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Antibacterial, quorum quenching and anti-biofilm formation activities of vinasse extracts against *Vibrio parahaemolyticus*

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Abstract *Vibrio parahaemolyticus* is a causing agent of vibriosis that can cause economic losses in aquaculture industry. The inhibitory activity of the extracts from vinasse to control *V. parahaemolyticus* isolated from white shrimp showing vibriosis clinical signs was investigated. The results showed that at the test concentration of 2% (w/v) of hexane and dichloromethane extracts, named H-V and DCM-V, respectively, showed antibacterial activities. An ethanolic extract (EtOH-V) exhibited quorum quenching activity that shorten the lag phase during the bacterial growth. No inhibitory activity was detected in an aquaes extract (H_2O-V) of vinasse. In addition, 4 vinasse extracts were tested for its anti-biofilm formation. The results obtained from crystal violet assay and scanning electron microscope (SEM) showed that the extracts of hexane, dichloromethane and ethanol were able to arrest the biofilm formation comparable to bacterial growth. This finding revealed that the vinasse extracts could be a good candidate of natural substances for bacterial control in sustainable aquaculture.

Keywords: Antibacterial, Anti-biofilm, Quorum quenching, *Vibrio parahaemolyticus*, Vinasse

Introduction

Vibriosis is bacterial diseases causing economic losses and is of concern for global shrimp production (Chandrakala and Priya, 2017; Abdel-Latif *et al*., 2022). Numerous speices belonging to the *Vibrio* genus, for example, *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. campbelli*, and *V. anguillarum*, are well documented as pathogens in shrimp (Chatterjee and Haldar, 2012; Aguiera-Rivera *et al*., 2019). Acute hepatopancreatic necrosis disease (AHPND) is one of the diseases that cause devastating losss in shrimp industry for almost a decade in particular white shrimp (*Litopenaeus vannamei*)

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(Tran *et al*., 2013; Peña-Navarro *et al*., 2020). Forthermore, outbreaks of white feces syndrome (WFS) associated with the abundant pathogenic *Vibrio* responsible for 10 to 15% loss in shrimp production has recently been reported (Hou *et al*., 2018; Dai *et al*., 2019).

Among the known *Vibrio* spp., the attribution of *V. paramaemolyticus* as the causing agent of the important shrimp diseases has been accounted. Specific strain of *V. parahaemolyticus* or Vp_{AHPND} that carries unique extrachromosomal plasmid containing the genes encoding the Photorhabdus insect-related (Pir) A and Pir B toxins is the classified pathogen of AHPND (Tran *et al*., 2013; Dangtip *et al*., 2015; Sirikharin *et al*., 2015; Xiao *et al*., 2017). In addition, our previous study demonstrated that a predominantly species found and isolated from the shrimps exhibiting WFS in southern part of Thialand is also *V. parahaemolyticus* (Bunserm *et al*., 2022). Shrimp farmers commonly use antibiotics and chemical substances to treat the bacterial diseases, however, this approach has been banned in several countries. This may be due to the negative effects of using anitibiotics, for instance, increase the risk of antibiotic resistant bacteria, causing the adverse effect on human health (Holmström *et al*., 2003; Cabello, 2006; Suzuki *et al*., 2019; Sun *et al*., 2020). As a result, many alternatives including natural subtances, medicainal herbal extracts, probiotics, and immunostimulants have been highlighted and developed (Citarasu *et al*., 2003; Soltani *et al*., 2019; Aribah *et al*., 2022; Citarasu *et al*., 2022).

The pathogenicity of *Vibrio* spp. including *V. parahaemolyticus* has been demonstrated to be associated with its cell-to-cell communication or quorum sensing (QS) since it controls several processes and certain phenotypic behaviors. It has been shown that QS contributes to the characters related to the strengthen and survival of the pathogens making it resists to the extreme environment conditions (Henke and Bassier, 2004; Jaques and McCarter, 2006; Liu *et al*., 2018; Prescott and Decho, 2020). Therefore, the approach to control or inhibit the QS signaling pathway of the pathogenic *Vibrio* spp. has currently been focused (Ye *et al*., 2008; Gode-Potratz and McCarter, 2011; Zhong *et al*., 2021). There has been previously demonstrated that the yeast extract product obtained from a byproduct of bioethanol distillation vinasse is able to inhibit the growth of AHPND-causing Vp_{AHPND} in the aspect of quorum quenching (QQ) effect (Tep-Ubon *et al*., 2020).

