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Trace metals supplementation enhanced microbiota and biohythane production by two-stage thermophilic fermentation

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ABSTRACT

The effect of trace metals supplementation into palm oil mill effluent on biohythane production and responsible microbial communities in thermophilic two-stage anaerobic fermentation was investigated. High biohythane yields were linked to Ni/Co/Fe supplementation (10, 6 and 20 mg L^{-1}, respectively) with maximum H_2 and CH_4 yields of 139 mL H_2 gVS^{-1} and 454 mL CH_4 gVS^{-1}, respectively. The Ni/Co/Fe supplementation resulted in higher numbers of *Bacillus* sp., *Clostridium* sp. and *Thermoanaerobacterium* sp. together with increasing hydrogenase expression level leading to increasing hydrogen yields of 90.4%. The numbers of *Methanosarcina*, *Methanomassiliicoccus*, and *Methanoculleus* were enhanced by Ni/Co/Fe addition, accompanied by 21.7% higher methane yields. No correlation between methyl coenzyme-M reductase expression level and methane yields was observed. The Ni/Co/Fe supplementation improved gas production in the two-stage biohythane process via enhancing a number of viable hydrogen-producing bacteria together with hydrogenase activity in H_2 stage and enhancing number methanogens in the CH_4 stage.

Introduction

Two-stage biohythane production is attracting attention as an environmentally friendly process for both waste treatment and energy production [1]. This process has several advantages compared to hydrogen production or biogas production. This includes an increase in the net energy balance and allowable organic loading rates along with an increase in the methanogenic activity leading to high production rates and

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chemical oxygen demand (COD) reduction efficiencies. The two-stage biohythane process is depended on the different function between hydrogen producers and methane producers in physiology, nutrition needs, growth rates, and sensitivity to pH [2]. The two-stage biohythane process is also characterized as by the short fermentation time and allowing for an optimized growth by control separately in two tanks [3]. This setup makes the process suitable for treating high organic matter substrates like palm oil mill effluent (POME) [4,5]. Mamimin et al. [6] reported a biohythane production performing by a two-stage anaerobic fermentation of POME with hydrogen production of 5 L H_2 L_{POME}^{-1} in the first stage and methane production of 17.6 L CH_4 L_{POME}^{-1} in the second stage using $NaHCO_3$ as a pH adjustment. In line with these prior findings, when oil palm fiber ash was used as a pH adjustment, a hydrogen production of 3.8 L H_2 L_{POME}^{-1} and a methane production of 14 L CH_4 L_{POME}^{-1} were achieved from a two-stage process [7]. Alkali material like ash obtained from waste-fired boilers can be a good alternative for alleviating the acidity in anaerobic digestion [8]. Banks and Lo [9] added bottom ash from municipal solid waste incinerators into biogas systems and found that ash addition had a positive effect on VS degradation efficiency and gas production. In addition, ash may contain metals, which can act as co-enzymes during anaerobic digestion and as such, apart from pH adjustment, could also help to enhance a microbial growth rate during the process [10]. More specifically, palm oil ash generally contains high concentrations of iron (Fe) and low concentrations of molybdenum (Mo), zinc (Zn), cobalt (Co) and nickel (Ni) [8,11,12]. Zhang and Jahng [13] found that the supplementation Co, Mo, Ni and Fe at a concentration of 2.0 mg L^{-1}, 5.0 mg L^{-1}, 10.0 mg L^{-1}, and 100 mg L^{-1}, respectively, improved the process stability of the biogas production from food waste by anaerobic digestion. The combined addition of Co, Mo, Ni and Fe at 1–200 mg L^{-1}, 5–500 mg L^{-1}, 5–1000 mg L^{-1}, and 100–10,000 mg L^{-1}, respectively, resulted in a high biogas yield reaching a value of 396 mL gVS^{-1} [14,15]. In contrast, a high concentration of trace metals might inhibit the methanogenic communities resulting in a low methane production [12]. Indeed, previous researchers reported a toxic effect of trace elements on methanogenic community and thus, on methane yields after the supplementation of Fe, Ni, Co at 1000 mg L^{-1}, 50 mg L^{-1} and 250 mg L^{-1} [15,16]. However, the application of oil palm ash as a mixture of trace metals increasing hydrogen and methane production from POME which is high lipid wastewater was reported by Mamimin et al. [6]. Therefore, it is necessary to determine whether or not trace elements can be added to improve hydrogen and methane production by two-stage anaerobic fermentation of POME.

Hydrogenases are key enzymes involved in hydrogen fermentation [17]. These enzymes can be classified depended on metal atoms present in the active site, and they include nickel–iron hydrogenase, iron–iron hydrogenase and iron hydrogenase [18]. Iron–iron [Fe–Fe] hydrogenase have around 10–100 times higher hydrogen production activities than Ni–Fe hydrogenases [15,19], and many hydrogen-producing microorganisms belong to Fe–Fe hydrogenase class. The *hydA* gene has been used as a biomarker to study the Fe–Fe hydrogenase distribution in hydrogen fermentation [20–22]. Moreover, Tolvanen et al. [23] studied *Clostridium butyricum* hydrogenase in continuous and open bioprocesses, where a change in the expression of *hydA* gene took place indicating that it can be considered as a target for monitoring the process performance. In the second stage, methyl coenzyme-M reductase (*mcrA*) is the important enzyme of methanogens is responsible for methane production [24,25]. The *mcrA* gene is present in all methanogens [26,27]. Trace elements (Co, Fe, Ni, Zn, Mo, and W) are known to be important for the activity of both enzymes [28]. Cai et al. [29] report that adding trace elements in biogas reactor changed the composition and diversity of bacterial resulting to high methane yield. Addition of a mix consisting of Co, Mo, Ni, Se, and W resulted in a more stable digestion performance. Daily trace element mix supplementation promoted the hydrogenotrophic *Methanoculleus bourgensis*, which is an ammonia tolerant methanogen [30]. However, to date, there is still scarce information about the linked effects of trace elements on process parameters with regard to the aforementioned enzymes and microbial community composition. As such, further investigation is needed to understand the links between the effect of trace metals supplementation on microbial composition, hydrogenase, methyl coenzyme-M reductase, biohythane production via a two-stage process.

Here, we wanted to test whether the supplementation of trace metals Co, Ni, Mo, and Fe or ash can increase hydrogen yields and methane yields produced in a two-stage biohythane process. The microbial communities producing the desired gas products were analyzed by combining PCR with denaturing gradient gel electrophoresis (DGGE) technique, community profiling by Illumina amplicon sequencing and quantification of specific genes relevant for the processes (*hydA*, *mcrA*) by real-time PCR. The microbial profile identified was linked back to trace metal or ash addition in order to identify on which process level (dis)advantageous process modulations occur. These data can increase our current understanding of process performance and support the optimization of process monitoring.

Materials and methods

Experimental design and optimization

The optimum concentration of trace metal for hydrogen and methane production was investigated using the design expert according to Mamimin et al. [31]. The design matrix of the variables and response was shown in Table 1. The two-stage fermentation for biohythane production of POME was assays described previously by Giordano et al. [32]. Different trace metals including ammonium molybdate ($(NH_4)6MO_7O_{24}$ $4H_2O$), iron (II) sulfate ($FeSO_4$ $7H_2O$), nickel sulfate ($NiSO_4$ $6H_2O$) and cobalt (II) chloride ($COCl_2$ $6H_2O$) were supplemented into POME at the concentrations indicating in Table 1. In the hydrogen reactors, 20% (v/v) of hydrogen producing inoculum was mixed with 80% (v/v) of POME according to the substrate to inoculum ratio (S:I) of 20:1 based on VS basis. The experiments were performed in 500 mL serum bottles and incubated at a temperature of 55 °C for 4 days. After 4 days, the reactors were opened under a nitrogen environment and 60% of methane inoculum was introduced according to S:I ratio of 2:1

Table 1 – A central composite experimental design for study effect of trace metal on two-stage hydrogen and methane production.

