

Microbial Dynamics in Laying Hen Waste and its Anaerobic Treatability at Mesophilic Temperature

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Abstract

Chicken litter consists of a large and diverse population of microorganisms. Wastes from poultry industries have been treated by anaerobic reactors for biogas production as a renewable energy source for many decades. In this study, anaerobic treatability of the chicken manure (total solids; TS \approx 5.5%) and its biogas potential were investigated. Moreover, the microbial diversity in the raw manure was also presented. According to the results; laying hen manure is an important renewable energy source and significant biogas productions might be possible from the farms producing high amounts of chicken wastes if they are managed/treated properly. Respective cumulative and average biogas productions of ca. 70 L and 650 mL per day were obtained from the bioreactor in about five months. The biogas yield was observed as ca. 0.47 L/gr VS_{added} although total ammonia nitrogen (TAN) concentration was measured around 2300 mg/L on average. The microbial diversity using Illumina Miseq sequencing revealed that *Bacilli*, *Clostridia*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, *Deinocci*, *Mollicutes*, *Thermotogae*, *Cyanobacteria* and *Archaea* were present in the raw chicken waste used in this study. Moreover, the most abundant two bacteria classes were identified as *Clostridia* and *Bacteroidetes* whereas *Bacilli*-like sequences were also characterized. A scarce sequence revealed similarity to *Archaea* (i.e., 3%) and pathogenic bacterial species were also identified in few numbers.

Keywords: Anaerobic treatment; Biogas; Chicken manure, Microbial diversity; Next Generation Sequencing

1. Introduction

Chicken litter is a mixture of feces, wasted feeds, bedding materials, and feathers [1]. More than 10 million tons of chicken litter is produced every year in Turkey, most of which is usually spread on land as a low cost organic fertilizer owing to its high nutrient content. Because, poultry manure contains significant amounts of nitrogen because of the presence of high levels of protein and amino acids [2]. Chicken litter is also the source of human pathogens, such as *Salmonella*, *Campylobacter jejuni*, and *Listeria monocytogenes*, that can potentially cause contamination that are frequently associated with foodborne outbreaks [3]. It was reported in several studies that chicken litter contains a large and diverse population of microorganisms. Gram-positive bacteria (e.g., *Actinomycetes*, *Clostridia/Eubacteria*, and *Bacilli/Lactobacilli*) account for nearly 90% of the microbial diversity that can reach up to 10¹⁰ CFU per gram of chicken litter. A variety of pathogens might be detected in chicken litter-based organic fertilizers, such as *Actinobacillus*, *Bordetella*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Escherichia coli*, *Globicatella*, *Listeria*, *Mycobacterium*, *Salmonella*, *Staphylococcus*, and *Streptococcus*. An investigation on pathogenic microorganisms in poultry litter was also performed with selective medium and it was found that *Staphylococcus xylosus* was the predominant species. Hence, for ensuring the absence of pathogens in the fresh chicken waste, poultry compost, or the physically heat-treated chicken litter; additional approaches such as physical, chemical, and biological treatments, should be considered for pathogen control [4-8].

Anaerobic digestion is a very efficient process for poultry litter producing a collectable biogas mixture with an average methane content of 60%. Yongabi et al. [9] designed a simple plastic anaerobic digester to disinfect the contaminated poultry feces while providing biogas and pathogen-free fertilizer. Following anaerobic digestion of poultry feces for 37 days, both coliform and *E. coli* counts decreased drastically. However, due to high nitrogen contents; the concentration of endogenous ammonia-nitrogen rises considerably during anaerobic digestion of poultry litter. While ammonium ions can be utilized by some anaerobic bacteria to a certain amount, an excess of ammonium might inhibit the destruction of organic compounds, the production of volatile fatty acids (VFAs) and methanogenesis [10, 11]. It was reported by Krylova et al. [12] that high levels of ammonium (>30 g/L) during anaerobic digestion of poultry litter also resulted in a decrease in the numbers of all physiological microbial groups as well as the composition of the methanogenic consortium changed. According to the authors, the NH₃ concentrations and VFAs also affected the dominant methanogenic cultures. For example, the dominance of *Methanosarcinaceae* in manure digesters was observed at high levels while *Methanosaetaceae* dominated in sewage sludge digesters with low levels of NH₃ and VFAs. This is attributed to

the fact that; acetate-utilizing methanogens offering thin filaments with a great surface seemed to be more sensitive to ammonia concentrations than hydrogenotrophic methanogens growing as rods (e.g., *Methanosarcinaceae* consisting of thick clumps). Hence, *Methanosaeta* is not observed as the dominant species in biogas reactors particularly treating manure like organic substrates [13-15]. In this respect; the chicken manure was often diluted prior to feeding to the system (i.e., to TS=3.00-8.25%) in order to eliminate the inhibition by reducing NH₃ concentrations in the feed. The highest biogas production rate was found to be 554 mL/gVS_{feed} while feeding chicken manure at the organic loading rate of 2.17 g VS/L.d (i.e., corresponding to 3.7% TS and 2.4% VS contents).

