Producing Active Secondary Metabolite Against Pathogenic *Vibrio* spp. by Actinobacteria-Sodium Alginate Co-Culture

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Abstract

Vibrio vulnificus, Vibrio parahaemolyticus and Vibrio harveyi have been found in aquatic environments and suspected as the primary trigger of WFD (White Feces Disease) outbreaks in aquaculture. This Vibrio spp. has an antibiotic resistance to Ampicillin, Co-Amoxiclav, Amoxicillin, Azithromycin, Actinobacteria and Ciprofloxacin HCL. Actinobacteria and alginate have been reported to increase the marine biota resistance against diseases through prebiotic and probiotic mechanisms. This study aims to discover and increase the secondary metabolite production of Actinobacteria-Alginate and its ability as anti-vibrio. Alginate extraction in the samples dated September 2020 originally from Teluk Awur Bays, Jepara, Central Java, Indonesia (33.73±1.84%) was considerably higher than in May 2021 (22.67±0.3%). Samples were taken from sediment and mangrove root. Actinobacteria strains are macroscopically and microscopically similar to the genus Streptomyces. The most well-known antibiotics were produced by Streptomyces spp. The anti-vibrio test was carried out by Kirby-Bauer disc diffusion. The results were observed by measuring the inhibition zone surrounding the paper disc using a digital calliper. Co-culture strain 90 together with alginate have an approved antibacterial activity against all Vibrio spp. in the concentration of 10.disc^{-1} mg and 5 mg.disc^{-1}. Co-culture Actinobacteria with alginate has remarkably changed the green-yellow color to olive green/dark red-orange (strains 3, 62, 63, 72, and 90), indicating the transformation of the formation alginate with pigments into other compounds through the biosynthetic pathway. Therefore, alginate enables to support of Actinobacteria by induction the active secondary metabolite as an anti-vibrio to counteract the bacterial pathogen diseases.

Keywords: *Alginate, Actinobacteria, Co-culture, Vibrio spp.*

Introduction

Vibrio spp. live in the high tolerate salinity of aquatic environments (Lee *et al.*, 2018) and mostly pathogens (Baker-Austin *et al.*, 2018). *Vibrio vulnificus* (Jones and Oliver, 2009), *Vibrio parahaemolyticus* (Yeung and Boor, 2004; Letchumanan *et al.*, 2014), and *Vibrio harveyi* (Liuxy *et al.*, 1996; Xu *et al.*, 2017) are pathogens. Predominated of *Vibrio* spp. instead of *Alteromonas* sp. and *Pseudoalteromonas* sp. caused White Feces Disease (WFD) outbreaks in the Pacific *Penaeus vannamei* (Alfiansah *et al.*, 2020) shrimp.

FAO data from 1998-2018 shows that the shrimp family has exported around 1,860,820-4,675,650 tons with a 5.58-5.64 USD.kg^{-1} selling price. Total production in 2012 was 3,349,620 and increased to 3,455,260 tons in 2018. Other data from FAO shows that cultivation yields reached 4,064,000 tons (12,394 million USD) in 2012 and increased to 6,004,000 tons (15,549 million USD) in 2018 (FAO, 2020). On the other hand, disease losses of AHPND and WSSV shrimp (Asia) in 2015 were as big as more than 26 million USD and more than 11 million USD, respectively (Shinn *et al.*, 2018). The bioeconomic model was used to see the impact of AHPND on the production of *Litopenaeus vannamei*. The seven worst-case scenarios of increasing severity are very extreme, with an average loss of -727.56 USD.ha^{-1}, a benefit/cost ratio of 0.52 with a probability loss of 95.9% (Estrada-Perez *et al.*, 2020).

Overuse of antibiotics in shrimp culture will lead to resistance (Davies and Davies, 2010). Schar *et al.* (2020) reported that people antibiotic consumption in the world in 2017 was 10,259 tons and tend to increase up to 33% (13,600 tons) in 2030. The Asia-Pacific region has the largest global antibiotic consumption (93.8%), and China

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contributed at 57.9%. Antimicrobial consumption per species group were catfish 157 mg kg^{-1} (UI 9-2751); tilapia 59 mg kg^{-1} (UI 21-169); shrimp 46 mg kg^{-1} (UI 10-224). Due to its resistance, so, therefore, the cultivars have to activate their immune system to survive. Prophylactic action with natural, environmentally friendly marine seaweed (*Sargassum* sp.) is an alternative solution. The application of prebiotic immunostimulants as natural ingredients, namely alginate (Yudiati *et al.*, 2016), have been reported.