Run	Parameter (mg L^{-1})				Response	
	Molybdenum (A)	Nickel (B)	Cobalt (C)	Iron (D)	Hydrogen yield (mL H_2 gVS^{-1})	Methane yield (mL CH_4 gVS^{-1})
1	5	5	3	10	83	290
2	5	0	3	10	88	293
3	10	10	0	0	87	315
4	5	5	3	0	83	373
5	5	10	3	10	81	374
6	5	5	3	20	85	303
7	10	0	6	20	86	447
8	0	10	0	20	85	346
9	5	5	3	10	87	343
10	0	0	0	0	61	378
11	5	5	3	10	86	312
12	0	0	6	0	84	336
13	5	5	0	10	84	344
14	10	5	3	10	85	339
15	10	10	6	0	88	346
16	0	5	3	10	87	357
17	0	10	6	20	90	275
18	5	5	6	10	85	315
19	10	0	0	20	89	341
20	5	5	3	10	86	347
21	5	5	3	10	90	348
POME	0	0	0	0	59	278

based on VS basis. The reactors were close with butyl rubber stopper and incubated at thermophilic conditions (55 °C) for 45 days. The chemical characteristics of POME, hydrogen inoculum, and methane inoculum were shown in Table 2. The optimum concentration of the trace metals was obtained by regression analysis. The quality of the model was shown by R^2 and statically significance analyzing by the F-test.

Confirmation effect of trace metal on hydrogen and methane production

Four optimum concentrations of trace metals for enhancing the hydrogen and methane production were chosen. Additionally, in two further treatments, 5% of para rubber wood ash (AR) and 5% of oil palm biomass ash (AP) were added to POME for studying the effect of mixed trace metals on hydrogen production and methane production (Table 3). All of the reactors were done in duplicate. The 200 mL working volume sequencing batch reactor and 1 L working volume upflow anaerobic sludge blanket was set up according to Mamimin et al. [6]. Schematic diagram of two-stage hydrogen and methane production reactors was shown in Fig. 1. Sludge samples from the exponents of each stage were collected for microbial community analysis. The biogas volume was determined by water replacement method [33]. Gas composition (H_2, CH_4, and CO_2) was analyzed by GC-TCD (8A Shimadzu) equipped with 2.0 m packed column (Shin-Carbon ST 100/120 Restek) as previously described [34]. Volatile fatty acids (VFA) content was measured by GC-FID (HP6850, Hewlett Packard) equipped with a Stabilwax-DA column [34]. The sludge from the hydrogen production process and methane production process were collected and keep at −20 °C for molecular and chemical analyses.

DNA extraction and PCR-DGGE analysis

Sludge samples from each treatment were taken from the reactors. Total genomic DNA was extracted from sludge samples by using the NucleoSpin® Soil kit (MACHEREY-NAGEL, Düren, Germany). Approximately 5 ng of each DNA template was added to a PCR cocktail at a final concentration of 1X MyTaqTM reaction buffer supplied by the manufacturer (BIOLINE, Luckenwalde, Germany) with 20 mg mL^{-1} BSA, 10 μM of each primers (Table 4), 0.9 U MyTaqTM DNA Polymerase (BIOLINE, Luckenwalde, Germany) and sterile water. For PCR-DGGE analysis, the 16S rRNA gene of bacteria was

Table 2 – Chemical characteristic of POME, H_2 inoculum and CH_4 inoculum.

Parameters	Concentrations		
	POME	H_2 Inoculum	CH_4 Inoculum
Temperature (°C)	88	55	55
Chemical oxygen demand (g L^{-1})	90.5	–	–
Total solid (g L^{-1})	66.4	1.4	23.5
Total volatile solid (g L^{-1})	55.2	2.1	12.4
Total carbohydrate (g L^{-1})	10.1	0.3	0.1
pH	4.5	4.8	7.5
Alkalinity (g $CaCO_3$ L^{-1})	0.5	0.8	5.6
Acetic acid (g L^{-1})	1.5	1.5	0.05
Butyric acid (g L^{-1})	0.4	0.8	0.02
Propionic acid (g L^{-1})	0.4	0.5	0.01
Ethanol (g L^{-1})	0.1	0.2	0.01
Total volatile fatty acid (g L^{-1})	2.6	2.8	0.1
Oil and grease (g L^{-1})	4.3	0	0.1

Table 3 – The performance of two-stage thermophilic fermentation for hydrogen and methane production.

Reactors	Trace element (mg L^{-1})				Response							
	Mo	Ni	Co	Fe	H_2 yield (mL H_2 g^{-1} VS)	H_2 increasing (%)	CH_4 yield (mL CH_4 g^{-1} VS)	CH_4 increasing (%)	H_2 production L H_2 L^{-1}	CH_4 production L CH_4 L^{-1}	COD removal (%)	VS removal (%)
R1	10	0	6	20	113±3[a]	54.7	445±5[a]	19.3	6.2	24.5	95.0	90.5
R2	0	10	6	20	139±3[b]	90.4	454±7[a]	21.7	7.8	25.1	97.3	92.3
R3	10	0	0	20	111±4[a]	52.1	403±3[b]	8.1	6.1	22.2	93.2	89.7
R4	5	5	3	10	129±2[b]	76.7	411±5[b]	10.2	7.1	22.7	95.1	91.5
AR	5% w/v Para rubber wood ash				39±2[c]	−46.5	365±5[c]	−2.1	2.2	20.2	87.6	82.4
AP	5% w/v oil palm fiber ash				90±1[ae]	23.2	402±3[b]	7.7	4.9	22.2	91.5	87.0
POME	0.32	0.02	0.12	40	73±3[e]	0	373±3[c]	0	4.1	20.6	89.6	84.4

Mean of three replicates ± standard deviation.
Mean sharing the same letter within a column were not significantly different ($p < 0.05$, $n = 3$)

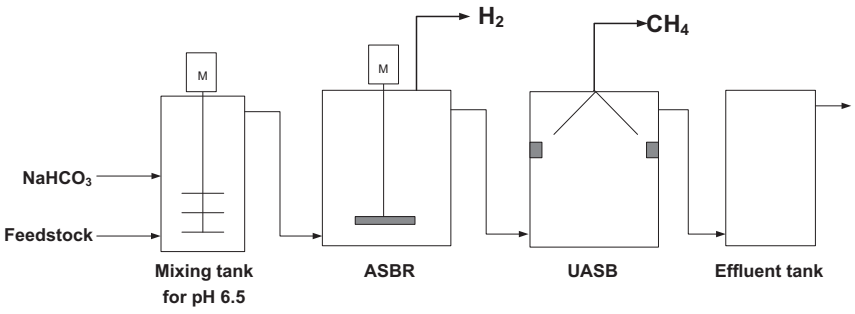

Fig. 1 – Schematic diagram of two-stage biohythane production experiments.

Table 4 – Oligonucleotide primers used for polymerase chain reaction (PCR) in this study.

