VNU Journal of Science: Earth and Environmental Sciences
Journal homepage: https://js.vnu.edu.vn/EES

Original Article

Evaluating the Sulfur Oxidation Capability of a *Rhodopseudomonas palustris* Strain by Gene and Enzyme Analyses for Potential Applications in Environmental Bioremediation

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Received 30 July 2024
Revised 10 September 2024; Accepted 23 October 2024

Abstract: Sulfur-oxidizing bacteria are capable of oxidizing various sulfur compounds including sulfide and thiosulfate in the environment. Hydrogen sulfide (H_2S), a product of anaerobic decomposition, is highly corrosive. With the typical smell of rotten eggs, the toxic gas H_2S causes many harmful effects on the environment and human health. Sulfide:quinone oxidoreductase enzyme (SQR) produced by sulfur-oxidizing bacteria plays an important role in the sulfide oxidation process, contributing to minimizing sulfide toxicity in the environment. In this study, the presence of sqr gene and SQR enzyme in the photosynthetic purple bacterium *Rhodopseudomonas palustris* (*R. palustris*) isolated from domestic wastewater was determined by gene amplification (PCR) using specific primer pairs following by sequencing and denaturing SDS-PAGE method combined with sulfide oxidase assay that measures $BaSO_4$ turbidity, respectively. The results showed that the sqr gene has 1254 bp in length, encoding for the SQR protein containing 417 amino acids with a molecular weight of 45.87 kDa and locating on the cell membrane. The enzyme operated optimally under the condition of pH 6.5 and temperature at 30 °C with sulfide oxidation activity recorded up to 20-21 U/ml. The study has initially shown the ability of the *R. palustris* bacterial strain to oxidize sulfide for further research using sulfur-oxidizing bacteria applied in environmental treatment.

Keywords: Sulfur oxidizing bacteria, sulfide, oxidation, gene, sulfide:quinone oxidoreductase, environmental bioremediation.

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https://doi.org/10.25073/2588-1094/vnuees.5194

1. Introduction

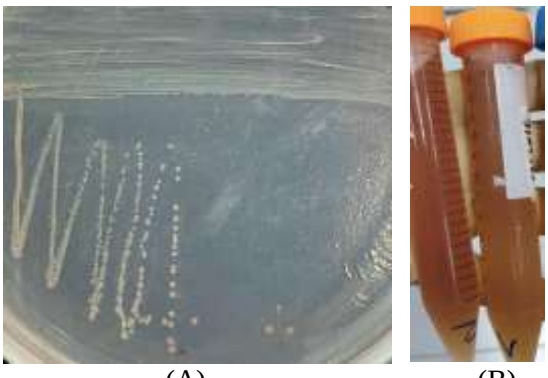
Hydrogen sulfide (H_2S), which is a product of anaerobic decomposition, is highly corrosive. With the typical smell of rotten eggs, the toxic gas H_2S causes detrimental effects on the environment and human health such as loss of consciousness, eye irritation and respiratory problems [1]. Sulfur-oxidizing bacteria (SOB) are versatile microorganisms known for their capability to oxidize various reduced sulfur compounds, namely, H_2S, thiosulfate ($S_2O_3^{2-}$), elemental sulfur (S^0), tetrathionate ($S_4O_6^{2-}$), and trithionate ($S_3O_6^{2-}$) to sulfate (SO_4^{2-}) as the final oxidation product [2]. SOB possess various enzymatic systems that play an important part in the conversion of H_2S to other sulfur compounds, including S^0, sulfite (SO_3^{2-}), sulfide (S^2), $S_2O_3^{2-}$, and $S_4O_6^{2-}$ [3, 4]. Among sulfur oxidation products, S^0 is the most ideal compound to be converted from H_2S due to its highly hydrophobic and generally non-toxic in the environment; hence, the sulfide oxidizing enzyme system that catalyzes the conversion of sulfide to elemental sulfur has a strong potential in H_2S detoxification.

Sulfide:quinone oxidoreductase (SQR) is a well-known enzyme that catalyzes the conversion of sulfide to elemental sulfur. SQR is a protein from the flavoprotein disulfide reductase (FDR) superfamily and generally related to the coenzyme FAD (flavin adenine dinucleotide) in biochemical studies [5].