Alginate is extracted from brown algae, ie. *Sargassum* sp. (Yudiati and Isnansetyo, 2017) and *Padina* sp. (Yudiati *et al.*, 2020). Alginate has been shown to the increase non-specific immunity of shrimp (Yudiati *et al.*, 2016; Yudiati *et al.*, 2019) and is capable of improving the resistance to diseases such as *V. parahaemolyticus* infection (Cheng and Yu, 2013), *V. harveyi* (Jiang *et al.*, 2017), *V. alginolyticus* (Cheng *et al.*, 2004; Cheng *et al.*, 2005; Cheng *et al.*, 2007; Cheng *et al.*, 2008), *Streptococcus agalactiae* (Van Doan *et al.*, 2016), *S. iniae* (Harikrishnan *et al.*, 2011), *White Spot Syndrome Virus (WSSV)* (Yudiati *et al.*, 2019), *Streptococcus* sp. and *Iridovirus* (Yeh *et al.*, 2008), respectively.

Probiotic bacteria are biological control agents in aquaculture (Verschuere *et al.*, 2000), which functionally strengthen the role of prebiotic alginate (Hindu *et al.*, 2018). In addition, the role of bacteria can also be used as alginate lyase (Wong *et al.*, 2000; Kim *et al.*, 2011; Zhu and Yin, 2015). Other studies have shown that Streptomyces sp. RL8 has a positive effect, similar to other known probiotics, namely *Lactobacillus graminis* and *Bacillus* spp. against *Vibrio* sp. (Mazón-Suástegui *et al.*, 2020). Actinobacteria have been applied as probiotic bacteria in aquaculture (Das *et al.*, 2008) with anti-vibrio abilities (You *et al.*, 2005; You *et al.*, 2007). Furthermore, Actinobacteria were also performed as the antibacterial, anti-fungal, anti-tumor, cytotoxicity, cytostatic, antiparasitic, anti-malarial, anti-viral, anti-inflammatory, antioxidant, as well as anti-angiogenesis (Manivasagan *et al.,* 2014). This study aims to discover and increase the secondary metabolite production of Actinobacteria-Alginate co-culture as an anti-vibrio.

Materials and Methods

Sample brown seaweed

Sargassum sp. as the source of alginate were collected at two different times, September 2020 and May 2021, at Teluk Awur Bays, Jepara, Central Java, Indonesia.

Strain bacteria

Vibrio spp. and Actinobacteria used in this study were obtained from the collection of the Biology Laboratory of the Faculty of Fisheries and Marine Science, Diponegoro University. The Vibrio bacteria isolated directly from shrimp ponds were *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio harveyi*. In our previous study, it has been shown that *Vibrio* spp. has antibiotic resistance to Ampicillin, Co-Amoxiclav, Amoxicillin, Azithromycin, and Ciprofloxacin HCL (Yudiati *et al.*, 2021). The actinobacteria used were derived from sediments (Strains 3, 14, 35, 45, and 46) and mangrove roots (Strains 62, 53, 64, 72, and 90).

Characterization of Actinobacteria

The Actinobacteria isolates used were observed for spore chain morphology. These were observed under the microscope with 400x magnification to examine the presence of spore chains and observe sporophores' nature (Priyanka *et al.*, 2019).

Screening of antivibrio

Antivibrio screening was carried out by overlay method referring to Shetty *et al*. (2014). Actinobacteria were grown in Gauze no 1 medium (Atlas, 2010) for 72 h at 28^{0}C. This medium has a composition of 20.0g Soluble Starch, 1.0g KNO_3, 0.5g K_2HPO_4, 0.5g $MgSO_4$, 0.5g NaCl, 0.01g $FeSO_4$, and 30.0g agar. The medium for growing the test bacteria was Nutrient Broth (Merck, USA). After growing, the test bacteria were prepared for 16-20 h before being planted into a semi-solid with a final concentration of agar less than 0.5%. Then, it was poured on a petri dish containing Actinobacteria as thin as possible and incubated for 24 h at 28^{0}C. An inhibition zone will be formed on the side of Actinobacteria, indicating the antibacterial activities.

Alginate extraction

Alginate preparation was carried out using the sodium alginate pathway referred by Yudiati and Isnansetyo (2017).

