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Detection of Sulfur Oxidizing Bacteria to Oxidize Hydrogen Sulfide in Biogas from Pig Farm by NGS and DNA Microarray Technique

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ABSTRACT

A high concentration of hydrogen sulfide (H_2S) released from pig farming is one of the major environmental problems affecting surrounding communities. In modern pig farms, the bioscrubber is used to eliminate H_2S, which is found to be driven mainly by the sulfur-oxidizing bacteria (SOB) community. Therefore, in this study, molecular biology techniques such as next-generation sequencing (NGS) and DNA microarray are proposed to study the linkage between enzyme activity and the abundance of the SOB community. The starting sludge (SFP1) and recirculating sludge (SFP2) samples were collected from the bioscrubber reactor in the pig farm. The abundance of microbial populations between the two sampling sites was considered together with the gene expression results of both *soxABXYZ* and *fccAB*. Based on the NGS analysis, the members of phylum Proteobacteria such as *Halothiobacillus, Acidithiobacillus, Thiothrix, Novosphingobium, Sulfuricurvum, Sulfurovum, Sulfurimonas, Acinetobacter, Thiobacillus, Magnetospirillum, Arcobacter,* and *Paracoccus* were predominantly found in SFP2. The presence of Cyanobacteria in SFP pig farms is associated with increased biogas yields. The microarray results showed that the expression of *soxAXBYZ* and *fccAB* genes involved in the oxidation of sulfide to sulfate was increased in *Halothiobacillus*, *Paracoccus*, *Acidithiobacillus*, *Magnetospirillum*, *Sphingobium*, *Thiobacillus*, *Sulfuricurvum*, *Sulfuricurvum*, *Arcobacter*, and *Thiothrix*. Both NGS and DNA microarray data supported the functional roles of SOB in odor elimination and the oxidation of H_2S through the function of *soxABXYZ* and *fccAB*. The results also identified the key microbes for H_2S odor treatment, which can be utilized to monitor the stability of biological treatment systems and the toxicity of sulfide minerals by oxidation.

INTRODUCTION

Next Generation Sequencing (NGS) is a nucleotide sequencing technology used to study the genetic variation associated with microbial traits using the highly variable regions within the conserved regions of 16S rRNA-encoded genes to identify phylogenetic relationships among prokaryotes (Gupta et al. 2019). NGS is a rapidly advancing technology that provides high throughput sequencing data, which can be further analyzed for sample identity (Besser et al. 2018). NGS can be applied in many research areas, including epigenetics, genetic variation discovery, genetic diversity, population validation, microbial community, and gene expression level. This technique is currently used for studying the microbial community structure and function of genes in environmental samples (Roh et al. 2010). DNA microarray also uses genome sequence information to analyze the structure and function of thousands of genes at a time. The microarray technique relies on hybridization between DNA targets and probes, which are specific to each position on the genome or chromosome. Microarray holds DNA probes that recognize other ssDNA from tested

samples. DNA microarray has been successfully applied to analyze functional microbial communities in various conditions (Roh et al. 2010) and monitor the function and cohabitation of hundreds to thousands of microbial populations at the same time.

DNA microarrays and NGS technologies are now being applied in microbial ecology studies as tools for the advanced understanding of the role of microorganisms in various environments (Roh et al. 2010). Microarrays for microbial detection can be divided into four different types: functional gene arrays (FGAs), community genome arrays (CGAs), phylogenetic oligonucleotide arrays (POAs), and metagenomics arrays (MGA), based on represented gene type on the array (Gentry & Zhou 2006). CGA arrays from the whole genomic DNA of 12 pure culture species were successfully used to detect and identify species of bacteria in acid mine drainage and bioleaching systems (Chen et al. 2009). The relative abundance of marine sediments involved in the nitrogen cycle genes (*amoA, nirS, and nirK*) was also analyzed by FGAs arrays (Wu et al. 2001). The *soxB, sqr,* and *dsrA* genes were used to classify the abundance and diversity of sulfur-oxidizing bacteria in the Pearl River water. *Dechloromonas, Limnohabitans, Paracoccus, Sulfuritalea, Sulfitobacter, and Thiobacillus* are the dominant SOB for *soxB* (Luo et al. 2018). The diversity and distribution of *fccAB* and *soxAXYZB* genes related to sulfur oxidation in 75 strains of *Thioalkalivibrio*, a group of haloalkaliphilic and chemolithoautotrophic SOB from soda lakes, were reported by Berben et al. (2019). *Paracoccus Versutus CM1* was identified by the *soxB* gene for H_2S removal efficiency up to 100% from biogas in the laboratory-scale system (Jirachaisakdeacha et al. 2020). Haosagul et al. (2021) designed a hybrid microarray covering both the whole genome sequences and specific gene regions (*soxAXBYZ* and *fccAB*) to identify the activity of sulfur-oxidizing bacterial strains that oxidize H_2S in biogas. However, the diversity of microbes is also changed according to time and environmental conditions. Thus, the combined study using microarray data and 16S rRNA analysis will provide comprehensive data for the role of microorganisms in their various environments. This research aims to study the functional role of SOB via the linkage between enzyme activity and the abundance of SOB in the bioscrubber system for treating H_2S in the biogas of pig farms using NGS and microarray techniques.

