

Comparative Performance and Microbial Community of Single-phase and Two-phase Anaerobic Systems Co-Digesting Cassava Pulp and Pig Manure

P. Panichnumsin, B. K. Ahring, A. Nopharatana, and P. Chaiprasert

Abstract—In this study, we illustrated the performance and microbial community of single- and two-phase systems anaerobically co-digesting cassava pulp and pig manure. The results showed that the volatile solid reduction and biogas productivity of two-phase CSTR were $66 \pm 4\%$ and $2000 \pm 210 \text{ ml l}^{-1} \text{ d}^{-1}$, while those of single-phase CSTR were $59 \pm 1\%$ and $1670 \pm 60 \text{ ml l}^{-1} \text{ d}^{-1}$, respectively. Co-digestion in two-phase CSTR gave higher 12% solid degradation and 25% methane production than single-phase CSTR. Phylogenetic analysis of 16S rDNA clone library revealed that the *Bacteroidetes* were the most abundant group, followed by the *Clostridia* in single-phase CSTR. In hydrolysis/acidification reactor of two-phase system, the bacteria within the phylum *Firmicutes*, especially *Clostridium*, *Eubacteriaceae* and *Lactobacillus* were the dominant phylogenetic groups. Among the Archaea, *Methanosaeta* sp. was the exclusive predominant in both digesters while the relative abundance of *Methanosaeta* sp. and *Methanospirillum hungatei* differed between the two systems.

Keywords—Anaerobic co-digestion, Cassava pulp, Microbial diversity, Pig manure.

I. INTRODUCTION

BIOMASS is widely available, and its utilization for renewable energy production is becoming increasingly essential, in order to reduce emissions from fossil fuel sources and consequently to prevent global warming. Among different conversion processes for biomass, biological anaerobic digestion is one of the most economic ways to produce biogas from biomass [1].

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Anaerobic digestion involves a series of metabolic interactions among various groups of micro-organisms. It is considered to be a complex biochemical process, comprising 4 steps including hydrolysis, acidogenesis, acetogenesis and methanogenesis. Since the acidogenic phase and methanogenic phase are greatly different in physiological and nutritional requirements, growth kinetics, and sensitivity to environmental stresses, a two-phase system takes advantage of phase separation, using separate units for acidogenesis and methanogenesis, in order to optimize environmental conditions for each phase [2, 3]. Much research has demonstrated that a two-phase system has several advantages over a conventional single-phase system, such as a higher organic degradation rate, methane production rate and process stability, as well as reducing significantly any risk of digester overloading [4-7]. Two-phase has been suggested for waste containing high amounts of readily organic compounds, in order to achieve a balanced process at a high organic loading rate [2]. In addition, results reported in the research literature have indicated that the two-phase system was able to achieve higher degradation of particulate organic compounds. The hydrolysis rate of ligno-cellulose could be enhanced by a slightly acidic pH, improving the working conditions for hydrolytic/acidogenic bacteria [8-10], and the produced VFA can improve the accessibility of hydrolytic enzymes [11, 12].

During starch production from cassava (*Manihot esculenta*), approximately 5.2 Mt of fresh cassava pulp is generated as a major solid waste annually in Thailand [13]. Since cassava pulp comprises 50-60% starch in dry matter and 60-70% moisture content [14], it has a major potential as raw material for biogas production. However, the low concentrations of nutrients such as nitrogen and the low buffering capacity of this waste, represent a difficulty for conversion of this material. In an anaerobic digestion process using cassava pulp, sufficient nitrogen is necessary to activate growth of microbes and to maintain buffering capacity, in order to improve process performance and stability. Generally, options to meet the above purposes are an addition of chemicals, such as urea, ammonium salt, and bicarbonate etc. or co-digestion, using waste containing high nitrogen content, which may be a more environmentally friendly alternative. In Thailand, with an average of 8 million pig produced per year, an approximate amount of 2.16 Mt of manure [13] is seen as a

great potential co-substrate. Co-digestion with manure would give the balance of nutrients, at an appropriate C/N ratio, and a stable pH needed to increase methane production [15].

