Continuous co-digestion of agro-industrial residues

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Abstract

Slaughterhouse waste (SB) has high potential to be utilized in anaerobic digestion due to its high protein and lipid content. However, these are also the limiting factors of system stability. Thus, co-digestion of slaughterhouse waste with other agro-industrial residues (manure (M), various crops (VC) and municipal solid waste (MSW)) was introduced in this study to overcome this problem. The main objective of the work was to determine the operating parameters and the methane yield in semi-continuous co-digestion of slaughterhouse waste with other agro-industrial waste streams. Four continuously stirring tank reactors (CSTRs) with different substrates and mixtures (SB, SB:M, SB:VC and SB:VC:MSW) were started up operating with hydraulic retention time (HRT) of 25 days in thermophilic conditions. The highest organic loading rates which could be achieved were 0.9 g VS/L·d in digestion of SB and 1.5 g VS/L·d for the co-digestion mixtures. In these cases, average methane yields of 300, 510, 587 and 426 ml/g VS were obtained from the digestion of SB, and the co-digestion of SB:M, SB:VC and SB:VC:MSW, respectively, with methane contents in the biogas of 60-85%. The highest average methane yield of 587 ml/g VS was found in co-digestion of SB:VC, which was in accordance with the value of 592 ml/g VS detected during the batch digestion of the same mixture. Moreover, batch assays with different substrates as well as 11 different mixtures of those were also set up to investigate the methane potential and the effect of second feeding. The results showed that the co-digestion of SB:VC, SB:VC:MSW and SB:M could provide high methane potentials, where the highest methane yields of 592, 522 and 521 ml/g VS, respectively were obtained. Moreover, increasing, similar or decreasing methane yields were determined from the second feeding depending on the substrates and substrate mixtures used.

Keywords: Biogas, slaughterhouse waste, co-digestion, semi-continuous experiment, CSTR
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<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Anaerobic digestion</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CHP</td>
<td>Combined heat and power</td>
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<tr>
<td>CH₃OH</td>
<td>Methanol</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>CH₃CH₂COOH</td>
<td>Propionate</td>
</tr>
<tr>
<td>(CH₃)₃-N</td>
<td>Methylamines</td>
</tr>
<tr>
<td>CH₄</td>
<td>Methane</td>
</tr>
<tr>
<td>C₂H₅OH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>C₆H₁₂O₆</td>
<td>Glucose</td>
</tr>
<tr>
<td>C/N</td>
<td>Carbon to Nitrogen ratio</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CSTRs</td>
<td>Continuous stirring tank reactors</td>
</tr>
<tr>
<td>d</td>
<td>Day</td>
</tr>
<tr>
<td>FVW</td>
<td>Fruit and vegetable waste</td>
</tr>
<tr>
<td>FYM</td>
<td>Farmyard manure</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic retention time</td>
</tr>
<tr>
<td>H₂</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>H₂S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kPa</td>
<td>KiloPascal</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long-chain fatty acid</td>
</tr>
<tr>
<td>M</td>
<td>Manure</td>
</tr>
<tr>
<td>m³</td>
<td>Cubic metre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MSW</td>
<td>Municipal solid waste</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NH₃</td>
<td>Ammonia</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonium ion</td>
</tr>
</tbody>
</table>
NH₄-N Ammonium nitrogen
NH₄HCO₃ Ammonium bicarbonate
OLR Organic loading rate
P Phosphorus
SB Slaughterhouse waste
SRT Solid retention time
TAC Total inorganic carbonate
TKN Total Kjedahl nitrogen
TS Total solids
VC Various crops
VFAs Volatile fatty acids
VS Volatile solids
Y_{CH₄} Methane yield
1. Background and Objectives

The increasing population in the world and the rapid economic growth lead to higher consumption and consequently the generation of large amounts of waste. In Thailand, it has been reported that livestock production has been increased from 8,945,000 to 16,394,000 tons between a period of 1980 to 2002 (FAO, 2005). According to a report of Pollution Control Department (PCD), the generated slaughterhouse waste is today mostly used as fish feed, water flea feed and for composting (PCD, 2012). However, anaerobic digestion could be an alternative solution for the utilization of these kinds of waste due to its environmental and economical benefits.

Anaerobic digestion (AD) can be defined as the conversion of biodegradable material to biogas which mainly consists of carbon dioxide (CO₂) and methane (CH₄). The process is performed by the activity of several different groups of micro-organisms in the absence of oxygen. The produced biogas can be utilized as vehicle fuel or for heat and electricity generation. Moreover, a nutrient rich digestate residual which is also generated can be utilized as fertilizer.

Slaughterhouse waste appears to be a good substrate for AD due to its high protein and fat content (Pagés Díaz et al., 2011; Palatsi et al., 2011; Salminen & Rintala, 2002). (Salminen & Rintala, 2002) reported specific methane yields of 0.52 to 0.55 m³ CH₄/kg VS which can be obtained during anaerobic degradation of these waste streams. However, AD of only the slaughterhouse waste fraction may generate low methane yield due to the accumulation of high amounts of fatty acids and ammonia produced by the degradation of fats and proteins (Cuetos et al., 2010; Palatsi et al., 2011). Moreover, the long lag phase reported for degradation of slaughterhouse waste is another drawback (Pagés Díaz et al., 2011).

In this study co-digestion has been therefore introduced to overcome these problems. Co-digestion is an anaerobic digestion technology using a combination of different wastes to stabilize the process and to increase the methane yield (Alvarez et al., 2008; Cuetos et al., 2011; Fernández et al., 2005; Liu et al., 2009). There are some studies performed previously on co-digestion of slaughterhouse waste with other waste streams (Alvarez et al., 2008; Bayr et al., 2012; Pagés Díaz et al., 2011). (Alvarez et al., 2008) reported a methane yield of 0.3 m³/kg VS during co-digestion of slaughterhouse waste with manure and fruit and vegetable waste in a semi-continuous process. Furthermore, (Bayr et al., 2012) reported that methane potential 0.26–0.57 m³ CH₄/kg VS could be obtained in semi-continuous co-digestion of rendering waste and slaughterhouse waste. Moreover, (Pagés Díaz et al., 2011) found by performing batch anaerobic digestion assays of co-digestion of slaughterhouse waste (SB), manure (M), various crops (VC) and municipal solid waste (MSW) that the mixture ratio of 1:1:1:1 gave the best performance with methane yield of 664 Nml CH₄/g VS.

The aim of this study was to investigate the utilization of slaughterhouse waste by co-digestion with different mixtures of other waste streams coming from agro-industrial activities. First different mixture ratios were investigated by using batch anaerobic digestion assays. In addition, the effects of a second feeding on the bacterial activity and
produced methane yields were also studied during the batch experiments. Furthermore, the long term effects in the process were determined during semi-continuous operation using continuously stirred tank reactors (CSTRs).

2. Literature review

2.1 Biogas Production and its utilization

Biogas is the main product from anaerobic digestion (AD), however, residues (biosolids and liquor) are also generated within the process (FOE, 2007; Luque et al., 2011). Biogas is a color less, odorless, flammable, stable and non-toxic gas (Hilkiah Igoni et al., 2008). The amount of methane present in biogas depends on the composition of substrate used. However, the biogas usually contains around 60% of CH₄ and 40% of CO₂.

After a process called upgrading to get the methane, biogas can mainly be used as vehicle fuel, energy generation purposes, and also can be injected to a natural gas grid (Hilkiah Igoni et al., 2008; Persson et al., 2006). Thus, biogas has to be purified to improve its quality due to the present of contaminants such as hydrogen sulfide, oxidation products, halogenated compounds, siloxanes, ammonia, humidity, dust and particles (Luque et al., 2011; Persson et al., 2006). According to the Swedish Standard for biogas as transportation fuel (SS 15 54 38), the methane content must be between 95-99 %, the water content less than 32 mg/Nm³, the oxygen content less than 1% and the total sulphur content less than 23 mg/Nm³ (Persson, 2006).

On the other hand, biogas can also be used as energy source for the generation of heat and electricity. In the case of small-scale AD plants, the produced biogas is usually used for cooking and lighting. In contrast, the produced gas from large-scale plants can be used in electricity generation or in combined heat and power plant (CHP) by using internal combustion, gas turbine or fuel cells technologies. Additionally, after upgrading, biogas can also be injected to the natural gas grid which has several benefits i.e. to avoid flaring, to mobilize the biogas further to the population, and to be used as gas back up increasing the local security supply (Persson et al., 2006).

