Effects of Ethanolic Vinasse Extract on Growth, Gene Expression, and Biofilm Formation of AHPND-Causing *Vibrio parahaemolyticus* (Vp_{AHPND}) Strain

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Abstract

The outbreaks of acute hepatopancreatic necrosis disease (AHPND) causes devastative losses of shrimp farming and production. The causative agent of AHPND is the specific strain of *Vibrio parahaemolyticus* (Vp_{AHPND}). Our previous study demonstrated that vinasse, a byproduct of bioethanol production, and vinasse extract using ethanol as a solvent are able to inhibit the growth of Vp_{AHPND} through anti-quorum sensing (QS) or quorum quenching (QQ) activity. Therefore, this study focused on the effects of ethanolic vinasse extract at various concentrations on growth, biofilm formation and expression of virulence-related genes of Vp_{AHPND}. The growth inhibitory effect was tested at 0.20%, 0.10%, 0.05% and 0.02% of the extract compared to that of the control (without the extract). The result showed that only 0.20% showed QQ activity while the lower amounts of the extract showed no effect. The concentrations of 0.20% and 0.02% were further evaluated for its effects on biofilm formation and the gene expression by using SEM and quantitative RT-PCR, respectively. Corresponding to the bacterial growth, 0.20% arrested the biofilm formation and significantly downregulated the Photorhabdus insect-related (Pir) A toxin, Pir B toxin compared to the control ($P<0.05$). However, the expression level of membrane-associated transcriptional factor (ToxR) was induced in 0.20% vinasse extract. Time course expression of signal generator (LuxI) and signal receptor (LuxR) in QS were also measured. The expressions of QS-related genes in the treatments of vinasse extracts were lower than that detected in the control, however, it seemed not to correlated to the result of bacterial growth. In conclusion, the inhibitory effects against Vp_{AHPND} depended on the amount or concentration of ethanolic vinasse extract. High enough concentration could play a role as quorum quencher while too low concentration exhibited no effect.

Keywords: Quorum quenching, Biofilm, $Vp_{AHPND,}$ Vinasse

Introduction

Vibrio spp. is known as the most common bacteria causing diseases in shrimp production. Recently, outbreaks of acute hepatopancreatic necrosis disease (AHPND) has served bad impact to shrimps farming industry. The agent that involve in AHPND has been reported as *V. parahaemolyticus* that carries Photorhabdus insect-related (Pir) like toxins encoded by pirA- and pirB genes (Han *et al*., 2015).

Previous study has reported that The $PirAB^{VP}$ toxins are the primary virulence factor of AHPND-causing bacteria, responsible for causing massive mortality in shrimp (Choi *et al*., 2017; Kumar *et al*., 2019). The virulence factors of Vp_{AHPND} include extracellular toxin (metalloprotease, extracellular capsular polysaccharide, serine proteases, type III secretion), flagella motility, and haemolysin and their expression are controlled by QS (QS) (Natrah *et al*., 2011). Pathogenicity of *Vibrio* spp. especially Vp_{AHPND} that infect shrimp is closely related to the release of factors regulated by QS. QS is bacteria communal behaviors are regulated by specialized cell-to-cell communication systems (Liu *et al*., 2018). This communication happens through the production, release and detection of signal molecules, also known as autoinducers. It can induce or regulate the virulence factors, for

example, gene expression and biofilm formation. The disruption of QS mechanism is known as quorum quenching (QQ).

QQ can reduce the pathogenicity of pathogenic bacteria without killing them. Theoretically, it could result in a situation in which there is less selective pressure on bacteria to develop resistance to the QQ agent than the development of antibiotic resistance after antibiotic treatment (Allen *et al*., 2014). Alternative substances including natural products from potent herbal plants and effective microorganisms responsible for the control of pathogenic bacteria have been interesting in order to replace antibiotics (Hai, 2015).

