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The potential of selected purple nonsulfur bacteria with ability to produce proteolytic enzymes and antivibrio compounds for using in shrimp cultivation

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ABSTRACT

This study was aimed to screen 22 purple nonsulfur bacteria (PNSB) isolates with the ability to secrete proteolytic enzymes and antivibrio compounds, including affecting factors on proteolytic enzyme production of the selected PNSB. Use of overlay diffusion method under aerobic dark conditions found that only 12 PNSB isolates (54.55%) were able to inhibit shrimp pathogenic *Vibrio* spp.; while 18 isolates (81.82%) could liquefy gelatin under conditions of aerobic dark and microaerobic light. Twenty-time freeze-dried culture supernatant concentrates collected from 12 PNSB grown under the microaerobic light conditions, with the use of agar well diffusion found that only strain PS342 was capable to inhibit all 6 tested shrimp pathogenic vibrios. Among 18 proteolytic PNSB, only 5 strains showed high activity of gelatin liquefaction and this included strain PS342. The strain PS342 was identified using 16 S rRNA gene as *Rhodovulum sulfidophilum*. Glutamate-malate (GM) medium supplemented with 1.5% NaCl was the most suitable medium by giving μ_{max} as 0.336 h^{-1}. Use of central composite design, the maximal proteolytic activity was an average of 14.52 unit/ml in the suitable medium containing 1% gelatin under aerobic-dark conditions with the optimal speed at 150 rpm. The results of the verify test showed that optimum conditions (pH 7.90, 1.30% NaCl and 29.50 °C) for the proteolytic activity (15.40 unit/ml) were very close to real conditions for shrimp cultivation. *R. sulfidophilum* PS342 has the potential to be used in shrimp cultivation for its activities as a good producer of both proteolytic enzymes and antivibrio compounds.

1. Introduction

Aquaculture has been rapidly increasing due to the growing world population, but there is a limitation in seafood resources worldwide. Among animal aquaculture, shrimp is one of the most popular cultivations with a high demand i.e. 400,000 MT Thai shrimp products have been roughly exported in 2011 (Reed and Royales, 2014), but only 300,000 MT in 2016 (Siam Canadian, 2017) due to shrimp diseases. The shortage of land for shrimp cultivation leads to intensive shrimp cultivation to achieve of shrimp demand. The high density of shrimp in ponds always causes high-stress condition for shrimp growth under improper management such as water quality and shrimp feeding. Hence, the outbreaks of shrimp pathogens, particularly from bacteria and viruses, have caused significant losses to shrimp industry (Rattanachuay et al., 2010, 2011; Dash et al., 2017). The most serious shrimp pathogens from bacteria are *Vibrio* spp. such as *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* (Rattanachuay et al., 2011; Chumpol et al., 2017a, b). Therefore, antibiotics or chemotherapeutic agents are commonly used to treat shrimp diseases caused by vibrios (Gräslund et al., 2002; Holmstrom et al., 2003). However, those agents remain in shrimp ponds as they are continuously and prophylactically applied to shrimp; the residues might have an adverse effect to the environment for developing antibiotic resistance genes as evidences occurring of antibiotic resistant bacteria (Le et al., 2005; Defoirdt et al., 2011).

Water quality is one of the important factors on shrimp health; thereby, bioremediator like proteolytic bacteria are attractive to be used for controlling water quality during intensive shrimp cultivation. Why? This is because shrimp feed contains a high content of protein and only roughly 85% of feed is eaten by shrimp (Goddard, 1996). Uneaten feed leads to residual protein in the water to cause unsuitable water for shrimp growth so proteolytic bacteria are necessary to improve water quality (Barman et al., 2015; Chumpol et al., 2017b). Several researchers reported the use of beneficial microbes, particularly bacteria as biocontrol agents to control water quality and also shrimp

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diseases (Rattanachuay et al., 2010; Cruz et al., 2012; Barman et al., 2015). Recently, beneficial microbes including purple nonsulfur bacteria (PNSB) have been studied for their properties to act as probiotics for applying in shrimp farm for controlling water quality including shrimp diseases caused by vibrios (Barman et al., 2015; Chumpol et al., 2017b). It would be worth to explore any proteolytic PNSB that are able to digest protein for their proliferation and inhibit shrimp pathogenic *Vibrio* spp. while they are applied in shrimp cultivation. Hence, the aims of this study were to select PNSB based on their activities for proteolytic and also antivibrio including an optimal condition for producing proteolytic enzymes by related to conditions of shrimp cultivation for consideration to use them as the concomitant bi-function in shrimp farm for improving water quality and controlling shrimp diseases.

2. Materials and methods

2.1. Bacterial preparation

A total of 22 PNSB isolated from various shrimp ponds in Southern Thailand were used to screen PNSB on the basis of their antivibrio and also proteolytic activities. They were maintained in glutamate-malate (GM) medium and kept in a refrigerator at 4 °C. One liter of GM consisted of 3.8 g sodium L-glutamic acid, 2.7 g DL-malic acid, 2.0 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.8 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.053 g $CaCl_2{\cdot}2H_2O$, 0.001 g nicotinic acid, 0.001 g thiamine hydrochloride, 0.01 g biotin, 0.012 g $MnSO_4{\cdot}5H_2O$, 0.025 g ferric citrate and 0.95 g $CoCl_2{\cdot}6H_2O$, pH 6.8. For testing, after twice subcultures, each PNSB isolate was grown in GM broth containing 1.5% NaCl and incubated under microaerobic as a little head space was set light conditions for 48 h. Each culture was adjusted to obtain cell density roughly 10^8 cells/ml (optical density at 660 nm, OD_{660} = 1); and it was ready to be used as inoculum.