Fermentation Actinobacteria with sodium alginate

Actinobacteria fermentation was carried out in media with and without alginate. The media used were 1/10 gauze no 1 (Atlas, 2010), media 27 (Ding *et al.*, 2015), and 0.3% sodium alginate (Wang *et al.*, 2013; Li *et al.*, 2016). Media 27 contain of 2% soybean powder, 2% glucose, 0.3% $CaCO_3$, and 0.5% NaCl. All fermented products were extracted with ethyl acetate, referring to Thirumurugan *et al.* (2018) with slight modifications. The supernatant

was extracted with an equal volume of ethyl acetate (1:1). The supernatant was separated from the mycelia using centrifugation (5000 rpm) for 15 mins and filtered (Whatman No.1). We collected 400 mL of the supernatant and then concentrated it with a rotary evaporator (50^{0}C).

Antivibrio tests

The compound was carried out by Kirby-Bauer disc diffusion test (Bauer *et al.*, 1966) with Whatman, no. 3 as paper disk filter (Valgas *et al.*, 2007). Mueller-Hinton Agar (Merck) was used as a test medium containing 300.0 g beef infusion, 17.5 g acid hydrolysate of casein, 1.5 g starch and 17.0 g agar (Mueller and Hinton, 1941; Atlas, 2010). Bacterial density in Nutrient broth (Merck) was adjusted to the standard 0,5 McFarland (1-2 x 10^8 CFU). The final concentration of the extract on the paper disc were 10, 5 and 1 mg.disc^{-1}.

Twenty mL of MHA in the petri dish was poured and waited until hardened. The bacterial cultured was then swabbed on the MHA surface and dry for 3-5 min (Bauer *et al.*, 1966). We put three paper discs as repetitions into a petri dish (Radjasa *et al.*, 2007), followed by incubation at 37^{0}C for 24 h (Jiang *et al.*, 2018). Gentamicin (10 µg.disc^{-1}) and ethyl acetate was used for positive and negative control. These results were observed by measuring the inhibition zone surrounding the paper disc using a digital calliper.

Result and Discussion

Alginate extraction

The Alginate yield from *Sargassum* sp. in September 2020 was higher than in May 2021 samples (Table 1). In Teluk Awur Bays, September is the peak season, while in May 2021, the growth of natural *Sargassum* sp. began. The smaller thallus width indicated this. Research by Bertagnolli *et al.* (2014) showed that alginate yield from *S. filipendula* seaweed collected during the autumn and spring were 17.0±0.1% and 17.2±0.3%, respectively. Meanwhile, the algae collected during the summer heat showed a lower amount (15.1±0.1%). Suboptimal growth had been confirmed. Algae collected during summertime (February) are characterized by their reproductive period. Summer algae showed lower alginate amounts in branches and stems (15.1%). The study by Zubia *et al.* (2008) on Sargassum mangarevense and Turbinaria ornata showed a significant interaction between seasonal factors and species that indicate seasonal varies. Another study by (Otaola *et al.*, 1969) on acid alginate at the reproductive and vegetative stages found *S. vulgare* and *S. polyceratium*. Acid alginate yield at the reproductive stage was lower when compared to the vegetative stage. In terms of the extraction method, the best yield values are recorded after 5 h of extraction at 60 or 80^{0}C. The value was 40% higher than the extraction yield at 25^{0}C (Torres *et al.*, 2007). The effect of temperature has also been confirmed by (Davis *et al.*, 2004) at 80^{0}C. *S. fluitans* from the Atlantic Ocean and *S. oligocystum* from Goold Island yielded up to 20.5% with alkaline methods at 80^{0}C.

Characterization of Actinobacteria

Actinobacteria have several types of macroscopic and microscopic characteristics. Macroscopic and microscopic characterization of Actinobacteria strain is presented in Table 2. All the Actinobacteria strains contain spores. Microscopically, these isolates were dominated by spiral shapes. A few verticillate spores and rectiflexibiles spore chains have appeared. Based on this, all the Actinobacteria are characterized to *Streptomyces* sp. (Nomi, 1960; Williams and Davies, 1967; Tashiro *et al.*, 1990; Nomi, 1992).

Screening of antivibrio

Screening Antivibrio had negative and positive results (Figure 1). All test results are presented in Table 3. In 50 years, there are more than 10.100 natural compounds of antibiotics produced from filamentous Actinomycetales and dominated by *Streptomyces* spp. (7.600 compounds) and rare Actinomycetes *(*2.500). Actinobacteria have represented the most significant part microbial bioactive metabolite (45%) from the known 22.500 antibiotics from microbes during this period. Amongst those, only 1% (150 compounds) have been developed into drugs (Berdy, 2005).

