Microbial community composition and reactor performance during hydrogen production in a UASB reactor fed with raw cheese whey inoculated with compost

E. Castelló, V. Perna, J. Wenzel, L. Borzacconi and C. Etchebehere

ABSTRACT

This study investigated the microbial community developed in a UASB reactor for hydrogen production and correlated it to reactor performance. The reactor was inoculated with kitchen waste compost and fed with raw cheese whey at two organic loading rates, 20 gCOD/Ld and 30 gCOD/Ld. Hydrogen production was very variable, using an OLR of 30 gCOD/Ld averaged 1.0 LH2/Ld with no methane produced under these conditions. The hydrogen yield was also very variable and far from the theoretical. This low yield could be explained by selection of a mixed fermentative population with presence of hydrogen producing organisms (Clostridium, Ruminococcus and Enterobacter) and other non-hydrogen producing fermenters (Lactobacillus, Dialister and Prevotella). The molecular analysis of the raw cheese whey used for feeding revealed the presence of three predominant organisms that are affiliated with the genera Buttiauxella (a low-yield hydrogen producer) and Streptococcus (a lactic acid-producing fermenter). Although these organisms did not persist in the reactor, the continuous addition of these fermenters could decrease the reactor’s hydrogen yield.

Key words | bio-hydrogen, cheese whey treatment, microbial community structure

INTRODUCTION

Cheese whey is an important by-product generated during cheese production and is composed primarily of sugars, proteins, and mineral salts (Panesar et al. 2007). Due to its low buffer capacity, treatment in a conventional anaerobic reactor frequently leads to acidification and inhibition of methanogenic activity (García et al. 1991). Therefore, treatment in a two-phase anaerobic reactor has been evaluated (Demirel et al. 2005; Saddoud et al. 2007). In recent years, with the development of hydrogen energy technology, the production of hydrogen using cheese whey in an acidogenic reactor has become an alternative method for increasing energy production through a two-stage anaerobic treatment (Hallenbeck & Ghosh 2009). Other researchers have reported working with dry whey powder as a substrate for bio-hydrogen production in continuous and batch modes using mixed consortia (Yang et al. 2007; Davila-Vázquez et al. 2008) or pure cultures (Ferchichi et al. 2005). However, knowledge about the production of hydrogen using raw cheese whey is still lacking.

This work is framed within the search for a solution for the complete treatment of cheese whey required by the industry in Uruguay. Considering this goal, we worked with non-sterile liquid whey in a UASB reactor. The UASB reactor was chosen because of its efficiency in terms of operating costs and the known applicability at full scale. In previous work we demonstrated the feasibility to produce hydrogen under these conditions but the hydrogen production was low due to the persistence of methanogenesis and the predominance of propionic fermentation (Castelló et al. 2009). In order to avoid these problems, a different inoculum (without methanogenic activity) and a higher starting organic loading rate (OLR) (20 gCOD/Ld) were tested. The aim of this work was then to study the microbial community that developed in the reactor under these new conditions and correlate it to its performance. As unsterilised raw cheese whey was used to feed the reactor, the effect of the fermenting organisms added during the feeding was evaluated.
MATERIALS AND METHODS

The reactor system and operation

Kitchen waste compost with no pre-treatment was used to inoculate a laboratory-scale UASB reactor (working volume 4.6 L, height 54 cm).

The system was operated at 30 °C at two organic loading rates (20 and 30 gCOD/Ld) with a hydraulic residence time (HRT) of 24 hours, which was kept constant.

Biogas production was measured with a water displacement meter.

The pH was controlled by the addition of NaHCO₃ to the feeding, and an average pH of 5.0 with a standard deviation of 0.7 (104 samples) was maintained at the outlet.

Substrate

Cheese whey was obtained from a local cheese production factory and stored at 4 °C until used. The whey was diluted to a COD concentration, according to the OLR, and supplemented with NaHCO₃ (0.3 g of NaHCO₃/gCOD). The diluted solution was maintained at room temperature while being added to the reactor at an OLR of 20 gCOD/Ld and was maintained at 4 °C after starting the operation at an OLR of 30 gCOD/Ld.

