BIOMETHANATION AND HOP ACIDS

By Sami Faour

Introduction

Methanogenesis or biomethanation is described by Angelidaki, Karakashev, Batstone, Plugge and Stams (2011) as a natural process by which organic material is degraded microbiologically under anaerobic conditions to generate biogas (a mixture of 60 percent methane and 40 percent carbon dioxide). It occurs naturally in landfills, ponds, and the intestines of ruminant animals. The anaerobic digestion (AD) or degradation is a multistep process carried out by a blend of three different groups of microorganisms: hydrolytic-acidogenic bacteria, anaerobic, acetogenic bacteria and methanogenic bacteria according to Hattori (as cited in Angelidaki et al., 2011).

Production of biogas from the decomposition of complex organic matter by anaerobic microorganisms has been utilized for centuries in man-made systems to produce energy. Methane generated from the AD of different organic wastes combines the production of renewable energy with environmental benefits, such as reduction of greenhouse emissions, controlled waste disposal and other (Angelidaki et al., 2011). Further, the digested material containing nutrients such as nitrogen and phosphorous could possibly be applied on the agriculture field as biofertilizer and thus replace artificial fertilizers.

AD is a complex multistep process that can be separated into four stages: Hydrolysis, Acidogenesis, Acetogenesis and Methanogensis (see Figure 1).

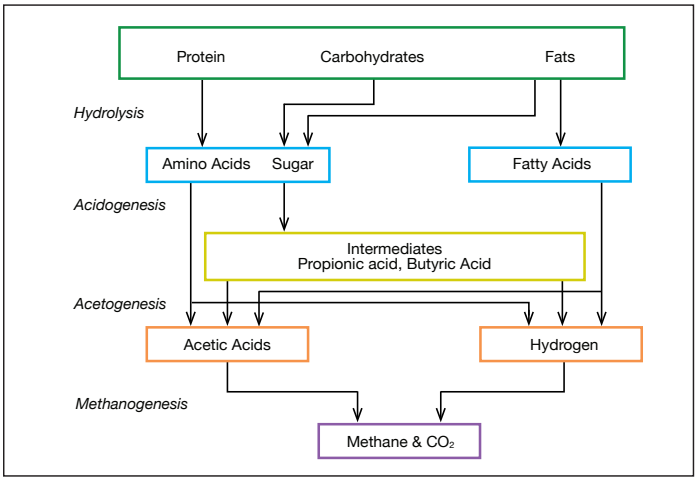

Figure 1. Breakdown steps of anaerobic digestion process.

Hydrolysis

In the first stage, the term hydrolysis is used to describe the depolymerization of insoluble complex organic compounds such as lipids, carbohydrates, and proteins into soluble monomers such as amino acids, monosaccharides and long chain fatty acids (Angelidaki et al., 2011). This stage alone is a complex multistep process carried out by strict anaerobes such as *bacteroides* and *Clostridia* as well as facultative bacteria such as *Streptococci* (Yadvika, Streekrishnan, Kohli and Rana, 2004). Equation one shows an example of a hydrolysis reaction where polysaccharides in organic waste is broken down into simple sugar (Ostrem, 2004).

Equation 1: $C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + H_2$

Acidogenesis

The second step which is called acid formation; the monosaccharides and amino acids produced from the hydrolysis step are transformed by the action of a wide range of microorganism but mostly *Clostridia* and other low guanine and cytosine (GC) Gram positive microorganisms into butyric acids, propionic acids, acetic acids, hydrogen and carbon dioxide (Angelidaki et al., 2011). It's interesting to note that the acetic acid, hydrogen and carbon dioxide will skip the third stage, acetogenesis and be utilized directly by the methanogenic bacteria in the final stage. Equation two below is an example of an acidogenesis reaction where glucose is converted to propionic acid:

Equation 2: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2COOH + 2H_2O$

Acetogenesis

In the third stage the short chain fatty acids (such as propionate and butyrate) and alcohols such as ethanol are oxidized by the action of obligate hydrogen producing acetogenic bacteria (see equation 3). Examples of hydrogen fermenting bacteria include anaerobic microorganisms such as *Clostridia* and *Syntrophobacter wolinii* (Boone, 1980). The level of hydrogen gas plays an important role in this process as energy will be derived by bacteria for growth only if the concentration of the hydrogen gas is kept low (Angelidaki et al., 2011). This is achieved by the presence of hydrogen scavenging bacteria such as methanogenic archaea, thus the partial pressure of hydrogen of a digester is an indicator of its health (Serna, 2009).

