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Technical Note

Elucidation of the thermophilic phenol biodegradation pathway via benzoate during the anaerobic digestion of municipal solid waste

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HIGHLIGHTS

- Phenol can be completely biodegraded during thermophilic anaerobic digestion.
- Benzoate was identified as metabolite during anaerobic digestion of phenol at 55 °C.
- Two kinds of degradation and gas production kinetics were observed.

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ABSTRACT

Anaerobic digestion makes it possible to valorize municipal solid waste (MSW) into biogas and digestate which are, respectively, a renewable energy source and an organic amendment for soil. Phenols are persistent pollutants present in MSW that can inhibit the anaerobic digestion process and have a toxic effect on microbiota if they are applied to soil together with digestate. It is then important to define the operational conditions of anaerobic digestion which allow the complete degradation of phenol. In this context, the fate of phenol during the anaerobic digestion of MSW at 55 °C was followed using an isotopic tracing approach ($^{13}C_6$-phenol) in experimental microcosms with inoculum from an industrial thermophilic anaerobic digester. With this approach, it was possible to demonstrate the complete phenol biodegradation into methane and carbon dioxide via benzoate. Benzoate is known to be a phenol metabolite under mesophilic conditions, but in this study it was found for the first time to be a phenol degradation product at thermophilic temperature.

1. Introduction

Anaerobic digestion (AD) for the treatment of municipal solid waste (MSW) is a process by which microorganisms break down biodegradable material in the absence of oxygen, creating value by transforming the waste into biogas and digestate, which constitute respectively a renewable energy source and an organic amendment for soil.

The MSW can contain phenols which are organic micropollutants that exhibit toxic properties. At high concentrations such compounds can inhibit organic fraction degradation, stopping the process of degradation and the biogas production (Fedorak and Hrudey, 1984; Hernandez and Edyvean, 2008). Moreover, if the degradation of phenol is not complete, the digestate used as fertilizer can contain this pollutant (Angelidaki et al., 2000; Levén et al., 2006) and have adverse effects on soil bacteria, representing a risk for the environment and human health. In this context, it is necessary to make sure that phenol is completely degraded into methane, thus avoiding the methanization inhibition and assuring that digestates contain no phenol when introducing into the soil ecosystem.

Up to now, several studies have shown that phenol degradation at 55 °C under methanogenic conditions was possible (Karlsson et al., 1999; Fang et al., 2005; Levén et al., 2006; Chen et al., 2008; Limam et al., 2013), but the knowledge regarding the thermophilic phenol metabolic pathway is limited due to the fast degradation rates which hamper metabolite accumulation and pathway elucidation. Nevertheless, today it is generally accepted that thermophilic phenol degradation occurs via caproate (Fang et al., 2005) and not through the benzoic acid pathway, generally used in the mesophilic condition (Knoll and Winter, 1987, 1989; Béchard et al., 1990; Gallert et al., 1991; Karlsson et al., 1999; Levén and Schnürer, 2005; Zhang et al., 2005). This study is focused on the elucidation of the phenol fate and metabolite identification during the AD at 55 °C. The elucidation of some intermediate metabolites of phenol degradation will shed light on the ways to improve phenol degradation in bioreactors under thermophilic methanogenic conditions.

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2. Materials and methods

2.1. Chemicals

The ethyl acetate, methanol, acetone, acetic anhydride, potassium bicarbonate, mercuric chloride and sodium chloride were obtained from Supelco (St. Quentin Fallavier, France). All the solvents and reagents were of analytical degree, and ultrapure water from Milli-Q system (Eschborn, Germany) was used throughout the experiments. The $^{13}C_6$-phenol (99%) was purchased from EuroIsotope Laboratory (France).

2.2. Inoculum preparation

The inoculum used in this study was prepared by the centrifugation of five fractions of 50 mL of an anaerobic sludge from thermophilic municipal household waste digesters. The centrifugation was carried out at 4 °C for 10 min at 8500 rpm (Allegra TM X-22 R Centrifuge, Beckman Coulter, France). Finally, 2% (volatile solids) were inoculated in each microcosm for the $^{13}C_6$-phenol study.

2.3. Microcosm studies

Two types of experimental microcosms, with and without MSW, were incubated under thermophilic conditions.

The microcosms containing MSW "waste-incubations" consisted of 5 330 mL glass bottles (Fischer Scientific Bioblock, Illkirch, France) filled with 210 mL of $NaHCO_3$ buffer solution at pH = 8.3 and 10 g of reconstituted French Solid Waste (MODECOM Composition) (Qu et al., 2009). The inoculum was then simultaneously added to the bottles, which were closed with a screw cap and a septum (Fischer Scientific Bioblock, Illkirch, France), and a nitrogen atmosphere was established (anaerobic treatment). The biogas composition (CH_4 and CO_2) was analyzed immediately after equilibration by connecting the bottle to a gas chromatograph (CP 4900 Micro-GC, Varian, France) equipped with two parallel chromatographic columns coupled to thermal conductivity detectors (TCD). The cumulative biogas production was monitored until it reached stable methanogenesis, after 140 d. $^{13}C_6$-phenol was then injected into the microcosms. 1.5 mg of phenol was added to each microcosm in order to obtain a concentration of 7200 μg L^{-1}. Three of the five microcosms were incubated with $^{13}C_6$-phenol. In one of them 300 mg of $HgCl_2$ was diluted in 15 mL sterilized water and later added to inhibit the biological activity (Tuominen et al., 1994). In another microcosm $^{12}C_6$-phenol was added to monitor the biogas isotopic composition when no enriched substrate was added. No phenol was added to the remaining microcosm.

