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# Study of microbial community plasticity for anaerobic digestion of vegetable waste in Anaerobic Baffled Reactor

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ABSTRACT

Anaerobic baffled reactor (ABR) provides a selective environment for the microbial community and their respective metabolic activities, which supports the physiochemical conditions required for an optimal performance of reactor. Hydrolysis and methanogenesis are rate limiting steps of anaerobic digestion which are very sensitive to changes in pH. Effluent recirculation provides buffering environment as well as prevents loss of some methanogenic population. In the present study, we used four chambered (C-1, 2, 3, and 4) anaerobic baffled reactor treating vegetable waste under three operating conditions (OCs); no effluent recirculation (OC I), 25% effluent recirculation (OC II), 100% effluent recirculation (OC III) and studied changes in microbial diversity along with selected parameters. OC I showed dominance of Bacteroidetes and Firmicutes in C-1 while remaining chambers were dominated by Proteobacteria, Bacteroidetes, Thermotogae, Spirochaetes and Chloroflexi. This demonstrated that the hydrolytic and fermentative taxa colonized chamber C-1 while syntrophic acetogenic population dominated the remaining chambers. However, a drastic change was observed during OC III, advocated by an increase in diverse population from Firmicutes and Actinobacteria in all chambers. Our results suggest plasticity in microbial population, which could ensure a better reactor performance under different OCs in ABR for methanogenesis.



## 1. Introduction

Anaerobic digestion (AD) is an alternative and efficient biological process of converting variety of organic wastes to renewable bioenergy with one of the management option [1]. Vegetable waste (VW) constitutes one of the major organic fractions of Municipal Solid Waste (MSW) having high moisture and organic content, which contributes to atmospheric pollution and green house gases [1,2]. Due to its high biodegradability, VW is prone to rapid acidification resulting in an accumulation of acids in the AD system, which inhibits the biogas production [1,3]. High efficiency in AD process can be achieved by understanding the microbial community dynamics, leading to sustainable $CH_4$ production. The AD process involves hydrolysis of polymeric organic materials to intermediate organic acids, which get converted to volatile fatty acids (VFAs) which serve as a substrate for methanogenesis. The microbial communities associated with these different steps have different growth rates and substrate requirements. Hence, overall performance towards stable $CH_4$ production can be achieved through physiological balance among the different communities. Various strategies have been deployed to achieve digester stability and better performance at higher organic loading rates of fruit and vegetable wastes (FVW) such as, addition of trace elements like tungsten [4], recirculation of reactor effluent in order to maintain pH balance of system [5,6] and co-digestion with high nitrogen and phosphorus containing wastes, which provides an optimum ratio of lignocellulose, proteins and fats [7–9].

Overcoming these problems, an Anaerobic Baffled Reactor (ABR) design was reported for digestion of a variety of low strength waste waters [10] and organic solid wastes such as FVW, kitchen waste and MSW [3,11–13] by providing an optimum condition for satisfying the requirements of each group of microbes. An ABR is

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designed to compartmentalize hydrolytic and organic acid producing bacteria from methanogens [14] with multiple chambers separated by a series of vertical baffles through which the liquid moves upward and downward along the reactor. Phase separation is the most significant advantage of ABR, which allows biomass retention, organic component concentration gradient, provides opportunity to different microbial groups for specific activity in each compartment and ultimately separates the hydrolysis/fermentation process from acetogenesis/methanogenesis [11,15]. Moreover, this spatial separation helps to keep away the more sensitive anaerobic population (acidogenic and methanogenic) from unfavorable environments in the initial compartments of the reactor [5].

To improve the reactor performance, a detailed understanding and knowledge of microbial community structure and its associated metabolic activities in AD process is required. Here, microbial plasticity in AD refers to variation in its structure and composition in response to available environment and substrate. The ABR design shapes the distribution curve of microbial communities involved in key steps of methanogenesis across the chambers supported by functional stabilization in different stages of AD [12]. The objectives of the present study were i) to look for identification of the hydrolytic and fermentative microbial populations present according to ABR design using Illumina sequencing; ii) to examine the applicability of effluent recycling on microbial plasticity throughout the chambers of ABR and whether they play a significant role in enhancing the reactor performance.

## 2. Materials and methods

### 2.1. Inoculum

Vegetable waste (VW) comprising of cabbage, tomato, capsicum, bitter gourd, radish leaves, cauliflower leaves and fenugreek leaves mixed in appropriate proportions, as described previously [13] was used as a substrate in the study. VW was shredded, grinded for 5–7 min using kitchen mixer-grinder resulting in a semisolid paste with particle size <3 mm, and stored at 4 °C until used. The characteristics of VW were: total solids (TS)- 8.9%; volatile solids (VS)- 77.2%; carbon (C)- 41.3%; nitrogen (N)- 4.0%; hydrogen (H)- 4.2%; and sulfur (S)- 1.2%. Inoculum consisted of anaerobic sludge from a food processing industry and residue from cow-dung digester (mixed in a 1:1 ratio) and having 5.1% TS; 68% VS; 37% C; 3.8% N; 4% H and 1% S.