Equation 3: $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$

Methanogenesis

According to Angelidaki et al. (2011) methanogenic microorganisms belong to the *Archaea* domain, phylum *Euryarchaeota*. Further, five phylogenetic orders of methanogens have were identified and include: *Methanosarcinales, Methanobacterials, Methanomicrobiales, Methanococcales* and *Methanopyrales*. The methanogenic archaea are responsible for the final stage in the AD process where methane is produced from either acetate and/or from carbon dioxide and hydrogen.

Further most methanogens are neutrophiles that flourish in growth at a pH of 7.0. Conrad (as cited in Angelidaki et al., 2011) state that there are three known main pathways for methane production: aceticlastic methanogenesis where the acetate is cleaved to carbon dioxide and methane; hydrogenotrophic methanogenesis where carbon dioxide is reduced to methane and methylotrophic methanogenesis where methylated C1 molecules are converted to methane Interestingly 70% of the methane produced anaerobically is generated from acetate and only ~30% of it is formed from carbon dioxide and hydrogen (see equation 4).

Equation 4: $CH_3COOH \rightarrow CH_4 + CO_2$

BetaTec® solves bio-digester problem for ethanol plant

A forty five million liter (twelve million gallon) North American ethanol plant was experiencing contamination issues during ethanol fermentation. In spite of utilizing chlorine dioxide, it was determined that they had a severe problem with microbial infection that was affecting the operation of the plant and their daily ethanol production. The bacterial issues began in November of 2013 when the ethanol plant started operating a bio-digester designed by Global Water Engineering (GWE) at the backend of the process. This digester is capable of producing up to 45,000 m^3/day of biogas by treating 800 m^3 of vinasse feed each day. The raw vinasse feeds into the equalization tank (hydrolysis & acidogenesis) which then gets transferred to the bio-digester that operates at a pH between 7.0-7.5 and at a temperature of 38 °C. Those conditions are optimal for the growth of the sludge microorganisms as outlined in Figure 2.

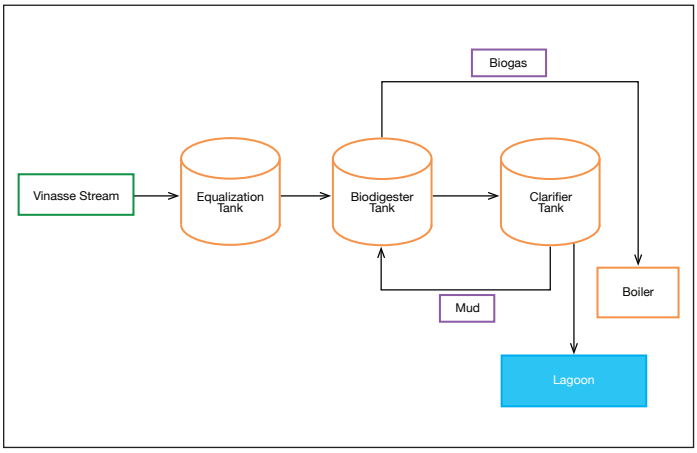

Figure 2. A schematic of the plant's bio-digester

The aim of installing a bio-digester was to reduce the high level of the chemical oxygen demand (COD) present in the vinasse to environmentally acceptable levels, allowing the plant to discharge the treated vinasse back to the lagoon as well as producing biogas to fuel the boilers of the plant. That's not surprising, as it's well documented in literature that vinasse (a byproduct of molasses ethanol fermentation) represents a major environmental challenge for the ethanol industry due to its high Chemical Oxygen Demand (COD) level. If disposed of without treatment it can cause damage to the aquatic life present in rivers streams and landfills (O., Sulieman and Elhardallou, 2013).

Subsequently, it was discovered that the cocktail of antibiotics applied in the fermentation process to control lactic acid bacteria affected the production of biogas during the anaerobic digestion of the vinasse. Various internal plant studies showed that the antibiotic residue present in the vinasse increased the level of the volatile free acids (VFA) above the maximum threshold level by inhibiting or partially inhibiting the acetogenic bacteria capable of converting the VFA's into acetate and hydrogen. This led to an irreversible decrease in the plant's biogas production.

The effect of various classes of antibiotics on the bio-digester micro-flora in other industries has been extensively reported in literature. According to Sanz, Rodriguez and Amils (1996) antibiotics found as contaminants in the liquid wastes from lives stock farms that are sent to the bio-digester for treatment affected the production of biogas during anaerobic digestion. Sanz et al. (1996) report that chlortetracycline inhibits methanogenesis because this group of antibiotics are strong inhibitors of methanogenic archaea.