The present work aimed to verify the effects of the extracted substances derived from vinasse on growth inhibition and production of virulence factor biofilm of *V. parahaemolyticus* that isolated from AHPND-diseased white shrimp. The growth of the pathogenic *V. parahaemolyticus* was observed while the crystal violet assay and scanning electron microscope (SEM) were

conducted in order to analyze the biofilm formation and bacterial cell structure and morphology.

Materials and methods

The extracts of vinasse

The vinasse used in this study was provided by the private company in Thailand. Sequential extraction using 4 solvents with different polarities including hexane, dichloromethane, ethanol, and water was conducted following the protocol adopted from Nguyen *et al*. (2022). The vinasse biomass (100 g) was firstly mixed with hexane (a lowest polarity solvent) with a ratio of 1:4 (w/v), stirred using a magnetic mixer then kept in dark overnight. The slurry was filtered through a Whatman No. 1 filter paper with the aid of a vacuum. The supernatant was separated (henceforth reffered as the H-V extract) and collected until further used while the residual pellet was recovered, dried and weighed. The obtained vinasse pellet was further extracted using the higher polarity solvents; dicholoromethane, ethanol and water, respectively, by repeating the previously despribed protocol. The extracts were named as DCM-V, EtOH-V and H_2O-V, respectively. The solvents were eliminated from all fractionated extracts under vacuum evaporation to remove hexane, dichloromethane and ethanol while lyophilization for water extract. The obtained extracts were weighed, calculated for the yield and then kept at -20 ℃ for further study.

All of the obtained extracts were prepared as a stock at the concentration of 20% (w/v). The extracts of H-V, DCM-V and EtOH-V were dissolved in a diluent solution containing 10% (v/v) DMSO, 5% (v/v) Triton X-100 and 10% (v/v) ethanol while the H_2O-V extract was dissolved in sterile distilled water. All extract stocks were filtered through a membrane filter pore size 0.45 μm (Whatman) and kept at -20 ℃.

V. parahaemolyticus and growth condition

The pathogenic *V. parahaemolyticus* was isolated from the AHPND-diseased shrimp from the private grow-out farm in Nakhon Si Thammarat Province, Thialand. Pure culture of the AHPND-causing *V. parahaemolyticus* strain was performed and kept at -80 ℃ 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 grown in tryptic soy broth (TSB, Difco) containing 1.5% (w/v) NaCl (TSB^+), and cultured with 150rpm-shaking at 37 ℃ for 18-24 h. The bacterial cells were then centrifuged 8,000 x g for 10 min, resuspended in sterile 1.5% (w/v) NaCl. The bacterial concentration was adjusted to a desired concentration of approximately 10^7 CFU/ml (OD_{600} ~ 0.5) prior to be using for further experiments.

Determination of the growth of V. parahaemolyticus

The growth of *V. parahaemolyticus* was measured real-time according to the modified methods (U-taynapun *et al*., 2018; Tep-Ubon *et al*., 2020). Briefly, the stock of *V. parahaemolyticus* was subcultured 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 antibacterial activity in terms of bacterial growth inhibition of the vinasse extracts (H-V, DCM-V, EtOH-V, and H_2O-V) was separately measured into an individual set. Each set comprised 3 different test media; (1) TSB^+ as a control, (2) TSB^+ with the diluent solution used for the extract dissolution referred as a mock control and (3) TSB^+ containing the test vinasse extracts at the final concentration of 2% (w/v). The starter of *V. parahaemolyticus* was added to the final concentration of 10^5 CFU/ml and 1.5% (w/v) NaCl was added to adjust the final volume of 10 ml in each test tube. The growth of *V. parahaemolyticus*, a growth curve, was monitored at 37°C for 24 h using RTS-1C Personal Bioreactor (Biosan). The number of bacteria after testing for 24 h was also examined by serial dilution and spread plate and the data was present as Log CFU/ml. This experiment was performed in triplicate.