MATERIALS AND METHODS

Microbial Samples

Fresh pig manure was used as the starting sludge in wastewater treatment systems to produce biogas on the SFP farm. The treatment sludge will then be fed into the bioscrubber as a substrate for SOB. The inlet H_2S concentration was 2,365±152 ppm, and the outlet H_2S was 20±5 ppm. This average concentration was measured in real time by a GFM416 biogas analyzer (Gas Data, England). The starting sludge (SFP1) was immobilized on the biofilter media in the form of biofilms to absorb dissolved hydrogen sulfide gas and cell survival. Water consisting of essential nutrients (pH 7) was constantly sprayed from top to bottom of the bioscrubber to maintain moisture for SOB in the system coupled with approximately 1.5±0.5% (v/v) in opposite directions of aeration. The biotreatment system has been operated until the H_2S removal efficiency is stable at 99%, which indicates the presence of product accumulations in the form of sulfate or sulfur. However, some loose sludge from the biofilm layer could be suspended along with the recirculating water in the system and settled at the bottom of the bioscrubber tank. Therefore, the settled sludge samples were collected as recirculating sludge (SFP2) for analysis of both functional genes and diversity of bacteria in comparison with the SFP1 sampling point. Both SFP1 and SFP2 sludge samples were transferred to clean tubes and centrifuged at 14,000 g (MX-301, Japan) for 15 min and stored in the freezer at −20°C before the genomic DNA extraction.

Genomic DNA Extraction for NGS Analysis

The starting sludge (SFP1) and recirculating sludge (SFP2) were defrosted at room temperature before use. SFP1 and SFP2 sludge were lysed in DNA extraction buffer and lysozyme (10 $mg.L^{-1}$) at a ratio of 1:1, followed by shaking at 180 rpm at 37°C for 60 min. Next, the other DNA binding proteins were released by adding 100 μL sodium dodecyl sulfate and incubated at 65°C for 30 min. DNA was precipitated with isopropanol (0.6 volume) and rinsed with ice-cold ethanol. Then, the DNA pellet was dissolved in the elution buffer, as described in Haosagul et al. (2020). The quality of gDNA was measured by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). The gDNA was prepared for NGS analysis based on the Illumina HiSeq 2500 platform according to the company's instruction using the specific primer named 515F-806R that targeted to V4 region of the 16S rRNA gene. The PCR amplification steps were described in Haosagul et al. (2021).

Microbial Community Study Using 16S rRNA Sequencing

The DNA extracts from SFP1 and SFP2 sludge were prepared into a library that could be processed in an Illumina HiSeq 2500 sequencing platform. In brief, the 16S rRNA gene segments for the PCR process were amplified using a primer set of 515F 5'- GTGCCAGCMGCCGCGGTAA-3'

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and 806R 5'-GGACTACHVHHHTWTCTAAT-3' for targeting the V4 hypervariable regions. The data analysis was conducted with QIIME2 (v.2020.8). Pair-end reads obtained from the HiSeq 2500 sequencing platform were merged, filtered, and quality-controlled to gain the clean sequences with the QIIME2-DADA2 plugin. The clean sequences were clustered into the amplicon sequence variant (ASV) table at the 97% similarity level (Bolyen et al. 2019). Phylogenetic taxonomy assignment was achieved using the Silva database version 138 classifier. PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) was utilized to predict the potential function of microbial communities via marker gene sequencing profiles. PICRUSt2 analysis was applied to predict the potential Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways by 16S rRNA data (Douglas et al. 2020).

Microarray Construction and Probe Designed

In brief, for probe design, a comparative genomic hybridization (CGH) microarray consisting of 61,788 probes was prepared on a glass slide. Each spot was a synthetic oligonucleotide of 60 bp/probe, which covered a list of SOB genera (42,248 probes) and other genera (NSOB) that were detected on SFP1 and SFP2 samples of bioscrubber from SFP pig farm by NGS technique. The genome sequences and genes related to sulfide oxidation (*soxAXBYZ* and *fccAB*) of each SOB species were obtained from the National Center for Biotechnology Information (NCBI) database and the Pathosystems Resource Integration Center (PATRIC) database for microarray probe design, according to the manufacturer instruction (Agilent Technologies 2019).

DNA Preparation for CGH Microarray

Fresh sludge from two sampling sites (SFP1 and SFP2) of bioscrubber from the SFP pig farm was collected and centrifugation at 10,000 g (MX-301, Japan) for 10 min. Total genomic DNAs (gDNA) from fresh sludge were extracted from the sludge pellet using a sodium dodecyl sulfate (SDS)--based DNA extraction method (Haosagul et al. 2020). Then, 20-40 µl of RNase A (10 mg/ml) was added and incubated at 37°C for one hour, followed by incubation at 65°C for 40 min. The GenepHlow™ Gel/PCR Kits purified the gDNA samples. The DNA intactness and molecular weight for each sample were checked using 0.8% (w/v) agarose gel electrophoresis. NanoDrop 2000 UV-VIS Spectrophotometer was primarily applied to measure the purity of gDNA at $A_{260}/_{280}$ and $A_{260}/_{230}$. The values of $A_{260}/_{280}$ and $A_{260}/_{230}$ of purified gDNA should be ≥ 1.8 and ≥ 1.0, respectively. A Qubit dsDNA BR Assay Kit then assayed the extracted dsDNA sample. The calculation of yield and dsDNA concentration were calculated according to Eq. 1 and Eq. 2 (Agilent Technologies 2019).