Determination of the structure of microbial communities in bioreactors is of importance for the achievement of high efficiencies during waste treatment. In this respect, development of culture independent molecular biological techniques of the 16S rRNA gene analysis facilitated investigations of microbial communities of waste treatment systems, where microbial diversity is extremely high. Application of molecular approaches to anaerobic reactors resulted in detection of the organisms related to both cultured and uncultured microorganisms [16]. Among them, microbial sequencing is a new tool in the field of molecular biology that has great potential for the development of environmental analysis. Because, microbial sequencing methods achieve high sequencing depth which makes controlling the changes in the structure of a microbial community possible with a more in-depth understanding especially in the bioreactors where biogas production occurs. Pyrosequencing, on the other hand, is an innovative next-generation sequencing (NGS) system with a promising position in environmental samples with remarkable genetic diversity. Recently, application of the Illumina sequencing technology has been accepted as the most successful and widely adopted NGS technology worldwide [17].

The main objective of this study was to investigate the anaerobic treatability of the diluted chicken manure and its biogas potential in a lab-scale semi-continuous anaerobic digester. Moreover, the microbial diversity in the raw manure was also identified using Illumina Miseq NGS and dominant cultures were presented.

2. Materials and Methods

Characterization of the chicken waste

Raw manure was taken fresh from a facility with a daily capacity of about 20000 eggs from 275,000 livestock. The waste produced in this industry was the manure from the laying-hen having average TS of ~28% (volatile content of ca. 56%). Before feeding the system; raw chicken manure was diluted with tap water in order to provide TS of ca. 5.5% according to an optimization study (data not shown). The composition of the diluted chicken waste used as the feed of the anaerobic digester was presented in Table 1.

Table 1. Characterization of the feed (diluted chicken waste)

Parameter	Unit	Value
TS	%	5.58
VS/TS	%	53
pH	-	8.00
Alkalinity	mg CaCO ₃ /L	15550
TAN	mg/L	2180
sCOD	mg/L	13325

Anaerobic reactor and the inoculum sludge

Anaerobic treatability was conducted in a N₂-flushed, 3 L glass reactor with an effective volume of 2.5 L at mesophilic condition (35°C) in a dark constant temperature room for about 5 months. The lab-scale bioreactor was operated in semi-continuous mode at a solids retention time of about 60 d. The granular inoculum sludge was taken from the mesophilic anaerobic Internal Circulation (IC) reactor treating the wastewater produced at a paper/cardboard industry. TS concentration of the granular seed was 95 g/L with a volatile solids (VS)/TS ratio of 50%. The reactor was inoculated in a 1:3 ratio (v/v).

Analytical procedure

The performance of the anaerobic reactor was investigated by measuring the following parameters both in the influent slurry and in the effluent digestate; Alkalinity, total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), TS, VS, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), and total ammonia nitrogen (TAN) concentrations according to Standard Methods [18]. Besides, daily biogas production was measured using the Ritter Milligas Counter 770991000 model gas meter (Ritter, Germany). pH measurements were done by using HI 2211-02 HANNA Model pH meter.

Molecular Analysis

The microbial diversity in chicken feces was analyzed using Illumina NGS. Total DNAs were isolated from the 1 mL sludge samples by using PureLink Genomic DNA extraction kits (Invitrogen, U.K.). NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the concentration. The V4-V5 hypervariable region of the 16S rRNA gene was reproduced with region-specific primers which were designed to contain Illumina adaptor and barcode sequences 518F-926R for bacteria, 518F-958R for archaea.

3. Results and Discussion

Microbial diversity in the raw manure

Although chicken litter is commonly used as a fertilizer; health and environmental concerns necessitate knowledge about the composition of the bacterial microflora present in the litter. The microbial community according to Illumina NGS analysis revealed that *Bacilli*, *Clostridia*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Alpha-proteobacteria*, *Beta-proteobacteria*, *Delta-proteobacteria*, *Epsilon-proteobacteria*, *Gamma-proteobacteria*, *Deinocci*, *Mollicutes*, *Thermotogae*, *Cyanobacteria* and *Archaea* were present in chicken fecal sample as seen in Figure 1. The most abundant class of bacteria (~21%) was identified as *Clostridia* whereas *Bacteroidetes* was the second most abundant (~18%) class of bacteria. Additionally, Bacilli-like sequences characterized ~16% of the bacteria classes. Other classes displayed resemblance to *Actinobacteria* (5.0%) and *Cyanobacteria* (3.4%) and to *Alpha-* (2.4%), *Beta-* (3%), *Gamma-* (4.7%), *Delta-*(4%), *Epsilon-*(3%) *Proteobacteria*. A scarce sequence (3%) also revealed similarity to *Archaea*. On the other hand, a few of pathogenic bacterial species (e.g. *E. coli*, *Listeria monocytogenes*, and *Salmonella enterica*) were existing in the laying hen feces. NGS analysis in genus level indicated that about 6% *Streptococcus sp.* (i.e., sphere-shaped gram-positive bacteria belonging to the phylum *Firmicutes*) was also present in the raw chicken feces (Figure 2).