The second type of microcosm contained no reconstituted MSW "non-waste incubations" but only the inoculum and the biochemical methane potential buffer (BMP) (ISO 11734, 1995) in order to evaluate the potential of the inoculum to degrade phenol. This set of microcosms also had 5 incubations, 3 with $^{13}C_6$-phenol (in one of them $HgCl_2$ was added to inhibit biological activity), one with natural phenol and the last with no phenol. The total volume of these incubations was 40 mL in 50 mL glass bottles.

The microcosms were then incubated in the dark at 55 °C under methanogenic conditions for 60 d for "waste-incubations" and 236 d for "non-waste incubations." Liquid samples (1 mL) were retrieved 3 times a week from the different microcosms in order to analyze the $^{13}C_6$-phenol.

2.4. Analytical methods

During the incubation time, the isotopic composition of biogas (CH_4 and CO_2) was measured by GC–IRMS. Moreover, a quantitative analysis of $^{13}C_6$-phenol was performed by GC–MS.

2.4.1. $^{13}C_6$-Phenol

Phenol concentrations were determined following a published analytical procedure (Limam et al., 2010). Briefly, after the dilution of 100 μL of the samples in 2 mL Milli-Q water, the obtained diluted sample was introduced into a 20 mL PTFE-capped glass vial, and 0.8 g of sodium chloride, 0.08 g $KHCO_3$ and 15 μL of acetic anhydride were added. A reaction time of 5 min at 40 °C was then necessary to obtain a complete derivatization reaction prior to the extraction step. The headspace extraction of derivatized $^{13}C_6$-phenol was performed with a 65 μm polydimethylsiloxane–divinylbenzene-solid phase microextraction (SPME) fiber at 40 °C, with a 500 rpm for 30 min. Each head-space extract was analyzed with a GC–MS. MS was operated in the electron impact ionization mode with a scan range of m/z 50–400 at 3.88 scans s^{-1}. The quantification was performed on the selected ion monitoring mode. Quantification ions were m/z 100 for $^{13}C_6$-phenol and m/z 99 for $^{12}C_6$-phenol. Xcalibur Software from Thermo Fisher Scientific was used for online data acquisition and processing.

2.4.2. $^{13}C_6$-phenol metabolites

$^{13}C_6$-phenol metabolites were obtained by a liquid–liquid extraction of 500 μL microcosm liquid phase. The pH of each sample was maintained at a value above 12 for 30 min by the stepwise addition of 1 N NaOH to hydrolyze putative thioester bonds. Each sample was then acidified to a pH below 2 with 12 N HCl. The samples were then extracted 3 times with 2.5 mL of ethyl acetate. The three fractions of ethyl acetate extracts were combined, dried through anhydrous sodium sulfate, and then concentrated to a volume of 500 μL under a stream of nitrogen gas. The ethyl acetate extract was then derivatized with N,O-bis-(trimethylsilyl)trifluoroacetamide. Each concentrated and derivatized extract was analyzed with the GC–IRMS system. Chromatographic conditions were the same as described above in $^{13}C_6$-phenol section. The elucidation of the chemical structure of the ^{13}C-labeled metabolites identified by the GC–IRMS analysis was carried out by a GC–MS using the same chromatographic conditions as for the GC–IRMS analysis.

2.4.3. Biogas isotopic composition

The biogas analysis of the CO_2 and CH_4 isotopic composition of carbon was carried out by injecting 10 μL of the headspace gas from each incubation, using a 100 μL Pressure-Lock gas tight syringe in a GC–IRMS system (Thermo Fisher Scientific, France). The GC–IRMS system consisted of a Trace GC Ultra attached to a Delta V plus isotope ratio mass spectrometer via a Finnigan GC combustion III unit. Typical $\delta^{13}C$ uncertainties, quantified by repeated measurements of a referential CH_4 and CO_2 mixture of a known isotopic composition, were ±0.1‰ for both gases.

3. Results and discussion

3.1. Anaerobic biodegradation of $^{13}C_6$-phenol under thermophilic conditions – incubations without solid waste (non-waste incubations)

In such incubations only the inoculum was present in the incubation medium. Hence, it was possible to measure the potential of a thermophilic MSW biodigester inoculum to degrade phenol.

The remaining $^{13}C_6$-phenol concentration over time is represented in Fig. 1a. Firstly, it was observed that phenol concentration for the abiotic incubation "Abiotic13C" remained stable over time without any diminution, whereas in the biotic ones the concentration decreases. This demonstrates that in the incubations the biological activity is responsible for phenol degradation. This result is in agreement with previous studies (Limam et al., 2013) where phenol biodegradation was observed at 55 °C but diverges from