Analytical methods

Chemical oxygen demand (COD), total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to Standard Methods (APHA/AWWA/WEF 1995). Hydrogen and methane levels were measured using gas chromatography (Chromatograph SRI 8610) with a molecular sieve 13 column (Chrompack) and TCD detector. Volatile fatty acids (VFA), lactic acid, ethanol, and lactose were determined using HPLC with the following conditions: polymeric column ORH-801, UV detector (Shimadzu 10AD) at 210 nm and IR detector (Shimadzu RID-10AD), mobile phase H₂SO₄ (0.005 mol/L), flow rate of 0.8 mL/min, and oven temperature of 45 °C.

Microbial community’s composition analysis

Microbial community composition was determined using T-RFLP analysis of the 16S rRNA gene. DNA was extracted from samples (20 mL) taken from the reactor biomass and from 1 g of the inoculum using an UltraClean Soil DNA Extraction Kit (MO BIO Laboratories Inc.) according to the manufacturer’s protocol. The PCR reaction (using primers 27 forward and 1,492 reverse), purification, digestion with restriction enzyme MspI, fragments separation and analysis were performed as described (Castelló et al. 2009). A 16S rRNA gene clone library was constructed for the sample obtained on day 71 as described (Castelló et al. 2009). Clones sequencing using the M13 forward primer was performed by Macrogen Ltd. sequencing service (Korea).

Sequences were compared to sequences from the NCBI database using the BLAST (nucleotide–nucleotide comparison) tool and from the Ribosomal Database Project (RDP) using the Classifier tool. Clones with 16S rRNA sequence similarity of more than 97% were grouped into an operational taxonomic unit (OTU). To compare the T-RFLP peaks with the sequences, the number of nucleotides of the 5’ fragment was determined using ‘in silico’ restriction analysis with the enzyme MspI.

Microbiological analysis of the inoculum

The number of hydrogen-producing bacteria from the inoculum (compost) was determined using MPN with an anaerobic glucose-containing medium (Sigma, 10 g/L) (PYG medium) as previously described (Castelló et al. 2009). The methanogenic activity of the inoculum was measured in batch tests in triplicate as previously described (Soto et al. 1993).

Isolation of lactic acid bacteria

Isolation was performed in agar plates with MRS medium (Difco) incubated at 35 °C in a CO₂-enriched atmosphere. The isolates were characterised using 16S rRNA gene sequence analysis. The capacity for producing hydrogen was tested in an anaerobic PYG medium.

Quantification and characterisation of bacteria during the feeding

Determination of microorganisms was conducted in a sample obtained as soon as the feeding was prepared and after one day of storage at room temperature or at 4 °C. Cells were enumerated using standard plate count with TSA (Difco) media incubated at 35 °C.

The microbial composition of the feeding was evaluated by T-RFLP analysis of the 16S rRNA gene and construction of a cloning library, performed as previously described. For DNA extraction, 2 L of the feeding stock was centrifuged at 4,000 rpm for 40 min; cells were harvested, and DNA was extracted as described.
Accession number of the sequences

16S rRNA gene sequences were deposited in the NCBI database under the accession numbers: JF281135–JF281143 (reactor library); JF281144–JF281146 (cheese-whey library); JF281149, JF431544 (lactic-bacteria isolates).

RESULTS AND DISCUSSION

Microbiological studies of the inoculum

The selection of the seed was focused on avoiding the heat treatment in order to reduce the energy input involved. Therefore, the inoculum should be free of methanogenic archaea and at the same time be rich in hydrogen production bacteria. These two properties were detected in the kitchen waste compost used as inoculum. The compost presented a high number of hydrogen-producing bacteria with MPN values of $46 \times 10^7$ cells/g, and the level of methanogenic activity was below the detection limit.

Biogas production

The UASB reactor was operated at an HRT of 24 h for 110 days under OLR of 20 gCOD/Ld with an intermediate stop due to operational problems. After that (day 196 from the start up), the OLR was increased to 30 gCOD/Ld and maintained in this condition for 30 days.