Analysis of biofilm formation

The biofilm production of *V. parahaemolyticus* was determined by crystal violet assay following the method of Lu *et al*. (2021) and Bunserm *et al*. (2022) with some modification. The reaction was measured using the 96-well microtiter plate. Sterile TSB^+ and each of the extracts (H-V, DCM-V, EtOH-V, and H_2O-V) in the raio of 1:1 were added into each well and then mixed well. The final concentration of the vinasse extracts was fix at 0.2%. Fifty µl of *V. parahaemolyticus* suspension were added at an initial concentration of 10^7 CFU/ml. Sterile 1.5% (w/v) NaCl solution was used as the control. The plate was inculated at 37 ℃ for 24 h, the planktonic cells were removed and washed three times with 250 µl saline. Afterwards, the plate was dehydrated by air-flow

for 15 min. Then 0.1% (w/v) crystal violet was added to every well to stain the produced biofilms; incubated for 10 min, and followed by rinsed three times with 250 µl saline. The dye was dissolved in 95% (v/v) ethanol for 10 min and the OD was measured at 570 nm.

Observation of bacterial cell structure and morphology

The structure and morphology of *V. parahaemolyticus* cells influcened by the vinasse extracts were determined through SEM images according to the method of Guo *et al*. (2019) and Bunserm *et al*. (2022). The bacterial cells cultured in TSB^+ at 37^oC for 18-24 h were adjusted to OD_{600} of 0.5 and transferred into the TSB^+containing the vinasse extract, H-V, DCM-V, EtOH-V, and H_2O-V. The cells were incubated at 37 ℃ for 24 h. Afterwards, 20 µl of the inoculated cell suspension was subjected to nuclear pore polycarbonate membranes, and fixed in 2.5% glutaraldehyde at 4^oC for 24 h. The process for bacterial cell dehydration was conducted by increasing concentrations of ethanol (from 30%, 50%, 70%, 80%, up to 100%). The dried cells were mounted onto stubs and coated with 40-60 nm of gold. The images of bacterial cells were observed by SEM (Zeiss/Merlin compact) and photographed.

Statistical analysis

The statistical differences of bacterial number and biofilm formation were analysed through one-way analysis of variance (ANOVA) using the SPSS statistics software version 16.0 (SPSS Inc.). Duncan's Multiple Range Test (DMRT) was used to analyzed the significant differences among data sets. Independent samples t test was carried on to evaluate the significance of differences in mean comparison of bacterial number in some treatments showing 2 data. Significant differences were stated at $P<0.05$.

Results

The vinasse extracts using different solvents

The yields of all extracts derived from the vinasse after extracted with different solvents including hexane, dichloromethane, ethanol, and water, are shown in Table 1. We found that the characters of H-V, DCM-V, EtOH-V, and H_2O-V were similar, brownish black in color and sticky. The extract obtained from ethanol as the extract solvent or EtOH-V gave highest yield of 48.88 ±

5.16% followed by those of H_2O-V, DCM-V and H-V with 6.84 ± 0.41%, 3.77 ± 0.64% and 0.04 ± 0.01%, respectively.

Table 1. The percentage yield of vinasse extracts obtained from different solvents

Solvent	Extract or sample name	Extraction yield (%)
Hexane	H-V	0.04 ± 0.01
Dichloromethane	DCM-V	3.77 ± 0.64
Ethanol	EtOH-V	48.88 ± 5.16
Water	H_2O-V	6.84 ± 0.41

The inhibitory effect of the vinasse extracts on the growth of V. parahaemolyticus

The antibacterial activity of H-V, DCM-V, EtOH-V, and H_2O-V extracts was tested *in vitro* by observation the growth of *V. parahaemolyticus* compared between the different medium conditions. The growth curve of *V. parahaemolyticus* was obtained by real-time detection of the OD_{850} every 10 min for 24 h (Figure 1). The effect of the diluent solution, which was used to enhance the dissolution of the extracts, on the bacterial growth was also analyzed (as the mock control). The result revealed a slight effect of the the diluent solution on the growth inhibition of the test bacteria. The bacterial growth was completely inhibited showing antibacterial activity in the media containing 2% (w/v) H-V and DCM-V (Figure 1A and 1B). Conversely, the H_2O-V showed minor inhibitory effect on the bacterial growth since the growth curve was similar to that of the control (Figure 1D). The kind of growth exhibiting QQ effect, shortern log phase and faster stationary phase, was detected in the EtOH-V treatment (Figure 1C).