Residuals are the remaining materials from AD including both solids and liquid; biosolids and liquor. Biosolids can be described as organic matter of unconverted substrates and grown microorganism (Luque et al., 2011). Liquor defines as dissolved organic matter and nutrients. Both products can be used as soil conditioner and low-grade fertilizer (FOE, 2007; Hilkiah Igoni et al., 2008; Luque et al., 2011).

2.2 Anaerobic digestion process

Anaerobic digestion (AD) is a biological process that naturally occurs when bacteria decompose the organic matter producing mainly methane (CH₄) and carbon dioxide (CO₂) in
oxygen-free environment (Arsova, 2010; FOE, 2007). The AD process normally consists of four steps; each of these is completed by different groups of bacteria. These are hydrolysis, acidogenesis, acetogenesis and methanogenesis (Polprasert, 2007). All reactions happen simultaneously and are interdependent. Nevertheless, the overall chemical reaction can be simplified to:

\[
\text{Organic matter} \rightarrow \text{CH}_4 + \text{CO}_2 + \text{H}_2 + \text{NH}_3 + \text{H}_2\text{S}
\]

2.2.1 Hydrolysis
The reaction in which large organic molecules: proteins, carbohydrates and fats, are enzymatically broken down into smaller constituents. The enzymes are produced by the hydrolytic and fermentative bacteria present in the environment or in the digester. The results of this stage are simple monomers, like sugars, amino acids, fatty acids and water (Arsova, 2010). The rate of the hydrolysis step depends on substrate characteristics, bacteria concentration, and also environmental factors such as pH and temperature (Polprasert, 2007).

- Protein \(\rightarrow\) Amino acids
- Carbohydrate \(\rightarrow\) sugars
- Fat \(\rightarrow\) long chain fatty acids

2.2.2 Acidogenesis
The monomeric compounds from the hydrolysis stage are converted further into simple organic compounds, mostly short-chain (volatile) fatty acids, such as propionic, butyric acid etc., by the action of acidogenic bacteria (Arsova, 2010; Polprasert, 2007). Moreover, methanol and other simple alcohols are also produced by breaking down the carbohydrates (Polprasert, 2007). Typical reactions occurring in this stage are presented below:

- Conversion of glucose into ethanol:

  \[
  \text{C}_6\text{H}_{12}\text{O}_6 \leftrightarrow 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2 + \text{heat}
  \]

- Conversion of glucose into propionate

  \[
  \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2 \leftrightarrow 2\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O}
  \]

2.2.3 Acetogenesis
In this stage acetogenic bacteria transform the resulting compounds from acidogenesis into acetic acid (acetates) and hydrogen (H\textsubscript{2}) and carbon dioxide (CO\textsubscript{2}) (Arsova, 2010). The concentration of hydrogen is very crucial because the reactions can only proceed when the hydrogen concentration is very low, therefore acetogenic
bacteria live in symbiosis with hydrogen consuming methanogens. In addition, the acetogenic bacteria are very sensitive to fluctuations of temperature (Polprasert, 2007).

2.2.4 Methanogenesis

The methanogens convert acetic acid, simple alcohols (methanol, ethanol) or carbon dioxide and hydrogen into methane. Approximately 70% of total methane production is acquired from the conversion of acetic acid or by fermentation of alcohols, while 30% of the methane production comes from the reduction of carbon dioxide by hydrogen (Arsova, 2010; Polprasert, 2007). The reactions which occur in methanogenesis step are the following:

- Conversion of acetic acid

\[ 2\text{CH}_3\text{CH}_2\text{OH} + \text{CO}_2 \leftrightarrow 2\text{CH}_3\text{COOH} + \text{CH}_4 \]

Followed by

\[ \text{CH}_3\text{COOH} \leftrightarrow \text{CH}_4 + \text{CO}_2 \]

- Conversion of methanol

\[ \text{CH}_3\text{OH} + \text{H}_2 \leftrightarrow \text{CH}_4 + \text{H}_2\text{O} \]

- Reduction of carbon dioxide by hydrogen

\[ \text{CO}_2 + 4\text{H}_2 \leftrightarrow \text{CH}_4 + \text{H}_2\text{O} \]

The amount of biogas produced from the digestion process depends on several parameters; like pH, temperature, composition of substrate, organic loading rate, and retention time. The pH is important because the methane producing methanogens are inhibited under acidic conditions. Moreover, the methanization potential depends on the concentration of four main components present in the substrate; proteins, lipids, carbohydrates and cellulose (Arsova, 2010). The highest methane yields can be obtained from systems with excess of lipids and operating at long retention times, while the fastest methanization can be achieved from systems with excess proteins, cellulose and carbohydrates, respectively. In addition, overloading of the system with substrates will result in low biogas yield. There are several ways to improve the efficiency of biogas production. One of these is known as co-anaerobic digestion, which may result in a better nutritional balance in the system.

2.3 Operating Parameters

There are many parameters that influence the performance of AD, such as temperature, pH and alkalinity, mixing conditions, nutrient composition, Carbon to
Nitrogen ratio (C/N), particle size, organic loading rate (OLR), and hydraulic or solid retention time.

2.3.1 Temperature

Temperature is an important parameter for AD because the bacteria (both acetate-forming bacteria and methane-forming bacteria) need the optimum temperature to grow and maintain their activity. There are a wide ranges of temperatures can be used for AD, like psychrophilic (5-25°C), mesophilic (30-35°C), thermophilic (50-60°C) and hyperthermophilic (>65°C) range. Generally, the operating temperature is maintained in mesophilic or thermophilic range (Gerardi, 2003; Monnet, 2003). Temperature of between 40-50 °C can be inhibiting because it is undesired temperature for both the mesophilic and the thermophilic bacteria (Gerardi, 2003; Polprasert, 2007).

Higher temperature can increase the rate of CH₄ production, because the reaction rates are higher, the decomposition of volatile solids is faster which give higher methane production rate (Arsova, 2010; Gerardi, 2003; Polprasert, 2007). However, a more complex and costly operating system is usually necessary for thermophilic processes, which can create more problems in the operation compared to that of mesophilic processes.

2.3.2 pH value and alkalinity

As mentioned above in an AD process, there are 4 different stages, which depending on the bacterial activity may need different pH optimum. Most of the anaerobic bacteria are active at pH 6.6-7.6 (Arsova, 2010; Gerardi, 2003; Monnet, 2003; Polprasert, 2007). Methane-forming bacteria are more sensitive to the decrease in pH, than acid-forming bacteria. Methane forming bacteria can only tolerate pH down to 6.2 while in the case of acid-forming bacteria the pH can decrease down to pH 5 (Gerardi, 2003). To get higher biogas yield, and to avoid the failure of the process, the pH has to be maintained within a stable range by controlling the alkalinity in the reactor.

The failure of the system may appear when pH decreases due to high organic loading rate leading to the accumulation of volatile fatty acid (VFA), decreased alkalinity, and the presence of toxic materials. In order to maintain the right pH and alkalinity in the system, chemicals such as sodium bicarbonate, potassium bicarbonate or lime can be added. In contrast, adverse effects, like foaming, pH change, toxicity may be occurred (Gerardi, 2003; Polprasert, 2007).

On the other hand, pH can be increased to higher than 8 due to high concentration of ammonia (NH₃) from rapidly increase of methanogens (Arsova, 2010) because some groups of methanogens grow on the methyl group substrates such as methanol (CH₃OH) and methylamines [(CH₃)₃-N]. The methanogens that
use \((\text{CH}_3)_3\text{-N}\) gives the \(\text{NH}_3\) as by product (Gerardi, 2003) as shows in following equation.

\[
4(\text{CH}_3)_3\text{N} - \text{N} + 6\text{H}_2\text{O} \rightarrow 9\text{CH}_4 + 3\text{CO}_2 + 4\text{NH}_5
\]

Then the reaction of \(\text{NH}_3\) and \(\text{CO}_2\) will form the ammonium bicarbonate \((\text{NH}_4\text{HCO}_3)\) which in turn can result in an increase of alkalinity and rise of \(\text{pH}\). It leads the restrictive in the system because acidogenesis is inhibited (Arsova, 2010; Gerardi, 2003).

2.3.3 Mixing

Mixing is required to provide better contact between the substrate, the anaerobic bacteria and the nutrients. Moreover, it also decreases the accumulation of settling solids; reduces the scum formation on the surface and equalizes the temperature. Moreover, mixing can also minimize toxicity caused by rapid digestion which may lead lower performance of the system (Gerardi, 2003; Monnet, 2003; Polprasert, 2007). Slow and gentle mixing is preferable because rapid or excessive mixing may disturb the microorganism’s activity leading to wash out (Gerardi, 2003; Monnet, 2003).

