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Hydrogen Sulfide Oxidizing Microbiome in Biogas-Stream Fed Biofilter in Palm Oil Factory

Siriorn Boonyawanich*, Peerada Prommeenate*, Sukunya Oaew***, Nipon Pisutpaisal*(**) and Saowaluck Haosagul**†**

*Department of Agro-Industrial, Food and Environment Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

**Biosensor and Bioelectronics Technology Centre, King Mongkut's University of Technology North Bangkok, Bangkok 10800, Thailand

***Biochemical Engineering and System Biology Research Group, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at King Mongkut's University of Technology Thonburi, Bang Khun Thian, Bangkok, 10150, Thailand

†Corresponding author: Saowaluck Haosagul; h_saowaluck@hotmail.com

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ABSTRACT

Hydrogen sulfide (H_2S) is highly corrosive to electric generators, which is the main problem of biogas utilization. The industrial scale of the biofilter system relies on the performance of sulfide-oxidizing bacteria (SOB) via the activity of sulfur oxidation (*soxABXYZ*) and flavocytochrome sulfide dehydrogenase (*fccAB*) enzymes to reduce to a concentration below 100 ppm before using in industrial machinery. The main purpose of this research is to investigate the SOB community in full-scale H_2S removal and their gene expression (*fccAB* and *soxABXYZ*) associated with H_2S elimination efficiency. In this study, SOB communities were obtained from 2 sampling sites of the full-scale biofilter of palm oil factory (PPG), comprising starting sludge (PPG1) and recirculating sludge (PPG2). The abundance of SOB strains was examined by next-generation sequencing analysis (NGS) based on the 16S rRNA gene. The changes in the expression of genes involved in sulfur oxidation, namely *soxABXYZ,* and *fccAB,* between the 2 sampling sites were evaluated by using a comparative genomic hybridization (CGH) microarray. The results indicate that the high abundance of SOB genera that could play a vital role in biofilters belonged mainly to *Sulfurovum*, *Paracoccus, Acidihalobacter, Acidithiobacillus*, *Thioalkalispira*, *Thiofaba*, *Caldisericum*, *Bacillus*, were rapidly increased in the biofilter tank. Interestingly, expressions of *soxAXYZ* gene cluster at PPG2 were increased in *Paracoccus pantotrophus* J40 and *Paracoccus alkenifer* DSM 11593 for 1.1188 and 1.0518-fold, respectively, while in *Acidihalobacter prosperus* F5, the expression of *fccAB* genes was up to 1.3704 fold in comparison with PPG1. Increasing both relative abundance and gene expressions at PPG2 were correlated with 95% H_2S removal efficiency. Hence, stabilization of the SOB microbiome is vital to H_2S removal in industrial-scale biogas applications.

INTRODUCTION

Palm oil is the 5th most important economic crop in Thailand. It occupies more than 70% of the acreage in Southern Thailand (Giz Agriculture and Food Cluster Thailand 2021). In 2020, palm oil production was approximately 3.78 million tonnes/yr., which accounts for 2.8% of the world market (United States Department of Agriculture 2021). Palm oil consumption is forecast to continue to increase by 5% in 2022. Apart from being used for consumption, palm oil has also been incorporated into the petroleum industry, diesel oil in particular. In order to comply with the circular economy plan, the palm oil industry in Thailand has to adopt a new policy on waste utilization along with energy efficiency. The wastewater from the palm oil industry is rich in biodegradable organic compounds that are suitable for biogas production. According to the Department of Alternative Energy Development and Efficiency (DEDE) has prepared the 10-year Alternative Energy Development Plan (AEDP 2012-2021), biogas can be used as a renewable energy source for electricity and heat power. Biogas is typically composed of 50-70% methane (CH_4), 30-40% carbon dioxide (CO_2) and 1-2% hydrogen sulfide (H_2S) (Elshiekh et al. 2016). H_2S is harmful to humans and can be toxic to methanogen,

