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Research article

Key microbial populations involved in anaerobic degradation of phenol and p-cresol using different inocula

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ABSTRACT

Background: Anaerobic digestion is an alternative bioprocess used to treat effluents containing toxic compounds such as phenol and p-cresol. Selection of an adequate sludge as inoculum containing an adapted microbial consortium is a relevant factor to improve the removal of these pollutants. The objective of this study is to identify the key microorganisms involved in the anaerobic digestion of phenol and p-cresol and elucidate the relevance of the bamA gene abundance (a marker gene for aromatic degraders) in the process, in order to establish new strategies for inocula selection and improve the system's performance.
Results: Successive batch anaerobic digestion of phenol and p-cresol was performed using granular or suspended sludge. Granular sludge in comparison to suspended sludge showed higher degradation rates both for phenol (11.3 ± 0.7 vs 8.1 ± 1.1 mg l^{-1} d^{-1}) and p-cresol (7.8 ± 0.4 vs 3.7 ± 1.0 mg l^{-1} d^{-1}). After three and four re-feedings of phenol and p-cresol, respectively, the microbial structure from both sludges was clearly different from the original sludges. Anaerobic digestion of phenol and p-cresol generated an abundance increase in *Syntrophorhabdus* genus and bamA gene, together with hydrogenotrophic and aceticlastic archaea. Analysis of results indicates that differences in methanogenic pathways and levels of *Syntrophorhabdus* and bamA gene in the inocula, could be the causes of dissimilar degradation rates between each sludge.
Conclusions: Syntrophorhabdus and bamA gene play relevant roles in anaerobic degradation of phenolics. Estimation of these components could serve as a fast screening tool to find the most acclimatized sludge to efficiently degrade mono-aromatic compounds.

1. Introduction

Phenols are the major organic constituents in effluents of coal conversion processes, coke ovens, petroleum refineries, phenolic resin manufacturing, herbicide manufacturing, fiberglass manufacturing and petrochemicals [1]. These pollutants are harmful for the environment, toxic to organisms and recognized as carcinogenic compounds [2]. Different biological technologies are available to treat effluents containing such compounds, one of these technologies is the anaerobic digestion.

The anaerobic digestion process is a complex procedure that involves different stages, each one catalyzed by different consortia of microorganisms. These steps involve the hydrolysis of complex molecules to monomers, which is followed by the steps of acidogenesis, acetogenesis, and methanogenesis [3].

The anaerobic technology has been implemented to treat phenols containing effluents due to the advantages it offers over other biological operations: withstanding high organic loading rates and low sludge generation, in addition to energy production [4]. Despite this, the stability and efficiency of anaerobic digestion depend on the microbial population, the biodegradability of the compounds and chemical characteristics [5]. The anaerobic treatment of wastewater containing toxic pollutants, can present low degradation rates of organic compounds, due to bacterial activity inhibition. Thus, the need of having bacteria with robust physiology is critical to the stability of the biological treatment process, because they can efficiently facilitate the removal of refractory organics, mitigate wastewater toxicity and thus, improve wastewater biodegradability [6].

A way to have bacteria with robust physiology and activity, during anaerobic process, is by choosing the proper sludge that is going to be used as inoculum when starting the system, which should contain an adapted microbial consortium for a specific substrate. It has been reported that inoculum source is important for starting up anaerobic digesters, especially when treating organic compounds with low

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biodegradability [7]. For systems that work with retained biomass, such as sequential batch reactors (SBRs), it has been demonstrated that the inoculum composition determines the microbial functions in terms of chemical product generation [8], that is, different inocula have different performances under the same operating conditions. According to this, the inoculum selection process to carry out the anaerobic digestion of phenols is relevant to establish an efficient process.

Different molecular approaches based on the analysis of 16S rRNA gene like fingerprinting, high throughput sequencing and quantitative PCR (qPCR) methods, have been used in order to identify the microbial populations present in anaerobic sludge on reactors treating phenolic compounds [4,9,10,11,12]. However, there are no studies regarding anaerobic digestion of phenols in which different sources of inocula are analyzed microbiologically and compared, in order to determine which are the microorganisms that effectively contribute to a better performance of the process.

Apart from 16S based methods, used to identify and estimate the microorganisms responsible for anaerobic degradation of phenols, complementary information can be gained by studying key functional genes [13].

In the anaerobic degradation of mono aromatic compounds (like phenol and p-cresol), via the 4-hydroxybenzoate to benzoyl-CoA pathway, the ring cleavage step of 6-oxocylcohex-1-ene-1-carbonyl-CoA is catalyzed by a hydrolase encoded by bamA gene [14,15]. This gene has been used as a biomarker of aromatic-degrading anaerobes under different redox conditions and has been correlated positively with the amount of degraded mono aromatic compounds, like toluene [16]. Therefore, the bamA gene amounts on different inocula could be related with different performances of these in terms of phenol degradation capabilities.