2.3.4 Nutrient composition and the Carbon to Nitrogen ratio (C/N)

Nutrients can be classified into 2 types; macronutrients and micronutrients. Macronutrients are needed for all bacteria in larger quantities, like carbon, nitrogen and phosphorus; while micronutrient refers to nutrients such as cobalt, iron, nickel, and sulfide that most of the bacteria require in small amounts (Gerardi, 2003). To achieve the good production of biogas, an adequate balance within the nutrients provided is needed.

The Carbon to Nitrogen ratio (C/N) refers to the relative amounts of carbon and nitrogen present in the organic substances. Depending on the composition of raw materials low C/N ratio can be found in for example human excreta, animal manure and sewage sludge, whereas agricultural residuals, sawdust and wood chips have a high C/N ratio. However, the combination of substrates with low and high C/N ratios is preferable to get the optimum gas production. Generally, bacteria take up carbon 25-30 time faster than nitrogen (Polprasert, 2007), thus the C/N ratio should be between 20-30 as several researchers such as (Gerardi, 2003; Polprasert, 2007; Verma, 2002) suggested.

Moreover, the supply of sufficient micronutrients should also be considered, because the lack of micronutrient will lead to lower methane production. The presence of micronutrients, especially trace metals are essential for the methane-forming bacteria, and their metabolism in converting acetate to methane. On the other hand, the present of high concentration of micronutrient can cause trace element precipitation and toxicity (Gerardi, 2003).
2.3.5 Particle size

The particle size of feedstock also affects the bacterial activity in the reactor. The degradation of organic material occurs at the surfaces so the large particle size which has less total surface area leads to difficulties in decomposition. Thus, the particle size should be reduced to an optimum size (Hilkiah Igoni et al., 2008).

2.3.6 Organic Loading Rate (OLR)

Organic loading rate (OLR) is the measurement of biological conversion capacity in term of added volatile solids to the AD system (Arsova, 2010; Gerardi, 2003; Monnet, 2003). The increase in OLR can reduce the hydraulic retention time (HRT), which is the average time that methane-forming bacteria use to decompose organic material (Arsova, 2010; Klemeš et al., 2008). However, overloading leads to lower biogas yield and system failure due to the accumulation of volatile fatty acid (VFA) which will inhibit the activity of methane-forming bacteria. On the other hand, if OLR is too low, there will not be enough substrate for the methane production (Polprasert, 2007). Thus, optimum OLR which depends on feedstock, type of digester and the operating conditions should be provided for the system.

2.3.7 Retention time

Retention time can be defined as time that organic matter uses to complete degradation. It can be divided into 2 types; solid retention time (SRT) and hydraulic retention time (HRT). Solid retention time (SRT) defines as the average time that bacteria or solids spend in the digester. It can be used to find the proper size of the reactor (Gerardi, 2003; Klemeš et al., 2008). Hydraulic retention time (HRT) describes as the average time that needed for the feedstock to be decomposed in the system. It can be calculated by dividing the operating volume by the substrate flow rate (Arsova, 2010; Klemeš et al., 2008).

HRT has to be long enough for the bacteria, especially for methane-forming bacteria (Polprasert, 2007) to be able to complete the decomposition of organic materials (Arsova, 2010; Gerardi, 2003; Klemeš et al., 2008). However, too long HRT can create accumulation of sludge or digested material in the digester. Additionally, there is no increase in the rate of reaction due to the longer HRT (Arsova, 2010; Gerardi, 2003), therefore an optimum HRT should be maintained. Optimum HRT depends on the characteristic of substrates, operation conditions (temperature), type of digester and environmental conditions. For example, at thermophilic conditions the HRT usually shorter, i.e. 12-14 days, than at mesophilic conditions (15-60 days), because volatile solids can decompose faster at higher temperature as it was mentioned above (Arsova, 2010; Monnet, 2003; Polprasert, 2007).

(Gerardi, 2003) pointed out that SRT is a more important parameter than HRT. The conversion of volatile solids to gas needs enough SRT (typically 12
days) to achieve complete decomposition. At longer SRT, higher bacteria concentration and increasing bacterial activity can be expected. On the other hand, methane-forming bacteria can be washed out from the system if SRT is too low.

2.4 Feedstock for biogas production

The variety of organic compounds, for example carbohydrates, proteins, lipids, and cellulose, can be digested by anaerobic bacteria (Luque et al., 2011). These compounds are mainly presented in different organic solid wastes that are commonly used as substrates for the anaerobic digestion. Some examples for organic solid wastes, which can be used as digestion substrates, are municipal solid waste, slaughterhouse waste, agricultural waste, animal manure, food waste, sewage sludge, etc. (Kacprzak et al., 2009). The methane content of the biogas produced depends on the carbon presented in the substrate. Higher methane content can be obtained from substrate with high carbon composition (Luque et al., 2011).

The slaughterhouse waste is considered as an ideal feedstock for biogas production, because this substrate is rich in proteins and lipids, and also contains high concentration of organic matter (Bayr et al., 2012). Slaughterhouse waste mostly contains untreated blood and the contents of rumen and stomachs (Alvarez et al., 2008). However, using slaughterhouse waste in AD is sensitive and prone to failure because of the high protein content. When the protein is degraded, two forms of ammonia; free ammonia (NH₃) and ammonium ion (NH₄⁺) can be generated depending on the temperature and pH (Pagés Díaz et al., 2011). Ammonium ions can be used as a nutrient source for nitrogen, while free ammonia is toxic to methane forming bacteria. It results in the inhibition of methane production (Gerardi, 2003). The releases of ammonia can raise the pH in the digester. It can be interrupt by the consumption of volatile fatty acids (VFAs) by methanogenic bacteria. The high fermentation of proteins and fats at above optimal pH leads to an accumulation of fatty acids. It can cause the process to collapse (EK et al., 2011).

Other waste streams, coming from municipalities, like waste water, garbage and sewage sludge, are also attractive feedstocks for anaerobic digestion. These waste fractions are also rich in lipids and proteins, like slaughterhouse waste, which result in high methane yield (Nges et al., 2012). However, the methane yield may be reduced due to too high protein and lipid contents. The long-chain fatty acids (LCFAs) that are degradation products from lipids can cause severe inhibition in methanogenesis. Moreover, the concentration of ammonia coming from the degradation of proteins can inhibit the aceticlastic methanogens (Nges et al., 2012). These all will lead to the failure of the process.

The animal manure in forms of both farmyard manure and liquid manure (farm slurry), mostly obtained from horses, cattle, pigs, chicken, etc., and contains urine and feces. The farmyard manure (FYM) includes also other materials especially straw, which has been used as bedding and has absorbed the urine and feces. Animal manure is known as a substrate with poor methane yield (Liu et al., 2009), but it is a great co-
substrate because of its high buffering capacity. Moreover, it is rich in a wide variety of nutrients needed for the optimal growth of bacteria (Panichnumsin et al., 2010). The qualities of the manure are different for each animal. More biogas per unit volume was obtained from the digestion of pig farmyard manure, because the solid fraction has higher organic matter that raw pig manure (Xie et al., 2012). Chicken manure contains high concentration of nitrogen (Abouelenien et al., 2009), while manure from animals that consume plant based food, like cattle and horses, has relatively low nitrogen content and the biodegradability is also low (Liu et al., 2009).

The fruit and vegetable waste (FVW), which is usually collected from the food market, mainly contain sugars, and hemicelluloses. The volatile solid content in this waste is between 8% and 18%. The utilization of this cellulose-poor waste fraction is limited by methanogenesis because of the high biodegradability of this waste (Garcia-Pena et al., 2011). The high biodegradability of the fruit and vegetable waste encourage the rapid production of volatile fatty acids (VFAs). The rapid production of VFAs will lead to a rapid pH drop, which inhibits the methanogenic activity. Moreover, FVW is defined as low nitrogen and phosphorus containing materials. Thus the methane yield that can be obtained from FVW would be lower than that from waste with higher nitrogen content (Callaghan et al., 2002). Therefore, mixing with other wastes, such as manure, that have high nitrogen content is preferable, since this way the acidification of the system can be avoided (Garcia-Pena et al., 2011).

