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Profile of sulfur oxidizing bacteria in full-scale Biotrickling filter to remove H_2S in biogas from in cassava starch industry

Saowaluck Haosagul[a,b], Sukunya Oaew[c], Peerada Prommeenate[c], Vanatpornratt Sawasdee[d], Siriorn Boonyawanich[a,b,*], Nipon Pisutpaisal[a,b,**]

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

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

[c] *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*

[d] *Program in Innovation of Environmental Management, College of Innovative Management, Valaya Alongkorn Rajabhat University Under The Royal Patronage Pathumthani 13180, Thailand*

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Abstract

The success of Next generation sequencing (NGS) technology over the past decades has led to a better understanding of complex microbial communities in such environment. Here, we employed NGS to monitor sulfur oxidizing bacteria (SOB) community from cassava starch industry (BPT), which can oxidize H_2S into sulfate or elemental sulfur under aerobic conditions. Microbial sludge was collected from two sampling points comprising starting sludge (BPT1) and recirculating sludge (BPT2) of the biotrickling filter. gDNA was extracted from BPT1 and BPT2 samples and removed other contaminations by using the GenepHarM Gel/PCR Kit (Geneaid). DNA concentrations and DNA purity were analyzed by using gel electrophoresis and NanoDrop^{TM} 1000 Spectrophotometer, respectively. Analysis of SOB species using NGS technique was based on the hypervariable regions V4 on the 16S rRNA gene. The comparative genomic hybridization (CGH) microarray probes for detection and identification of SOB species-strain that high expression of *soxAXBYZ* and *fccAB* genes in the cassava starch industry. For the recirculating sludge (BPT2), the bacterial communities were dominated by *Sulfurimonas* (13.7%) followed by *Synechococcus* (9.4%), *Hydrogenophaga* (6.5%), *Methanosaeta* (2.4%), and *Acidithiobacillus* (2.1%), the same dominant genera were also found in the starting sludge (BPT1), but with slightly different abundancy. The heatmap revealed that *Acidithiobacillus caldus* species (33.9%–49.9%) could play a vital role in eliminating H_2S in biogas from the cassava starch industry. The CGH microarray indicated that *Thiothrix, Paracoccus, Sulfurimonas, Hydrogenophaga, Magnetospirillum, Rhodoplanes, Syntrophomonas, Pseudomonas, Sulfuricurvum,* and *Arcobacter* were dominant genus, that has a high expression of sulfur oxidation genes in biotrickling filter.

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Keywords: Biogas clean-up; Biotrickling filter; Hydrogen sulfide; Cassava starch industry; Sulfur oxidizing bacteria; 16S rRNA gene; NGS

* Corresponding author.
** Corresponding author at: Department of Agro-Industrial, Food and Environment Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand.
E-mail addresses: siriorn2002@yahoo.com (S. Boonyawanich), nipon.p@sci.kmutnb.ac.th (N. Pisutpaisal).

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Nomenclature

BPT1	Starting sludge
BPT2	Recirculating sludge
SOB	Sulfur oxidizing bacteria
NGS	Next generation sequencing
gDNA	genomic DNA
A_{260}/A_{280} ratio	Nucleic acid and proteins have an absorbance at 260 nm and 280 nm
fccAB	Flavocytochrome c sulfide dehydrogenase
soxAXBYZ	Sulfur oxidation genes