especially during anaerobic digestion. It can cause severe corrosion to boilers and engines, which is the main problem of biogas utilization. To overcome these limitations, it is necessary to reduce the concentration of H_2S to less than 100 ppm before being used in industrial machinery (Tilahun et al. 2018). Several biological processes such as biofilter, biotrickling filter, and bioscrubber have been used to treat H_2S without causing secondary pollution. In this case, it is known that the H_2S elimination process is driven by sulfur oxidation enzyme (encoded by *soxABXYZ* genes) and flavocytochrome c sulfide dehydrogenase (encoded by *fccAB* genes) produced from sulfur-oxidizing bacteria (SOB) (Friedrich et al. 2005, Sander et al. 2006, Chan et al. 2009, An et al. 2016).

A number of studies have focused on the diversity of the SOB community in bioreactors and their ability to oxidize thiosulfate and H_2S in biogas. Omri et al. (2013) developed a biofilter to treat H_2S (inlet H_2S of 0.2-1.3 ppm) from wastewater odor. The dominant SOB in the biofilter were identified as heterotrophic bacteria that belong to the genus *Bacillus*, *Pseudomonas*, and *Xanthomonas*, with the highest removal efficiencies of 99%. Zhou et al. (2015) reported the predominant chemoautotrophic SOB in biotrickling filters, which include the genus *Thiothrix*, *Acidithiobacillus*, *Thiomonas*, *Sufurimonas*, *Halathiobacillus* and *Thiobacillus* which play an important role in treating H_2S (inlet conc. 2000-7,800 ppm) and enhance methane content in biogas. In the steady state of the anaerobic biotrickling filter system, H_2S concentrations between 2,500 to 10,000 ppm were removed with 100% efficiency by activities of phylum Proteobacteria and Actinobacteria (Quijano et al. 2018). However, many researchers have mainly investigated the SOB populations based on 16S ribosomal RNA genes only in laboratory or pilot scales. The profile of SOB in full-scale H_2S removal coupled with the change of gene expression associated with H_2S elimination was rarely observed.

Hence, the purpose of this research is to study the abundance and diversity of SOB strains in a full-scale biofilter, which is used to treat H2S in biogas from palm oil factories using 16SrRNA together with next-generation sequencing. The changes in the expression of flavocytochrome c sulfide dehydrogenase (*fccAB*) and Sox multi-enzyme (*soxAXBYZ*), the key enzymes for sulfide/thiosulfate oxidation, were also investigated.

MATERIALS AND METHODS

The Full-Scale Biofilter System and Samples Collection

The full-scale biofilter system consists of a packed column of pall ring media, which motivates biofilm growth through humidifying biogas that is pumped into the system. In the biofilter system, microbes are coated on porous media in the form of biofilm, and H_2S in biogas will be adsorbed on the porous media and interact with a biofilm of sulfur-oxidizing bacteria (SOB). Biodegradation processes occur in both the biofilm area and the porous media (Fig. 1). The portable H_2S detector can sense the H_2S inlet concentration in the range of 2,500-3,000 ppm, and the H_2S outlet concentration was observed at ~150 ppm. The H_2S removal efficiency was over 95%. Therefore, the SOB was collected from the

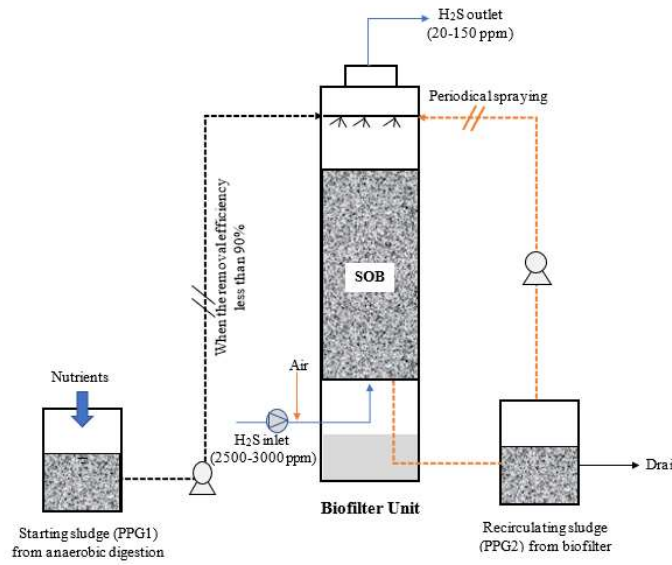

Fig. 1: Schematic diagram of the biofilter system in the PPG factory.