Several strategies have been proposed as QQ agent to prevent AHPND, such as the usage of plant-derived compounds, probiotic, phages, nanoparticles, and recombinant immune-related proteins with antibacterial effects (Kumar *et al*., 2020). Recently, exploring substance derived from byproduct has chosen, one of the is byproduct vinasse. Vinasse is waste products from ethanol production and microorganisms that have potential produce compound to display QQ in pathogen. As yeast biomass production, vinasse that produce by cultured in industry could be valuable. The utilization of vinasse extract has been used as mixture feed addictive that was tested in Nile tilapia (Chirapongsatonkul *et al.*, 2019). Previous study also reported that vinasse is able to inhibit the growth of *V. parahaemolitycus* and AHPND-causing *Vp*$_{AHPND}$ in the aspect of QQ effect (Tep-ubon *et al.,* 2020; U-taynapun, 2022). It has demonstrated that ethanolic extract of vinasse is really effective to inhibit bacterial activity and showing QQ against *V. parahemolyticus*. Therefore, the effects of the extracted substance derived from vinasse on growth inhibition, gene expression, and biofilm formation of *Vp*$_{AHPND}$ was observed.

Materials and Methods

Vinasse extraction

The vinasse, provided by the private company in Thailand, was extracted using ethanol as the extraction solvent following U-taynapun *et al*. (2022). Firstly, 100 g of vinasse biomass was mixed with ethanol with a ratio of 1:4 (w/v), stirred by using a magnetic mixer then kept in dark overnight. The mixture was filtered through a Whatman No. 1 filter paper with the aid of a vacuum. The supernatant was separated and ethanol was removed by evaporation. The obtained crude extract was dried, weighed and kept at -20^{o}C until used. For further study, the ethanolic vinasse extract was prepared as stock (2.0%) by dissolving in 20% (v/v) ethanol then filtered through the membrane filter pore size 0.45 µm and kept at -20°C.

Vp$_{AHPND}$ and growth condition

Vp$_{AHPND}$ was isolated from the AHPND-diseased shrimp from the private farm in Nakhon Si Thammarat Province, Thailand. Pure culture of *Vp*$_{AHPND}$ was performed and kept at -80°C followed the protocol previously described by Bunserm *et al*. (2022) in a house culture collection (Aquatic Animal Health Management Research Unit, Department of Agricultural Science, Faculty of Agriculture, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat Campus). The bacterial cells were cultured in tryptic soy broth (TSB, Difco) containing 1.5% (w/v) NaCl (TSB+) with 150 rpm-shaking at 37^{o}C for 18-24 h. Then it centrifuged 8,000 x g for 10 min and resuspended in sterile 1.5% (w/v) NaCl. The bacterial concentration was adjusted to concentration of approximately 10^{7} CFU/ml (OD_{600} ~ 0.5) prior to be using for further experiments.

Observation of the growth of *Vp*$_{AHPND}$

The growth of *Vp*$_{AHPND}$ was measured real-time according to the methods of U-taynapun *et al*. (2022). Firstly, the stock of *Vp*$_{AHPND}$ was sub-cultured twice onto tryptic soy agar (TSA, Difco) containing 1.5% (w/v) NaCl

(TSA+), then transferred into the freshly prepared TSB+ and adjusted to a final concentration of 2 x 10^6 CFU/ml. The bacterial growth was measured in TSB+ medium containing the ethanolic vinasse extract at various final concentrations (0.20%, 0.10%, 0.05%, and 0.02%). The starter of *Vp*$_{AHPND}$ was added to the final concentration of 10^5 CFU/ml while 1.5% NaCl was added to adjust the final volume of 10 ml for each tube and used replace the extract in the control treatment. The growth curve of *Vp*$_{AHPND}$, was recorded at 37°C for 24 h using RTS-1C Personal Bioreactor (Biosan). This experiment was performed in triplicate.

Analysis of bacterial cell and biofilm formation by scanning electron microscopy (SEM)

The morphology of *Vp*$_{AHPND}$ cells and biofilm formation influenced by the vinasse extracts was determined through SEM images according to the method of Guo *et al*. (2019). The bacterial cells cultured in TSB+ at 37°C for 18-24 h were adjusted to OD_{600} of 0.5 and transferred into the TSB+ containing the ethanolic vinasse extract at the final concentration of 0.02% and 0.2% while 1.5% NaCl replacing the extract was used as the control. The cells were incubated at 37°C for 24 h. Afterwards, 20 µl of the inoculated cell suspension was continued to nuclear pore polycarbonate membranes, and fixed in 2.5% glutaraldehyde at 4°C for 24 h. The process for bacterial cell dehydration was conducted by increasing serial concentrations of ethanol (from 30%, 50%, 70%, 80%, up to 100%). The dried cells were affixed onto stubs and coated with 40-60 nm of gold. The images of bacterial cells were observed by SEM (Zeiss/Merlin compact) and photographed.

