Screening and Characterization of Photosynthetic Bacteria (PSB) for Hydrogen Sulfide Removal

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Abstract

Hydrogen sulfide (H_2S) is considered as a toxic substance produced under anaerobic conditions in aquaculture systems. Its accumulation can cause negative effects on aquatic organisms. This research focused on the screening and characterization of photosynthetic bacteria (PSB) that is capable to remove hydrogen sulfide in the laboratory experiment. Five isolates of PSB; G1-O, G1-OBRI, GNew3-BO, RG-YB, and RS13-Y, collected from sludges and sediments of shrimp pond, waste canal and public canal in shrimp culture areas in Nakhon Si Thammarat province were tested for its ability of hydrogen sulfide removal. Colony morphology on an agar plate and Gram stain was examined. The test bacteria were grown photoautotrophically in a modified Ormerod medium containing sulfide equivalent to 13 mg/L and determined for the amount of sulfide, sulfite and sulfate. The results showed that only 2 isolates, GNew3-BO and RS13-Y, could reduce sulfide while increased sulfite and sulfate were detected in the media during their growth. Moreover, the existence of sulfur functional genes (*sqr*, *pdo*, *sor*, and *sox*) was investigated. Comparable to the hydrogen sulfide removal activity, these genes were detected in GNew3-BO and RS13-Y with a pattern profile of $sqr^+/pdo^+/sor^+/sox^+$ and $sqr^+/pdo^+/sor^+/sox^-$, respectively. Our findings provide a viable organism for environmentally friendly removal of hydrogen sulfide that can be applied to reduce pollutant accumulation in aquaculture system.

Keywords: hydrogen sulfide, photosynthetic bacteria (PSB), aquaculture, waste treatment

Introduction

Aquaculture effluents have been known to produce numerus amounts of waste materials containing high organic materials, nutrients, ammonia, nitrite, and other contaminants (Ghaly *et al*., 2005). The produced waste matters lead to the deterioration of water quality and emerging diseases. The pollutants, for example ammonia (NH_3), nitrite (NO_2^-), and hydrogen sulfide (H_2S), are considerably noticed to cause negative effects on aquatic organisms and environment. Hydrogen sulfide produced under anaerobic conditions in aquaculture systems with a specific odor of "rotten eggs" (Thulasi *et al*., 2020). Its accumulation at elevated levels can affect not only the survival but also negatively impacts the physiological and immunological activities of aquatic organisms. There have been reported that hydrogen sulfide binds reversibly to cytochrome c oxidase (COX) in mitochondrial electron transport chain, resulting in the inhibition of oxidative phosphorylation and ATP production (Bagarinao, and Vetter, 1989; Cochrane *et al*., 2019). The levels the animals tolerant are varied depending on species and exposure condition, however, toxicity of hydrogen sulfide has been rarely reported in aquaculture-reared species (Letelier-Gordo *et al*., 2020). Smith and Oseid (1974) have reported LC_{50} values of hydrogen sulfide in some fishes, for example, at 0.013 mg/L in 48 h for walleye (*Sander vitreus*), 0.025 mg/L at 72 h for goldfish (*Carassius auratus*), 0.030 mg/L at 72 h for bluegill (*Lepomis macrochirus*), 0.026 mg/L in 96 h for northern pike (*Esox Lucius*), and 0.031 mg/L in 96 h for brook trout (*Salvelinus fontinalis*). Some researchers have reported that the level of hydrogen sulfide should be lower than 0.03 mg/L for shrimp culture while the higher level may lead to more pathogen susceptibility in shrimp (Andrew, 2007; Hsu and Chen, 2007). It is noted that around 10% of crop loss in shrimp culture is associated with sulfide production, and 4-million metric tons

of shrimp may have been lost as a result of sulfide outgassing (Thulasi *et al*., 2020). Despite the lethal effect of sulfide is 100-time higher than that of nitrogenous compounds, it is not practically measured in aquaculture farms due to the difficulty and measure cost. In addition, the symptom caused by sulfide exposure is quite similar to that of hypoxia (Thulasi *et al*., 2020).