The OLR presented variation from the expected values (20 and 30 gCOD/Ld) mainly due to variations in raw cheese whey composition, affecting the feed concentration (Figure 1(a)). To a lesser extent, these variations respond to natural fermentation of the feed during storage and variations in the volumetric flow rates. VSS in the reactor was maintained at an average concentration of 10 g/L by purging the excess biomass. The food to microorganism ratio ($F/M$), calculated as the OLR by the VSS concentration in the reactor, was on average $2 \ gCOD/gVSSd$ and $3 \ gCOD/gVSSd$ for the two OLR conditions. Methane was only detected at days 55, 58 and 63 (Figure 1(b), maximum value 0.25 L/d). The hydrogen production was not stabilised during operation (Figure 1(b)). The higher values were observed when the system was operated at an OLR of 30 gCOD/Ld but without a clear tendency. At this OLR the hydrogen production was on average $4 \ LH_2/d$ ($1 \ LH_2/Ld$) achieving punctual values of $9 \ LH_2/d$. The fluctuations observed did not respond only to punctual variations in the OLR (Figures 1(a) and (b)). It could be related neither to pH variations nor VSS content in the reactor as both values were maintained at set values. However, a constant concentration of VSS doesn’t assure the constant composition of the biomass. Then, variation in

Figure 1 | (a) OLR and VSS in the reactor during reactor operation and (b) Hydrogen yield, $H_2$ and $CH_4$ production during reactor operation. From day 0 to 197: OLR = 20 gCOD/Ld; from days 198 to 230: OLR = 30 gCOD/Ld.
H₂ production could be due to the competition of different metabolic pathways with no predominance of hydrogen producing bacteria, as will be discussed later.

The yield was determined as the ratio between the moles of H₂ produced and the COD entering the reactor, converted to moles of lactose (lactose was determined in five samples and related to the COD values). The calculated yield values presented also high variability (Figure 1(b)). This fluctuation could respond to the changes in the metabolic pathways and also to the intrinsic variability of the cheese whey composition (% of proteins, grease and oil, lactic acid). This factor could have an influence in the variation of the relation between hydrogen produced and substrate entering. Due to this high variability, an average could not be calculated but it can be observed that the values are far from the theoretical.

In our previous work, the maximum values achieved for VHPR (volumetric hydrogen producing rate) were 0.1 LH₂/Ld and methane was also present in the biogas. So, despite the variations in the VHPR observed in the new conditions, its value increased 10 times from the one obtained in our previous study with no methane produced after day 63.

Biomass developed in the reactor

The biomass in the reactor became whitish in colour and presented positive settling properties, which allowed it to be easily retained in the reactor. Biomass was observed at an electronic magnifying glass (64×) and, as in our previous work, granulation was not evident, although small aggregates of less than 1 mm were observed (images not shown).

Metabolic products

The substrate was fermented, producing primarily butyric and acetic acid (Figure 2), obtaining an HAc : HBu ratio of 0.4, on average, during the operation at both OLRs (SD 0.1); this result is similar to those obtained in other studies using cheese whey (Davila-Vázquez et al. 2009; Venetsaneas et al. 2009). In addition, this result is consistent with the low hydrogen yield obtained, suggesting that the predominant metabolic pathways were not the most efficient for hydrogen production.

Despite the high lactic acid and acetic acid concentrations (7,600 and 2,370 mg/L, respectively) that were observed in the feeding when the feedstock was maintained at room temperature, lactic acid was not abundant in the reactor’s out-stream (accounting for less than 15% of the product). This result could be explained by the conversion of lactic acid inside the reactor. The property of transforming lactic acid to butyric acid in several Clostridium genus fermenters (Cato et al. 1986) was previously reported. It was also reported that Clostridium diolis produces hydrogen using acetic and lactic acids as substrates (Matsumoto & Nishimura 2007). The authors of that study suggest that this ability may be shared by other species of the Clostridium genus.

The production of ethanol was not detected during the operation, and propionic acid was only detected in the first two weeks and after the re/start up of the reactor (day 188 and 191). Lactose at the outlet was determined in five samples and a removal above 99% was verified.

Microbial composition of the biomass

T-RFLP analysis of 16S rRNA bacterial genes indicated that the bioreactor community changed over time. A total of 32 peaks (T-RFs) were detected, and eight of them were present in more than half of the samples, indicating their persistence in the reactor (Figure 3). T-RFs of 91 and 177 nucleotides increased their relative abundance during the operation at an OLR of 30 gCOD/Ld.