starting sludge (PPG1) and recirculating sludge (PPG2) of the full-scale biofilter tank (under a stable operation period of ~3 months) at Palm Power Green Company (Prachuap Khiri Khan, Thailand). Sludge samples were transported to the laboratory and transferred to a centrifuge bottle (250 mL). Bacteria cells were settled by centrifugation at 10,000g for 15 min. The pellet was kept in a 20°-C freezer before the genomic DNA (gDNA) extraction.

DNA Extraction and Purification

Samples from starting sludge (PPG1) and recirculating sludge (PPG2) were defrosted at room temperature. For gDNA extraction, cells were lysed with 600 µL of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 1.5 M NaCl) and 600 µL of lysozyme10) mg/l). The mixture was shaken at 180 rpm at 37°C for 1 h (Dezhbord et al. 2019). Next, 100 µL of sodium dodecyl sulfate (20%w/v) was added and incubated at 65°C for 30 min. DNA precipitation was carried out with the addition of 0.6 volume of isopropanol and then subsequently washed with 1 mL of cold ethanol (-20ºC) 2 times. The precipitated DNA was dried at room temperature and dissolved in 30 µL of elution buffer (10 mM Tris-HCl, pH 8.5 at 25°C). Finally, the DNA was purified using the GenepHlow™ Gel/PCR Kit (Geneaid) as described in Haosagul et al. (2020). DNA was extracted from three duplicate samples per sampling site and then merged for further study. DNA purity was evaluated using both gel electrophoresis and the A_{260}/A_{280} ratio. The gDNA is considered to be pure if A_{260}/A_{280}>1.8 and A_{260}/A_{230}>1.0. The purified DNA (50 ng/µL) was stored at -20°C for NGS analysis.

Next-Generation Sequencing

In this analysis, next-generation sequencing (NGS) is used to infer the phylogenetic relationships among the phyla and species in the same genus. The gDNA was used as a DNA template for PCR amplification. The specific primer, 515F806-R, was used to target the V4 region of the 16S rRNA gene. PCR reactions were carried out in the 19 µL of PCR Master Mix (1U/µL of *Taq* DNA polymerase, 0.02 mM of dNTPs, 3 mM of $MgCl_2$, 1X PCR buffers), 0.5 µM of forward and reverse primers, and 1 µL of 10 ng template DNA. The PCR condition was carried out as previously reported (Haosagul et al. 2020). The PCR products were examined in 0.8% (w/v) of agarose gel electrophoresis in 1x TAE (Tris 40 mM, Acetate 20 mM, EDTA 2 mM) buffer and stained with 0.5 µg/mL of ethidium bromide. The NEB Next® Ultra™ II DNA Library Prep Kit for Illumina was used to generate a sequencing library. The sequencing reactions were performed using Illumina HiSeq 2500. The low-quality sequences in raw data were removed using QIIME (Version 1.7.0). The remaining sequences at 97% identity were clustered into the same operational taxonomic units (OTUs). Species richness was identified using Chao1 and ACE estimators. Community diversity was identified using Shannon and Simpson indexes. The sequencing result was characterized by Good's coverage. These alpha diversity metrics were calculated by QIIME.

Expression of the Sulfur Oxidation Gene

The comparative genomic hybridization (CGH) microarray probes with 61,788 spots were designed for the detection and identification of SOB species in the palm oil factory. Database of genes related to sulfur oxidation (*soxAX, soxYZ*, *soxB*, and *fccAB),* including genome sequences of SOB species, were compiled from PATRIC and NCBI databases (Haosagul et al. 2021). The raw signal intensities from the microarray were calculated into log-2 ratios of PPG2/PPG1. The expression of sulfur oxidation genes between the recirculating sludge (PPG2) and starting sludge (PPG1) was investigated. Gene expression related to H_2S oxidation of SOB in the biogas was also studied.

