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Original Article

Investigation of the *sqr* and *fcc* Genes Associated with the Sulfur Oxidation Activity of a *Rhodobacter capsulatus* Strain Isolated in Vietnam

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Abstract: Flavoproteins sulfide:quinone oxidoreductase (SQR) and flavocytochrome c sulfide dehydrogenase (FCC), two key enzymes involved in the sulfide oxidation pathways, are responsible for the hydrogen sulfide (H_2S)-metabolizing capability of various bacterial strains employed in the biological treatments of the toxic gas H_2S. Previous studies mainly focus on isolating sulfur-oxidizing bacteria (SOB) and evaluating the total sulfur oxidation activity. Thus, in this study, an in-depth investigation of the *sqr*, *fcc* genes, the encoded proteins, and their association with the sulfide oxidation activity in an SOB bacterial strain, *Rhodobacter capsulatus*, was carried out. Firstly, the specific primers were designed and used to amplify *sqr* and *fcc* genes of a *R. capsulatus* strain isolated in Vietnam. In addition, the bacterial cells were fractionated to obtain periplasmic, membrane, and cytoplasmic fractions for denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and activity assay. The bands corresponding to the calculated molecular weights of SQR (approximately 47 kDa in the membrane fraction) and FCC (approximately 35 kDa in the periplasmic fraction) could be observed on the SDS-PAGE gel. Significant sulfide oxidation activity could be detected in the periplasmic and the membrane fractions compared to the cytoplasmic fraction (2 and 20 times higher, respectively). More importantly, sulfide oxidation activities in these fractions were inhibited by the specific flavoprotein inhibitor KCN, indicating a possible association of SQR and FCC with the recorded activity. The study has initially established the evidence supporting the link between the presence of *sqr*, *fcc*-encoded proteins, and the sulfide oxidation activity in the studied *R. capsulatus* bacterial strain for further research utilizing SOB in environmental treatment applications.

Keywords: Sulfur-oxidizing bacteria, sulfide, sulfide:quinone oxidoreductase, flavocytochrome c sulfide dehydrogenase, flavoprotein, KCN.

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1. Introduction

The impact of hydrogen sulfide (H_2S) on human health has been examined based on its concentration. The gas has an unpleasant rotten-egg odor at low concentrations, ranging from 0.0005 to 0.03 ppm. However, at higher concentrations (>100 ppm), it causes damage to multiple organs, and gradual or sudden deaths may occur at the concentration of 500 ppm onwards, depending on dosage and exposure time [1]. Currently, different methods, generally divided into three main categories: physical, chemical, and biological, have been implemented for the treatment of H_2S. Compared to physical and chemical methods, the major advantages of biological-based methods are environmentally friendly and have usage flexibility. Within this category, microbial technology is possibly the most promising method, with the example of sulfur-oxidizing bacteria (SOB), which are capable of converting toxic H_2S gas into non-toxic forms [2]. Various studies have shown the potential applications of photosynthetic bacteria to treat H_2S in wastewater. Using a combination of primarily two strains of sulfur and non-sulfur bacteria, Ferrera et al. demonstrated that those bacterial strains were able to treat 95-97.9% of wastewater with an input sulfide level of 26-112 mgS/L [2]. The photosynthetic purple bacteria *Ectothiorhodospira magna* achieved a sulfide removal rate of 99.99% after 72 hours of testing at pH 8.0, 35 °C [3]. These remarkable capabilities are associated with diverse sulfur-metabolizing pathways, including multiple enzyme systems.

Compared to the other oxidation products, elemental sulfur (S^0) is the most ideal compound to be converted from H_2S; hence, the sulfide-oxidizing enzyme system that catalyzes the conversion of sulfide to elemental sulfur has a strong potential for bioremediation of H_2S pollution. This enzyme system consists of two main enzymes: sulfide:quinone oxidoreductase (SQR) and flavocytochrome c-sulfide dehydrogenase (FCC). SQR is a membrane-bound protein, discovered as a single polypeptide chain with a molecular mass of approximately 50-55 kDa assessed by SDS-PAGE [4]. FCC also belongs to the class of oxidoreductase enzymes and uses the sulfur group as an electron donor. Based on the peptide chain composition, FCC was identified as a soluble enzyme, mostly found in the periplasm. FCC is composed of a flavoprotein (fccB), which is for FAD binding, and a diheme cytochrome (fccA). The FCC molecular mass is determined separately, with the flavoprotein subunit having a molecular mass of approximately 40 kDa and the fccA subunit at approximately 21 kDa [5].