Based on the results, Table 3 demonstrates that Strain 3, 62, 63 dan 90 has antibacterial activity against all *Vibrio* spp. On the other hand, merely strain 72 is resistant against all *Vibrio* spp. and some variation from other results. *S. californicus* strains MNM-1400 from marine sediments (10 shrimp farms) showed antivibrio activity against V. *alginolyticus, V. anguillarum, V. harveyi,* and *V. parahaemolyticus* (Gozari *et al.*, 2016). Seven isolates of *Streptomyces* sp. from sponges have abilities against *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* (Dharmaraj, 2011). Research by Sarveswari *et al.* (2020) found that *Micromonospora* sp. RMA46 (rare Actinobacteria) from mangrove soil has an anti-infective against *V. cholerae*. Eighteen strains of Actinobacteria (Streptomyces, Nocardioides, and Glutamicibacter) from 8 samples of shrimp-pond water have antibacterial activity against *V.*

parahaemolyticus (Phuong and Diep, 2020). Other research by Su *et al.* (2014) found that *Streptomyces* sp. HSN054 and *Nacardiopsis* sp. HSN054 from specimens of the marine sponge *Mycale* sp. have the ability against *V. diabolicus* and *V. parahaemolyticus*. Actinobacteria associated with red algae *Gelidiella acerosa* have exhibited activity against *V. alginolyticus* (Ulfah *et al.*, 2017; Ulfah *et al.*, 2021) and *V. parahaemolyticus* (Ulfah *et al.*, 2021).

Fermentation and extraction of Actinobacteria-sodium alginate co-culture

The fermentation (Figure 2A-C.) and extraction (Figure 2D.) were carried out for five days (Figure 2.). The different color of the ethyl acetate extract was shown from the fermentation using alginate and without alginate (Figure 2E-I). Generally, co-culture with the alginate has colors that are darker than the non-alginate extract. As shown in Figure 3, the alginate extract color was green-yellow (EtOAc and ethanol). EtOAc extract from water (WEE) and ethanol (EWE) extraction were dominant in yellow, while direct dried in ethanol (EtOH) was dominant in green. Crude extracts from alginate and fermented Actinobacteria are presented in Figure 4. EtOAc extract from water (WEE) and ethanol (EWE) was 0.01 g in weight, while direct dried extract of ethanol (EtOH) was 0.18 g. All co-culture alginate extracts had a higher weight than without alginate.

Sargassum sp. has many pigments, namely chlorophylls and carotenoids (Erulan *et al.*, 2009; Yip *et al.*, 2014). Fucoxanthin was identified as the carotenoid which presents in *S. binderi* (Yip *et al.*, 2014). Another study reported that *S. arnuticum* was dominated by chlorophylls and xanthophyll, with some seasonal changes (Lewey and Gorham, 1984). It is postulated, that pigments can be trapped together with alginate during extraction. In this study, the color was changed from green-yellow to olive green/dark red-orange, pursuing formation from polar to semi or non-polar. Chlorophyll and Xantophyll are polar pigments (Schertz, 1928; Schertz, 1938), while xanthophylls are polar carotenoids containing the essential pigment called lutein (yellow) and zeaxanthin (orange) (Grudzinski *et al.*, 2017). In this study, there was some indication that the pigments turn to beta carotene (yellow) or astaxanthin/ echinenone/ adonirubin/ chataxantine/ adhonaxantin (orange-dark orange) (Minyuk and Solovchenko, 2018) and increased the carotenoids (provitamin A) content (Provitamin A) (Yao *et al.*, 2018). Dambek *et al.* (2012) reported another carotenoid biosynthesis pathway in *Phaeodactylum tricornutum* from beta-carotene to diadinoxanthin and fucoxanthin end products. Several types of bacteria, such as *Massilia* sp., can produce fucoxanthin (Shen *et al.*, 2018). Fucoxanthin is brown or olive-green color (Miyashita *et al.*, 2011). EtOAc and EtOH (1:1, v/v) were the best extraction solvent to carry out Astaxanthin (Liu *et al.*, 2011). *Streptomyces* sp. has cellulase-producing for bioethanol production (Hsu *et al.*, 2011). Astaxanthin has a polar-nonpolar structure (Stachowiak and Szulc, 2021). The biosynthesis of xanthophylls derives from the synthesis of non-polar carotenoids such as beta-carotene, astaxanthin and fucoxanthin (Alcaino *et al.*, 2014).