SQR has been discovered as a single polypeptide chain with a molecular mass of approximately 50-55 kDa determined by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) method [5]. As a membrane-bound protein, SQR has been found in both chemotrophs and phototrophs. Sulfide is oxidized by SQR and electrons are transferred to quinone, then the cytochrome b6f complex re-oxidizes quinol and reduces plastocyanin, and electrons are transferred to the photosystem I [4]. As a result, a proton gradient is formed across the membrane, thereby conserving energy for photosynthesis and respiration in bacteria and archaea. The structure and activity of SQR in some bacteria such as *Acidianus ambivalens*, *Aquifex aeolicus*, *Rhodobacter capsulatus* and *Acidithiobacillus ferrooxidans* have been previously investigated [6-8]. Thanks to its function of converting sulfide to a non-toxic sulfur form, the enzyme SQR could contribute to H_2S detoxification and sulfide homeostasis [8]. *Rhodopseudomonas palustris* (*R. palustris*) belonging to the purple non-sulfur bacteria which are widely distributed in water environments with sufficient light such as lakes, rivers and swamps [9]. *R. palustris* can utilize various carbon sources (e.g., lignin, aromatic compounds) and inorganic compounds (e.g., sulfide, chloride, nitride) under aerobic or anaerobic conditions [9, 10]. Due to the versatile metabolisms, *R. palustris* has potential application in wastewater treatment and bioremediation [10]. *R. palustris* has been applied in treatment of swine wastewater and aquaculture wastewater [9, 11]. *T*he present study investigated the sulfide oxidation ability of the bacterium *R. palustris* (PAM 36) via gene and enzyme analyses to understand the underlying molecular mechanisms and provide scientific knowlege for application, contributing to H_2S treatment and environmental bioremediation.

2. Materials and Methods

2.1. Materials



(A) (B)

Figure 1. The *R. palustris* (PAM36) bacterium grown on agar plate (A) and in liquid medium (B).

The strain *R. palustris* (PAM 36) used in this study was provided by GREENLAB (Laboratory of Bioelectrochemical Technologies for Green Growth), University of Science, Vietnam National University, Hanoi [12]. Fig. 1 shows the *R. palustris* bacterium grown on agar plate and in liquid medium.

2.2. Methods

2.2.1. Growth of Purple Non-sulfur Bacteria

Bacterial colonies were picked from the plate and then grown in 50 ml falcon tubes with the enrichment medium containing the ingredients (g/l): KH_2PO_4 - 0.33; $MgSO_4.H_2O$ - 0.33; NaCl - 0.33; NH_4Cl - 0.5; $CaCl_2$ - 0.038; $(CH_2COOH)_2$ - 1.5; Yeast extract - 1.02 with pH adjusted between 6.8 - 7.2 [12]. Bacteria were cultured under still conditions and continuous yellow light. After approximate 5 to 7 days, when the color changed to dark orange, the culture was stopped and the cells were harvested for further experiment.

2.2.2. Total DNA Extraction

3-5 ml liquid culture of *R. palustris* were centrifuged at 12000 rpm for 5 min, then the supernatant was discarded before the addition of 200 µl of CTAB buffer. Then, 20 µl of 30 mg/ml lysozyme were added with gentle mixing and incubated at 37 °C for 1 h. Next, 60 µl of 10% SDS and 10 µl of 20 mg/ml proteinase K were added; the mixture was incubated at 55 °C for 2 h. Subsequently, 300 µl of CI solution (Chloroform: Isoamyl with a ratio of 24:1) were added and mixed gently. The sample was centrifuged at 12000 rpm, 4 °C, for 10 min. The supernatant was aspirated and transferred to a new 1.5 ml microcentrifuge tube. One equal volume of cold isopropanol was added and the mixture was incubated on ice for 1 h before centrifuging at 12000 rpm, 4 °C, for 10 min. The supernatant was discarded and the pellet was washed with 1ml of cold 70% ethanol. The DNA precipitate was air-dried at room temperature and then dissolved in 30 µl of TE buffer and stored at -20 °C [13].