$$\text{dsDNA concentration} = \text{QF value} \times (200/y) \quad \ldots(1)$$

where,

QF value = the value given by the Qubit Fluorometer

y = the volume of the sample added to the assay tube.

$$\text{Yield } (\mu g) = \frac{\text{gDNA concentration } (ng/\mu L) \times \text{sample volume } (\mu L)}{1000\ ng/\mu g} \quad \ldots(2)$$

The gDNA with good quality was applied further for the gDNA labeling step. In brief, gDNA was purified using the reaction purification column provided by the SureTag Complete DNA Labeling Kit and SureTag DNA Labeling Kit. The DNA Labeling Kit contained Cyanine3 (Cy3) and Cyanine5 (Cy5) dyes that were used to label recirculating sludge (SFP2) and starting sludge (SFP1), respectively. The specific activity was calculated according to Eq. 3 (Agilent Technologies 2019).

$$\text{Specific Activity (pmol dyes/}\mu g \text{ gDNA)} = \frac{\text{pmol per } \mu L \text{ of dye}}{\mu g \text{ per } \mu L \text{ gDNA}} \quad \ldots(3)$$

Hybridization of labeled DNA fragments from both samples (SFP1 and SFP2) and probes were performed on the CGH microarray slide for 24 hr at 65°C with agitation at 20 rpm in an Agilent hybridization oven. Unbound DNA was washed off with buffer 1 for 5 min at room temperature and washed once with buffer 2 for 1 min at 37°C. The microarray slide was analyzed for the signal intensity of each spot by the Agilent High-Resolution Microarray Scanner (C-model). The signal intensity of 61,278 probes was calculated into log2 ratios of SFP2/SFP1. QC metrics from the microarray set, such as background noise, signal intensity, signal-to-noise, reproducibility, and DLRSD (Derivative Log2 Ratio Standard Deviation), are criteria for signal quality analysis and indicate processing errors.

RESULTS AND DISCUSSION

Quantity and Quality of gDNA

The gDNA of starting sludge (SFP1) and recirculating sludge (SFP2) from the SFP pig farm was successfully extracted. Double-stranded DNA concentrations of SFP1 and SFP2 were found in the range of 44–418 ng/µl. SFP samples had the A_{260}/A_{280} ratio of ≥ 1.8 and the A_{260}/A_{230} ratio of ≥ 1.0 (Table 1). These results confirmed the quality and quantity of both samples before they were submitted to 16S rRNA sequencing using the Illumina HiSeq 2500 platform in the next step.

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Table 1: Double-stranded DNA concentration.

No.	Sample Name	Description	A_{260}/A_{280}	A_{260}/A_{230}	Qubit Concentration
1	SFP1	Starting sludge-Pig farm	1.76	1.21	418.0
2	SFP2	Recirculating sludge-Pig farm	1.92	1.95	43.6

Microbial Community of SOB Detected by NGS Analysis

The abundance of microbial community was analyzed from the 16S rRNA sequencing (based on the V4 region). A total of 28 phyla were classified from the starting sludge (SFP1) and recirculating sludge (SFP2) of a full-scale bioscrubber from SFP farms. The top three most abundant phyla detected in the SFP farm were Proteobacteria, Cyanobacteria, and Actinobacteria. The community structure of these three species was markedly changed according to H_2S eradication between SFP1 and SFP2. Before the elimination of H_2S (SFP1), Cyanobacteria, Actinobacteria, and Proteobacteria were among the top 3 most abundant phyla, with a relative abundance of 25.56%, 25.46%, and 25.33%, respectively. During the H_2S removal, the community structure changed dramatically. Proteobacteria replaced Actinobacteria with 12.95% relative abundance, which accounted for 39.79% of the total relative abundance in SFP2 (Fig. 1A). The relative abundance of Cyanobacteria was observed to be stable between the SFP1 (25.56%) and SFP2 (25.87%). Cyanobacteria have been found in the wastewater treatment of pig farms, biogas slurry, and bioenergy production (Lu et al. 2020, Stewart et al. 2011). Especially *Synechococcus elongatus,* which can increase intracellular sucrose accumulation, leading to increased yields of both biohydrogen and biomethane (Vayenos et al. 2020, Samiotis et al. 2021). The presence of cyanobacteria in both collecting points indicated their function in the wastewater treatment of pig manure. In the class level, Betaproteobacteria (13.69-26.08%), unidentified Cyanobacteria (25.31-25.52%), and Gammaproteobacteria (3.91-6.22%) were three dominant members within phylum Proteobacteria and Cyanobacteria (Fig. 1B), which increased more than 50% of the relative abundance when collected as SFP1 to SFP2. At the order level, Burkholderiales (21.53%) has increased over 45% of the total relative abundance in SFP2 samples (Fig. 1C). Meanwhile, Thiotrichales (185-fold) was fast-growing and increasing inside the bioscrubber tank (SFP2) when compared to the starter point (SFP1). These members have been shown to play an important role