1. Introduction

in Thailand, wastewater from starch industry, palm oil mill industry, food industry, and ethanol industry could efficiently be used to produce biogas, particularly, in the starch industries where the highest potential biogas production was reported up to 41% [1]. In general, cassava starch industries have the maximum production capacity of 250–350 tons of starch/d. In this case, wastewater from washing starch process is produced up to 4,000–5,000 m^3/d, which in turn could produce biogas of about 20,000 m^3/d [1]. Typically, biogas is composed of methane (50%–75%) and carbon dioxide (25%–50%), however, it may sometimes contain small amounts of moisture, and H_2S (1%–2%) that serves as impurities in the biogas [2]. As alternative energy in industry, contamination of H_2S is the main problem of using biogas as it caused severe corrosion in boilers, pipelines, instruments, meters, and combustion engines. Hence, biotrickling filter, one of the most widely used biological treatment processes, is normally installed to eliminate H_2S in the biogas on-site industrial. The principle of the biotrickling filter for the treatment of H_2S depends on the performance of sulfur oxidizing bacteria (SOB) species which convert sulfur compounds into elemental sulfur or sulfate under aerobic conditions [3]. Previous studies reported the critical role of SOB communities in oxidizing H_2S in biogas by biological processes. *Pseudomonas, Bacillus*, *and Xanthomonadaceae were* found to dominate the SOB heterotrophic communities, with the ability to remove up to 99% H_2S in the municipal wastewater system [4]. *Thiothrix* spp., on the other hand, were detected under aerobic conditions, and are responsible for the oxidation of elemental sulfur to sulfate [5]. *Acidithiobacillus* sp. was the most SOB species in the bioreactor tank that eliminate H_2S under acidic conditions [6]. The sulfide oxidation process involves two genes: flavocytochrome c sulfide dehydrogenase (*fccAB*) and sulfur oxidation genes (*soxAXBYZ*), in which their electron transport mechanisms can be described in Eqs. (1) and (2) [7–9].

$$S^{2-} + \text{cytc}_{550(\text{ox})} \xrightarrow{\text{fccAB}} S^0 + \text{cytc}_{550(\text{red})} \tag{1}$$

$$S_2O_3^{2-} + 5H_2O \xrightarrow{\text{soxAXBYZ}} 2SO_4^{2-} + 8e^- + 10H^+ \tag{2}$$

The knowledge of gene function and coexistence in the SOB communities plays a significant role in driving the sulfide oxidation process in biogas and helps stabilizing and balancing the systems. Hence, the purpose of this research is to study the profiling of sulfur oxidizing bacteria in the cassava starch industry by 16S ribosomal RNA gene next generation sequencing analysis. Qualitative expression of flavocytochrome c sulfide dehydrogenase (FccAB), a key enzyme in sulfide oxidation, and Sox multi-enzyme (SoxAXBYZ), a key enzyme in thiosulfate oxidation was investigated

2. Materials and methods

The experimental procedures to identify sulfur oxidizing bacteria in full-scale biotrickling filter to remove H_2S in biogas from cassava starch industry are shown in Fig. 1. The detail description as of followed.

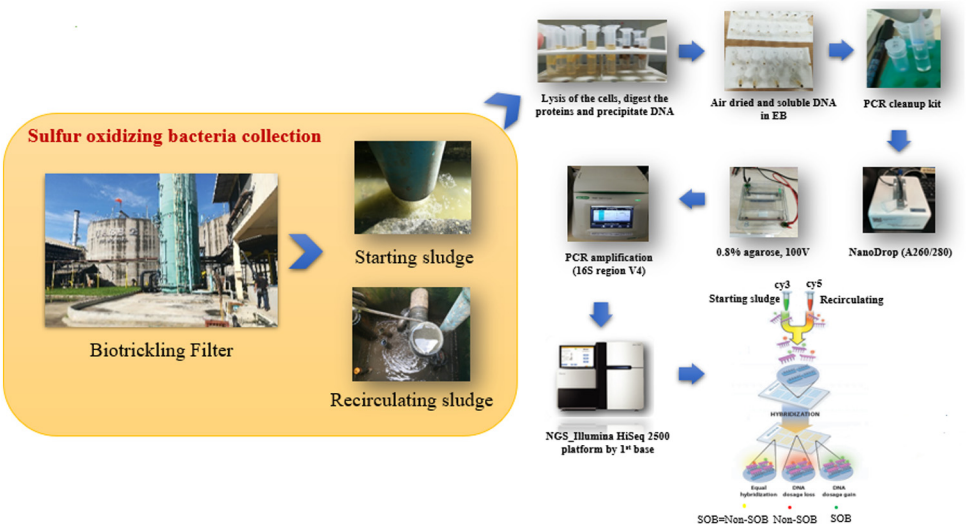

Fig. 1. Workflow for identifying the SOB communities to removing H_2S in biogas from in cassava starch industry.