Photosynthetic bacteria (PSB) are a diverse group of microorganisms that are capable of converting light energy into chemical energy through the process of photosynthesis. PSB generate chemical compounds by using light as energy driver and use various substrates; organic matters, nitrite, ammonia and sulfide (S^{2-} or $S_2O_3^-$) to metabolize via multiple pathways (Lu *et al*., 2011). In addition, they release oxygen as a byproduct (Chen *et al*., 2020). Based on its unique characteristics and potentials, PSB have been explored as a promising solution for waste treatment in several systems including aquaculture. Recently, several groups of PSB including anoxygenic purple phototrophic bacteria (PPB) and anoxygenic green sulfur bacteria (GSB) have been gained more attention for their ability to remove sulfide from wastewater and environment mainly to reduce the pollutant and odor control purposes (Egger *et al*., 2020). These bacteria have been focused as a good candidate for biologically sulfide removal because of fewer resource requirement and the growth conditions of photoautotroph or chemolithotroph using sulfide as electron donor to reduce inorganic carbon for growth (Syed *et al*., 2006).

This study aimed to screen and characterize the PSB from sludges and sediments of shrimp pond, waste canal and public canal in shrimp culture areas that exhibited the efficiency to reduce hydrogen sulfide in the synthetic medium. The existence of functional genes involved in sulfur oxidizing process was examined in these bacteria in order to discover the feasible connection between the gene encoding enzymes and products in sulfur oxidation pathway. The target genes included genes encoding sulfide:quinone oxidoreductase (*sqr*), persulfate dioxygenase (*pdo*), sulfur oxygenase reductase (*sor*), and sulfur oxidizing enzyme (*sox*).

Materials and Methods

Isolation of PSB

For isolation of the PSB feasible to remove hydrogen sulfide, sludge and sediment with malodor of rotten eggs were collected from 5 different collection habitats of shrimp reservoir, shrimp pond, waste canal in shrimp farm, sediment waste pond, and public canal in shrimp culture areas located in Nakhon Si Thammarat province, Thailand. Samples were isolated by dilution plate method onto Starkey medium and thiosulfate medium containing 1.5% NaCl followed the method of Vidyalakshmi and Sridar (2006) and Hidayat *et al.* (2017). After incubation at 37°C for 7 days, a single colony was transferred onto modified tryptic soy agar (TSA) with 1.5% NaCl or TSA^+ and incubated for 7 days to get pure culture. All pure isolates were subsequently prepared for storage at -80°C as a bacterial collection stock in Aquatic Animal Health Management Research Unit (AAHMRU). Five isolates of PSB, 1 isolate from each collection habitat named, G1-O, G1-OBRI, GNew3-BO, RG-YB, and RS13-Y, were randomly selected from the stock for further study.

Observation of bacterial colony and cell morphology

The pure colony of bacteria grown on TSA^+ after incubation at 37°C for 7 days was carried out for colony morphology by observation of the colony shape. Each pured isolate was Gram-staining followed by microscopic observation. Gram stain and cell shape were then recorded.

Screening of hydrogen sulfide removal and sulfur compounds detection

To determine the efficiency of the test bacteria on hydrogen sulfide removal, the *in vitro* test was performed following a modification of Egger *et al.* (2020) and Xin *et al*. (2020). A single colony of each PSB isolate was cultured in tryptic soy broth (TSB) with 1.5% NaCl (TSB^+) and incubated at room temperature with shaking (200 rpm) for 7 days. The cells were collected by centrifugation (8,000 x g) for 5 min, resuspended in sterile saline solution, and then adjusted to an OD_{600} of approximately 1.0. A modified Ormerod medium

(Ormerod *et al*., 1961) with $Na_2S \cdot 9H_2O$ (equivalent to 13 mg-S/L) was prepared. To avoid the interference with sulfate measurement, all sulfate salts were replaced with chloride salts. The medium was collected for sulfide, sulfite and sulfate measurement as the sample at 0 day. Afterward, the medium was transferred to the 1-L Duran bottle. Fifty mL of the suspension was added into the bottle containing 750 mL of the modified Ormerod medium. All bacteria were cultured under 16/8-h light/dark cycle. The test was performed in triplicate.