Six shrimp pathogens; *V. harveyi* PSU2015, *V. parahaemolyticus* strains SR1 and SR2 were obtained from Department of Microbiology, Faculty of Science, Prince of Sonkla University (PSU); while *V. harveyi* KSAAHRC32, *V. parahaemolyticus* KSAAHRC46, and *V. vulnificus* KSAAHRC3 were kindly supported by Aquatic Animal Health Research Center, Faculty of Natural Resources, PSU, Hat Yai, Songkhla, Thailand. The strains used in this study, *V. harveyi* and *V. parahaemolyticus* are causative agents for vibriosis and early mortality syndrome (EMS) and known as acute hepatopancreatic necrosis disease (AHPND), respectively (Rattanachuay et al., 2011; Chumpol et al., 2017a). Each *Vibrio* sp. was cultured in trypticase soy broth (TSB) plus 1% NaCl for 24 h and the culture broth was adjusted for obtaining approximately 10^9 cells/ml (OD_{660} = 1).

2.2. Screening of PNSB with ability to produce proteolytic enzyme

Twenty-two PNSB isolates were checked for their proteolytic activity. Gelatin hydrolysis was used to test the proteolytic activity of each PNSB by stabbing active culture PNSB after twice sub-cultures and separated incubating under conditions of aerobic dark and microaerobic light at room temperature for 4 days. The gelatine hydrolysis by PNSB was monitored every day for 4 days to check gelatin liquefaction at room temperature. The proteolytic activity was ranked on the basis of detection of liquefying at day 4, 3 and 2 for +, ++ and +++, respectively; while - indicates no detection of liquefying after 4 days incubation.

2.3. Screening of PNSB with ability to inhibit shrimp pathogenic Vibrio spp

The antivibrio activity of 22 PNSB was also parallel tested using overlay assay, 5 µl of each PNSB was spotted (3 points as a triangle) on trypticase soy agar (TSA) containing 1% NaCl and incubated under aerobic dark conditions for 48 h. Then, cell suspension of each *Vibrio* spp. in melted TSA was poured on a PNSB plate to provide an initial cell density of 10^6 cells/ml; and all PNSB plates were incubated with the same condition as previously described. After incubating for 24 h and 48 h, inhibition zones were measured to interpret the antivibrio activity of each PNSB; and any strain that showed high ability to inhibit vibrios was selected to investigate their released antivibrio compounds using agar well diffusion. As PNSB can grow under both incubating conditions, aerobic dark and microaerobic light, so 10% of each selected strain were inoculated in GM broth containing 1.5% NaCl and incubated under the latter condition for 48 h. The culture broth was centrifuged at 8000 rpm for 20 min to obtain culture supernatant and concentrate using a freeze-dryer. A freeze-dried sample was adjusted to obtain varying culture supernatants at 5, 10 and 20 folds; and antivibrio activity of each concentration was tested against the growth of 6 shrimp pathogenic vibrios strains.

2.4. Bacterial identification

The selected strain was identified using 16 S rRNA gene by growing the culture in GM broth containing 1.5% NaCl and incubated under microaerobic light conditions until reached the late log phase at 48 h. The culture broth was centrifuged at 4000 rpm for 15 min to collect cell pellet for DNA extraction using the DNA isolation kit (PowerSoil; Mo Bio, Carlsbad, CA, USA). The universal primers 27 F (5′-AGAGTTTGA TCMTGGCTCAG-3′) and1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for the amplification of 16 S rRNA gene by PCR technique. There were three steps: the initial step, denaturation at 95 °C for 60 s; an annealing step at 55 °C for 30 s; and the final extension step at 72 °C for 120 s. The PCR products were purified using the Gel/PCR DNA fragment extraction kit (Geneaid, Qiagen, Taiwan) according to the manufacturer's instructions. The purified PCR product was sequenced by an automated DNA sequencer at Macrogen DNA Sequencing Service (Macrogen, Seoul, Korea). Then the corrected sequence of the strain PS342 was submitted to the Genbank database in nucleotide BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison with known sequences available in the database. The sequences of the strain PS342, seven closely related strains, and an out-group strain; *Rhodopseudomonas thermotolerans* JA576^T were evaluated using multiple sequence alignments in CLUSTALW (Hall, 1999). A neighbor-joining phylogenetic tree was reconstructed using MEGA software, version 4 (Tamura et al., 2007), wherein evolutionary distance matrix was calculated using Jukes-Cantor model, and topologies of the neighbor-joining trees were calculated by bootstrap resampling method based on 1000 replicates.

2.5. Investigation of a suitable growth medium and culture conditions

According to the results of the previous experiments, the promising PNSB which showed both strong activities of antivibrio and proteolytic was investigated for a suitable growth medium. To prepare inoculum, the selected strain was grown in GM broth containing 1.5% NaCl and incubated under microaerobic light conditions for 48 h; bacterial cells were twice washed with 0.85% NaCl (normal saline solution: NSS) and then adjusted the OD_{660} to 0.5 using NSS. A 10% inoculum was inoculated into the following media, glutamate acetate (GA), GM, G5 and basal isolation medium (BIM), and each medium containing 1.5% NaCl (see medium composition in Nunkaew et al., 2012, this study, Ormerod et al., 1961; Chumpol et al., 2017a, respectively), and incubated under microaerobic light conditions without shaking for 48 h. Bacterial growth was measured using a spectrophotometer at a wavelength of 660 nm every 6 h for 24 h and then every 12 h until 72 h. Growth curves were used to determine maximum specific growth rate (μ_{max}) and generation time (h) as described by Slater (1979) for obtaining the suitable medium. The active culture of a selected strain was grown in the optimal growth medium for investigating optimal speed by shaking speeds at 50, 100, 150 and 200 rpm under aerobic dark conditions. These conditions were studied to follow the conditions of shrimp