The performance of an anaerobic digester is primarily related to the microbial community present in the digester. In contrast, the operational and environmental parameters of the process evidently affect the behavior, performance and finally the fate of the microbial community [1]. The evolving succession of the microbial community or the dominant population thus controls overall performance of the system autonomously. Therefore, monitoring and understanding the consortia diversity would entail understanding information, for controlling the performance of the reactor under different conditions.

Molecular biology methods, based on the sequence comparison of small subunit (SSU) ribosomal RNA (rRNA) molecules, have been widely used to determine the microbial community structure in the anaerobic digestion process, especially the construction of 16S rDNA clone libraries and, subsequently, sequence analysis [16-19]. Anaerobic co-digestion of solid wastes has been exclusively studied, in order to evaluate the synergy or adverse effect on the performance and stability of bioreactors. However, very little information has been reported, for us to reach an understanding of the microbial community of systems operated for the co-digestion of waste. Therefore, this study is aimed at determining the microbial community within anaerobic co-digestion bioreactors, both single-phase and two-phase systems, and to discuss the relationship between the reactors performance to the microbial community determined. Above all, the knowledge gained could be of important for optimizing reactor performance.

II. MATERIALS AND METHODS

A. Reactor Operation and Chemical Analysis

A single-phase anaerobic reactor was carried out in a 5 l continuously stirred tank reactor (CSTR), with 3 l working volume. The two-phase system comprised two CSTR with different volumes. The hydrolysis/acidification phase was performed in a reactor with 0.5 l active volume. The methanogenic phase was carried out in a 5 l reactor, with 3 l active volume. Single- and two-phase CSTR were inoculated with digestate collected from an industrial scale plug flow reactor at a pig farm. The reactors were semi-continuously fed with a withdraw/feed method once a day and mechanically stirred at 100 rpm by an electric motor for 15 minutes, at intervals of 15 minutes, and maintained at $37 \pm 1^\circ\text{C}$. The reactors were fed with co-substrate at cassava pulp to pig manure ratio (CP:PM ratio) of 50:50 based on volatile solid (w/w). The reactors were operated at an OLR of $3.5 \text{ kg VS m}^{-3} \text{ d}^{-1}$, with a total retention time of 15 days. The retention times of the hydrolysis/acidification reactor and methanogenic reactor were 2 and 13 days, respectively. The characteristics of the substrates are shown in Table I. Volatile solid (VS) and pH were analyzed, according to the Standard Method [20]. Total alkalinity was measured by titration to pH 4.0, with $0.1 \text{ mol l}^{-1} \text{ H}_2\text{SO}_4$. Biogas production was measured, using the liquid displacement method [20]. The percentage of methane

and carbon-dioxide in biogas was analyzed by gas chromatography (Shimadzu, Class-GC 14B, Japan), fitted with a thermal conductivity detector (TCD). The determination of volatile fatty acid (VFA) was done using the same gas chromatograph, equipped with a flame ionization detector (FID).

TABLE I
 CHARACTERISTICS OF SUBSTRATE

TS (%)	6.2 ± 0.3
VS (%)	5.2 ± 0.1
pH	7.22 ± 0.2
VFA (g COD. l^{-1})	1.68 ± 0.05
Ammonium N (g l^{-1})	0.09 ± 0.01
TKN (g l^{-1})	1.18 ± 0.05
C/N	27

B. DNA Extraction

The genomic DNA was extracted, using a method which involved bead beating and precipitation of impurities in sludge samples with ammonium acetate, as described previously [21]. The concentration and size of DNA were estimated by electrophoresis on a 1% (w/v) agarose gel, and viewed by ethidium bromide with ultra violet emissions. The purified DNA was used as a template for PCR amplification.