The suspension sample was taken at 1, 3, 5, and 7 days for sulfur compound detection. The suspension was centrifuged at 10,000 x g for 5 min, then the supernatant was collected for detection. Sulfide, sulfite and sulfate were detected using kits and MD 600 multi-parameter photometer (Lovibond) according to manufacturer instructions for M365, M368 and M355, respectively. The detection limits of sulfide, sulfite and sulfate are 0.04-0.5 mg/L S^{2-}, 0.1-12 mg/L SO_3 and 5-100 mg/L SO_4^{2-}, respectively. The sample was diluted 100 times with deionized water prior to use for sulfide measurement.

DNA extraction and determination of sulfur functional genes

Five PSB isolates were analysed for the genes involved in sulfide removal. DNA were isolated and purified from 5-ml of bacterial culture by using Presto™ Mini gDNA Bacteria Kit (Geneaid). The extraction procedure was carried out following the manufacturer's instructions with slight modification. The extracted DNA was measured spectrophotometrically using a BioDrop DUO UV/Vis spectrophotometer (BioDrop). The quality and quantity were determined through calculating the ratio of absorbance at 260 nm and at 230 nm (260/230) for guanidine contaminant. The ratio of absorbance at 260 nm and at 280 nm (260/280) was measured to detect the contaminated protein in the extracted DNA. The ratio in a range of 1.8–2.0 is accepted as good DNA quality. The obtained DNA were stored at -80°C until use.

The DNA of 5 PSB candidates was determined for the existence of the genes involved in the sulfide oxidizing process (*sqr*, *pdo*, *sor*, and *sox*) through a qPCR technique according to Chen *et al.* (2023) with slight modification. The specific primers used for qPCR analysis are shown in Table 1. Each qPCR reaction mixture (total 20 µl) contained the extracted DNA (fixed at 500 ng for each reaction), HOT FIREPol® EvaGreen® (Solis Biodyne), primers and the desired final volume was adjusted with DEPC-treated water. The reactions were performed in a CFX96 Touch™ Real-Time PCR (Bio-Rad) in triplicate. Cycling conditions were as follows: 95°C for 12 min followed by 45 cycles of 15 s at 95°C, 20 s at 50-55°C (depending on the primer pairs) and 20 s at 72°C. Negative control using DEPC-treated water replacing DNA template must be conducted for every detection. The Ct value of < 40 was interpreted as the positive result that referred to the existence of the target genes while the Ct of the negative control must be > 40.

Table 1 Primers used in this study.

Gene	Primer	Sequence (5′– 3′)	Annealing temperature (°C)	Product size (bp)	References
sqr	*sqr*145F	TGGACCCTGGTGGGCGSSGG	52	360	Pham *et al.* (2008)
	Sqr490R	TTCWKCGGCGCGCCSSCGCA			
pdo	*pdo*1F	CGACTTTCACCGTCTCCTATGT	50	155	Xin *et al.* (2020)
	*pdo*1R	CACCTCAAGCCCGTTCTCC ´			
sor	*sor*C1F	GTIGGICCNAARGTNTGY	50	230	Chen *et al.* (2007)
	*sor*H1R	RTGCATNTCYTCRTGRTC			
sox	*sox*B432F	GAYGGNGGNGAYACNTGG	55	239	Petri *et al.* (2001)
	*sox*B1446B	CATGTCNCCNCCRTGYTG ´			

Statistical analysis

The statistical difference of sulfur compound content 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).

Results

Isolation and characterization of the test PSB

The colony and cell morphological characterization of 5 PSB isolates is present in Table 2. Figure 1 and Figure 2 showed the colony grown on TSA^{+} for 7 days and Gram stain result of each isolate. Diverse colony morphologies were observed, in addition, all test PSB also showed different colors. These 5 PSB isolates were Gram-negative with 2 types of cell shape, coccus for G1-O and G1-OBRI and bacilli or rod shape for GNew3-BO, RG-YB and RS13-Y.

Table 2 Colony and cell morphology of 5 isolated PSB.