2.1. Biotrickling filter system

The wet–dry biotrickling filter units (Fig. 2) were used to eliminate H_2S in biogas. The raw gas and microbial sludge from the cover lagoon were used to set in a biotrickling filter tank (size 4.5 m × 6 m). The microbial sludge was immobilized on packing media into wet phases for H_2S absorption. Bad air was decomposed by immobilized microbes on the packing media and suspended microbes in water. Some recirculating water was continually flowing down to the bottom of the system. Pure biogas was sent to the dry unit to remove moisture before used. The component of the biotrickling filter, and the H_2S removal efficiency from the starch industry can be described in Table 1.

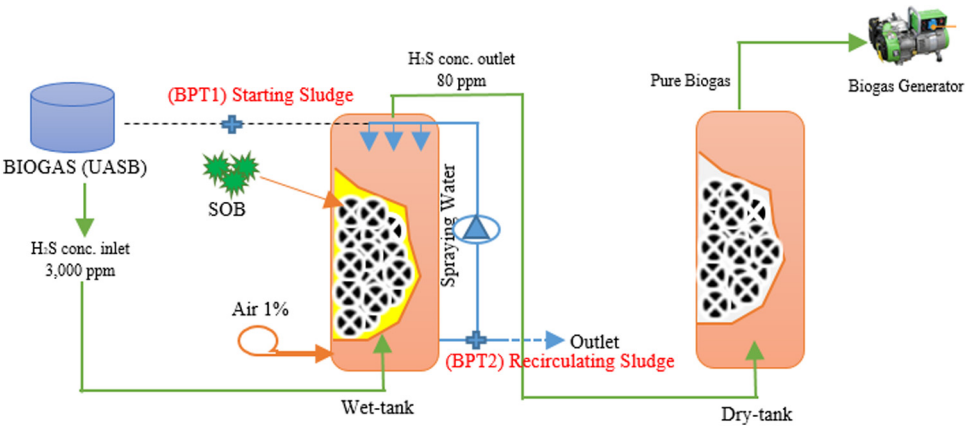

Fig. 2. Process diagram of the wet-dry biotrickling filter units.

2.2. Sulfur oxidizing bacteria collection

Microbial sludge from the cassava starch industry was collected from 2 sampling points: starting sludge and recirculating sludge of the biotrickling filter. After samples were delivered to the laboratory, 2 L of total sludge was transferred to a centrifuge bottle, and settling bacteria by centrifugation at 10,000g for 10 min. The pellet was

Table 1. Operating condition of biotrickling filter in cassava starch industry.

Operating conditions	Details
Biotrickling no.	2 (wet and dry)
H_2S conc. inlet (ppm)	3000
H_2S conc. outlet (ppm)	80
H_2S removal (%)	97.3
Spay water	Continued
Packing media	Pall Ring Media (PE)
Surface area (m^2/m^3)	105
pH outlet	6.66
CH_4 purity (%)	65

transferred into phosphate buffered saline (PBS) solution (pH 7.4), and kept in a −20 °C freezer prior to genomic DNA (gDNA) extraction.

2.3. Extraction of genomic DNA

The starting sludge and recirculating sludge from the freezer were defrosted at room temperature, to prepare for gDNA extraction. To begin with, 600 μl of the extraction buffer was mixed with each sludge, followed by 600 μl of lysozyme (10 mg L^{-1}). The mixture was shake at 180 rpm at 37 °C for 1 h [10]. Next, 100 μl of sodium dodecyl sulfate (20%w v^{-1}) was added and incubated at 65 °C (30 min). Later, 0.6 volume of isopropanol was added to precipitate DNA that was subsequently washed with cold ethanol 2 times. The precipitated DNA was dried at room temperature and dissolved with 30 μL of elution buffer as described in Haosagul et al. [11]. Finally, the color in gDNA samples was removed using the GenepHlow ™ Gel/PCR Kit (Geneaid).

2.4. Quality and quantity checking

DNA concentrations were quantified by gel electrophoresis. The ratio of A_{260}/A_{280} (Thermo Scientific NanoDrop™ 1000 Spectrophotometer) was used to assess the DNA purity. The ideal ratio for pure gDNA is $A_{260}/A_{280} > 1.8$, and $A_{260}/A_{230} > 1.0$, and gDNA concentration of 50 ng/μl (50 μl) are generally accepted as pure DNA for NGS analysis.

