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An Introduction of Autoinducer Biosensor for *Vibrio parahemolyticus*, Pathogenic Bacteria in White Shrimp (*Litopenaeus vannamei*) Intensive Culture

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ABSTRACT

One of the bacteria that causes Acute Hepatopancreases Necrotic Disease (AHPND) is *Vibrio parahaemolyticus*. Factors for the occurrence of pathogenicity in *Vibrio* sp. caused Auto Inducer (AI) in the form of AHL (Acyl Homoserine Lactone), which functions as quorumsensing communication tool for these bacteria. To prevent the achievement of quorum is to detect the presence of Vibrio sp. with β-galactosidase from *Agrobacterium tumifaciens*. The purpose of this research was to make a nanobiosensor to detect *V. parahemolitycus*. based on bacterial AI. This study lasted for 1 year, where 1) screening and isolation of V. parahemolitycus bacteria from shrimp ponds affected by AHPND were carried out, 2) FTIR test for the content of V. parahemolyticus bacteria, and 3) AHL biosensor test with *A. tumifaciens*. The results obtained that the bacterial metabolite extract of *V. parahemolitycus* contained AHL from the amine and amide groups. Its presence can be detected by *A. tumifaciens* containing β-galactosidase.

Key words*: AHL, Vibrio parahemolitycus, Quorumsensing, β-galactosidase*

Introduction

Vibriosis in shrimp often causes mass mortality and crop failure in shrimp ponds in Indonesia and other shrimp-producing countries. Vibriosis disease that often attacks shrimp in the larval, post-larval and young shrimp stages is caused by several species of *Vibrio* sp. including: *V. parahaemolyticus, V. alginolyticus V. angguillaru, V. vulnivicus* and *V. flupialis.* The bacterium V. parahaemolyticus is a gram-negative bacterium which is known as a very acute and malignant disease. *V. Parahaemolyticus* is a native marine bacteria that can be isolated from marine biota, seaweed, sea water and brackish water. The application of technology in efforts to control bacterial diseases has been widely carried out, ranging from prevention to treatment actions using various antibiotics. The habit of using antibiotics has a negative impact, not only causing pathogenic bacteria to become resistant, but also has a negative impact on the environment and surrounding communities. These various Vibrio genera cause various diseases, including: White Feces Syndrome (WFS), Early Mortality Syndrome (EMS), sometimes followed by attacks of secondary diseases such as viruses that cause high mortality of up to 100% in larvae and juveniles (Sukenda *et al.*, 2005; KKP. 2015).

The pathogenicity of *Vibrio* sp. begins with the

quorum sensing process which begins with the release of a molecular substance known as an autoinducer. (AI). Types of Vibrio bacteria can secrete different AI. Based on the released AI, the presence and level of attack will be known.

Many methods have been developed to detect the presence of viruses in body cells or aquatic environments but all of them take time to get results. To shorten the time and efficiency of work can be done with nanosensors, which work based on biological components of nanotechnology has emerged a tremendous potential to improve cultivation with new nanotools. resulting in advances in the application of nanoparticles and emulsion-based systems for fish disease prevention, water purification and nutrient delivery.

This study was aimed to get a nanobiosensor for AHL detector based on autoinducer which can be used to inhibit pathogenic bacteria increasing especially *V. parahaemolyticus* in white shrimp (*Litopenaeus vannamei*) culture.

Methods

Screening and identification of *V. parahemolitycus*

The implementation stage was carried out based on Saejito and Haditomo (2016), including taking bacterial isolation from shrimp ponds in Banyuwangi, East Java; bacterial purification, bacterial culture, bacterial identification. Isolation of bacteria using TCBS (Thiosulphate Citrate Bile Salt) agar medium which is specific for *Vibrio* sp., while for slanted media using TSA (Triptic Soy Agar) with streak method. The target organ for bacterial isolation was hepatopancreas, and purification was carried out to obtain isolates of *V. parahaemolyticus.*

Isolation of *V. parahemolyticus* was carried out three times by taking hepatopancreas samples from shrimp suspected of having AHPND. The first and second identifications were carried out at the BKIPM Laboratory, Juanda, Surabaya; with conventional methods; while the third identification was done in the Lab. BBAP, Bangil; using BD Microbact® kits for bacterial identification

FTIR test for the content of *V. parahemolyticus* bacteria

Bacterial centrifugation of *V. parahaemolyticus*

V. parahaemolyticus bacteria that had been diluted were then centrifuged with a Hettich EBA 20 centrifuge according to Radzi *et al.* (2015) at 6000 rpm for 15 minutes. The supernatant was then poured into an erlenmeyer for further extraction.

Production of *V. parahaemolyticus* Extract

Supernatant of *V. parahaemolyticus* was macerated using ethyl acetate solvent in a ratio of 1:1 in one night (Garcia-Aljaro *et al.,* 2011). The bacterial macerate was evaporated using a digital IKA HB10 rotary evaporator at a temperature that is 40 °C for 7 hours. The AHL extract was then stored at room temperature prior to analysis.