Other antibiotics such as tylosin and penicillin generate a partial inhibition of methane production, probably due to their inhibitory effect on acetogenic bacteria. Further, Lallai, Mura and Onnis (2002) report that antibiotics have negative effects on the mixed populations of anaerobic bacteria present in the bio-digester, as they could reduce the rate of the growth and have detrimental effects on the degradation of the organic load of the waste and on the biogas production.

The irreversible drop in biogas production coupled with a gradual increase in the VFA levels combined with the literature review lead the ethanol plant in early 2014 to discontinue the use of antibiotics and switch to other chemicals that could control the lactic acid infection during must fermentation without affecting the micro-flora of the biomethanantor. Though chlorine dioxide was determined not to affect the production of biogas, the product failed to keep the infection under control and maintain the yeast health for a prolonged time as was observed with antibiotics. The lack of sufficient bacterial inhibition in the fermentation process steered the plant to seek other alternatives that are capable of resolving their contamination issues.

Hop Acids

The antimicrobial properties of hop acids that have been known and used in the brewing industry for more than a millennium, and constitute a legitimate naturally derived alternative. Furthermore, hop acids (BetaTec's IsoStab®) have been successfully used in dry mill and wet mill ethanol plants since 2004. By adding the minimum inhibitory concentration of hop acids to the yeast propagation tank and/or to the fermentation vessel, one can inhibit the growth of lactic acid bacteria during the production of ethanol and entirely avoid the need for antibiotics and/or other chemicals.

The mode of action for hop acids is based on the fact that the un-dissociated form of the hop acid is toxic to bacteria. Decreasing the mash or must pH increases the concentration of the un-dissociated form of hop acid, which is permeable to the cell membrane. The latter form is soluble in the phospholipid of the membrane and diffuses passively into the cell. Because of the higher pH inside the cell, the acid then dissociates and the liberated proton acidifies the cytoplasm, and as a result dissipates the pH gradient across the cytoplasmic membrane. This leads to the inhibition of nutrient uptake and, eventually, the bacteria starve to death.

Solution employed

The ethanol plant experiencing infection issues considered the utilization of hop acids seriously particularly after it was communicated by the manufacturer of the bio-digester that hop acids do not have detrimental effect on the micro-flora of the bio-methanator. Further, they were more encouraged to learn that the pH of the bio-digester is too alkaline for the hop acid extract utilized in the trial to be functional against the mix of bacteria present in the anaerobic bio-reactors.

The plant followed the feed rate recommendation for hop acid extract and applied the product directly to the yeast propagator. Fermentation analysis of the plant data under the hop acid extract program showed positive results. The data showed a reduction of 41 percent in lactic acid concentration and a relative increase of at least 18 percent in ethanol yield (see Figure 3 & 4). Further the hop acid extract treatment allowed the plant to build a fresh propagator every fifteen days vs. seven days with the chlorine dioxide treatment.

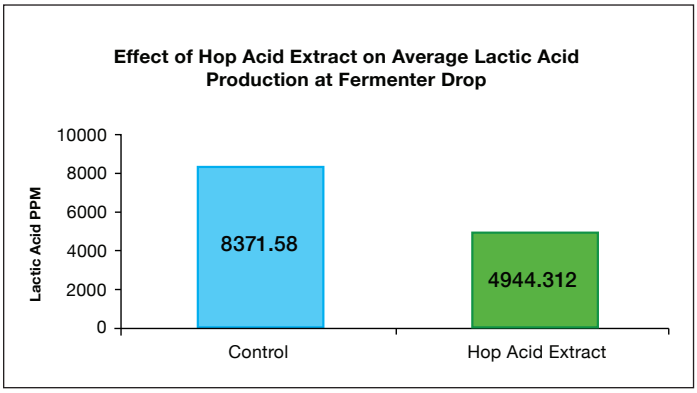

Figure 3. Effect of hop acid extract on the lactic acid concentration.