Analysis of gene expression

To analyze the gene expression, total RNA was extracted from *Vp*$_{AHPND}$ cells cultured in the medium containing vinasse extract at concentrations of 0.20%, 0.02% and control (without the extract) for 48 h in personal bioreactor system (RTS, Biosan) using Presto™ Mini RNA Bacteria Kit (Geneaid) following the manufacturer's instruction. The RNA was checked for the quantity and integrity by spectrophotometer (BioDrop) for further analysis. One µg of total RNA was used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturing instruction in the T100TM Thermal Cycler (BioRad). The relative mRNA expression level of 3 virulence genes; PirA, Pir B and ToxR, and 2 genes involved in QS system; signal generator (LuxI) and signal receptor (LuxR), was quantitatively analyzed through a qPCR technique by using 16S rRNA as a reference gene. Each qPCR reaction mixture (total 20 µl) contained cDNA, HOT FIREPol® EvaGreen® (Solis Biodyne), primers (the details were shown in Table 1) and the final volume was adjusted with DEPC-treated water. The reactions were performed in a CFX96 Touch™ Real-Time PCR (Bio-Rad) in triplicate in a 96-well plate. Cycling conditions were as follows: 95°C for 12 min followed by 40 cycles of 15 s at 95°C, 20 s at 58-65°C (depending on the primer pairs) and 20 s at 72°C. Melting curves were also analyzed for all amplification products. The fold change of the relative expression of all virulence genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

The statistical difference of expression levels of virulence- and QS-related genes was analyzed by one-way analysis of variance (ANOVA) via the SPSS Statistics software version 16.0 (SPSS Inc.). The significant differences among treatments were analyzed using a multiple comparison by Duncan's Multiple Range Test (DMRT).

Table 1 Primer used for quantities RT-PCR (qRT-PCR) analysis of the gene expression

Primer name	Primer sequence	Target	References
PirA-1_F143	5'-GTGGGGAGCTTACCATTCAA3'	*Pir A toxin*	Pragthong *et al.*, 2020
PirA-1_R310	5'-CACGACTAGCGCCATTGTTA3'		
PirB-11_F514	5'-TACATGGCTTGTGGTCTGGA3'	*Pir B toxin*	Pragthong *et al.*, 2020
PirB-1_R715	5'-ACCAACTACGAGCACCCATC3'		
ToxR2VPF105	5'-AGGAAGCAACGAAAGCCGTA-3'	*membrane-associated transcriptional factor* (ToxR)	Pragthong *et al.*, 2020
ToxR2VP – R314	5'-TAGCCTCGTTTTGGAACGGT-3'		
LuxI-qrt-F	5'-AATGGTGCAAACCTGGTCGAT-3'	*LuxI*	Yu *et al.*, 2020
LuxI-qrt-R	5'-CTCGCGAAATGCCTCATCCT-3'		
LuxR-qrt-F	5'-CTTTGAGCTGTGCACTGGGA-3'	*LuxR*	Yu *et al.*, 2020
LuxR-qrt-R	5'-ATGACGGTTTCGGTGCTGAT-3'		

Results

Effect of ethanolic vinasse extract on the growth of Vp_{AHPND}

The inhibitory activity of various concentrations of ethanolic extract was tested *in vitro* by observation of Vp_{AHPND} growth. The growth curve of the bacteria was obtained by real-time detection of the OD_{850} every 10 min for 24 h (Figure 1). According to the results, various concentrations of the ethanolic extract showed diverse effects on on Vp_{AHPND} antibacterial activity. At the concentration of 0.20% ethanolic vinasse extract showed QQ activity against Vp_{AHPND}. The kind of growth exhibiting QQ effect, short log phase and faster stationary phase, was detected in the 0.20% ethanolic extract while 0.10% of the extract slightly decreased the growth of bacteria but not effective to inhibit the growth. The control, 0.05% and 0.02% of extract showed no inhibitory activity.

