Short Communication

Two amplicon sequencing strategies revealed different facets of the prokaryotic community associated with the anaerobic treatment of vinasses from ethanol distilleries

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HIGHLIGHTS

- Diversity of a microbial consortium from anaerobic digestion of vinasses is studied.
- DNA amplified from the V4-16S region by two different sets of primers is compared.
- Each set of primers detected a limited diversity of the vinasses digesting consortia.
- One set of primers identified *Archaea* whilst the other set captured more diversity.

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ABSTRACT

The prokaryotic consortium from a pilot-scale UASB reactor fed with vinasses from ethanol distilleries was evaluated by means of amplicon sequencing of the 16S rRNA gene. Two different sets of primers targeted to overlapping regions of the V4-16S region were used to gain a broad picture of such community and to perform a comparative analysis. From the two datasets obtained, prevalent phyla were *Firmicutes*, *Verrucomicrobia* and *Thermotogae*. Interestingly, one set of primers captured variability in both the bacterial and archaeal portions of the community, whilst the other one revealed a more diverse community structure, but only in the Bacteria domain. Although a certain level of agreement between the two strategies was observed, sharp differences indicate that different facets of the community were disclosed by each approach.

1. Introduction

In Northwest Argentina there are a growing number of large-scale distilleries integrated into sugar mills for alcohol production. Following molasses fermentation by yeasts, vinasses are a highly polluting waste typically produced in the range of 9–14/l of ethanol obtained and as a result, enormous amount of wastes frequently remains poorly treated in the region causing serious environmental problems. The anaerobic fermentation is an effective treatment technology since it improves both the economic and ecological results of biomass utilization (Dererie et al., 2011).

In the present work we examined the structure of the prokaryotic community performing the anaerobic digestion of a pilot scale up flow anaerobic sludge blanket type reactor (UASB) fed with vinasses by amplicon sequencing of the 16S rRNA gene, a methodology that has proven to be a valuable tool to get a glimpse of microbial communities in several environments (Liu et al., 2008). As a second goal, a comparison between two community profiles produced by two alternative strategies was performed. Indeed, two sets of primers were used to target different yet overlapping sequences of the hypervariable region V4 of the 16S rRNA gene to achieve a meaningful coverage of the prokaryotic diversity. The resulting data are thus comparable and provide an a priori similar taxonomic utility. Furthermore, among several dispersed regions of the bacterial 16S rRNA gene, the V4 region was found to be particularly informative for short reads sequencing studies (Cole et al., 2009; Bergmann et al., 2011; Ghyselinck et al., 2013).

To date, microbial consortia from a number of anaerobic digestion processes have been analyzed using next generation sequencing (NGS) platforms (Nettmann et al., 2010; Rademacher et al., 2012). However, wastes from ethanol distilleries have not been similarly examined. With the exception of a global 16S rRNA gene library of a laboratory scale reactor (España-Gamboa et al., 2012),

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this is the first pyrosequencing approach to evaluate the microbial consortium growing with vinasses as the sole substrate, by two complementary approaches.

2. Methods

2.1. Reactor operation and wastewater description

Sludge samples were obtained from an anaerobic digester type UASB with a working volume of 180 l. The reactor was configured for continuous operation with recirculation, thus modifying it to an expanded granular reactor with an ascending rate of 1 m h⁻¹. The inoculum used for setting up the processes was municipal household waste. The digester's working parameters were temperature 30 °C and pH 6.55. Operation was stable at a pH value of 6.55, and occasional acidification caused by volatile fatty acid (VFA) accumulation was controlled by adjusting the influent pumping. (For a detailed description of the reactor set up see the Supplementary material.)

2.2. Total community DNA preparation and amplicon sequencing of metagenomic DNA

The activated sludge samples were taken from three sampling points of the reactor (at 1.00, 2.00, and 2.50 m) and were mixed immediately after collection. After centrifugation for 15 min at 7000×g, the sludge pellets were washed three times with PBS (Phosphate Buffered Saline) plus EDTA 10 mM and stored at −20 °C until processing. Aliquots of the pellets were used for total community DNA extraction using the MO BIO PowerSoil® DNA Isolation Kit. DNA concentration and integrity were estimated spectrophotometrically and by gel electrophoresis. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using two sets of primers (named RK and RDP) modified for 454 pyrosequencing (Table S1). PCR amplifications and sequencing services were carried out on a Genome Sequencer FLX (Roche Applied Science) at the INDEAR genome sequencing facility (Argentina).