2.5. Next generation sequencing

Next generation sequencing (NGS) was used to infer the phylogenetic relationships among the phyla while also using the comparison among species in the same genus. The gDNA was used as a DNA template for PCR amplification using the specific primer named 515F-806R that targets the V4 region of the 16S rRNA gene. PCR reactions were carried out in a 20 μl reaction with 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. *Escherichia coli* (*E. coli*) and water were used as the positive and negative controls in this reaction, respectively. The thermal cycle of PCR procedure was previously report [11]. The PCR products were examined in 0.8% (w/v) of agarose gel electrophoresis. Statistical analyzes (Chao1, ACE, Shannon, Simpson, Goods coverage) were used to classify the genus and species obtained from the NGS analysis.

2.6. Visualization of the sulfur oxidation gene

The genic region of *soxAXBYZ* and *fccAB* genes distribution in SOB were collected from the Pathosystems Resource Integration Center (PATRIC) database, and Genome sequences of SOB species were compiled from the National Center for Biotechnology Information (NCBI) database. To design comparative genomic hybridization (CGH) microarray probes for detection and identification of SOB species in the cassava starch industry. The raw signal data from the microarray scanner (TIFF image) was calculated into log-2 ratios based on Cy3 over Cy5. The

copy number of 2 target genes (*soxAXBYZ* and *fccAB*) be evidence of the change in the expression of genes in the recirculating sludge (BPT2) versus the starting sludge (BPT1) related to the SOB metabolism that oxidized H_2S in the biogas.

3. Results and discussions

3.1. Genomic DNA concentrations

DNA concentrations of starting sludge (BPT1) and recirculating sludge (BPT2) were found in the range of 329–414 ng/μl, and the DNA purity (A_{260}/A_{280} nm) were greater than 1.8. If the ratio is lower 1.8, it indicates the presence of protein, phenol, or other contaminants that absorb light near 280 nm in the sample. The result of the PCR amplification was shown in Fig. 3. The band size of 250 bp appeared in all lanes. Therefore, both samples were subjected to sequencing using the Illumina HiSeq 2500 platform in the further step.

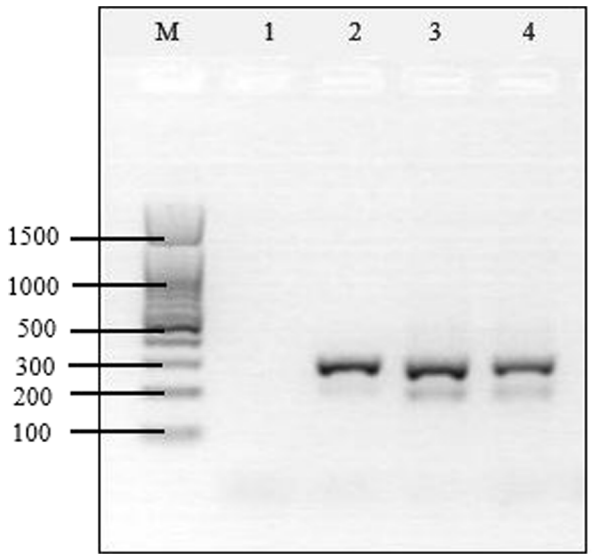

Fig. 3. PCR products of bacterial 16S region V4 (size 250 bp) were run on 0.8%TAE agarose gel. The amplified products in lane M is 100bp DNA ladder, lanes 1 and 2 are negative control (no DNA template) and positive control (*E. coli*), lanes 3 and 4 are starting sludge (BPT1) and recirculating sludge (BPT2).

3.2. Sulfur oxidizing bacteria community based on 16S rRNA gene

The analyzes of the bacterial communities from both sampling points of the biotrickling filter were conducted by the Illumina HiSeq 2500 platform to generate 250 bp paired-end raw reads of the 16S rRNA gene amplicons. The sequences were analyzed by using an UPARSE software version 7.0.1001 [12] for all effective tags. A total of 135,413 reads were obtained from both sampling points (data not shown). For diversity indices, species richness was estimated based on Chao1 and ACE whereas, community diversity was identified based on Shannon and Simpson (Table 2). We found that the starch industry has a higher diversity of bacterial community at the starting sludge (BPT1) than the recirculating sludge (BPT2), as demonstrated by the Shannon value (6.295), and Simpson values (0.961) had increased significantly. Based on the Chao1 and ACE values, the trend for species richness in each sample was likely to be similar to the community diversity. The Goods coverage of > 0.995 indicates that number of readable sequences encompassed almost 100% of the entire population of bacteria in the sample. The Good's coverage value changed during the treatment of H_2S in the biogas while the starting sludge at BPT1 observed varied species more than the recirculating sludge BPT2.