### 2.5 Co-digestion

Co-anaerobic digestion or co-digestion is anaerobic digestion performed on a mixture of at least two different substrates (Alvarez et al., 2008; Cuetos et al., 2011; Fernández et al., 2005; Liu et al., 2009). Co-digestion offers many possible ecological, technological and economical benefits (Alvarez et al., 2008). For example, co-digestion of slaughterhouse waste with low nitrogen and/or lipid containing substrates provides better process stability and higher methane yields. Slaughterhouse waste is rich in proteins and lipids, so the digestion of this waste tend to failure due to the production of ammonia, volatile fatty acids (VFAs) and long chain fatty acids (LCFA) at inhibitory high levels (Bayr et al., 2012).

The advantages and limitations of co-digestion are expressed in Table 1.1
Table 1.1 Advantages and limitations of co-digestion technology (Wu, 2007)

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Improved nutrient balance within the digestion</td>
<td>• Additional pre-treatment requirements</td>
</tr>
<tr>
<td>• Additional biogas collection</td>
<td>• Increased digester effluent Chemical Oxygen Demand (COD)</td>
</tr>
<tr>
<td>• Equalization of particulate, floating, settling, Acidifying, etc. wastes, through dilution by manure or sewage sludge</td>
<td>• Increased mixing requirements</td>
</tr>
<tr>
<td>• Additional fertilizer (soil conditioner)</td>
<td>• High utilization degree required</td>
</tr>
<tr>
<td>• Reclamation</td>
<td>• Wastewater treatment requirement</td>
</tr>
<tr>
<td>• Renewable biomass (&quot;Energy crops&quot;) disposable for digestion in agriculture</td>
<td>• Decreasing availability and rates</td>
</tr>
<tr>
<td>• Possible gate fees for waste treatment</td>
<td>• Restrictions of land use for digestate</td>
</tr>
<tr>
<td></td>
<td>• Economically critical dependent on crop costs and yield</td>
</tr>
</tbody>
</table>

Ref: (Wu, 2007)

2.6 Anaerobic digestion and co-digestion of slaughterhouse waste.

There are several studies found in the literature on AD of slaughterhouse wastes (bovine, swine and poultry) and the utilization of this waste in co-digestion within continuous processes. Previous studies showed that slaughterhouse wastes have feasibility in anaerobic digestion due to its high protein and lipid content. However, suitable conditions have to be first investigated for a successful process performance. OLR and HRT are critical parameters to run a stable digester. Recently, in anaerobic digestion of slaughterhouse wastes, OLR of 0.5-1.7 kg VS/m$^3$ d (Alvarez et al., 2008; Bayr et al., 2012; Cuetos et al., 2008; Salminen & Rintala, 2002) and in co-digestion of slaughterhouse waste with other waste streams OLR of 0.3 -3.7 kg VS/m$^3$ d (Alvarez et al., 2008; Cuetos et al., 2008) were suggested. Moreover, the composition of the microbial community is also an important factor for a successful process. Additionally, mesophilic operation was found to give a more stable process performance than operation at thermophilic conditions (Bayr et al., 2012).

(Salminen & Rintala, 2002)) studied the effects of HRT and OLR on anaerobic digestion of solid poultry slaughterhouse waste at mesophilic conditions. They have found that stable process could be achieved when the loading was 0.8 kg VS/m$^3$ day and HRT 50-100 days resulting in high methane yields of 0.52- 0.55 m$^3$ CH$_4$/kg VS. When OLR was increased and HRT decreased, the process failed, due to overloading and inhibition indicated by a drop in pH and a reduced methane production. These effects were explained by the accumulation of VFAs and LCFA which inhibits the methanogenesis. Therefore the OLR had to be decreased to recover the digester performance. However, even these conditions could cause that the stability of the digester in long-term operation to be unsure. It was indicated by the slightly increase in ammonia, soluble COD, LCFA and/or VFA concentrations which could lead to further accumulation and process failure in the future.
(Alvarez et al., 2008)) studied the co-digestion of slaughterhouse waste as main substrate with manure and fruit and vegetable waste within a mesophilic semi-continuous process. They have found that the methane yield increased when the OLR increased up to 0.34 kg VS/m$^3$ d, however, at higher OLR there was a breakpoint when the pH dropped, due to the accumulation of VFAs and the yield of methane started to decrease. It was also obtained, that the mixture of only two substrates resulted in a good process performance, although the mixture of all the three components gave better methane yield, VS reduction and biogas productivity than single or two substrates. Moreover, co-digestion of these substrates resulted in methane yield of 0.3 m$^3$/kg VS with 54-56% methane composition in the biogas and 50-65% of VS reduction using OLRs of between 0.3-1.3 kg VS/m$^3$ d.

(Cuetos et al., 2008)) studied the co-digestion of solid slaughterhouse waste with the organic fraction of municipal solid waste (OFMSW) in a semi-continuous process at mesophilic conditions, using HRT of 25 days. They found, when digesting the solid slaughterhouse waste without adding OFMSW that the failure of the process occurred with of OLR 1.7 kg VS/m$^3$. Moreover, in co-digestion of solid slaughterhouse waste with OFMSW, the system could work up to OLR of 3.7 kg VS/m$^3$. When the system was stable, decreasing of HRT and increasing OLRs between 0.9-1.7 kg VS/m$^3$ d in the case of the digestion of solid slaughterhouse waste, while in the case of co-digestion with OFMSW OLRs between 0.85-3.70 kg VS/m$^3$ d were possible. Moreover, co-digestion gave better VS reduction, i.e. 83% compared to 61% for slaughterhouse digestion, and also the biogas yield was two times higher than that of slaughterhouse waste alone. However, the methane yield from the digestion of slaughterhouse waste was higher than from the co-digestion process, ranging between 0.6-0.7 m$^3$ CH$_4$/kg VS and 0.4-0.5 m$^3$ CH$_4$/kg VS, respectively, with slightly higher CH$_4$ content of 66% and 65%, respectively.

(Palatsi et al., 2011)) reported that the main limiting factors in digestion of slaughterhouse wastes, are the lipid concentration and the composition of the microbial community used. The lipid rich substrate showed slower degradation kinetic than protein rich substrate, consequently high lipid concentration could cause LCFA inhibition leading to long lag phase in CH$_4$ formation and uncompleted substrate degradation. Moreover, LCFA can only be degraded by specific syntrophic microorganism groups thus the composition of microbial community is also important for a successive process.

(Bayr et al., 2012)) studied the effect of temperature (mesophilic and thermophilic) to biogas production in semi-continuous co-digestion of rendering and slaughterhouse wastes including bovine, swine and poultry wastes. They have found that in the batch assays, gave similar methane yields of between 0.26 - 0.57 m$^3$ CH$_4$/kg VS in both temperature ranges. However, the data obtained in the semi continuous co-digestion process showed that the mesophilic process was more stable than the thermophilic one in terms of process performance, methane yield, methane content and VS reduction. Moreover, it was found that the initial OLR of 1.5 kg VS/m$^3$ day
was too high for these substrates resulting in reduction of methane production, accumulation of VFAs and LCFAs. The OLR had to therefore be decreased to 0.5 VS/m³ day, which gave the highest methane yield of 0.97 m³ CH₄/kg VS at mesophilic conditions, while at thermophilic conditions the highest methane yield obtained was 0.77 m³ CH₄/kg VS with OLR of 1.5 VS/m³ day. However, the process started to get unstable after it had been operated for longer than one HRT. Thus, low OLRs and long HRTs are the critical factors to start up the process because the microorganisms need to adapt well to these substrates. Furthermore, it was also found that NH₄-N content in the thermophilic process was almost 2 times higher than that in the mesophilic process thus, the high ammonia (NH₄-N) concentration would inhibit the methanogenesis. It was concluded, that the mesophilic process was found more suitable in long-term co-digestion of high lipid and protein containing waste streams than the thermophilic process.

3. Methodology

3.1 Substrates and inoculums

The different substrates; slaughterhouse waste (SB), animal manure (M), various crops residues (VC), and municipal solid waste (MSW) were taken from different sites outside Borås, Sweden. The SB consisting of cow rumen and blood was obtained from a biogas plant near by Helsingborg. The M was a mixture of three different animal manures – pig manure (50%), horse manure (25%) and cow manure (25%), which were taken from different farms outside Borås. The VC was a combination of fruits and vegetable waste (70%) and straw (30%). The MSW was provided by the full-scale biogas plant, Sobacken in Borås, Sweden. All substrates were prepared, blended or cut to reduce the particle size and well mixed in the laboratory and stored at temperature below 0 °C before usage.