C. Clone Library Construction

Two clone libraries, BS5 and AS5, were constructed for bacteria and archaea communities present in the single-phase CSTR, respectively. For the two-phase CSTR, a bacterial clone library (BT5 library) was constructed from 16S rRNA genes, derived from hydrolysis/acidification reactor, while an archaeal clone library (AT5 library) was constructed from a 16S rRNA gene, obtained from a methanogenic reactor. Bacterial 16S rRNA genes were amplified from the purified DNA by PCR amplification, using a primer pair (U-1509r, EUB-8f: [22]). 16S rRNA genes from the archaea domain were targeted with the archaeal primers (ARC-1100r, ARC-1f: [23]). Each reaction tube contained $0.1 \mu\text{mol l}^{-1}$ of each primer, 100-200 ng of purified template DNA, 1X Q solution PCR buffer (Qiagen, Germany), 0.25 mol m^{-3} of deoxynucleotide triphosphate, 2.5 mol m^{-3} of MgCl_2 and 1U of taq DNA polymerase (Qiagen, Germany) in the total volume of 50 μl . The PCR was performed with a Mastercycle (Eppendorf, Germany). After an initial denaturation at 94°C for 5 min, 25 temperature cycles were performed for bacteria (94°C for 30s, 59°C for 30s and 72°C for 2 min) and 30 temperature cycles for archaea (94°C for 30s, 58°C for 1 min and 72°C for 2 min). Finally, one step of 72°C for 7 min was used for all PCRs. The PCR products were purified by micro-column method, according to the manufacturer's instructions (A&A Biotechnology, Gdynia, Poland).

The purified PCR products were cloned into the pGem-T Easy vector plasmid from the DNA Ligation Kit (Promega, USA), according to the instructions of the manufacturer. The ligation product was transformed into *Escherichia coli* DH5 α -competent cell. The transformed cells were plated onto a Luria-Bertani (LB) medium, containing $50 \mu\text{g ml}^{-1}$ ampicillin and 0.1 mol m^{-3} X-Gal (5-bromo-4-chloro-3-indolyl- β -d-

galacto-pyranoside), to identify white-colored recombinant colonies [24].

D. Clone Screening by PCR-DGGE Analysis

For each library, 70 positive clones were screened for different inserts by PCR-DGGE [16]. Clones were checked for their inserts by colony PCR with the vector specific primers T7 and SP6. White colonies were randomly selected and resuspended in 25 μ l sterilized, deionized water, followed by cell lysis, by heating at 94°C for 2 min and centrifuging at 83.33 Hz for 5 min. Two micro-liters of supernatant were used as template DNA for PCR. PCR amplification was performed, as follows: after an initial denaturation at 95°C for 5 min, 30 temperature cycles were performed (95°C for 50s, 55°C for 30s and 72°C for 2 min). The reaction was completed after a 7 min extension at 72°C.

The PCR products of positive clones were used as a template in a nested PCR with a primer pair 338GCf-518r [25, 26] for a bacterial rRNA gene, and with the following thermal cycle conditions: pre-incubation at 95°C for 5 min, 30 cycles of 95°C for 50s, 60°C for 30s and 72°C for 50s, followed by a final extension at 72°C for 7 min. For the Archaea rRNA gene, a primer pair 344GCf-522r [27, 28] was used. The thermal cycle conditions were as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 50s, 58°C for 30s and 72°C for 50s, followed by a final extension at 72°C for 7 min.

The PCR products from each positive clone were loaded on DGGE gel, to determine the electrophoretic migration. A DGGE was conducted, using a CBS Scientific Co. (California, USA). PCR products were loaded onto 6.5% (w/v) polyacrylamide gel with the linear gradient of denaturants (urea and formamide), ranging from 40% to 55% for bacteria and 30% to 70% for archaea. Electrophoresis was performed at a constant voltage of 200 V and temperature of 60°C for 5 hours, in an 1X TAE buffer [29]. After electrophoresis, gels were stained, using ethidium bromide, and observed in a UV illumination device and photographed, using Geldoc (Vilber-Lourmat). Gel images were produced with Bio1D++ analysis software (Vilber-Lourmat).

The total number of clones with different rDNA inserts was estimated from the total number of DGGE bands, with different electrophoretic positions relative to the bands of the reference marker. The marker was prepared by mixing the PCR product of a number of differently migrating clones. A clone with a different electrophoresis position on a DGGE gel, was defined as an operational taxonomic unit (OTU). Clones that showed different migration positions in the gel, were sequenced and phylogenetically classified.

E. Sequence Analysis

The representative clone fragments were amplified by a primer set T7-SP6 and purified for sequencing with Favorprep™ (Favorgen, Taiwan), according to the manufacturer's instructions. The sequencing was performed by 1st BASE (Malaysia). The derived nucleotide sequences were compared with available sequences in the GenBank (NCBI) database. Phylogenetic trees were constructed with PHYLIP in the Ribosomal Database Project II. The Jukes-

Cantor correction was used for distance matrix analyses, and the trees were reconstructed using the Neighbor-joining method.