### 2.2. Bioreactor design and setup

An anaerobic baffled reactor (ABR) with 39 L working volume was used in the study which consisted of four chambers (C-1, C-2, C-3, and C-4) with design, setup and operating conditions similar to that described previously [13]. Initially, reactor was seeded with inoculum for a week, followed by daily feedings at organic loading rate (OLR) of 0.5 g VS/L/d with a hydraulic retention time (HRT) of 30 d using mixed vegetable waste (VW) as a substrate. The overall anaerobic digestion was performed in mesophilic (30 ± 5 °C) condition. The study was divided into two parts; i) to investigate the bacterial diversity in the four chambers of ABR after achieving a stabilized state of operation wherein the reactor was fed with VW slurry prepared in tap water as diluent (operating condition I-OC I); ii) to study the effect of effluent recycling on bacterial diversity in these chambers under conditions wherein VW slurry was prepared using 25% and 100% effluent from chamber C-4 of ABR as diluent (operating condition II and III- OC II & OC III respectively). Under all these conditions, the amount of VW taken was the same so as to achieve constant VS of substrate (0.5 g L/d) and only the diluent used for preparing the feed slurry was different (either tap water or effluent from 4th chamber).

Following parameters were estimated to indicate the performance efficiency of the reactor viz., pH, TS, VS, Total and Soluble COD (TCOD, SCOD), which were analyzed by Standard Methods [16]. Elemental composition (C, H, N, S) was determined by a CHNS analyzer (Elmentar, Vario EL III, Germany) while volatile fatty acids (VFAs), and biogas composition were estimated by methods described previously [13].

### 2.3. Metagenomic DNA extraction and sequencing

DNA from sludge was extracted at 90 d (OC I), 130 d (OC II) and 250 d (OC III), for constructing the 16S rRNA gene amplicon library. 1–2 ml sludge samples were centrifuged at 7000 *g* for 10 min at 4 °C, and supernatant was decanted carefully to obtain a sediment sample (500 mg net weight) for DNA extraction. Metagenomic DNA was extracted using the Fast DNA SPIN Kit for soil (MP Biomedicals, USA) following the manufacturer's instructions and then visualized on 1% gel electrophoresis. Its concentration and purity was determined by spectrophotometry (NanoDrop-1000, Thermo Scientific, USA). The bacterial diversity of four chambers of the reactor at three time points was identified by amplifying and sequencing the V3 region of the 16S rRNA gene from the metagenome. The amplicon consisted of primers, sequences adaptors, and dual-index barcodes. The V3 conserved region was targeted by using the forward primer 5′ CCTACGGGAGGCAGCAG 3′ and reverse primer 5′ ATTACCGCGGCTGCTGCTGG 3′ [17]. Libraries were normalized and sequenced on the MiSeq platform (Illumina).

### 2.4. Bioinformatics analysis

Illumina sequencing data were pair assembled using PANDAseq [18] with an assembly quality score of 0.9, which is the most stringent option to reduce errors. We then followed the Mothur Illumina MiSeq SOP [19] to align, filter, trim, remove chimeras, and classify and assign taxonomy. Sequences were aligned against the Greengenes reference sequences [20], and then the chimeric sequences were removed using UCHIME [21] but only after the preclustering command was run to remove erroneous sequences generated due to sequencing errors. Each operational taxonomic unit (OTU), defined at a ≥97% cutoff of sequence similarity, was classified based on the Greengenes database [20]. As read length for Illumina is more limited than that for pyrosequencing, OTU identity can most accurately be seen as indicating genus equivalents [22]. We performed all statistical analyses on a subsample of 12,000 reads per sample. There were a total of 12 samples (four chambers samples from each three time point) but we created 4 artificial subsamples from each sample at a size of 12,000 reads per sample leading to a total of 48. Richness and diversity indices and dissimilarity matrices (Bray-Curtis, and unweighted-UniFrac) were estimated using Mothur (Supplementary Table S1). All sequences obtained were deposited under the following GenBank SRA Accession numbers SRX709005, SRX709902, and SRX709903.

### 2.5. Statistical analysis

To obtain a complete randomized design (CRD) with four replications, we artificially created 4 subsamples from all 12 samples (using subsample command in Mothur [23] to yield a total of 48 samples. Two-way analysis of variance (ANOVA) was performed to determine the effects of operating conditions (no reactor effluent, 25% reactor effluent, and 100% reactor effluent) and chambers (C-1, C-2, C-3, C-4) on the bacterial community composition in the AD. All statistical analyses were performed using different packages in