The inoculum was also provided by Sobacken; the large-scale biogas plant operating at thermophilic conditions in Borås. It was filtered with 2 mm porosity sieve to remove the big and undigested particles such as fibers and stored in the incubator at 55 °C for 3 days for its stabilization before usage. The characteristic of substrates and inoculum are shown in Table 3.1.

3.2 Experimental procedure

3.2.1 Batch experiment

The batch experiment was performed according to a method described by Hansen et al., (2004). The VS ratio of substrates and inoculum was maintained at ratio of 1: 2 for all experiments. Additionally, cellulose was also used as reference substrate to examine inoculums quality. 400 ml of inoculum and the appropriate amount of substrates or substrate mixtures were added to 2 L glass reactors which were then sealed with rubber septum and metallic cover (Apodan Nordic, Copehagen, Denmark).
To ensure anaerobic conditions, a gas mixture of 80% of N\textsubscript{2} and 20% of CO\textsubscript{2} were flushed through the headspace of each reactor for 3 minutes. After that the reactors were kept in an incubator at 55°C (±1°C) and were shaken once a day during the whole experimental period. The reactors were fed again, each with the same amount of substrate, as second feeding when the gas production achieved 80% of the gas production obtained during the first feed obtained previously by Pagés Díaz, (2012) as shown in Table 3.1. Then, the reactors were sealed, and flushed again with the mixture of N\textsubscript{2} and CO\textsubscript{2}, and then kept at 55°C (±1°C) and shaken once a day as mentioned above for an additional 30 days of incubation.

For measurements of the gas compositions; methane (CH\textsubscript{4}) and carbon dioxide (CO\textsubscript{2}), gas samples were collected regularly from the headspace through the septum with a 0.25 mL pressure-tight syringe (VICI, Precision Sampling Inc., Baton Rouge, LA, USA) to ensure that the gas samples could be taken at the actual pressure. Then, the gas samples were directly analyzed regarding CH\textsubscript{4} and CO\textsubscript{2} content via gas chromatography (GC). To avoid building up high pressure in the reactors, once the gas samples were collected, a needle was injected to the rubber septum to release the excess gas, and a sample was taken from each reactor again and the gas analysis was repeated. The difference in the methane content measured before the release of the actual measurement occasion and after the release of the gas from the previous measurement occasion will give the amount of produced methane between these two measurement occasions. The gas analyses in this way were performed 15-20 times within the incubation period. Moreover, volume corrections were taken into account to remove the methane production that generated from the inoculum. Additionally, all gas volumes results are presented at normal conditions; 0°C, 101.325 kPa according to ISO 10780:1994 (ISO, 1994).
Table 3.1 Characteristics of substrates and substrate mixtures used in the batch experiment and the date of the second feeding

<table>
<thead>
<tr>
<th>Substrate or Mixtures</th>
<th>Ratio</th>
<th>Total Solid (TS), (%)</th>
<th>Volatile Solid (VS), (%)</th>
<th>TKN (% of VS)</th>
<th>Protein (% of VS)</th>
<th>Fat (% of VS)</th>
<th>Carbohydrate (% of VS)</th>
<th>Date of the Second feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB (M1)</td>
<td>-</td>
<td>26.04</td>
<td>24.74</td>
<td>2.18*</td>
<td>13.53*</td>
<td>3.38*</td>
<td>81.46*</td>
<td>Day 25th</td>
</tr>
<tr>
<td>M (M2)</td>
<td>-</td>
<td>35.47</td>
<td>14.03</td>
<td>1.55*</td>
<td>9.59*</td>
<td>1.47*</td>
<td>66.37*</td>
<td>Day 20th</td>
</tr>
<tr>
<td>VC (M3)</td>
<td>-</td>
<td>24.06</td>
<td>21.69</td>
<td>1.03*</td>
<td>6.53*</td>
<td>0.62*</td>
<td>89.30*</td>
<td>Day 15th</td>
</tr>
<tr>
<td>MSW (M4)</td>
<td>-</td>
<td>18.39</td>
<td>15.46</td>
<td>3.04*</td>
<td>19.29*</td>
<td>8.90*</td>
<td>57.12*</td>
<td>Day 15th</td>
</tr>
<tr>
<td>SB: M (M5)</td>
<td>1:1</td>
<td>30.76</td>
<td>19.39</td>
<td>1.87**</td>
<td>11.56**</td>
<td>2.43**</td>
<td>73.92**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>M: VC (M6)</td>
<td>1:1</td>
<td>29.77</td>
<td>17.86</td>
<td>1.29**</td>
<td>8.06**</td>
<td>1.05**</td>
<td>77.84**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>VC: MSW (M7)</td>
<td>1:1</td>
<td>21.23</td>
<td>18.58</td>
<td>2.04**</td>
<td>12.91**</td>
<td>4.76**</td>
<td>73.21**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>SB: VC (M8)</td>
<td>1:1</td>
<td>25.05</td>
<td>23.22</td>
<td>1.61**</td>
<td>10.03**</td>
<td>2.00**</td>
<td>85.38**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>M: MSW (M9)</td>
<td>1:1</td>
<td>26.93</td>
<td>14.75</td>
<td>2.30**</td>
<td>14.44**</td>
<td>5.19**</td>
<td>61.75**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>SB: MSW (M10)</td>
<td>1:1</td>
<td>22.22</td>
<td>20.10</td>
<td>2.61**</td>
<td>16.41**</td>
<td>6.14**</td>
<td>69.29**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>SB: M: VC (M11)</td>
<td>1:1:1</td>
<td>28.24</td>
<td>19.95</td>
<td>1.57**</td>
<td>9.78**</td>
<td>1.81**</td>
<td>78.25**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>M: VC: MSW (M12)</td>
<td>1:1:1</td>
<td>25.72</td>
<td>16.89</td>
<td>1.69**</td>
<td>10.62**</td>
<td>3.30**</td>
<td>63.84**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>SB: VC: MSW (M13)</td>
<td>1:1:1</td>
<td>22.60</td>
<td>20.42</td>
<td>2.06**</td>
<td>12.99**</td>
<td>4.26**</td>
<td>75.20**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>SB: M: MSW (M14)</td>
<td>1:1:1</td>
<td>26.37</td>
<td>17.90</td>
<td>2.23**</td>
<td>14.00**</td>
<td>4.54**</td>
<td>67.63**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>SB: M: VC: MSW (M15)</td>
<td>1:1:1:1</td>
<td>25.99</td>
<td>18.98</td>
<td>1.95**</td>
<td>12.24**</td>
<td>3.59**</td>
<td>73.56**</td>
<td>Day 20th</td>
</tr>
<tr>
<td>Cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculum_average</td>
<td>-</td>
<td>3.18</td>
<td>1.78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data obtained by AKO lab, Borås, Sweden

**Data calculated on the bases of mixture ratios
### 3.2.2 Continuous experiment

Four continuously stirred tank reactors (CSTR) with a total volume of 5 liters and working volume of 3 liters were used in the semi-continuous co-digestion experiments. Reactor 1 (R1) was used as control reactor and was fed with only SB (M1). Reactor 2 (R2), 3 (R3) and 4 (R4) were used to examine the co-digestion process using mixtures of SB: M (M5), SB: VC (M8) and SB: VC: MSW (M13), respectively. The mixture ratios were the same as used in the batch experiment and are presented in Table 3.1. The stirring blades rotated at 90-110 rpm. The temperature was controlled to keep thermophilic conditions (55 ± 1 °C) by circulating hot water from a water bath into the water jacket of each reactor. The daily produced gas was determined by connecting the gas tube with the Automatic Methane Potential measuring system (Bioprocess control, Sweden). Finally, the obtained gas volumes were converted to normal conditions; 0°C, 101.325 kPa according to ISO 10780:1994 (ISO, 1994).

Substrates were fed once a day after that the same volume of effluents were taken out from the reactors to keep constant working volume of 3 L. Initial OLR for all reactors were set to 0.5 g VS/L·d with HRT of 25 days. Depending of the performance obtained, the OLR were increased gradually to 0.9 g VS/L·d for R1 and to 1.5 g VS/L·d for R2, R3 and R4, respectively. Two of the effluent samples taken in each week were kept in low temperature (< 0 °C) before analyses regarding to the following parameters; TS, VS, Volatile Fatty Acids (VFAs), and Ammonium-Nitrogen (NH$_4^+$-N). The VFA/alkalinity ratios were evaluated twice a week by Nordmann-titration method according to Lossie & Pütz, (2012) to control the stability of the system. Gas samples were collected and the biogas composition was measured by GC once a day. Furthermore, the pH was also determined every day.