Group	Primer name	Sequence (5′→3′)	Method	Reference
Bacteria	984F-GC	GC clamp-AACGCGAAGAACCTTAC	DGGE	[63]
	1378R	CGGTGTGTACAAGGCCCGGGAACG		
Archaea	357F-GC	GC clamp-CCCTACGGGGCGCAGCAG	DGGE	[64]
	691R	GGATTACARGATTTCAC		
	GC clamp	CGCCCGCCGCGCGCGGCGGGCG	DGGE	[65]
		GGGCGGGGGCACGGGGGG		
Hydrogenase gene	*hydA*-F	GCTKGGCGAATCMTCYGGTG	Real-time PCR	[36]
	hydA-R	GGCTGWCCRCCGCCCATTAT		
Methyl coenzyme-M reductase gene	*mcrA*-F	GGTGGTGTMGGDTTCACMCARTA	Real-time PCR	[37]
	mcrA-R	CGTTCATBGCGTAGTTVGGRTAGT		

amplified using the primer 984F with GC-clamp and 1378R. The archaeal 16S rRNA gene was amplified using the primer set 357F with GC-clamp and 691R. The PCR products were checked for quality by electrophoresis on 1% agarose gels. DGGE analysis of the PCR products was carried out by the INGENYphorU® electrophoresis system for 16 h at 100 V and a temperature of 60 °C. PCR products of 60 ng were loaded in an 8% (v/v) polyacrylamide gel with a denaturing gradient of 40%–70% for bacteria and 45%–60% for archaea. DGGE gels were silver stained and scanned for subsequent image analysis by using GelCompar® II software (Version 4.0, Applied Maths, Belgium). The DGGE bands were cut from the polyacrylamide gel and sequenced. The sequences were identified by database searches in Gene Bank using BLAST [35].

Real-time quantitative PCR

Real-time PCR was performed on the Rotor-Gene 6000 Thermal Cycler (Corbett Research, Sydney, Australia) with the Rotor-Gene Series Software 1.7. For the detection of hydrogenase (*hydA*) gene from *Thermoanaerobacterium thermosaccharolyticum*, the primers *hydA*-F and *hydA*-R were used and designed by Hniman et al. [36]. For the detection of the methyl coenzyme-M reductase (*mcrA*) gene from methanogens, the primers *mcrA*-F and *mcrA*-R were used [37]. PCR products from *T. thermosaccharolyticum* PSU2 (bacteria) and *Methanosarcina barkeri* (archaea) was quantified and constructed standard curves. Assays reaction and real-time PCR programs were prepared according to Hniman et al. [36] for the *hydA* gene and

according to Friedrich [27] for *mcrA* gene. To check for product specificity and primer–dimer formation, the PCR were completed with a melting analysis starting from 60 °C to 99 °C with temperature increments of 0.2 °C and a transition rate of 5 s (*mcrA* gene) and 65 °C–90 °C with temperature increments of 0.2 °C and a transition rate of 0.2 s (*hydA* gene). The purity of PCR products was also checked by 1% agarose gel, the presence of a single band of the expected 350 (*hydA* gene) and 469 (*mcrA* gene) base.

Next-generation sequencing

DNA extracts from parallel reactors and both hydrogen and methane stages were pooled prior to sequencing. Sequencing was operated by the Illumina Miseq platform by the amplicon sequencing 250 bps paired-end approach at Microsynth GmbH, Switzerland. Obtained reads were analyzed using mothur v.1.37.6 (updated 6/20/2016) [38]. Briefly, forward and reverse reads were combined, low-quality sequences were eliminated from the dataset. PCR and sequencing errors were removed following Miseq SOP [39]. Sequences were screened (maximum length 300 bp, minimum length 230 bp, no ambiguities, a maximum homopolymer of eight nucleotides) and unique sequences were aligned to the SILVA database (release 102). Chimeric sequences were eliminated using uchime [40] and OTUs were generated on 97% similarity. Final OTU table was subsampled to the smallest sample size after removing OTUs made up by less than five reads. Core community was extracted on minimum read abundance of 1% and OTU presence in at least three samples. Sequences and OTUs were classified using the trainset reference database at a cutoff of 80. Alpha diversity was analyzed in mothur.

Statistical analyses

The data were evaluated for normality and variance prior to ANOVA analysis using the Shapiro-Wilks and Levene's tests, respectively, followed by statistical analysis using Statistica 9 software. Hydrogen and methane production were analyzed by a paired sample *t*-test with a 95% confidence ($P < 0.05$). Significant differences in the gene copy numbers of hydrogenase (*hydA*) and methyl coenzyme M reductase (*mcrA*) among treatments (R1, R2, R3, R4, AP, AR, and POME) were analyzed by ANOVA. Post-hoc analyses were performed with the Tukey HSD test. The relations between gas yield and gene copies of *hydA* and *mcrA* were analyzed by Pearson correlation.

Results and discussion

Optimization of trace metal concentration

The most common heavy metals necessary for the AD are Ni, Co, Mo, and Fe [12]. The different concentrations (0–20 mg L^{-1}) of trace metals were added into POME as a supplement in order to enhance thermophilic hydrogen fermentation and methane production. Optimization concentration of trace metal was determined the model from experimental data. The significance model was used for predicted response values. The ANOVA results of trace metal optimization for hydrogen and methane production presented in Table 5. Molybdenum (A) and cobalt (C) has no significant individual effect two-stage biohythane production. Nickel (B) and iron (D) has a significant individual positive effect on two-stage biohythane production ($p < 0.05$). Combination of molybdenum (A) and nickel (B), molybdenum (A) and cobalt (C) and molybdenum (A) and iron (D) has the significant interactive effect to enhance hydrogen and methane production ($p < 0.05$). Statistical analysis predicted that addition of molybdenum (A) combined with nickel (B), cobalt (C) and iron (D) and concentration of (10, 0, 6 and 20 mg L^{-1}), (0, 10, 6, and 20 mg L^{-1}), (10, 0, 0 and 20 mg L^{-1}) as well as (5, 5, 3 and 10 mg L^{-1}) could enhance hydrogen yield, hydrogen production rate and methane yield in two-stage biohythane production process. The addition of trace metal at optimum concentration increase hydrogen yield 2 times compared to without addition. The addition of trace metal at optimum concentration was increased hydrogen yield to 90 mL H_2 gVS^{-1} (Table 1.). While without the addition of trace metal addition has a hydrogen yield of 59 mL H_2 gVS^{-1}. The methane yields from POME hydrogenic effluent were ranged between 290 and 447 mL CH_4 gVS^{-1}. The addition of trace metal at optimum concentration was increased methane yield to 447 mL CH_4 gVS^{-1} from hydrogen effluent (Table 1.). While without the addition of trace metal addition has methane yield of 278 mL CH_4 gVS^{-1}. Zhang and Jahng [13] also found that the supplementation trace metal at a concentration of 2.0 mg L^{-1}, 5.0 mg L^{-1}, 10.0 mg L^{-1}, and 100 mg L^{-1} of Co, Mo, Ni, and Fe, respectively, improved biogas production from food waste. Zhang et al. [14] showed that the combined addition of 1–200 mg L^{-1}, 5–500 mg L^{-1}, 5–1000 mg L^{-1}, and 100–10,000 mg L^{-1} of Co, Mo, Ni, and Fe, respectively, resulted in a high biogas yield reaching a value of 396 mL gVS^{-1}. Demirel and Scherer [41] also report those trace elements can positively influence the anaerobic process such as the addition of Fe, Ni, Co, and others, that are otherwise deficient in the substrate. Trace elements are an essential part of enzymes and co-factors involved in methanogenesis and therefore can directly affect microbial activity and consequently the resulting degradation efficiency and process stability in an anaerobic digestion system [42].