Based on the above discussion, the objective of this study was to evaluate the adaptation process of different inocula during the anaerobic digestion of phenol and p-cresol and determine, by high throughput sequencing of the 16S rRNA gene, what microorganisms are involved in the anaerobic digestion process of these compounds. In addition, the bamA gene was quantified in order to elucidate if initial amounts on the inocula are determinants of anaerobic digestion performance.

2. Material and methods

2.1. Experimental set-up

Batch anaerobic digestion tests were performed at 37 ± 1°C by adding mineral medium [4] and 183 ± 23 mg l^{-1} of phenol (three successive batches) or 106 ± 7 mg l^{-1} p-cresol (four successive batches) in 500 ml bottles. The concentrations of phenol and p-cresol chosen were similar to those used previously by Fang and Zhou [17] to emulate a wastewater containing medium-strength phenolic pollutants. As inoculum 4 g l^{-1} of volatile suspended solids (VSS) of sludge obtained from an up-flow anaerobic sludge blanket (UASB) reactor treating tobacco industry effluents or a continuous stirred-tank reactor (CSTR) treating aerobic sludge from a municipal treatment plant was used. The successive batches were carried out by re-feeding the inoculum with phenol or p-cresol once previous addition of the aromatic compound was depleted. Assays were performed in triplicate and included a negative control where only mineral medium was added.

2.2. Analytical methods

Degradation of phenolic compounds and methane production was monitored by sampling the liquid and gaseous phase, respectively every 1–3 d. Phenol and p-cresol concentration in liquid samples (1 ml) was determined by high performance liquid chromatography (HPLC) with an UV detector (Perkin Elmer, 200 series). The column used for chromatographic separation of compounds was a C_{18} Interstil® ODS 3 (250 x 4.6 mm, 5 um, GL Science Inc.). The mobile phase was composed of Milli Q water:acetonitrile, 50:50 *v*/v running at 25°C with a flow rate of 1.5 ml min^{-1}. The eluate was monitored at 280 nm and quantification was made by the external standard method using phenol and p-cresol as standards (Sigma-Aldrich, 99%).

2.3. Maximum rates of phenolic degradation and methane production

Maximum rates of phenolic degradation were estimated on the first and last batch of phenol and p-cresol by calculating the maximum slopes obtained from degradation curves over time for each compound.

2.4. Sludge sampling and total DNA extraction

Sludge samples were taken before inoculating the bottles (inoculum) and after the first and last batch for each compound. DNA was extracted from 0.5 g of pellet obtained after centrifuging the samples at 10,000 × g for 10 min at 4°C using the Power Soil DNA Isolation Kit (MoBio) following the manufacturer's guidelines. For sequencing analysis, extracted DNA from triplicate bottles were mixed in equal volumes in order to establish the mean microbial community present on samples.

2.5. 16S rRNA amplicon gene sequencing

The 16S rRNA gene V4 variable region PCR primers 515/806 [18] with barcode on the forward primer were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products are checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Samples are pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples are purified using calibrated Ampure XP beads. Then the pooled and purified PCR product is used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were joined, depleted of barcodes then sequences <150 bp removed, sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes [19].

2.6. qPCR of bamA gene and Syntrophorhabdus

The quantification assay was conducted in an AriaMX real-time PCR cycler (Agilent) using the following primer sets: Bam-sp9/Bam-asp1 for *bamA* gene [11] and primers 5′-GAAAGCCTGACCCAGCG-3′ (forward) and 5′-CCCACCTTCCTCCGCATTAA-3′ (reverse) for *Syntrophorhabdus* genus quantification [20]. Each 20 μl PCR reaction contained 10 μl of Takyon Rox SYBR MasterMix dTTP Blue (Eurogentec), 0.9 μM of each primer and 2 μl of DNA template. Thermal program consisted of an initial denaturation (95°C, 3 min) and 44 cycles of amplification (95°C, 3 s; 60°C, 40 s). Melting curves were constructed from 65°C to 95°C, read every 0.5°C for 5 s. Calibration curves (10^0–10^6 gene copies μl^{-1}) were prepared using different standards. Genomic DNA from *Thauera aromatica* (DSM 6984) was used to quantify *bamA* gene assuming a genome size of 4.6 Mb [21] and one copy number of this gene per genome. *Syntrophorhabdus* genus was quantified using as standard

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