RESULTS AND DISCUSSIONS

Quality of gDNA and NGS Analysis

The genomic DNA band of starting sludge (PPG1) and recirculating sludge (PPG2) were successfully extracted and separated on the agarose gel. gDNA concentrations of PPG1 and PPG2 were found in the range of 462.45-761.08 $ng.\mu L^{-1}$, and with the DNA purity (A_{260}/A_{280}) greater than 1.8, which indicated the absence of protein, phenol, and other contaminants in the samples (Brescia et al. 2012, Hashemipetroudi et al. 2018). The 250 bp PCR products that appeared in lanes 3 and 4 represent the 16S rRNA gene, as shown in Fig. 2. While in lane 1, there is no band on the agarose gel, indicating some contamination during the PCR reaction. Therefore, both samples were subjected to sequencing using the Illumina HiSeq 2500 platform. The sequence analysis was performed using Uparse software version 7.0.1001 (Edgar 2013). A total of 139,348 NGS reads, and 1,846 OTUS were obtained from 2 sampling sites of the palm oil factory. According to the alpha diversity analysis, the observed species, community diversity, and species richness at PPG1 are more diverse than at PPG2 (Table 1). A number of readable sequences encompassed almost 100% of the entire population of bacteria in the sample according to Goods coverage of 0.997. These results indicated that bacterial communities in PPG2 have less bacterial strain diversity, which is possibly due to the anaerobic strains in PPG1 (e.g., Methanogen, SRB) being unable to resist O_2 and H_2S concentrations in the biofilter system.

Diversity of Bacterial Community

Analysis of the microbial population's diversity showed that the majority of microorganisms found in a full-scale biofilter system from the palm oil factory belong to phylum Firmicutes, Proteobacteria, and Euryarchaeota (Fig. 3A), which account for 24-28%, 19-20%, and 11-22% of the total microbial community, respectively. The populations of phylum Firmicutes and Euryarchaeota were significantly decreased in the recirculating sludge (PPG2). In contrast, phylum Proteobacteria and Spirochaetes were found to increase by about 1.6-2.0-fold of the starting sludge (PPG1), which comprise 3 main classes: Epsilonproteobacteria (14.14%), Deltaproteobacteria (6.11%), and unidentified_ Spirochaetes (3.74%) (Fig. 3B). The high abundance of the Proteobacteria phylum was observed to degrade H_2S in biogas (Quijano et al. 2018, San-Valero et al. 2019, Haosagul et al. 2020) while phylum Firmicutes were commonly detected in activated sludge fed to biogas reactor (Schnürer et al. 2016, Schnürer & Jarvis. 2018). Gammaproteobacteria and Epsilonproteobacteria were detected on biofilm surfaces that were fed with H_2S in a bioreactor and contained various phenotypes (Konishi et al. 2013). Interestingly, Clostdiales was the dominant order of the phylum Firmicutes found at PPG1 (23.91%) and PPG2 (20.64%) (Fig. 3C). Westerholm et al. (2016) also reported that Clostridiales and Methanosarcinales dominated the anaerobic microbial community of biogas production. Relative abundance analysis indicated that Helicobacteraceae, a family of Epsilonproteobacteria, was the stable member in the biofilter system, while the Methanosaetaceae, a family of Methanomicrobia, was the unstable major member over time (Fig. 3D).

The outstanding 35 genera that work together in the biofilter system of palm oil factory are shown in an abundance heatmap (Fig. 4A). The following genera were dominated in biofilter at the PPG factory, *Sulfurovum, Sphaerochaeta, Syntrophomonas, Methanosaeta, Mesotoga,* Christensenellaceae which could play a vital role. They are members of ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), methanogenic bacteria (MB), sulfur-reducing bacteria (SRB), and sulfur-oxidizing bacteria (SOB). These results indicate that the variation in bacterial communities in the biofilter depends on nutrients and operating conditions that affect growth and proliferation. For example, *Methanosaeta* was the predominant Archaeal community that prefers low acetate/COD concentration in anaerobic digesters (Pavlostathis 2011, Tang et al. 2015).