In Vietnam, several studies related to SOB have been carried out to treat wastewater contaminated with H_2S. An in-depth research carried out by Lien et al., [7] described 35 strains of photosynthetic purple bacteria isolated from mud and water samples in aquaculture water bodies around Vietnam [6]. These isolated strains were identified, cloned, and used as samples to investigate the *sqr* gene characteristics. Among them, three of the highest recorded growth and the sulfide removal activities were then applied in the testing and production of bioproducts to treat polluted water sources with positive results when the toxic content was significantly reduced [7]. However, the molecular studies of SOB group, especially information on SQR and FCC, up to now, are limited. Hence, in this study, we designed primers to amplify genes encoding significant enzymes in sulfur metabolic pathways, analyzed the sequences, and characterized the sulfur-oxidizing activity of a *Rhodobacter capsulatus* strain isolated from wastewater samples in Vietnam.

2. Experimental

2.1. Materials

The strain *Rhodobacter capsulatus* (PAM 34) was isolated and stored at GREENLAB (Laboratory of Bioelectrochemical Technologies for Green Growth), University of Science, Vietnam National University (HUS-VNU), Hanoi,

Vietnam [8]. *sqr* and *fccb* sequences were then submitted to NCBI database with the accession numbers PV432528 and PV432527, respectively.

2.2. Methods

2.2.1. Bacterial Culture

Bacterial colonies were transferred from the streaked plate to 50-ml conical tubes filled with the enrichment medium containing the following ingredients 0.33 g/l KH_2PO_4; 0.33 g/l $MgSO_4.H_2O$; 0.33 g/l NaCl; 0.5 g/l NH_4Cl; 0.0377 g/l $CaCl_2$; 1.5 g/l $(CH_2COOH)_2$; 1.02 g/l yeast extract; with pH adjusted between 6.8 - 7.2 [8]. The bacteria were grown at room temperature under still conditions and continuous yellow light. The cells were harvested after approximately 5 to 7 days, when the color changed to dark orange, and used for further analysis.

2.2.2. Total DNA Isolation

The cell pellet was harvested from a 3-5 ml liquid culture of *R. capsulatus* after centrifugation at 12000 rpm for 5 min. After the addition of 200 µl of CTAB buffer, 20 µl of 30 mg/ml lysozyme was added with gentle mixing, and the mixture was incubated at 37 °C for 1 hour before adding 60 µl of 10% SDS and 10 µl of 20 mg/ml proteinase K. After a 2-hour incubation period at 55 °C, 300 µl of CI solution (Chloroform: Isoamyl with a ratio of 24:1) were added, and the sample was centrifuged at 12000 rpm, 4 °C for 10 min. The supernatant was transferred to a new 1.5-ml microcentrifuge tube containing an equal volume of cold isopropanol. The mixture was incubated on ice for 1 hour, and then centrifuged at 12000 rpm, 4 °C for 10 min. After removing the supernatant, the pellet was washed with 1 ml of cold 70% EtOH. The DNA pellet was air-dried at room temperature, dissolved in 30 µl of TE buffer, and then stored at -20 °C.

2.2.3. Target Gene Amplification

The *sqr* and *fccB* genes were amplified by PCR using specific primers, including forward primers (SQR-F: 5'-TGCCATGATGGCCGGT TCTCC-3' and FCC-F: 5'-ATCTAYCGCCCG ATCCATCCC-3') together with reverse primers (SQR-R: 5'-CGGAATGGCAGAAGAAGATC GCC-3' and FCC-R: 5'-CCGATCTGGGYGAT GAAAATGTCG-3') at 10 µM (0.5 µl each primer per reaction), respectively, with other PCR reagents consisting of 6 µl of 2x DreamTaq Mastermix (Thermo Scientific, United States), 1 µl of extracted DNA, and 4 µl of sterile deionized water. The thermal cycle used for amplification was 95 °C for 3 min and 35 cycles (95 °C/45s; 60.2 °C/30s; 72 °C/1m30s) and 72 °C for 5 min. The PCR products were verified by gel electrophoresis and were sent for sequencing at the 1st BASE (Malaysia). The sequencing results were used as templates to construct phylogenetic trees using the Maximum Likelihood method with bootstrap replications of 250 in Molecular Evolutionary Genetics Analysis (MEGA), version 11.