Table 5 – Analysis of variance for quadratic polynomial model on two-stage hydrogen and methane production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	29267.33	14	2090.52	3.26	0.0769
A-Molybdenum	189.42	1	189.42	0.3	0.6063
B-Nickel	3949.99	1	3949.99	6.16	0.0476
C-cobalt	9.42	1	9.42	0.015	0.9075
D-iron	2936.66	1	2936.66	4.58	0.0461
AB	3028.89	1	3028.89	4.73	0.0327
AC	9173.02	1	9173.02	14.31	0.0091
AD	8948.29	1	8948.29	13.96	0.0097
BC	1478.96	1	1478.96	2.31	0.1796
BD	1011.42	1	1011.42	1.58	0.2558
CD	289.26	1	289.26	0.45	0.5267
A^2	714.7	1	714.7	1.11	0.3316
B^2	0.83	1	0.83	1.29E-03	0.9725
C^2	46.38	1	46.38	0.072	0.797
D^2	75.09	1	75.09	0.12	0.7438

Effects of trace metals on two-stage biohythane production

The optimum concentrations of trace metals for enhancing the hydrogen and methane production were chosen for studying the effect of mixed trace metals on hydrogen production and methane production in continuous reactor operation. The addition of Ni, Co, Mo and Fe of 10, 0, 6, and 20 mg L^{-1}, respectively was referred to as reactor R1. The addition of Ni, Co, Mo and Fe of 0, 10, 6, and 20 mg L^{-1}, respectively was referred to as reactor R2. The addition of Ni, Co, Mo and Fe of 10, 0, 0, and 20 mg L^{-1}, respectively was referred to as reactor R3. The addition of Ni, Co, Mo and Fe of 5, 5, 3, and 10 mg L^{-1}, respectively was referred as reactor R4, while AR and AP were added 5% of para rubber wood ash and oil palm fiber ash, respectively. POME without trace metal supplement was used as a control. In the first stage, hydrogen gas was produced from POME with and without trace metal addition within 48 h. The concentration of hydrogen ranged between 17 and 42% with carbon dioxide as the second most abundant gas fraction, while no methane was detected during the hydrogen stage. Hydrogen yields from R1, R2, R3, R4, AR, AP and raw POME were 113, 139, 111, 129, 39, 90 and 73 mL H_2 gVS^{-1}, respectively (Table 3) which corresponds to a hydrogen production of 6.2, 7.2, 6.1, 7.1, 2.1, 4.9 and 4.1 L H_2 L_{POME}^{-1}, respectively. The hydrogen yields from trace metal addition reactors were higher ($p < 0.05$) than the raw POME reactor. The highest yield (139 mL H_2 gVS^{-1}) was achieved for R2 that is at a trace metal concentration of 10 mg L^{-1}, 6 mg L^{-1} and 20 mg L^{-1} of Ni, Co, and Fe, respectively. R4 also gave a high hydrogen yield of 129 ± 5 mL H_2 gVS^{-1} with a trace metal concentration of 5 mg L^{-1}, 3 mg L^{-1}, 10 mg L^{-1} and 5 g L^{-1} of Ni, Co, Fe, and Mo, respectively. Hydrogen production in R2 and R4 was 2-fold higher than in raw POME ($P < 0.05$). The addition of Ni, Fe, and Co in R2 and R4 significantly improved the hydrogen yields ($p < 0.05$) compared to raw POME and also compared to R1 and R3 that were characterized by no addition of Ni. R1 and R3 still produced more hydrogen compared to POME ($p < 0.05$). The Ni/Co/Fe supplementation was increased hydrogen yields by up to 90.4%. The micronutrients (Fe, Co, and Ni) were found critical to optimize the process of hydrolysis and acidogenesis as the trace elements supplementation had increased the COD solubilization and organic acids production [43]. Logically, this can be explained that the hydrolysis and acidogenic bacteria have benefited from Ni/Co/Fe supplementation as the growth factor and hydrogenase enzyme activity. Due to the fact that hydrogenase enzymes containing Ni–Fe play a key in hydrogen production by dark fermentation [18], Ni and Fe might have enhanced the activity of this enzyme and resulted to enhancing the hydrogen yields. The addition of iron seemed generally beneficial for the amount of hydrogen produced since all trace metal supplementations included iron and resulted in elevated gas yields compared to raw POME. The highest hydrogen yield was obtained from R2 which was also the only reactor without Mo addition, suggesting that Mo had no effect on hydrogen production. In a study by Evranos and Demirel [44], Mo addition had been demonstrated to have a negative impact on solid removal and methane production. Nickel and iron could effect on both bacteria growth and hydrogenase activity, while molybdenum and cobalt could effect on only bacteria growth. The VFAs present in the hydrogen effluent were mainly acetic and butyric acid with a concentration of 4.0–12.7 and 4–5.93 g L^{-1}, respectively (Table 6). The overall VFA concentrations obtained from reactors R1-R4 were 10.0–12.7 g L^{-1} of acetic acid, 4.4–5.2 g L^{-1} of butyric acid and 0.2–0.46 g L^{-1} of propionic acid corresponding to hydrogen yields of 111–132 mL H_2 gVS^{-1}. Theoretically, hydrogen yield is 4 and 2 mol of hydrogen per mole glucose when acetic and butyric acid as end-products in the fermentation system. Taking into consideration the two-stage character of the production process, a high VFA concentration in the substrate after the hydrogen production stage is favorable since VFAs are converted into methane in the methane production step following hydrogen production [21].

The hydrogen effluent from the first stage was easily degradable as indicated by immediate methane production. Higher methane yields (403–458 mL CH_4 gVS^{-1}) were achieved in POME supplemented with trace metals (R1-R4) (Table 3) compared to raw POME ($p < 0.05$) resulting in 8.1–21.7% higher methane yields. Methane yields from R1, R2, R3, R4, AR, AP and raw POME were 445, 454, 403, 411, 365, 402 and 373 mL CH_4 gVS^{-1}, respectively (Table 3) which corresponds to a methane production of 24.5, 25.1, 22.2, 22.7, 20.2, 22.2 and 20.6 L CH_4 L_{POME}^{-1}, respectively. The methane yields from trace metal addition reactors were higher ($p < 0.05$) than the raw POME reactor. The highest yield (454 mL CH_4 gVS^{-1}) was achieved for R2 that is at a trace metal concentration of 10 mg L^{-1}, 6 mg L^{-1} and 20 mg L^{-1} of Ni, Co, and Fe, respectively. R1 also gave a high methane yield of 445 mL CH_4 gVS^{-1} with a trace metal concentration of 6 mg L^{-1}, 20 mg L^{-1} and 10 g L^{-1} of Co, Fe, and Mo, respectively. The addition of Ni, Fe, and Co in R2 significantly improved the methane yields ($p < 0.05$) compared to raw POME. The Ni/Co/Fe supplementation was increased methane yields by up to 21.7% with maximum yields of 454 mL CH_4 gVS^{-1}. The COD and VS removal of trace element supplementation was 93.2–97.7% and 89.7–92.3%, respectively. The COD and VS removal of raw POME was 89.6% and 84.44%, respectively. Addition of this Ni/Co/Fe (R2) resulted in enhanced degradation efficiency, higher biogas production. Trace elements supplementation is effective to improve the performance of the anaerobic digestion process by applying necessary (sub) optimum dosage. The positive impacts include the longer term digester stability with greater organic

Table 6 – Soluble metabolites from hydrogen stage of palm oil mill effluent (POME) with different trace element additions.

Reactors	Acetic acid (g L^{-1})	Butyric acid (g L^{-1})	Propionic acid (g L^{-1})	pH	Alkalinity (g $CaCO_3$ L^{-1})
R1	12.77	5.20	0.46	5.1	2.2
R2	11.78	4.93	0.33	5.2	2.3
R3	12.31	5.10	0.36	4.9	2.4
R4	10.62	4.44	0.21	5.3	2.1
AR	4.96	4.10	0.28	5.8	4.5
AP	6.32	5.93	0.40	5.4	4.8
POME	5.89	2.52	0.00	5.6	2.2

Table 7 – Impact of trace element supplementation on anaerobic digestion process.