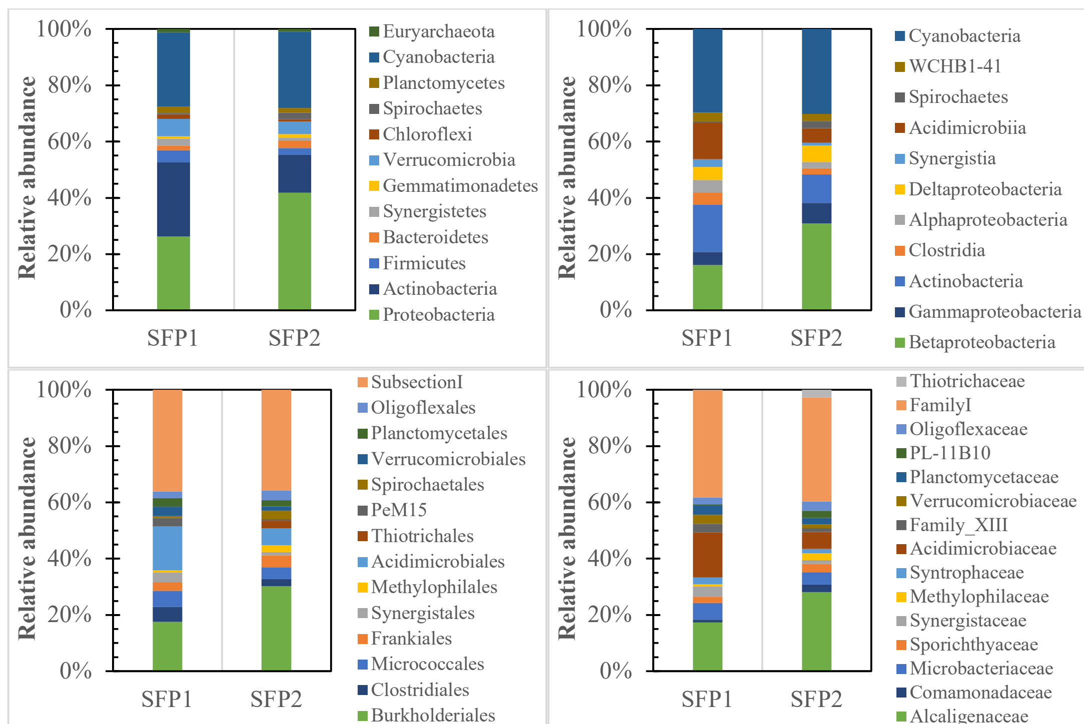

Fig. 1: Phylum (A), class (B), order (C), and family (D) levels showed the microbial dynamics during the starting sludge (SFP1) and recirculating sludge (SFP2) collected from a bioscrubber in the pig farm.

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Table 2: Microbial diversity and gene expression related to eliminating H_2S in biogas.

Genus	SFP1 (%)	SFP2 (%)	Growth (%)	*soxABXYZ*	*fccAB*	Classified
Novosphingobium	0.315	0.344	0.030	D	ND	SOB
Gemmatimonas	0.289	0.493	0.204	D	ND	SOB
Sphingobium	0.027	0.032	0.006	D	D	SOB
Acinetobacter	0.018	0.060	0.042	D	ND	SOB
Sulfurovum	0.016	0.063	0.047	ND	D	SOB
Sulfurimonas	0.010	0.056	0.046	D	D	SOB
Syntrophomonas	0.010	0.018	0.008	ND	D	SOB
Sulfuricurvum	0.009	0.336	0.327	D	D	SOB
Thiothrix	0.007	1.853	1.846	D	D	SOB
Arcobacter	0.002	0.020	0.018	D	D	SOB
Paracoccus	0.002	0.005	0.003	D	D	SOB
Acidithiobacillus	0.001	0.498	0.497	D	D	SOB
Halothiobacillus	0.001	0.015	0.014	ND	D	SOB
Magnetospirillum	0.000	0.017	0.017	D	D	SOB
Thiobacillus	0.000	0.009	0.009	D	D	SOB
Synechococcus	20.953	23.248	2.295	ND	ND	NSOB
MWH-UniP1_aquatic_group	10.008	18.791	8.782	ND	ND	NSOB
CL500-29_marine_group	9.734	3.502	-6.232	ND	ND	NSOB
Candidatus_Aquiluna	1.670	2.068	0.398	ND	ND	NSOB
Alsobacter	1.093	0.150	-0.943	ND	ND	NSOB
Desulfomonile	0.977	0.261	-0.716	ND	ND	NSOB
Ilumatobacter	0.822	0.684	-0.138	ND	ND	NSOB
Acidibacter	0.806	1.312	0.505	ND	ND	NSOB
Thiolamprovum	0.718	0.523	-0.195	ND	ND	NSOB
Rhodopirellula	0.706	0.361	-0.345	ND	ND	NSOB
Aeromicrobium	0.668	0.730	0.062	ND	ND	NSOB
Pirellula	0.612	0.221	-0.390	ND	ND	NSOB
Methanospirillum	0.563	0.521	-0.042	ND	ND	NSOB
Syntrophus	0.421	0.108	-0.313	ND	ND	NSOB
Methanosaeta	0.403	0.129	-0.274	ND	ND	NSOB
Roseiflexus	0.280	0.262	-0.018	ND	ND	NSOB
Dinghuibacter	0.255	0.194	-0.061	ND	ND	NSOB
Syntrophorhabdus	0.198	0.518	0.320	ND	ND	NSOB
Alpinimonas	0.197	0.193	-0.005	ND	ND	NSOB
Aminivibrio	0.175	0.113	-0.063	ND	ND	NSOB
Ignavibacterium	0.172	0.507	0.335	ND	ND	NSOB
Peredibacter	0.125	0.270	0.145	ND	ND	NSOB
Smithella	0.122	0.393	0.271	ND	ND	NSOB
vadinBC27_wastewater-sludge_group	0.121	0.303	0.182	ND	ND	NSOB
Hydrogenophaga	0.108	0.462	0.354	ND	D	NSOB
Luteolibacter	0.071	0.211	0.140	ND	ND	NSOB
Opitutus	0.062	0.176	0.114	ND	ND	NSOB
Ferrovibrio	0.059	0.217	0.158	ND	ND	NSOB
Mesotoga	0.019	0.058	0.039	ND	ND	NSOB
Sphaerochaeta	0.014	0.053	0.039	ND	ND	NSOB