2.2.4. Cellular Fractionation

The cell pellet harvested was used as the starting material for the fractionation procedure described by Klotz and Hutcheson [9] to obtain three different cellular fractions: the periplasmic, cytoplasmic, and membrane fractions. Ethanol precipitation was used to concentrate the periplasmic and cytoplasmic fractions. Protein concentration was determined by the Bradford method [10] for all of the samples, including the three extracted fractions and the total cell lysate. The protein pattern was visualized using SDS-PAGE followed by Coomassie blue staining [11].

2.2.5. Sulfide Oxidase Activity Assay

The sulfide oxidation activity was measured by tracking the formation of the reaction product in the form of sulfate precipitates [12]. The reaction mixture containing 0.45 ml of 0.1 M sodium acetate (pH 5.6), 0.1 ml sample, 0.05 ml freshly prepared sodium sulfide solution (0.06 g Na_2S, 0.16 g NaOH, 0.02 g $Na_2EDTA.2H_2O$, 2 ml glycerol, and 40 ml distilled water) was incubated at 30 °C for 30 minutes. The reaction was stopped by adding 0.15 ml of NaOH 1.0 M before the addition of 0.005 M barium chloride solution. After vortexing constantly for 60 s, the turbidity of the mixture was measured using the spectro-

photometer at 450 nm [13]. The value of turbidity obtained was proportional to the sulfate concentration in the sample. One unit of sulfide oxidase activity was defined as the amount of enzyme required to generate 1 µmol sulfate/hour/ml (U/ml). An experiment with the flavoprotein inhibitor KCN was performed simultaneously, in which the sample was mixed with 0.02 M KCN for 5 min before performing the aforementioned procedure.

3. Results and Discussion

3.1. Amplification of the Target Genes and Sequence Alignments

With the specific primer pairs detailed above (section 2.2.3), the calculated lengths of the amplified products from *sqr* and *fccB* genes were 1374 bp and 1216 bp, respectively. As shown in Figure 1, using the described thermal cycle, two single bands at the expected positions could be observed for both of the target genes, indicating successful amplification of *sqr* and *fccB* genes from the total DNA extracted from the *R. capsulatus* strain PAM34.

Further alignments of the DNA sequences obtained from the resulting PCR products using NCBI BLAST tool indicated high similarities of the studied *sqr* gene (including the coding region of 1131 bp, encoding for 376 amino acids) to those of several *R. capsulatus* strains (nearly 96%) (Figure 2, upper). Similarly, comparing the amplified *fccB* sequence (1026-bp-long coding region, encoding a 342-residue protein) with other published *fccB* sequences in NCBI indicated high similarities to the same *R. capsulatus* strains (Figure 2, lower). The phylogenetic analysis for further confirmation showed that both genes of strain PAM34 were most closely related to *R. capsulatus* strains SB 1003, A12, and 37b4. However, in the study that reported the sequence of strain SB 1003 [14], the authors only predicted the function and characterized neither protein localization nor its activity. The other sequences (Accession numbers CP061202, CP119563) were submitted to NCBI GenBank, yet not been officially published.

The translated protein sequences were also aligned with published SQR and FCCB. The results shown in Figure 3 indicated the presence of highly conserved amino acid residues in all three FAD binding motifs 1-3 with the conserved residues marked with asterisks. In SQR FAD binding motif 2, A103 and G105, written in red and green, respectively, were unchanged in the compared sequences. These amino acids were indicated in stabilizing the FAD structure and thus were highly conserved. Similarly, the residues highlighted in red and written in blue, corresponding to D317 and G328 in FCCB FAD binding motif 3, respectively, were also conserved in *R. capsulatus* strain PAM34.