The predominant organisms were identified using a 16S rRNA gene library, which was constructed with the sample obtained on day 71. Sequences from 89 clones were analysed and grouped into nine different OTUs. According to the sequence analysis, the predominance of fermenters was verified, with some of them producing hydrogen (Clostridium, Ruminococcus and Enterobacter) whereas others did not (Table 1).

Six predominant T-RFLP peaks were correlated with sequences from clones, according to in silico restriction analysis. Based on these sequences, T-RFs that correlated with Lactobacillus, Clostridium, Dialister and Prevotella sequences were detected in several samples.
Table 1  
<table>
<thead>
<tr>
<th>Sample</th>
<th>OTU</th>
<th>Closer relative</th>
<th>Fermentation products</th>
<th>Reference</th>
<th>T-RFLP peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor</td>
<td>otu 1</td>
<td><em>Enterobacter</em> sp. EF498450 (0.775)</td>
<td>H₂, formic, acetic, lactic, ethanol, butanediol</td>
<td>Shin <em>et al.</em> (2007)</td>
<td>Nd</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 2</td>
<td><em>Acetobacter orientalis</em> AB117965 (0.758)</td>
<td>Not fermenter</td>
<td>Cleenwerck <em>et al.</em> (2002)</td>
<td>Nd</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 3</td>
<td><em>Bifidobacterium minimum</em> AY174103 (0.638)</td>
<td>Lactic, acetic</td>
<td>Meile <em>et al.</em> (1997)</td>
<td>Nd</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 4</td>
<td><em>Lactococcus</em> sp. EF204371 (0.934)</td>
<td>Lactic, acetic</td>
<td>Schleifer <em>et al.</em> (1985)</td>
<td>565</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 5</td>
<td><em>Lactobacillus delbrueckii</em> AY050172 (0.968)</td>
<td>H₂ (not all), lactic</td>
<td>Kandler &amp; Weiss (1986)</td>
<td>177</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 6</td>
<td><em>Clostridium ljungdahlii</em> GU139552 (0.780)</td>
<td>H₂, acetic, butyric</td>
<td>Cato <em>et al.</em> (1986)</td>
<td>515</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 7</td>
<td><em>Ruminococcus bromii</em> X85099 (0.765)</td>
<td>H₂, acetic, ethanol, formic/lactic</td>
<td>Naikoua <em>et al.</em> (2008)</td>
<td>Nd</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 8</td>
<td><em>Dialister invisus</em> AY162469 (0.750)</td>
<td>Acetic, succinic</td>
<td>Morotomi <em>et al.</em> (2008)</td>
<td>181</td>
</tr>
<tr>
<td>Reactor</td>
<td>otu 9</td>
<td><em>Prevotella albensis</em> AJ011683 (0.591)</td>
<td>Acetic, lactic</td>
<td>Wu <em>et al.</em> (1992)</td>
<td>94</td>
</tr>
<tr>
<td>Feeding whey-otu1</td>
<td></td>
<td><em>Buttaiauxella izardii</em> AJ233404 (0.969)</td>
<td>H₂, formic, acetic, lactic, succinic, ethanol</td>
<td>Müller <em>et al.</em> (1996)</td>
<td></td>
</tr>
<tr>
<td>Feeding whey-otu2</td>
<td></td>
<td><em>Buttaiauxella brennerae</em> AJ233401 (0.952)</td>
<td>H₂, formic, acetic, lactic, succinic, ethanol</td>
<td>Müller <em>et al.</em> (1996)</td>
<td></td>
</tr>
<tr>
<td>Feeding whey-otu3</td>
<td></td>
<td><em>Streptococcus thermophilus</em> AY188354 (0.991)</td>
<td>Lactic</td>
<td>Hols <em>et al.</em> (2005)</td>
<td>557</td>
</tr>
</tbody>
</table>

*Accession numbers of the sequences, and S-ab, a score indicating the homology between the sequences by RDP Seq-match tool, is shown in brackets. Nd: not determined because the sequences were only obtained in the reverse orientation.*
The capacity of organisms affiliated with the genus, *Lactobacillus*, to produce hydrogen was reported for some species (Kandler & Weiss 1979), and more recently, a highly efficient hydrogen-producing *Lactobacillus* was reported by Yang et al. (2007).