D=Detected, ND=Not Detected, % represent the signal intensity of the sulfide oxidation gene of the SOB genus.

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in the degradation of H_2S in biogas from pig farms (Dubinina et al. 2011, Xu et al. 2020, Xu et al. 2021). On the other hand, the relative abundance of CL500-29 marine group bacteria, which belonged to the family Acidimicrobiaceae, was found to be decreasing. In contrast, the representatives of the family Alcaligenaceae, FamilyI, Thiotrichaceae, and Comamonadaceae were increased in the bioscrubber tank (Fig. 1D). These results strongly support functional roles in the oxidation of H_2S of FamilyI, Comamonadaceae, and Alcaligenaceae as discussed in the previous study (Kersters et al. 2006, Wang et al. 2018, Li et al. 2020, Flood et al. 2021). These members were found to be predominant in the pig gut microbiota. More details on functional pig gut microbiota were reported by Wylensek et al. (2020). The member of Proteobacteria and Cyanobacteria (e.g., *Thiothrix, Synechococcus, Sulfuricurvum*) plays an important role in the elimination of H_2S, which was increased when H_2S decreased (Table 2).

At the genus level, a total of 45 genera were revealed, with a relative abundance of ≥ 0.015%. The coexistence genera in the bioscrubber tank of the SFP pig farm is shown in Table 2. The bacterial communities of *Synechococcus, MWH-UniP1_aquatic_group, CL500-29_marine_group, Thiothrix,* and *Sulfuricurvum* were mainly detected in both SFP1 and SFP2. However, the differences in the relative abundance between the two collecting points might be related to cell multiplication and gene expression associated with sulfide treatment (Stewart et al. 2011, Dong et al. 2017). In particular, the genera *Thiothrix*, *Sulfuricurvum*, and *Acidithiobacillus* in SFP2 revealed the expression of 16S rRNA genes, sulfur oxidation gene (*soxAXBYZ*), and flavocytochrome c sulfide dehydrogenase (*fccAB*) genes during H_2S removal (Friedrich et al. 2005, Hong et al. 2013, Dong et al. 2017) which also correlated with the relative abundance in the sample.

Functional Gene Predicted by PICRUSt2 and Correlation with Microbial Community

Functional gene prediction was performed using PICRUSt2 based on 16S rRNA data of SFP1 and SFP2. In total, 7,429 genes of SFP1 and SFP2 were assigned to 434 functional KEGG pathways. All genes were mapped to different KEGG pathways. Only the functional genes/pathways involved in sulfide oxidizing bacteria were focused on in this study. The functional gene prediction revealed the metabolism of sulfide oxidizing bacteria, which is involved in the super pathway of sulfate-sulfur assimilation, cysteine biosynthesis, sulfate reduction I (assimilatory), and sulfur oxidation (*Acidianus ambivalens*) with gene abundance of 0.50%, 0.44%, and 0.07% in SFP1, respectively, and 0.47%, 0.40%, and 0.13% in SFP2, respectively. Several numbers of predicted genes were assigned to several KEGG categories that related to protein degradation (reduction of sulfate to H_2S). Based on the presented gene involved in the sulfate assimilation reduction pathway (SO_4^{-2}), which incorporates sulfur to amino acids and the sulfide (S^{2-}) assimilation to remove sulfur from biogas (Treu et al. 2018), it can be hypothesized that the microbial community of SFP1 and SFP2 has the potential ability in chemotrophic desulfurization.