Identification of Compounds using Fourier Transform Infra Red (FTIR)

FTIR test was carried out in October 2019 at the Organic Chemistry Laboratory, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University (UIN) Malang. Fourier Transform Infrared (FT-IR) is a measurement method for detecting the molecular structure of a compound by identifying the functional groups that make up the compound. The advantage of testing using FTIR is that it does not require complicated sample preparation and can be used to identify samples in various phases (solid, liquid or gas). The sample was put into an FTIR spectrophotometer (Varian 1000 FT-IR Scimitar Series), with a wavelength range of 400 to 4000 cm-1 with a resolution of 4 cm-1. The wavelengths that appear can be seen on the annotated spectrum. The infrared absorption spectrum is then interpreted to determine the chemical bonds of the molecule.

AHL biosensor test with *A. tumifaciens*

Media creation - Agrobacterium tumifaciens stock was cultured in 250 ml Erlenmeyer. A total of 200 ml. aquadest is needed to dissolve 6 grams of Trypton Soya Broth (TSB) and 2% NaCl (4 grams) for environmental manipulation of *A. tumifaciens*. The media was wet sterilized by conventional autoclaving at a temperature of 121^{o}C at a pressure of 1 atm/ 0.15 Mpa for 15-20 minutes. The medium was cooled at room temperature. Bacterial culture was carried out by inoculation of bacterial samples on new media that had been prepared using an ose loop with ose loop fixation before taking bacterial samples. The inoculated bacterial stock was then incubated for 18 hours at 30 ^{o}C.

Harvesting. The culture results of *A. tumifaciens* that have been incubated are put into a 1mL appendorph

tube until all the bacterial stock is used up using a micropipette and blue tip. Centrifugation at 12,000 rpm for 10 minutes was repeated 6 times until all tubes were centrifuged. The pellets were taken and transferred to a sterile falcon. The pellet was resuspended with 1mL KH_2PO_4 (100mM, pH 7.4). Sonification is carried out with 50% waves for 30 seconds 3 times with the aim of stirring the sample particles to break up the particles so that they are nano-sized with the effect of sonic waves provided by the sonicator. The sample was transferred to an appendorph tube for re-centrifugation at 12,000 rpm 40C for 30 minutes. The supernatant was transferred to the falcon and obtained "Cell-free lysate".

Biosensor test using *A. tumifaciens*

AHL (N-butyl homoserine lactone) was diluted using PBS in a ratio of 1:1. 50 µL AHL was loaded into 96 well-plates A1, A2, A3 and A6, A7, A8 (control). 50 L of cell extract and 100 L of KH2PO4 (20mM, pH 7.0) were added to the A1, A2, A3 well-plates. 50 L KH_2PO_4 (20mM, pH 7.0) was added to A6, A7, A8 (control). Incubate at 30 °C for 2 hours. The addition of X-Gal to A1, A2, A3 was carried out after incubation and re-incubating at a temperature of 30 °C for 1 hour

Results

The bacteria found in shrimp suspected of being infected with AHPND are *Vibrio parahaemolyticus*, *V. anguilarum*, and *Pseudomonas angiostensin*. This research was focussed in V. parahaemolyticus as research object.

2) FTIR result can be seen in Figure 1 and Table 1. 3) The color density of the test and control samples was significantly different, this was because the bacterial cell content given to the test sample showed a pinkish yellow color, while the control was clear. The absorbance value was measured using a spec-

