that can inhibit *E. coli* and *B. cereus* growth, knowing genus of active bacteria, and growth inhibition against contamination bacteria in Belanak *Mugil subviridis*. Total count of contaminant bacteria in Belanak fish will illustrate the potency of gastropod association bacteria.

MATERIALS AND METHODS

The research was conducted in February-July 2018 at Tropical Marine Biotechnology, Marine Science and Oceanography Laboratory, Diponegoro University, Semarang, Indonesia. The procedure of research was sample preparation, association bacteria isolation, antibacterial test, biochemical identification, and potency to inhibit bacterial contamination in Belanak fish *Mugil subviridis*.

Sample Preparation

Five different species of Gastropods samples were collected at Mangrove Education Park, Tugu, Semarang. Sampling was done by purposive sampling methods. Gastropods were handed in directly by picking hand methods. The samples were collected in sterile Ziplock plastic and then put it on the cool box. Gastropods sample were brought in

to laboratory for association bacteria isolation and followed by antibacterial test.

Association Bacteria Isolation

Gastropod association bacteria were isolated by some serial dilution. One gram of gastropods inner tissue was diluted at 9 ml sterile sea water. Some serial was administered at 10^{-1} – 10^{-5} . Serial dilution at 10^{-3} , 10^{-4} , 10^{-5} were cultivated in 2216 Zobell agar, incubated for 3 days at 37° C, and then isolated to get the single pure culture. Purified gastropod association bacteria were kept at slant Nutrient Agar and incubated for 2 days at 37° C.

Antibacterial Test

The bioactivity of purified association bacteria was screened by overlay methods against *Bacillus cereus* and *Escherichia coli* [7]. Association bacteria were cultured by dotting in 2216 Zobell agar, and then incubated for 2 days at 37° C. In the first day of incubation, *Bacillus cereus* and *Escherichia coli* were cultivated in 5 ml Nutrient Broth, and then shakes at 120 rpm for 24 hours. *Bacillus cereus* and *Escherichia coli* (1% total volume of Soft Agar) were cultivated in warm Soft Agar Zobell, and then pour it on to association bacteria culture in 2216 Zobell Agar, incubated for 1 – 2 days at 37° C. Active association bacteria were cultured at 10 ml Nutrient Agar and shaken for 5 days at 120 rpm. The 5 days culture were then centrifugated at 3000 rpm. Antibacterial assay was done by Kirby-Bauer or Paper Disk methods [8]. The 10 μ l active association bacteria supernatant were taken and then pour it on to 6 mm paper disk. Prior to this, 2216 Zobell Agar were cultured by pathogenic bacteria. This was then followed by incubation for 1 and 2 days at 37° C.

Biochemical Identification

Identification of active association bacteria was done by biochemical methods. Biochemical identification based on Bergeys Manual of Determinative Bacteriology 9th edition [9]. Serial biochemical tests including gram staining, catalase test, oxidase test, nitrate reduction test, sulphides hydrogen test,

MR test, VP test, urea hydrolysis test, starch hydrolysis test [10], sugar fermentation test, tryptophan hydrolysis test [11], and gelatine hydrolysis [12], were done.

Application Test in Belanak fish *Mugil subviridis*

Application test of active bacteria supernatant was done by soaking 200-gram flesh of Belanak fish for 5 hours on the active bacteria supernatant. We used Belanak fish without any soaking of supernatant as negative control. Contaminant bacteria was counted by Total Plate Count method. One gram of soaked coded Belanak fish were isolated by some serial dilution and then cultivate by spread plate methods in 2216 Zobell Agar. Contamination bacteria were counted and compared to the negative control.

Contaminant bacteria were accounted by this formula below:

$$\text{CFU/ml} = \text{Mean of total bacteria} \times (1/\text{Dilution factor}) \quad (1)$$

RESULT AND DISCUSSION

Gastropods sample *Certhideopsilla alata*, *Cerithidea quoyii*, *Cassidula aurisfelis*, *Cassidula nucleus*, *Telescopium telescopium* were collected at Mangrove Education Park, Tugu, Semarang. There were 61 bacteria managed to isolate from those five species of Gastropods. Thirteen isolates were able to inhibit *E. coli* and eight to *B. cereus*. As seen in Table 1, supernatant of GMT 3.2.5 and GMT 4.1.3 have the most widely inhibition zone (8,48 mm and 7,84 mm).