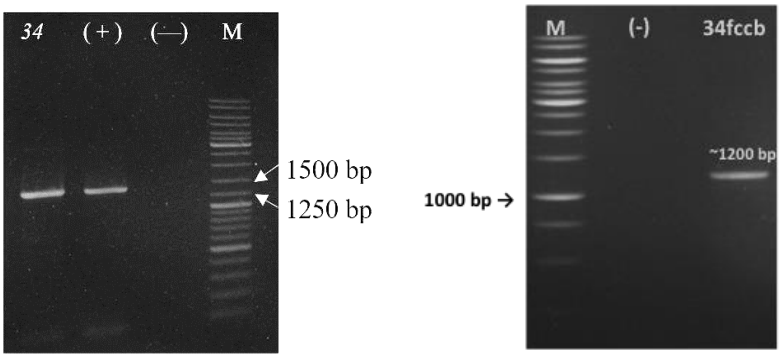

Figure 1. Products after PCR amplification of the *sqr* and *fccB* genes. (-): negative control, (+): positive control, M: 1 kb ladder, 34: PCR product for *sqr* gene, 34fccB: PCR product for *fccB* gene.

sqr gene

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Rhodobacter capsulatus strain A12 chromosome, complete genome	Rhodobacter capsulatus	1816	1816	99%	0.0	95.74%	3616128	CP061202.1
Rhodobacter capsulatus strain 37b4 chromosome, complete genome	Rhodobacter capsulatus	1812	1812	100%	0.0	95.58%	3912233	CP119563.1
Rhodobacter capsulatus SB 1003, complete genome	Rhodobacter capsulatus SB 1003	1724	1724	100%	0.0	94.16%	3738958	CP001312.1
Rhodobacter sp. LPB0142, complete genome	Rhodobacter xanthinilyticus	699	699	97%	0.0	78.52%	3462926	CP017781.1
Thermomonas sp. XSG chromosome, complete genome	Thermomonas sp. XSG	185	185	30%	1e-41	76.66%	3047478	CP061497.1

fccb gene

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Rhodobacter capsulatus SB 1003, complete genome	Rhodobacter capsulatus SB 1003	1299	1299	100%	0.0	89.60%	3738958	CP001312.1
Rhodobacter capsulatus strain A12 chromosome, complete genome	Rhodobacter capsulatus	1291	1291	100%	0.0	89.39%	3616128	CP061202.1
Rhodobacter capsulatus strain 37b4 chromosome, complete genome	Rhodobacter capsulatus	1286	1286	100%	0.0	89.29%	3912233	CP119563.1
R.capsulatus genes rnfA - rnfF, fdxC, fdxN, Orf14 and Orf 10	Rhodobacter capsulatus	885	885	100%	0.0	83.16%	12480	X72888.1