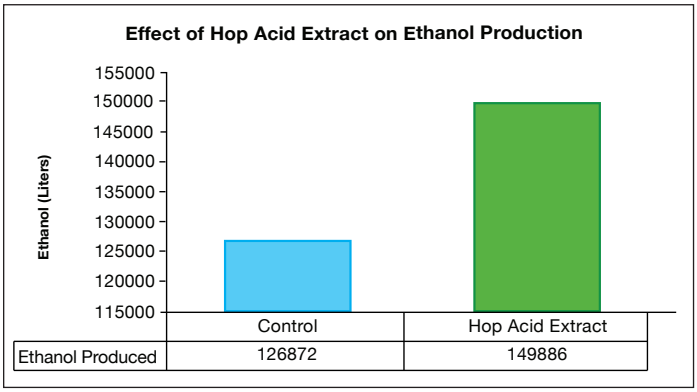

Figure 4. Effect of hop acid extract on the ethanol production.

Concurrently, it was communicated to plant personnel that the intent was to validate that the hop extract being applied to the fermentation process has no ill effects on the viability of the bio-digester micro-flora. Composite stream samples were pulled from selected points of the process and both a microbiological evaluation and a hop acid extract residue analysis were run.

Validation Method

Composite samples from the raw vinasse stream, acidification tank and bio-digester tank were pulled and shipped to the research and development center (R & D) for further analysis. The objective of the analysis was to achieve the following:

1. Quantify the hop acid extract residue in each sample according to QAM 05.09-Hop acid extract-HPLC.
2. Isolate the anaerobic bacteria either from the bio-digester or the equalization tank according to a basic culture plate method.
3. Conduct a susceptibility - minimum inhibitory concentration (MIC) study of the isolate/s to the hop acid extract being utilized at the plant.

Results & Discussion

The microbiological evaluations of the samples received yielded the following results:

1. An anaerobic gram positive rod was isolated from the bio-digester sample.
2. The microbiologist was not able to cultivate any bacteria from the acidification sample.

The failure was due to the fact that some of those anaerobes are sensitive to oxygen and if those microorganisms were exposed to air during the sampling and shipping process, they would get poisoned by oxygen leading to their death.

The anaerobic susceptibility study to evaluate the inhibitory effect of Hop acid extract on the isolated strain was conducted under conditions similar to the one present in the bio-digester (pH=7 & temperature ~37 ºC). Table 1 indicates that hop acid extract failed to inhibit the growth of the bacteria even at concentrations higher than 89 ppm, which is roughly five times more than what's theoretically expected to be found in the raw vinasse. Moreover, the fact that the theoretical concentration of hop acid extract (18 ppm) in the raw vinasse entering the bio-digester is below the MIC level capable of inhibiting gram positive bacteria coupled with the aforementioned findings is further evidence that hop acid extract does not affect the mirco-flora of the bio-digester.

MIC Results for Anaerobic Gram Positive Rods Isolate from	Hop Acid Extract Product Concentration
Bio-digester Tank 1	> 89 ppm
Bio-digester Tank 2	> 89 ppm

Table 1. Anaerobic Susceptibility Testing-MIC

Concurrently, the samples were also analyzed for hop acid extract residue by a modified HPLC method. The hop extract residue was only detected in the raw vinasse sample that's being fed into the bio-digester, and at very low levels (6.60 ppm product). The rest of the samples including the bio-digester did not carry detectable levels of hop acid extract residue (Table 2). Trace amounts of product was not found in subsequent streams (bio-digester or the equalization tank). This was thought to be due to sedimentation and/or to the product dilution triggered by the presence of sludge and other ingredients.

Sample #	Sample Type	Hop Acid Extract Product Concentration (PPM)
1	Bio-digester Tank 1	ND (<<0.1)
2	Bio-digester Tank 2	ND (<<0.1)
3	Equalization Tank 1	ND (<<0.1)
4	Raw Vinasse	6.60

*ND: Not Detected.

Table 2. Hop Acid Extract Residue Analysis

The absence of detectable levels of hop acid extract residue in both the bio-digester and equalization samples is further evidence that the risk of utilizing hop acid extract in the fermentation process is almost nonexistent and our findings are in line with what's reported in literature. E.P. 172, 1871, A1 (2006) reports that the presence of hop acids effectively suppresses the lactic acid bacteria which negatively affect the activity of hydrogen fermenting microorganisms and does not inhibit the growth of the hydrogen fermenting bacteria leading to more methane production.

Conclusion

The results of the study indicate that the hop acid extracts are an effective product to inhibiting both the lactic acid & acetic acid formation without affecting the yeast metabolism and the micro-flora of the bio-digester during the production trial. Moreover, the absence of detectable levels of hop acid extract in the bio-digester coupled with failure of hop acid extract to inhibit the isolated bacteria from the bio-digester even at levels higher than 89 ppm; is further proof that hop acid extracts could be utilized to control contamination issues in the fermentation processes without affecting biogas production.

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