### 3.3 Analytical methods

Total solids (TS), volatile solids (VS), biogas composition, VFA/alkalinity ratio, VFAs, and NH$_4^+$-N were investigated by different methods. TS and VS were analyzed according to Sluiter et al., (2008) and Sluiter et al.,(2005) respectively, where the empty crucibles were first put in the oven and kept at 105±3 °C for 24 hours to ensure that they dry. The oven-dried crucibles were then placed into a desiccator to cool down and weighted. Samples of 20 mL were added in the prepared crucibles and then the weight was measured again. The crucibles containing samples were dried in 105±3 °C for 24 hours. After that, cooled down and weighted again. To determine the VS, the crucibles with the dried sample were put into a muffle furnace at 575±25 °C for minimum 4 hours. Then, the crucibles were taken out to cool down in a desiccator before weighting. The TS and VS were calculated by the following equations;
The biogas composition was determined by using a gas chromatograph (Auto System, Perkin Elmer, USA) equipped with a packed column (Column 8000 PKD, Perkin Elmer, USA) and a thermal conductivity detector (Perkin Elmer, USA) with inject temperature of 150°C. The carrier gas was nitrogen operated with a flow rate of 20 mL/min at 60°C.

Alkalinity as referred to total inorganic carbonate (TAC) was determined by Nordmann-titration method according to Lossie & Piitz, (2012). The samples were centrifuged (10000 rpm for 15 minutes) and 20 mL of the supernatant was titrated with 0.1N sulfuric acid (H₂SO₄) to pH 5. As mentioned above, VFA/alkalinity ratio was determined to control the system, thus to find VFA concentration which was considered as acetic acid equivalent, the titration was continued using 0.1N of H₂SO₄ to reach pH 4.4. Empirical equations were used to calculate the VFA/alkalinity ratio as it is summarized below;

\[
\text{Alkalinity} = \frac{\text{mL of } H_2SO_4 \text{ from start to pH 5.0}}{250} \times 100 \\
VFA = \left( \frac{\text{mL of } H_2SO_4 \text{ from pH 5.0 to pH 4.4} \times 1.66 - 0.15}{500} \right) \times 500
\]

For the VFAs and NH₄⁺-N determinations, the samples were first centrifuged at 10000 rpm for 15 minutes and the supernatants were centrifuged again at 14000 rpm for 5 minutes. After that the samples were filtrated with 0.20 µm porosity cellulose acetate syringe filter. The concentration of different VFAs were then analyzed by high performance liquid chromatography (HPLC, Waters 2695, Millipore, Milford, USA) equipped with a refractive index (RI) detector (Waters 2414) and an ion-exchange column (Aminex HPX-87H column, Bio-Rad, USA), at 60 °C using 5 mmol/L H₂SO₄ as eluent with a flow rate of 0.6 mL/min.

The NH₄⁺-N was determined by ion exchange chromatography (IC, 819 IC detector and 820 IC separation Center, Metromh AG, Herisau, Switzerland). First the prepared samples were diluted with the eluent solution (4 mmol/L tartaric acid and 0.75 mmol/L dipicolinic acid) to a ratio of 1:10. Then, 10 µl of these diluted samples were injected to the column with a flow rate of 0.50 mL/min at 35-40°C, and pressure of 8-9 MPa.

3.4 Calculations

Organic loading rate (OLR), (g VS/L·d) and Hydraulic retention time (HRT), (d) were calculated based on actual daily feed. Specific methane yield (Y(CH₄)) (mL/g VSadded) was defined as produced CH₄ volume per amount of VS substrates added at given time in the batches experiment. Moreover, in the semi-continuous experiment, Y(CH₄) were presented on the basis of daily CH₄ production and amount of substrate fed daily (mL/g VS·d).
4. Results and discussion

4.1 Batch experiments

Within the batch experiment different substrates and substrate mixtures (Table 3.1) were investigated for their methane potential and for the effect of a second feed. The VS ratio of substrates and inoculum was maintained at ratio of 1:2 in each set up and the experiments were carried out under thermophilic conditions. The same amount of substrates and its mixtures were fed again as second feeding, when the gas production obtained during the first feed reached 80% of the maximum value. The methane yield of first and second feeding are shown in Table 4.1.

Table 4.1. Summary of the results obtained during the batch assays: pH measurements after first and second feeding and methane yields (ml/g VS) obtained after 1st and 2nd feeding.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Date of the Second feeding</th>
<th>pH After first feeding</th>
<th>Methane Yield (ml/g VS)</th>
<th>Methane Yield (ml/g VS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>After second feeding</td>
<td>First feeding*</td>
<td>Second feeding*</td>
</tr>
<tr>
<td>M1</td>
<td>Day 25th</td>
<td>8.59</td>
<td>7.50</td>
<td>740</td>
</tr>
<tr>
<td>M2</td>
<td>Day 20th</td>
<td>8.06</td>
<td>7.67</td>
<td>326</td>
</tr>
<tr>
<td>M3</td>
<td>Day 15th</td>
<td>8.16</td>
<td>7.75</td>
<td>312</td>
</tr>
<tr>
<td>M4</td>
<td>Day 15th</td>
<td>8.15</td>
<td>8.21</td>
<td>497</td>
</tr>
<tr>
<td>M5</td>
<td>Day 20th</td>
<td>8.18</td>
<td>8.43</td>
<td>576</td>
</tr>
<tr>
<td>M6</td>
<td>Day 20th</td>
<td>8.13</td>
<td>7.72</td>
<td>322</td>
</tr>
<tr>
<td>M7</td>
<td>Day 20th</td>
<td>8.12</td>
<td>8.19</td>
<td>413</td>
</tr>
<tr>
<td>M8</td>
<td>Day 20th</td>
<td>8.20</td>
<td>7.82</td>
<td>539</td>
</tr>
<tr>
<td>M9</td>
<td>Day 20th</td>
<td>8.21</td>
<td>7.82</td>
<td>356</td>
</tr>
<tr>
<td>M10</td>
<td>Day 20th</td>
<td>8.25</td>
<td>7.83</td>
<td>613</td>
</tr>
<tr>
<td>M11</td>
<td>Day 20th</td>
<td>8.22</td>
<td>7.89</td>
<td>478</td>
</tr>
<tr>
<td>M12</td>
<td>Day 20th</td>
<td>8.03</td>
<td>7.82</td>
<td>380</td>
</tr>
<tr>
<td>M13</td>
<td>Day 20th</td>
<td>8.16</td>
<td>7.87</td>
<td>535</td>
</tr>
<tr>
<td>M14</td>
<td>Day 20th</td>
<td>8.09</td>
<td>7.86</td>
<td>502</td>
</tr>
<tr>
<td>M15</td>
<td>Day 20th</td>
<td>8.09</td>
<td>7.91</td>
<td>466</td>
</tr>
</tbody>
</table>

* Methane yield when 80% of substrates were consumed.
** Maximum methane yield

During the first feeding, best methane yields of 740, 613 and 576 ml/g VS were achieved in digestion of SB (M1), and co-digestion of SB: MSW (M10) and SB: M (M5), respectively while in the second feeding the best methane potentials were 506, 521, and 493 ml/g VS from the digestion and co-digestion of MSW (M4), SB: VC: MSW (M13) and SB: VC (M8) when 80% of the substrates were consumed, as it is shown in Table 4.1.
Figure 4.1  Summary of methane yields obtained from batch digestion assays after a period of time when 80 % of substrates were consumed in first feeding compared to that of second feeding – SB (M1), M (M2), VC (M3), MSW (M4), SB: M (M5), M: VC (M6), VC: MSW (M7), SB: VC (M8), M: MSW (M9), SB: MSW (M10), SB: M: VC (M11), M: VC: MSW (M12), SB: VC: MSW (M13), SB: M: MSW (M14), and SB: M: VC: MSW (M15).