The metabolic pathways involved in organic sulfur metabolism shown in Fig. 2 might play an important role in the bioscrubber system. The sulfide oxidizing bacteria have complex sulfur metabolism for the oxidizing and/or reducing sulfurs of various oxidation states. Flavocytochrome c sulfide dehydrogenase (*fccAB*) is one of the central enzymes in the respiratory chain of sulfur-oxidizing bacteria. This enzyme catalyzes the oxidation of sulfide and polysulfide ions to molecular sulfur, together with electron transfer to cytochrome c (Tikhonova et al. 2021). In this study, the predicted gene abundance of flavocytochrome c sulfide dehydrogenase (*fccB*) [EC:1.8.2.3] was increased from 0.01% in SFP1 to 0.02% in SFP2, which corresponded to an increase of sulfide oxidizing bacteria in Class Betaproteobacteria (0.261%) and Gammaproteobacteria (0.062%) of SFP2. Grim et al. (2021) also found that genes encoding for sulfide oxidation via flavocytochrome c sulfide dehydrogenase (fcc) were observed in both Betaproteobacteria and Gammaproteobacteria. Additionally, genes for chemolithotrophic thiosulfate oxidation via the Sox pathway (Lu-Kelly–Friedrich pathway) were observed in this study. Thiosulfate is directly oxidized to sulfate without forming any free intermediate. A complex multienzyme system involved in the complete oxidation of thiosulfate composed of *soxA*; L-cysteine S-thiosulfotransferase [EC:2.8.5.2], *soxX*; L-cysteine S-thiosulfotransferase [EC:2.8.5.2], *soxY*; sulfur-oxidizing protein, *soxZ*; sulfur-oxidizing protein and *soxB*; S-sulfosulfanyl-L-cysteine sulfohydrolase [EC:3.1.6.20] (Alam et al. 2021). In SFP2, gene abundances for *soxA, soxX, soxY, soxZ,* and *soxB* were 0.02%, 0.019%, 0.025%, 0.021%, and 0.019%, respectively, and 0.01%, 0.01%, 0.014%, 0.012% and 0.01%, respectively, for SFP1. Increasing Sox gene abundances in SFP2 support the assumption that the Sox gene was presented in chemotrophic species that form sulfur globules during thiosulfate oxidation (*Thiothrix, Beggiatoa*, and *Thiobacillus*) (Meyer et al. 2007). The relative abundances of Thiothrix (1.85%), Acidithiobacillus (0.49%), and Thiobacillus (0.009%) suggest that sulfur-oxidizing bacteria were dominant in SFP2 over SFP1. The presence of these SOBs in the SFP2 microbial community could be related to high activity in bioscrubber, which was also reported by Tilahun et al. (2018) and Alinezhad et al. (2019). The

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Fig. 2: Metabolic pathways of organic sulfur compounds play an important role in the bioscrubber. The assimilatory pathway [MD: M00176] (blue arrows), dissimilatory pathway [MD: M00596] (green arrows), and sulfur oxidation pathway [MD: M00595] (red arrows) were plotted with K number of microbial communities detected on starting sludge (SFP1) and recirculating sludge (SFP2).

reaction of H_2S oxidation occurring in SOB was shown in Eq. 4 and Eq. 5 (Tilahun et al. 2018). However, the route of H_2S oxidation depended on oxygen concentration, i.e., oxidation to elemental sulfur (S^o) or sulfate occurred under oxygen-limiting and non-limiting conditions, respectively.

$$HS^- + 0.5O_2 \leftrightarrow S^0 + OH^- \quad \ldots(4)$$

$$HS^- + 2O_2 \leftrightarrow SO_4^{-2} + H^+ \quad \ldots(5)$$

Abundance of SOB Detected on DNA Microarray

In this study, DNA microarrays containing a total of 61,278 probes for specific genes that drive the process of sulfide oxidation by the SOB community have successfully been constructed. The abundance of sulfur-oxidizing bacteria (SOB) was detected in both starting sludge (SFP1) and recirculating sludge (SFP2), as shown in Fig. 3. The main sulfide oxidizing bacteria in the SFP2 belonged to the phyla Proteobacteria (23.95%) and Cyanobacteria (23.25%) (Fig. 3A), while the dominant phyla in SFP1 was a member of Euryarchaeota (0.40%) (Fig. 3B). Proteobacteria was previously reported as the main phylum with H_2S eliminating the potential for biogas production. This phylum is composed of various genera such as *Acidithiobacillus*, *Thiothrix*, *Novosphingobium*, *Sulfurovum*, *Acinetobacter*, *Thiobacillus*, *Magnetospirillum*, and *Arcobacter* (Kodama et al. 2004, Hong et al. 2013, Haosagul et al. 2020). The three most abundant phyla detected by the NGS technique (Proteobacteria, Cyanobacteria, and Actinobacteria) had slightly different SOB communities compared to the DNA microarray technique. Probes specific for Proteobacteria and Cyanobacteria on the microarray were more prominent and diverse at the genus level than the NGS analysis technique. Moreover, the members of the genus belonging to phylum Actinobacteria and Euryarchaeota were detected in both techniques. Still, they did not play a key role in H_2S elimination due to only the 16S rRNA gene was expressed (Narihiro et al. 2008).

The top 40 strains of sulfur-oxidizing bacteria based on different protein expressions of sulfide oxidation between SFP1 and SFP2 are shown in Table 3. SOB communities in SFP2 were dominated by *Halothiobacillus hydrothermalis* strain DSM 7121, *Paracoccus denitrificans* JCM 21484, *Paracoccus alkenifer* strain DSM 11593, *Paracoccus pantotrophus* J40, *Paracoccus versutus* DSM 582, *Acidithiobacillus* sp. SH, *Halothiobacillus* sp. 39-53-45, *Magnetospirillum magnetotacticum* MS-1. The expression of the sulfur oxidation gene (*soxAXBYZ*) that oxidized thiosulfate to sulfate [EC:2.8.5.2; EC:3.1.6.20] had been increased in these genera.