Moreover, the number of bacterial cells after incubation in the test media for 24 h was determined. The result showed that the number of cells was correlated with the growth curve pattern present in Figure 1. Control and mock control displayed no significant in the bacterial numbers in all sets of treatment ($P>0.05$). As expected, there was no bacteria grown on the TSA^+ in the treatment of H-V and DCM-V while the bacterial number was significantly decreased in the EtOH-V treatment ($P<0.05$) (Table 2).

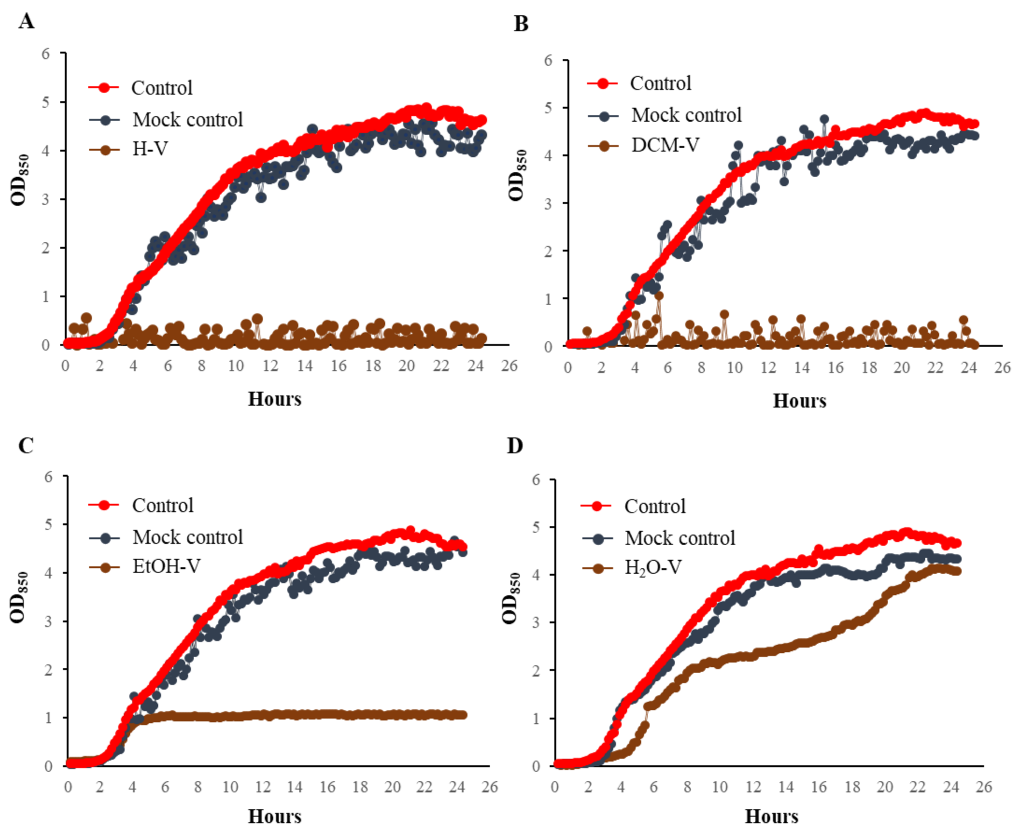

Figure 1. Growth curve of *V. parahaemolyticus* cultured in the media containing the vinasse extract derived from different solvents; **(A)** hexane or H-V extract, **(B)** dichloromethane or DCM-V extract, **(C)** ethanol or EtOH-V extract, and **(D)** water or H_2O-V extract. The bacteria were grown at 37°C for 24 h. Sterile 1.5% (w/v) NaCl solution and the diluent solution used for the extract dissolution was used as the control and mock control, respectively.

Table 2. The number of *V. parahaemolyticus* in different vinasse extract treatments

Vinasse extract	**Log CFU/ml (mean ±SD)**[1]		
	Control	**Mock control**	**Extract treatment**
H-V	9.63 ±0.07	9.53 ±0.03	ND
DCM-V	9.63 ±0.05	9.57 ±0.04	ND
EtOH-V	9.65 ±0.11[b]	9.54 ±0.04[b]	4.82 ±0.05[a]
H_2O-V	9.49 ±0.06	9.49 ±0.02	9.31 ±0.19

[1/] Determined by spread plate technique and ND means not detected. Values in the same row with different letters are signifcantly different (*P<0.05*).