F. Nucleotide Sequence Accession Number

Archaeal and bacterial 16S rDNA partial sequences, obtained in this study, were deposited in the nucleotide Genbank database, under the accession numbers: GQ458195-458206, GQ458220-458221, GQ458236-458243, GQ458246-458249, 458252-458253.

III. RESULTS AND DISCUSSION

A. Performance of Single-and Two-Phase Anaerobic Co-Digestion System

The performance data of the single- and two-phase CSTR are shown in Table II. In comparison to single-phase CSTR, co-digestion in two-phase CSTR enhanced the solid degradation and methane production 12% and 25%, respectively. In addition, two-phase CSTR obtained higher methane content in biogas than single-phase CSTR.

TABLE II
 PERFORMANCE AND STABILITY

	Single-phase CSTR	Two-phase CSTR	
		Hydrolysis reactor	Methanogenic reactor
pH	7.11 \pm 0.10	4.46 \pm 0.1	7.22 \pm 0.08
VFA (g COD l ⁻¹)	0.20 \pm 0.02	18.07 \pm 1.93	0.20 \pm 0.04
VFA/alkalinity ratio	0.06	Not determine	0.06
VS reduction (%)	59 \pm 1	29 \pm 4	66 \pm 4
% CH ₄	57 \pm 0	12 \pm 2	66 \pm 1
Biogas productivity (ml l ⁻¹ d ⁻¹)	1670 \pm 60	940 \pm 260	2000 \pm 210

The VS reduction efficiency of the single-phase CSTR was 59 \pm 1%, while the biogas productivity and CH₄ content were 1670 \pm 60 ml l⁻¹ d⁻¹ and 57 \pm 0%, respectively. The reactor performance was considered to be stable, as can be seen from the VFA/alkalinity ratio, and the pH values of 0.06 and 7.11, respectively. The two-phase CSTR comprised hydrolysis/acidification and methanogenic reactors. The hydrolysis/acidification reactor had the VS removal efficiencies of 29%. The gas production rate was 940 \pm 260 ml l⁻¹ d⁻¹ with 12 \pm 2% CH₄, as the pH of the reactor was 4.46. The methanogenic reactor showed a better performance, in terms of VS reduction and methane productivity, compared to a single-phase CSTR. The VS removal efficiencies were 66 \pm 4%. The biogas productivity was 2000 \pm 210 ml l⁻¹ d⁻¹, and the CH₄ content was 66 \pm 1%. The pH stabilized at 7.22, when the VFA concentration and VFA/alkalinity ratio were as low as 0.2 g COD l⁻¹ and 0.06, respectively, indicating that the reactor had good stability.

B. Microbial Community in Single- and Two-Phase Anaerobic Co-Digestion System

As shown in Fig. 1, the phylogenetic analysis of the representative bacterial clones revealed that micro-organisms in the phyla *Firmicutes* and *Bacteroides* were observed in both libraries. The phylum *Bacteroidetes* was the predominant group in the BS5 library (55.3%), but was only detected at very low frequency in the BT5 library (7.7%). In contrast, the *Firmicutes* were the predominant phylum in BT5 library (88.5%) while they were the second dominant phylum in the BS5 library (26.8%). The distinctive phyla presented in the BT5 and BS5 libraries were *Actinobacteria* (1.9%), and *Planctomycetes* (3.6%) respectively. *Proteobacteria* was found in BS5 (8.9%) and BT5 (1.9%), while the unclassified Bacteria were observed in the BS5 (8.9%).

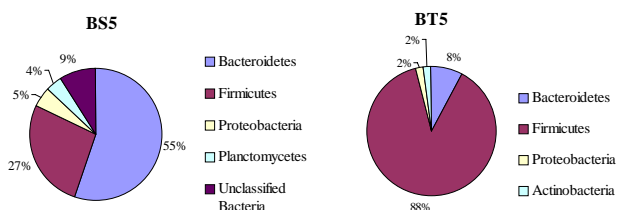


Fig. 1 Bacterial 16S rDNA Clone Distribution of BS5 and BT5 Libraries

Micro-organisms within the class *Clostridia* and *Bacteroidales* have been frequently reported to be important throughout various anaerobic habitats, and have the ability to degrade a wide variety of complex organic molecules, including proteins and carbohydrates [30, 31]. *Clostridia* and *Bacteroides* species isolated from rumen, digesters and natural habitats hydrolyze cellulose, hemi-cellulose and pectin to produce volatile fatty acids, alcohol, lactic acid CO₂ and H₂ [32]. In this study, the identified micro-organisms within these classes are in agreement with other community analyses in anaerobic digesters, and demonstrate the importance of these phylogenetic groups, for the degradation of complex organic matter in anaerobic digestion systems.