The horizontal bar graph (Fig. 4B) shows the percentage of relative abundance between starting sludge (PPG1) and recirculating sludge (PPG2). SOB was found to be the most

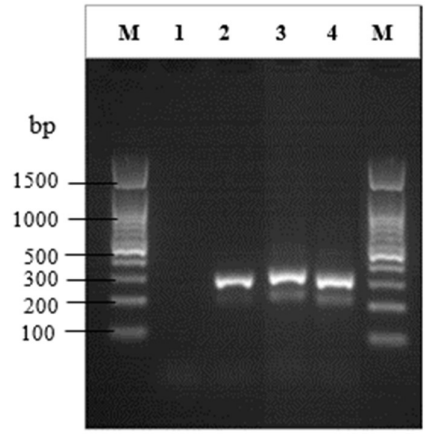

Fig. 2: The PCR products (approximated size 250 bp) were run on 0.8%TAE agarose gel. Lane M was 100bp DNA ladder, and lanes 1 and 2 were negative control (no DNA template) and positive control (*E. coli*), respectively. Lanes 3 and 4 were the amplified products from starting sludge (PPG1) and recirculating sludge PPG2), respectively.

Table 1: Community diversity and species richness.

Sample name	Observed Species	Community diversity		Species richness		Goods Coverage
		Shannon	Simpson	Chao1	ACE	
PPG1	1597	6.317	0.947	1788.482	1826.687	0.997
PPG2	1530	6.026	0.938	1747.562	1772.613	0.997