PSB isolate	Bacterial colony and cell morphology				
	Collection habitat	Colony morphology	Colony color	Gram	Cell shape
G1-O	Shrimp reservoir	Circular, flat	Light orange	Negative	Coccus
G1-OBRI	Shrimp pond	Circular, raised	Bright orange	Negative	Coccus
GNew3-BO	Waste canal in shrimp farm	Circular, flat	Orange	Negative	Bacilli/Rod
RG-YB	Sediment waste pond	Circular, raised	Yellow	Negative	Bacilli/Rod
RS13-Y	Pubic canal in shrimp culture area	Circular, flat	Yellow	Negative	Bacilli/Rod

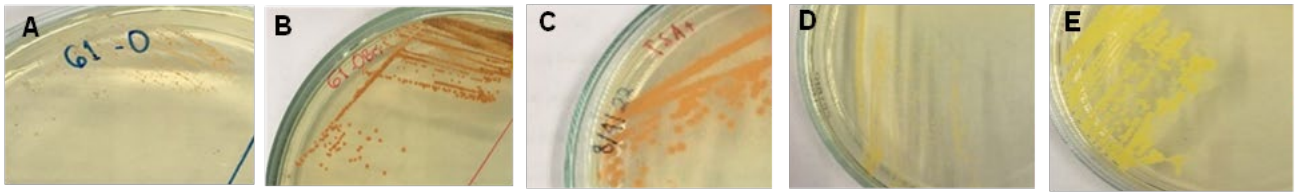

Figure 1 Colony characters of 5 PSB isolates on TSA plus 1.5% NaCl (TSA^{+}) for 7 days; **(A)** G1-O, **(B)** G1-OBRI, **(C)** GNew3-BO, **(D)** RG-YB, and **(E)** RS13-Y.

Removal of sulfide and the concentration of sulfur compounds in PSB culture

The ability of the test PSB to remove hydrogen sulfide was determined by detection of the level of sulfur compounds associated in sulfur oxidation pathway including sulfide, sulfite and sulfate in the culture of PSB. Only 2 isolates, GNew3-BO and RS13-Y, could oxidize sulfide to sulfite and sulfate because the reduction of sulfide occurred simultaneously with the increase of sulfite and sulfate was observed during the experimental period (Figure 3). Sulfide levels were significantly decreased ($P<0.05$) 1 day after inoculation of GNew3-BO and RS13-Y compared to that of the control. The concentration of sulfide was continually declined. Among the 2 PSB isolates exhibiting sulfide oxidizing activity, the significantly lower level of sulfide was detected in GNew3-BO ($P<0.05$) (Figure 3A). Comparable results were obtained for sulfite and sulfate produced in GNew3-BO and

RS13-Y as shown in Figure 3B and 3C, respectively. Sulfite was statistically higher in RS13-Y. Conversely, the highest level of sulfate was detected in the culture of GNew3-BO.