Table III shows the Top three distributions of bacterial clones in both libraries. For the single-phase CSTR, the most detected OTU (BS5_15), representing 28.5% of the total clones, was closely related to uncultured bacterium; FP_F8 (FJ769480) with 99% similarity and affiliated with uncultured *Cytophagales* bacterium clone; TDNP USbc97 180 1 43 (FJ516910) with 94% similarity. BS5_22 and BS5_96 were the second most detected OUTs, accounting for 10.7% of the clones affiliated with uncultured *Clostridium* sp.; EHFS1_S16b (EU071533) and uncultured bacterium; E58 (EU864457) within the genus *Clostridium* and unclassified *Bacteroidales*, respectively. The third dominant OTUs, which corresponded to 8.9% of the total clone, were BS5_110 related to uncultured bacterium; SJA-63 (AJ009471) within *Syntrophaceae-Smithella* and BS5_108 affiliated with uncultured bacterium BS14 and uncultured *Fibrobacteres* bacterium 290cost002-P3L-1635 (EF454079).

For the two-phase CSTR, BT5_35 was the most abundant sequence (26.9% of total clones), affiliated with uncultured

bacterium; SMB3 (AM183027), within the genus *Clostridium*. BT5_12 was the second dominant species (23.2% of total clones), affiliated with *Eubacteriaceae* bacterium; DJF_B077 (EU728704), within the *Clostridiales* while BT5_82 was the third dominant OTU (13.5% of total clones) closely related to *Lactobacillus mucosae*; 40E (DQ471799).

The analysis of bacterial composition and their distribution in both single- and two-phase CSTR, revealed the existence of the micro-organisms in these digesters. They were similar to the dominant groups of fermentative bacteria in pig slurry, such as the *Eubacteria-Clostridium*, the *Bacillus-Lactobacillus-Streptococcus* subdivision, the *Mycoplasma* and relatives and the *Flexibacter-Cytophaga-Bacteroides*, as reported by Peu et al. [33] and Snell-Castro et al. [34].

Analysis of bacterial communities in this study demonstrated clear differences in both dominant groups and phylogenetic distribution between single-phase CSTR and hydrolysis/acidification CSTR. The analyzed results revealed that in the hydrolysis/acidification reactor (2 days HRT), the diversity and distribution of micro-organisms within the *Bacteroidetes* decreased, compared to that of single-phase CSTR (15 days HRT). *Clostridia*, such as *Clostridium* and *Eubacteriaceae*, represented the exclusive dominant phylogenetic group, suggesting a major impact of these bacteria on the conversion of organic matter, at short retention time and acidic pH (4.5) conditions (Table II). Bacteria in the order *Clostridiales* have demonstrated a considerable concentration of cellulolytic capabilities in the anaerobic digestion of cellulosic material [35]. Most of the members of the genus *Clostridium* are strictly anaerobic, producing ammonia, H₂S and large amounts of H₂ and ferment carbohydrates. Their fermentation products include acetate, butyrate, lactate, ethanol, acetone, CO₂ and H₂ [36]. Therefore, the higher VS reduction, obtained in a two-phase CSTR rather than single-phase CSTR, might be due to the high abundance of these bacteria.

The presence and dominance of *Lactobacillus* was dependent on pH values. *Lactobacillus* was the third dominant species in the hydrolysis/acidification reactor, where the pH was 4.5, while it was detected in very low numbers at pH 7.1, inside the single-phase CSTR. As the pH changes, shifts of *Lactobacillus* species were observed [37, 38]. They grow intensively at pH 4-6, but grow slowly at pH 7 and 8 [39].