The NGS analysis, based on the V4 region of the 16S rRNA sequences, revealed that *Proteobacteria* (48%–52%) and *Cyanobacteria* (21%–24%) were the dominant phyla of SOB in the starch industry (Fig. 4). *Thermotogae* was rapidly increased in the recirculating sludge (BPT2), starting from 0% at BPT1 to 0.78% at BPT2. On the contrary,

Table 2. Diversity indices.

Sample name	Observed species	Community diversity		Species richness		Goods coverage
		Shannon	Simpson	Chao1	ACE	
BPT1	1729	6.295	0.961	1998.297	2070.096	0.996
BPT2	1498	6.007	0.951	1748.952	1744.651	0.997

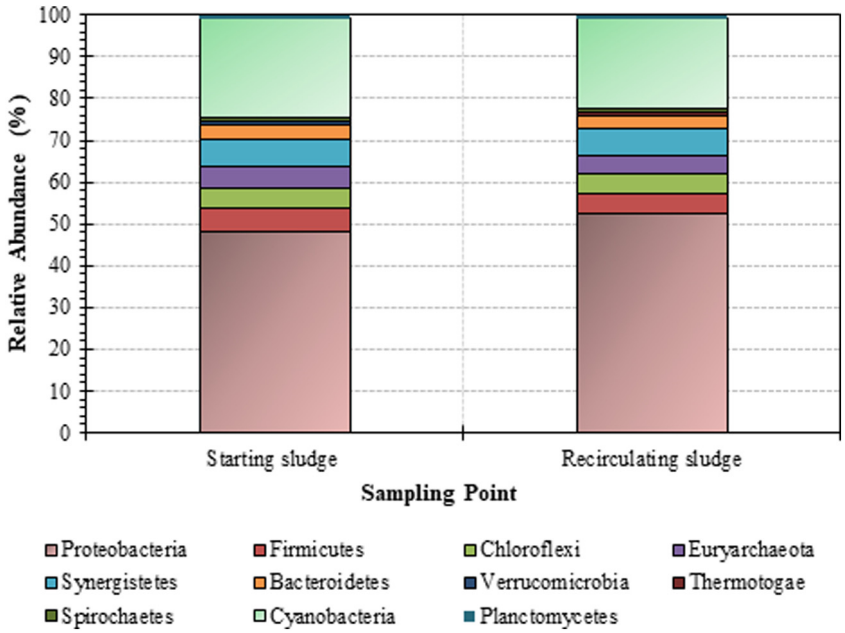

Fig. 4. Distribution of SOB phyla in biotrickling filter.

the bacteria belonging to the *Cyanobacteria* was rapidly dropped down from 24% at BPT1 to 21% at BPT2. These results indicate that bacterial communities in the biotrickling filter consisted of various bacterial phyla based on H_2S concentration and operating condition.

The relative abundance of the top 20 genera presented in the cassava starch industry was shown in Fig. 5. The recirculating sludge of BPT (BPT2) was dominated by *Sulfurimonas* (13.7%) *Synechococcus* (9.4%), *Hydrogenophaga* (6.5%), *Methanosaeta* (2.4%), and *Acidithiobacillus* (2.1%). The same dominant genera were also found in the starting sludge of BPT (BPT1), with slightly different in the abundance proportion. In this case, *Sulfurimonas*, *Synechococcus,* and *Hydrogenophaga* belong to the obligately chemoautotrophic SOB that help oxidize H_2S in the biogas. Several studies have found that various factors such as carbon sources, substrates, and operation conditions could affect the structure of the microbial community [13–15]. The concentration of H_2S is a very important factor that could alter the SOB communities in the biotrickling filter as reported by Dong et al. [13], which might be the reason of detectable *Sulfurimonas* at low H_2S.