Substrate	Operation condition	Trace element and added concentration	Improvement	References
Food waste	CSTR with OLR 6.8 kgVS m^{-3} d^{-1}, temperature 55 °C	Fe: 10 mg L^{-1} Ni: 1 mg L^{-1} Co: 1 mg L^{-1}	Biogas production +12.7%	[45]
Grass silage	CSTR, temperature 37 °C, HRT 19 days, OLR 4.0 kgVS m^{-3} d^{-1}	Fe: 74.4 mg L^{-1} Ni: 2.48 mg L^{-1} Co: 0.13 mg L^{-1}	Methane yield +12%	[46]
Food waste	CSTR, temperature 37 °C, HRT 30 days, OLR 2 gVS L^{-1} d^{-1}	Fe: 100 mg L^{-1} Co: 1 mg L^{-1} Ni, Mo: 5 mg L^{-1}	Methane yield +18.7%	[61]
POME	Two-stage ASBR with HRT 2 days and UASB with HRT 15 days, temperature 55 °C,	Fe: 20 mg L^{-1} Ni: 10 mg L^{-1} Co: 6 mg L^{-1}	Hydrogen yield +90.4% Methane yield +21.7%	This study

matter degradation, low VFA level, and higher biogas production [16]. The high COD removal and higher solid destruction were also found in the co-digestion system of food waste and swine wastewater after the addition of Fe/Ni/Mo/Co [14]. The Ni/Co/Fe supplementation increased hydrogen yields by up to 90.4% with maximum yields of 139 mL H_2 gVS^{-1}. The Ni/Co/Fe supplementation also increased methane yields by up to 21.7% with maximum yields of 454 mL CH_4 gVS^{-1}. The two-stage reactor system reached a mixed hydrogen and methane (biohythane) production of 32.3 L biogas L $POME^{-1}$ with a biogas composition of 57% CH_4, 10% H_2 and 33% CO_2, respectively. The improvement of biogas production was in agreement with a previous study by Qiang et al. [45], who found that the supplementation of food waste with Fe, Ni, and Co could enhance biogas production up to 12.7%. Wall et al. [46] also report that the addition of Fe, Ni, and Co into grass silage could improve methane yield up to 12% (Table 7). All in all, it indicates that POME supplemented with trace metals (Ni, Co, and Fe) resulted in improved hydrogen production and methane production.

When POME was supplemented with complex mixed trace elements, such as oil palm fiber ash and para rubber wood ash, lower hydrogen and methane yield were reached than with Ni/Co/Fe supplementation. Ash supplementation attained hydrogen and methane yields of 39–90 mL H_2 gVS^{-1} and 365–402 mL CH_4 gVS^{-1}, respectively, while Ni/Co/Fe supplementation reached hydrogen and methane yields of 111–139 mL H_2 gVS^{-1} and 403–454 mL CH_4 gVS^{-1}, respectively. The 5% w/v oil palm fiber ash supplementation had 20% higher hydrogen yields (90 mL H_2 gVS^{-1}) than those obtained by POME alone, while 5% w/v para rubber wood ash addition, showed around 50% lower hydrogen yields (39 mL H_2 g VS^{-1}) (Table 3). The 5% w/v oil palm fiber ash supplementation had 7% higher methane yields (402 mL CH_4 g VS^{-1}) than those

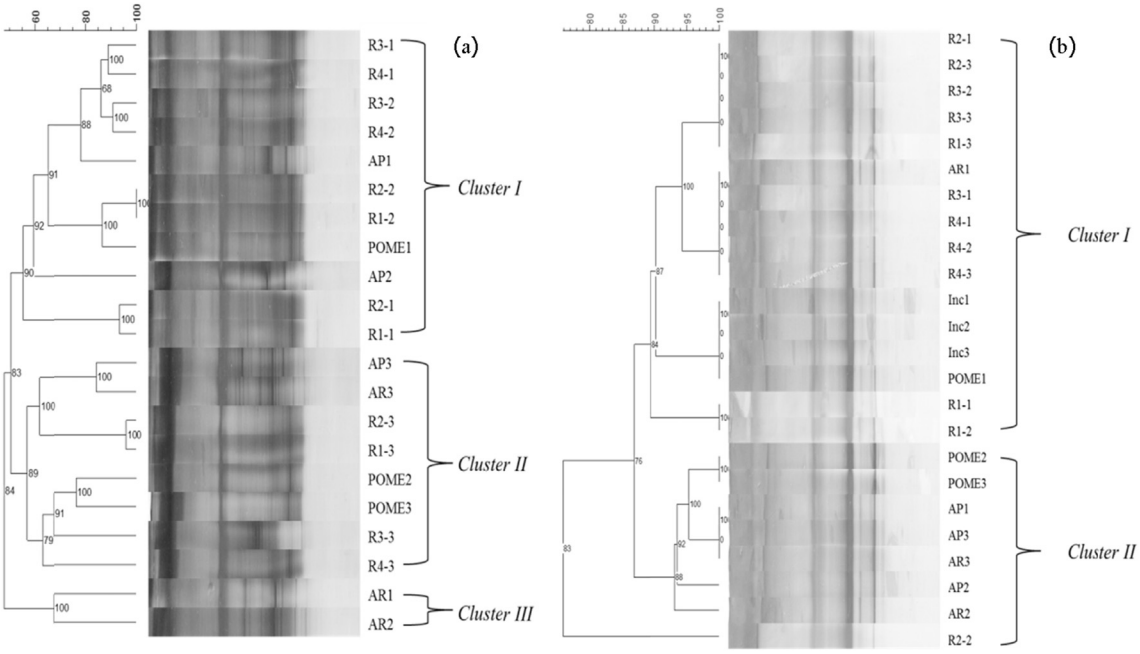

Fig. 2 – Cluster analysis of the hydrogen producing bacterial community in the first stage (a) and methanogenic community in the second stage (b) based on 16S rRNA gene fragments. Values at the branches indicate the percentage of similarity, according to the Ochiai correlation coefficient.

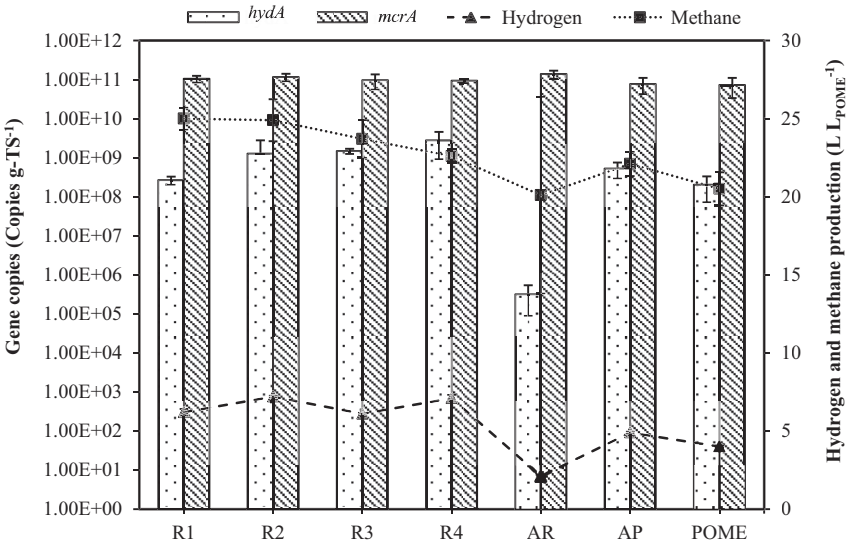

Fig. 3 – Hydrogen, methane production and gene copies of *hydA* (hydrogenase gene) and *mcrA* (methyl coenzyme-M reductase) gene from the two-stage thermophilic digestion of palm oil mill effluent (POME) with different trace element additions.