2.3. Sequence analyses, graphs and other statistical analyses

Analyses were performed using QIIME software package (Caporaso et al., 2010) with slight variations of the standard pipeline, using default parameters and options of the scripts unless specifically stated. In short, reads were assigned to each strategy (RK or RDP) and then were clustered into Operational Taxonomic Units (OTUs). Representative sequences were aligned using PyNast (Caporaso et al., 2010) and chimeric sequences were removed with ChimeraSlayer. Alpha diversity metrics Chao, Shannon and PD_distance, and rarefaction curves were calculated as implemented in QIIME. Taxonomical characterization of each OTU was performed with the 'RDP classifier' using the version of the Ribosomal Database Project database (Cole et al., 2009). Phylogenetic inference was performed using PhyML (Guindon et al., 2010). (For a detailed version see the Supplementary material.)

3. Results and discussion

3.1. Microbial consortium according to RDP and RK amplicon sequencing schemes

A strategy using different library construction schemes with two sets of primers was applied in order to select the methodologies to explore the prokaryotic community from an anaerobic digester fed with vinasses from ethanol distilleries. One set, RDP, is well defined by the online matching tool RDP probe match (Table S1). The second set, RK, was selected since it has shown to achieve good coverage with no significant bias against several bacterial and archaeal groups (Bates et al., 2011; Bergmann et al., 2011). The sequences amplified covered overlapping regions of the V4-16S rRNA region and produced reads with an average length of 220 and 270 bp, where RDP amplified sequences are nested within RK ones (Table S1). A total of 3048 V4-16S rRNA gene sequences were obtained after quality trimming, filtering and chimera detection. Of these, 1803 reads resulted from amplicon sequencing using RDP primers and 1246 from the RK primers. Fig. 1a and b show prevalent phyla detected in the UASB sampled. The RDP primers produced sequences that matched almost exclusively to the Bacteria domain (with the exception of 3 reads). Instead, RK primers revealed a population comprising 82% of *Bacteria* and 17.8% of *Archaea*, as was observed in other microbial communities from anaerobic digesters (Schlüter et al., 2008; Rademacher et al., 2012) and consistent with the decreased organic content of the treated waste obtained (data not shown).

Most bacterial groups were differentially detected by each strategy. However, for both datasets, prevalent phyla were *Firmicutes*, *Verrucomicrobia* and *Thermotogae*. On the other hand, *Synergistetes*, *Choloroflexi*. *Bacteroidetes* and *Proteobacteria* occurred less frequently. Minor populations, grouped as other bacteria, included *Spirochaetes*, *Panctomycetes* and MPV-15 (⩽1%) as well as *Acidobacteria*, *Amatimonadetes*, *Cyanobacteria*, *Deferribacteres*, *Elusimicrobia* and *Lentisphaerae* (Fig. 1a and b). Most of the reads were assigned at the class rank; only a small number of reads could be classified at the genus rank, which is a limitation commonly reported for 16S rRNA based NGS studies (Liu et al., 2008; Rademacher et al., 2012).

3.2. Bacterial representatives involved in the anaerobic vinasses digestion

Firmicutes was noted as the dominant phylum when total diversity is considered. However, this phylum was unequally detected, comprising 40.3% and 12.4% of assignments in the RDP and RK-based reads, respectively (Fig. 1a and b). *Clostridiales* were mostly identified while *Bacilli* were barely represented by the order *Lactobacillales*, thus seem to play a less important role in this anaerobic consortium.

An unexpectedly high abundance of the phylum *Verrucomicrobia* was observed. Its members were similarly detected by both amplicon sequencing schemes applied (36.1% and 35.0% from the RK and RDP-based reads, respectively). *Verrucomicrobia* are rarely cultured yet are commonly detected in metagenomic libraries from various environments. Among well-studied members of this phylum, different fermentation patterns leading to acetate over of propionate production were observed when *Opitutus terrae* was grown in a mixed culture with a hydrogenotrophic partner (Chin and Janssen, 2002). Accordingly, the *Verrucomicrobia* representatives might contribute to the acidogenic stages in the UASB sampled here, and some members might also establish a syntrophic association with *Archaea* for acetate oxidation. Moreover, Bergmann et al. (2011) described a higher frequency of *Verrucomicrobia* in soils analyzed using the RK primers. These observations might also explain the high abundance of *Verrucomicrobia* observed.

Thermotogae and *Synergistetes* phyla exhibited abundance rates of 11.9% and 6.7%, and 10.4% and 1.2% from the RK and RDP-based reads, respectively (Fig. 1a and b). The thermotogales comprised 8.63% of the total number of sequences. All of them were clustered in only one OUT and taxonomically related *Kosmotoga* spp. (Fig. 2). This could simply be explained by its dominance, but also by the fact that there are a limited number of cultivated species from this taxon (Ben Hania et al., 2013). The thermotogales detected might