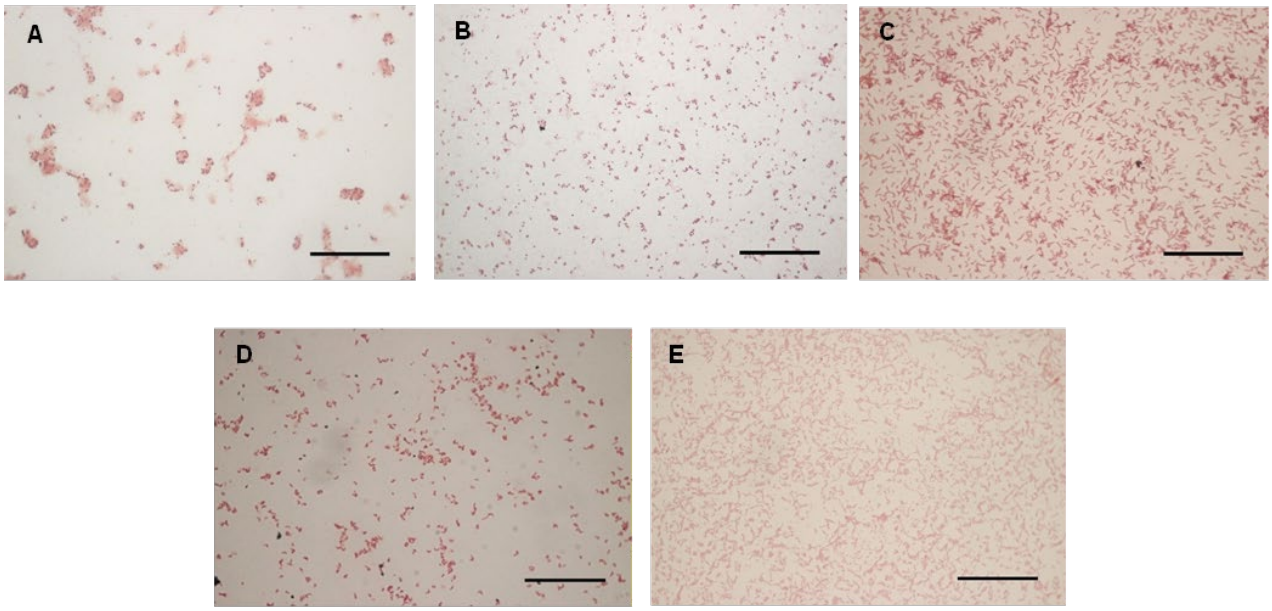

Figure 2 Gram staining result of 5 PSB isolates grown on TSA plus 1.5% (TSA^{+}) for 7 days; **(A)** G1-O, **(B)** G1-OBRI, **(C)** GNew3-BO, **(D)** RG-YB, and **(E)** RS13-Y. Scale bar = 100 µm.

The existence of sulfur functional genes in the test PSB

The appearance of genes encoding sulfur oxidation-related enzymes were studied in this work. Four target genes are distributed in different parts of sulfur oxidizing pathway. The qPCR results showed that the sulfur oxidizing genes were positively detected in GNew3-BO and RS13-Y while there was no examined gene in other 3 isolates (G1-O, G1-OBRI and RG-YB). However, the pattern of gene existence in 2 PSB isolates was different (Table 3). All 4 target genes were present in GNew3-BO while *sox* gene was not detected in RS13-Y. This result suggested the pattern profiles of the sulfur functional genes were $sqr^{+}/pdo^{+}/sor^{+}/sox^{+}$ and $sqr^{+}/pdo^{+}/sor^{+}/sox^{-}$, for GNew3-BO and RS13-Y, respectively.

Table 3 Distribution of gene putatively involved in oxidative sulfur metabolism.

PSB isolate	Functional genes involved in sulfur-oxidizing process*			
	Sqr	*pdo*	*sor*	*Sox*
G1-O	-	-	-	-
G1-OBRI	-	-	-	-
GNew3-BO	+	+	+	+
RG-YB	-	-	-	-
RS13-Y	+	+	+	-

* + indicates the presence of a gene (Ct value < 40); – indicates the absence of a gene (Ct value > 40).