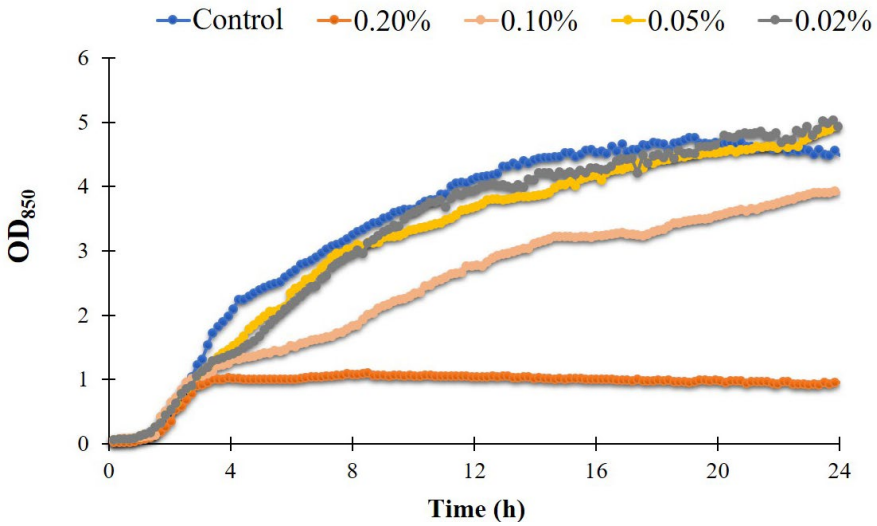

Figure 1 Growth curve of of Vp_{AHPND} cultured in the media containing various concentrations of ethanolic vinasse extract (0.20%, 0.10%, 0.05%, and 0.02%) observed for 24 h.

Examination the biofilm formation of Vp_{AHPND}

Based on QQ inhibitory result, ethanolic vinasse concentration that used for biofilm formation analysis were control (0% of the extract), 0.02%, and 0.20% representing the concentration unable to inhibit and able to inhibit the growth. The morphology as well as biofilm formation of Vp_{AHPND} treated with the vinasse extracts was observed through SEM as present in Figure 2. The results of control and 0.02% revealed no negative effect

on bacterial cell and biofilm formation since dense biofilm was found. However, the cells in the treatment of 0.20% ethanolic vinasse were disrupted, in addition, the number of cells was visibly decreased compared with the control.

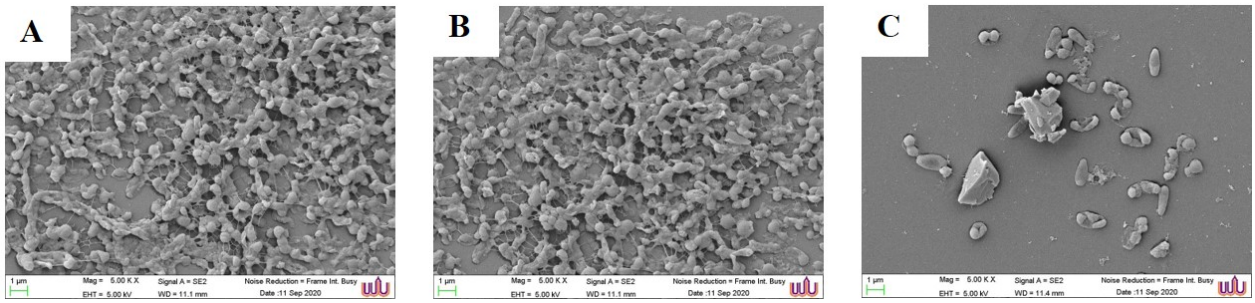

Figure 2 SEM images of *Vp*$_{AHPND}$ treated with various concentrations of ethanolic vinasse extracts; **(A)** Control, **(B)** 0.02% and **(C)** 0.2% for 24 h.