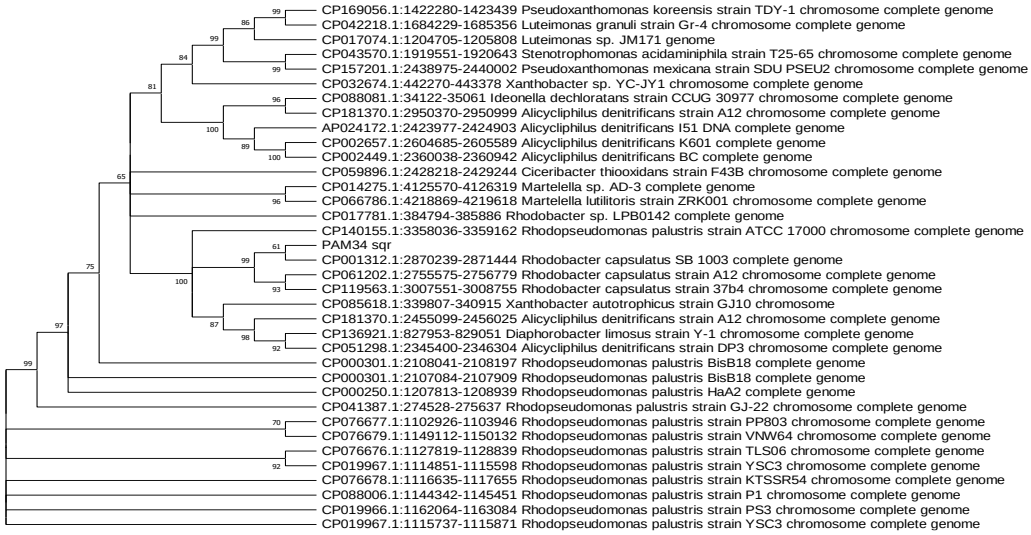

Figure 2. Comparing sequences using BLAST function in NCBI and phylogenetic tree for the *sqr* gene (upper) and the *fccB* gene (lower). The phylogenetic trees were based on *sqr* and *fccB* sequences of the isolated strain *R. capsulatus* PAM34 with the Maximum Likelihood method by MEGA11, the number of bootstrap replications was 250 with the Hasegawa-Kishino-Yano model.

The degree of coservation among amino acid position from identical (*) to strongly (:) and weakly (.) conserved, respectively.

Figure 3. Amino acid sequence comparison using the translated protein sequences corresponding to the *sqr* gene (left) and the *fccB* gene (right).

3.2 Presence of SQR and FCCB Proteins in the Fractionated Samples

The deduced SQR protein sequence from *R. capsulatus* PAM34 was 376 amino acids in length and approximately 47 kDa in weight. The total protein content measured by the Bradford method indicated that the membrane fraction had the highest concentration at 25.58 mg/ml, followed by the total cell lysates at 4.32 mg/ml, and then the cytoplasmic fraction at 1.61 mg/ml (data not shown). The lowest concentration was recorded at the periplasmic fraction with 0.9 mg/ml. The protein pattern of the isolated fractions from the total cell lysate was demonstrated in Figure 4. Consistent with the protein concentration results, the TS and M fractions showed sharp bands with higher intensity, and there were only a few faint bands in the P fraction, with a single dominant band. In the M fraction, there were multiple bands of approximately 50 KDa that might include the SQR protein. There was a band of approximately 40 kDa in the periplasmic fractions that could be the FCCB protein (342 amino acids) [15].

Since different proteins could migrate the same distances on the SDS-PAGE [16] and certain proteins might appear at unexpected positions depending on their charges, further experiments are needed to confirm the presence of the enzymes SQR and FCCB.

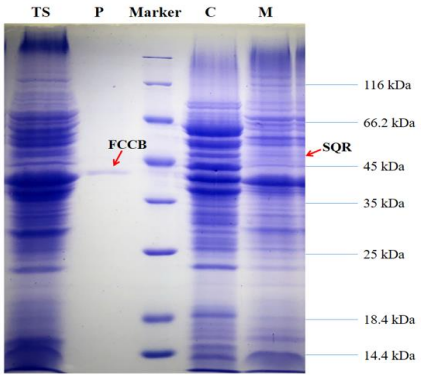

Figure 4. SDS-PAGE electrophoresis of proteins in different cellular fractions. Marker: Pierce™ Unstained Protein MW marker (Thermo Fisher Scientific); TS: Total cell lysate; P: Periplasmic fraction; C: Cytoplasmic fraction; M: Membrane fraction.

3.3 The Sulfide Oxidation Activities of the Isolated Fractions

Compared to the other fractions, the membrane fraction had the strongest activity measured at 1.448 U/ml, and the lowest activity was found in the cytoplasmic fraction, with an activity of 0.058 U/ml (Figure 5). This result indicated the possible existence of SQR as a membrane-associated protein.

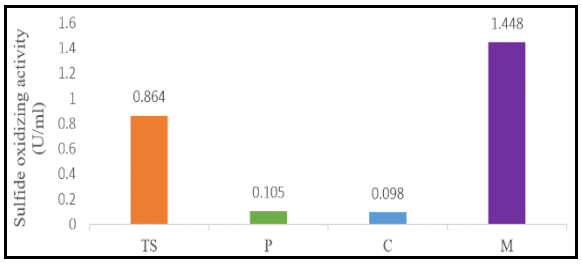

Figure 5. Enzyme activities recorded from different fractions of *R. capsulatus* PAM34 strain; TS: Total cell lysate; P: Periplasmic fraction; C: Cytoplasmic fraction; M: Membrane fraction.