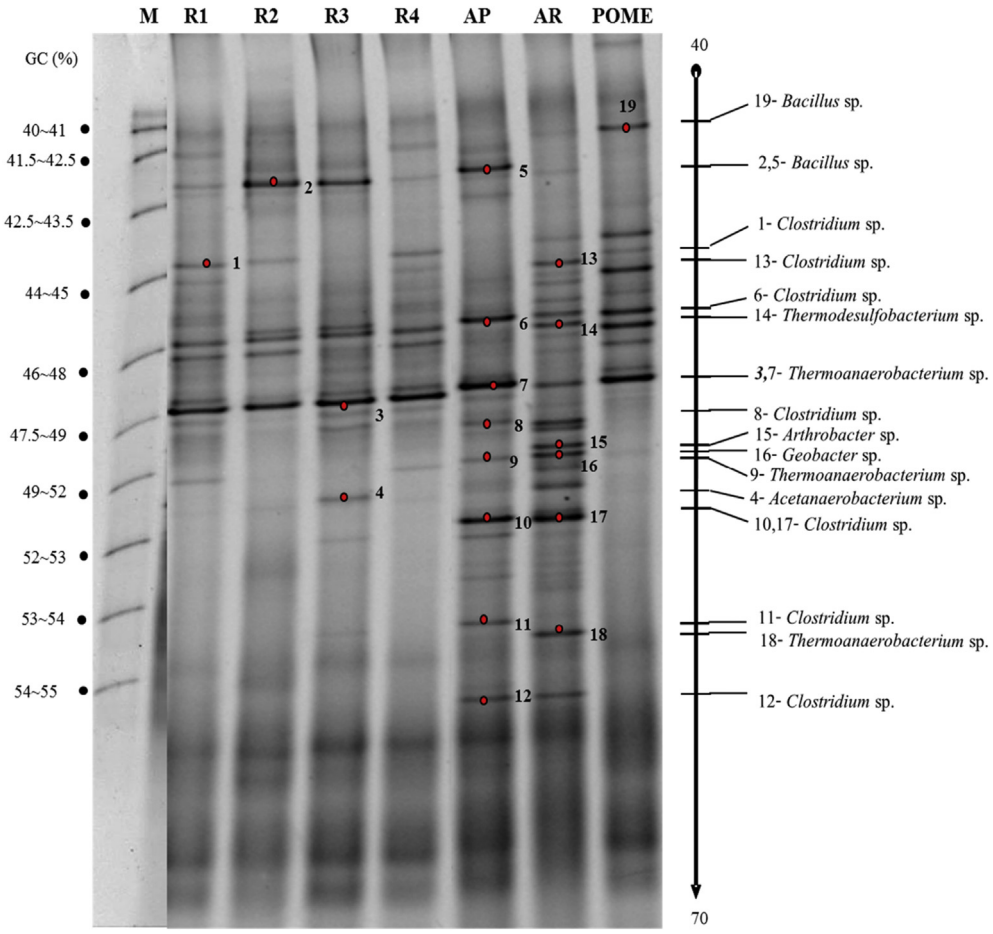

Fig. 4 – DGGE profile of bacterial community in hydrogen stage from the two-stage thermophilic anaerobic digestion of palm oil mill effluent (POME) with different trace element additions.

obtained by POME alone (373 mL CH_4 gVS^{-1}), while 5% w/v para rubber wood ash exhibited similar methane yields (365 mL CH_4 gVS^{-1}) to those obtained by POME alone (373 mL CH_4 gVS^{-1}). Palm oil fiber ash contained SiO_2, CaO, K_2O, P_2O_5, MgO and Fe_2O_3 in a percentage of 44.84%, 12.01%, 4.99%, 4.48%, 3.22% and 2.13%, respectively. Para rubber wood ash mainly comprised SiO_2, CaO, K_2O, P_2O_5, MgO and Fe_2O_3 in a percentage of 4.84%, 48.59%, 5.52%, 0.53%, 2.30% and 1.08%, respectively. Both ashes contained only Fe but not contained Ni and CO resulting to lower hydrogen and methane yield than Ni/Co/Fe supplementation. The lower gas yield in reactors with ash addition might also be due to higher CaO content which can lead to toxicity and inhibition of the methanogenic activity [47,48]. Podmirseg et al. [11] attributed the negative effect on biogas production of ash addition to hydrogen sulfide formation. This result was similar to that registered in the hydrogen stage by Lo and coworkers [47], who also recorded lower biogas yields with ash addition.

Microbial community changes

The microbial community composition in biohythane process was evaluated by PCR-DGGE. A UPGMA algorithm was applied to a similarity matrix of Ochiai correlation coefficients generated from the resulting DGGE banding patterns. The cluster dendrograms from bacteria and methanogens are illustrated in Fig. 2. The DGGE profiles of the bacterial community from all sludge samples of the hydrogen stage were grouped into two main clusters (Fig. 2a). AR reactors were separated from R3, R4, R2, R1, AP, and POME. Interestingly,

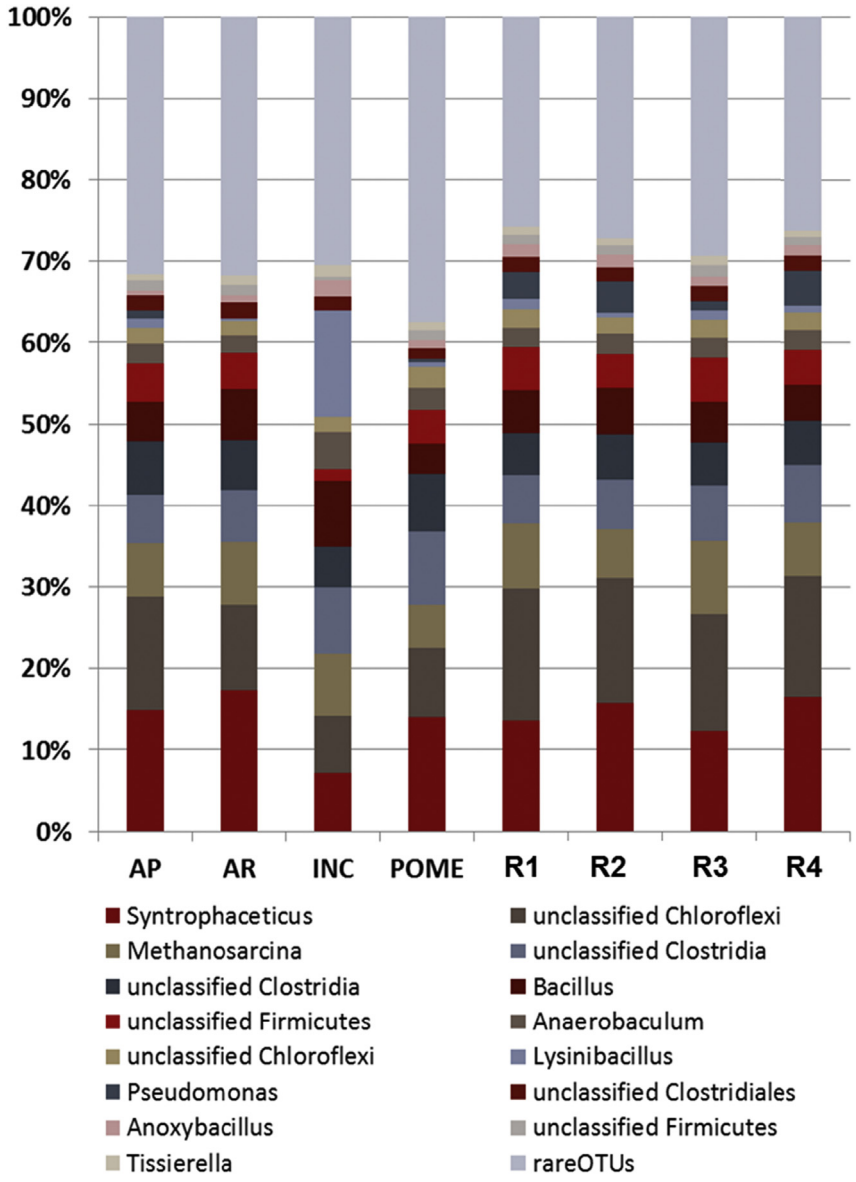

Fig. 5 – Microbial community structure of two-stage hydrogen and methane production with different trace element addition was investigated by Illumina amplicon sequencing. Community structure was analyzed using Illumina Miseq amplicon sequencing of V3/V4 16S DNA using primers specific for bacteria and archaea. Sequences were grouped to OTUs according to 97% sequence similarity. Core OTUs were defined as OTUs detected in at least three samples with at least 1% abundance based on reads. OTUs not matching these criteria were summarized as "rare OTUs".