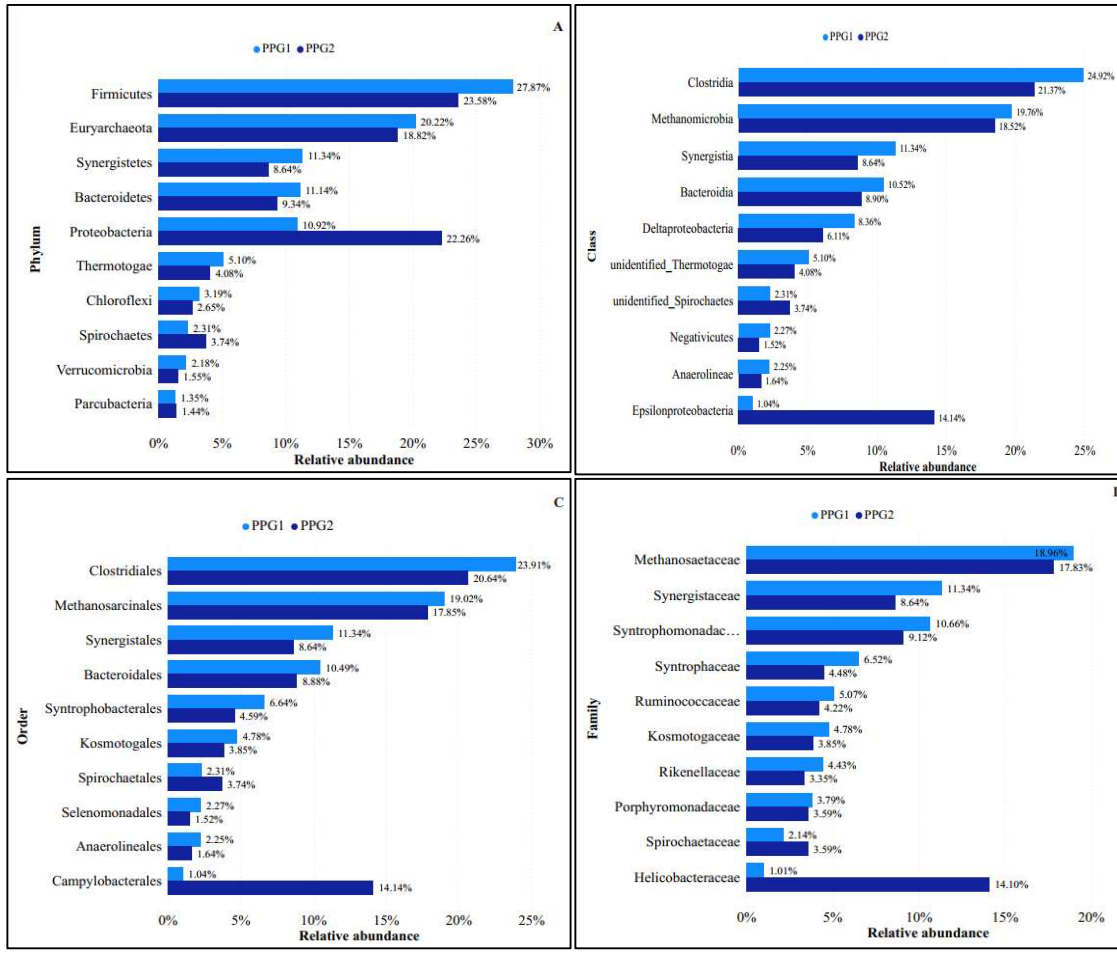

Fig. 3: (A) phylum, (B) class, (C) order, and (D) family of bacterial community in the biofilter system of palm oil factory.

abundant group covered in both sampling sites. They comprise mainly *Sulfurovum* (94.1%), *Acidithiobacillus* (91.0%), *Thioalkalispira* (95.7%), *Thiofaba* (90.9%), *Caldisericum* (100%), and *Bacillus* (100%), were rapidly increased at PPG2. On the contrary, the bacteria belonged to the *Synechococcus* (94.3%), *Acinetobacter* (100%), *Aquaspirillum* (100%), *Chlorobium* (100%), *Pararhodospirillum* (100%), and *Thiothrix* (100%) were commonly found at PPG1. The high abundance might indicate their significant function for the utilization of inorganic/organic compounds as an energy source and CO_2 as the carbon source of obligately/facultative chemoautotrophic bacteria found in the communities. Kovács et al. (2015) reported that *Bacillus* strains, which naturally display the ability to digest protein, were applied to enhance the hydrolysis during biogas production. Furthermore, the concentration of H_2S is a main factor that affects the variation of SOB communities, especially at the H_2S exposure site (Dong et al. 2017). For example, genus *Sulfuritalea, Thiobacillus, Sulfurovum, Sulfuricurvum, Sulfurimonas*, and *Sulfuricella* were typically found at a low H_2S site, while *Acidithiobacillus, Sulfobacillus, Sulfurospirillum, Thiomonas*, and *Halothiobacillus* were presented at a high H_2S site.

Correlation of Expressed Genes Profile in SOB Species-Strain with H_2S Elimination in Biogas Treatment System

The SOB communities are responsible for the sulfide/thiosulfate oxidation process and are of great importance for treatment system optimization in H_2S biological degradation. Many species of SOB contained the thiosulfate oxidizing multienzyme gene subunits (*soxB*, *soxAX*, and *soxYZ*) and the flavocytochrome c sulfide dehydrogenase genes (*fccA* and *fccB*) for sulfide oxidation and related to the diversity of species-strains of SOB. The involvement of these pathways in sulfide/thiosulfate oxidation by sulfur oxidation bacteria (SOB) was described in Haosagul et al. (2021a). Expression of sulfide/thiosulfate oxidation genes of SOB species-