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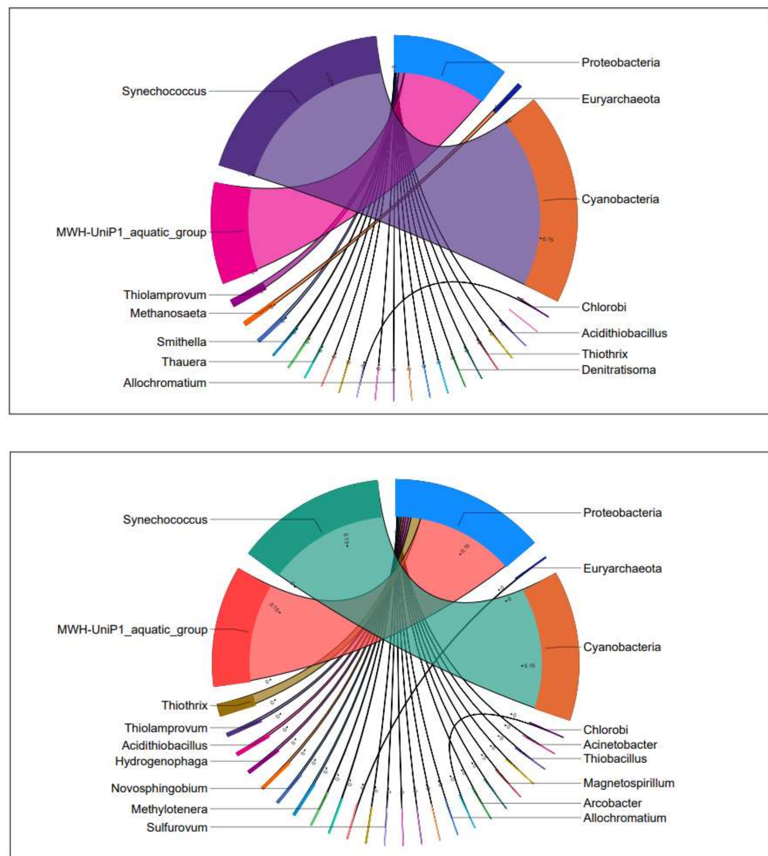

Fig. 3: Relative abundance of sulfur-oxidizing bacteria of starting sludge (SFP1) (A) and recirculating sludge (SFP2) (B) fed into bioscrubber, which was detected on a DNA microarray.

In addition, the dominant strains including *Sphingobium japonicum* UT26S, *Thiobacillus denitrificans* strain UBA4768, *Paracoccus* sp. UBA3880, *Thiobacillus denitrificans* strain UBA4768, *Sulfuricurvum* sp. UBA5598, *Sulfuricurvum* sp. MLSB, *Arcobacter cryaerophilus* strain LMG 10229, *Hydrogenophaga flava* NBRC 102514, *Paracoccus denitrificans* strain DSM 413, and *Thiothrix lacustris* strain A8; have sulfide oxidation genes (*fccAB*) [EC:1.8.2.3] for *in vitro* oxidation of H_2S with cytochrome c molecules as electron acceptors (Zhou et al. 2015). The final product of sulfide oxidation is elemental sulfur (Gregersen et al. 2011, Zhou et al. 2015). Moreover, non-sulfur oxidizing bacteria (NSOB) like *Sphaerochaeta pleomorpha* str. grapes and *Mesotoga prima* MesG1.Ag.4.2 were also increased in SFP2. Although *Sphaerochaeta pleomorpha* cannot use thiosulfate or sulfide, it reduces Fe^{3+} to Fe^{2+} in the glucose-supplemented medium (Ritalahti et al. 2012). On the other hand, *Mesotoga* was associated with high COD removal efficiency and high CH_4 yield. This genus was one of the significant members of waste sludge (Shin et al. 2019). For these reasons, both types of NSOBs were presented as the SOB strains under the same condition.

CONCLUSIONS

This research has successfully identified sulfur-oxidizing bacteria (SOB) communities that play an important role in the biological elimination of H_2S (~2,300 ppm) in biogas production of the pig farm based on NGS and microarray techniques. The members of phyla Proteobacteria and Cyanobacteria including *Acidithiobacillus, Thiothrix, Novosphingobium, Sulfuricurvum, Sulfurovum, Sulfurimonas, Acinetobacter, Thiobacillus, Magnetospirillum, Arcobacter, Paracoccus,* and *Synechococcus* were all shown to express the H_2S functional involvement of SOB in odor removal and H_2S oxidation via *soxABXYZ* and *fccAB*. These findings not only uncovered the crucial microorganisms for H_2S odor treatment but may also be used to track the reliability of biological treatment systems and the toxicity of sulfide minerals through oxidation. Moreover, the key microbes in this study can be further applied for biogas quality improvement by H_2S reduction in the future.

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Table 3: Microarray expression results showed the abundance of SOB species based on sulfide oxidation protein.