To determine whether organisms from the *Lactobacillus* genus produce hydrogen in our reactor, lactic acid bacteria were isolated from a sample obtained at the end of the operation. Two strains were isolated and characterised as belonging to the genus *Lactobacillus*; the isolates did not produce hydrogen during fermentation in the PYG liquid medium. Next, in our reactor, the presence of *Lactobacillus* was not associated with hydrogen production. According to these findings, the capacity of *Lactobacillus* genus for hydrogen production should be investigated further.

**Microbial fermentation of the feedstock**

Cheese whey stored at 26 °C was rapidly fermented, as evidenced by the pH decrease to 4.77, the increase in the lactic acid content and the increase in the number of cells (Table 2).

The number of cells entering the reactor were estimated using this formula, $2 \times 10^{-15}$ g of cells/CFU (Etchebehere et al. 2001) (Table 2).

The biomass yield during the operation at an OLR of 20 gCOD/Ld was estimated at an average of 0.1 gVSS/g lactose, after which a growth rate of 3 gVSS/d could be estimated. Therefore, the biomass added with the feeding represents a maximum of 1.5% and 22% of the biomass growth when stored at 4 and 26 °C, respectively. The average biomass content in the reactor was 46 gVSS; according to these observations, the maximum quantity of cells added to the reactor per day was always less than 12% of the total biomass in the reactor.

To identify the microorganisms added during the feeding, a sample was obtained from the raw cheese whey and T-RFLP analysis of the 16S rRNA gene and construction of a cloning library were performed. Both analyses revealed the presence of three different microorganisms (Figure 3) (Table 2). Their sequences were related to *Buttiauxella* and *Streptococcus* sequences (Table 2). These organisms were previously detected in milk products; in fact, *Streptococcus thermophilus* is widely used as a starter culture in the manufacture of dairy products (Hols et al. 2005), and organisms from the *Buttiauxella* genus have been associated with milk spoilage (He et al. 2009). However, despite the constant addition of these microorganisms to the reactor, their dominance in the reactor biomass was not observed; indeed, the T-RFs of 78, 486 and 557 nucleotides were detected in low amounts in the reactor samples (Figure 3). Nevertheless, these fermenters could compete for the substrate in the reactor, causing a decrease in the hydrogen yield.

To minimise this effect, it was decided to keep the feedstock at 4 °C while it was added to the reactor at the OLR of 30 gCOD/Ld.

**A Link between reactor performance and microbial composition of the biomass**

It was previously suggested that both the inoculum and the OLR have an important effect on the selection of high-yield hydrogen producers. In 2010, Hafez et al. described a correlation between the OLR and the microbial community selected in the reactor. The authors proposed an optimal operational OLR of 103 gCOD/Ld in the conditions tested (a CSTR reactor fed with glucose and an HRT of 8 h); they also showed a correlation with the F/M ratio, with an optimum of between 4.4 and 6.4 gCOD/gVSSd. An extremely high OLR (154 and 206 gCOD/Ld) resulted in the selection of non-hydrogen fermenters.

In our study, the use of a suitable inoculum without methanogens and enriched in hydrogen-producing fermenters, as well as the appropriate OLR during operational start-up, led to an improvement in hydrogen production.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Lactic acid (mg/L)</th>
<th>Acetic acid (mg/L)</th>
<th>Butyric acid (mg/L)</th>
<th>Number of cells in diluted cheese whey (cfu/mL)</th>
<th>Number of cells entering the reactor (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW, as it arrived from the factory</td>
<td>7.17</td>
<td>1,047</td>
<td>186</td>
<td>356</td>
<td>$41 \times 10^5$</td>
<td>0.004</td>
</tr>
<tr>
<td>CW, stored at 4 °C one day after arriving from the factory</td>
<td>7.37</td>
<td>1,290</td>
<td>201</td>
<td>400</td>
<td>$40 \times 10^6$</td>
<td>0.04</td>
</tr>
<tr>
<td>CW, stored at 26 °C one day after arriving from the factory</td>
<td>4.77</td>
<td>7,260</td>
<td>317</td>
<td>336</td>
<td>$63 \times 10^7$</td>
<td>0.6</td>
</tr>
</tbody>
</table>
The F/M in our operation was 3 gCOD/gVSSd, which was close to the optimum reported by Hafez et al. (2010). This improvement could also be attributed to the non-selection of propionic fermenters and the absence of methanogenesis.