2.2.3. Gene Amplification

The *sqr* gene was amplified by PCR using specific primers, including forward primer (FW) 5'- TGCCATGATGGCCGGTTCTCC-3' and reverse primer (RW) 5'-CGGAATGGCAGAAGAAGATCGCC-3' with component of 6 µl 2x DreamTaq Mastermix, 0.5 µl FW/RW 10 µM, 1 µl DNA and 4 µl water. Thermal cycle of PCR was 95 °C for 3 min and 35 cycles (95 °C /45 s; 60,2 °C/30 s; 72 °C/90 s) and 72 °C for 5 min. The PCR products were checked by gel electrophoresis and were sent for sequencing at the company 1st Base (Malaysia).

2.2.4. Fractionation of Bacteria

Bacteria were harvested from cultures when the OD_{600} reached 1 and then followed procedure as previously described by Klotz and Hutcheson (1992) to collect three different cellular fractions: the periplasmic, cytoplasmic, and the membrane fractions. The periplasmic and the cytoplasmic fluids were concentrated using ethanol precipitation. The Bradford method was carried out to determine the protein concentration of all the samples, including three extracted fractions and the total protein fluid. To examine the presence of these enzymes in their localization, the fractions were subjected to SDS-PAGE [14].

2.2.5. Sulfide Oxidase Assay

The enzyme activity was determined through measuring of the enzymatic reaction product in the form of sulfate precipitates [15]. The reaction mixture contained 0.45 ml of 0.1 M sodium acetate (pH 5.6) and 0.1 ml sample contained the fraction, then 0.05 ml freshly prepared sodium sulfide (Na_2S) solution (0.06 g Na_2S, 0.16 g NaOH, 0.02 g EDTA $Na_2.2H_2O$, 2 ml glycerol, and 40 ml distilled water). The mixture was incubated at 30 ^oC for 30 min and stopped with the addition of 0.15 ml of 1.0 M NaOH. 0.75 ml of 0.005 M barium chloride solution was added and the solution was vortexed constantly for 60 s to form barium sulfate precipitates. The concentration of sulfate ions formed during sulfide oxidase assay was assessed through the turbidity of the precipitates using the

spectrophotometer at 450 nm [16]. The amount of turbidity formed is proportional to the sulfate concentration in the sample. One unit of sulfide oxidase activity was defined as an amount of the enzyme required to produce 1 µmol sulfate/h/ml (U/ml).

An experiment with an inhibitor was performed paralelly, in which the protein fraction was mixed with 0.02 M KCN for 5 min before performing the previously described procedure.

The pH activity profile of SQR was determined between pH 4.5 and 9.5 through the adjustment of sodium acetate buffer at 30 °C. The optimum pH value obtained from this assay was used in the temperature activity profile. The effect of temperature on SQR activity was measured between 20 °C and 90 °C.

3. Results and Discussion

3.1 Amplifing and Sequencing of Sqr Gene

The total DNA of *R. palustris* strain was extracted and used as template for amplification of the *sqr* gene using the PCR method. Electrophoretic examination showed that the amplification product was approximate 1580 bp as expected size (Fig. 2). There were no nonspecific bands. Thus, the *sqr* gene has been sucessfully amplified from DNA extracted from *R. palustris* strain.

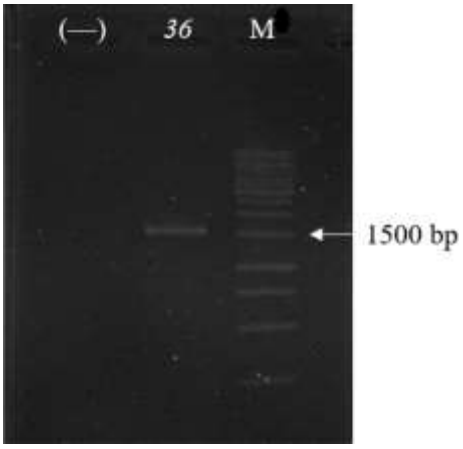


Figure 2. Gel electrophoresis of PCR products amplified the *sqr* gene (-) is negative control, 36: PCR product, M: 1 kb ladder.

Then, PCR products were sent for sequencing and the results showed the good results of clear peaks (Fig. 3A). The *sqr* gene is obtained with 1254 bp in length. Comparing the obtained *sqr* sequence with other published *sqr* sequences in NCBI indicated highly similarity to that of *R. palustris* strain GJ-22 (98%) (Fig. 3B).