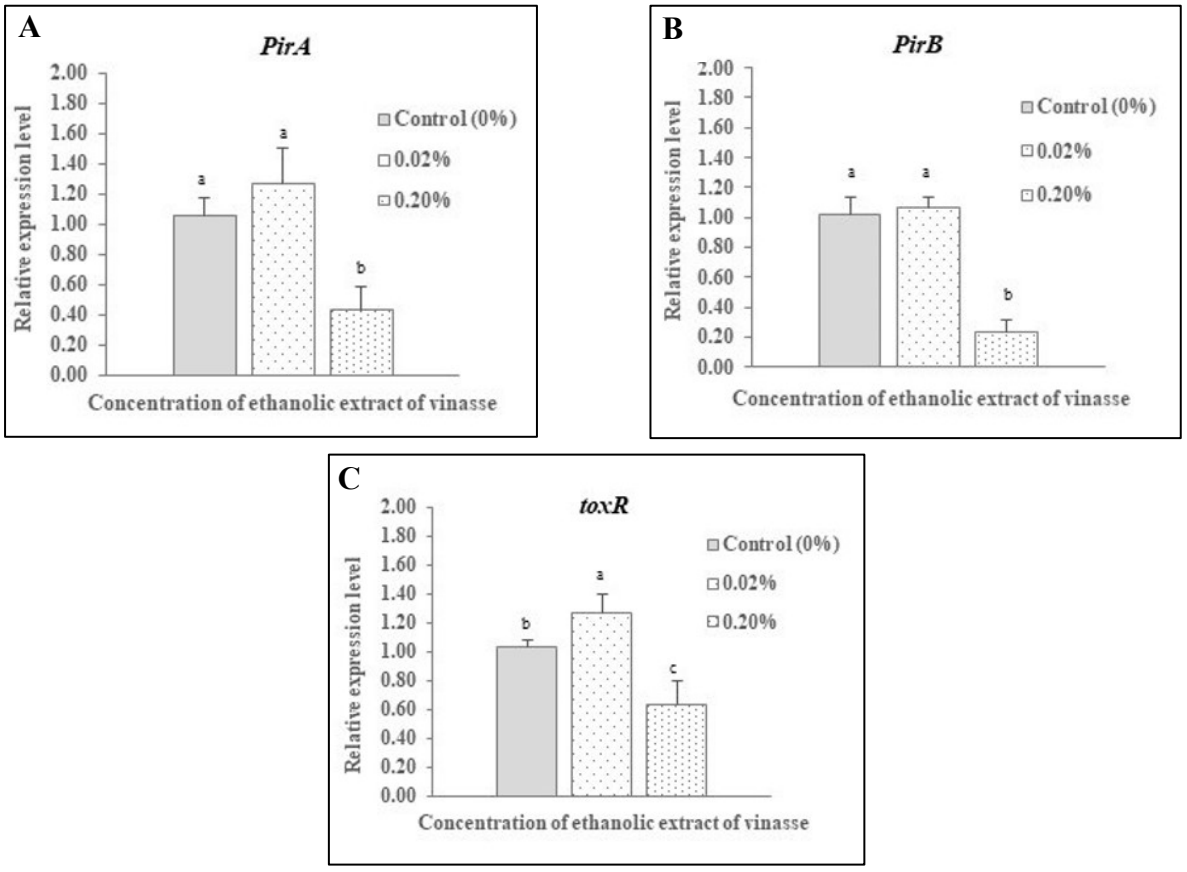

Figure 3 Expression of virulence-related genes of *Vp*$_{AHPND}$ when cultured in medium containing ethanolic vinasse extract at concentrations of 0.20% and 0.020%. The results were expressed as the mean ± SD. Bars and different letters stand for statistically significant differences (P<0.05) between groups.

Expression of virulence- and QS-related genes of *Vp*$_{AHPND}$

The effect of vinasse extract on the gene expression of *Vp*$_{AHPND}$ was revealed by qRT-PCR. The results showed that the levels of PirA were significantly higher (P<0.05) in the bacteria treated with 0.20% extract

compared with control, meanwhile 0.02% were not different (Figure 3A). Similar pattern and statistical differences were obtained for the expression of PirB gene (Figure 3B). The expression level of ToxR was significantly induced in the bacteria grown under different concentration whereas 0.02% ethanol increased and 0.20% decreased (Figure 3C).

The expression level of genes associated with the QS was shown in Figure 4. The expressions of LuxI and LuxR that is responsible to protein catalyzes of N-acil-homoserine lactone (autoinducer) and bond to autoinducer to contribute on QS activity were measured. The expression of LuxI was increased in ethanolic vinasse and reached the highest level at 24 h. However, there was significantly differed in 0.02% treatment, compared with those observed in 0.20%. Similar with LuxI, the pattern of ethanolic vinasse showed LuxR increased the level expression at 24 h. However, treated with 0.20% extract showed the lowest expression at log phase or exponential compared with control.