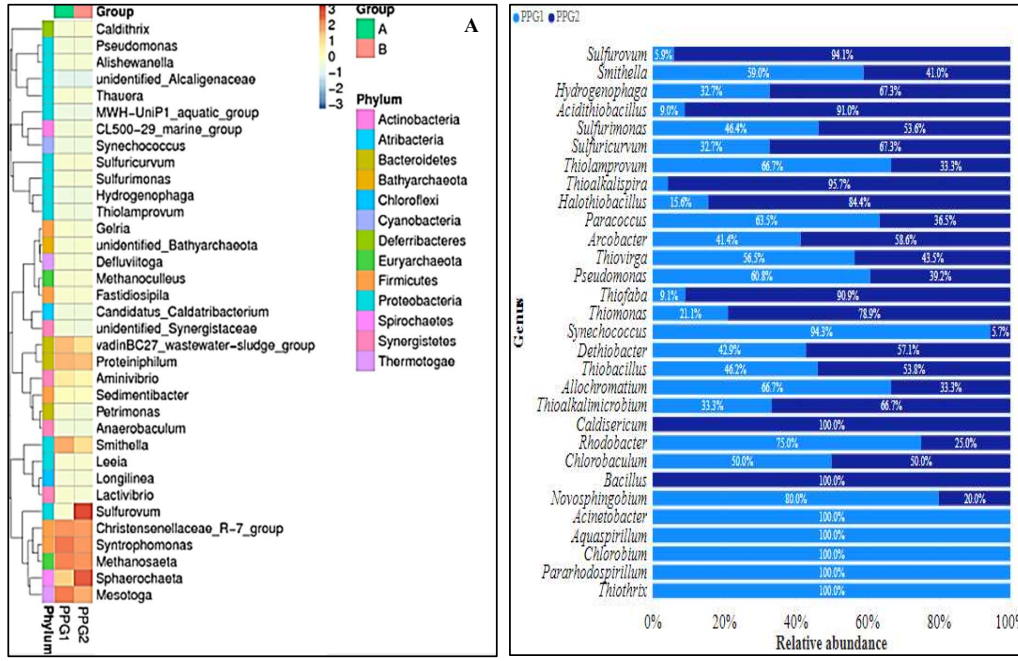

Fig. 4: (A) The abundance heatmap depicting the dominance of the top 35 genera in the biofilter, (B) A comparative horizontal bar graph between starting sludge (PPG1) and recirculating sludge (PPG2).

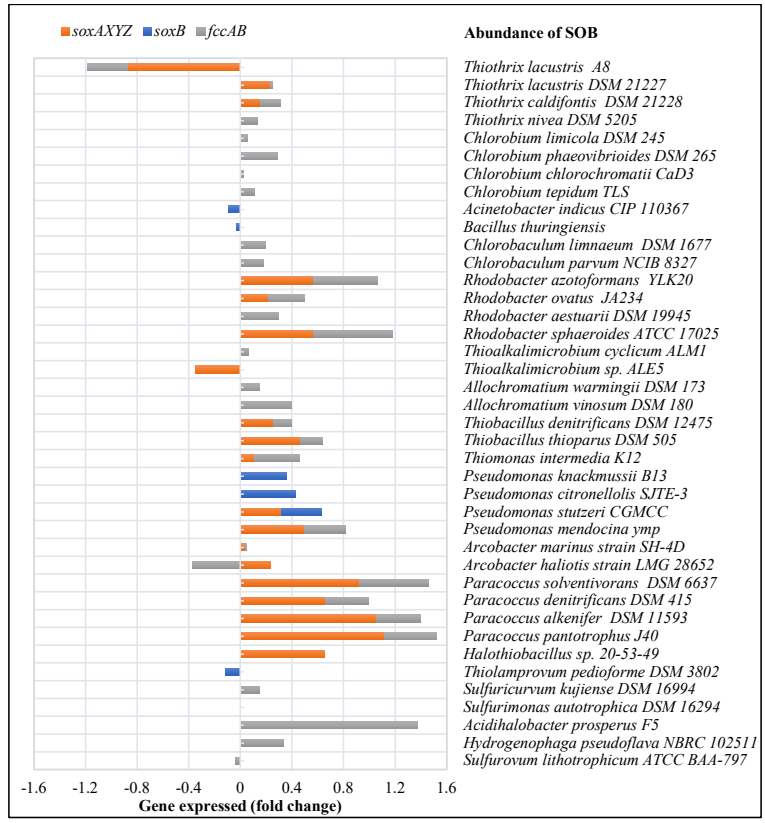

Fig. 5: Abundance of SOB species based on sulfide/thiosulfate oxidation gene expression.