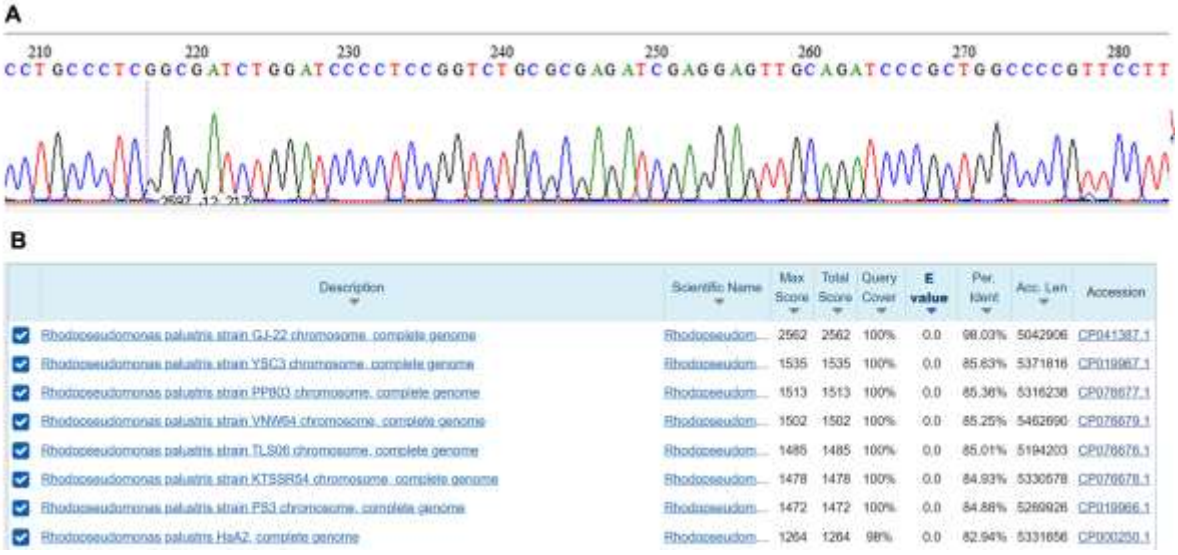


Figure 3. Part of sequencing result of the *sqr* gene (A), comparing sequences using blast function in NCBI (B).

3.2. Evidence for the Presence of SQR in Cell Membrane

The deduced SQR protein is 417 amino acids in length and 45.87 kDa in weight. The SQR protein is well known for being located on the cell membrane. Therefore, the bacterial cells were fractionated to obtain different cell components. From the fractionation procedure with the total cell lysates as the starting material (T), three fractions were obtained, designated periplasmic (P), cytoplasmic (C), and membrane (M). The fraction concentration was determined using Bradford assay. The results show that the membrane fraction had the highest concentration at 7.52 mg/ml, followed by the total cell lysates at 6.06 mg/ml, and then the cytoplasmic fraction at 0.15 mg/ml. The lowest concentration was recorded at the periplasmic fraction with 0.06 mg/ml. The protein composition of the isolated fractions above is shown in Fig. 4. Consistent with the protein concentration results, the T and M fractions showed bands with higher intensity, and there were only a few faint bands in the P fraction. In M fraction, there was a band of approximately 50 KDa that might be SQR protein.

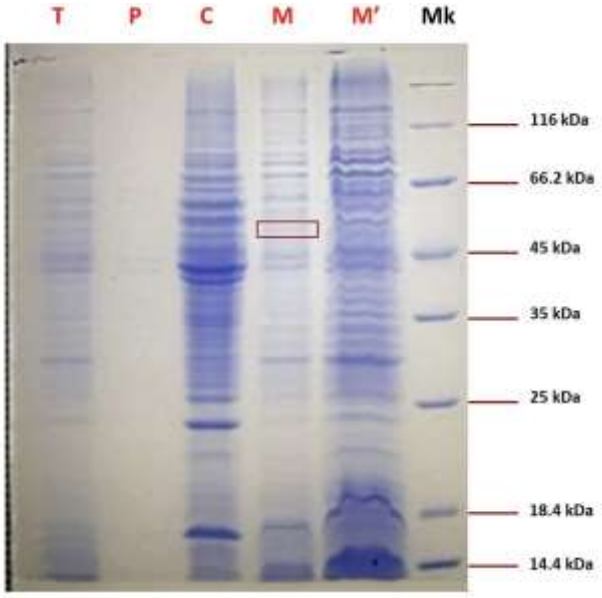


Figure 4. SDS-PAGE electrophoresis of proteins in different cellular fractions.
Mk: Protein marker; T: Total cell lysate; P: Periplasmic fraction; C: Cytoplasmic fraction;
M and M': original and concentrated membrane fractions

However, different proteins with similar sizes can possibly appear at the band of 50 KDa. Thus, even though SDS-PAGE electrophoresis initially demonstrate the presence of SQR in protein fractions, further experiments were needed to confirm the presence of the enzyme SQR.