Similarly, the low sulfide oxidizing activity measured in the periplasmic fraction suggested the presence of FCCB. Consistent with the gene-deduced protein analyses and the fraction electrophoresis, this provided the first evidence for the co-existence of both sulfur-oxidizing enzymes in *R. capsulatus*, instead of having only SQR as reported by Schütz et al., [17]. In addition, since the fractionation procedure employed sonication without harsh detergent, the high level of activity remained in the membrane fraction supported the previously established localization of SQR as a peripheral protein.

It is important to note that both SQR and FCCB belong to the flavoprotein family that uses FAD as a cofactor, thus, is sensitive to KCN, a compound reported to be able to induce FAD release from proteins [18]. As demonstrated in Figure 6, after the addition of KCN, the specific flavoprotein inhibitor, the sulfide oxidizing activity of fractions was reduced significantly.

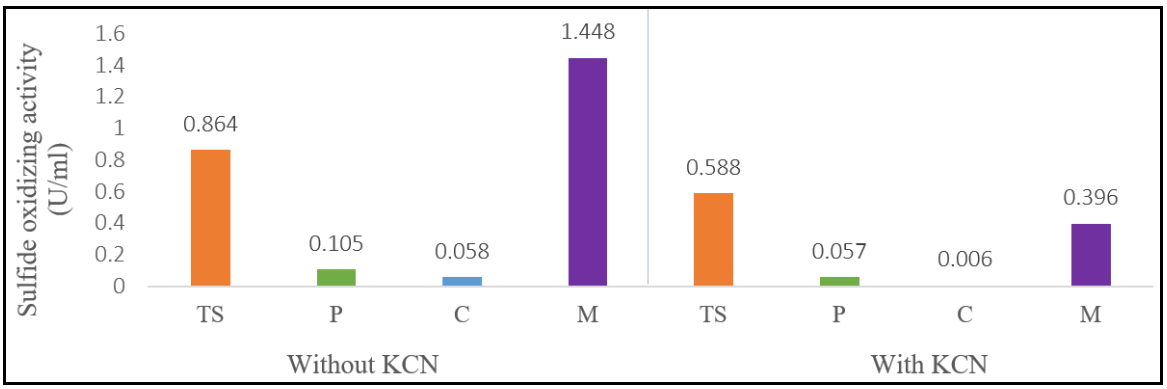

Figure 6. Sulfide oxidation activities without (left) and with (right) inhibitor KCN. TS: Total cell lysate; P: Periplasmic fraction; C: Cytoplasmic fraction; M: Membrane fraction.

Briefly, the sulfide oxidizing activity in the membrane fraction sample decreased significantly from 1.448 to 0.396 U/ml (27% remaining activity). This provided another evidence for the activity originating from a flavoprotein bound to the cell membrane, potentially SQR. The reaction catalyzed by several SQRs has been previously shown to be inhibited by KCN up to 42% [19]. Similarly, approximately 50% of the activity in the periplasmic fraction was inhibited, supporting the hypothetical presence of FCCB in this fraction. A FCC isolated from *Thiobacillus* sp. strain was also reported to be inhibited by cyanide, possibly through the formation of an adduct with the flavin moiety [20]. However, since both fractions with higher sulfide oxidation activity, especially the membrane

fraction, contained other proteins, it is necessary to further separate or purify the proteins using size exclusion chromatography or differential centrifugation employing filter units having different molecular weight cut-offs.

4. Conclusion

In this study, the presence of *sqr* and *fccb* genes in the *R. capsulatus* PAM34 strain isolated from wastewater in Vietnam was determined, showing highly conserved sequences of the FAD-binding domain. Preliminarily, the sulfide-oxidizing activity of PAM34 was determined under the tested conditions, which indicated the expected localization of the enzymes SQR and FCCB. The highest activity (1.448 U/ml) was observed in the membrane fraction, where SQR is located, and lower activity was found in the periplasmic fraction, where FCCB is situated. With the information regarding the possible mechanism of sulfide oxidizing capability in *R. capsulatus* PAM34, this bacterial strain could be further optimized regarding working conditions for applications of H_2S bioremediation.

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