Genome ID	Genome Name	Avg. Log 2 Ratio (Cy3/Cy5)	Protein Expression				SOB/ NSOB
		SFP2.SFP1	SoxXA	SoxYZ	SoxB	FccAB	
AFRZ01000001.1	*Sulfurimonas gotlandica* GD1	-0.1973	D	D	D	D	SOB
AJ294325.1	*Halothiobacillus hydrothermalis* strain DSM 7121	0.6745	ND	ND	D	ND	SOB
AJ294332.1	*Halothiobacillus neapolitanus* strain DSM 581T	-0.2024	ND	ND	D	ND	SOB
AP010803.1	*Sphingobium japonicum* UT26S	0.1509	ND	ND	ND	D	SOB
BBFH01000969.1	*Paracoccus denitrificans* JCM 21484	0.5292	D	D	D	ND	SOB
CP003155.1	*Sphaerochaeta pleomorpha* str. Grapes	0.8403	ND	ND	ND	ND	NSOB
CP003532.1	*Mesotoga prima* MesG1.Ag.4.2	2.1989	ND	ND	ND	ND	NSOB
DCWD01000005.1	*Thiobacillus denitrificans* strain UBA2171	0.5991	ND	ND	ND	D	SOB
DGGG01000247.1	*Paracoccus* sp. UBA3880	0.5394	ND	ND	ND	D	SOB
DHHQ01000013.1	*Thiobacillus denitrificans* strain UBA4768	0.6037	ND	ND	ND	D	SOB
DILG01000074.1	*Sulfuricurvum* sp. UBA5598	0.1277	ND	ND	ND	D	SOB
FNXG01000003.1	*Paracoccus alkenifer* strain DSM 11593	0.8909	ND	D	ND	ND	SOB
JAEM01000011.1	*Paracoccus pantotrophus* J46	0.6060	ND	D	ND	ND	SOB
JAGK01000007.1	*Paracoccus pantotrophus* J40	0.6208	ND	D	ND	ND	SOB
JQGL01000140.1	*Sulfuricurvum* sp. MLSB	0.2709	ND	ND	ND	D	SOB
JRKO01000033.1	*Paracoccus versutus* DSM 582	0.5789	ND	D	ND	ND	SOB
LNTC01000035.1	*Arcobacter cryaerophilus* strain AZT-1	-0.5250	ND	ND	ND	D	SOB
LRUS01000073.1	*Arcobacter cryaerophilus* strain L399	-0.4044	ND	ND	ND	D	SOB
LRUT01000068.1	*Arcobacter cryaerophilus* strain L400	-0.5188	ND	ND	ND	D	SOB
LRUU01000074.1	*Arcobacter cryaerophilus* strain L401	-0.2732	ND	ND	ND	D	SOB
LRUV01000056.1	*Arcobacter cryaerophilus* strain L406	-0.5123	ND	ND	ND	D	SOB
MIBP01000017.1	*Sulfuricurvum* sp. RIFCSPHIGHO2	-0.8204	ND	ND	ND	D	SOB
MTEJ01000680.1	*Thiothrix lacustris* strain A8	0.0522	D	ND	ND	ND	SOB
MTEJ01000720.1	*Thiothrix lacustris* strain A8	0.5289	ND	ND	ND	D	SOB
MXAV01000010.1	Acidithiobacillus sp. SH	0.1522	D	D	D	ND	SOB
NCFS01000047.1	*Halothiobacillus* sp. 35-54-62	0.5356	ND	ND	D	ND	SOB
NCHH01000032.1	*Sulfuricurvum* sp. 24-42-5	-0.3833	ND	D	ND	ND	SOB
NCJI01000088.1	*Halothiobacillus* sp. 39-53-45	1.5084	ND	D	ND	ND	SOB
NXGD01000002.1	*Arcobacter cryaerophilus* strain LMG 10229	0.2472	ND	ND	ND	D	SOB
NXGJ01000010.1	*Arcobacter cryaerophilus* strain LMG 9861	-0.2068	ND	ND	ND	D	SOB
NXGK01000016.1	*Arcobacter cryaerophilus* strain LMG 24291	-0.4705	ND	ND	ND	D	SOB
NZ_AAAP01000572.1	*Magnetospirillum magnetotacticum* MS-1	0.6233	ND	D	ND	ND	SOB
NZ_BCTF01000035.1	*Hydrogenophaga flava* NBRC 102514	0.5581	ND	ND	ND	D	SOB
NZ_BCTF01000067.1	*Hydrogenophaga flava* NBRC 102514	-1.0481	ND	ND	ND	D	SOB
NZ_CGIH01000043.1	*Syntrophomonas zehnderi* OL-4	-0.2280	ND	ND	ND	D	SOB
NZ_FNEA01000052.1	*Paracoccus denitrificans* strain DSM 413	0.5429	ND	ND	ND	D	SOB
NZ_FNEA01000064.1	*Paracoccus denitrificans* strain DSM 413	0.6220	ND	ND	ND	D	SOB
NZ_FNEA01000070.1	*Paracoccus denitrificans* strain DSM 413	0.9407	ND	ND	ND	D	SOB
NZ_FNXG01000001.1	*Paracoccus alkenifer* strain DSM 11593	0.2015	D	D	D	ND	SOB
NZ_FOYK01000064.1	*Paracoccus denitrificans* strain DSM 415	0.5959	ND	ND	ND	D	SOB

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