Table 1. The Result of Antibacterial Test Gastropods Association Bacteria

Bacteria Code	Inhibition Zone (mm)			
	<i>E. coli</i>		<i>B. cereus</i>	
	24 hours	48 hours	24 hours	48 hours
GMT 4.1.3	5.125 ± 0.95	7.845 ± 2.0	0	0
GMT 4.1.5	3.95 ± 1.48	5.765 ± 2.07	3.38 ± 0.25	0
GMT 5.2.6	5.15 ± 0.63	4.68 ± 0.67	5.065 ± 1.32	3.1 ± 0.28
GMT 3.2.5	4.65 ± 0.07	8.48 ± 0.77	3.48 ± 0.16	5.68 ± 0.91
GMT 5.1.2	4.18 ± 0.82	5.215 ± 1.29	0	0
GMT 5.1.3	4.03 ± 0.8	5.465 ± 0.47	0	0
GMT 2.2.9	3.75 ± 0.49	0	0	0
GMT 4.2.7	0	0	0	0
GMT 4.1.2	2.765 ± 0.33	0	2.7 ± 0.7	3.845 ± 1.67
GMT 4.1.1	2.7 ± 0.56	0	0	0
GMT 3.2.3	0	0	0	0
GMT 3.2.1	3.2 ± 0.98	0	0	0
GMT 5.1.4	3.815 ± 0.58	0	0	0
GMT 5.1.1	4.4 ± 0	5.85 ± 0.35	3.565 ± 0.65	4.04 ± 1.4
GMT 2.3.12	3.98 ± 0.25	0	0	0
GMT 5.2.5	3.065 ± 0.47	0	0	0
GMT 4.1.4	3.9 ± 0.14	0	0	0
GMT 2.5.14	0	0	0	0
Amoxicilin	23.75 ± 0.21	23.93 ± 0.18	7.405 ± 0.07	6.6 ± 0.28
Sterile NB	0	0	0	0

Footnote: Bold font: The most potential culture

The biochemical identification shown that GMT 3.2.5 has an identical characteristic

to genus *Micrococcus* sp. and GMT 4.1.3 to genus *Bacillus* sp. Total plate count (cfu/ml)

of bacteria from Belanak fish flesh that soaked by *Micrococcus* sp. GMT 3.2.5 and *Bacillus* sp. GMT 4.1.3 supernatant were 1150×10^3 and 265×10^3 colony (Table 2). It has proven, that the colony were less than the negative control (2312×10^3 colony).

Table 2. Total Plate Count of Contaminant Bacteria in Belanak fish

Num.	Supernatant Code	Bacteria Colony Total (CFU)
1.	GMT 4.1.3	265×10^3
2.	GMT 3.2.5	1150×10^3
3.	Negative Control	2312×10^3

Identified gastropods were *Cerithideopsis alata*, *Cerithidea quoyii*, *Cassidula aurisfelis*, *Cassidula nucleus*, and *Telescopium telescopium*. Identification was done based on Identification Book [13]. *Cerithideopsis alata* is a member of Potamididae family, shell length 2,5 cm, blackish brown color, and pointed apical whorl. The habitat of gastropod was in coastal, estuary, and mangrove ecosystem [14]. Secondly species, *Cerithidea quoyii* is Potamididae family. *Cerithidea quoyii* has shell length 3,5 cm, yellowish brown color, spiral cone shell, convex body whorl, and blunt apical whorl. Gastropod *C. quoyii* which live in root in root and stem of mangrove [15]. *C. aurisfelis* is a member of Potamididae family. Gastropod *Cassidula aurisfelis* has shell length 2,5 cm, dark brown color with yellow line, convex body whorl, and tight aperture. The *C. aurisfelis* shell has a unique right spiral pattern. Their habitat is in mud substrate in mangrove ecosystem. Gastropod *Cassidula nucleus* was lived in mud substrate in mangrove ecosystem, Ellobidae family, 2 cm in shell length, brown color with spiral line, convex body whorl, pointed apical whorl, and tight aperture. *C. nucleus* lives in mangrove substrate [14]. The last species is *Telescopium telescopium*. Potamididae family, has 7 cm in shell length, blackish brown colour, spiral pattern. This gastropod has bold and hard shell, widen aperture, and pointed apical whorl. *T. telescopium* live in brackish water and mud substrate in mangrove ecosystem [16].

We succeed to isolate 61 association bacteria and 21 of those were active. 13 bacteria were able to inhibit pathogen bacteria. Antibacterial experiments were done

by agar diffusion (modified Kirby-Bauer). Inhibition zone of GMT 4.1.3 to *E. coli* were 7,48 mm. In other hand, GMT 3.2.5 was succeeded to suppress the *E. coli* growth in 8,48 mm inhibition zone. Even though, the Amoxicillin inhibition zone was greater (23 mm), GMT 3.2.5 has a potency to reduce the contaminate bacteria such as *E. coli*. As we were only centrifuge the bacteria culture, there will be much more information can be explored to find the more powerful compounds and several methods on extraction [17].