As well as *Bacteroidetes* and *Clostridia*, a small fraction of *Syntrophaceae* was also detected in the single-phase CSTR. The *Syntrophaceae* comprises the genera *Syntrophus*, *Desulfomonile*, *Desulfobacca*, and *Smithella*. Bacteria within the genera *Syntrophus* and *Smithella* grow in syntrophic association with H₂-utilizing micro-organisms, and oxidize substrates incompletely into acetate [40]. *Syntrophus* are slow-growing syntrophs, and has been shown to be capable of oxidizing butyrate, as well as some longer fatty acids, in syntrophic association with hydrogenotrophic methanogens [41]. *Smithella* sp., such as *Smithella propionica*, degrades propionate to produce acetate and butyrate [42]. Their slow-growing characteristics might result in their disappearance from the hydrolysis/acidification reactor operating at 2 days HRT.

TABLE III
TOP THREE PREDOMINANT BACTERIAL OTUS OF SINGLE-PHASE CSTR (BS5) AND TWO-PHASE CSTR (BT5)

Library	OTU	Closest relative	% Similarity	% Frequency
BS5	BS5_15	Uncultured bacterium; FP_F8; FJ769480: <i>Cytophaga</i> sp.	99	28.5
	BS5_22	Uncultured <i>Clostridium</i> sp.; EHFS1_S16b; EU071533	99	10.7
	BS5_96	Uncultured bacterium; E58; EU864457	98	10.7
BT5	BT5_35	Uncultured bacterium; SMB3; AM183027: <i>Clostridium</i> sp.	99	26.9
	BT5_12	<i>Eubacteriaceae</i> bacterium DJF_B077; EU728704	99	23.2
	BT5_82	<i>Lactobacillus mucosae</i> ; 40E; DQ471799	99	13.5

TABLE IV
PHYLOGENY AND FREQUENCY OF THE 16S rDNA ARCHAEAL CLONES OF SINGLE-PHASE CSTR (AS5) AND TWO-PHASE CSTR (AT5)

OTU	Closest relative	% Similarity	% Frequency	
			AS5	AT5
ARC_50	uncultured <i>Methanosaeta</i> sp.; SMS-T-Pro-5; AB479409	99	54.3	18.6
ARC_17	<i>Methanosaeta</i> <i>concilii</i> ; X16932	99	21.4	50.0
ARC_3	<i>Methanospirillum</i> <i>hungatei</i> ; JF1; AY196683	98	24.3	31.4

As shown in Table IV, ARC_50 was the most abundance archaeal 16S rRNA gene sequences of the AS5 library, which was closely related to the sequence of uncultured *Methanosaeta* sp.; SMS-T-Pro-5, accounting for 54.3% of the total clones. The second dominant sequences (ARC_3) were very close to known species, *Methanospirillum hungatei*, representing 24.3% of the total clones. In the AT5 library, ARC_17, affiliated with *Methanosaeta concilii*, was the most dominant sequence, corresponding to 50.0% of the total clones while ARC_3 was the second dominant OTU, accounting for 31.4% of the total clones.

Methanosaeta sp., which is an acetoclastic methanogens, has been found to be a dominant Archaea in a stable bioreactor and is very frequently detected in anaerobic digesters, where acetate concentrations are low. The acetate concentrations presented in our digesters were at a very low level (< 3 mM) (data not shown). *Methanosaeta* sp. has a Km value for acetate of 0.8 to 0.9 mM [43]. *Methanospirillum hungatei*, which is a H₂/CO₂ or formate utilizer, was more established in the methanogenic reactor than in the single-phase CSTR. An increase in the methane production and CH₄ content in biogas of the two-phase CSTR partly corresponded to the higher in the distribution of *Methanospirillum hungatei*. Syntrophic association between acetogens and hydrogenotrophic methanogens plays an important role in anaerobic digester performance and stability. Syntrophic degradation of propionate and butyrate is thermodynamically favorable, only when the H₂ partial pressure is low enough (<10⁻⁴ atm) [44, 45]. McMahon et al. [46] reported that well established hydrogenotrophic methanogens possibly allowed the syntrophic VFA oxidizers to grow more quickly, and to degrade propionate more rapidly, resulting in a more rapidly stabilizing digester.

ACKNOWLEDGMENT

The authors wish to thank the Joint Graduate School of Energy and Environment and the Royal Golden Jubilee Ph.D. Program for financial support.

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