Pagés Díaz et al., (2011) found that adding of second feed after 31 days degradation of the first feed resulted in a long lag phase, especially in the case of SB substrate due to stress conditions appeared for the bacteria. However, it was also found that in some other batches; MSW, VC and the mixture of SB: M:VC: MSW at mixing ratio of 2:3:3:0, the methane yield increased because of the adaptation of microorganisms to the substrate. Thus, to be able to further investigate the effect of second feeding on bacterial activity, the same amount of the different substrates were added to each assay as second feeding in this study at day 15, day 20 or day 25, when 80 % of the substrates were consumed from the first feed (Field et al., 1988), as it is shown in Table 3.1. Figure 4.1 illustrates the comparison of the achieved methane yields when 80% of substrates were consumed. The results indicate that the second feeding resulted in higher methane yield than the first feeding in co-digestion of M: VC: MSW (M12) because the microorganisms still worked actively. In contrast, lower methane potentials were obtained after the second feed from the digestion of M (M2), VC (M3) and from the co-digestion of SB: M (M5), M: VC (M6), VC: MSW (M7), SB: VC (M8), SB: MSW (M10), SB: M: VC (M11), SB: M: MSW (M14) and SB: M: VC (M15). Moreover, the similar methane yield was found in the digestion of MSW (M4), and the co-digestion of M: MSW (M9) and SB: VC: MSW (M13). However, introducing of second feeding did not work at all in the digestion of SB (M1) due to that some inhibition occurred in the system, resulting in a loose of activity of the methanogenic bacteria.
Figure 4.2 Comparison of methane production rates (ml/g VS) in the batch experiments; (a) digestion of mono-substrates, (b) Co-digestion with two different substrates and (c) Co-digestion with three or more different substrates

Figure 4.2 clearly illustrates how the methane production rates behaved during the first and second feeding in each sample. During second feeding, the methane production rate was negative in the digestion of SB (M1). Pagés Díaz et al., (2011) reported also lower degradation rate of SB previously, compared to other substrates and substrate mixtures (1). In the digestion of M (M2), VC (M3), MSW (M4) (Figure 4.2 (a)) and in the co-digestion of M: VC (M6), VC: MSW (M7), M: VC: MSW (M12), and SB: VC: MSW (M13) (Figure 4.2 (b), (c)), the methane production rate rapidly increased even after the second feed because the microorganisms still worked actively. In contrast, as it is shown on Figure 4.2 (b) and (c) lag phase occurred in the co-digestion of SB: M (M5), SB: VC (M8), M: MSW (M9), SB: MSW (M10), SB: M: VC (M11), SB: M: MSW (M14) and SB: M: VC (M15), these may be explained by stress conditions which may appear for the bacteria (Pagés Díaz et al., 2011).

In summary, the maximum methane yield of 592, 573, 522 and 521 ml/g VS were obtained from the digestion and co-digestion of SB: VC (M8), MSW (M4), SB: VC: MSW (M13) and SB: M (M5), respectively as shown in Table 4.1. Moreover, the mixture of SB: M (M5), SB: VC (M8) and SB: VC: MSW (M13) gave an excellent methane yield in both first and second feedings. On the basis of these results, these substrates were subjected to further studies in the continuous system to evaluate the long term effects.

4.2 Continuous experiments

To study the long term effects in co-digestion system of agro-industrial wastes, continuous stirred tank reactors (CSTR) were operated at thermophilic conditions with 3 liters working volume. Four reactors were fed with different substrates, using SB as substrate for the control reactor (R1) and a mixture of SB: M for R2, a mixture of SB:
VC for R3 and a mixture of SB: VC: MSW for R4, respectively. The HRT for each reactor was kept at 25 days.

4.2.1 Feeding rate

The organic loading rates applied during the experiment are shown on Figure 4.3. First, the initial OLR of all reactors were 0.5 g VS/L·d and this was increased gradually to 1.5 g VS/L·d until day 8. However, the feeding had to be stopped for several days to let the system to be recovered. Due to substrate overloading indicated by pH drop, low methane production, a decrease in biogas volume produced and high VFA/Alkalinity ratio were observed. Then at day 17, the reactors were started to be fed again using initial OLR of 0.3 g VS/L·d for all reactors, which was then gradually increased to OLR 0.9 g VS/L·d for R1, and to 1.5 g VS/L·d for R2, R3 and R4 until day 40 and then the reactors were kept under those operation conditions until day 56 (when our experimental period for this thesis work had to finished. However, the reactors are operated further by our supervisor, Jhosane Pages Diaz, PhD student at School of Engineering, University of Borås).

According to the obtained results we can conclude, that the start-up stage is really important in a continuous system; the overloading due to non adapted microorganisms can lead to the failure of the process (Alvarez et al., 2008; Cuetos et al., 2008; Salminen & Rintala, 2002). Moreover, Figure 4.3 also shows that pH was around 8.50 at the beginning of the process for all reactors. Then, the pH started to decrease until 6.7 due to the accumulation of fatty acids, which are intermediary products from the hydrolysis and conversion of organic matters. After that, the feed was stopped to allow the system to recover itself and the pH started to rise again. The substrates were then fed again and the pH was maintained between 7.0 and 8.0 in all reactors. However, we can also see that the pH in R1, which is fed with only SB, started to decrease again towards the end of the experimental period, indicating that this system will collapse again. Additionally, foam building was also observed in R1. It could be caused by many operational conditions such as increasing of alkalinity, insufficient mixing, fluctuation of temperature and rising of fatty acid concentration (Gerardi, 2003). Cuetos et al., (2008) reported the formation of floating aggregates with high lipid concentration. Salminen et al., (2001) also observed a tendency of layer formation in the study of semi-continuous AD of poultry slaughterhouse waste when using short HRTs of 13-50 days with OLRs of 1.0–2.1 VS/L·d. Additionally, Cuetos et al., (2008) had the same observation in semi-continuous reactors operating with ORL of 1.70 and 3.70 g VS/L·d and HRT of 25 days during the digestion of solid slaughterhouse waste and during its co-digestion with the organic fraction of municipal solid waste. There were foam formations on the top of both reactors even though the systems were mechanically stirred.
Figure 4.3 Organic Loading Rate (OLR) and pH of the continuously stirred tank reactors (CSTR) digesting different substrates

4.2.2 Gas production

The daily biogas volume of all reactors increased continually because the OLR was increased as shown in Figure 4.4. However, the produced biogas volume dropped when new OLR was applied and then gradually increased again. Cuetos et al., (2008) mentioned previously, that when new conditions are introduced, the system needs some time to adjust to these new conditions. Moreover, during the end of experiment when the OLR was kept at the same level for all reactors the biogas production stabilized. However R1, there SB is digested alone, shows still unstable operation which may lead to the collapse of the system in the future.
Normally, the biogas contains about 60% of methane (CH₄) and 40% of carbon dioxide (CO₂) in the anaerobic digestion. However, the content of methane varies with the type of substrates because each substrate has different composition. In the earlier stage of the experiment, the CH₄ content in the biogas produced in all reactors was in between 35-50% due to instabilities during the startup period. Then, after the feeding was stopped to allow the system to recover, and starting up the feeding again from low OLR, the CH₄ content increased to more than 60% in all reactors as illustrated in Figure 4.5, indicating that the microorganisms have been adapted to the given substrates. The remaining organic matters were consumed and the accumulation of VFAs decreased by around 40-60% as it is shown in Figure 4.8. During the last period of the experiment, when constant OLR had been used, average methane contents of 69, 76, 71 and 71% was observed for R1, R2, R3 and R4, respectively.
Figure 4.5 Methane content in the biogas produced in the continuously stirred tank reactors (CSTR) digesting different substrates

Figure 4.6 shows the methane yield obtained during the continuous experiment. From day 40, the same OLR of 0.9 g VS/L·d in R1 and of 1.5 g VS/L·d in R2, R3 and R4, respectively, had been kept. The methane yields from R2, R3 and R4 were almost stable, but the methane yield decreased in R1. Between day 40 to day 56, average methane yields of 300, 510, 587 and 426 ml/g VS were observed in R1, R2, R3 and R4, respectively. However, higher methane yields were obtained when the OLR was lower, i.e. 910 ml/g VS, methane was produced at OLR of 0.5 g VS/L·d in R1, and 599, 706 and 866 ml/g VS at OLR of 0.6 g VS/L·d in R2, R3 and R4, respectively. In comparison, 0.4-0.5 m³/kg VS and 0.3-0.9 m³/kg VS methane yields were reported during the digestion and co-digestion processes of slaughterhouse waste in previous studies (Alvarez et al., 2008; Bayr et al., 2012; Cuetos et al., 2008; Salminen & Rintala, 2002).
Figure 4.6 Methane yields obtained from the continuously stirred tank reactors (CSTR) digesting different substrates.