There are several methods to separate subcellular fractions, with chromatography, isoelectric focusing, and gel electrophoresis being the most common. The advantage of these methods is that they allow the targeted proteins to be isolated effectively; however, purified SQR are often unstable due to their structure, and there were studies prove that the specific activity of the heterogeneously expressed SQR was 15 times higher than that of the directly purified SQR from *R. capsulatus* [17]. Therefore, the osmotic shock method used in this study is preferable to identify SQR at their localized subcellular compartments. This method was successfully employed to extract the periplasmic and membrane fractions in various studies [18].

3.3. The Sulfide Oxidation Activities of the Isolated Fractions

To further investigate the presence of targeted SQR enzyme, the enzyme activity of each fraction was subjected to the sulfide oxidation assay. The targeted enzyme catalyzed the production of sulfur, which can be then converted to sulfate. The sulfate was then combined with $BaCl_2$ to create a $BaSO_4$ precipitate, which was used to estimate the enzyme activity of each fraction. 30 min after the addition of the substrate (Na_2S), the sulfate products were determined using a spectrophotometer at 450 nm. The concentration of the product was then determined based on the SO_4^{2-} standard curve (Fig. 5).

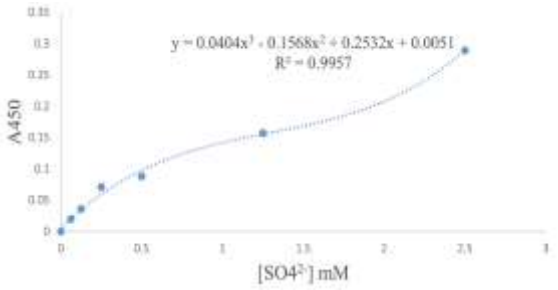


Figure 5. Standard curve for determined sulfate concentration (mM)
(y: the absorbance at 450 nm (A450) of a sample;
x: the corresponding sulfate concentration).

Based on the sulfate concentrations, the enzyme activity of each fraction was fitted with a third–order polynomial line, and the results are demonstrated in Fig. 6. The R^2 value of the standard curve is greater than 0.996, proving the resulting equation was suitable for further use.

Overall, the membrane fractions have the highest activity with 19.96 U/ml, and the lowest records were found in cytoplasmic fractions, with an activity of 0.12 U/ml. This result approves the presence of SQR in the cell membrane with the highest activity. To confirm the enzyme activity, the well known inhibitor KCN which inhibits the flavoprotein group of SQR was used. Before doing the sulfide oxidation assay, 20 mM KCN was added to the samples. After incubation time, the enzyme activity was determined with the same method, and the results are demonstrated in Fig. 7.

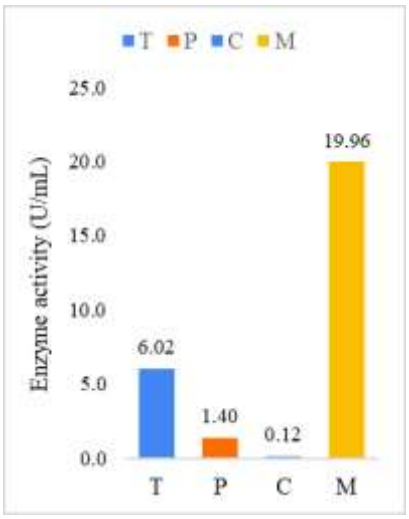


Figure 6. Enzyme activities of different fractions from the studied bacteria; T: Total cell lysate; P: Periplasmic fraction; C: Cytoplasmic fraction; M: Membrane fraction.