Determination of biofilm formation

To evaluate the anti-biofilm activity of the vinasse extracts, the formation of biofilm was examined 24 h after incubation of *V. parahaemolyticus* in various vinasse extracts. The biofilm formation in each treatment was comparatively compared to that measured in the control and reported in terms of % Inhibition. The result achieved by cryatal vioet assay revealed that mock control showed less than 5% inhibition which was not significant to the level of control ($P>0.05$). More than 90% of biofilm formation was inhibited in H-V, DCM-V and EtOH-V treatment which was consistent with its growth inhibitory activity. Production of biofilm was reduced in the H_2O-V treatment; biofilm was formed approximately 80% compared to the control (Figure 2). This result together with the bacterial growth suggested the anti-biofilm of EtOH-V. The inhibition phenomena observed in H-V and DCM-V may be due to the low number of living bacteria.

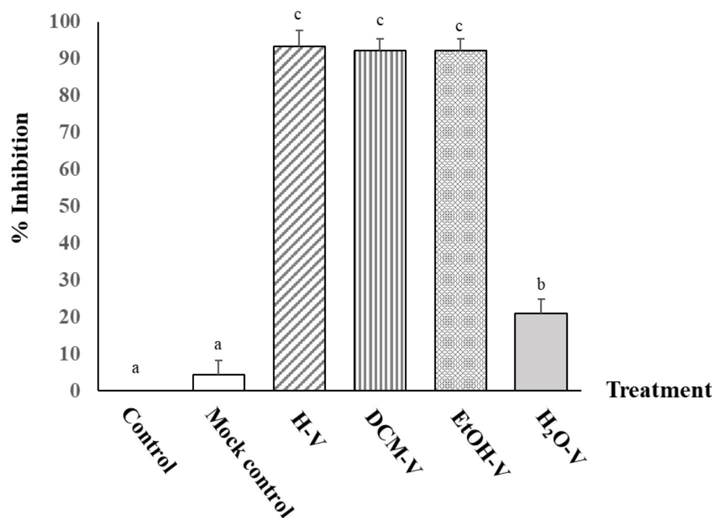

Figure 2. Biofilm formation by *V. parahaemolyticus* after stained with crystal violet stain. Bar graph represents the inhibition of biofilm upon treatment treated with various vinasse extracts. Error bar represents standard deviations. Different letters indicate significant difference ($P<0.05$).

SEM observation of bacterial cell morphology

The morphology and biofilm formation of *V. parahaemolyticus* treated with the vinasse extracts was observed through SEM as present in Figure 3.

Intact bacterial cell with dense biofilm and extracellular metrix was detected in the control while some of cell lysis was shown in the mock control. However, the cell distrubtion was apparently observed in H-V and DCM-V treatment. Much lower number of bacterial cells was observed in the image of EtOH-V treatment compared to the control. Indeed, most cells were lysed and individual cells were loosely attached on the surface.