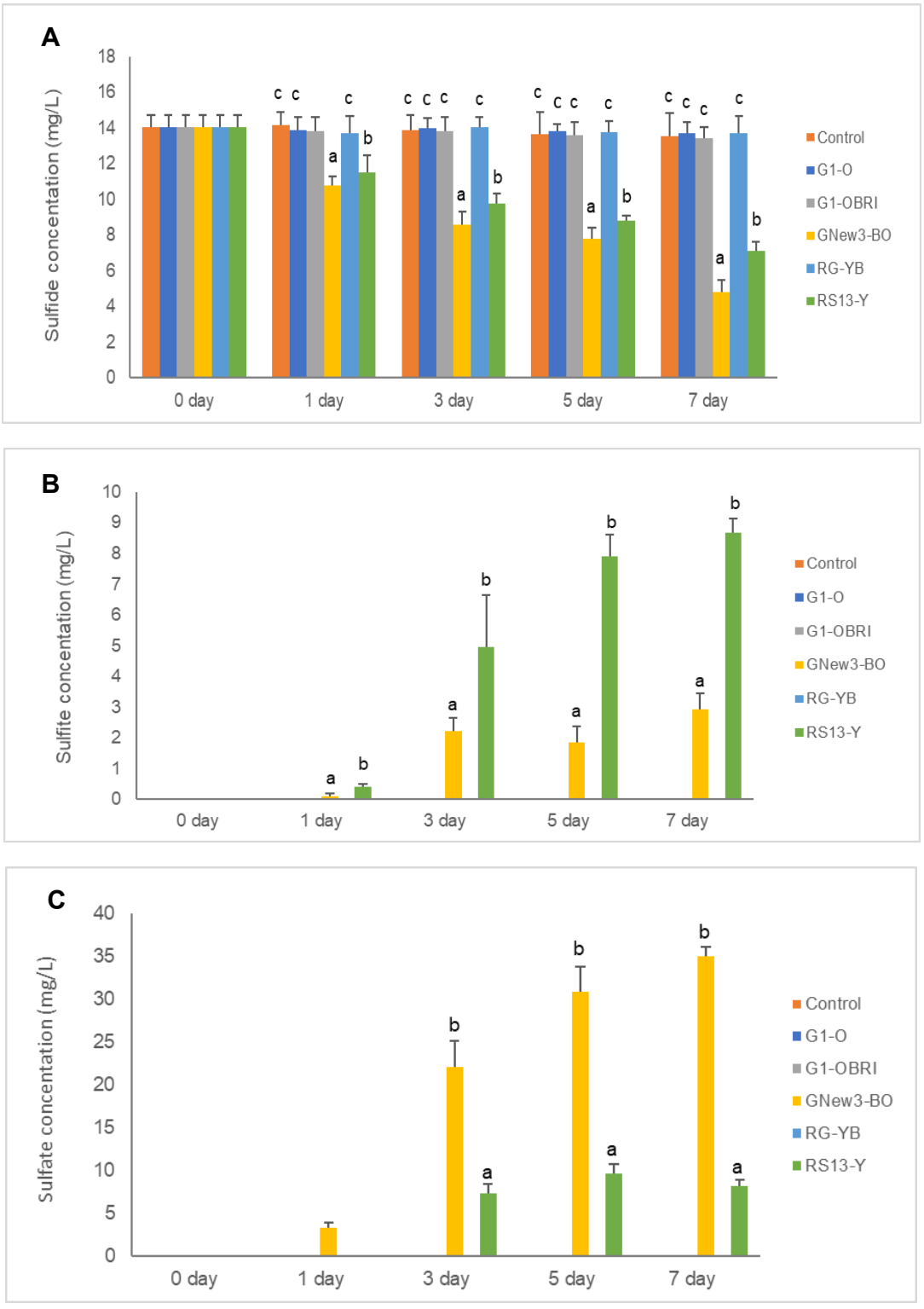

Figure 3 Sulfur compounds in culture media of 5 PSB isolates (G1-O, G1-OBRI, GNew3-BO, RG-YB, and RS13-Y); **(A)** Sulfide (S^{2-}), **(B)** Sulfite (SO_3), and **(C)** Sulfate (SO_4^{2-}). The results were expressed as the mean ± SD. Bars and different letters stand for statistically significant differences (P<0.05) between groups.

Discussion

In this present work, 5 isolates of PSB were screened to remove hydrogen sulfide. This substance is preferable removed because of its toxic and undesirable odor. The high level of sulfide has been demonstrated for its deleterious impacts on aquatic animals, for example, it interferes aerobic respiration, causes many

cytotoxic effects, disturbs the normal function of ATPase, and reacts with hemoglobin to form hematin which leads to respiratory pigment dysfunction (Vetter *et al*., 1987; Vismann and Hagerman, 2008; Thulasi *et al*., 2020). Biological means mainly of functional microorganisms have been more attractive for solving the sulfide in various circumstances because of their lower operation requirement and flexibility (Lin *et al*., 2018). Sulfur-oxidizing bacteria (SOB) and sulfur-reducing bacteria (SRB) are 2 major groups of microorganisms involved in biological sulfur cycle. SOB used inorganic reduced sulfur species including sulfides (S^{2-}, HS^-, H_2S), polysulfides ($^-S\text{-}S_n\text{-}S^-$), elemental sulfur ($S^0$), sulfite ($SO_3^{2-}$) and thiosulfate ($S_2O_3^{2-}$) as energy sources obtained from sulfur oxidation process. SRB on the other hand uses oxidized sulfur compounds (SO_4^{2-}, SO_3^{2-} and S^0) as electron acceptors and produces sulfide while oxidizing organics for energy generation and cell growth (Muyzer and Stams, 2008; Hao *et al*., 2014). Due to the products gained during the sulfur metabolisms, SOB seems to be more useful in aquaculture circumstance, particularly for the treatment of effluent wastes. Phototrophic SOB, for instance green sulfur bacteria (GSB) and purple phototrophic bacteria (PPB) have been discovered for versatile application in hydrogen sulfide removal process (Lin *et al*., 2018; Egger *et al*., 2020).