The heatmap depicts the abundance of species-strains classification (Table 3) of the starting sludge (BPT1) and recirculating sludge (BPT2). Abundance of *Acidithiobacillus caldus*, *Beta proteobacterium MWH-UniP1*, *Bacteroidales bacterium C*, and *Desulfovibrio alcoholiv* were found increasing at the end of the removal of H_2S process (BPT2), which indicate their vital role in biotrickling filter. Specifically, *Acidithiobacillus caldus* was normally applied to enhance hydrolysis in the biogas production due to its high affinity to protein degradation [16]. The *Acidithiobacillus caldus,* a member of *Acidithiobacillus* genus, found in both sampling points of the cassava starch industry was consistent with the report by Dong et al. [13]. The bacterial strains preferred acidic conditions that adapted well in the pH range 3–7 and aerated conditions like in biotrickling tanks, where the final product of the sulfide deformation is sulfate [17]. The *soxAXBYZ* gene overexpression of *Acidithiobacillus caldus* (CP026328, region 2050999-2212793) confident that *Acidithiobacillus caldus* found in both sampling points of the cassava

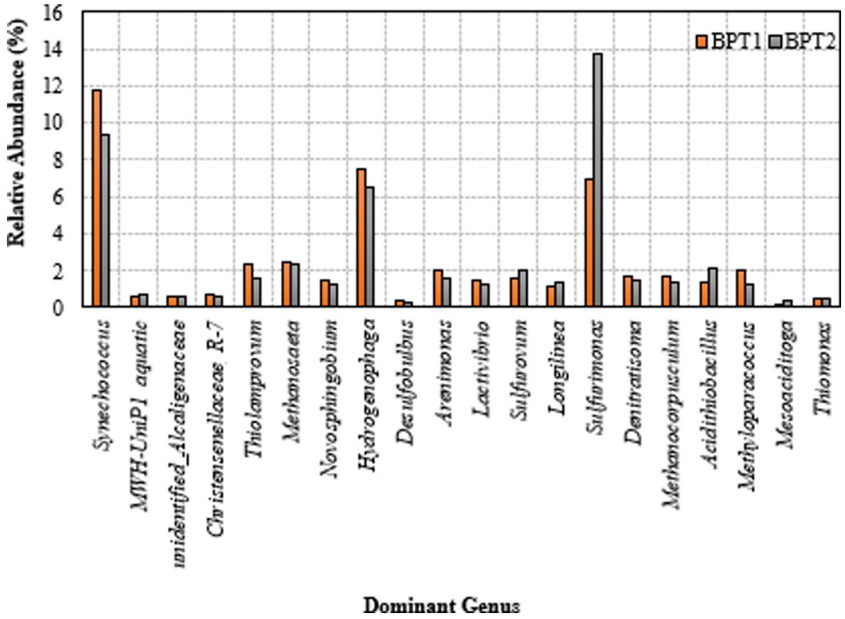

Fig. 5. Top 20 genera richness in the starch industry.

Table 3. The heatmap depicting the dominant of the top 20 species-strains.

BPT1 (%)	BPT2 (%)	Species classification
7.84	6.43	*Bacterium enrichment culture clone R4-41B*
3.68	2.71	*Methanobacterium formicicum*
4.97	3.13	*Spirochetales bacterium enrichment culture clone D2CL Bac 16S Clone1*
33.88	49.87	*Acidithiobacillus caldus*
2.76	0.00	*Veillonellaceae bacterium 6-15*
5.66	4.69	*Burkholderiales bacterium Beta 02*
15.66	14.33	*Kerstersia gyiorum*
5.21	6.03	*Beta proteobacterium MWH-UniP1*
17.41	7.07	*Synergistetes bacterium enrichment_culture_clone_DhR^2/LM-F01*
2.92	3.04	*Bacteroidales bacterium CF*
0.00	2.71	*Desulfovibrio alcoholiv*

starch industry were SOB species. In addition, the log2 fold change between BPT2 and BPT1 of *Acidithiobacillus* sp. SH (MXAV01000034, region 112318-116498), indicating the overexpression of *soxAXBYZ* gene was 0.2127. In conclusion, the abundance of the genus *Acidithiobacillus* play an important role in the sulfide–thiosulfate oxidation process of the cassava starch industry.