despite differences in hydrogen yields, the dominating bacterial OTUs in the hydrogen stage were similar in trace metal amended reactors and non-amended ones. The increase in hydrogen yield is thus not due to a change in the microbial community composition, but rather to a stimulation of hydrogenase activity of the present microbiota. High butyric and acetic acid concentrations in hydrogen fermentation were linked to higher gas yields and a more diverse microbial community [34]. The clustering of bacterial communities in this study indicates that hydrogen yields were not mainly related to microbial diversity but dependent on metabolisms or enzyme activity. This agrees with prior findings of O-Thong et al. [49] who also showed a low microbial diversity in the thermophilic hydrogen production process where only *Thermoanaerobacterium* species played a major role. The addition of Fe at 100 mg L^{-1} can increase hydrogen production from *Thermoanaerobacterium* species by 20%. The phenomena in AR indicated that POME supplemented with para rubber wood ash negatively affected hydrogen producers leading to low hydrogen yield. The cluster analysis of methanogens showed that archaeal communities were clearly separated into two main groups (76% similarity). As expected, the cluster associated with high methane yields included R2, R3, R4, Inoculum, and R1. The second cluster referred to lower methane yields and it included POME, AP, and AR. All in all, this indicates that the elevated methane yields by trace metal addition were related to the shifts in archaeal community composition as also stated by Westerholm and co-workers [50].

Real-time quantitative PCR of hydrogenase gene (hydA) and methyl coenzyme-M reductase gene (mcrA)

Hydrogenase and methyl coenzyme-M reductase are the most important enzymes in hydrogen-producing bacteria and methanogens. Therefore, they are likely to influence hydrogen and methane yields. Quantitative analyses of *the hydA* gene in hydrogen stage and *mcrA* gene in methane stage were performed by real-time PCR (Fig. 3). The highest *hydA* gene copy number was recorded following trace metal addition, in particular in R2 and R4 treatments (Fig. 3), which refer to Ni, Co and Fe addition and showed a 90.4% and a 21.7% increased hydrogen and methane yields respectively (Table 3). The addition of Ni at a concentration of 0.2 mg L^{-1} enhanced hydrogen production rate [51]. O-Thong et al. [52] also reported the addition of Fe at a concentration of 247 mg L^{-1} increasing the hydrogen production by 50% compared to raw POME. Since both [FeFe]- and [NiFe]- hydrogenases found in most *Clostridium* and *Thermoanaerobacterium* species, Ni and Fe

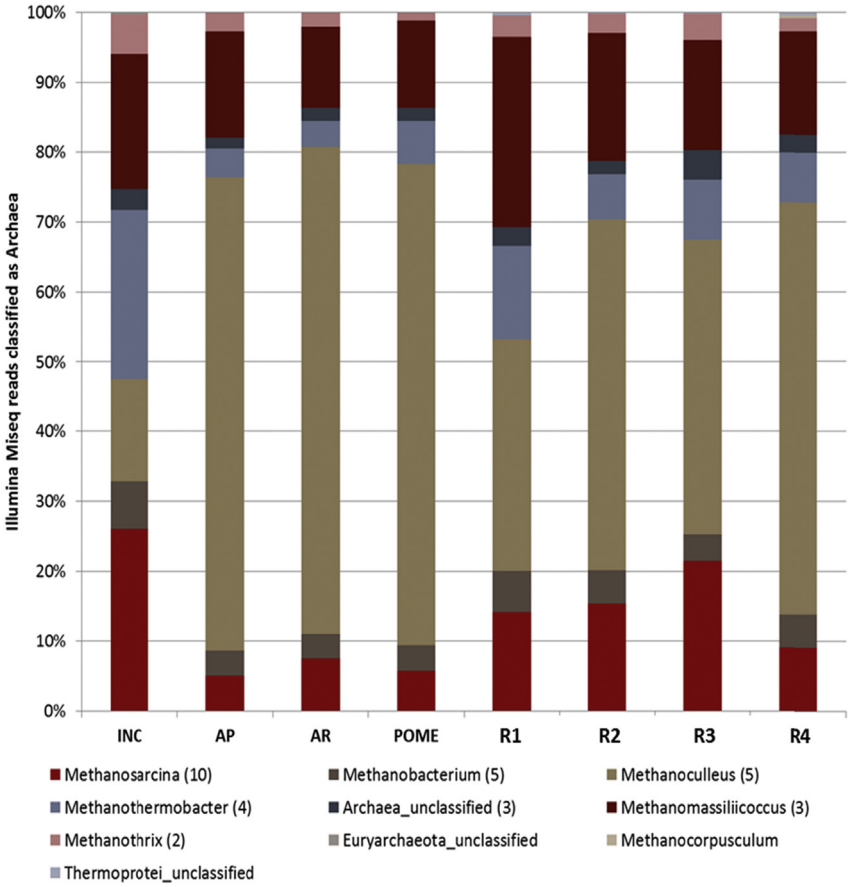

Fig. 6 – Microbial community structure of the read fraction assigned to archaeal taxa during fermentation of POME, POME supplemented with different trace elements (R1-R4) and POME supplemented with ash (AR, AP), according to Table 3. Community structure was analyzed using Illumina Miseq amplicon sequencing of V3/V4 16S DNA using primers specific for bacteria and archaea. Sequences were grouped to OTUs according to 97% sequence similarity. Values in brackets indicate richness on OTU level.

influencing the activity of these enzymes. Both [FeFe]- and [NiFe]-hydrogenases were also identified in dark hydrogen fermentation systems by a proteomic approach [22]. Here, a significant positive correlation between *hydA* gene copy numbers and hydrogen yields ($R^2 = 0.97$, $p < 0.05$) was observed. The hydrogen production from glucose fermentation by *C. butyricum* was related to the activity of *the hydA* gene [53]. The quantification of *hydA* genes by real-time PCR in a hydrogen fermentation system can be a useful molecular marker for a rapid bioprocess monitoring activity of hydrogen-producing bacteria in reactors. Further research is still required to investigate the reaction time of copy numbers to process performance. In the second stage, there were no differences in the copy numbers of the *mcrA* gene (Fig. 3). Moreover, no significant correlation was found between the *mcrA* gene copy number and the methane yields ($R^2 = 0.22$).

Community composition of anaerobic microbiota in hydrogen and methane reactors at different trace metal concentrations

Trace metal addition improved hydrogen and methane production from POME. According to Illumina sequencing, *Syntrophaceticus*, *Chloroflexi*, *Methanosarcina* and *Firmicutes* joint abundance in the two-stage hydrogen and methane process (Fig. 4). Using DGGE techniques and band identification by sequencing, *Firmicutes*, mainly *Clostridium* and *Bacillus*, were identified as the dominant genera and responsible for hydrogen production. In reactors with high hydrogen production (R1, R2, R3, and R4) two strongly stained, distinctive bands seemed characteristic and were phylogenetically related to *Bacillus* sp. and *Thermoanaerobacterium* sp. (Fig. 5). The trace metal additions seemed to influence the bacterial community composition, with *Clostridium* sp. and *Bacillus* sp. populations responding by abundance increase to the addition of Ni/Co/Fe. *Clostridium* species has [FeFe]-hydrogenases response for hydrogen production [54]. Prasertsan et al. [55] studying thermophilic hydrogen fermentation from POME, found that the hydrogen productivity was correlated with responsible microbial that was mainly represented by *Firmicutes* including *Thermoanaerobacterium* sp., *Clostridium* sp. and *Bacillus* sp. These microorganisms have previously been shown as efficient thermophilic hydrogen producers that have the capacity to degrade carbohydrates [55–57]. *T. thermosaccharolyticum* strain PSU-2 isolated from a biohydrogen reactor has an optimum pH range from 5 to 6 that renders high yields of 2.53 mol H_2 mol^{-1} hexose [52].