4.2.3 VFA and Alkalinity; VFA/Alkalinity ratio,

VFA are important intermediate products that would be converted to CH₄ during anaerobic digestion. In this study, two different measurements of VFA were applied; by titration method and by HPLC. The titration method was performed regularly and the results were used as indicator of process stability, due to that this method is fast and easy to perform. It measures the concentration of volatile organic acids and gives the results as acetic acid equivalent, while the alkalinity refers to total inorganic carbonate (TAC) (Lossie & Pütz, 2012). In addition, to determine the various VFA presented in the system; i.e. acetate, propionate, butyrate, caproate, isobutyrate, valerate and isovalerate, HPLC was used.

4.2.3.1. VFA/Alkalinity ratio

The VFA/alkalinity ratio was investigated to control the stability of the reactors. For a well performing digestion process the VFA/alkalinity ratio should be between 0.3-0.4. If it is higher than 0.4, the system is going into an unstable performance, so the amount of substrate in the feed have to be decreased or stopped. In contrast, if the VFA/Alkalinity ratio is lower than 0.3, more substrate can be added, since the system in this case is operating under its capacity (Lossie & Pütz, 2012). According to Lossie & Pütz, (2012), an optimum ratio of 0.4-0.6 is usually required for the degradation of most of the renewable raw materials used in biogas production, however, each plant has its own optimum.

Figure 4.7 illustrates the VFA/alkalinity ratio obtained in the four different reactors during this study. In the beginning these ratios in all reactors were higher than 0.6, indicating unstable conditions in the reactors, so that the feeding had to be stopped. After a stop in feeding for several days, when the feeding has started again low initial OLRs were applied with slightly and gradually increase. which This resulted in a stable system, with VFA/alkalinity ratios of between 0.2 and 0.6. Moreover, after day 40 when the constant OLRs has been introduced at kept at 0.9
gVS/L·d for R1 and 1.5 g VS/L·d for all of the other reactors, the VFA/Alkalinity ratio could be still maintained at low level in R2, R3 and R4, while it started to increase again in R1 (Figure 4.7).

4.2.3.2. Volatile Fatty Acids (VFAs)

During the starting up period, when the initial OLR of 0.5 g VS/L·d was increased to 1.5 g VS/L·d in all reactors, the accumulation of VFAs led to stress condition in the reactors (Figure 4.8). Between day 9 to day 16, the total VFAs were higher than 10 g/L which according to Wang et al.,  (2009) can inhibit the activity of methanogenic bacteria. Thus, the substrate feeding was stopped to allow the methanogens to recover also to be adapted. Then, when the reactors was started to be fed again with low OLR, it was found that the concentrations of VFAs were decreased for all of the reactors, as it is shown in Figure 4.8.

Moreover, acetic acid was the most abundant VFA found in all reactors. This indicates that the acetoclastic methanogenesis is the rate-limiting step, similarly as it was reported in previous studies (Bayr et al., 2012; Cuetos et al., 2008; Padilla-Gasca et al., 2011; Salminen & Rintala, 2002). The propionic acid was also observed at lower concentrations. It is produced by the conversion of hydrolysis products such as sugars, amino acids, and long chain fatty acid (LCFA) in the acidogenesis phase (Arsova, 2010; Polprasert, 2007). According to Wang et al., (2006) the accumulation of propionic acid can also result in low efficiency of the methanogenic phase. The highest propionic acid concentrations observed were 3.293, 1.787, 3.493, and 3.113 g/L in R1, R2, R3 and R4, respectively; however this was not the main VFA to destabilize the anaerobic process in this study. The concentrations of butyric acid, isobutyric acid, valeric acid and isovaleric acid were lower than 1 g/L and caprionic acid were not detected in any of the reactors.
Figure 4.8 Volatile Fatty Acids (VFAs) concentration obtained in the continuously stirred tank reactors (CSTR) digesting different substrates: (a) R1 (SB), (b) R2 (SB: M), (c) R3 (SB: VC) and (d) R4 (SB: VC: MSW)
4.2.3.3. Alkalinity

Alkalinity is the natural buffering system in the digester. At the beginning the alkalinity in all reactors was between 7300 - 8000 mg /L, as it is presented in Figure 4.9. Then it started to decrease during the first stage of the process in all reactors. The decreasing of alkalinity resulted in a rapid change in pH, due to the organic acid accumulation, which can inhibit the activity of methane forming bacteria (Gerardi, 2003). After the system had been recovered, the total alkalinity increased again indicating an increased capacity in acids neutralization. Thus, the total alkalinity should be maintained in the optimum range considering the VFA concentrations in order to neutralize the system. Cuetos et al., (2008) reported alkalinites of 9342.8±200 and 12166.7±150 mg/ L during anaerobic digestion of slaughterhouse waste with OLR of 1.7 g VS/L·d and during the co-digestion of slaughterhouse waste and municipal solid waste with OLR of 3.7 g VS/L·d, respectively, using HRT of 25 days.

Figure 4.9 Alkalinity measured in the continuously stirred tank reactors (CSTR) digesting different substrates

4.2.4 Ammonium-Nitrogen (NH\textsubscript{4}+-N) concentration

Ammonium ions (NH\textsubscript{4}+) are the dissolved form of ammonia, which forms during the biodegradation of amino acids. The preferable nitrogen (N) source for methane forming bacteria is NH\textsubscript{4}+-N, however they can take N from another sources as well. The lower concentration in the effluent indicates that NH\textsubscript{4}+-N was consumed by the microorganisms (Gerardi, 2003).

Figure 4.10 shows that NH\textsubscript{4}+-N concentrations in the effluent of all reactors investigated in this study were declined towards the end of the experimental period. As it was mentioned previously, this indicates that the nitrogen source had been consumed by the microorganisms.

Moreover, depending on the pH and temperature, NH\textsubscript{4}+ can be converted to ammonia (NH\textsubscript{3}), which reacts with CO\textsubscript{2} to form ammonium bicarbonate
(NH₄HCO₃), which in turn can result in an increase of alkalinity. Even though the alkalinity is the natural buffering of the system, excessive alkalinity should be avoided because it may cause free ammonia toxicity (Gerardi, 2003). According to Straka et al., (2007), the methane production was inhibited when the concentration of NH₄⁺-N excided about 1.2 g N/L. Moreover, Benabdallah El Hajd et al., (2009) had found when treating MSW at the thermophilic conditions that the methane production reduced by 50% at 5.6 g NH₄⁺N/L concentration. However, the ammonium nitrogen concentrations in our system remained lower than 250 mg/L in all reactors.

![NH₄⁺-N Concentration vs Time](image)

**Figure 4.10 Ammonium-Nitrogen concentrations measured in the continuously stirred tank reactors (CSTR) digesting different substrates**

5. Conclusions

In this study, the co-digestion of different agro-industrial wastes were investigated. The potential of methane production and the effects of a second feed were determined in batch anaerobic digestion experiments. It was shown that co-digestion of SB:VC, SB:VC:MSW and SB:M provides high methane potentials. The highest methane yields obtained were 592, 522 and 521 ml/g VS, respectively in these samples. Moreover, the second feeding could increase the methane yield of some of the substrate mixtures, due to building up an active microbial consortium. In contrast, decreasing yields or inhibition was detected in some other substrate mixtures.

Furthermore, a continuous experiment using four continuously stirred tank reactors (CSTR) was also started to study the long term effects during co-digestion of agro-industrial wastes focusing on slaughterhouse waste. In a continuous process the start-up stage is really important, the OLR should be low and then it should be slightly increased gradually for avoiding overload in the system and for the adaptation of microorganisms to the substrate. VFAs, alkalinity and ammonium-N concentrations were used as control parameters for the operation of the continuous systems. The methane content of the produced biogas during the digestion and co-digestion of
slaughterhouse waste were obtained between 60-85% (lower in the beginning and higher towards the end) and the highest methane content of 76% were found from co-digestion of SB:M towards the end of the operation. Towards the end of the investigation period, average methane yields of 300, 510, 587 and 426 ml/g VS were obtained in the digestion of SB, and co-digestion of SB:M, SB:VC and SB:VC:MSW, respectively. The highest average methane potential of 587 ml/g VS was found in co-digestion of SB:VC and it is comparable to the result of 592 ml/g VS obtained from the batch digestion of the same mixture.

6. Future work

- Fed batch system can be set up before start of the continuous experiment to help the adaptation of microorganisms to the substrates.
- Optimization of HRT and OLR should be investigated further.
- In this study, the equal proportions of different substrates in the mixtures were used, thus the effect of biogas production with different ratios in the mixtures can be further explored.
- Two phase anaerobic digestion systems can be introduced, especially in case of short HRT.
- The economic aspects of the anaerobic digestion process using these kinds of substrates should be also considered in the future.
7. References


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