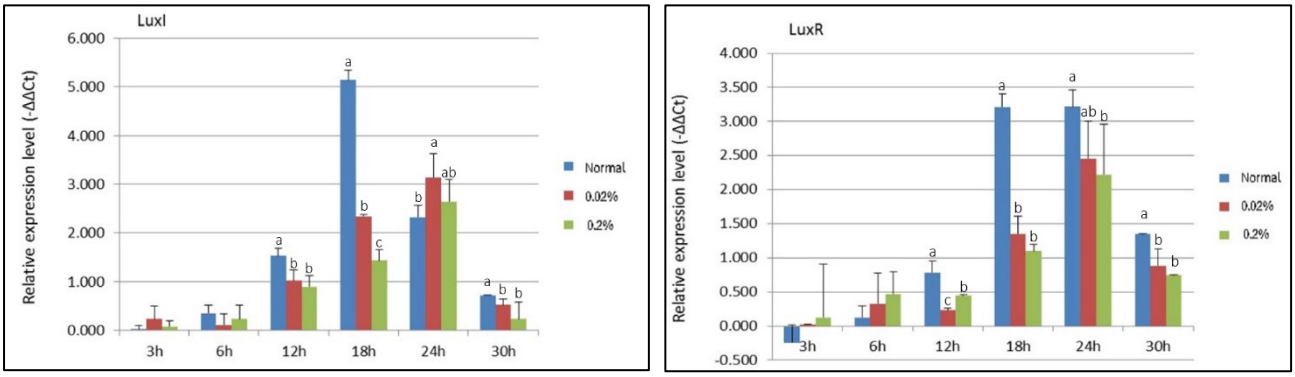

Figure 4 Expression of QS-related genes of *Vp*$_{AHPND}$ when cultured in medium containing ethanolic vinasse extract at concentrations of 0.20% and 0.020% by time variation until 30 h.

Discussion

One of pathogen that usually being major concern for shrimp culture is particularly those belonging to the genus of *Vibrio*. The emergence of a bacterial disease called AHPND has led to a decline in shrimp production and severe economic losses is caused by *V. parahaemolyticus* strain *Vp*$_{AHPND}$ (Hong *et al*., 2016). Utilization of bioactive compounds as antibacterial activity against bacterial pathogen has been reported, recently. Our previous work has constructed that the crude extract product (Teb-Ubon *et al*., 2020) and mixtures containing the vinasse and yeast extracted with organic solvent with fixed concentration, 2% (U-taynapun *et al*., 2022) showed antibacterial activity against the Gram-negative bacteria, in the kind of QQ effect). By using organic solvent, it reported that vinasse substance under differential solvent polarity condition was showed ethanolic vinasse as the most effective solvent to inhibit *V. parahaemolyticus* growth and biofilm formation without killing them. Therefore, in this present work scenario we were focused to decrease the concentration of ethanol for long-term investment with same ability including can inhibit *Vp*$_{AHPND}$ growth, biofilm formation and modulate the virulence-related genes by *In Vitro*, effectively.

Based on the result, ethanolic vinasse concentration 0.20% showed growth inhibition activity QQ because the log phase or exponential phase of the test *Vp*$_{AHPND}$ cultured decrease and the stationary phase appeared earlier than the control and other treatment. At low cell density (LCD), when autoinducer concentration is low, QS promotes gene expression programs that benefit individual bacteria although at high cell density (HCD), when autoinducer concentration exceeds the threshold required for detection, it beneficial to the community (Jemielita *et al*., 2018). Previous study has been reported that yeast extract has ability to

degraded N-acyl homoserine lactone (AHL) signaling moleculs as QS signal was one of way to activated QQ (Ghani *et al*., 2014; Wong *et al*., 2013).

After 24 h, the bacterial number was decreased around 4.5-log in ethanolic vinasse 0.20% concentration compared to that measured in control. This finding was similar to the ethanolic vinasse extract at 2% in our previous study (U-taynapun *et al*., 2022). These observations led us to know more whether morphological changes would be induced upon growth of Vp_{AHPND} grown using scanning electron microscopy by comparing specific concentration (control, 0.02%, and 0.20%).