A significant increase in the abundance of *Chloroflexi* (10–15% of total bacterial sequences) in trace metal addition reactors in comparison with POME, AR and AP ($P = <0.05$). In particular, there was a trend to increased abundance of *Syntrophaceticus* (7–9% of total bacterial sequences) relative to the inoculum, which is known to degrade phenol to benzoate and subsequently to acetate and hydrogen in syntrophic association with hydrogenotrophic methanogens. In terms of archaea, *Methanosarcina*, *Methanoculleus*, and *Methanomassiliicoccus* were the dominant archaeal players (Fig. 6). In line with the productivity of the reactors, the relative abundances of *Methanosarcina*, *Methanomassiliicoccus*, and *Methanoculleus* were influenced by the addition of trace metals. *Methanosarcina* is common in methane production processes

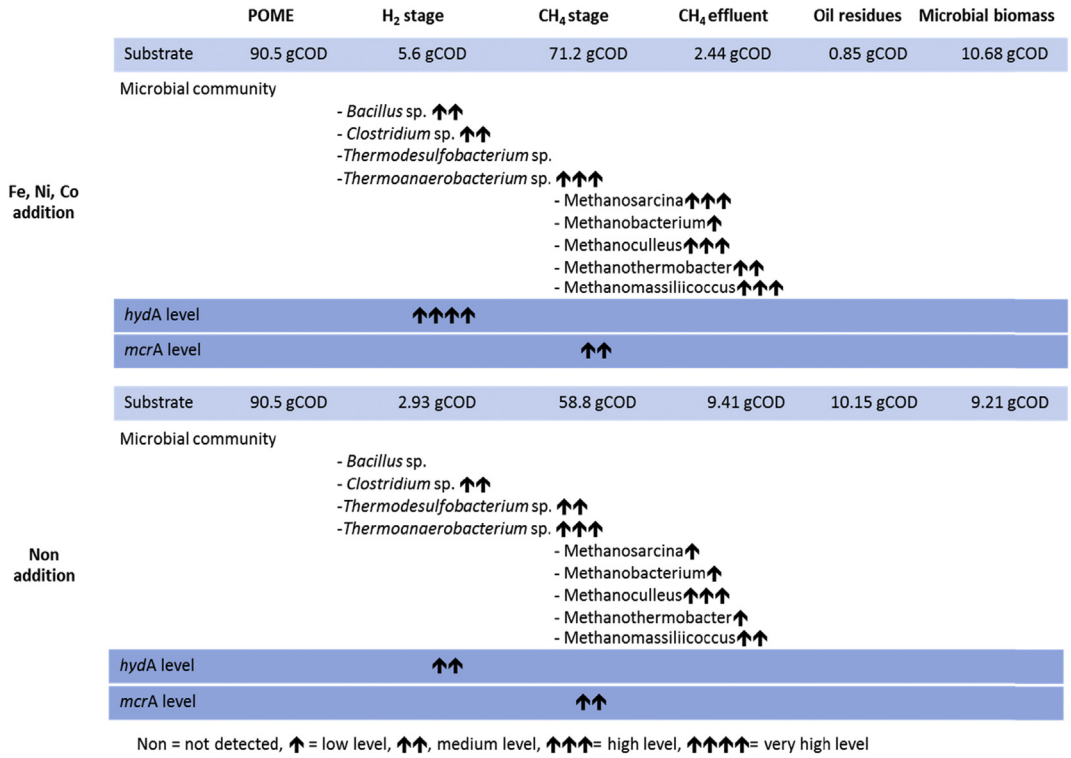

Fig. 7 – The COD balance, responsible microbial community and enzyme activity in two-stage biohythane production from POME with and without trace element addition.

[58] and considered an indicator for well-functioning processes [59]. Its abundance was significantly increased in reactors with trace metal addition compared to the reactors POME, AR, and AP ($P = 0.02$). Also, *Methanomassilicoccus* was enriched by trace metal addition ($P < 0.08$). In reactors POME, AR and AP, *Methanoculleus* dominated the archaeal community in terms of abundance and was significantly higher compared to those reactors with trace metal addition ($P < 0.05$). *Methanoculleus* normally dominant in the biogas reactor feed with high ammonium concentration feedstock [60].

Proposed mechanism for enhanced biohythane production by trace element addition

The Ni/Co/Fe supplementation increased *hydA* gene copy numbers and hydrogen yields by up to 90.4% with maximum yields of 139 mL H_2 gVS^{-1}. The Ni/Co/Fe supplementation increased methane yields by up to 21.7% with maximum yields of 454 mL CH_4 gVS^{-1}. Together, with increasing hydrogenase activity in the hydrogen stage, it resulted in higher numbers of *Bacillus* sp., *Clostridium* sp. and *Thermoanaerobacterium* sp. (Fig. 7). Thus the proposed mechanism for enhanced hydrogen production by Ni/Co/Fe supplementation is via increasing number of viable hydrogen-producing bacteria and hydrogenase expression level. COD in Ni/Co/Fe amended reactors was better converted to hydrogen and methanogenic microbial biomass than in non-supplemented ones. Also according to Zhang et al. [61], the addition of a Fe/Ni/Co mixture resulted in higher biomass content in the digester. Similarly, Speece et al. [62] suggested that Ni, Fe and/or Co should be supplemented into the digester in order to achieve high volatile suspended solids (VSS) contents. *Methanosarcina*, *Methanomassiliicoccus*, and *Methanoculleus* made up the highest percentage of archaeal taxa in the process and their abundance was influenced by Ni/Co/Fe addition. *Syntrophaceticus* was the most abundant bacterial taxon in the second stage. The possible mechanism of enhanced methane production via Ni/Co/Fe supplementation is through increased numbers of viable acetate-oxidizing bacteria that improve syntrophic associations with hydrogenotrophic methanogens and through increased numbers of viable *Methanosarcina, Methanomassiliicoccus,* and *Methanoculleus*. The Ni/Co/Fe supplementation could further improve methane production in the second stage by enhancing acetate-oxidizing bacteria and growth rate of methanogens.

Conclusions

The addition of trace metals into a two-stage production process increased yields of both hydrogen and methane. The combined addition of Ni/Co/Fe into POME doubled the maximum gas production compared to raw POME. An increase in hydrogen production was found to be strongly correlated with hydrogenase enzyme gene *(hydA)* copy numbers underlining its suitability for thorough process monitoring. Members of the phylum *Firmicutes*, more specifically *Bacilli* and *Clostridia*, were found relevant for efficient hydrogen production. Trace metal addition leading to higher gas yields in the second stage affected the composition of the methanogenic community. A decreased abundance of *Methanoculleus* was observed by trace metal addition while the abundance of *Methanosarcina* and *Methanomassiliicoccus* increased. The copy numbers of methanogenic archaea did not correlate with methane produced. Further studies are necessary to shed light on the complex dynamics of archaeal community dynamics following trace metal addition. The addition of ash was not advantageous for the production process. Para rubber ash (AR) decreased hydrogen production by nearly half and changed the microbial community compared to all of the other reactors during hydrogen production stage. Both palm biomass ash and para rubber ash affected the microbial community during methane production stage and did not increase methane production. On the whole, the addition of selected trace metals can boost gas production in the two-stage biohythane process while the addition of ash is not beneficial, despite its known buffering capacity and rich trace metal contents.

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