This present study strongly indicated that Streptomyces sp. strains 3, 62, 63, 72 and 90 can use pigments and alginate polysaccharides for fermentation as a pathway for biosynthesis. Supporting this fact, *S. griseus* using plasmids and genetic techniques for gene crtU that assigns to encode a unique desaturase responsible for converting L-carotene via L-isorenieratene to isorenieratene by a desaturation/ methyl-transferation mechanism (Krügel *et al.*, 1999). In addition, a purified carotenoid fraction from *S. coelicolor* wild-type cells grown for two days under blue light illumination identified as beta-carotene and isorenieratene by HPLC analysis (Takano *et al.*, 2005). Other studies, *Streptomyces* sp. HJC-D1 can remove chlorophyll a (anti-cyanobacterial) (Kong *et al.*, 2014). A similar result was strengthened by Hua *et al.* (2009) and Zhang *et al.* (2014) using *S. grisovariabilis* and *S. alboflavus* as anti-cyanobacterial.

Antivibrio test

Antibacterial test against *Vibrio* spp. showed positive and negative results (Figure 5A) with positive and negative controls (Figure 5B). All extracts and negative controls had negative results except strains 90-Alg. Zone of inhibition against *Vibrio* spp. presented in Figure 6. The 90-Alg strain had positive results at concentrations of 10 mg and 5 mg.disc^{-1} with different inhibition zones. During the screening, our study found that *Streptomyces* sp strains 3, 62, 63, and 90 had antibacterial activity against *Vibrio* spp. though the concentration was shallow. Strain 90 denoted that alginate was successfuly increased the antibacterial performance. Supporting facts was reported. After adding mannan oligosaccharides at 24 h, the maximum increase in specific productivity of penicillin G compared to control cultures was 130% (Ariyo *et al.*, 1998). Another similar research was done by Asilonu *et al.* (2000). The researchers added 50 to 100 g.ml^{-1} acid hydrolyzed alginic oligosaccharides and enzyme hydrolyzed pectin oligosaccharides to the *Penicillium chrysogenum* culture. This co-culture increased chrysogenin production by more than 30 to 40% in shaken flasks

and bioreactors. Another study was also showed that alginate oligosaccharides extracted from brown algae (*Sargassum* sp.) act as alginate-derived elicitors on beta-glucan production in cauliflower mushrooms (*Sparassis latifolia*). Some oligosaccharides also promote biomass formation but are not used as a carbon source (Kim *et al.*, 2020).

Fosfomycin is an antibiotic from *Streptomyces* sp. One of the antibacterials targetted to the bacterial cell wall is Phosphonic Acid (Fosfomycin) (Hendlin *et al.*, 1969). Fosfomycin is bactericidal that inhibits the biosynthesis of peptidoglycan in gram-positive and harmful bacteria (Kahan *et al.*, 1974; Skarzynski *et al.*, 1996; Karadb *et al.*, 2014). This antibiotic acts as phosphoenolpyruvate (PEP) analog that will bind to an essential enzyme of peptidoglycan biosynthesis, namely MurA (UDP-GlcNAc enolpyruvyl transferase) (Brown and Wright, 2016). This then catalyzes the transfer of the enolpyruvyl moiety of PEP to the 3'-hydroxyl group of UDP-N- acetylglucosamine (UNAG) (Eschenburg *et al.*, 2005). The formation of N-acetylmuramic acid from the reaction between N-acetyl-glucosamine and phosphoenolpyruvate, resulting in bacterial cell lysis and death (Castañeda-García *et al.*, 2013; Sastry and Doi, 2016). In addition, antibiotics have mechanisms of action such as peptidoglycan synthesis, bacterial ribosomes (targeting the 30s subunit and 50s subunit), DNA (replication and transcription), folate biosynthesis, tRNA synthesis, and fatty acids acid and mycolic acid biosynthesis, bacterial membranes, and agents that exert pleiotropic effects or unknown effects (Lange *et al.*, 2007).

Some studies have shown that feed enriched with three probiotic (*Streptomyces* sp. RL8, *Lactobacillus graminis* and *Bacillus* spp.) formulations resulting in a significant stimulation when challenged with *V. parahaemolyticus* due to the Bacterovorax population and other antimicrobial producing agents (Mazón-Suástegui *et al.*, 2020). Furthermore, the hydrolysis of polysaccharides of actinobacteria suggests the potential of these microbes for industrial applications. Therefore, continuous studies on the identification and characterization of potential genes and their encoding enzymes targetting different industrial applications are required to develop sustainable and cost-effective production for commercialization (Salwan and Sharma, 2018).

Conclusion

Streptomyces spp. strain 90 with alginate co-culture develop the anti-vibrio activity. The co-culture strains 3, 62, 63, 72, and 90 have remarkably changed the green-yellow color to olive green/dark red-orange, indicating alginate and pigments transformation to other compounds through the biosynthetic pathway. Therefore, alginate supports Actinobacteria to induce the active secondary metabolite as an anti-vibrio to counteract the bacterial pathogen diseases.

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