The performance of inhibition zone shows that gastropod association bacteria produce antibacterial compound. There are several steps on antibacterial mechanism. Firstly, those bacteria were inhibit synthesizing the pathogen bacteria cell wall, increasing cell membrane permeability, and finally, irritate cell protein synthesise [18].

The supernatant of GMT 3.2.5 and GMT 4.1.3 were inhibiting *E. coli* greater, rather than *B. cereus*. Our postulate is, *E. coli* is negative gram bacteria and have a thinner cell wall compare to positive gram bacteria such as *B. cereus*. Negative gram bacteria cell wall contains 10% peptidoglycan, lipopolysaccharide, and 11 – 22 % lipid. Antibacterial compound of association bacteria can easily damage the cell wall of *E. coli* and inhibit *E. coli* growth, rather than *B. cereus*. Reported that *B. cereus* cell wall contain of 60 – 100 % peptidoglycan, and 1 – 4 % lipid [19]. The function of cell wall is in relation with the protection of bacteria osmotic pressure. Cell wall is also having a role in cell fission [20].

Inhibition zone that created by GMT 3.2.5 and GMT 4.1.3 were not clear but there some colony of pathogen inside. This is an indication that GMT 3.2.5 and GMT 4.1.3 are bacteriostatic. The bacteriostatic mechanism is to inhibit protein synthesis from pathogenic bacteria, it binds to the ribosome of the bacteria. The bonding was temporary and not strong enough, so ribosome will be released again when antibacterial compound concentration was decreased. So, therefor, the bacteria will grow up again [21]. Antibacterial strength, indicated by inhibition zone, were divided in to four groups, there were very strong (≥ 20 mm), strong (10 – 20 mm), intermediate (5 – 10 mm), and weak (≤ 5 mm)

[22]. Bacteria GMT 3.2.5 and GMT 4.1.3 were in intermediate antibacterial strength zone.

Biochemistry identification shown that the GMT 3.2.5 and GMT 4.1.3 have an identic characteristic with genus *Micrococcus* sp. and *Bacillus* sp. (Table 3). Bacteria genus *Micrococcus* sp. was positive gram bacteria, coccus, irregular position, and lived-in land or water. *Micrococcus* sp. has $\pm 0,5 - 2,5 \mu\text{m}$ cell size [23]. *Micrococcus* sp. was organic decomposer bacteria. It can decompose organic material in euphotic area. *Bacillus* sp. is a positive gram bacterium, bacillus, obligate aerobe, and can be found in any environment. *Bacillus* sp. can produce endospore when the environment was not in good condition [24]. *Bacillus* sp. is an organic decomposer bacterium [25].

Table 3. Biochemistry Identification

Test	GMT 3.2.5	GMT 4.1.3
Catalase	+	+
Oxidase	+	+
Fermentation:		
Glucose	-	A
Xylose	-	-
Lactose	-	-
Mannitol	-	A
Hydrolysis:		
Gelatine	+	+
Starch	-	-
Urea	-	+
Tryptophan	-	-
Nitrate	-	+
Reduction	-	+
H ₂ S Product	-	+
MR	-	-
VP	+	-

Footnote:

(+) = Positive test result

(-) = Negative test result

Total plate count shown that GMT 3.2.5 and GMT 4.1.3 can inhibit contaminant bacteria in Belanak fish. Total contaminant bacteria in Belanak fish soaked by association bacteria supernatant were less than negative control. This indicated that inhibition occurred during soaking process [26]. This research positively suggest that GMT 3.2.5 and GMT 4.1.3 have antibacterial compound against contaminant bacteria in Belanak fish. In conclusion, GMT 3.2.5 and GMT 4.1.3 have some potency as natural material for preservative in fish product. Moreover, some

more research need to be done to optimize this potency.

CONCLUSION

Association bacteria of gastropod GMT 3.2.5 and GMT 4.1.3 were able to inhibit *E. coli* and *B. cereus*. Biochemistry identification shown that GMT 3.2.5 has identic characteristic with genus *Micrococcus* sp. and GMT 4.1.3 has identic characteristic with genus *Bacillus* sp. The supernatant from these bacteria can inhibit contaminant bacteria in Belanak fish (*M. Subviridis*) based on Total Plate Count result.

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