strains was detected as shown in Fig. 5. We found that both *soxAXBYZ* and *fccAB* genes predominantly expressed in the palm oil factory belong to *Thiothrix* sp., *Chlorobium* sp., *Rhodobacters* sp., *Thioalkalimicrobium* sp., *Allochromatium* sp., *Thiobacillus* sp., *Thiomonas* sp., *Pseudomonas* sp., *Arcobacter* sp., *Paracoccus* sp., *Halothiobacillus* sp., *Thiolamprovum* sp., *Sulfuricurvum* sp., *Sulfurimonas* sp., *Acidihalobacter* sp., *Hydrogenophaga* sp. and *Sulfurovum* sp., which correlated well with the H_2S removal efficiency of over 95%. A variety of electron donors (e.g., sulfide, sulfur, thiosulfate, sulfite) and acceptors (e.g., oxygen nitrate nitrite) play a pivotal role in the presence of SOB species in biofilter tank, including fold change of gene copy number variation that observed between the 2 sampling sites (Haosagul et al. 2021b).

Especially, the expression of the *soxAXYZ* gene cluster of *Paracoccus pantotrophus* J40 and *Paracoccus alkenifer* DSM 11593 has been increased by 1.1188 and 1.0518-fold, respectively. In contrast, the expression of *fccAB* genes was up to 1.3704-fold in *Acidihalobacter prosperus* F5 at PPG2, in agreement with their relative abundance study. The expression of six genes (*soxX*, *soxY*, *soxZ*, *soxA*, *soxB*, and *soxCD*) of *P. pantotrophus* GB17 involved with H_2S biodegradation was confirmed by Ramadhani et al. (2017). It has been reported that the halotolerant acidophile *Ac. prosperous* F5 revealed the industrial potential applicability in bioleaching. Catalyzing the dissolution of sulfide minerals (Dopson et al. 2017, Khaleque et al. 2017) and obtaining energy source from sulfide oxidation to sulfur by sulfide cytochrome c reductase (EC:1.8.2.3). Rana et al. (2020) reported the potential for odors elimination due to the release of undesirable H_2S from wastewater using the following species; *C. limicola*, *A. ferooxidans*, *T. thiolates*, *T. denitrificans*, *P. putida,* and *F. thiosulatophilum*. These microorganisms, at low microbial counts, can oxidize H_2S with a high removal rate at ambient temperature and neutral pH. In-depth aspects, the ortholog groups containing genes related to sulfur/sulfide/thiosulfate oxidation in all strains could be found in the Kyoto Encyclopedia of Genes and Genomes, KEGG database (M00595-M00596, K17229-17230) (KEGG GENES 2022).

CONCLUSIONS

This study successfully demonstrated the correlation between SOB strains' abundance and the expression of sulfide and thiosulfate oxidation genes in a full-scale biofilter treating H_2S in biogas from palm oil factory sites. The high abundance of SOB genera that play a vital role in biofilters belonged mainly to *Sulfurovum*, *Paracoccus, Acidihalobacter, Acidithiobacillus*, *Thioalkalispira*, *Thiofaba*, *Caldisericum*, *Bacillus* that appear both *soxAXBYZ* and *fccAB* genes in the metabolic pathway. The results also confirmed that the stability of the SOB microbiome is vital to H_2S removal in industrial-scale biogas applications. The data collection of SOB species-strains and genes associated with sulfide oxidation in this research can be further applied to many issues, for example, biogas purification, evaluation of system efficiency, selection of microbial species in the start-up stage, reduction of clogging problem on the media, and helping save maintenance costs, etc. The limitations of the study also lacked a link between other genes that promote the activity of *sox* and the *fcc* gene to eliminate H_2S.

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ORCID DETAILS OF THE AUTHORS

Saowaluck Haosagul: https://orcid.org/0000-0002-9448-5840