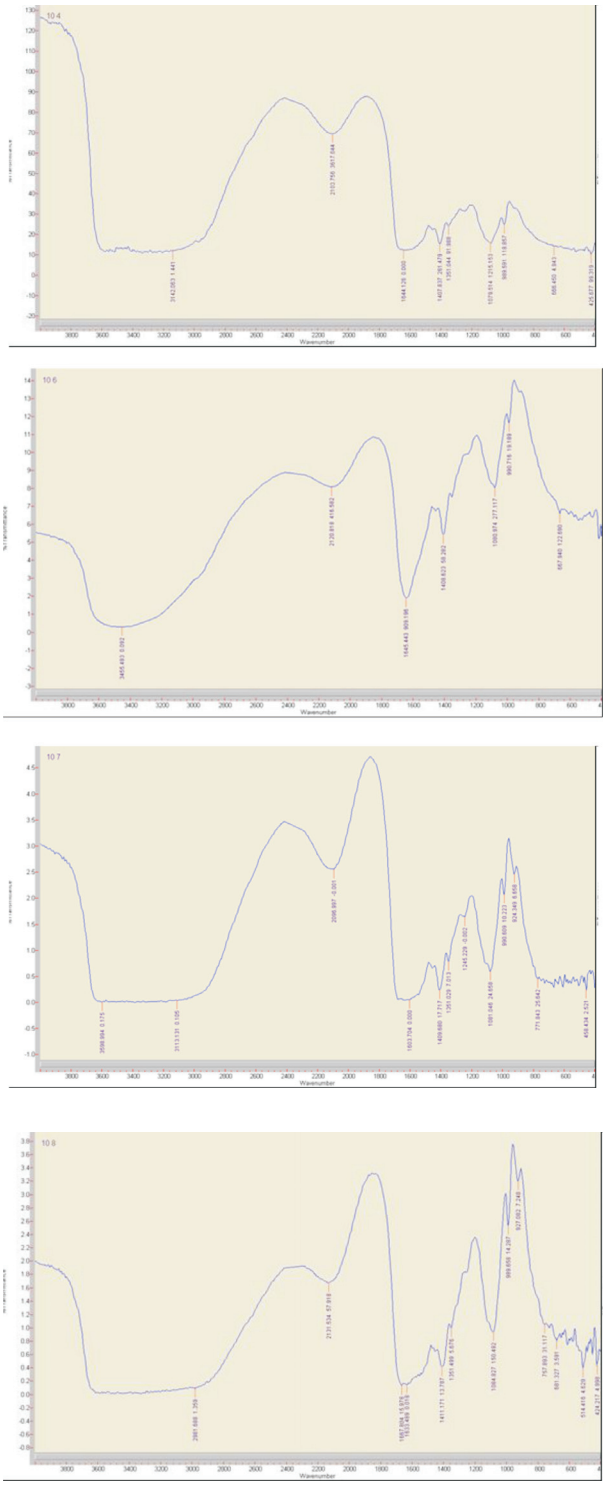

Fig. 1. FTIR result of V. parahaemolyticus in defferent densities; (a) 104, (b) 105, (c) 106, (d) 107, and (e) 108 cfu/mL

Table 1. FTIR test results extract of *V. parahaemolyticus* in different density

Density	Wave Number (cm^{-1})	Bond	Functional Group
10^4	3142,063	sp2 C-H	Aromatic
	2103,756	Ca≡C	Alkuna
	1644,126	C=O	Amida
	1407,837	C-H3	Alkana
	1351,044	C-N	Amina
	1079,514	C-O	Keton
	989,591	C-H	Alkena
	666,450	C-Br	Bromida
	425,677	C-I	Iodida
10^5	3410,051	O-H	Fenolic
	2103,756	Ca≡C	Alkuna
	1647,155	C=O	Amida
	1409,504	C-H3	Alkana
	1106,580	C-O	Keton
	1082,066	C-O	Keton
	668,367	C-H	Alkena
	539,333	C-I	Iodida
	419,394	C-I	Iodida
10^6	3455,493	O-H	Fenolic
	2120,818	Ca≡C	Alkuna
	1645,443	C=O	Amida
	1408,623	C-H3	Alkana
	1080,974	C-O	Keton
	990,716	C-O	Keton
	667,940	C-H	Alkena
10^7	3598,994	O-H	Fenol
	3113,131	sp2 C-H	Aromatik
	2096,997	Ca≡C	Alkuna
	1603,704	C=C	Aromatik
	1409,680	C-H3	Alkana
	1351,029	C-N	Amina
	1245,229	C-O	Keton
	1081,046	C-O	Keton
	990,609	C-O	Keton
	924,349	=C-H	Alkena
	771,843	N-H	Amina
	458,434	C-I	Iodida
10^8	2981,688	O-H	Fenol
	2131,534	Ca≡C	Alkuna
	1667,804	Ca≡O	Amida
	1633,489	C=O	Amida
	1411,171	C-H3	Alkana
	1351,499	C-N	Amina
	1084,827	C-O	Keton
	989,658	C-O	Keton
	927,082	C-O	Keton
	757,893	=C-H	Alkena
	681,327	C-Br	Bromida
	415,416	C-I	Iodida
	424,417	C-I	Iodida

trophotometer using a 635 nm wavelength. The absorbance values obtained with A1, A2, A3 are 0.92; 0.367; 0.188 while the control A6, A7, A8 is 1.209; 0.62; 0.348.

Discussions

V. parahaemolyticus which found in shrimp pond is AHPND or Early Mortality Syndrome (EMS), causes bacteria. EMS usually infects shrimp in 40 DOC (day of culture) or earlier.

The amine and amide functional groups appeared in the FTIR results of the *V. parahaemolyticus* extract. AHL has amine and amide groups in its series, so it can be assumed that in the extract of *V. parahaemolyticus* there is AHL whose number increases with increasing bacterial density.

AHL can be detected using cell free lysate of *A. tumifaciens*. Of all the AHL biosensors available to date, *A. tumefaciens* showed the widest sensitivity to AHL in the lowest concentrations. This b-galactosidase-based biosensor is very suitable for TLC analysis and is very sensitive for many AHLs which only require a small volume of AHL extract from supernatant (Farrand *et al.*, 2002).

Using of cell free lysate system was eliminated the time consuming steps for biosensor cell culture conditioning, which are required prior to whole-cell bioassays. This significantly reduced assay times from greater than 24 h to less than 3 h, while maintaining high sensitivity. Assay lysate may be prepared in bulk and stored (80 °C) over 6 months for future use. The present protocol can be adapted for use with other biosensor strains and be used in high-throughput AHL screening of bacteria or metagenomic libraries.

Conclusion

V. parahemolitycus can be detected in AHPND case, its autoinducer can be presumed in functional group of amida and amina, and detected with *A. tumifaciens*.

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