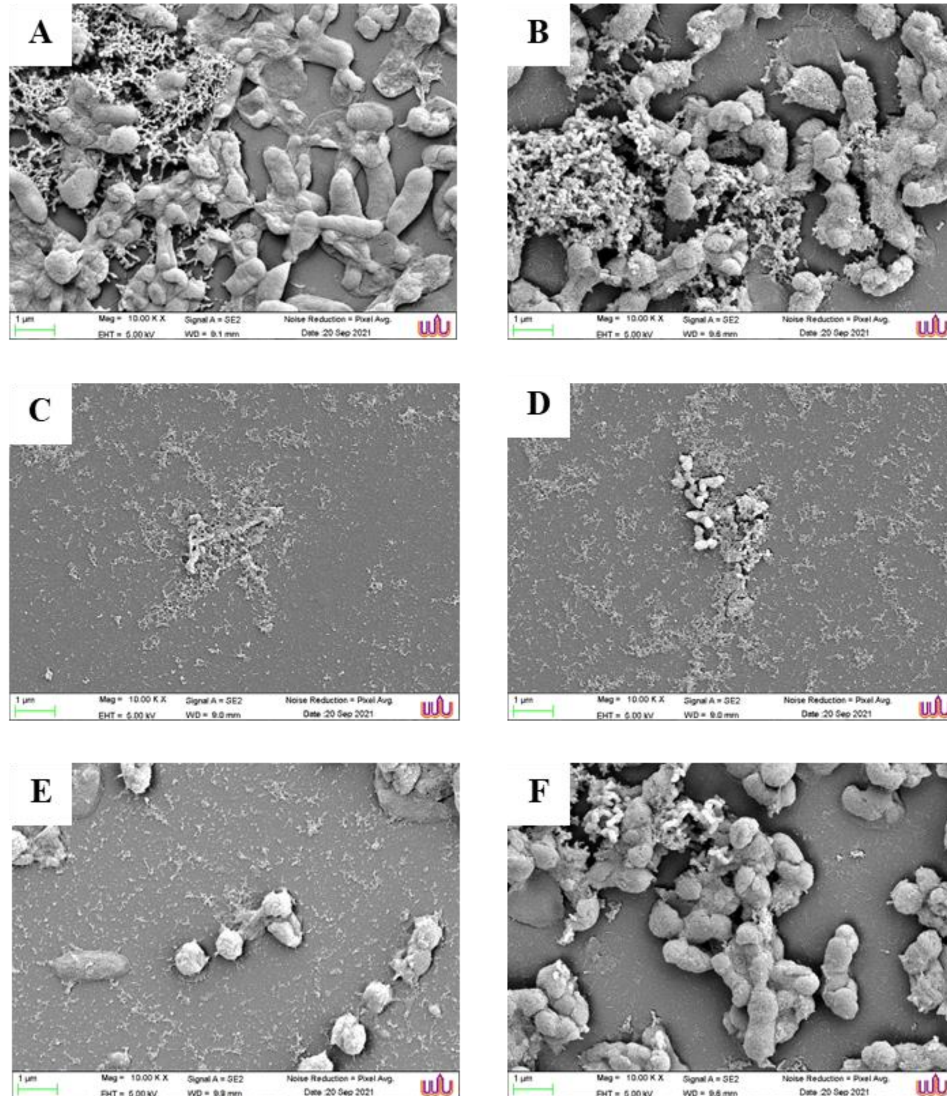

Figure 3. SEM images of bacterial cell morphology of *V. parahaemolyticus* treated with various vinasse extracts; **(A)** Control, **(B)** Mock control, **(C)** H-V, **(D)** DCM-V, **(E)** EtOH-V, and **(F)** H_2O-V for 24 h.

Discussion

V. parahaemolyticus is the bacteria that can be found in diverse aquatic habitats because of its versatile and diverse mechanisms for survival (Gode-Potratz and McCarter, 2011). Several bioactive compounds exhibiting antibacterial activity against this microorganism have been reported. However, one mechanism noticeably involved in the bacterial survival, virulence and pathogenicity is quorum sensing (QS) of bacterial communication. There has been highlighted on several new strategies to overcome the pathogenic bacteria by modulation or control of QS. This procedure reduces the threaten's strength and pressure onto the bacteria so it can also redeuce the development of resistance (Tay and Yew, 2013). Thus, development of the natural substances exhibiting QS inhibition or quorum quenching (QQ) has been currently focused. Indeed, the ability to produced certain virulence factors including biofilm formation has been reported to be related to the pathogenicity of *Vibrio* spp. (Ng and Bassler, 2009; Yildiz and Visick, 2009; Zheng, *et al*., 2010; Gode-Potratz and McCarter, 2011).