3.3. Abundant SOB species-strain based on soxAXBYZ and fccAB genes expression

Fig. 6 represents the top 20 abundance of SOB species based on the presence of sulfide-thiosulfate oxidation genes. The high level of *soxAXBYZ* and *fccAB* genes expression in the log 2 ratio of the recirculating sludge (BPT2) over the starting sludge (BPT1) was observed. *Thiothrix, Paracoccus, Sulfurimonas, Hydrogenophaga, Magnetospirillum, Rhodoplanes, Syntrophomonas, Pseudomonas, Sulfuricurvum,* and *Arcobacter* were detected as the dominant genus, these bacteria have to expressed genes and proteins for surviving in biotrickling filter. Specifically, species diversity within the genus *Sulfurimonas* and *Hydrogenophaga* were increasingly relative abundance and had the high expression of 16S rRNA gene, *soxAXBYZ,* and *fccAB* genes, corresponding to the removal efficiency of H_2S (97.3%). *Thiothrix* nivea considered to be a filamentous SOB, obtaining energy source from the sulfide oxidation to sulfur catalyzed by sulfide cytochrome c reductase (EC:1.8.2.3) [18] and carbon source

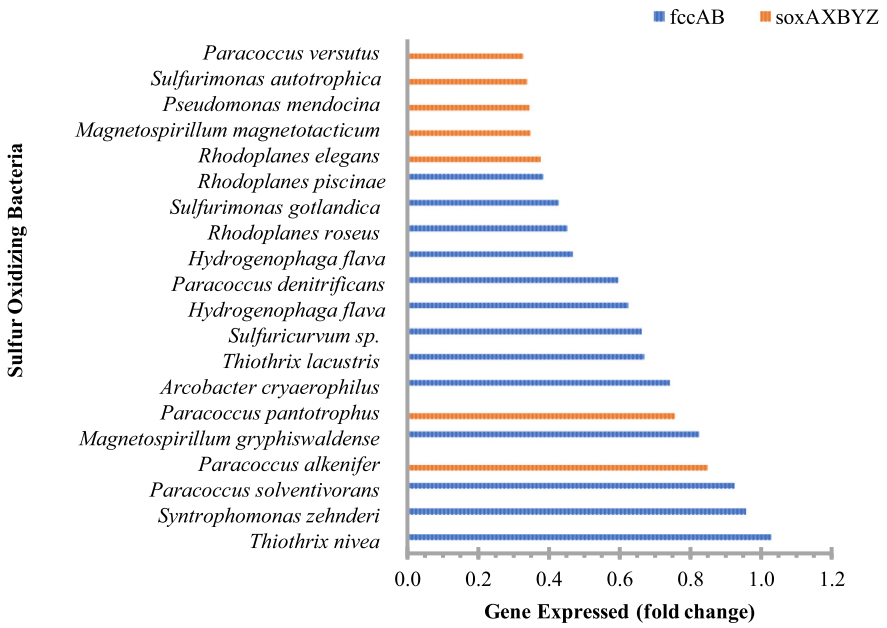

Fig. 6. Abundance of SOB species based on sulfide-thiosulfate oxidation genes expressed.

from CO_2 and organic compounds [19]. These species were the most commonly discover in bioreactors, containing sulfide-rich wastewater like biotrickling filter. The wide 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 bioreactor tank, including fold change of copy number variation that observed between two sampling points. *Sulfurimonas*, *Hydrogenophaga,* and *Thiothrix* species can grow with a variety of electron donors and acceptors, which possibly leading to their proliferation [20].

4. Conclusions

We have successfully used the NGS technology to identify the sulfur oxidizing bacteria (SOB) in the cassava starch industry. The knowledge profile of SOB members can be adopted to both anaerobic conditions in the biogas production and the aerobic conditions in the biotrickling tank. This information would help to better understand the synergy of the SOB members in both systems. We found that *Sulfurimonas*, *Hydrogenophaga*, and *Acidithiobacillus* are dominant genera that play a crucial role in both the production of biogas and the treatment of hydrogen sulfide odor in the cassava starch industry. SOB population in biogas digester can be used as a regulator of biogas purity and yield. If the fermentation tank is designed to contain methanogenic bacteria working with the anaerobic SOB group, it will result in decreased H_2S and increased methane yield. Helps to reduce the cost of building and maintaining a H_2S treatment tank and also reduce generator repair cost.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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