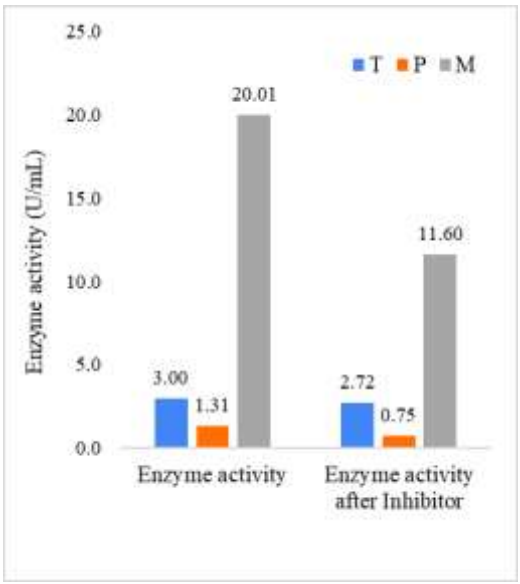


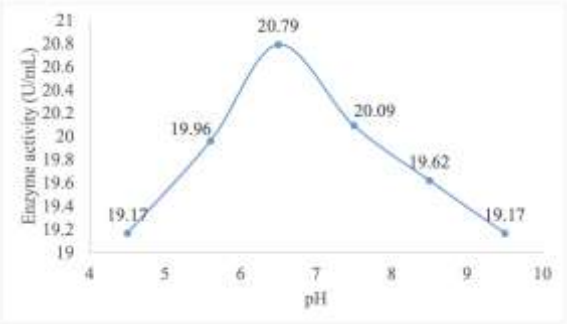
Figure 7. Sulfide oxidation activities in the presence of inhibitor KCN.

In membrane fractions, the activity decreased from 20.01 to 11.60 U/ml, equal to 42% of the membrane fraction was inhibited.

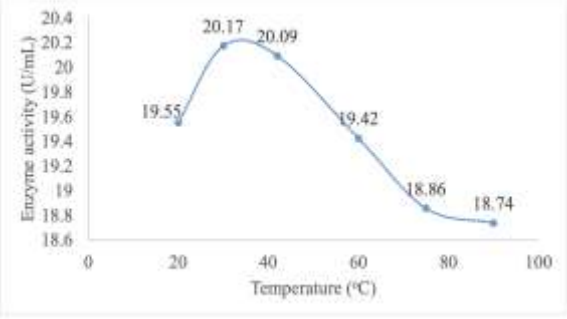
These results confirm the presence of SQR in the membrane fraction, being inhibited by KCN. In the study of B. Arieli et al., (1994), the SQR actitivities of *Oscillatoria limnetic* were shown to be inhibited by KCN and also by other inhibitors that are quinone analogues [19].

3.4. Optimal pH and Temperature for Sulfide Oxidation Activity

The membrane fraction was tested through a range of pH (from 4.5 to 9.5) and temperature (20 °C and 90 °C). This range was created by changing the pH of sodium acetate used in enzyme assays. The results are shown in Fig. 8.



(A)



(B)

Figure 8. Effect of pH (A) and temperature (B) on sulfide oxidation activity.

It appeared that SQR in the membrane fractions was still active at a broad pH range, with the highest activity at pH = 6.5. The growth medium of the studied bacteria has a pH range from 6.8 to 7.2, slightly off the optimum pH, but still helping the enzyme work better. The pH of wastewater generally varies from 6 to 8, so the enzyme SQR from the studied bacteria can work its best to remove H_2S. The highest enzyme activity was 20.17 U/ml, and this value was achieved at 30 – 40 ^oC, the same range for the bacterial best growth condition. At this temperature range, it is assumed that the enzyme SQR of the studied bacteria can work effectively anywhere in Vietnam. In a prior study, the highest activity of SQR was found at 70 ^oC and pH 6 in a thermoacidophilic *A. ambivalens* [6] which grows optimally at 85 ^oC and pH 2.

4. Conclusion

In this study, the presence of *sqr* gene and SQR enzyme in the photosynthetic purple bacterium *R. palustris* was determined. Under the optimum conditions (pH = 6,5 and temperature in the range of 30 – 40 ^oC), sulfide oxidation activity was higher than 20 U/ml in the membrane fractions where enzyme SQR is located. Thus, the *R. palustris* bacterial strain could be promising for application in remediation of H_2S pollution.

Acknowledgments

This research has been done under the research project QG.21.04 “Study on SQR, FCC genes and enzymes in sulfur oxidizing bacteria for application in environmental treatment” of Vietnam National University, Hanoi.

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