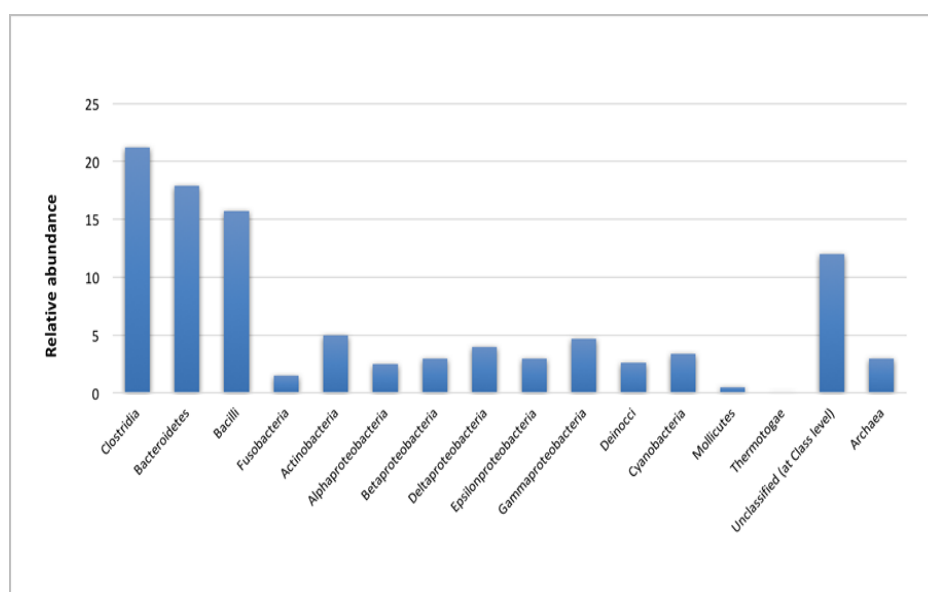


Figure 1. Relative abundance of bacterial classes in chicken feces.

Anaerobic treatability and biogas production

Results indicated that influent and effluent sCOD concentrations were observed as 12560 ± 1660 and 6610 ± 2715 mg/L, respectively with an average removal of around 47%. Moreover, influent TS and VS concentrations were observed as 55950 ± 9595 and 29050 ± 4300 mg/L whereas effluent TS and VS concentrations were as 33620 ± 8590 and 14930 ± 4045 mg/L, respectively. Hence, average TS and VS removals were calculated about 38% and 47%, respectively. Alkalinity and pH results in the influent and effluent were as 16190 ± 2140 and 15900 ± 5040 mg CaCO₃/L and as 7.91 ± 0.24 and 7.87 ± 0.20 , respectively.

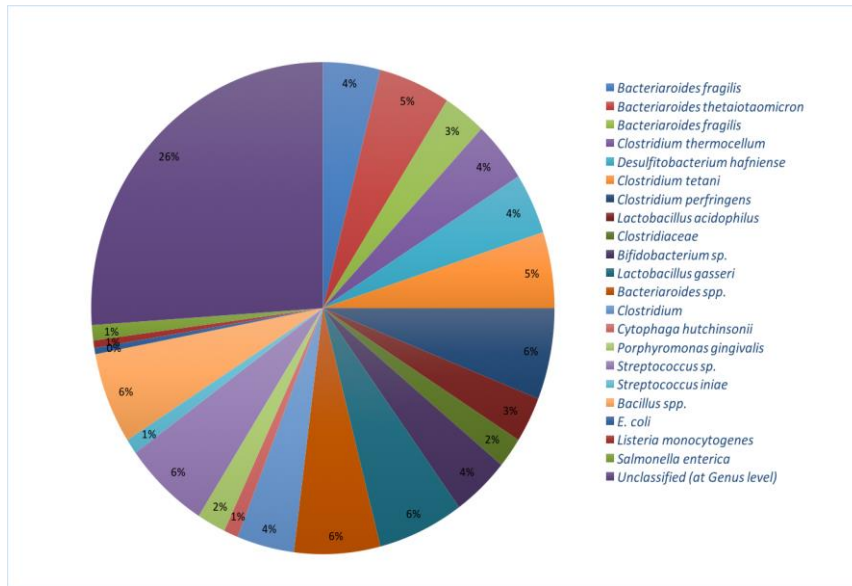


Figure 2. Relative abundance of bacterial species in chicken feces.