Our results showed that there were 2 isolates, GNew3-BO and RS13-Y, that were capable of reducing the amount of sulfide in laboratory scale. These PSB were obtained from malodor sludge and sediment samples from waste canal in shrimp farm and public canal in shrimp culture area. Based on the photoautotrophic condition of growth and the procedure for sulfide removal experiment which let some oxygen in bacteria culture condition, our interesting PSB might be favorable categorized in the group of SOB. Another evidence that supported the assumption was the dynamic change of sulfur compounds in this work. Sulfide in the form of Na_2S (in the modified Omerod medium) was transformed into sulfite and sulfate via oxidative process. Moreover, the functional genes encoding enzymes contributing in sulfur oxidizing pathway were found in the PSB isolate GNew3-BO and RS13-Y. Many researchers have described the functions of enzymes and putative genes in sulfur oxidizing pathway. The gene *sqr* encodes enzyme sulfide: quinone oxidoreductase which is the first enzyme action in the first step of oxidation process converting sulfide to elemental sulfur. Afterwards, the products obtained from the first step would be further oxidized to sulfite by persulfate dioxygenase enzyme encoded by *pdo* gene. Both sulfur oxygenase reductase and sulfur oxidizing enzymes encoded by *sor* and *sox*, respectively, are involved in the conversion of oxidizing sulfite to sulfate (Li *et al.,* 2019; Xin *et al*., 2020; Chen *et al*., 2023). The sulfur compounds detected in this work were consistent with the existence of the genes of enzymatic proteins contributing in sulfur oxidizing process described above. The level of sulfate in GNew3-BO culture was highest and significantly higher than that present in RS13-Y ($P<0.05$). In contrast, the sulfite was greater in the PSB isolate RS13-Y. This may be due to the fact that GNew3-BO had all 4 functional genes mentioned above which gradually convert sulfite to sulfate by the action of enzymes encoded by *sor* and *sox* while RS13-Y lacked *sox*. The correlation between the expression of the functional genes responsive to the conversion of the sulfur compounds have been demonstrated (Xin *et al*., 2020; Chen *et al*., 2023). However, our present work determined the existence of the gene but not the gene expression, further work in the gene expression analysis in couple with measurement of the produced sulfur compounds is necessary to get more insight.

There has been reported that the habitats of SOB are diverse. This bacteria group can be found and isolated from mud oil, canal water, palm oil wastewater plant, solid waste landfills, sulfide polluted water (Vidyalakshmi and Sridar, 2006; Pudi *et al*., 2022). Overmann (2008) has suggested that natural habitats for phototrophic SOB are usually aquatic environments because of the available reduced sulfur and penetrated light in the anoxic layers. Therefore, the matters collected from the accumulated sludge or effluent from aquaculture environments could be the good candidate for isolation and screening of phototrophic SOB which is one of a huge community of PSB. Moreover, the findings obtained in this work suggesting that detection of the appearance of functional genes of sulfur oxidizing process can be used as a biological marker for screening of the SOB.

Conclusions

In conclusion, 2 isolates of PSB, GNew3-BO and RS13-Y, were able to reduce sulfide and converted to sulfite and sulfate. These bacteria would be a good candidate for hydrogen sulfide removal since its growth condition was not complicated and no special elements required. However, identification and classification to gain more detail about these bacteria would be carried out. The mixed PSB should also be tested for the efficiency and feasibility of application in water treatment. In addition, evaluation of the bacterial biodiversity in the habitat and the influences that functional bacteria have on microbial community are crucial issue for sustainability approach.

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