One of the virulence factors produced by the pathogenic *Vibrio* spp. and causing the disease is biofilm formation. In addition, the morphological structures of the biofilm were obviously differed among the test concentrations. Mechanisms of QQ activity in controlling bacterial biofilm formation were: (1) inhibit AIs synthesis; (2) degrade or inactivate AIs by AHL-lactonases, oxidoreductases, antibodies, etc.; (3) interfere with the signal receptors using AI antagonists; (4) interfere with the response regulators thus disturbing signaling cascade; (5) reduce the extracellular AIs accumulation by inhibiting AIs efflux hence inhibited cell-to-cell signaling (Zhou *et al*., 2020). The effectivity to inhibit biofilm formation was determined on this work whereas we found the most powerful anti-biofilm formation in Vp_{AHPND} referred to the ethanolic vinasse concentration at 0.20% measured by SEM. These circumstances were relatable with the growth inhibition of the vinasse extracts previously described.

The virulence of Vp_{AHPND} is conferred by the pVA1 plasmid that includes the operon encoding homologs genes Photorhabdus insect-related (Pir) toxins that contribute at pore-forming including Pir toxin, PirA, and PirB (Lee *et al*., 2015). Our results of gene expression of treatment at 0.20% compared with control showed downregulated of virulence-related genes including PirA, PirB, and ToxR significantly. Meanwhile, ToxR expresses level of 0.02% treatment were showed interesting case whereas the expression was upregulated. This condition was corelated with previous study about NaCl and pH comparation that reported not optimal condition can cause stress condition that prevents adaption to additional stresses that so ToxR upregulated (Whitaker *et al.,* 2010). The $PirAB^{VP}$ and toxins are the primary virulence factors of AHPND-causing bacteria that mediates AHPND and mortality in shrimp. ToxR is the gene encoding protein that acts as the virulence regulator. Zhang *et al*. (2018) have reported that virulence factors of *V. parahaemolyticus* can be activated via direct repression of T3SS1 genes but activates *Vp*-PAI (T3SS2 and *tdh2*) genes where it binds to the multiple promoter-proximal DNA regions within the T3SS1 locus and biofilm formation also under the control ToxR,

QS in *Vibrio* spp. regulated by the production of virulence associated elements. *LuxI* and *LuxR* as autoinducer is really important on QS especially in *V. parahaemolyticus*. The utilization ethanolic vinasse extract gave an impact to *LuxI* and *LuxR* production. In signaling of *LuxI*, ethanolic vinasse demonstrated the highest expression at 24 h. Same case with ToxR, 0.02% significantly upregulated the level expression compared with control and 0.2% not significant. However, in our present work the level expression of LuxR actively upregulated at 24 h while ethanolic vinasse 0.20% showed significantly lower expression compared with control. LuxI protein is the AHL synthases as signal generator and produced signaling molecules (Dong *et al*., 2017) while LuxR protein is a transcriptional regulator that binds the AHLs as a ligand or signal receptor (Ball *et al*., 2017).

LuxI, the autoinducer synthase produced the AHL molecule that diffuses freely across the cell membrane and is accumulated with the increase of cell density. If AHLs reach a threshold concentration, they form a complexed with the LuxR receptor and activate the transcription of the downstream genes (*luxI* and *luxR* in this case) (Kong *et al*., 2014). Group behaviors of biofilm formation, secretion, motility, metabolisms and cyclic di-GMP signalling (Ball *et al*., 2017) and specific virulence genes (Ruwandeepika *et al*., 2010) are regulated

by *Vibrio* LuxR regulons. In summary, all the downregulated expression at 0.20% can supported QQ activity of Vp_{AHPND}. This data also supported production of ethanolic vinasse with lower concentration around ten times from 2% to 0.20%. It might be related to the investment of business whereas lower material will decrease the production cost.

Conclusion

In conclusion, ethanolic vinasse extract at the concentration of 0.20% can be QQ agents as the result of the present work that revealed in bacterial growth and biofilm formation of *V. parahaemolyticus* isolated from AHPND (Vp_{AHPND}). The substances also regulated and decreased the expression level of virulence factors, PirA, PirB, ToxR. Moreover, it could block signaling virulence genes via QS of bacteria since the QS-related genes, LuxR, and LuxI was reduced which involving in pathogenicity causing disease. Our results correlated with the strategies for the decrease of bacterial number and reduce the activity of virulence related genes. It has been proposed that the milder the bacterial control procedure the less pressure the bacterial resistance induction. Therefore, ethanolic vinasse extract at 0.20% shown here could be a good candidate for bacterial control towards environmental sustainability. However, the concern about the proper amount of substance used for inhibit the pathogenic bacteria was noticed, high enough concentration could serve the expected role of QS inhibitor or quorum quencher while too low concentration exhibited no effect.

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