Since ammonia inhibition is especially distinct when digesting raw poultry manure, TAN and free ammonia nitrogen (FAN) contents in the influent and effluent were also monitored periodically. The FAN is suggested to be the active component causing inhibition on which pH has a significant effect. Although the chicken waste was diluted with tap water in order to provide the TS content of the influent as ca. %5.5; the influent slurry still comprised high amounts of TAN. In this study, FAN inhibition was not evident due to the fact that pH levels did not rise above 8.00. Influent and effluent TAN concentrations were measured as 2360 ± 285 mg/L and 2300 ± 105 mg/L whereas FAN was 285 mg/L in the effluent on average.

Cumulative biogas production was observed as about 70 L whereas daily biogas production rate was ca. 650 ± 385 mL/day in the bioreactor (Figure 3). Respective biogas yield was observed as ca. 0.47 L/gr VS_{added}. Although the effect of anaerobic digestion on the removal of pathogenic bacterial species was not investigated in this study; Yongabi et al. [9] reported that both coliform and *E. coli* counts decreased drastically following anaerobic treatment of poultry feces which was operated for 37 days to disinfect contaminated poultry feces and to provide biogas and pathogen-free fertilizer.

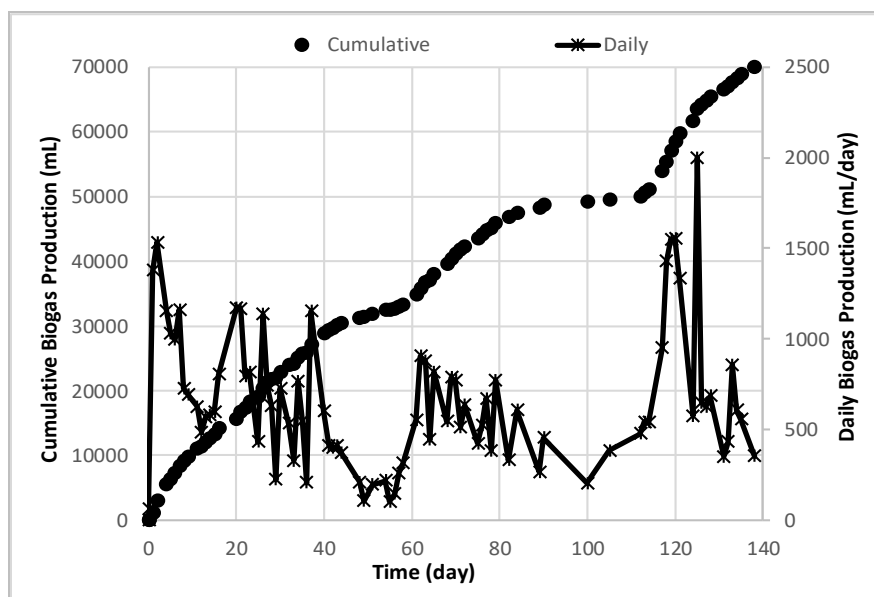


Figure 3. Biogas production from mesophilic anaerobic digestion of laying hen waste

4. Conclusions

Anaerobic digestion of high strength organic wastes such as chicken manure is considered as one of the most appropriate treatment alternatives due to biogas and nutrient recovery. In the present study, anaerobic treatability of the diluted chicken manure (TS ~5.5%) and its biogas potential were examined. Moreover, the microbial diversity in chicken feces was also identified. The experimental findings indicated the following conclusions;

- Laying hen manure is an important renewable energy source and if it is managed/treated properly; significant biogas productions might be possible from the farms producing high amounts of chicken wastes.
- Effective performance of semi-continuously fed anaerobic digester treating the diluted laying hen manure regarding VS removal was about 47% on average.
- Respective biogas yield was observed as about 0.47 L/gr VS_{added} from the bioreactor although TAN concentration was around 2300 mg/L on average.
- FAN inhibition during the operating period of this study was not significant because the pH in the system did not increase to >8.0.
- The microbial diversity in chicken feces revealed that *Bacilli*, *Clostridia*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Alpha-proteobacteria*, *Beta-proteobacteria*, *Delta-proteobacteria*, *Epsilon-proteobacteria*, *Gamma-proteobacteria*, *Deinocci*, *Mollicutes*, *Thermotogae*, *Cyanobacteria* and *Archaea* were present.
- The most abundant two bacteria classes were identified as *Clostridia* and *Bacteroidetes* whereas *Bacilli*-like sequences were also characterized.
- A scarce sequence revealed similarity to *Archaea* (i.e., 3%) and pathogenic bacterial species were also identified in few numbers.

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