The quantity of hydrogen production obtained by these new conditions was close to that obtained by Venetsaneas et al. (2009). These authors operated a CSTR that was fed with unsterilised raw cheese whey at an OLR of 60 gCOD/Ld and an F/M of 7.4 gCOD/gVSSd and obtained a VHPR of 2.9 LH2/Ld and a hydrogen yield of 2.9 LH2/L cheese whey (0.89 mol H2/mol lactose). In both cases, the yield is far from the optimum theoretical yield.

Other authors have reported higher rates of hydrogen production using a similar substrate (dry powder cheese whey). In 2009, Davila-Vázquez et al. obtained a maximum VHPR of 16.9 LH2/Ld while operating at a high OLR (92.4 gLactose/Ld) and an F/M ratio of approximately 18 gLactose/gVSSd. Organisms from the Clostridium genus predominate in this reactor. The VHPRs achieved in this study were higher than the values previously reported and more than 20 times higher than that obtained in our work. This difference could be explained by the development of a stable microbial community with high predominance of organisms from the Clostridium genus. The higher OLR applied and the use of dry cheese whey added with micro- and macro-nutrients could contribute to the development of this high hydrogen production community.

In our reactor, the low yield could be associated with the prevalence of metabolic pathways with low hydrogen yields (such as mixed acid or butanediol fermentations associated with Enterobacteria) and the presence of fermenters without the capacity to produce hydrogen. In particular, organisms from the Prevotella and Dialister genera persisted in our reactor and were previously detected in other hydrogen-producing ecosystems (Shin et al. 2004; Kim & Shin 2008; Castelló et al. 2009). These fermenters may outcompete other organisms in the operation conditions applied to produce hydrogen (low pH, high HRT). A high variation in the microbial composition of the biomass was detected in our reactor, this could also explain the variation in the hydrogen production, there is not a predominant organism and we detect fermenters with different metabolic pathways. The hydrogen production will depend on the prevalence of the hydrogen producing fermentation pathways.

The organisms added with the raw cheese whey used in the feeding could cause the dilution of the biomass in the reactor and the reduction in the amount of lactose available for hydrogen production. This effect could be significant, especially in reactors that are operated at a high F/M (gCOD/gVSSd).

CONCLUSIONS

Kitchen waste compost is a suitable and low cost source of inoculum to seed UASB hydrogen producing reactors, avoiding the high energy requirements of heat treatments. With this inoculum and using a high OLR during start up, methanogenesis and propionic fermentation persistence was avoided. The UASB reactor that was demonstrated to be suitable for hydrogen production, presented a biomass with positive settling properties with no problems to be retained in the reactor.

Although the performance was better than in our previous work, the yield obtained was still lower than other work using similar substrates. This low yield could be explained by selection of a mixed fermentative population with presence of hydrogen producing organisms (Clostridium, Ruminococcus and Enterobacter) and other non-hydrogen producing fermenters (Lactobacillus, Dialister and Prevotella). Further work is necessary to understand the competition among these fermenters and to determine if it is possible to use this raw effluent to select the microorganisms with the most efficient metabolic pathways.

The microorganisms added during the feeding were characterised as belonging to the genera Buttiauxella (a low-yield hydrogen-producing fermenter) and Streptococcus (a lactic acid fermenter). Although these organisms were not dominant in the reactor biomass, they could compete for the substrate and decrease the hydrogen yield. This negative effect should be evaluated so that this technology can be applied to industrial wastewaters.

Cheese whey treatment in two stages, an acidogenic phase with hydrogen production and a methanogenic step in two UASB reactors could be a feasible alternative for real scale application that should be further studied to improve the efficiency.

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