The present work focused on exploring the anti-QS or QQ effect, the anti-biofilm formation and antibacterial activity of the substances derived from the byproduct vinasse. Our previous works have demonstrated that the test mixtures containing the yeast extract and vinasse at certain concentrations showed antibacterial activity against the Gram-negative bacteria, in the kind of QQ effect (Chirapongsatonkul *et al*., 2019; Tep-Ubon *et al*., 2020). The QQ activity inactivating the QS singnals, N-acyl homoserine lactone (AHL) signal, in yeasts has also been reported by Leguina *et al*. (2018). The potential of the extracts obtained from several solvents with different polarities to inhibit or control the pathogenic bacteria *V. parahaemolyticus* was then investigated. Diverse effects on bacterial control was detected. The vinasse extracts obtained using hexane and dichloromethane as the solvent exhibited the robust antibacterial and anti-biofilm formation activity since no bacteria and no biofilm detected in the media containing these extracts. Several studies have demonstrated the antibacterial activity of secondary metabolites derived from yeast, medicinal plants and marine organisms extracted with organic solvents including hexane, chloroform, ethyl acetate, and dichloromethane (Alves *et al*., 2020). Apolar and low polarity solvents could extract the hydrophobic substances, for example, neutral polar lipids, sterol esters, sphingolipids, terpenes, and some of fatty acids. The aforementioned compounds have been reported for its antibacterial potentials against several Gram-postive and Gram-negative pathogenic bacteria. Halogenated sesquiterpenes extracted from the red macroalgae *Laurencia* spp. have been shown to inhibit 6 Gram-negative

bacteria including *V. parahaemolyticus* (Vairappan *et al*., 2008). Makky *et al*. (2021) have demonstrated that the ethyl acetate extract of yeast *Saccharomyces cereviciae* was able to inhibit the growth of *Staphylococcus aureus* and *S. epidermidis*. The identified bioactive compounds with low to neutral polarities that possess the antibacterial activity includes benzeneethanol, 4-hydroxy-, 1-Methyl-3,3-diphenylurea, 9-hexadecenoic acid, hexadecanoic acid, and octadecanoic acid (Makky *et al*., 2021). However, the yield of extracts obtained from low polarity solvents is mostly lower than that from more polar solvents (actone, ethanol and methanol) (Alves *et al*., 2020). The extracted yields of hexane and dichloromethane extracts in our work showed similar result, less than 5% yields were achieved in either H-V or DCM-V.

The ethanolic extract of vinasse (EtOH-V) also exhibited the antibacterial potential, however, the inhibitory activity seemed to be different from those displayed in H-V and DCM-V. The log phase or exponential phase of the test *V. parahaemolyticus* cultured in EtOH-V was diminished and the stationary phase occurred earlier than the control. The bacterial gowth curves upon treatment with H-V and DCM-V were almost constant throughout the culture period while similar gowth curve was presented in H_2O-V treatment. The bacterial numbers after 24 h treatment was decreased around 4.5-log in EtOH-V treatment compared to that measured in the control and mock control while no living bacteria was detected in H-V and DCM-V treatments. At present, the QQ effect to bacterial control was existed in EtOH-V treatment. This finding was similar to the yeast QQ to control other organisms previously demonstrated by Leguina *et al*. (2018), Christwardana *et al*. (2019) and Tep-Ubon *et al*. (2020).

Biofilm is one of the virulence factors produced in the pathogenic *Vibrio* spp. It is noticeably demonstrated to initiate the bacterial aggregation and enhance the bacterial resistance toward extreme environments, including that contains antibiotics or disinfectants, leading to the increased virulence (Overhage *et al*., 2008; Guzman *et al*., 2022). The efficiency on the inhibition of biofilm formation was thus determined in this study. We found the strong anti-biofilm formation in *V. parahaemolyticus* treated upon H-V, DCM-V and EtOH-V measured by both crystal violet staining and SEM. These phenomena were consistent with the growth inhibition potential of the vinasse extracts previously described. Moreover, the SEM images showed damaged cells especially in H-V and DCM-V tretments suggesting that the substances in these extracts were able to cause bacterial cell leakage or lysis. However, some demaged and altered cell morphology without biofilm was observed in EtOH-V treatment supporting the QQ effect of this extract.

In summary, the present work provided the evidence of diverse effects of byproduct vinasse extracts on the inhibition and control of *V.*

parahaemolyticus, the important pathogenic species associated with losses in aquaculture. Different effects including antibacterial, QQ effect and anti-biofilm formation activity was detected among the natural substances obtained from sequential extraction using various solvents. Our results correspond with the strategies for bacterial number reduction or inactivation of bacterial growth. It is proposed that the milder the bacterial control procedure the less pressuse the bacterial resistance induction. Therefore, the potent vinasse extracts could be a good candidate for bacterial control towards environmental sustainability.

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